ENGINEERING NANOPARTICLES FOR PHARMACEUTICAL APPLICATIONS: 
FORMULATION AND FREEZE-DRYING TECHNIQUES

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ABSTRACT

This dissertation discusses the use of Flash NanoPrecipitation, a continuous rapid precipitation method, to fabricate pharmaceutical nanoparticles. After introducing the precipitation technique and reviewing the current understanding and applications in the literature of Flash NanoPrecipitation, three chapters exhibiting a methodology for formulating active pharmaceutical ingredients are presented. The main focus is the adaption of Flash NanoPrecipitation techniques for producing final dosage forms that could be feasibly administered at a therapeutic dose, which has not been emphasized in the past. Guided by scientific principles, systematic evaluations of critical formulation and process parameters achieve formulations with maximized loading efficiencies.

This treatment is first given for the production of lyophilized progesterone-loaded nanoparticles for emergency treatment of a traumatic brain injury, followed by a model study investigating how to generate lyophilized nanoparticles loaded with a crystalline weak base, GW771806X. These two chapters are aimed for parenteral administration of nanoparticulate therapeutics, which is why freeze-drying is employed to obtain dry dosage forms. The last chapter focusing on rational formulation details the production of nanoparticles suitable for oral dosage forms, for which traditional Flash NanoPrecipitation formulations would not be cost-effective.

Finally, an evaluation of freeze-drying nanoparticles is presented. This fundamental study elucidates on the effects of various formulation and process parameters on underlying phenomena in the freeze-drying process. The results underscore the need for a rational development of a lyophilized nanoparticle formulation in order to achieve a dosage form that can
be easily reconstituted at therapeutic concentrations, starting with nanoparticle composition, choice of protectant excipients, and freeze-drying conditions.

Overall, the methodology put forth by this dissertation exemplifies some of the necessary steps for the Flash NanoPrecipitation technique to be adapted into industrial pharmaceutical practice. While the technique has been highly characterized and promoted as a viable option for the formulation of active pharmaceutical ingredients that have traditionally been difficult to formulate, there has yet to be significant use of Flash NanoPrecipitation in the industry. Therefore, in attempting to marry the particle engineering with the requirements of pharmaceutical formulation development, this dissertation aims to establish a starting point for future industrial adaption of Flash NanoPrecipitation.
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CHAPTER 1 - INTRODUCTION

1.1 MOTIVATION

In the area of pharmaceutical research and development, the last few decades have been revolutionized due to the influx of hit compounds that have been generated from high-throughput screening. It has been estimated that roughly 40% of these compounds have poor water solubility [1], which may translate to a high partition or distribution coefficient, logP or logD, respectively. Based on experimental and computational analysis, the famous Lipinski’s rule of five [2] has been used in the selection and optimization of lead compounds; one of the components of this proposed guide for improved bioavailability is that the compound have a logP no greater than 5. Considering that high-throughput screening techniques rely on biochemical [3, 4] or phenotypic assays where potency is determined by the degree of binding to protein targets or localization in a cellular compartment [5-7], compounds of increased logP may be able to yield higher potencies [8]. More hydrophobic compounds can be problematic in the development process, as compounds with higher logP values have tended to be discontinued before progressing to the next phase in development [9], which is usually attributed to their poor bioavailability. For compounds that pass through lead optimization, become candidate drugs and meet requirements for marketing, it has been shown that there has been an increase in molecular weight over the past thirty years [10]. For various datasets of leads and launched drugs, an increase in molecular weight has corresponded with a decrease in solubility [11, 12], and thereby an increase in logP and possibly a decrease in bioavailability. Clearly, there is a need for enhancing the bioavailability of potent active pharmaceutical ingredients (APIs), such that they can continue in the development cascade.
To address this issue, many formulation techniques have been developed in order to increase the solubility of APIs, which include polymorph formation, salt formation, co-crystallization, complexation, micelle solubilization, solid dispersions, and particle size reduction. These various techniques attempt to modify the dissolution rates of an API through the modulation of one or more parameters in the diffusional layer model for a spherical particle [13], presented below.

\[
\frac{dm}{dt} = 4\pi r^2 D c_r \left( \frac{1}{r} + \frac{1}{h} \right)
\]

(1.1)

Here, \( m \) is the mass of an API particle that is dissolving at time \( t \), \( r \) is the radius of the particle, \( D \) is the diffusion coefficient of the API, \( c_r \) is the solubility of the API particle of radius \( r \), and \( h \) is the thickness of the boundary layer for diffusion. This model has various assumptions, including isotropic dissolution of the particle, a diffusional boundary layer of constant thickness, pseudo-steady-state being established with only minimal particle dissolution, the API concentration at the interface between the particle and the fluid being saturated, the diffusion coefficient being constant throughout the boundary layer, and the bulk API concentration in solution being zero.

The boundary layer thickness has been suggested to take the following form, where \( a \) and \( b \) are constants, \( U \) is the fluid velocity, and \( \nu \) is the fluid kinematic viscosity [14].

\[
h = ar + b \sqrt{\frac{U}{\nu D}} \left( \frac{\nu}{D} \right)^{1/3} r^{3/2}
\]

(1.2)

Furthermore, the solubility of a particle with radius \( r \) is described by the Kelvin equation as follows, where \( c_{eq} \) is the saturated equilibrium solubility of the API, \( \gamma \) is the interfacial tension between the solute and fluid, \( v \) is the API molar volume, \( R \) is the universal gas constant, and \( T \) is absolute temperature.
\[ c_r = c_{eq} \exp \left( \frac{2\gamma v}{RT_r} \right) \]  

(1.3)

However, when considering an API dose of a specific mass, it is more important to consider the total dissolution rate of the dose and not just that of one particle. The number of spherical particles, \( n_p \), of radius \( r \) in a dose of \( m_{tot} \) can be expressed as shown below, where \( \rho \) is the API density.

\[ n_p = \frac{3m_{tot}}{4\pi \rho r^3} \]  

(1.4)

Finally, through substituting equations 1.2 and 1.3 into 1.1 and multiplying the resulting expression by equation 1.4, the final expression for the total dissolution rate below is derived.

\[
\frac{dm}{dt}_{tot} = n_p \frac{dm}{dt} = \frac{3Dc_{eq}m_{tot}}{\rho r^2} \left( 1 + \frac{1}{a + b \sqrt{\frac{rU}{\nu} \left( \frac{\nu}{D} \right)^{1/3}}} \right) \exp \left( \frac{2\gamma v}{RT_r} \right)
\]

(1.5)

This final equation shows that only a few parameters can be practically varied in order to increase the total dissolution rate, namely particle radius and API solubility in the fluid.

The techniques for increasing bioavailability can be broadly categorized into two groups: one that modifies the saturated solubility of the API and the other which increases the specific surface area of the API particles. For example, polymorph formation aims to increase the saturated solubility of the API by increasing the energy state of the compound, where amorphous material tends to be in the highest energy state, such that it can more easily dissolve [15, 16]. Salt formation and co-crystallization also increases API solubility, generally by the introduction of a pairing acid or base or a coformer ligand that either shifts the pH to a range where the solubility of the active is higher [17, 18] or leads to API supersaturation from a transient amorphous form [19, 20], respectively. Both complexation and surfactant solubilization increase solubility by.
essentially sequestering the API into an environment (e.g. cyclodextrin central cavity, micelle core) within a dispersed phase in aqueous media; this effectively increases the API concentration that is dissolved or dispersed in the medium, as compared to the saturation solubility. While these two techniques do not inherently increase the solubility of the API, stable supersaturated concentrations can be achieved. Contrastingly solid dispersions involve the formation of a solid solution or the distribution of fine API crystals within a hydrophilic polymer matrix [21, 22]; in reducing the dimension of the API phase, the surface area per volume is substantially increased, resulting in a higher dissolution rate. With particle size reduction, the concept is similar, where the particle size of the drug substance is made smaller, such that the specific surface area increases.

However, another very prominent formulation technique, which is a growing field of research, is that of nanotechnology. A large number of different nano-constructs have been developed which make use of the aforementioned formulation techniques and concepts to increase the bioavailability of poorly soluble APIs. Nanoparticles include nano-crystal suspensions [23-25], lipid nanoparticles [26-28], dendrimers [29-31], liposomes [32, 33], and polymeric nanoparticles [34, 35]. With the abundance of nano-formulation methods available, it can become difficult to decide on which technique to use. Any technique chosen must be capable of being scaled up from laboratory scale to production scale. This is a key point which is usually not addressed by academic literature reports presenting novel formulation methods.

One method which is of particular interest because of this point is Flash NanoPrecipitation (FNP), developed by Johnson and associates [36, 37]. This is a rapid precipitation technique making use of directed assembly to kinetically freeze supersaturated API dispersions in polymer-stabilized nano-sized particles. Since the production of these API
nanoparticle dispersions is accomplished by micromixing of various streams through a mixer of specific geometry [37-39], the output rate of the liquid dispersion is controlled by the input flow rates of the streams, allowing for the use of the same mixer in a laboratory or plant setting. Based on a typical laboratory effluent flow rate of 140 mL/min with an API concentration in the effluent dispersion of 1 mg/mL, production rates of ~200 kg/day of nanoparticulate API could be accomplished. While much work has already been done using FNP, which will be summarized in the next section and discussed in detail in Chapter 2, there is still a great need to properly adopt the production technique for API formulation, which is the emphasis of this dissertation.

1.2 PREVIOUS RESEARCH

Flash NanoPrecipitation has been described as a competitive precipitation process of an API and a stabilizing polymer [40]. Typically, one or more APIs and a stabilizing polymer, and possibly excipients, are dissolved in a water-miscible organic solvent. This organic phase comprises the organic stream that is mixed against an aqueous anti-solvent stream. In order to obtain tight particle size distributions, the mixing must be such that homogeneous precipitation kinetics are achieved by the organic solutes, which means that the characteristic time scale for mixing, $\tau_{\text{mix}}$, must be faster than the overall precipitation time scale of the solutes, $\tau_{\text{flash}}$ [36]. Once the organic and aqueous streams have started to mix, the competitive precipitation of the polymer and the other solutes will proceed based on the supersaturation of the organic species. Depending on the relative difference between the time scales for polymer assembly or aggregation, $\tau_{\text{agg}}$, and the nucleation and growth of the solutes, $\tau_{\text{ng}}$, the particle size can be modulated [36, 41]. This allows for facile control of particle size.
Johnson, Liu, and Saad conducted much of the characterization with respect to the actual precipitation process of FNP through model experiments using "Bourne" reactions and β-carotene-loaded nanoparticles stabilized by various amphiphilic block copolymers [37, 41-43]. Effects of mixer types, mixing conditions, and polymer aggregation kinetics were the main focus. Similarly, Zhu et al extended this characterization for β-carotene nanoparticles using polyelectrolytes as the stabilizing polymer [44]. While some basic understanding has been developed for these model systems, this preliminary approach has some limitations. For example, in selecting β-carotene as the model API, which is very hydrophobic (ALOGPs: 9.72; refer to Appendix A for details on ALOGPs), the findings might not be easily translatable to more therapeutically relevant APIs with relatively lower logP values. Furthermore, in using an API that has no ionizable groups, the model system has not considered that developmental APIs usually have acidic, basic, or zwitterionic moieties; such solutes generally require excipients to obtain stable nanoparticles and thus the precipitation process might be different in comparison to the model system.

Most of the early work was done as proof of concept studies, with later generations of studies focusing on trying to bridge the technological and scientific aspects of FNP with pharmaceutical applications. Liu, Saad, and Pustulka discovered that for therapeutic APIs rifampicin and paclitaxel, with ALOGPs values of 3.81 and 3.20 respectively, FNP with only an amphiphilic block copolymer did not yield stable nanoparticles, since the API would quickly recrystallize and precipitate out of the nanoparticles [39, 42, 45] through Ostwald ripening [46]. Their solution to this problem was to conjugate cleavable hydrophobic moieties onto the parent APIs in order to increase the logP and thus decrease the relatively high solubility that led to Ostwald ripening. The same approach was used by Gindy to produce nanoparticles loaded with
lysozyme conjugates, since FNP with pure lysozyme and a block copolymer resulted in micron-sized particles [47]. While this approach was successful in vivo for nanoparticles loaded with paclitaxel conjugates, resulting in enhanced therapeutic activity compared to commercial formulations [48], the concept of API conjugation has limitations because the conjugate constitutes a new chemical entity requiring FDA approval.

Subsequent work shifted to other techniques for formulating APIs of low logP values into nanoparticle form using FNP without chemical modification. Abkulut and Kumar introduced the concept of using a hydrophobic excipient as a core "co-solute" that could serve to stabilize the API or interest [49, 50]; although their efforts were for producing fluorophore-loaded nanoparticles, the technique was used by D’Addio in the formulation of SQ-641 [51], a tuberculosis developmental compound with a ALOGPs value of 1.97. Using both α-tocopherol and cyclosporin A, D’Addio succeeded in formulating the API into a nanoparticle dispersion with comparable therapeutic activity to that of the first-line medication for tuberculosis treatment. In attempting a different formulation technique, Pinkerton reported the formation of ion pairs as a technique for the loading of ionic APIs into nanoparticle form; using hydrophobic pairing excipients, cinnarizine, clozapine, and α-lipoic acid were successfully formulated into nanoparticle dispersions [52].

However, most of this work has only produced liquid dispersions as the final dosage form. Considering that the shelf-life of dispersions is appreciably enhanced when converted to a dry solid form [53], it is desirable to convert liquid dispersions into a stable dried state. Only limited work has been reported with respect to this aspect of nano-formulations produced using FNP. For parenteral formulations, only freeze-drying has been considered. Saad freeze-dried nanoparticles loaded with paclitaxel conjugate but had 30% loss of the API and some particle
size growth; also, no optimization or a systematic study of the freeze-drying conditions were reported [42]. D'Addio and co-workers presented results for the freeze-drying of β-carotene nanoparticles and showed acceptable redispersibility only at sucrose concentrations that would be unacceptable for intravenous administration [54]. For inhalable formulations, Liu spray dried rifampicin conjugate nanoparticles and showed excellent reconstitution for the dry powders [39]. D'Addio compared spray drying and spray freeze drying of model cholesterol nanoparticles [55]. For oral formulations, Liu and co-workers produced spray dried powders containing nanoparticles loaded with SR13668, an anti-cancer agent, which have performed better than a commercial formulation [56, 57]. With the exception of the SR13668 case, none of these studies have focused on producing dry dosage forms that are feasible for therapeutic administration.

Overall, from the pertinent research concerning the use of FNP for development of relevant APIs, there is a lack of examples that focus on methodology for practical formulation of an API from neat drug substance to liquid nanoparticle dispersion to dry dosage form. As discussed next, this dissertation aims to provide a basis for rational formulation platforms that feasibly adapt FNP into the industrial development of APIs that are challenging to formulate with traditional methods.

1.3 OBJECTIVES FOR DISSERTATION

This dissertation attempts to address the need for better integration of the FNP process in formulation development of therapeutic APIs. First, in Chapter 2 a review of the fundamental understanding of FNP is presented, detailing the effects of various parameters in the process on particle size distributions and overall techniques for producing stable nanoparticle dispersions.
Furthermore, the review attempts to tie together results from previous publications into a unified understanding, where previously there have been contradictory explanations and theory.

Next, three cases studies are given in which the scientific and engineering principles presented in Chapter 2 are used for systematic evaluations of various formulation and process parameters to determine optimal formulations of APIs with low logP values. The first two are targeted for parenteral administration. In Chapter 3, progesterone was formulated into a lyophilized powder that could be reconstituted to ~300 nm nanoparticles with 25 wt% API loading and could be administered at API concentrations of ~30 mg/mL; in vivo parenteral administration showed quick peak plasma concentrations, which is desirable for emergency traumatic brain injury. Chapter 4 presents a model study for an ionizable API, GW771806X, in which the ion pairing technique was used to produce lyophilized formulations that could be reconstituted to ~170 nm with 33 wt% API loading and at API concentrations of ~8 mg/mL. Next, since most FNP work has been centered on parenteral administration, which is less attractive from the patient compliance perspective than oral formulations, Chapter 5 focuses on a general oral formulation for the model APIs indomethacin, fenofibrate, and cinnarizine. Using one base formulation, liquid dispersions with average particles sizes < 200 nm, API loadings of ~10 wt%, and API concentrations of ~3 mg/mL were produced, which could be used for subsequent conversion to dry powders. These case studies highlight the importance of systematic evaluation of various parameters to obtain optimal formulations, which has not been normally done in past FNP publications.

Furthermore, to this day, no fundamental studies have been reported in which systematic evaluations of process parameters have been conducted to better understand the phenomena of drying FNP nanoparticles. While Chapters 2 - 4 discuss the use of freeze-drying to obtain dried
nanoparticles, the emphasis is not on understanding the freeze-drying process, which mirrors the treatment of freeze-drying in previous FNP publications. Thus, in the final chapter, investigations into the process of freeze-drying are presented, elucidating on the phenomena that govern conversion liquid dispersions to dry powders. In Chapter 6, the freeze-drying of PEG-stabilized nanoparticles produced through FNP is the focus. The importance of block copolymers, core physical states, concentrations, protectant excipients, and process parameters demonstrate the superiority of PEG-based excipients for parenteral lyophilized formulations. Through these findings, a basic understanding of the phenomena occurring during freeze-drying can be developed such that future freeze-drying endeavors can be begun from a rational starting point.

Clearly, there is a need for FNP to make further inroads into the pharmaceutical culture. The focus on producing pharmaceutically relevant final dosage forms has not been central to previous studies on FNP. By describing scientifically-guided systematic FNP formulation optimization and drying methods, as well as the introduction of FNP into oral applications, this work provides a starting point for these necessary steps. It is hoped that this dissertation can serve to continue to improve FNP developmental techniques.

1.4 NOMENCLATURE

API active pharmaceutical ingredient
FNP Flash NanoPrecipitation
\( m \) mass of a particle at time
\( t \) time
\( r \) radius of a spherical particle
\( D \) diffusion coefficient
$c_r$  solubility of a spherical particle of radius $r$,

$h$  thickness of boundary layer for diffusion

$a, b$  constants

$U$  fluid velocity,

$\nu$  fluid kinematic viscosity

$c_{eq}$  saturated equilibrium solubility

$\gamma$  interfacial tension between a solute and fluid

$\nu$  API molar volume

$R$  universal gas constant

$T$  absolute temperature

$n_p$  number of spherical particles of radius $r$

$m_{tot}$  total mass dose of an API

$\rho$  material density

$\tau_i$  a characteristic time scale

1.5 REFERENCES


42. Saad, W., Drug nanoparticle formation via flash nanoprecipitation: conjugation to encapsulate and control the release of paclitaxel, in Chemical Engineering2007, Princeton University.
CHAPTER 2 - FLASH NANOPRECIPITATION: FUNDAMENTALS & APPLICATIONS

ABSTRACT

With the advent of nanoparticles in biotechnological and pharmaceutical research, many production methods for synthesizing nanoparticulates have been reported. Here, we focus on Flash NanoPrecipitation, which is a rapid precipitation, or bottom-up, approach that is typically used for the production of nanoparticles loaded with water-insoluble solutes and/or colloids. Polymer stabilizers are used to stabilize the particles and arrest growth during precipitation. The method boasts scalable production of tight particle size distributions with easily tunable average particle sizes from 50 - 500 nm and generally high loading efficiency of solutes. In this review, we cover the current fundamental understanding of polymer-stabilized rapid precipitation process (and specifically FNP), beginning with solute nucleation and growth, followed by the importance of homogeneous mixing, and finally polymer assembly and effects on particle formation. Other topics covered include current applications for FNP, existing FNP formulation techniques for producing nanoparticle dispersions, as well as how to improve stability of these dispersion. This treatment is meant as a compilation of the existing literature on FNP and related research on nucleation, assembly and polymer adsorption to provide a unified understanding of this class of nanoparticle formation process.

2.1 MOTIVATION

In the past decades, there has been growing interest in nanotechnology for various applications, as evidenced by a huge surge in publications on the area, growing by over 200%
from 2000 to 2006 in the United States alone [1]. The biotechnology and pharmaceutical fields have been no exception, where the introduction of nanoparticles has led to the marketing of novel therapeutic and diagnostic products [2]. Nanoparticles offer many advantages over other formulation techniques for drug delivery and bio-imaging applications. First, for hydrophobic compounds, because of the nano-sized dimensions these constructs provide, fast dissolution rates and enhanced bioavailability can be achieved [3, 4]. While the use of other formulation methods, such as those discussed in Chapter 1, are also capable of improving bioavailability of water-insoluble therapeutics, they are typically only useful for oral administration of drugs. However, when using nanoparticulate formulations, other administration routes can benefit from the improved bioavailability and tap into other advantages. For example, since many types of nano-constructs load the active pharmaceutical ingredient (API) into an internal domain of the particle, the compound is protected from rapid metabolism and clearance [5], potentially increasing the residence time of the drug in vivo. A common strategy for nanoparticle design is to have a stealth stabilizing layer on the surface, with a structured core in which the API is contained; usually, poly(ethylene glycol) (PEG) is used as a stealth material for nano-surfaces because of its ability to prevent protein adsorption to the particles which can induce bodily clearance [6-8]. In fact, dense PEG coatings on nanoparticles allow them to be able to penetrate through mucus membranes without appreciably associating with mucus networks [9-11], as well as extend circulation times in the bloodstream [12, 13]. Furthermore, these constructs are amenable to the introduction of ligands on the surface, such that a targeting modality can be included in the formulation. Such a possibility opens doors for improved safety profiles, increased maximum tolerated doses, and more efficient treatments. While the last two properties are not exclusive to nanoparticles, since microparticles and conjugated APIs can also have PEG stabilization and
attached ligands, the intermediate size of nanoparticles is a great compromise for colloidal stability, since microparticles are more likely to sediment or phase separate, and improved protection, since molecular conjugation does not sequester an API within a separate phase. Moreover, for intravenous cancer treatments, the nano-sized dimensions of nano-constructs is perfectly matched for taking advantage of passive targeting. Due to leaky vasculature from angiogenesis, tumors can have a porous vasculature into which particulates with characteristic sizes of roughly 100 nm can accumulate [14-16]; this effect allows for passive targeting, termed enhanced permeability and retention, that can be used for engineering nanoparticles [17, 18]. While nano-constructs for bio-imaging applications do not necessarily require high bioavailability, the capability for inclusion of imaging modalities into pharmaceutical nanoparticles is attractive for better understanding the fate and activity of such formulations.

Clearly, there is potential for nanoparticles to play a significant role in next-generation treatments and diagnostics. However, for this to become reality, there is a need for economical production methods that reproducibly yield dispersions with the small sizes and tight distributions required to take advantage of the potential of nano-formulations. While in the pharmaceutical industry the use of top-down methods, in which bulk drug substances are reduced in size through techniques such as wet media milling, is becoming commonplace, bottom-up methods, such as precipitation-based techniques, are less well-established. Here, we present fundamentals and applications of rapid precipitation techniques, specifically Flash NanoPrecipitation (FNP), which have the potential to fill the need for a cost-effective nanoparticle production method. In the next sections, we will discuss the fundamental process of the technique and highlight its simplicity, scalability, and its excellent control over particle size distributions. FNP is an attractive method not only from a laboratory perspective, but also
industrially, because it is a robust process and can reproducibly produce nanoparticles of tight size distributions, which can be critical for various applications.

2.2 PRECIPITATION KINETICS

Various bottom-up methods exist, of which many are ultimately controlled by thermodynamics, as opposed to kinetics, because of their slow characteristic time scales. Although labeled under many different names, most of these methods involve some emulsification process of an organic phase, many times rich in a hydrophobic polymer or oil and contain a smaller amount of a solute, within a surfactant-laden aqueous continuous phase [19]. While emulsification may not be thermodynamically-limited due to rapid time scales, the removal of the organic solvent, through evaporation or extraction, is relatively slow. A key limitation of these characteristic methods is the relatively low solute loadings due to thermodynamic equilibrium, or saturation solubility, between the solute and the nano-construct. The concentration of solute achieved in such a formulation is determined by the equilibrium solubility of the solute in the core phase of the polymer/surfactant construct. Phase-separated solute is usually ejected from the final particles and is reported as "unincorporated" solute. This has been analyzed and the details for the equilibria involved have been presented by Kumar and Prud'homme [20]. In contrast, when using a rapid precipitation process, the precipitated solute can be captured by a steric stabilizing polymer in a kinetically trapped state, which enables higher API loadings. The fast time scales in the FNP process are crucial to this kinetic trapping, which enable high solute loadings. In developing the understanding for this, we first discuss some of the fundamentals of these precipitations, beginning with nucleation and growth processes.
By definition, precipitation is the formation of a phase induced by supersaturation, or solute concentrations reaching levels higher than the saturation solubility. This can be achieved through various means, such as solvent quality, temperature, and/or pH change. We consider classical crystallization theory in describing the precipitation process [21]. As the solute experiences supersaturation, molecular clusters will begin to form, possibly becoming nuclei on which more solute molecules can deposit and grow. Depending on the size of these clusters, the excess free energy between the solid particle and the solution may be such that the cluster re-dissolves if below a critical size or continues to grow if larger than this threshold size. The critical nucleus radius defines the required critical excess Gibbs free energy to form stable nuclei. A nucleation rate can be expressed in an Arrhenius form that depends on the critical excess Gibbs free energy as an activation energy, as shown below where $J$ is the nucleation rate, $A$ is a pre-exponential factor, $\Delta G$ is the critical excess Gibbs free energy, $k_B$ is the Boltzmann constant, and $T$ is absolute temperature.

$$J = A \exp\left(\frac{-\Delta G}{k_B T}\right)$$  \hspace{1cm} (2.1)

Through solving for the critical nucleus radius and expressing it in terms of experimental parameters, the following expression can be derived. The full derivation can be found elsewhere [21].

$$J = A_{homo} \exp\left[-\frac{16 \pi \gamma_{cl}^3 v_0^2}{3 k_B^3 T^3 (\ln S)^2}\right]$$  \hspace{1cm} (2.2)

Here, $A_{homo}$ is a pre-exponential factor which is a function of the precipitation conditions, $\gamma_{cl}$ is the interfacial tension between the crystallizing solute and the liquid, $v_0$ is the solute molar volume, and $S$ is the supersaturation ratio. The supersaturation ratio is a function of time as
shown below, since the solute concentration that has not precipitated, \( c \), will drop as the precipitation continues.

\[
S \equiv \frac{c(t)}{c_{eq}}
\]  \hspace{1cm} (2.3)

Here, \( c_{eq} \) is the saturated equilibrium solubility of the solute. When substituting the pre-exponential factor and an expression for the interfacial tension as a function of other solute properties that are easier to measure, the complete nucleation rate expression becomes as follows, where \( D_{AB} \) is the diffusion coefficient of solute A in solvent B and \( \beta \) is a numerical factor that depends on the shape of nuclei [22].

\[
J = \frac{3}{2} D_{AB} \left( v_0 c^3 \right)^{1/3} \sqrt{-\beta \ln \left( v_0 c_{eq} \right)} \exp \left\{ \frac{16\pi \left[ \beta \ln \left( v_0 c_{eq} \right) \right]^3}{3 \ln^2 S} \right\}
\]  \hspace{1cm} (2.4)

However, equation 2.2 is valid for homogeneous nucleation events, where foreign surfaces or other substances do not contribute to the nucleation. To account for heterogeneous nucleation, the activation energy in equation 2.1 is modified as follows, where \( \theta \) represents the contact angle between the crystallizing solute and foreign substance [23]. It is important to note that the pre-exponential factor for heterogeneous nucleation, \( A_{hetero} \), is in fact different than that for homogeneous nucleation, \( A_{homo} \), and can be many orders of magnitude lower [24, 25].

\[
J = A_{hetero} \exp \left\{ -\frac{\Delta G}{k_B T} \left[ \frac{(2 + \cos \theta)(1 - \cos \theta)^2}{4} \right] \right\}
\]  \hspace{1cm} (2.5)

The contact angle may be expressed in terms of interfacial tensions between phases, as follows, where the subscripts \( c, l \), and \( s \) denote crystallizing solute, liquid, and foreign solid surface.

\[
\cos \theta = \frac{\gamma_{cl} - \gamma_{cs}}{\gamma_{cl}}
\]  \hspace{1cm} (2.6)
The additional coefficient in the activation energy term delineates three possible situations. In the case of a $0^\circ$ contact angle, there is no energy barrier to nucleation, which is the case when the foreign surfaces are crystal seeds already present in the supersaturated solution, such that growth can occur immediately. When the contact angle is $180^\circ$, the addition of a foreign substance in the precipitation medium has made no change to the activation energy of the crystallizing solute; this means that there is no affinity between the substance and the solute, such that pure crystals will form. Lastly, there is the intermediate case, where there is some affinity between the crystallizing solute and the other substance; the presence of the foreign substance will help to reduce the energy barrier for nucleation of the solute, thereby facilitating earlier nucleation. However, the actual rate will still be lower than that of homogeneous nucleation, as pointed out earlier.

Nucleation will continue as long as stable nuclei can form with a radius larger than the critical nucleus radius; this is dependent on how long it will take for crystal growth from existing nuclei to deplete the supersaturated solution. Growth of nuclei is generally considered a two-step process, whereby the solute first needs to be transported to the nuclei surface either through diffusive or convective processes. Next, the individual solute molecules must displace solvent molecules and integrate into the crystal surface. As Söhnel and Garside point out, it is likely that crystal growth be made up of a series of different growth mechanisms or various mechanisms in parallel, making growth a difficult step to model [23]. Although there are many different models attempting to explain the various growth mechanisms [21], we consider the case where the integration step is relatively fast in comparison to the transport step, thereby being a diffusion-limited model; this is characteristic of precipitation at high supersaturation ratios [26]. The details of the derivation can be found elsewhere [23], but the main growth expression is shown
below, where $G$ is the growth rate, $N_{Av}$ is Avogadro's number, and $r$ is the radius of a spherical particle.

$$G = 2v_0N_{Av}c_{eq} \left( \frac{D_{AB}}{r} \right) (S - 1) = 2v_0N_{Av} \left( \frac{D_{AB}}{r} \right)(c - c_{eq}) \quad (2.7)$$

Note how the growth rate is linearly dependent on how far the system is from equilibrium (i.e. $S = 1$). The expression can also be expressed in terms of the concentration of supersaturated solute that has not yet precipitated, which has a $c^1$ dependence. In contrast, the homogeneous nucleation rate has a stronger dependence on the supersaturation ratio, which is present in the exponential argument, even though the pre-exponential factor has a stronger dependence on the solute concentration of $c^{7/3}$. However, it is very important to consider that nucleation and growth rates cannot be compared directly because they are inherently very different values, where the nucleation rate is a measurement of the number of crystal nuclei created per unit volume and time and the growth rate is a measurement of the linear velocity at which nuclei expand perpendicularly outward from a crystal face.

While it is rare to find cases of precipitations conducted at high supersaturation ratios in the literature, the work of Mahajan and Kirwan is one of the few reports with supersaturation ratios above 2. They investigated the precipitation kinetics of two model compounds, L-asparagine and lovastatin, through supersaturation ratio ranges of 1.17 - 4.1 and 1.25 - 8.8, respectively, and determined that the measured experimental nucleation rates fit the classical expressions well [27]. The experimental and fitted results are shown in Figure 2.1. In fact, they observed a transition from heterogeneous to homogeneous nucleation at a crossover point when increasing the supersaturation ratio from low to high values.
Figure 2.1 Induction times and nucleation kinetics for two model solutes: a) asparagine at 23°C, 50 vol% 2-propanol; b) lovastatin at 23°C, 60 vol% methanol. The abscissa is proportional to the exponential argument in equation 2.2, where larger values indicate lower supersaturation ratios. A crossover from heterogeneous to homogeneous nucleation is observed for both systems. Taken from reference [27].

Through independent measurements, they also determined the induction times, or the characteristic time for a solid to form and reach a detectable size (2 µm), for the same conditions; the same crossover behavior was observed as with the nucleation data. The data showed much faster, or smaller, inductions times for homogeneous, as opposed to heterogeneous, nucleation. This means that at higher supersaturation ratios, while nucleation rates will be drastically higher, growth rates will also be faster, such that larger particle sizes will be achieved per unit time as compared to low supersaturation ratios. Mahajan and Kirwan were able to model growth rates as a function of the solute's chemical potential driving force in the following form, where $B$ is a constant, $R$ is the universal gas constant, and $x$ is a growth kinetic order determined through experimental fit.

$$G = B \left( RT \ln S \right)^x$$  \hspace{1cm} (2.8)

This growth rate expression was valid for the entire supersaturation ratio ranges investigated. Through use of the Nielsen surface nucleation growth model, an excellent fit was also achieved for both model systems. This model explains growth as the formation of surface layers by the
integration of solute molecules at kink sites where the maximum number of contacts on the crystal can be achieved instead of depositing on an edge of a growing face [28]. For this model, diffusion is not the limiting factor, but rather solute integration into the growing nuclei, which is reasonable for the relatively low supersaturation ratios used in the study. It is also important to note that while a supersaturation ratio is used in the expressions presented, this is used to describe a bulk parameter and not the actual supersaturation which is function of time. Therefore, only an average growth rate was measured and fit to the models.

From this cursory overview, it becomes clear that there are various important considerations for particle sizes when using a precipitation-based nanoparticle production method. First, both nucleation and growth rates can be strong functions of supersaturation that increase drastically as concentration is increased. For the purposes of producing small particles, it is important that nucleation dominate over growth. Since the two competing rates cannot be compared directly, it is important to find appropriate experimental conditions for the system under investigation. In the cases of poor nucleation due to high saturation solubility, techniques involving heterogeneous nucleation pathways might be useful and will be discussed in more detail in section 2.8. Furthermore, in achieving efficient precipitation processes, the supersaturation ratio must be high enough that the fraction of solute that remains in solution (i.e. a saturated solution) is minimal from a yield or recovery perspective. Finally, precipitation kinetics are entirely dependent on the spatial conditions present; as described later, in order to obtain tight particle size distributions, it is important that nucleation and growth kinetics be uniform throughout the mixing vessel being used.
2.3 FLASH NANOPRECIPITATION

Precipitation processes have traditionally been carried out in batch-wise operation modes, where a feed stream with the dissolved solute is injected into a stirred tank, resulting in a product of considerable polydispersity due to relatively poor mixing [29], since the feed must be mechanically dispersed through the mixing volume. In contrast to these traditional methods, nanoprecipitation techniques have attempted to bypass this problem by incorporating in a mixing tee where the feed stream with the solute comes into contact with the anti-solvent [30]; this provides better mixing of the two streams, since all material must pass through the tee mixing point. However, there are still limitations to this, such as the requirement of equal momenta of the two streams at the mixing point, generally resulting in a dilution by half of the feed solute stream; when considering an organic feed stream consisting of a water-miscible solvent and an aqueous anti-solvent, the effluent will then consist of 50 vol% organic solvent, which might yield low supersaturation ratios and compromise particle stability [31]. Furthermore, in comparison to confined impinging jet mixers, which are similar in concept, tee mixing requires very high pressure drops to achieve efficient mixing because of the lack of an ample mixing chamber where the kinetic energy of the two incoming jets can dissipate and yield turbulence [26].

Flash NanoPrecipitation overcomes the challenges of poor mixing and low supersaturation ratios. In typical FNP production, a water miscible-organic solvent is used to prepare a solution consisting of a hydrophobic solute and a stabilizing amphiphilic material, as well as possible excipients; this solution is then micro-mixed against anti-solvent in carefully designed mixing geometries. Through the use of a multi-inlet vortex mixer (MIVM) [32], up to four separate streams can be mixed at desired flow rates resulting in an effluent with a tunable amount of organic solvent; furthermore, all streams must pass through a mixing chamber where a
great degree of mixedness can be achieved continuously. Furthermore, since the same mixer can be used from the laboratory scale up to production plant scale, FNP boasts robust scalability. The process is quite versatile in that it allows for the incorporation of various actives, including small hydrophobic APIs [33-41], fluorescent imaging agents [42-44], peptides and proteins [45, 46], small inorganic nano-structures [46], and large inorganic colloids [47, 48]. Furthermore, simultaneous loading of more than one active has been demonstrated [34, 46, 48, 49], allowing for multifunctional particles and/or delivery of drug cocktails.

The introduction of a stabilizing amphiphilic material is crucial in rapid precipitation techniques for producing nanoparticles, since the adsorption of the polymer on the growing nuclei will arrest growth, preventing macroscopic crystallization, and sterically prevent particle aggregation. Typically, amphiphilic di-block copolymers have been used for this purpose in FNP [50, 51]. If there is some affinity between the hydrophobic block and the solute, the polymer can attach to small nuclei such that the final particle consists of several polymer/nuclei aggregates [37]; otherwise, if there is little or no affinity, the polymer chains will preferentially assemble into micelles, although some chains will aggregate and adsorb in patches on the surfaces of large nuclei and sterically prevent any further growth. Simulations have shown that once the di-block copolymers have assembled into these nanoparticulate structures, they are kinetically frozen; that is, the energy barrier for dynamic exchange of the individual polymer chains between the solution and the particle surface is too great for it to occur [52]. Since it is the polymer adsorption onto growing nuclei that will arrest further growth, the resulting nanoparticles are not at thermodynamic equilibrium and much higher solute loadings can be achieved than the example mentioned at the beginning of section 2.2.
In the FNP process, the time scales involved in the various steps must be considered, as depicted in Figure 2.2. Most importantly, the characteristic mixing time, $\tau_{\text{mix}}$, must be faster than the induction, or formation time, of a nanoparticle, $\tau_{\text{flash}}$, in order to ensure homogeneous precipitation kinetics [32, 53]. The induction time of a nanoparticle can be considered as an overlay of two competing reactions, where there is a characteristic aggregation time of the amphiphilic polymer, $\tau_{\text{agg}}$, and the nucleation and growth times of the solute, $\tau_{\text{ng}}$ [53]. In comparing the two precipitation time scales, there are three cases. In the extreme where $\tau_{\text{agg}} > \tau_{\text{ng}}$, the nucleation and growth kinetics of the solute are faster than that of the polymer, allowing for particles to reach large sizes before growth is arrested by the polymer. When $\tau_{\text{agg}} < \tau_{\text{ng}}$, the polymer aggregation is faster than solute particle formation; this could result in a large polymer micelle population with small or no solute loading and a significant loss of precipitated solute.

**Figure 2.2** A schematic highlighting the time scales involved in the Flash NanoPrecipitation of a simple two-component system. For homogeneous precipitation to occur, and to achieve the least polydispersity, the mixing time scale, $\tau_{\text{mix}}$, must be faster than that of the rapid precipitation, $\tau_{\text{flash}}$. Proper balancing of the two competing precipitations, solute and polymer, is necessary to achieve a monomodal size distribution. Taken from reference [53].
Finally, when the two time scales are similar, it is more likely to form solute-loaded particles of an intermediate size, where size can be modulated by the absolute values of the time scales. Therefore, in a well mixed process, FNP allows particle size distributions to be easily tuned by adjusting the precipitation time scales of the particle components. In the following sections, we describe various process and formulation parameters that are critical to the production of nanoparticle dispersions via FNP.

2.4 MIXING CONDITIONS

2.4.1 Mixer types

FNP can be carried out in two general modes, each utilizing a different type of mixer. When first reported, FNP made use of confined impinging jets where one water-miscible organic stream, containing the solute and amphiphilic polymer, and an anti-solvent stream collide, forming an impingement plane of high turbulent energy dissipation [53]. When the two jet streams collide, the kinetic energy of each stream is converted into turbulent motion that redirects the flow; this energy dissipation results in excellent micro-mixing, with rapid mixing times on the order of microseconds [54]. The properly designed confined impinging jets mixer (CIJM) allows for this turbulent mixing to proceed without significant amounts of fluid bypassing the region of high mixing intensity, as shown in Figure 2.3a. The fast mixing time scales allow for precipitation kinetics to not be transport-limited. However, one of the main shortcomings in this operation mode is that due to the necessity of the two feed streams having equal momenta to result in the high energy dissipation, low supersaturation ratios may be obtained in the 50/50 solvent/anti-solvent mixture.
The 50/50 mixture has two disadvantages. First, the low supersaturation ratio will decrease the maximum achievable loading efficiency. Also, the higher solute solubility in the 50/50 mixture will increase the Ostwald ripening rate, resulting in particle size growth, for particles left in this mixed solvent (see section 2.9.1). To mitigate these problems, the effluent from the CIJM can be directed into a larger volume anti-solvent to further decrease solute solubility. The original CIJM was driven by computer-controlled syringe pumps; this enabled precise control of mixing conditions to develop the fundamental mixing rules and study scale-up [54]. Han et al [55] used the same CIJ mixing head geometry, but used hand-driven disposable syringes to enable nanoparticle formation using very small amounts of sample. The confined impinging jets mixer with dilution (CIJM-D) is shown in Figure 2.3b with the effluent directed into a reservoir containing anti-solvent to produce lower solvent/anti-solvent ratios, such as 10/90. This advance greatly facilitates lab-scale screening studies where many formulations can be quickly evaluated.

Following the use of the CIJM, the MIVM was introduced to FNP. While the CIJM was designed as a cylindrical vessel with two carefully placed inlets and an outlet, the MIVM design involves four inlets that are directed perpendicularly from individual chambers into a cylindrical central mixing chamber leading to the exit [56] (Figure 2.3c). Likewise, while the CIJM had the highest energy intensity at the central impingement plane, the MIVM experiences the highest energy intensity at the exit [32, 57]. Because the momentum from each stream independently contributes to the micro-mixing that is experienced in the mixing chamber, it is possible to achieve the required micro-mixing time scale while operating the inlet streams at different volumetric flow rates. The MIVM also allows for the introduction of the various nanoparticle components in separate streams if they cannot be incorporated into one stream because of
solubility issues, or a desire to keep components separate rather than premixing all of the hydrophobic species.

**Figure 2.3** Mixers used in Flash NanoPrecipitation. a) confined impinging jets mixer, b) confined impinging jets mixer with dilution (lab-scale shown), c) multi-inlet vortex mixer. Taken from references [54], [55], [56], respectively.

### 2.4.2 Mixing characterization

Various aspects of the mixing conditions achieved through the CIJM and MIVM have been characterized by comparing experimental competitive reaction results to computational
fluid dynamics (CFD). Johnson [54] and Liu [32, 57] made use of competing Bourne reactions, in which a fast and slow reaction both involve a limiting reagent, HCl.

fast reaction: $\text{OH}^- + \text{H}^+ \xrightleftharpoons[k_1]{k_2} \text{H}_2\text{O}$
slow reaction: $\text{CH}_3\text{C(OCH}_3)_2\text{CH}_3 + \text{H}^+ + \text{H}_2\text{O} \xrightleftharpoons[k_2]{k_1} \text{CH}_3\text{COCH}_3 + 2\text{CH}_3\text{OH} + \text{H}^+$

The fast reaction is orders of magnitude faster than the slow reaction ($k_1 \gg k_2$); also the slow reaction is catalytic, meaning that it will not consume the limiting reagent. When conducted under conditions where the characteristic mixing time is faster than the reaction time of the fast reaction, homogeneous kinetics will be experienced, resulting in minimal conversion for the slow reaction; however, when the reaction time is comparable or faster than the mixing time, local depletion of the hydroxide ion will allow for the acid to proceed along the slow reaction pathway, resulting in detectable amounts of methanol. Therefore, by measuring the conversion of 2,2-dimethoxypropane (DMP) to methanol, the extent of mixing can be determined. Johnson showed that for the CIJM, the onset of turbulent-like flow occurred at a jet Reynolds number ($Re_{jet}$) of ~90, with increasing jet Reynolds number achieving less DMP conversion. Micro-mixing times of < 9.5 ms were measured. For the MIVM, Liu showed that for inlet total Reynolds numbers ($Re_{tot}$) > 1600, DMP conversion was minimized and did not practically vary with total Reynolds number for reactions with reaction times > 50 ms. While the two analyses were done using different Reynolds number definitions (CIJM: $Re_{jet}$ = Reynolds number of one inlet stream; MIVM: $Re_{tot}$ = sum of Reynolds numbers of all inlet streams), both results show the same dependence of DMP conversion on mixing efficiency, where conversion dropped as flow became more turbulent. Johnson also used the ethylchloroacetate competitive reactions and obtained the same mixing kinetics as with the DMP reactions [54].

Through the use of computational fluid dynamics, Liu and Fox compared simulations to experiment for both mixer types. For the CIJM, there was good agreement between experimental
and simulated DMP conversion for $200 \leq Re_{jet} \leq 4000$ and for reaction times $> 9.5$ ms as shown in Figure 2.4a, since the simulation model used was for turbulent flow [57]. Micro-particle image velocimetry revealed that for $Re_{jet} > 300$ and especially at $Re_{jet} > 600$, chaotic behavior was observed for the flow field inside the CIJM, such that many small eddies interacted with the incoming streams, rather than a well defined impingement plane [58]. The CFD simulations, micro-particle image velocimetry measurements, and competitive reaction measurements all indicated rapid micro-mixing for $Re_{jet} > \sim 200 - 300$. The Bourne reaction data used to compare experiment to CFD essentially showed that while excellent micro-mixing is achieved beyond $Re_{jet} > 200$, the DMP conversion continued to decrease with an increasing Reynolds number as the macro-mixing improved and the flow approached full turbulence [54]. For the MIVM, there was generally satisfactory agreement between CFD and experiment for the range of total Reynolds numbers studied, which was $800 \leq Re_{tot} \leq 5000$ (Figure 2.4b). The decrease in DMP conversion as the total Reynolds number increased was captured well. Other CFD work has confirmed the formation of turbulent flow in this total Reynolds number regime as well [59, 60].

![Figure 2.4 Comparison of Bourne reactions conducted experimentally and through simulations on a) CIJM, b) MIVM. The plots depict DMP conversion versus Reynolds number. In a), the reaction time scale was varied by changing inlet concentrations of the components. In b) the two cases considered correspond to different configurations of the inlet streams. Taken from references [57] and [32], respectively.](image)
However, various studies, including simulations using coupled CFD and bi-variate population balance equations for FNP of a hydrophobic solute and an amphiphilic block copolymer, have shown an interesting behavior in the MIVM. While turbulent flow can be achieved at high Reynolds numbers, complete mixing is not observed at the mixing chamber outlet [59, 61], which has also not been observed for the CIJM [62]; this suggests that complete mixing can only be achieved until exiting the mixer. Likewise, while fast micro-mixing times can be achieved, various aggregation zones will form in the mixing chamber where different solvent qualities exist and different kinetics will be experienced. The mixing intensity is always greatest at the center of the mixing chamber; this is where the characteristic macro-mixing time, $\tau_{mac}$, is the fastest. Various bands surround the center with decreased mixing intensity, resulting in slower macro-mixing times and non-uniform solvent quality. Although the degree of macro-mixing of the aggregation zones can be improved at higher Reynolds numbers, the change is quite minimal [59], as shown in Figure 2.5. These results suggest that while high Reynolds numbers can reduce particle size, such that micro-mixing times can become comparable or faster to aggregation time scales, limitations with macro-mixing will always contribute to greater polydispersity in the nanoparticle sizes.

2.4.3 Examples in nanoparticle production

In practical terms, the descriptions above have shown that in order to obtain characteristic time scales that will result in "homogeneous" precipitation kinetics, it is important for FNP to be done at fast flow rates, regardless of the mixer type used. The effect of Reynolds number on particle size has been studied using a CIJM-D, where β-carotene nanoparticles stabilized by poly(D,L-lactide)-block- poly(ethylene glycol) (PLA-b-PEG) (MW: 10kD-b-5kD) were produced by Han and co-workers [55]. As a nomenclature note, XXXYkD-b-PEGZkD will
represent a block copolymer with a block XXX of molecular weight YkD and a PEG block of ZkD. They noticed that similar particle sizes were produced on the CIJM-D as on an MIVM under the same total Reynolds numbers. For both devices, particle sizes decreased with increasing total Reynolds numbers until reaching a critical value of ~2000.

**Figure 2.5** Kinetic modeling of nanoprecipitation using computational fluid dynamics coupled with a population balance for a hydrophobic solute and amphiphilic block copolymer in a multi-inlet vortex mixer. Characteristic micro-mixing times and macro-mixing times are displayed on the top and bottom, respectively, for a jet Reynolds number of 240 (left) and 475 (right). While almost doubling the jet Reynolds number results in increased homogeneity for micro-mixing, it has little impact on macro-mixing. Taken from reference [59].

This behavior has also been verified on the MIVM through various examples. In producing β-carotene nanoparticles stabilized by polystyrene-\textit{block}-poly(ethylene glycol) (PS-\textit{b}-PEG) (MW: 1kD-\textit{b}-3kD) or poly(ε-caprolactone)-\textit{block}-poly(ethylene glycol) (PCL-\textit{b}-PEG) (MW: 3.6kD-\textit{b}-5kD), Shen and co-workers demonstrated a similar trend as that of the DMP conversion [63]. They showed that particle size decreased as the total Reynolds number increased until a critical total Reynolds number of ~2000, where particle size was no longer a
function of the mixing conditions. In producing poly(ethylene imine)/poly(acrylic acid)-block-poly(ethylene glycol) complexed nanoparticles, Shen also observed the same trend; however, in this case the particle size increased until reaching the critical total Reynolds number, since more electrolytic complex can form when mixing improves, increasing particle size. This demonstrates the impact of relative time scales, where the micro-mixing time scale required for homogeneous particle aggregation was not reached until mixing was turbulent enough. The value of the Reynolds number that must be achieved to make assembly times longer than micro-mixing times depends on the kinetics of the assembling system. Zhu and associates observed a critical total Reynolds number ~3000 when producing β-carotene nanoparticles without a stabilizer [64]. β-carotene is highly hydrophobic and without a stabilizer to slow assembly, this is expected to be a rapid assembly process. As a rule of thumb, for most nanoparticle systems with amphiphilic stabilizing polymers, a total Reynolds number of 2000 is high enough that assembly is no longer strongly sensitive to micro-mixing.

In a different study, solvent yellow 98 (SY98) particles stabilized by PLA3.7kD-b-PEG5kD (1:1 SY98:polymer by mass) were produced on an MIVM with varying fractions of organic solvent, tetrahydrofuran (THF), in the dispersion solvent (solvent/anti-solvent) mixture, while maintaining the solids concentration constant; the particle size, span range ($\Delta = d_{90} - d_{10}$) and loading efficiency of the solute are displayed in Figure 2.6. Refer to Appendix A for experimental protocol and Appendix B for commentary on how size distributions have been reported. The peak mean diameter data showed the same trend observed by Shen, where particle size decreased as the total Reynolds number increased up to $Re_{tot} > 2500$. The span range followed the same trend, indicating that less polydisperse particles, or tighter size distributions were achieved with improved macro-mixing. The loading efficiency, which is a measure of the
fraction incorporated into the nanoparticles, was near 100% for all cases when THF was present at 15 or 20 vol%; however, for 5 and 10 vol% THF, the maximum loading was only reached once the critical total Reynolds number was passed. The effect of the THF content will be further discussed in the next section, since it is a supersaturation issue.

![Graphs showing particle size distribution and loading efficiency](image)

**Figure 2.6** Flash NanoPrecipitation of solvent yellow 98 nanoparticles stabilized by PLA3.7kD-b-PEG5kD at a nominal solids concentration of 2 mg/mL with a 1:1 mass ratio of components. The results emphasize the dependence of particle size distribution with degree of mixedness, as represented by the total Reynolds number.
2.5 IMPACT OF SUPERSATURATION ON PARTICLE SIZE

In the production of nanoparticles through precipitation processes, the solubility of the solute in the dispersion solvent mixture, or effectively the supersaturation ratio, determines the upper limit of the loading efficiency of the solute. For example, if the solubility of an API in a 10 vol% THF/water mixture is 0.5 mg/mL and a THF stream containing 10 mg/mL API is mixed against water at a 1:9 THF:water ratio, the maximum achievable loading efficiency is 50%; in this case, the supersaturation ratio is only 2. The remaining 50% of the API will remain dissolved in the solvent phase. By the same reasoning, supersaturation ratios of 10 and 100 in the final solvent mixture are necessary for having maximum achievable loading efficiency of 90 and 99%, respectively. Of course, the supersaturation ratio also greatly influences the nucleation and growth rates of the components, thereby affecting particle size distributions as well. Therefore, it is imperative to carefully design the FNP process such that an appropriate supersaturation ratio is used to produce desired nanoparticle properties.

From the examples in the current literature, three different situations have been considered for FNP:

(I) an organic stream with a fixed solute concentration is mixed against water at different dilutions, resulting in decreasing solute concentrations in the final dispersion of variable composition

(II) an organic stream with a variable solute concentration is mixed against water at different dilutions, resulting in a constant solute concentration in the final dispersion of variable composition
(III) an organic stream with a variable solute concentration is mixed against water at a constant dilution, resulting in an increasing solute concentration in the final dispersion of constant composition.

We first consider the three situations and then discuss the importance of supersaturation in these practical examples.

The work of Shen et al [63], presented in section 2.4.3, and Han et al [55] fall under case I. Shen observed for β-carotene that decreasing the solute concentration in the final dispersion from 4.4 to 3.0 to 2.2 to 1.8 mg/mL while decreasing the THF fraction from 50 to 33 to 25 to 20 vol% resulted in a continual decrease in particle size at constant Reynolds numbers [63]. Han et al also reported a similar observation with the β-carotene concentrations in the final dispersion decreasing 3.3 to 2.0 to 0.9 to 0.5 mg/mL while decreasing the THF fraction from 33 to 20 to 9 to 5 vol% at constant Reynolds numbers [55]. Shen attributed the results to an increasing supersaturation ratio which increased the nucleation rate. However, even at 20% THF, the resulting nanoparticles were unstable, as they grew in size by 60% over the course of two weeks when not dialyzed [31]. It is expected that the samples produced with higher THF content would be more unstable.

In contrast to the aforementioned examples, two examples of case II are summarized below. In continuing the solvent yellow 98 example from section 2.4.3, we consider the effect of THF content in the dispersion solvent mixture on supersaturation. Due to its insolubility in water, it was not possible to determine the saturation solubility of SY98 in 0%, 5%, 10%, and 15% THF; however, its solubility is 0.1 µg/mL and 52 mg/mL at 20% and 100% THF, respectively. Therefore, while the supersaturation ratios cannot be determined for most of the cases presented, it is reasonable to claim that the supersaturation ratio increased as the THF content was reduced,
since the total solids concentration was kept constant. In observing the results at fixed Reynolds numbers in Figure 2.6, an increase in supersaturation yielded larger particle sizes with a broader distribution; this is indicative of faster growth kinetics as the supersaturation increased. While the effect on loading efficiency was not pronounced for \( Re_{tot} \geq 3300 \), at lower Reynolds numbers, the increase in supersaturation yielded poorer active loading. Likewise, in Chapter 4, GW771806X nanoparticles prepared through ion pairing with pamoic acid and stabilized by PLA3.7kD-b-PEG5kD were produced on an MIVM at a constant total solids concentration with varying dimethyl sulfoxide (DMSO) content, at 20, 10 and 6.25 vol%. Again, it was expected that the supersaturation ratio was higher for the lower DMSO case, since both solutes are water insoluble. It was shown that while the API loading remained at ~80% for all cases, 20 vol% DMSO resulted in the smallest peak mean particle diameter and span range, with larger and broader size distributions for the case with 6.25% DMSO. In these examples, although the supersaturation ratio was increased, the particle size increased. Coarse-grained particle dynamics simulations have shown similar results, where an decrease in solute solubility at a constant concentration (i.e. increase in supersaturation) led to larger particle sizes when the solute had some affinity for the hydrophobic block of the stabilizing amphiphilic block copolymer [65].

Case III has had the most reported examples, where total solids concentrations have been increased while maintaining the dispersion solvent mixture composition constant, which is equivalent to increasing the supersaturation ratio. The effect of increasing solids concentrations in FNP has been evaluated on a CIJM for β-carotene [53, 66], paclitaxel conjugated to PCL [37], α-tocopherol [37], a mixture of α-tocopherol succinate and paclitaxel conjugated to α-tocopherol succinate [37], PS [67], a mixture of α-tocopherol and progesterone [68], a cinnarizine/pamoic acid ion pair [40], and on a MIVM for rifampicin conjugated to α-tocopherol succinate [36],
bifenthrin [69], gold nanoparticles [49], cobalt ferrite nanoparticles [46], a GW771806X/pamoic acid ion pair (Chapter 4), and α-tocopherol/fenofibrate and α-tocopherol/cinnarizine mixtures (Chapter 5). In all of these cases, there has been an observed increase in particle size as the solute concentration was increased. Although there been some cases where an increase in concentration has not resulted in larger particles, such as for a mixture of α-tocopherol and indomethacin (Chapter 5), these are quite rare. Coarse-grained particle dynamics simulations have shown that depending on the affinity of the solute with hydrophobic block of the stabilizing amphiphilic block copolymer, the increase in particle size as component concentrations are increased at constant stoichiometries are different [65]. When there is affinity between the two species, the increase is slight, but is quite pronounced when there is no affinity.

These examples demonstrate different effects on particle size as supersaturation ratios were increased. Considering that for case I the increase in supersaturation ratio was achieved while decreasing the solute concentration, it is possible that the growth rate was decreased sufficiently to allow nucleation to dominate. In case II, while the supersaturation ratio increased by reducing the organic solvent content, the solute concentration was maintained constant; here, the increase in particle size might be explained as growth dominating nucleation, since nucleation alone would dictate that smaller particle sizes should have been observed. Finally, in case III, the increase in solute concentration gives rise to an increase in supersaturation and particle size, which is the same explanation as for case II. For the three cases, nucleation rates are less important than growth rates in FNP; however, adequate supersaturation and nucleation rates are always required for successful nanoparticle formation through FNP. Decreasing solute concentrations in the final dispersion generally results in smaller particle sizes, as shown by cases I and III, even though it can result in lower supersaturation ratios in case III. Although
lower concentrations resulted in smaller particle sizes and higher supersaturation ratios in case I, case II shows that if supersaturation is reduced at a constant solute concentration, the particle size can be reduced further. Therefore, it is seen that by reducing the supersaturation ratio, smaller particle sizes can be generated, but at the expense of a decreased maximum loading efficiency. In a production application, where the maximum solute concentration in the nanoparticle dispersion is the goal, a compromise between particle size and loading efficiency will need to be made. When a high solute concentration is required, it is recommended that the supersaturation ratio be modulated by considering different dispersion solvent mixture compositions or modifying solute solubility, as opposed to further increasing solute concentrations in FNP since significant increases in growth rates are expected at higher solute concentrations. Various formulation techniques, presented in section 2.8, can also help to reduce solute solubility and thus may allow for smaller particle sizes at higher solute concentrations.

2.6 POLYMER STABILIZERS

2.6.1 Choice of stabilizer

While the emphasis so far has been on the hydrophobic solute, the amphiphilic polymer is also of great importance since it will determine the stability of the nanoparticle dispersion in liquid and solid form. The stability of a nanoparticle dispersion will depend on anchoring energy of the polymer, affinity between solute and the polymer, and surface coverage provided by the polymer; these will be addressed in detail. It is desirable to use approved or generally regarded as safe (GRAS) polymers by the United States Food & Drug Administration (FDA). However, in specific applications, an efficacy advantage may dictate the use of a polymer that would involve additional safety testing. For example, the significantly longer circulation times observed for
paclitaxel nanoparticles stabilized by PS3kD-\(b\)-PEG2kD was considered a significant advantage for passive targeting [33]. However, if a nanoparticle formulation is desired purely to increase bioavailability without regard to the actual release profile, then long-term \textit{in vivo} stability is not necessarily a priority. In this case, as long as a GRAS formulation can provide \textit{in vitro} stability for manufacturing, the formulation could be feasible.

\textbf{2.6.1.1 Homopolymers vs. block copolymers: Adsorption & anchoring energy}

Some of the most commonly used polymers in pharmaceutical applications, such as polyvinylpyrrolidone (PVP), are homopolymers; that is, the entire polymer chain is composed of one monomeric entity. Cellulosic materials are similar in that they are composed of a repeating glucose ether unit, but with some monomers having hydrophobic substitutions (i.e. hydroxypropyl, ethyl) at hydroxyl groups. Polyvinyl alcohol (PVA), another commonly used polymer, is produced from polyvinyl acetate and hydrolyzed to some extent, meaning the main monomer present is the vinyl alcohol group, with a few vinyl acetate groups present. Gelatin, a partially denatured derivative of the water-insoluble protein collagen, is also widely used for various application. The preceding polymers are generally considered to be hydrophilic, but they have been used as stabilizers for nanoparticles because hydrophobic residues in the chains allow them to adsorb onto hydrophobic surfaces [70-74]. This adsorption lowers the surface energy of hydrophobic nanoparticle cores and provides steric barriers on the particle surfaces. However, for stabilization to be maximized, there must be strong adsorption of the polymer to the particle surface, while having most of the chain extending away from the surface into the solvent to provide steric repulsion. It has been estimated that at least 10\% of the polymer segments must act as anchoring sites to achieve maximum adsorption [75], which requires careful selection of homopolymers. In FNP, both PVP and PVA have been used to formulate
bifenthrin, a water insoluble pesticide, into nanoparticle form; however, these formulations experience > 100% growth in particle size over an 18-day and 29-day period, for PVP and PVA respectively [69]. Particles loaded with ibuprofen or curcumin and stabilized by PLA-b-PEG of different molecular weights and either PVA, PVP, or hydroxypropyl methylcellulose have also been produced by FNP [39]. Other homopolymers that have been used include polyelectrolytes, such as chitosan, poly(ethylene imine), ε-polylysine [64] and gelatin [76]; in this case, electrostatic stabilization is afforded due to the dual steric and electrostatic stabilization from the polymer. A potential limitation of homopolymers as stabilizers for FNP is that a single chain may adsorb to multiple nanoparticles and thereby become a flocculating agent rather than a steric stabilizer [77, 78].

Block copolymers, in contrast to homopolymers, have distinct chain segments or blocks composed of different monomeric entities. Feasible block copolymers will generally have a distinct hydrophobic block(s) and hydrophilic block(s). The hydrophobic block creates strong van der Waals attractions between the water-insoluble polymer segments and the particle surface, resulting in high adsorption. This adsorption must balance out the entropic penalty of having the polymer tethered at the particle surface. On the other hand, the hydrophilic block should be strongly solvated and extend away from the particle surface, providing a sufficient barrier from aggregation or interaction with other species or surfaces. Di-block copolymers have the added advantage that the anchoring of the hydrophobic block on the nanoparticle surface prevents the chain from being able to bridge between two particles. Block copolymers with very low critical micelle concentrations (CMCs) (≤ 10^{-3} wt%), due to significant hydrophobicity, will have low levels of unimer exchange between particles due to the high free energy penalty required to expel a chain from a nanoparticle surface [79]. The various poloxamers (marketed as
Pluronics or Lutrols), which are also used commonly for pharmaceutical applications, are triblock copolymers of poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) of various block molecular weights; although many of these polymers have low CMCs, the hydrophobic block is fluid [80], reducing the energy penalty for individual chains to participate in dynamic equilibrium [81]. Appropriate amphiphilic di-block copolymers result in the kinetically frozen nanoparticles discussed in section 2.3. This frozen structure should provide improved integrity to nanoparticles when in vivo, which is especially important for intravenous formulations requiring long release times. Johnson & Prud'homme have shown that in contrast to surfactant micellization which experiences dynamic unimer exchange at equilibrium [82], the rapid precipitation of amphiphilic di-block copolymers results in a two-step assembly process [79], as shown in Figure 2.7. First, unimers will essentially follow nucleation and growth kinetics leading to unimer aggregates; next these aggregates fuse under diffusion-limited conditions to form un-equilibrated micelles with a sufficient insertion barrier to prevent growth. The formation of a steric brush layer on the surface will also prevent unimer exchange resulting in frozen structures. Coarse-grained simulations and analysis through mean-field population balance models have observed this assembly behavior [83, 84]. Thus, selection of an amphiphilic di-block copolymer requires a hydrophobic block with insignificant water solubility to result in a very low CMC and a hydrophilic block that will form a dense brush layer to minimize unimer exchange.

For parenteral nanoparticle formulations, di-block copolymers with a PEG hydrophilic block and a bio-degradable hydrophobic block that is FDA-approved for parenteral use have been used, such as PCL [12, 36, 37, 46, 47, 49, 63, 85, 86], PLA [12, 35, 39-41, 47, 48, 68, 86], and poly(lactide-co-glycolide) (PLGA) [12, 41, 47, 86, 87]. Other hydrophobic blocks, such as
PS, have also been used [12, 26, 33-35, 42, 53, 63, 88, 89]. D-α-tocopheryl poly(ethylene glycol) 1000 succinate, although not truly a block copolymer since the tocopherol segment is monomeric, is another example of an amphiphilic polymer that is commonly used in the industry. The use of D-α-tocopheryl poly(ethylene glycol) 1000 succinate is investigated in Chapter 5 for the production of indomethacin-loaded nanoparticles.

Figure 2.7 Mechanism of di-block copolymer assembly in Flash NanoPrecipitation. (I) First, the high supersaturation created by rapid solvent change will induce nucleation and growth of the hydrophobic blocks. (II) Polymer aggregates will fuse with each other until sufficient insertion barriers form from the overlapping hydrophilic corona. (III) Remaining chains or aggregates must fuse with particles or aggregates with a low enough insertion barrier. Taken from reference [79].

Pluronic F127 (MW: 4.4kD-b-3.8kD-b-4.4kD) was also evaluated in Chapter 5, as well as in the formulation of bifenthrin aforementioned [69], but resulted in poor particle stability as observed through macroscopic phase separation in both cases. Kumar and co-workers also observed rapid instability for itraconazole particles stabilized by Pluronic F127. They explained the observations by considering that the equilibrium solubility of itraconazole was increased 5.5-fold in a 6 mg/mL F127 solution in comparison to pure water. Since Pluronic F127 is labile due to the fluid
hydrophobic block, individual polymer chains can participate in dynamic equilibrium between dissolved, micelle, and adsorbed phases, allowing for active entrainment of API molecules from particle surfaces into Pluronic F127 micelles, as shown in Figure 2.8 [89]. This highlights the importance of selecting appropriate block copolymers for a nanoparticle formulation.

![Figure 2.8](image.png)

**Figure 2.8** The labile nature of Pluronic F127 is thought to allow for dynamic unimer exchange to occur, allowing for active entrainment of solute from the initial nanoparticle structure to leave and result in particle instability. Taken from reference [89].

### 2.6.1.2 Affinity of solute and polymer

As was mentioned before, for a polymer to properly stabilize a hydrophobic particle, sufficient adsorption must balance out the entropic tendency of the polymer. Adsorption depends on the affinity of the hydrophobic residues of the polymer for the precipitating solute. Considering that during nucleation of the solute, the presence of a foreign substance with sufficient affinity can induce heterogeneous nucleation, it is important to consider how the attraction or repulsion between the two precipitating species will influence particle formation. The coarse-grained simulations of Chen [45] and Spaeth [65] revealed that when there is little or no interaction between the two species, di-block copolymers will preferentially form empty
micelles instead of stabilizing the solute particles. Furthermore, the adsorption of the polymer on particles is in a patchy manner where clusters of hydrophobic blocks will adsorb at a few sites [45]; this patchiness implies exposed hydrophobic surfaces that can then aggregate primary particles, resulting in larger particle sizes as compared to the case of higher affinity [65]. However, in the case when there is interaction between the two species, aggregates of unimers with solute will fuse and assemble into the final particles. Although Cheng and co-workers have reported that population balance equations suggest that heterogeneous nucleation is favored for a case of species attraction [84], it is unlikely that this is the case since activation energies for nucleation were not implemented in the model. In cases where the solute is more hydrophobic than the hydrophobic block of the block copolymer or in cases where the molar concentration of solute is much higher than that of the stabilizing polymer [49, 59, 63], the solute will experience nucleation and growth prior to polymer assembly. Thus, it is more likely that single polymer chains will adsorb onto nuclei and thus incorporate the solute into the final structures.

Regardless, of the actual assembly mechanism, there is involved incorporation of the species into the particle, which results in a more uniform distribution of the polymer on the surface [45]; this was used to explain the smaller particle size when affinity is increased, since growth by aggregation is arrested by uniform steric layers covering the particle surface [65].

Although no systematic studies have been reported where polymers with varying affinities for a solute but with similar assembly dynamics were evaluated, we expect that differences in particle sizes and stability could be observed in such a study. Furthermore, the aforementioned examples of Pluronic F127 demonstrates the case of a labile stabilizer with sufficient affinity for the solute. In this case, affinity between the two species is not desirable, since entrapment of the solute on unimers resulted in solute depletion from the initial particles.
and unstable loading into micelles and macroscopic precipitate. When Kumar et al produced odanacatib particles stabilized by Pluronic F127, they observed less of a stability issue, particularly because the equilibrium solubility of the API was increased only 1.6-fold in a 6 mg/mL F127 solution in comparison to pure water, while itraconazole's solubility increased 5.5-fold [89]. In considering solubility parameters, it was observed that there was smaller difference between the values of the poly(propylene glycol) block and itraconazole than for the polymer block and odanacatib. This solubility parameter, or the Flory-Huggins interaction parameter, analysis is useful in determining the affinity between the hydrophobic residues of stabilizing polymers and solutes.

2.6.1.3 Surface coverage

In the assembly process of the polymer, the final conformation of the chains on the particle surface will determine the surface coverage of the particle. This, in turn, is critical for the stability of the dispersion. The conformation on the surface will primarily depend on the molecular weight of the polymer. In particular, the molecular weight of the entire polymer chain can be crucial to achieving small particle sizes. Larger polymer chains will result in longer assembly times because of the smaller diffusion coefficient. However, by the same reasoning, more hydrophobic residues or a longer hydrophobic block will also reduce the CMC of the polymer [90], which will increase the supersaturation ratio and the aggregation rates. It is important that the aggregation rates of the polymer, related to $\tau_{agg}$, are matched to the precipitation kinetics of the solute, as mentioned in section 2.3. Therefore, an optimal molecular weight will balance out the decreasing diffusion coefficient with the increasing rate to aggregate. Coarse-grained simulations showed that when there is no affinity between the solute and the hydrophobic block (di-block copolymer), a hydrophobic block of higher molecular weight will
result in larger particle sizes when kept at a constant molar concentration; contrastingly, when affinity exists, the difference in aggregation kinetics attributed to hydrophobic block molecular weight can result in different particle sizes depending on the growth mechanisms, favoring adsorption onto solute nuclei at low molecular weights and eventually transitioning to fusion of solute/polymer aggregates at higher molecular weights [65]. Another factor that also needs to be accounted for is how the molecular weight of the hydrophobic domains of the polymer affects unimer exchange; the polymer should have a low enough CMC and poor mobility in the dispersion solvent mixture to prevent this.

Furthermore, the physical state of the hydrophobic residues, particularly for di-block copolymers, can be a function of molecular weight. A good example is that of PCL, which has a glass transition temperature of -71°C [91] and will crystallize at different temperatures depending on the molecular weight. A study by He and associates investigated the physical state of PCL-\(b\)-PEG block copolymers with a PEG of constant molecular weight (5kD) and PCL of varying molecular weights (2-33kD); they observed that crystallinity of PCL became more prominent at molecular weights comparable to that of the PEG5kD block and dominated at higher molecular weights [92]. Budijono et al [47] and Zhu [86] have reported crystallinity causing instability in nanoparticle dispersions stabilized by PCL7kD-\(b\)-PEG5kD and PCL12kD-\(b\)-PEG5kD, respectively. Scanning electron micrographs of both formulations are shown in Figures 2.9a & b. Budijono explained that the tendency of PCL to crystallize could result in dense crystal clusters on particle surfaces, leaving exposed regions that aggregate, or possibly the lamellar structure of the PCL crystals having exposed hydrophobic edges could induce aggregation. The crystallinity of PCL has been used to explain the high complement activation (Figure 2.9c), which is responsible for clearance mechanisms \textit{in vivo} [93], and low circulation
rates of nanoparticles loaded with paclitaxel prodrug and stabilized by PCL9kD-b-PEG5kD [12] (Figure 2.9d).

![Figure 2.9](image)

**Figure 2.9** Use of PCL-b-PEG block copolymers in the Flash NanoPrecipitation literature. a) Scanning electron micrograph of polymer stabilized upconverting nanophosphors, with a peak mean diameter of ~300 nm. Large precipitates are present, confirmed to be due to polymer aggregation. b) Scanning electron micrograph of polymer stabilized β-catorene nanoparticles, with an initial particle size of 42 nm. When left to age for a few hours, the crystalline structures in the micrograph formed, which was verified to be due to PCL crystallization. c) Complement activation assay results for various α-tocopherol nanoparticle formulations, ranging from 60 - 100 nm in diameter and using the different polymers listed. Only the PCL9kD-b-PEG5kD was observed to strongly activate complement. d) In vivo circulation of various paclitaxel prodrug nanoparticle formulations, ranging from 60 - 100 nm in diameter and using the different polymers listed. It was observed that for polymers with a 5kD PEG and a PCL block, increasing the PCL molecular weight resulted in a lesser amount of the dose in circulation after 4 hours, correlating with the increased PCL crystallinity. Taken from references [47], [86], [12] (c & d), respectively.
In contrast, PLA, PLGA, and PS are all glassy polymers with glass transition temperatures higher than room temperature [94, 95] and thus, are not expected to destabilize nanoparticle dispersions through crystallinity.

In terms of developing stealth nanoparticles for intravenous formulations, the molecular weight of the hydrophilic block must also be considered. As mentioned in the introduction, one of the most commonly used stealth materials is PEG. FNP simulations suggest that increasing the molecular weight of the PEG block at fixed polymer and solute molar concentrations and hydrophobic block molecular weight should result in smaller particle sizes [65]. However, various studies have suggested that an optimal molecular weight for PEG for stealth purposes is 5kD [96, 97]. For PEG to impart stealth properties to nanoparticles, it is required that the PEG coating be as dense as possible. We will now consider the case of di-block copolymers with a PEG5kD as the hydrophilic group. In order for PEG to be present in a conformation similar to a brush, the anchoring area of the hydrophobic block must be small enough to allow for close packing of the anchors, thereby extending the PEG chains laterally. D'Addio and associates experimentally measured the blob size, or the diameter of statistical spheres which compose a polymer, of PEG chains from PEG di-block copolymers coated on PS latex beads and found that it increased as the molecular weight of the hydrophobic block increased [12]. This finding implies that the PEG layer is less dense as the anchoring area of the hydrophobic block increases. In general, we have found that for more hydrophobic polymers such as PCL, PLA, and PS, the molecular weight should not be considerably larger than that of the PEG block (5kD).

Lastly, for block copolymers, the degree of micro-phase separation of the various blocks must be considered. Since FNP will result in non-equilibrium structures, two blocks that have some miscibility may end up being kinetically frozen into one phase, as is discussed in Chapter 6.
for PCL7kD-\textit{b}-PEG5kD and PLA3.7kD-\textit{b}-PEG5kD block copolymers. In these cases, a lower PEG concentration was measured than what was expected, which accounted for the increased aggregation during freeze-drying in comparison to PS1.6kD-\textit{b}-PEG5kD which fully micro-phase separates. Zhu also observed that PLA10kD-\textit{b}-PEG5kD micelles were stable in water, but the introduction of 1 wt% saline led to precipitation of the particles [86]; he attributed this to the micelles being only electrostatically stabilized, which suggests that the PEG surface density was low and a significant amount of PEG was trapped within the PLA matrix. While sufficient stability may be afforded \textit{in vitro}, even when some of the stabilizing block is trapped within the hydrophobic block domain, different performance may be observed \textit{in vivo} because of the increase of species (i.e. proteins) that can adsorb to the particle surface and the change in ionic strength. This matter will be studied in more depth in the future.

### 2.6.2 Effect of polymer on particle size

In the previous sub-sections of 2.6, we have covered several aspects that must be considered in selecting a polymer for FNP formulations. When using FNP to produce a nanoparticle dispersion, aside from the mixing conditions and dispersion solvent mixture composition, the formulation parameters (i.e. polymer choice, relative ratio of polymer to solute, and absolute concentrations of the polymer and solute) will have an important impact on the product. We now present how the formulation composition, particularly how the absolute and relative concentrations of polymer with respect to the solute, affect particle size.

With respect to relative ratio of polymer to solute, if the total solids concentration does not change, then increasing the polymer ratio will result in less solute available for loading. In this case, since the supersaturation ratio of the solute will decrease through a decrease in concentration, it is expected that particle sizes will decrease. Similarly, because the concentration
of polymer will increase, the length scale for unimers to diffuse and assemble into aggregates and micelles will decrease, allowing for formation of smaller particles. However, if the time scale for polymer assembly, $\tau_{agg}$, becomes much faster than the time scale associated with solute nucleation and growth, $\tau_{ng}$, empty micelle formation can occur; this can be difficult to diagnose. A dynamic light scattering measurement to determine particle size can report a unimodal distributions skewed towards a smaller particle size if the deconvolution of the autocorrelation function cannot distinguish the true nanoparticle distribution from the micelle distribution. In this case, the abundance of polymer with respect to solute will also reduce the maximum achievable solute loading, which is generally not favorable. Thus, while increasing the relative ratio of polymer to solute should result in smaller particle sizes, care must be exercised to not result in empty micelle formation and decreased solute loading.

Spaeth et al have shown through simulations that at a constant solids volume fraction, decreasing the polymer to solute ratio resulted in larger particle sizes [65]. Figueroa observed similar results for production of β-carotene nanoparticles stabilized by PS1.6kD-b-PEG with three different PEG molecular weights [98]. Produced at a nominal solids concentration of 2 mg/mL with varying polymer to solute mass ratios, the peak particle sizes decreased with an increasing polymer fraction. When re-plotted to express the formulations as molar ratios of polymer to solute (Figure 2.10), the trend still holds, but it shows that the block copolymer with the larger PEG molecular weight (5.3kD) resulted in smaller particle sizes, which is in agreement with Spaeth's simulations. Pustulka et al have reported a similar decrease in particle size when the relative ratio of polymer to solute was increased for tetramethyl silicate nanoparticles, with a relatively smooth transition from 200 nm at 0 wt% polymer to 75 nm at 100 wt% polymer [41].
Figure 2.10 Peak mean diameters of β-carotene nanoparticle formulations stabilized by PS-b-PEG of different PEG molecular weights. All dispersions had a nominal solids concentration of 2 mg/mL. The data shows that increasing the relative ratio of polymer to solute decreased the particle size, with the polymer with a larger PEG molecular weight resulting in smaller sizes than the smaller PEG molecular weights.

In comparison, when the relative ratio of polymer to solute is kept constant and the solids concentrations are increased, Spaeth reported larger particle sizes, which is consistent with results given in Chapters 3, 4 and 5 and by Pinkerton for cinnarizine/pamoic acid nanoparticles stabilized by PLA3.7kD-b-PEG5kD [40]. However, the case when the solute concentration is kept constant, but the polymer concentration is varied, is similar to the case where relative ratios are varied at a constant solids concentration. Although the solids concentration will increase in this case, the solute concentration will not, thereby resulting in similar solute nucleation and growth rates regardless of the polymer presence. When the concentration of polymer is increased, the particle size decreases until additional polymer only results in the formation of unloaded micelles. This trend was observed for progesterone-loaded particles in Chapter 3, where a constant concentration in the organic stream of progesterone at 200 mg/mL and α-tocopherol at 100 mg/mL resulted in a peak mean diameter of 350 nm with a span range of 627
nm when the PLA3.7kD-b-PEG5kD concentration was 133 mg/mL. Upon increasing the polymer concentration to 200 and 267 mg/mL, the size decreased to 245 nm, with only a change to the span range (319 nm and 248 nm, respectively). Shen et al also reported the same trend for β-carotene nanoparticles stabilized by PCL3.6kD-b-PEG5kD, where increasing the polymer concentration five-fold decreased the particle size by roughly a half [63]. However, when keeping the polymer concentration constant and increasing the solute concentration, larger sizes can be expected because the increase in solute concentration will drive the solute growth rate up. Gindy observed this trend when increasing the loading of colloidal gold in PCL7kD-b-PEG5kD stabilized nanoparticles and could linearly correlate the increase in size as a function of the polymer volume fraction to the -1/3 power. These examples show that varying the stabilizing polymer concentration and relative ratio in comparison to the solute are two other methods for tuning particle size in FNP.

2.7 APPLICATIONS

2.7.1 Pharmaceutical nanoparticulates

FNP has had the largest application for formulating APIs into nanoparticulates. Nanoparticle dispersions have been produced with the following APIs loaded into the nanoparticles: rifampicin and hydrophobic prodrugs [34, 36], paclitaxel hydrophobic prodrugs [33, 37, 86], itraconazole [39, 89], odanacatib [89], nitric oxide prodrugs [35], SQ641 [34], cyclosporin A [34], progesterone [68], cinnarizine [40], clozapine [40], α-lipoic acid [40], ibuprofen [39], curcumin [39], doxorubicin [39], flurbiprofen [39], SR13668 [38], GW771806X (Chapter 4), indomethacin (Chapter 5), and fenofibrate (Chapter 5).
Out of these formulations, a few have been developed into dry dosage forms. Liu demonstrated the capability of converting liquid nanoparticle dispersions loaded with a rifampicin-tocopherol prodrug to a spray dried powder targeted for inhaled aerosol delivery [36]. Kumar et al lyophilized nitric oxide prodrug nanoparticles and found similar anti-proliferative activity between the dry nanoparticulate dosage and the unformulated prodrugs [35]. As discussed in Chapter 3, freeze-dried nanoparticles loaded with progesterone have been produced and were found to have similar a similar pharmacokinetic profile in rats as a DMSO solution of progesterone when administered intravenously and produced a faster maximum plasma concentration than standard oil-based formulations when administered intramuscularly. Furthermore, Chapter 5 discusses how GW771806X nanoparticles have been produced and lyophilized to yield formulations that can be reconstituted to ~8 mg/mL API. Shen and co-workers have produced a spray dried nanoparticulate formulation of SR13668 that has demonstrated roughly a three-fold increase of API concentrations in plasma when administered orally to mice [38]. These examples show that FNP-produced liquid dispersions can be formulated into dry dosage forms applicable for at least inhaled, parenteral, and oral administration.

2.7.2 Imaging capabilities

In the current literature, there have not been many reports of imaging applications of FNP, though this is an active area of research within the Prud'homme group at the moment. The work of Gindy, in which she produced PCL9kD-b-PEG5kD stabilized nanoparticles loaded with cobalt ferrite nano-crystals, demonstrated that the magnetic nanoparticles possess relaxivity values suitable for magnetic resonance imaging (MRI) [46] and representative samples are shown in Figure 2.11. Gindy also reported the simultaneous loading of cobalt ferrite nano-
crystals and gold colloids into nanoparticles through FNP, allowing for both MRI and computed X-ray tomography functionalities into one nanoparticle formulation [46]. Ongoing work is focusing on the incorporation of APIs and fluorescent agents for near-infrared into the aforementioned formulations. Current research is also investigating the production of nanoparticles loaded with metal chelates for positron emission tomography and single-photon emission computed tomography.

Figure 2.11 Cobalt-ferrite loaded nanoparticle samples. The top image shows various dilutions of a nanoparticle dispersion at 2.1 mg/mL total solids with 4.2 wt% loading of iron (1.4 mM iron) cast into a gelatin mold for magnetic resonance imaging. The iron concentration is denoted in the image. The bottom shows the magnetic resonance imaging (T$_2$-weighted) of the respective samples from the above image. Taken from reference [46].
2.7.3 Targeting functionalities

The possibility of introducing active targeting functionalities to nanoparticles has been and is a topic of great interest [14, 18, 99], particularly for the localization of APIs/imaging agents at disease sites. FNP presents an elegant way in achieving such functionalities. The strategy that has been reported in the literature is to first functionalize the PEG block of a diblock copolymers with a reactive end group; this functionalized polymer, in conjunction with unfunctionalized polymer, is then used to produce nanoparticles with a specific fraction of polymer with reactive end groups on the surface. Once the functionalized nanoparticles have been produced, targeting ligands are conjugated onto the reactive sites. This strategy allows for accurate characterization of the maximum achievable number of targeting moieties on a particle surface, since the polymer has been functionalized prior to particle formation. Reactive groups that have been used include maleimide (MAL), \( p \)-nitrophenol (pNP), and azide, which react with thiol, amine, and alkyne functionalities, respectively. Depending on the chemical structure of the targeting ligand, an appropriate reactive group can be functionalized on the PEG block.

To validate this technique, Gindy et al [100], Ji et al [85], and Akbulut et al [43] have reported the production of particles stabilized by PCL-\( b \)-PEG-MAL. Ji showed that for particles stabilized by polymers of molecular weight 8.6kD-\( b \)-4.6kD and 4.6kD-\( b \)-4.6kD, 51 and 67% of the maleimide end groups were accessible. Gindy reported that when attempting to conjugate bovine serum albumin onto particles stabilized by a polymer with a molecular weight of 7kD-\( b \)-5kD, up to a 22% conversion efficiency of the maleimide groups was achieved when using 2.7 thiol equivalents. Akbulut, using the same polymer as Gindy, conjugated lutenizing hormone-releasing hormone to the surface and demonstrated greater active uptake of the targeting particles.
in comparison to non-targeting particles, as shown by a 2-fold increase in cell fluorescence (since the particles were loaded with the fluorophore Nile Red).

With respect to \( p \)-nitrophenol functionalized particles, Budijono [101], Liao [102], and Tse [103] have all reported the use of PS-\( b \)-PEG-pNP to produce functionalized particles. Budijono, in conjugating a fluorophore Alexa Fluor 350 to particles stabilized by a polymer of molecular weight \( 1.5\text{kD-}b\text{-}5\text{kD} \), was able to achieve an 81% conversion efficiency of the \( p \)-nitrophenol groups when using 100 amine equivalents with an almost linear relation between conversion efficiency and amine equivalents. When Budijono conjugated monoclonal antibody 2C5 to the particles stabilized by the same aforementioned polymer, loaded with upconverting nano-phosphors and a photosensitizing agent [48], she observed 44% more HeLa cell death for targeted particles in comparison to non-targeting particles. Liao, in attempting to conjugate Alexa Fluor 350-labeled monoclonal antibody trastuzumab to particles stabilized by the same polymer used by Budijono, reported a maximum of 40% conversion efficiency when using 100 equivalents of the antibody. Tse, in using a smaller PS-\( b \)-PEG-pNP (1.6kD-\( b \)-2.5kD) to produce nanoparticles, observed 100% conversion efficiency in conjugating either Alexa Fluor 350 or superfolder green fluorescent protein to the \( p \)-nitrophenol groups at \( \sim 60 \) amine equivalents. When Tse instead conjugated monoclonal antibody C225 at 0.4 equivalents, 16% conversion efficiency was achieved and \textit{in vitro} testing showed roughly a 70-fold increase in cellular uptake of the targeted particles in comparison to the same particle formulation with no targeting ligand.

Finally, Zhang et al have reported the use of azide-terminated PLA5kD-\( b \)-PEG5kD to form nanoparticles; alkyne-derivatized folate or protein A1 were conjugated to the particles through click chemistry [104]. For the folate and protein A1 present at 1 and 0.2 alkyne equivalents, respectively, 10% and \( \sim 100 \) conversion efficiency were observed, respectively.
Another technique that has been used for small molecule targeting ligands is to perform the conjugation of the ligand to the PEG block prior to particle formation. D’Addio and co-workers have reported the functionalization of PS1.5kD-b-PEG5kD with an alkyne end group on the PEG; an azide derivatized mannose was synthesized to carry out the click reaction to conjugate the two species [105]. The mannose-functionalized polymer was then used in producing nanoparticles. It was observed that active cellular uptake of particles was optimal at 9 mol% of the polymer being functionalized, which showed a three-fold increase in uptake to non-functionalized particles.

These examples highlight the capability of incorporating targeting ligands onto nanoparticles produced through FNP. Current research is investigating in vivo performance of targeting nanoparticles.

2.8 FORMULATING SOLUTES THROUGH FNP

When attempting to formulate a solute into nanoparticle form through FNP, the solute solubility in the final dispersion solvent mixture will most likely determine the quality of the nanoparticles produced. Not only will it affect the maximum achievable loading efficiency and supersaturation as discussed before, it can also affect the stability of the resulting dispersion. Although solute solubility cannot always be reliably predicted, it is can sometimes help to use a partition coefficient, such as logP (the negative logarithm of the partition coefficient of the solute between octanol and water), as a surrogate property, since logP values can be readily calculated, as they are for pharmaceutical applications. Refer to Appendix C for more information on logP. The clogP (or calculated logP) is a measurement of lipophilicity that can be used to gauge the loading extent of a material in a hydrophobic nanoparticle core, where for FNP clogP values ≥ 4-
5 are preferred. However, this property is only a starting point to determine suitability of a solute for FNP nanoparticle formation, since it does not capture the degree of ionization, crystallinity, and other properties that will affect the formation and stability of loaded nano-constructs.

If a solute can be ionized, its ionization depends on the dielectric constant of the medium and pH. In organic media with lower dielectric constant, solutes are less ionized or not ionized at all. However, in the mixed solvent resulting from FNP, ionization can result in an effectively higher solute solubility. Such ionization enhances Ostwald ripening of a nanoparticle dispersion, where smaller particles will tend to dissolve and larger particles will tend to grow until thermodynamic equilibrium based on particle curvature is reached [26, 31]. Since Ostwald ripening is dependent on the solubility of the solute in the dispersion solvent mixture, it is recommended that the either the solute be extremely insoluble in the dispersion solvent mixture containing organic solvent or that the fraction of organic solvent be quickly reduced after FNP. Ostwald ripening is discussed in further detail in section 2.9.

Moreover, rapid precipitation experienced under high supersaturation ratios can lead to the formation of non-equilibrium phases. This can mean higher-energy polymorphs or even amorphous phases. For example, amorphous material β-carotene was observed by Zhu et al when using FNP to produce nanoparticles with and without polyelectrolytes as stabilizers [64]. Ostwald's law of stages, although not universal, suggests that for various materials, an unstable system will undergo a series of transformations until eventually reaching an equilibrium state [21]. Therefore, the most stable crystalline state of a solute will not necessarily be formed during FNP; the precipitate could be of a higher energy state, and thus have higher solubility, which could enhance Ostwald ripening and result in redissolution and recrystallization outside of the nano-constructs.
For an API with a relatively high clogP ($\geq 5$) and no ionizability, it is typical to begin FNP formulation attempts by precipitating the API with an appropriate amphiphilic polymer (1:1 mass ratio) at a relatively low concentration (~1 mg/mL API in the final dispersion). This preliminary screening is generally done with a low organic solvent content in the dispersion solvent mixture (~10 vol%). If the resulting product is a dispersion that is stable for a time period in which size and loading characterization can be done, subsequent optimization of the FNP can begin. However, if poor short-term stability is observed, or immediate macroscopic phase separation, this is an indication that the solubility of the API may be too high in the dispersion solvent mixture. While studies can be conducted to determine FNP conditions to decrease API solubility (i.e. choice of organic solvent, choice of anti-solvent, final dispersion solvent mixture composition), if the quality of the product cannot be significantly improved, various techniques can be considered. Several methods have been used in the literature to formulate solutes with low water solubility, but poor property profiles for FNP. Depending on the chemical structure, three general strategies have been reported.

First, if the solute has a moiety to which a hydrophobic structure can be reactively linked, conjugation to form a hydrophobic prodrug may be a suitable option. The parent solute must be regenerated through degradation of a linker and the resulting by-products should not be toxic. The concept of this technique is to decrease the solubility of the solute through attachment of low solubility moieties, such that supersaturation is increased and stability issues can be reduced. Liu, in attempting to formulate rifampicin, an antibiotic used for tuberculosis treatment, could not form stable particles through FNP because of observed recrystallization in a matter of days [36]. However, in conjugating a tocopherol or PCL2kD through a succinate linkage or 2-ethylhexyl vinyl ether through an acetal linkage to a hydroxyl group from the parent API, three hydrophobic
prodrugs were synthesized that could be used to produce nanoparticle dispersions that were stable for at least a month. Likewise, Ansell et al produced hydrophobic prodrugs of paclitaxel, an anti-cancer agent, that were used in producing nanoparticles [33] since FNP with the parent API did not form stable dispersions [37]; depending on the chemistry of the prodrug (i.e. linker and hydrophobic moiety), the release rate of the prodrug was modulated in vivo. However, the disadvantage to this technique is that conjugation results in a new chemical entity in the pharmaceutical industry, which requires further approval from the FDA; considering that companies are keen on reducing the time from discovery to launch, this added delay is not attractive.

Another strategy, when conjugation is not possible or desirable, is that of dispersion or solubilization within a nanoparticle core, through the addition of a hydrophobic excipient that can be loaded into nanoparticles through FNP. For successful application of this technique, it is important that the solute have high affinity for the additive used, since the solute should be molecularly dissolved or stably dispersed throughout the excipient matrix. This is discussed in further detail in Chapters 3 and 5, as well as later in section 2.9. While the incorporation of cosolute in the nanoparticle core will reduce the maximum solute loading, this strategy can be useful in reducing Ostwald ripening and recrystallization by reducing the driving force for smaller particles to re-dissolve and precipitate as a new phase. Since an appropriate co-solute will promote heterogeneous precipitation kinetics because of the affinity between the two solutes, this may enhance incorporation of the solute of interest into the nanoparticles. This method can be attempted with solutes with clogP values < 5, as shown in Chapter 5. Chen et al showed through coarse-grained simulations that when there is low or no affinity between a solute and the hydrophobic block of a stabilizing amphiphilic block copolymer, large solute particles
will form and aggregate, possibly leading to macroscopic precipitates; however, when there was affinity between the two species, particles were smaller, less polydisperse, and had a larger fraction of the solute in the core [45]. This dispersion technique has been used by Kumar for the production of pyrene and solvent yellow 98 loaded nanoparticles, using cholesterol, PS or PCL as co-solutes. For the case where there was high affinity (pyrene/PS), the fluorescence quenching of pyrene was decreased, suggesting a dispersed pyrene within the PS phase [42]. Similarly, as shown in Chapter 3, through the addition of α-tocopherol, progesterone particles could be produced through FNP in which progesterone was largely dissolved. The limitation of this technique is that high affinity between the API and the additive is required, which is not always possible with excipients that are GRAS by the FDA.

Lastly, ion pairing has been used to create stable salts from an ionic solute and a relatively hydrophobic counter-ion. The concept of this method is the addition of a hydrophobic excipient with a charge opposite to the solute of interest; this can be simply a GRAS additive or part of the stabilizing structure of the nano-construct. The additive must easily ionize (i.e. low $pK_a$ for an acid or high $pK_a$ for a base); also, the resulting ion pair must be hydrophobic so that solubility is low in the dispersion solvent mixture, allowing for efficient loading into nanoparticles. Not only should the effective supersaturation ratio be increased, proper pairing should reduce the extent of Ostwald ripening and recrystallization. Like the solubilization technique, the ion pairing method can be used for solutes with low clog$P$ values. Pinkerton investigated the use of this technique and observed that a $pK_a$ difference of 2 units was necessary for the formation of stable nanoparticles loaded with ion pairs of cinnarizine, a weak base used to treat motion sickness, and a counter-ion acid [40]; she was also able to form nanoparticles loaded with an ion pair of the water soluble α-lipoic acid, an antioxidant. This technique is used in
Chapter 4 for the production of nanoparticles loaded with a paired GW771806X, a receptor tyrosine kinase inhibitor that is a weak base. However, this technique is not limited to small molecules, as this has been used to load small interfering RNA (siRNA) into lipid nanoparticles through FNP. Kumar produced lipid vesicles using a cationic lipid and anionic siRNA, such that > 90% loading efficiency of the siRNA was achieved [88]. In this case, the ion pair was formed using a structural component of the nano-construct.

While not formally reported yet in the literature, another technique exists, which is applicable for solutes which can form coordination complexes. Ongoing research in the Prud'homme lab is focusing on the production of hydrophobic complexes of gadolinium and other inorganic contrast agents used in various imaging modalities. The only reported instance of this method is the production of zinc-loaded nanoparticles through the use of pyrithione zinc [88]. The use of this technique should increase for imaging nanoparticles soon.

2.9 DOWNSTREAMING ISSUES

2.9.1 Stability

In using FNP to produce a nanoparticulate formulation, the immediate product will be in the liquid state. Liquid dispersions of nanoparticles are difficult to keep stable for extended periods; the high interfacial areas and energies promote destabilization by Ostwald ripening and recrystallization, particle aggregation, and solute degradation. Of great concern is Ostwald ripening because the immediate dispersion will still contain organic solvent which can increase the solubility of the precipitated solute. In considering the discussion on nucleation (section 2.2), it was mentioned that a critical nucleus radius is necessary for growth to begin; nuclei with radii smaller than the critical radius will dissolve. The Kelvin equation, shown below, expresses the
solubility of a sphere, \( c_r \), as a function of, among other values, the bulk equilibrium solubility of the solute in the medium and the particle radius.

\[
c_r = c_{eq} \exp \left( \frac{2\gamma_{cl}v_0}{RT} \right) \tag{2.9}
\]

This equation highlights two main points for a system in which precipitation has been completed:

1. smaller particles will have increased solubility, particularly when dealing with a particle distribution with a small average size (≤ 50 nm); (2) an increased bulk equilibrium solubility will increase the solubility of the particles. From the first point, smaller particles, which have higher surface energy due to increased specific surface area, tend to dissolve, resulting in supersaturation once more. In order to reduce the free energy of the system, the dissolved solute can either form new larger particles or deposit on larger particles, which will depend on the local fluctuations in the dispersion. If new particles form, these will not be stabilized by polymer, as the polymer chains will be kinetically frozen on the previously formed particles; thus, un-arrested growth will lead to precipitation of the solute without surface stabilizers, which is observed as macroscopic precipitates. Furthermore, there will be an increase in average particle size, due to the disappearance of smaller particles from the distribution.

If the dissolved solute deposits on existing larger particles, a redistribution of mass will lead to a shifting in the particle size distribution. Liu et al, in following the treatment of Hoang et al of classical Lifshitz, Slyozov, and Wagner theory of Ostwald ripening [106], has shown that the average radius growth rate for a particles through this process is as given below [31].

\[
\frac{d\bar{r}^3}{dt} = \frac{8\gamma_{cl}v_0}{9RT} D_{AB} c_{eq} \tag{2.10}
\]

Through numerical simulations considering cases with monomodal and bimodal initial size distributions, as well as varying dispersion solvent mixture compositions, Liu showed that
monomodal distributions tend to have a slower growth rate in comparison to those that are bimodal. Similarly, in comparing dispersions that were not processed after production (i.e. the organic solvent was not removed), those made with less organic solvent experienced less growth. These simulation results were in agreement with experiment, as shown below in Figure 2.12, which considered β-carotene particles stabilized by PS1kD-b-PEG3kD. This work also showed that while the stabilizing polymer layer prevents aggregation of nanoparticles, the possibility exists (as was in this case) that this layer is not a significant diffusion barrier for the inner solute core; this suggests that the stabilizer will not have an important effect on solute release and dissolution.

![Figure 2.12](image-url)

**Figure 2.12** Ostwald ripening of β-carotene nanoparticles stabilized by PS1kD-b-PEG3kD produced at varying organic solvent compositions in the final dispersion solvent mixture. The simulated results are defined by the solid lines, as predicted using LSW theory; experimental sizes are displayed as symbols denoting the volume fraction of organic solvent in the final dispersion solvent mixture as follow: triangles - 20%, circles - 17%, filled diamonds - 13%, squares - 9%, open diamonds - 5%. Taken from reference [31].

However, the above treatment does not consider the case when the solute exists in a dissolved state in a co-solute that is loaded in nanoparticle cores. In such a state, the chemical
potential (or solubility) of the solute in the external aqueous phase is lowered. This is why the stabilization technique discussed in section 2.8 works, since the solution of the solute and the co-solute in the particle cores will result in a lower energy state than a pure solute phase that will tend to re-dissolve. Furthermore, since the co-solute should be highly hydrophobic, the net equilibrium solubility of the core solution will be lower than that of the pure solute, reducing the dissolution rate and growth rate.

In contrast to Ostwald ripening, particle aggregation is of less concern for FNP formulations because appropriate selection of the polymer stabilizer should yield dense hydrophilic layers on the particle surfaces. Such layers should effectively screen out any hydrophobic attractions amongst particles. However, if no affinity exists between the solute and the hydrophobic block of the chosen di-block copolymer, as was explained before, spotty surface coverage can result, which may lead to particle aggregation over time. Furthermore, for large particle sizes affected by gravity, sedimentation and/or flocculation can lead to phase separation, which may induce particle aggregation as fluctuations in the stabilizing layer will statistically present exposed hydrophobic surfaces over time.

With respect to solute integrity, proper caution must be exercised if the solute can be exposed to degradation pathways when dispersed. For example, Tien, in using the cholecalciferol nanoparticle formulation presented in Chapter 6, observed that the dialyzed liquid dispersion (i.e. no organic solvent in the dispersion) when stored at 4°C for 19 days experienced a statistically significant increase in size from 119 nm to 147 nm [107], without any change in the span range. This uniform increase in size was also accompanied with a decrease in UV-detectable cholecalciferol concentrations and no decrease in solids concentrations. Considering that cholecalciferol is known to undergo oxidative reactions, particularly in humid environments
[108]. Tien's observation most likely reflects the degradation of the solute into a UV-inactive product that produced structural changes within individual particles such that they grew in size. This undesirable change to the formulation was avoided by quickly freeze-drying the dispersion after dialysis.

2.9.2 How to improve stability

While several stability issues with the immediate FNP product have been discussed, solutions to these problems will be presented in the remaining subsections.

2.9.2.1 Liquid formulations

If the intended dosage form for the nanoparticulate formulation is a liquid dispersion, it is important to ensure that the organic solvent used in FNP be removed from the dispersion; this is necessary not only from a safety perspective, but also to mitigate the possibility for Ostwald ripening. It should be noted that while using a smaller fraction of organic solvent in the dispersion solvent mixture will slow down the ripening rate, using less organic solvent in the FNP process will also tend to increase particle size (section 2.5). Therefore, it is recommended that if the organic solvent accounts for an appreciable amount of the dispersion solvent mixture volume that it be removed quickly after FNP. Three methods for doing this have been considered in the literature. The first is dialysis against an aqueous bath. This is the traditional approach in the Prud'homme group for obtaining a dispersion in a completely aqueous medium. Since the organic solvents used in FNP must be water-miscible if the anti-solvent is aqueous, by dialyzing against a sufficiently large aqueous bath and replenishing the bath several times, the organic solvent can be reduced to minimal levels [109]. While this method is acceptable for the lab scale, it is not readily scalable. Thus, the second method used is a scalable version of dialysis, diafiltration. This method has not been reported in the existing literature for FNP formulations,
although ongoing research has been investigating optimal conditions for effectively removing organic solvent from FNP dispersions. This method has been used for other nanoparticle production techniques, such as the emulsification/solvent stripping method used by Bind Therapeutics [110]. The other method that has been used is flash evaporation. In this process, the introduction of the FNP effluent to a vessel with reduced pressure and increased temperature will result in the evaporation of a volatile organic solvent; more than one stage (or vessel) can be used to further remove residual solvent. Kumar & Prud'homme showed that for an initial feed with 9 wt% THF, a feed temperature of 57°C to the first stage, followed by a feed temperature of 45°C to the second stage, with a pressure of 2.96 kPa at both stages resulted in a reduction in the amount of THF in the dispersion to 0.37 wt%; this process design accomplished 96% THF removal and reached a THF level below the FDA approved limits [109]. However, flash evaporation requires that the organic solvent be sufficiently volatile and not form an azeotrope with water.

Considering that the existing FNP literature has not focused on the stability of a dosable liquid formulations, there are still various facets that need to be investigated. Of particular importance is storage under high temperatures, similar to the stability testing of dry dosage forms. Higher temperatures tend to increase the solubility of solutes, increasing Ostwald ripening growth rates. Such temperature effects on liquid FNP nanoparticle dispersions have not been investigated and might determine the feasibility of liquid formulations as a final dosage form.

2.9.2.2 Dry powder formulations

Contrastingly, dry powder formulations offer an alternative dosage form that can mitigate or avoid most problems associated with liquid formulations. Since nanoparticles, and their components, in a dry form have significantly reduced mobility, Ostwald ripening and
aggregation are irrelevant; nonetheless, solute integrity could still be of concern, depending on the stability of the solute upon storage. Moreover, since the dry nanoparticle powders must be reconstituted at some point for proper administration, it is imperative that the nanoparticles properly redisperse, which is one of the central focuses in drying nanoparticles.

While there exist many different techniques for converting liquid nanoparticulate dispersions into dry forms, the ones that been considered for FNP formulations in the literature are freeze-drying, spray freeze-drying, and spray drying. Freeze-drying and spray freeze-drying are similar techniques, which involve freezing of the liquid dispersion, followed by various heating steps under reduced pressures to sublime the ice and evaporate associated water and residual volatile organic solvent in the cryo-concentrate. More information on freeze-drying can be found in Chapter 6, as well as elsewhere [111, 112]. For spray freeze-drying, one step prior to freezing exists: the liquid is sprayed through a nozzle into a cryogenic liquid (usually liquid nitrogen), which is then boiled off before proceeding to the drying steps. The only advantage to spray freeze-drying is the production of spherical micron-sized aggregates of nanoparticles embedded in an excipient matrix, which can be beneficial for aerosol formulations [113]; the structure of these micro-aggregates is essentially determined by the composition within the frozen droplet. Spray drying, on the other hand, consists of spraying the liquid dispersion through a nozzle to create droplets that fall with (co-current) or into (counter-current) a hot gas that dries the droplets into micron-sized structures, determined by droplet dynamics while drying [114]. While spray drying can be carried out in a continuous mode, which freeze-drying cannot, the use of elevated temperatures may be detrimental to nano-construct integrity. All three methods for conversion to dry powders generally require the addition of excipients to prevent nanoparticle aggregation, particularly during drying, though in some cases, protection is required...
During the freezing steps in the respective methods. Since drying will involve the total removal of the solvent mixture, nanoparticles will be concentrated to the point that intimate contact, which can induce entanglements [115] or crystallization (as hypothesized in Chapter 6) of stabilizing polymers, can irreversibly bridge particles. Thus, proper formulation design is necessary to achieve dry powders that can be easily reconstituted to liquid nanoparticle dispersions with the same initial particle size distribution. However, this is beyond the scope of this review.

In the current literature, freeze-drying has only been considered for parenteral FNP formulations. Unfortunately, most examples have been lacking in full characterization of the lyophilized formulations and have only consider the redispersed particle size after reconstitution to gauge the feasibility of the formulation [34, 35, 87]. In contrast, the work described in Chapters 3, 4, and 6 has also involved measuring the percent recovery of API after reconstitution. However, various important findings exist in the current literature. For example, the work of D’Addio et al demonstrated that sucrose is not a great protectant excipient for β-carotene nanoparticles stabilized by PLGA7kD-PEG5kD, as a 60:1 sucrose to particle solids mass ratio was required to properly redisperse a lyophilized sample to the initial average particle size [87]. Kumar et al, in following the report of Layre et al for the lyophilization of polymeric nanoparticles produced using a thermodynamically-limited synthesis method, have shown that the dual use of Pluronic F68 and trehalose enabled particle size recovery of PS1.5kD-PEG5kD stabilized particles loaded with nitric oxide prodrugs [35]. Although requiring a considerable amount of Pluronic F68 and trehalose (9:1 and 11.4:1 excipient:particle mass ratios, respectively), this finding highlighted the possibility of polymeric excipients providing better protection to nanoparticles during lyophilization than sucrose. The work presented in this thesis
contributes greatly to the understanding of freeze-drying nanoparticles with a stabilizing PEG-layer produced using FNP. Not only is the superiority of polymeric excipients proved, it is shown that excipients that do not induce phase separation between excipient and particle are required for efficient protection during drying, since freezing does not induce appreciable particle aggregation. Furthermore, PEG-based excipients allow for more robust lyophilized formulations, since drying conditions are not as critical as with formulations containing sugars. Finally, the large difference in osmolarity contributions between polymeric and sugar excipients underline the potential in using polymeric excipients for producing highly concentrated formulations for parenteral administration. Please refer to the aforementioned chapters for more details.

With respect to spray freeze-drying and spray drying, D'Addio and associates have compared the use of both techniques to produce mannitol powders with embedded PLA3.7kD-b-PEG5kD stabilized cholesterol nanoparticles [116]. They showed that with spray freeze-drying, larger, more porous microspheres were produced in comparison to spray drying as shown in Figures 2.13a-d, which correspondingly reduced the extent of particle aggregation by increasing the inter-particle distance in the final solid structure. Reconstitution of a spray freeze-dried formulation containing equal masses of particles and mannitol produced only a 2.5-fold increase in particle size in comparison to the initial size when hand agitated for 30 seconds and sonicated for 1 minute, while reconstitution of a spray dried formulation consisting of 1:9 particles to mannitol (by mass) produced a 5.3-fold increase in particle size (Figures 2.13e-f). While spray drying involves the concentration of solids as the droplet size shrinks from solvent evaporation [117], thereby increasing particle-particle contacts, spray freeze-drying freezes the homogeneous distribution of particles and excipient from the liquid state resulting in less particle aggregation.
However, this does not mean that spray drying is not a viable method for conversion to dry powder. Shen and collaborators have produced SR13668-loaded PLGA nanoparticles of 150 nm in diameter through FNP which were then spray dried with a combination of trehalose and leucine as excipients [38].

Figure 2.13 Comparison of spray freeze-dried and spray dried nanoparticle aerosol carriers containing cholesterol-loaded PLA3.7kD-b-PEG5kD stabilized nanoparticles produced at a nominal solids concentration of 10 mg/mL. Scanning electron micrographs of formulations composed of 1:1 nanoparticles to mannitol by mass are shown at low magnification (a & c) and at high magnification (b & d) of spray-freeze-dried material (a-b) and spray dried material (c-d). Reconstitution of various dry formulations (with different nanoparticle loadings) at 10 mg/mL solids through 30 seconds of manual shaking followed by 1 minute of sonication in a bath sonicator at room temperature resulted in the sizes shown for e) spray-freeze-dried material and f) spray dried material. Taken from reference [116].

Spray drying of these particles was challenging because the PLGA used had a glass transition temperature of ~60°C and inlet temperatures in the range of 70 - 150°C were necessary for
sufficient drying; this resulted in various formulations with redispersed particle sizes of > 500 nm. However, they observed that to obtain the largest amount of sub-200 nm redispersed particles, it was necessary to introduce ethanol into the feed such that evaporative cooling would maintain a low enough temperature so that the particles would not become fluid and aggregate; using this technique, when using an inlet temperature of 75°C, a reconstituted dispersion with peak mean diameters of 622 nm and 139 nm were obtained, with 31% and 69% intensity attributed to the respective peaks. While API recovery was not assessed, in vivo data showed higher mice plasma concentrations of the API compared to another formulation in the literature when both were administered orally. Thus, spray drying can be feasibly used for dry FNP formulations, but requires that either particle size growth upon reconstitution be acceptable or the formulation must not have components with low glass transition temperatures or melting points.

2.10 CONCLUSIONS

We presented an overview of the current understanding of the rapid precipitation technique, Flash NanoPrecipitation, and various aspects of how a nanoparticulate formulation produced through FNP can be engineered. The potential of FNP to produce nanoparticles loaded with hydrophobic solutes at tunable sizes and at high loading efficiencies through a scalable process is quite attractive from a developmental perspective. Effective engineering and optimization of a formulation can allow for the administration of APIs at concentrations that hitherto have been impossible, such as the administration of progesterone for emergency traumatic brain injury [68]. Nonetheless, there are still various facets of FNP that currently require more research to produce improved formulations, such as how to improve mixing through aggregation zone reduction and the selection of the hydrophobic block in an amphiphilic
di-block copolymer. Likewise, the release and dissolution of APIs from FNP-produced nanoparticles is also a very important area of research that has yet to be addressed, which should be given more attention in order to assess the feasibility of formulation techniques for desired release profiles. Most likely, this phenomenon, which will depend on the structure and stability of the nanoparticle cores, will be intertwined with the actual production process of the nanoparticles that has been addressed here. Thus, we hope that this review can serve as a building block for future generations of FNP research.

### 2.11 NOMENCLATURE

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>API</td>
<td>active pharmaceutical ingredient</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>FNP</td>
<td>Flash NanoPrecipitation</td>
</tr>
<tr>
<td>MIVM</td>
<td>multi-inlet vortex mixer</td>
</tr>
<tr>
<td>CIJM</td>
<td>confined impinging jets mixer</td>
</tr>
<tr>
<td>CIJM-D</td>
<td>confined impinging jets mixer with dilution</td>
</tr>
<tr>
<td>CFD</td>
<td>computational fluid dynamics</td>
</tr>
<tr>
<td>DMP</td>
<td>2,2-dimethoxypropane</td>
</tr>
<tr>
<td>PLA</td>
<td>poly(D,L-lactide)</td>
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<tr>
<td>PS</td>
<td>polystyrene</td>
</tr>
<tr>
<td>PCL</td>
<td>poly(ε-caprolactone)</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactide-co-glycolide)</td>
</tr>
<tr>
<td>SY98</td>
<td>solvent yellow 98</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
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DMSO  dimethyl sulfoxide
GRAS  generally recognized as safe
FDA   United States Food & Drug Administration
PVP   polyvinylpyrrolidone
PVA   poly(vinyl alcohol)
CMC   critical micelle concentration
MAL   maleimide
pNP   \( p \)-nitrophenol
\( J \)  nucleation rate
\( A_i \)  pre-exponential factor, where \( i \) will denote the respective factor for
homogeneous or heterogeneous nucleation
\( \Delta G \)  excess Gibbs free energy
\( k_B \)  Boltzmann constant
\( T \)  absolute temperature
\( \gamma_{ij} \)  interfacial tension between phases \( i \) and \( j \), where possible subscripts include \( c, l, \) and \( s \), denote crystallizing solute, liquid, and foreign solid surface
\( v_0 \)  molar volume
\( S \)  supersaturation ratio
\( c \)  concentration
\( c_{eq} \)  saturated equilibrium solubility
\( D_{AB} \)  diffusion coefficient of solute A in solvent B
\( \beta \)  numerical factor that depends on the shape of a nuclei
\( \theta \)  contact angle between a crystallizing solute and a foreign substance
$G$  growth rate

$N_{Av}$  Avogadro's number

$r$  radius of a spherical particle

$B$  a constant

$R$  universal gas constant

$x$  a growth kinetic order determined through experimental fit

$\tau_{mix}$  characteristic mixing time

$\tau_{flash}$  induction, or formation time, of a nanoparticle

$\tau_{agg}$  characteristic aggregation time of an amphiphilic polymer

$\tau_{ng}$  rate-limiting nucleation and growth time of a solute

$k_i$  a reaction kinetics constant

$Re_{jet}$  jet Reynolds number, or the Reynolds number of one inlet stream

$Re_{tot}$  total Reynolds number, or the sum of Reynolds numbers of all inlet streams

$\Delta$  span range ($\Delta = d_{90} - d_{10}$)

$d_{10}$  10% of the intensity-weighted size distribution is smaller than this diameter

$d_{90}$  90% of the intensity-weighted size distribution is smaller than this diameter

$\mu$  kinematic viscosity

$\rho$  density

$M_A$  molecular weight

$pK_a$  logarithm of an acid dissociation constant

$c_r$  solubility of a sphere particle of radius $r$
2.12 REFERENCES


37. Saad, W., *Drug nanoparticle formation via flash nanoprecipitation: conjugation to encapsulate and control the release of paclitaxel*, in *Chemical Engineering*2007, Princeton University.


CHAPTER 3 - PROGESTERONE-LOADED NANOPARTICLES FOR
EMERGENCY TRAUMATIC BRAIN INJURY TREATMENT

Note: This chapter contains sections from the publication, "A Highly-Loaded Nanoparticulate Formulation of Progesterone for Emergency Traumatic Brain Injury Treatment. Therapeutic Delivery 3(11), 1269-1279 (2012)" and is used with permission.

ABSTRACT

Progesterone, a promising therapeutic for treating traumatic brain injury, has been difficult to formulate into a high dose/low volume form for emergency parenteral administration due to its hydrophobicity and crystallinity. This work demonstrates the use of Flash NanoPrecipitation through a lab-scale confined impingement jets mixer to produce ~300 nm progesterone-loaded nanoparticles with ~24 wt% loading using only components that are generally recognized as safe by the FDA. Approximately 80% of the loaded progesterone is dissolved into a fluid D-α-tocopherol nanoparticle core. For prolonged stability, the nanoparticles are freeze-dried with Pluronic F68 and can be reproducibly reconstituted by hand agitation for 1 minute without particle aggregation to produce injectable formulations with ~30 mg/mL progesterone, which is more than 10 times higher than has been previously reported. Rat plasma pharmacokinetics showed high progesterone bioavailability with peak concentrations being achieved less than an hour after administration. This formulation should allow for emergency administration of therapeutically viable concentrations of progesterone, which has been impossible with all previously reported nanoparticulate formulations because of low progesterone loadings and concentrations.
3.1 INTRODUCTION

The Centers for Disease Control estimates that 1.7 million people sustain a traumatic brain injury (TBI) annually, causing 52,000 deaths, which accounts for close to a third of all injury-related deaths in the United States [1]. There are 275,000 hospitalizations annually because of TBIs, which resulted in medical and indirect costs of $56.3 billion in 1995 [2] and $60 billion in 2000 [3] in the United States alone. Alarmingly, most intervention therapies used to manage the injuries focus on reducing intracranial pressure from inflammation and do not appreciably reduce the risk of death or disability [4]. Recently, neurosteroids have been identified as promising therapeutics that actually treat the inflammation. Progesterone is of particular interest because of its neuroprotective and neuroregenerative properties [5, 6] and its ability to reduce post-injury cerebral edema [7, 8], neuronal loss [9, 10], necrosis [11, 12], and secondary inflammation [11, 13, 14]. Human trials are currently being conducted to determine whether progesterone can be safely and effectively administered for TBI treatment [6, 15].

However, administering progesterone has been challenging because of its insolubility in water and tendency to crystallize, as well as the high doses needed for therapeutic activity. For emergency TBI treatment, it is anticipated that an intramuscular (i.m.) injection of at least 1 mg/kg progesterone may be required, translating to at least a 20 mg/mL dose, which is comparable to what has been dosed via i.m. bolus injections [6] and intravenous continuous infusions [15] in clinical trials. In attempt to formulate the neurosteroid such that it can be administered more easily, several nanoparticulate formulations of progesterone have been reported. For example, crystalline nanosuspensions stabilized by stearic acid have been produced, but at progesterone concentrations less than 2.7 mg/mL [16]. The most common approaches involve lipid nano-constructs loaded with progesterone or a steroid-cyclodextrin...
complex. Both Cavalli and Duchêne describe the complexation of the active pharmaceutical ingredient with 2-hydroxypropyl-β-cyclodextrin in order to increase the water solubility of progesterone [17, 18]. They utilize this technique to produce solid lipid nanoparticles with progesterone loadings of only 2 wt%. Yuan et al fabricated nanostructured lipid constructs with 12 wt% loadings at progesterone concentrations up to 2.6 mg/mL [19]. Memisoglu and colleagues produced nanospheres, using progesterone/β-cyclodextrin complexes, with less than 12 wt% loadings [20]. Polymeric nanoparticles have been made, however with loadings of less than 10 wt% [21]. None of these formulations report progesterone concentrations higher than 2.7 mg/mL, which means they fail to provide a feasible means for emergency TBI treatment in the form of a single i.m. injection.

The work presented here has two main goals. First, we describe a new progesterone formulation that addresses the current need for a feasible emergency TBI treatment. Lyophilized progesterone-loaded nanoparticle dispersions were produced that can easily be reconstituted in a clinical setting to achieve progesterone concentrations of up to ~30 mg/mL, which is suitable for emergency injectable dosing at a therapeutic level. Furthermore, since this formulation uses only FDA-approved biocompatible amphiphilic polymers and co-solutes, the use of toxic excipients (i.e. β-cyclodextrin [22]) and/or organic solvents (e.g. ethanol, DMSO) are not necessary to formulate vehicles for progesterone delivery.

Second, we consider the engineering processes involved in the production of the nanoparticle dispersions. The progesterone nanoparticles are produced by Flash NanoPrecipitation (FNP) which is a kinetically controlled, block copolymer directed assembly process. Progesterone is an interesting compound to consider because it of its low hydrophobicity and its high crystallinity. These two properties have previously proven
problematic in the production of nanoparticles through FNP because of low loadings and poor control over particle size due to high solubility in a mixed organic/aqueous dispersion solvent mixture and poor stability due attributed to Ostwald ripening. While there exist three different techniques to circumvent these problems, mentioned in section 2.8 of Chapter 2, one involves synthesizing a more hydrophobic derivative, which cannot be done in this case since progesterone has no reactive groups to which cleavable linkages can be attached. Another technique is only useful for compounds with ionizable groups, which again is not applicable to progesterone. Thus, we show how the neurosteroid can be made into nanoparticle form through FNP employing the technique of solubilization. Furthermore, the process for optimizing the nanoparticle formulation on a laboratory-scale confined impinging jet mixer with dilution (CIJM-D) is described, leading to freeze-drying and reconstitution of the best formulation. Attempts at translating formulations from the CIJM-D to a multi-inlet vortex mixer (MIVM) are also described.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Cholesterol (95%), progesterone (≥ 99%), and D-α-tocopherol (AT) (97%) were purchased from Sigma-Aldrich (USA). Tetrahydrofuran (THF) (HPLC grade) and acetone (ACS grade) were purchased from Fisher Scientific (USA). Poly(D,L-lactide)-block-poly(ethylene glycol) (PLA-b-PEG) (MW: 5kD-b-3.7kD) was generously supplied by Evonik Inc. (USA). Poly(ε-caprolactone)-block-poly(ethylene glycol)s (PCL-b-PEG) (MW: 3kD-b-5kD and 4kD-b-5kD) were synthesized by Dr. Margarita Herrera-Alonso based on the method by Shuai et al [23]. Poly(lactide-co-glycolide)-block-poly(ethylene glycol) (PEG-b-PLGA) (MW: 8kD-b-5kD)
was synthesized by Dr. Adam Wohl based on a novel synthesis method [24]. Pluronic F68 was supplied by BASF (Germany). Ultrapure water (MilliQ water) (18 MΩ·cm) was generated from a Barnstead Nanopure purification system.

### 3.2.2 Solubility Measurements

The solubilities of progesterone and AT were determined in MilliQ water and a 10 vol% THF in MilliQ water mixture. Each organic compound was first dissolved in sterile-filtered THF and then the organic solution was added to sterile filtered MilliQ water to result in 10 vol% THF in water. To pure sterile filtered MilliQ water, a small amount of the pure substances were added. The mixtures were stirred on a magnetic stir plate overnight. The samples were then filtered through 0.2 µm syringe filters (nylon; Pall Corporation, USA) and freeze-dried on a VirTis Benchtop 3.3 manifold lyophilizer (SP Scientific, USA) for 1 day (vacuum pressure < 200 mTorr, condenser temperature < -70°C). The dry solids were then dissolved in THF and analyzed through UV/visible spectroscopy.

### 3.2.3 Nanoparticle Formation

Block copolymer stabilized nanoparticles were produced using two different methods. The first made use of a laboratory-scale CIJM-D reported by Han et al [25]. Briefly, as an example, 1 mL of the organic stream containing the dissolved progesterone, block copolymer, and possibly co-excipients in THF in a disposable plastic syringe (National Scientific Company, USA) was simultaneously manually injected into the mixer at a syringe jet velocity of ~35 m/s against 1 mL of MilliQ water (or buffer) and collected into an 8 mL diluting reservoir of the aqueous phase. The resulting dispersion was 10 vol% THF. The nanoparticle samples were then either filtered through a 5.0 µm syringe filter (nylon; National Scientific Company, USA) and immediately freeze-dried (frozen within 5 minutes of manufacture) or had the THF removed via
a rotary evaporator (Rotovapor R-251, Büchi Labortechnik AG, Flawil, Switzerland) (101 torr, 37°C, ≥ 200 RPM, 20 minutes) and filtered.

The second method made use of a four-jet, or multi-inlet, vortex mixer (MIVM) described by Liu et al [26]. Depending on the amount of organic phase present in the final solvent mixture, different flow rates were used for the different streams, although all at turbulent mixing regimes. Three 50 mL gas tight glass syringes (SGE Analytical Science, Australia) were filled with MilliQ water and one 25 mL gas tight glass syringes (SGE Analytical Science, Australia) contained the organic phase. The streams were injected into the mixer using digitally controlled PHD 2000 syringe pumps (Harvard Apparatus, USA). The initial ~5 mL of effluent were discarded and then collection was started. Effluent collection was stopped prior to stopping the pumps. This ensured that only dispersion produced at steady state operation were collected.

3.2.4 Nanoparticle Dispersion Characterization

3.2.4.1 Particle size distribution measurement

After nanoparticle production and each processing step, the dispersions were characterized for particle size using a Malvern Zetasizer ZS ZEN 3600 (UK). Samples were prepared by diluting the dispersions at least 40-fold in MilliQ water and were contained in disposable 1.5 mL plastic cuvettes (Fisher, USA). All measurements were made with a 633 nm laser at a scattering angle of 173°. Sizes reported are those obtained using the general purpose normal resolution analysis mode of the intensity-weighted distribution. The peak means and span ranges ($\Delta = d_{90} - d_{10}$) are given. Refer to Appendix C for a discussion on why this reporting protocol is used. Usually, at least triplicate measurements were made and the metrics are given of at least two measurements that were averaged.
3.2.4.2 Progesterone concentrations

Progesterone concentrations were measured through UV/visible spectroscopy. An Evolution 300 UV/visible spectrophotometer (Thermo Electron Corporation, England) was used to construct a calibration curve for progesterone in the concentration range of 5-20 µg/mL ($R^2=0.996$) at $\lambda=230$ nm, yielding an extinction coefficient $\varepsilon=0.052$ cm$^2$/mg (path length of 1 cm). The solutions used for the calibration curve had the block copolymer and D-α-tocopherol present at the same concentrations as the progesterone. A THF solution containing only pure progesterone was made (10 µg/mL) and the absorbance spectra were compared to determine that there was no overlap of the progesterone peak with any of the other components (see Figure 3.1).

![Figure 3.1 Absorbance spectra of components.](image)

To determine the progesterone loading efficiency, a nanoparticle dispersion was taken immediately after formation and the THF was removed via a rotary evaporator. The dispersion was then filtered through an appropriate filter size; PVDF 0.45 µm syringe filters (Fisher Scientific, USA) were used for particles with a peak mean intensity diameter close to 100 nm and nylon 5.0 µm (National Scientific Company) for particles with a peak mean size close to 300 nm. The concentration, $c_{\text{measured}}$, was then measured as described previously. This concentration
were compared to the concentration in the organic stock solution used in the FNP process, \( c_{stock} \), which was also measured in the same manner. The loading efficiency (LE) of progesterone was calculated as follows:

\[
LE(\%) = \left( \frac{c_{measured}}{c_{stock}/10} \right) \times 100\%
\]  

(3.1)

To confirm that the progesterone measured in the filtered dispersions was in nanoparticle form, as opposed to in solution, the following control experiment was done. A THF stock solution (1 mL) of approximately 10 mg/mL progesterone was manually injected into the CIJM-D against an equal volume of MilliQ water and the effluent was collected into a water reservoir, resulting in 10 mL of 10 vol% THF. The THF was subsequently removed via a rotary evaporator (101 torr, 37°C, ≥ 200 RPM, 20 minutes) and aliquots were filtered through either PVDF 0.45 µm or nylon 5 µm syringe filters.

The initial THF progesterone stock solution was measured to have a progesterone concentration of 10.9 mg/mL; to measure this concentration, a sample was diluted to 13.6 µg/mL. The filtrates were diluted to have a nominal progesterone concentration of 12.1 µg/mL, assuming all progesterone had passed through to the filtrate. The resulting absorbance spectra did not show the characteristic progesterone peak (see Figure 3.2). From the absorbance values measured, the 5.0 µm and 0.45 µm filters resulted in filtrate concentrations that were 4.2% and 1.7% of the expected nominal concentrations, respectively. Therefore, the assay is expected to have < 5% error, which is acceptable.

For quantifying the loading of progesterone in the nanoparticle phase, a specific amount of freeze-dried nanoparticle powder without protectant excipients was weighed and dissolved in THF to a total solids concentration, \( c_{solids} \). UV/visible spectroscopy was used to determine the
concentration of progesterone, $c_{\text{measured}}$. The progesterone loading in the nanoparticles was then calculated as follows:

$$\text{loading} \, (\%) = \frac{c_{\text{measured}}}{c_{\text{solids}}} \times 100\% \quad (3.2)$$

**Figure 3.2** Comparison of absorbance spectra for progesterone stock solution and progesterone dispersions after THF removal and filtration.

### 3.2.5 Nanoparticle Dispersion Processing

#### 3.2.5.1 Freeze-drying

In order to obtain a dry nanoparticle powder, the dispersions were freeze-dried. First, aliquots of $\leq 3$ mL of the nanoparticle dispersions were loaded into 5 mL cryogenic vials (VWR, USA) and then submersed in a dry ice/acetone bath for at least twenty minutes. The vials were transferred into a pre-cooled ($<-70^\circ C$) flask, which was then hooked up to a VirTis Benchtop 3.3 manifold lyophiliizer (SP Scientific, USA). A condenser temperature of $<-70^\circ C$ was maintained, as well as a vacuum pressure $< 200$ mTorr. Samples were lyophilized for at least 1 day and then removed and immediately analyzed. In the case that nanoparticles were to be reconstituted after freeze-drying, Pluronic F68 powder was dissolved into the dispersions prior to freezing.
3.2.5.2 Reconstitution of lyo-cakes

When the lyo-cakes were to be reconstituted, the appearance of the cakes was first noted. Subsequently, the powders were rehydrated with MilliQ water or phosphate buffered saline (Fisher Scientific, USA) at specified volumes and then mildly manually agitated (~180-210 shakes per minute) for 1 minute. The reconstituted dispersions were then filtered (5.0 µm, nylon) and characterized for particle size distributions and progesterone concentrations.

To gauge the degree of redispersibility, the following metrics were calculated and compared. The redispersibility ratio, \( S_r/S_i \), is the ratio of the reconstituted peak mean intensity diameter of the main population to that of the initial dispersion prior to freeze-drying. Similarly, the span range ratio, \( \Delta r/\Delta i \), after reconstitution to prior freeze-drying is also considered. See Appendix D for more information on how these ratios were calculated and reported.

3.2.6 Imaging of Nanoparticles

Scanning electron microscopy was used to obtain micrographs of the nanoparticles. An XL30 FEG-SEM digital scanning microscope (FEI, USA) was used with a cold field emission cathode operated at 5 kV. Prior to imaging, nanoparticles were either drop-cast onto carbon tape and allowed to dry in a fume hood for at least 12 hours or the particles were freeze-dried and applied to carbon tape. The samples were then coated with a 5 nm thick iridium layer.

3.2.7 X-Ray Diffraction

Data was collected on an X’Pert Pro MPD (PANalytical, the Netherlands). The incidence beam optics consisted of 0.04 radian Soller slits, a \( \frac{1}{4}^\circ \) divergence slit, a 10 mm beam mask, and a \( \frac{1}{2}^\circ \) anti-scatter slit. The diffracted beam optics were an anti-scatter shield with a 5 mm anti-scatter slit, 0.04 radian Soller slits, a Nickel filter, and a Real Time Multiple Strip X’Celerator detector. Samples were lightly packed in a rectangular shallow chamber of an aluminum sample holder. All samples were scanned in Bragg-Brentano (\( \theta \)-2\( \theta \) scan) configuration using a step size
CHAPTER 3  Progesterone-Loaded Nanoparticles

of 0.017° at 10 s/step. The scan range was 5-52° in 20 The X-ray radiation was a Cu Kα, λ = 1.5419 Å. Data reduction and analysis was performed with MDI JADE 9+ software (Materials Data Incorporated, USA).

3.3 RESULTS AND DISCUSSION

3.3.1 Flash Nanoprecipitation Without a Co-Solute

In attempting to formulate progesterone into nanoparticle dispersions, FNP was first tried using only progesterone and an amphiphilic block copolymer, PLA3.7kD-b-PEG5kD. This block copolymer was used because the lactide monomer and PEG are generally recognized as safe (GRAS) by the FDA [27] and both polymers are approved for parenteral use [28], which would be the ideal administration route. A THF solution containing 200 mg/mL of progesterone and 200 mg/mL of PLA-b-PEG was mixed against MilliQ water on a CIJM-D, diluting the components to 20 mg/mL. The resulting mixture was cloudy and large aggregates were present. Subsequent filtration through a nylon 5.0 µm syringe filter yielded a filtrate that was slightly opalescent with no visual precipitate. Particle size measurements on the filtrate revealed only one population, consisting of micelles with a peak mean size of 38 nm and a span range of 38 nm (Figure 3.3a). However, these micelles contained relatively low levels of progesterone with only a 9.5% loading efficiency.

The poor loading efficiency achieved when FNP is attempted with only the steroid and an amphiphilic block copolymer can be understood by considering the physical properties of progesterone. Table 3.1 shows that while the aqueous solubility of progesterone is less than 10 µg/mL, it is surprisingly soluble in a 10 vol% THF in water mixture at approximately 0.42 mg/mL. This high solubility in the final dispersion solvent mixture can help for progesterone transiently loaded in a nanoparticle to leach out through the stabilizing polymer shell and...
recrystallize into macro-precipitates. Furthermore, the rapid precipitation process might initially trap progesterone in a disordered state that can crystallize over time as the fraction of THF drops locally through diffusion, finally resulting in destabilization of the particles and macro-precipitation.

![Figure 3.3 Particle size distributions for progesterone FNP formulations.](image)

**Figure 3.3** Particle size distributions for progesterone FNP formulations. Full symbols represent the particles after FNP, while open symbols are after THF removal and filtration. The individual formulations are as follows: a) no co-solute (macroscopic progesterone crystals formed during FNP and no size measurement was possible without filtration), b) cholesterol (2:1:1), c) prednisone cosanyl diglycolate (2:1:1), d) D-α-tocopherol (2:1:1 is represented with squares and 1:1:1 is represented with circles). The ratios given represent the mass ratio of block copolymer : progesterone : co-solute.

There is also low affinity between progesterone and the hydrophobic polymer block PLA3.7kD, as the 3D distance between the Hansen solubility parameters is 8.4; this explains why so little of the initially polymer-stabilized compound remained in the micelles. Please consult Appendix E for more information on solubility parameters. Considering that the ALOGPs of progesterone is
relatively low (3.58), the behavior observed here is reasonable, as FNP of compounds with low log $P$ values and high crystallinity is challenging. Refer to Appendix A for more information on log $P$ values and ALOGPs.

<table>
<thead>
<tr>
<th>Table 3.1</th>
<th>Solubilities of progesterone and D-α-tocopherol in solvents used in FNP. Values reported are the mean, followed by the standard deviation, of n=2 measurements.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compound</strong></td>
<td><strong>MilliQ Water (µg/mL)</strong></td>
</tr>
<tr>
<td>Progesterone</td>
<td>9.0 ± 0.6</td>
</tr>
<tr>
<td>D-α-Tocopherol</td>
<td>0.0 ± 0.2</td>
</tr>
</tbody>
</table>

### 3.3.2 Incorporation of Co-Solutes for Improving Progesterone Loadings

In evaluating methods of achieving higher progesterone loadings, the incorporation of co-solutes to stabilize the steroid in the particle cores was assessed. Hydrophobic co-solutes could increasing the rate of incorporation of progesterone through heterogeneous nucleation [29], thereby reducing recrystallization of progesterone into macro-precipitates. Initially, three compounds with relatively high log $P$ values (ALOGPs > 7) that can be loaded into block copolymer-stabilized nanoparticles were evaluated: cholesterol, prednisone cosanyl diglycolate, and D-α-tocopherol. The co-solutes were also chosen such that there should be high affinity between the co-solutes and progesterone based on solubility parameters. Previous work has demonstrated that FNP can be used to produce stable nanoparticles loaded with cholesterol [30] and D-α-tocopherol [30, 31]. Prednisone cosanyl diglycolate, which is a hydrophobic derivative of the corticosteroid prednisone made using the conjugation scheme published by Ansell and coworkers [32] (see Appendix F), was also attempted as a co-solute. The compounds, along with progesterone are displayed in Figure 3.4.

For these screening studies, THF solutions of 20 mg/mL PLA-$b$-PEG, 10 mg/mL progesterone, and 10 mg/mL co-solute (2:1:1 PLA-$b$-PEG : progesterone : co-solute) were mixed against MilliQ water using a CIJM-D, resulting in a 10 vol% THF in water mixture.
Figure 3.4 Compounds used in the dilute FNP trials. a) progesterone, b) cholesterol, c) prednisone cosanyl diglycolate, d) D-α-tocopherol.

Table 3.2 Estimated logP and solubility parameters for compounds from Figure 3.4. $\delta_d$, $\delta_p$, $\delta_h$ are the 3D Hansen solubility parameters for dispersion forces, dipolar interactions, and hydrogen bonding, respectively; $\delta_{tot}$ is the 1D collapsed Hansen solubility parameter; $r_{p,i}$ is the distance between the 3D solubility parameters of progesterone and co-solute $i$; $|\delta_p - \delta_i|$ is the distance between the 1D solubility parameter of progesterone and co-solute $i$. Refer to Appendices C & E for information on ALOGPs and solubility parameters, respectively.

| Compound                        | MW     | ALOGPs | $\delta_d$ (MPa$^{1/2}$) | $\delta_p$ (MPa$^{1/2}$) | $\delta_h$ (MPa$^{1/2}$) | $\delta_{tot}$ (MPa$^{1/2}$) | $r_{p,i}$ (MPa$^{1/2}$) | $|\delta_p - \delta_i|$ (MPa$^{1/2}$) |
|---------------------------------|--------|--------|--------------------------|--------------------------|--------------------------|---------------------------|--------------------------|----------------------------------|
| Progesterone                    | 314.51 | 3.58   | 18.6                     | 3.9                      | 3.8                      | 19.3                      | -                        | -                                |
| Cholesterol                     | 386.73 | 7.02   | 17.5                     | 1.2                      | 7.2                      | 19.0                      | 4.5                      | 0.4                              |
| Prednisone cosanyl diglycolate  | 755.15 | 7.74   | 18.2                     | 2.5                      | 8.1                      | 20.1                      | 4.6                      | 0.7                              |
| D-α-tocopherol                  | 430.79 | 8.84   | 17.6                     | 1.6                      | 7.4                      | 19.2                      | 4.4                      | 0.2                              |
For the cases of cholesterol and prednisone cosanyl diglycolate, the resulting dispersions were opalescent, indicating small particle sizes, and there was no visual precipitation. However, during the removal of the THF, there was rapid macro-crystallization which was most likely progesterone drug substance. Figures 3.3b and c show the particle size distributions for the nanoparticle formulations. After THF removal and subsequent filtration of the progesterone precipitate, the size distributions were less polydisperse. The use of steroidal co-solutes did not significantly help to load progesterone in nanoparticle form. It may be that the nucleation that occurred during FNP was not really heterogeneous, leading to formation of particles solely loaded with either solute or distinct solute domains within the particle cores; either case would not reduce the chance of leaching from the core and recrystallizing as macro-precipitates.

Contrastingly, D-α-tocopherol is a GRAS oil [33] that not only can act as a nucleating agent, it also provides a liquid core in which progesterone can remain in a stable dissolved state, preventing recrystallization. Both Peat [34] and Sonne [35] have reported significant solubility of progesterone in α-tocopherol of at least 25 wt%. The FNP trial incorporating AT resulted in an opalescent dispersion and size measurements showed a narrow monomodal distribution with a peak mean size of 69 nm and a span range of 67 nm. This experiment was repeated using 10 mg/mL PLA-b-PEG in the organic stream in order to increase the progesterone loading in the nanoparticles. There was a slight increase in peak mean size to 84 nm and in span range to 85 nm. In both cases, while some macroscopic crystals formed upon THF removal, the particle size distribution did not change, implying that the crystallization was most likely due to solubilized progesterone in the THF/water mixture that precipitated upon THF removal. The loading efficiency was measured to be 43% in the 1:1:1 PLA-b-PEG : progesterone : AT formulation.
There was also no significant change in particle size after being stored at T=4°C for one day after THF removal.

These experiments show that while all co-solutes had relatively similar affinity to progesterone and sufficiently high logP values for satisfactory nanoparticle formation alone, it was only the oil that was able to reduce recrystallization of progesterone. Table 3.2 presents the estimated properties. Interestingly, while the 3D distances between the Hansen solubility parameters of the co-solutes and progesterone are between 4.4 and 4.6, the values 1D distance (|δ_p - δ_i|), where p is progesterone and i is the co-solute, are between 0.2 and 0.7. See Appendix E for commentary on solubility parameters. Considering how the polarity of progesterone is not particularly strong, the collapsed solubility parameters were considered instead, since a Hildebrand-type parameter should be valid when dipolar interactions are not significant. These solubility parameters predict that all co-solutes should have similar results in terms of stabilizing progesterone within a nanoparticle core. However, being that cholesterol and prednisone cosanyl diglycolate precipitate as solids, if progesterone did not heterogeneously nucleate with these co-solutes, recrystallization of the neurosteroid would not be halted. Contrastingly, even if progesterone does not experience heterogeneous precipitation with AT during FNP, growing nuclei can partition into the hydrophobic D-α-tocopherol domains prior to block copolymer stabilization stopping growth. Therefore, although solubility parameters can help in predicting whether two compounds are compatible, they do not give any information regarding the manner in which the compounds will precipitate.

3.3.3 Optimization of the D-α-Tocopherol Nanoparticle Formulation

In the optimization of a nanoparticle formulation, it is important to set output variable criteria that will decide the best formulation. For example, in this work, while particle size
should be such that the dispersions can be feasibly injected into patients (< 5.0 µm), it is not
critical that the particles be O(100 nm) which is desirable for cancer treatments to make use of
the enhance permeability and retention effect. However, distributions should have only one clear
size population present. Furthermore, since the practical application of the study is a deliverable
progesterone dose, the loading efficiency should be as high as possible, such that the
progesterone loading in the nanoparticles is maximized. These criteria were most important for
the work presented. Factors that can be optimized for nanoparticle formulations include choice of
cosolute, choice of stabilizing polymer, relative amounts of components, total solids
concentrations, choice of organic solvent, and the fraction of organic solvent in the dispersion
solvent mixture. We have already presented how the ideal cosolute was chosen. Next, we
present the optimization of the other formulation parameters; the last two factors, which are
process parameters, were not evaluated.

While the experiments described in the previous section proved that D-α-tocopherol can
be used to produce nanoparticles with improved progesterone loadings, the dispersion was
relatively dilute in terms of progesterone (< 0.6 mg/mL). To produce more concentrated
dispersions, formulation parameters were investigated to make particles with higher progesterone
loadings. Solutions were prepared at the concentrations listed in Table 3.3 and mixed against
MilliQ water using a CIJM-D resulting in 10 mL of a 10 vol% THF in water dispersion. It should
be noted that organic solutions were made by first dissolving the D-α-tocopherol in THF,
resulting in an increased volume of solvent (AT is a liquid), and then dissolving the progesterone
and block copolymer in the mixed organic solvent; thus, the amount of component per mL of
THF is reported for simplicity.
Table 3.3 Compositions used in FNP optimization trials. Component concentration units are defined as milligrams of component per milliliter of THF added.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Progesterone (mg/mL)</th>
<th>AT (mg/mL)</th>
<th>PLA-b-PEG (mg/mL)</th>
<th>Peak Mean Diameters (nm)</th>
<th>Span Range (nm)</th>
<th>LE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>10</td>
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<td>200</td>
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<td>451</td>
<td>69</td>
</tr>
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<td>300</td>
<td>200</td>
<td>410, 3645</td>
<td>3196</td>
<td>74</td>
</tr>
<tr>
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<td>400</td>
<td>200</td>
<td>1106, 832, 4850</td>
<td>5128</td>
<td>71</td>
</tr>
</tbody>
</table>

3.3.3.1 Total solids concentration

The first parameter that was evaluated was the concentration of the components while maintaining a 1:1:1 mass ratio of PLA-b-PEG : progesterone : AT. The results show that as concentrations in the organic stream increased, the particle size, span range, and loading efficiency increased (Figure 3.5a-b). Similar size behavior was observed in Chapter 4 for GW771806X/pamoic acid nanoparticles stabilized by the same PLA-b-PEG, as well as in Chapter 5 for indomethacin, fenofibrate, or cinnarizine-loaded AT nanoparticles stabilized by D-α-tocopheryl polyethylene glycol 1000 succinate. Likewise, in the literature, comparable observations were made for cinnarizine/pamoic acid particles stabilized by PLA-b-PEG [36] and particles composed of D-α-tocopherol succinate and paclitaxel conjugated to D-α-tocopherol succinate stabilized by PCL7kD-b-PEG5kD [37].

An increase in size due to increasing total solids concentrations can be explained by accelerating growth kinetics as described in section 2.5 of Chapter 2. The increase in solids content is equivalent to increasing the supersaturation ratio experienced in the FNP process.
Equation 3.3 defines the supersaturation ratio, $S$, as the ratio of total solute concentration added to the precipitation, $c_{\text{total}}$, to the solubility of the solute in the medium, $c_{\text{eq}}$.

$$S = \frac{c_{\text{total}}}{c_{\text{eq}}} \quad (3.3)$$

Equation 3.4 shows the classical expression for the nucleation rate $J$ as a function of a pre-exponential factor $A$, the interfacial tension between the solute and the fluid $\gamma$, the solute molar volume $v$, the Boltzmann constant $k_B$, absolute temperature $T$, and the supersaturation ratio.

$$J = A \exp \left[ -\frac{16\pi\gamma^3v^2}{3k_B^2T^3(\ln S)^2} \right] \quad (3.4)$$

Figure 3.5 Optimization of the progesterone nanoparticle formulation, where in a) & b) the concentration of all components were varied while maintaining a 1:1:1 mass ratio for the three components, and in c) & d) the D-$\alpha$-tocopherol concentration was varied while keeping the PLA-b-PEG and progesterone concentrations at 200 mg of component per 1 mL of THF.
For homogeneous nucleation, classical precipitation theory predicts an increase in nucleation rates when there is an increase in the supersaturation ratio (equation 3.4), which should then result in a faster consumption of the supersaturated material as the co-solutes nucleate from the solvent mixture medium. This should translate to a larger number of smaller particles. However, as this was not the case for the experimental results, it is probable that the increase in supersaturation resulted in much faster growth as compared to nucleation. Based on these results, it was decided to continue with the most concentrated nanoparticle formulation, trial 4; although the size distribution of trial 4 was broader than trial 3 while having the same loading efficiency, the progesterone concentration was two times higher for trial 4.

### 3.3.3.2 Relative concentration of D-α-tocopherol

Next, the effects of AT concentration on the particle size distribution and loading efficiency were investigated while maintaining the individual concentrations of PLA-b-PEG and progesterone at 200 mg per mL of THF. Increasing D-α-tocopherol concentration yielded broader size distributions with larger particle sizes (Figure 3.5d), as well as higher loading efficiencies (Figure 3.5c). Beyond 200 mg of AT per mL of THF, the particle size distributions became more polydisperse, up to the point where at 400 mg it was very hard to control the size. By increasing the concentration of AT in the FNP process, the length scale for AT molecules to meet and form droplets decreased, allowing for more growth before polymer stabilization. These results highlight the same behavior as was discussed for the optimization of total solids concentrations.

Furthermore, the addition of AT into the nanoparticle composition should have reduced the fraction of soluble neurosteroid in the dispersion solvent mixture. Since progesterone is soluble in D-α-tocopherol, it will partition into the oil phase which will precipitates as
nanoparticles, resulting in a lower progesterone concentration in the aqueous solution that could macroscopically precipitate. However, the increase in D-α-tocopherol did not allow for a loading efficiency higher than ~70%. It was predicted that as more AT was added, a larger fraction of the progesterone would be solubilized by the oil, to the point where trial 9 should have resulted in all the progesterone being solubilized (refer to section 3.3.5 for solubility data). Since there was no increase in loading efficiency beyond a 1:1 mass ratio of AT to progesterone, a mismatch in the precipitation kinetics would explain why ~30% of the progesterone was always lost beyond 200 mg of AT per mL of THF; the nucleation might have been homogeneous for each species, with partitioning of the steroid into AT droplets explaining how progesterone remained in the particle cores. However, in terms of optimization, it was desirable to use as little D-α-tocopherol as possible, while reaching the maximum loading efficiency. Therefore, trials 4 or 7 were the best.

It is important to note that while both trials had identical formulation compositions, different particle size distributions were obtained; this is attributed to irreproducible syringe expression into the CIJM-D, which is one of the limitations of using this mixer.

3.3.3.3 Choice of di-block copolymer

The choice of the stabilizing block copolymer was subsequently evaluated. Based on the two previous optimization studies, it was important to have progesterone and AT present at a 1:1 mass ratio and at a concentration of 200 mg of component per mL of THF. A comparison to the initial 1:1:1 composition at a nominal progesterone concentration of 1 mg/mL (section 3.3.2) using PCL3kD-b-PEG5kD instead resulted in a higher loading efficiency (81% vs. 43%), but the particle size was also larger (peak mean size: 117 nm vs. 90 nm; span range: 118 nm vs. 88 nm). The difference in particle size distributions was markedly greater when scaling up the concentration. Using 200 mg PCL4kD-b-PEG5kD, 113 mg progesterone, and 113 mg AT per
mL of THF resulted in a polydisperse distribution with peak mean sizes of 1540, 223, and 4927 nm with a span range of 2747 nm. Similar results were observed at high concentrations when evaluating a PLGA8kD-PLA-b-PEG5kD. Since the PLGA3.7kD was the best hydrophobic block for the block copolymer when performing FNP at high solids concentrations, it was used for all subsequent experiments.

3.3.3.4 Relative concentration of block copolymer

Lastly, the concentration of the stabilizing block copolymer was investigated. Taking trial 6 as an example, conditions were attempted with 133 and 267 mg/mL PLA-b-PEG instead. As is shown in Table 3.4 and in Figure 3.6, increasing the polymer concentration yielded tighter distributions without affecting the loading efficiency. This suggests that the polymer only contributed to halting particle growth in the FNP process; it was not a critical factor in the loading process of the progesterone into the particle cores, once again suggesting little interaction between the PLA block and the solutes. Consequently, larger ratios of block copolymer could be used to produce smaller nanoparticles. However, since the block copolymer was the most expensive component in the nanoparticle formulation and since it was desirable to have higher progesterone loadings, all further studies used the 1:1:1 mass ratio.

From the optimization experiments, it was concluded that using 200 mg of each component (D-α-tocopherol, PLA-b-PEG, and progesterone) (1:1:1) per 1 mL of THF in the organic stream yielded the dispersion with the optimal progesterone concentration and loading efficiency at the cost of larger particle size distributions. At these conditions, FNP resulted in a dispersion that was very turbid due to the high concentration and there were some macro-precipitates present. However, after THF removal and filtration, there was a monomodal distribution with an peak mean size of O(300 nm). The progesterone loading efficiency was
measured to be 69%, yielding a loading of ~24%, and a progesterone concentration in the dispersion of ~10 mg/mL.

Table 3.4 FNP trials highlighting the effect of block copolymer concentrations on particle size distributions and progesterone loading efficiencies. Component concentration units are defined as milligrams of component per milliliter of THF added.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Progesterone (mg/mL)</th>
<th>AT (mg/mL)</th>
<th>PLA-b-PEG (mg/mL)</th>
<th>Peak Mean Diameters (nm)</th>
<th>Span Range (nm)</th>
<th>LE (%)</th>
</tr>
</thead>
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<td>100</td>
<td>133</td>
<td>350, 4199</td>
<td>627</td>
<td>43</td>
</tr>
<tr>
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<td>200</td>
<td>100</td>
<td>200</td>
<td>243</td>
<td>319</td>
<td>45</td>
</tr>
<tr>
<td>11</td>
<td>200</td>
<td>100</td>
<td>267</td>
<td>245</td>
<td>248</td>
<td>47</td>
</tr>
</tbody>
</table>

Figure 3.6 Particle size distributions for trials from Table 3.4.

3.3.4 Freeze-Drying and Reconstitution of Progesterone-Loaded Lyo-Cakes

Considering that nanoparticle dispersions produced through FNP are metastable, since they are kinetically frozen constructs, it is reasonable that they are generally not stable over long periods of time. For pharmaceutical products, long shelf life is desirable for patient convenience and compliance, as well as for reproducible efficaciousness. Thus, it is common for nanoparticle dispersions to be isolated into a dry state, where mobility is greatly reduced and stability can be prolonged. For this work, we used freeze-drying to obtain dry lyophilizates.
A nanoparticle dispersion with a peak mean size of 319 nm and a progesterone concentration of 9.9±0.6 mg/mL was split into two different aliquots: one was filtered (nylon 5.0 µm) and one was not. For freeze-drying, Pluronic F68 was added into each batch at 40 mg/mL. Pluronic F68 was used as a protectant excipient because it is a GRAS [38], high molecular weight polymer that allows for good redispersability of nanoparticles while not making the dispersion hypertonic (see Chapter 6). Three 2 mL aliquots from each batch were freeze-dried.

Each lyophilized sample was rehydrated with 2 mL MilliQ water and hand agitated for 1 minute. The dispersions were then filtered (nylon 5.0 µm) and expressed through 25-gauge needles. The particle size distributions and progesterone concentration were measured in between each step. The filtration step would be done for parenteral administration in order to avoid macro-aggregates causing blockages or embolisms in blood vessels. The 25-gauge needle was chosen since a fine gauge needle is anticipated for emergency TBI therapy. When comparing the samples that were filtered prior to freeze-drying to those that were not, there was no statistically significant difference in progesterone concentration, peak mean size, or span range (p > 0.05); this suggests that any macro-precipitates present in the non-filtered samples did not induce more particle aggregation. The lyo-cakes reconstituted well to an average peak mean size of 320 ± 23 nm with an average span range of 415 ± 72 nm, as compared to the initial peak mean size of 319 ± 14 nm and span range of 453 ± 20 nm. There was also no statistically significant change in particle size distribution after subsequent filtration (nylon 5.0 µm) (p > 0.05) or expression through 25-gauge needles (p > 0.05).
Figure 3.7 Average particle size distributions from Table 3.5 for: a) trials A-F, b) trials I-III.

Table 3.5 Reconstitution results of lyophilized progesterone dispersions. Recon = after reconstitution, Filt = after subsequent filtration through 5.0 µm nylon syringe filter, Expr = after expression through a 25-gauge needle

<table>
<thead>
<tr>
<th>Trial</th>
<th>Filtered Before Freeze-Drying?</th>
<th>Progesterone Concentration (mg/mL)</th>
<th>S/Si</th>
<th>Δ/Δi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recon</td>
<td>Filt</td>
<td>Expr</td>
<td>Recon</td>
</tr>
<tr>
<td>A</td>
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<td>9.3</td>
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<td>9.8</td>
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<td>II</td>
<td>Y</td>
<td>-</td>
<td>31.4</td>
<td>26.7</td>
</tr>
<tr>
<td>III</td>
<td>Y</td>
<td>-</td>
<td>32.2</td>
<td>32.3</td>
</tr>
</tbody>
</table>
Figure 3.7a displays the average particle size distributions upon reconstitution. Moreover, the samples achieved average progesterone concentrations of 10.7±0.2 mg/mL after reconstitution, 9.5±0.4 mg/mL after filtration, and 9.5±0.4 mg/mL after expression. There was no statistically significant difference in concentration between filtration and expression (p > 0.05). There was a ~10% loss of progesterone when filtering, which is most likely due to aggregates that cannot be broken up with hand agitation. Individual concentrations for each trial are listed in Table 3.5 (trial A-F), where there was little variability amongst all samples and all trials resulted in redispersibility and span range ratios < 1.1 once expressed through the needles.

The stability of reconstituted dispersions was also evaluated, as it is important that the dispersions maintain constant physicochemical properties from reconstitution until administration. Four lyophilized samples were reconstituted in PBS by hand agitation for 1 minute. Two samples were kept at room temperature on a bench top, while two samples were stored at T=4°C in a refrigerator. Progesterone concentrations and particle size distributions were monitored over time. Figure 3.8 shows the ratios of the metrics at different times compared to the initial time point. While the effect of temperature on particle size distributions was not statistically important (p > 0.05), the drop in concentration was statistically greater at the lower temperature. There was a 9% loss of progesterone over the course of 48 hours at room temperature, while it was a 19% loss at T=4°C. This higher loss at lower temperature may be due to a possible decrease in progesterone solubility in AT, resulting in rejection of insolubilized progesterone from the particle cores. Nonetheless, for practical emergency TBI treatment, the time between reconstituting a powder and administering the resulting dispersion should be minutes rather than days. Thus, the reconstituted dispersions should be administered within the
first 6.5 hours after reconstitution for there to not be any statistically significant difference in dispersion properties.

![Figure 3.8](image)

**Figure 3.8** Stability of reconstituted progesterone dispersions at room temperature and at T=4°C. Values displayed are defined as follows: $x_t/x_i$ is the ratio of the metric $x$ at time $t$ to the initial time point $i$, where $c$ is progesterone concentration, $S$ is peak mean intensity diameter, and $\Delta$ is span range.

Since the lyophilized powders were successfully reconstituted to the original progesterone concentrations, the next step was to determine if reconstitution could be used to reach higher concentrations for therapeutic use. Using the same nanoparticle formulation, but with a lower Pluronic F68 ratio of 1:2 protectant to particles for freeze-drying, powders were prepared and subsequently reconstituted in PBS at ~30 mg/mL progesterone, filtered (5.0 µm), and expressed (25 gauge). Figure 3.7b shows that this formulation had the same excellent redispersability with no particle aggregation.
Concentrations of ~30 mg/mL progesterone were reached (Table 3.5, trials I-III), which is higher than what was administered in the aforementioned clinical trials. While the variability amongst the three samples was much more than in the trials with lower reconstituted concentrations, this may be attributed to the use of less stabilizing protectant and error associated with the greater dilution necessary for characterization as compared to the previous trials. However, the mean redispersibility and span ranges were < 1.1 after needle expression, which is excellent for a lyophilized formulation.

3.3.5 Solid State Characterization

In order to have a better understanding of the physical nature of the progesterone particles formed through FNP, it was important to perform solid state characterization. Scanning electron microscopy was used to verify the spherical geometry of the particles. A 10 mg/mL progesterone nanoparticle dispersion was imaged before and after freeze-drying. The prismatic shape of the progesterone crystal that crystallized from solution during FNP without incorporation into an AT domain (Figure 3.9b) reflects the crystal habit of progesterone. The spherical shape of the particles in the dried dispersion (Figure 3.9c) and in lyophilized powders (Figure 3.9d) indicate the fluid nature of the D-α-tocopherol rich core into which the progesterone is solubilized. After freeze-drying with Pluronic F68, the progesterone nanoparticles are embedded in a polymeric matrix that isolated individual primary particles, which is seen in Figure 3.9d. This matrix does not allow for widespread particle aggregation and enables redispersion. Without protectant excipients, nanoparticle aggregation is generally observed during freeze-drying [39, 40].

X-ray powder diffraction was then used to determine the state of the progesterone within the nanoparticles. The diffraction pattern of the lyophilized nanoparticle powders revealed that some progesterone was present in a crystalline state in the nanoparticle cores, as the
characteristic diffraction peaks of the bulk progesterone at 10.6°, 12.7°, 14.6°, 15.3°, 16.9°, and 26.5° (2θ) were also present for the freeze-dried powder (Figure 3.9a). The diffraction patterns match the pattern of the α-form of progesterone [41, 42], which is the more thermodynamically stable polymorph when compared to the β-form [42, 43]. Furthermore, analysis of peak areas indicated that ~19% of the 70% loaded progesterone was crystalline. Considering that the steroid is very soluble in the tocopherol core, the remaining ~81% non-crystalline progesterone was most likely in a dissolved state, resulting in a ~36 wt% progesterone solution in the core. This value for the solubility limit of progesterone in α-tocopherol is reasonable, as UV/vis spectroscopy and HPLC yielded values of ~37 wt% and ~38 wt% solubility, respectively.

**Figure 3.9** Solid state characterization of progesterone nanoparticles. a) X-ray powder diffraction of bulk progesterone (top - dotted/dashed line), Pluronic F68 (upper middle - dotted line), a physical mixture of the formulation components with no α-tocopherol (lower middle - dashed line), and lyophilized progesterone nanoparticles (bottom - solid line). SEM micrographs of (b) crystallized progesterone that has precipitated from nanoparticles, (c) nanoparticles before freeze-drying, where the black marking encircles a primary particle, (d) lyophilized progesterone nanoparticles in the Pluronic matrix.
3.3.6 Scale-Up Using a Multi-Inlet Vortex Mixer

While the bulk of the work presented here was performed using a CIJM-D particle formulation, some experiments were done attempting to translate the nanoparticle formulation to a MIVM. Metrics for selected representative trials are presented in Table 3.6. Trial i is the direct translation of the optimal CIJM-D nanoparticle formulation onto the MIVM.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Progesterone (mg/mL)</th>
<th>AT (mg/mL)</th>
<th>PLA-b-PEG (mg/mL)</th>
<th>THF (vol%)</th>
<th>Peak Mean Diameter (nm)</th>
<th>Span Range (nm)</th>
<th>LE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>10</td>
<td>519, 4469</td>
<td>949</td>
<td>-</td>
</tr>
<tr>
<td>ii</td>
<td>113</td>
<td>113</td>
<td>200</td>
<td>10</td>
<td>387, 4767</td>
<td>546</td>
<td>-</td>
</tr>
<tr>
<td>iii</td>
<td>56</td>
<td>56</td>
<td>100</td>
<td>10</td>
<td>332, 5015</td>
<td>459</td>
<td>-</td>
</tr>
<tr>
<td>iv</td>
<td>38</td>
<td>38</td>
<td>50</td>
<td>10</td>
<td>260</td>
<td>278</td>
<td>96</td>
</tr>
<tr>
<td>v</td>
<td>178</td>
<td>178</td>
<td>321</td>
<td>6.25</td>
<td>354</td>
<td>360</td>
<td>58</td>
</tr>
</tbody>
</table>

The main peak mean diameter was ~60% larger, while the span range was > 100% larger. The poor translation from CIJM-D to MIVM is most likely due to the different supersaturation rates that are achieved on the two mixers. While the CIJM-D first has a 1:1 mixing of organic and antisolvent streams and then a subsequent dilution into more antisolvent, the MIVM has an immediate mixing to the final dilution. Therefore, the rate of change of supersaturation is much faster on the MIVM, which should therefore result in more particle growth, at least at concentrations where the length scale for precipitation should be strongly affected by supersaturation. Due to poor control over the size distribution in trial i, all subsequent trials made use of lower solids concentrations and relatively higher amounts of block copolymer to avoid large, broad distributions. While the particle size distributions were shifted to smaller peak mean sizes and tighter span ranges, the nominal concentrations of progesterone also decreased. For trial iv, although a 96% loading efficiency was achieved, the measured progesterone concentration was only 3.6 mg/mL. Although decreasing the amount of organic solvent in the
final solvent mixture can result in larger particle sizes as shown in Chapter 2 and 4, trial v was attempted with 6.25 vol% THF instead of 10 vol%. Component concentrations were also increased in order to offset the greater dilution. While the increase in peak mean diameter from the optimal CIJM-D formulation was only ~11% with a smaller span range, the loading efficiency was lower at 58%. Nonetheless, the nanoparticulate progesterone concentration in the mixer effluent was increased to 5.0 mg/mL.

Overall, translation to the MIVM was not a simple task. More work is required to obtain a feasible nanoparticle formulation. Well-planned studies that systematically investigate the effects of various parameters are recommended. In order to obtain better results, it is suggested that the organic solvent be increased, as this should reduce and tighten the particle size distributions and it would allow for the use of less concentrated stock solutions.

3.3.7 in vivo Pharmacokinetics of Nanoparticle Formulations

A pharmacokinetics study was conducted by the Emory Institute for Drug Discovery using Harlan Sprague Dawley male rats to determine pharmacokinetic parameters of the lyophilized formulation. A sterile progesterone dispersion was produced using the MIVM conditions of trial v and was freeze-dried with roughly 1:1 Pluronic F68 to particle solids. The lyophilized samples were reconstituted to an approximate progesterone concentration of 10 mg/mL. The formulation was tested intravenously (i.v.) and intramuscularly, using four animals in each set. The i.v. administration was done at 1 mg/mL using 2 mL/kg and blood samples were taken at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8 hours. Similarly, the i.m. administration was done at 10 mg/mL using 1 mL/kg with blood samples being taken at 0.25, 0.5, 1, 2, 4, 6, 8 hours. The blood was assayed for progesterone levels in the plasma. The study protocol is present in Appendix G. The blood pharmacokinetic profiles are shown below in Figure 3.10.
From the results, it is clear that there is faster clearance of progesterone from the plasma when administered i.v. as compared to i.m. This rapid release of active pharmaceutical ingredients has been observed for nanoparticle formulations of proprietary compounds produced using the techniques presented in Chapter 4 (unpublished). Considering that both cases made use of the PLA-b-PEG that is shown in Chapter 6 to have poor PEG steric stabilization, it could be that most of the particles dispersed in the blood interact with serum proteins and induce clearance [44], leading to seemingly quick release of the active compound, although the nano-constructs might really just be cleared from the bloodstream before releasing the therapeutic. Nonetheless, the researchers from the Emory Institute claimed that the pharmacokinetic profile of the i.v. administration of the lyophilized formulation was comparable to that of a DMSO solution of progesterone. Contrastingly, i.m. administration resulted in a more prolonged release of progesterone into the bloodstream. This is, in part, attributed to the steroid first diffusing out of the nano-construct and then being absorbed from the depot site into the muscle and into the bloodstream [45]; this is a much longer process for release into the plasma than for the i.v. counterpart. Also, i.m. administration yielded a bioavailability of 47% in comparison to the i.v.
route. The Emory researchers also commented that the lyophilized formulation resulted in a faster presence of progesterone in the plasma in comparison to oil-based formulations, when administered i.m. Nonetheless, both administration routes for the formulation achieved fast $T_{max}$ values, under an hour, which is beneficial for a formulation with emergency treatment applications. Pharmacokinetic parameters are also presented in Table 3.7.

<table>
<thead>
<tr>
<th>Dose</th>
<th>$T_{max}$ (hr)</th>
<th>$C_{max}$ (ng/mL)</th>
<th>$T_{1/2}$ (hr)</th>
<th>$AUC_{0-t}$ (hr-ng/mL)</th>
<th>$AUC_{0-\infty}$ (hr-ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v. 2 mg/kg</td>
<td>0.08</td>
<td>794</td>
<td>1.6</td>
<td>287</td>
<td>321</td>
</tr>
<tr>
<td>i.m. 10 mg/kg</td>
<td>0.56</td>
<td>442</td>
<td>0.94</td>
<td>583</td>
<td>755</td>
</tr>
<tr>
<td><strong>Bioavailability ($F$)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>47%</td>
</tr>
</tbody>
</table>

While it is not certain how effective the actual formulation is for emergency TBI treatment, the progesterone in the bloodstream is expected to partition through the blood-brain barrier and reach equilibrium in the brain, as has been seen in previous studies [46]. More studies would be need to test the feasibility of the formulation for use in humans.

3.4 CONCLUSIONS

This work showed that progesterone can be formulated into nanoparticles that are stabilized by a co-solute, D-α-tocopherol, and an amphiphilic block copolymer, PLA-β-PEG, through FNP on a lab-scale CIJM-D. Using these FDA-approved GRAS components, the formulation optimized for loading efficiency yielded particles of approximately 300 nm in diameter with a drug loading of 24% and at progesterone concentrations higher than any previously reported nanoparticulate formulation. Subsequent freeze-drying with Pluronic F68 allowed for reproducible reconstitution by mild hand agitation for 1 minute. Reconstituted
dispersions exhibited size distributions similar to before freeze-drying and concentrations of ~30 mg/mL progesterone concentrations were achieved.

The use of Pluronic F68 as a protectant excipient to produce lyophilizates that can be reconstituted merely using hand agitation is a significant result. In an emergency TBI treatment, the lyophilized material could be easily and reproducibly administered. The osmolarities of the reconstituted dispersions at 30 mg/mL progesterone, freeze-dried with Pluronic F68, are estimated to be ~200 mOsM, which is below the average human serum osmolarity of 289 mOsM [47]; through the use of a sterile saline solution for rehydration, the redispersed lyophilized powders could be expressed parenterally in humans through 25-gauge needles.

While it was shown that work is still required to obtain an optimal nanoparticle formulation using a MIVM, a lyophilized formulation of ~350 nm particles with ~5 mg/mL progesterone was used for a pharmacokinetic study in rats. The in vivo study demonstrated promising results in the form of fast peak concentrations in the plasma, which is necessary for effective emergency TBI treatment. Currently, alternate injectable formulations of progesterone are being investigated for treating TBI. Two examples are the formulations administered in the phase 2 clinical trials that have been conducted. For example, the US trial, which used i.v. administration, prepared the doses by mixing a progesterone solution in 95% ethanol with an Intralipid 20% fat emulsion [15]. While Intralipid is FDA-approved for parenteral use, our lyophilized nanoparticle formulation, which uses only GRAS components, avoids having organic solvent in the final dose, thereby providing a less toxic alternative. For i.v. administration, injection volume is not an important parameter and thus high concentrations of progesterone are not critical in the formulation. In contrast, the Chinese trial involved i.m. administration of a progesterone solution in camellia oil [6]. There are various problems with this oil-based
formulation. First, camellia oil is not FDA-approved and can oxidize over time [48], leading to decreased shelf life, which should be less of an issue with lyophilized formulations. Furthermore, for i.m. administration, injection volume is important. For the oil-based formulation, the progesterone concentration injected was 20 mg/mL, thereby requiring a 3.5 mL dose to deliver 1 mg/kg to a 70 kg adult. On the other hand, the FNP particles can be administered at 1.5 mg/kg, allowing for a 2.3 mL dose to deliver the same mass of progesterone. Since we can deliver higher concentrations, this will allow for a greater mass of progesterone to be administered in a small volume. Overall our novel formulation provides an advance in the safe and efficient delivery of progesterone.

Through the development of novel progesterone formulations that can deliver large amounts of the water-insoluble neurosteroid in small volumes, treatment for traumatic brain injury can hopefully progress and minimize the risk of death and disability. Further in vivo testing of the nanoparticles is necessary before effectiveness of such a treatment is determined. While the presented steroid formulation was motivated by the need for improved TBI therapies, such nanoparticulate constructs could be adapted for delivery of other insoluble steroids that currently cannot be formulated at high concentrations.

3.5 ACKNOWLEDGEMENTS

Thanks go out to Dr. Paul Reider from the Department of Chemistry, Princeton University for his helpful advice and insight on practical goals for this project. We are grateful to Dr. A. Alan Pinkerton and Dr. Pannee Burckel from the Department of Chemistry, University of Toledo for conducting the X-ray diffraction measurements. Also, many thanks to Dr. Michael
George Natchus, Dr. Randy Howard, and others involved from the Emory Institute for Drug Discovery for planning and running the pharmacokinetics study.

### 3.6 NOMENCLATURE

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>i.m.</td>
<td>intramuscular</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>GRAS</td>
<td>generally recognized as safe</td>
</tr>
<tr>
<td>FNP</td>
<td>Flash NanoPrecipitation</td>
</tr>
<tr>
<td>AT</td>
<td>D-α-tocopherol</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>PLA</td>
<td>poly(D,L-lactide)</td>
</tr>
<tr>
<td>PCL</td>
<td>poly(ε-caprolactone)</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(D,L-lactide-co-glycolide)</td>
</tr>
<tr>
<td>PXXYkD-b-PEGZkD</td>
<td>poly(XX)-block-poly(ethylene glycol) of MW: YkD-b-ZkD</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>CIJM-D</td>
<td>confined impinging jets mixer with dilution</td>
</tr>
<tr>
<td>MIVM</td>
<td>multi-inlet vortex mixer</td>
</tr>
<tr>
<td>$R^2$</td>
<td>linear coefficient of determination</td>
</tr>
<tr>
<td>$\Delta$</td>
<td>span range ($\Delta = d_{90} - d_{10}$)</td>
</tr>
<tr>
<td>$d_{10}$</td>
<td>10% of the intensity-weighted size distribution is smaller than this diameter</td>
</tr>
<tr>
<td>$d_{90}$</td>
<td>90% of the intensity-weighted size distribution is smaller than this diameter</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>wavelength</td>
</tr>
</tbody>
</table>
CHAPTER 3

Progesterone-Loaded Nanoparticles

\( \varepsilon \)  
\text{molar extinction coefficient}

\( c_i \)  
\text{concentration of } i

\( LE \)  
\text{loading efficiency}

\( S_f/S_i \)  
\text{redispersibility ratio, or the ratio of peak mean sizes before and after freeze-drying/reconstitution}

\( \Delta f/\Delta i \)  
\text{ratio of span ranges before and after freeze-drying/reconstitution}

\( \delta_d \)  
\text{Hansen solubility parameter accounting for dispersion forces}

\( \delta_p \)  
\text{Hansen solubility parameter accounting for dipolar interaction}

\( \delta_h \)  
\text{Hansen solubility parameter accounting for hydrogen bonding}

\( \delta_{tot} \)  
\text{1D collapsed Hansen solubility parameter}

\( r_{ij} \)  
\text{distance between the Hansen solubility parameters of two materials \((i,j)\) as defined in a 3D space}

\( S \)  
\text{supersaturation ratio}

\( J \)  
\text{nucleation rate}

\( A \)  
\text{pre-exponential factor for nucleation rate}

\( \gamma \)  
\text{interfacial tension between a solute and a fluid}

\( \nu \)  
\text{molar volume}

\( k_B \)  
\text{Boltzmann constant}

\( T \)  
\text{absolute temperature}

3.7 REFERENCES

CHAPTER 3

Progesterone-Loaded Nanoparticles


37. Saad, W., *Drug nanoparticle formation via flash nanoprecipitation: conjugation to encapsulate and control the release of paclitaxel*, in *Chemical Engineering* 2007, Princeton University.


CHAPTER 4 - PRODUCTION OF LYOPHILIZED NANOPARTICLE DISPERSIONS OF A WEAKLY BASIC API OF LOW HYDROPHOBICITY: A MODEL STUDY

ABSTRACT

With the abundance of manufacturing techniques for producing pharmaceutical nanoparticulate dispersions, it is uncommon for new methods to be considered for the development of a pharmaceutical product from conception of a liquid dispersion to a dosable solid form. We present a model study for the formulation of GW771806X, a weak base of low hydrophobicity, through Flash NanoPrecipitation, a facile rapid precipitation method that is scalable from the lab-scale to the production level. Optimization is used through the various phases of the formulation process, from preliminary lab-scale screening to translation to larger-scale production and finally to freeze-drying, to yield a stable lyophilized nanoparticle formulation that can easily be reconstituted to a dose of 8 mg/mL GW771806X. We describe the selection of the appropriate pairing acid at the optimal stoichiometry, as well as the block copolymer stabilizer for the formation of a nanoparticle dispersion designed for high loading efficiency. Also, the importance of process parameter optimization, for parameters such as choice of organic solvent, the fraction of organic solvent in the final dispersion, and total solids concentration, is detailed. Lastly, freeze-drying experiments show the superiority of polymeric excipients over sugars for the reconstitution of the dried nanoparticle dispersions. By presenting this methodology for produce a final dosage form for a model weakly basic active pharmaceutical ingredient of low hydrophobicity, we demonstrated that Flash NanoPrecipitation is amenable to the development of therapeutically relevant compounds.
4.1 INTRODUCTION

With the advent of nanoparticulate formulations for pharmaceutical applications, many different synthesis methods have been reported and compared [1, 2]. One such manufacturing route for producing nanoparticle dispersions is Flash NanoPrecipitation (FNP) [3, 4], a rapid precipitation method resulting in kinetically frozen, polymer stabilized nano-constructs. FNP boasts easy scale-up [5], excellent control over size distributions, and high active pharmaceutical ingredient (API) loadings [6]. However, when using FNP, the API(s) must be able to achieve a state that will be stable through solvent quality change in order to produce a stable nanoparticle dispersion. While it is difficult to predict whether an API will form satisfactory dispersions through FNP based only on the physical properties, some characteristics that are representative of excellent FNP candidates are water insolubility (many times denoted by a high partition coefficient such as logP) and ionizability (from pKa). While poor water solubility is becoming common in new pharmaceutical compounds [7], it is also quite common for APIs to have an acidic or basic moiety that can become charged. Generally, it is difficult to highly load such an API into a nanoparticulate because, when charged, the compound will have increased water solubility, which may lead to rapid dissolution from the nanoparticle and result in macroscopic crystallization. For example, block copolymer micelles with a hydrophilic poly(ethylene glycol) (PEG) block created through equilibrium nanoprecipitation techniques were only capable of reaching API loadings of ~10 wt% for the weak acid indomethacin [8]. Likewise, for the weak base procaine, loadings < 5 wt% were achieved in poly(glycolic acid-co-lactide) nanoparticles [9].

In order to deal with the challenges posed by weak acids or bases of low hydrophobicity, several strategies have been developed which were discussed in section 2.8 of Chapter 2. Two of
the techniques that can be employed to prevent recrystallization of poorly hydrophobic APIs are solubilization or dispersion in a hydrophobic core, and ion pair formation to neutralize charges. Solubilization involves the API being dissolved into a hydrophobic oil phase that can be well loaded into nanocapsule form stabilized by a polymer. Dispersion is similar to solubilization but is accomplished with hydrophobic phases that are not necessarily liquid and thus the API might be dispersed in a polymer or inert filler matrix, as Kumar showed for pyrene stabilized in a polystyrene core [10]. However, the effectiveness of the solubilization/dispersion technique is limited by how soluble the API is in the oil or the affinity of the API for the hydrophobic dispersion matrix in the nanoparticle core. This technique is not ideal for weak acids or bases, since it is difficult to find oils or matrix materials that load well into nanoparticles and in which the highly polar or charged API species will be soluble. For these reasons, it is more common for ion pair formation to be successful. The aforementioned studies for indomethacin and procaine observed 4 times higher API loadings when utilizing ion pairs [8, 9]. With respect to FNP, the ion pairing technique has been used for the loading of polyelectrolytes, such as siRNA [11, 12] and for small molecules, such as cinnarizine and clozapine [13]. Pinkerton and co-workers characterized the nano-constructs formed through FNP for the weak base cinnarizine and various acids and suggested rules for how to obtain satisfactory nanoparticle dispersions [13]. However, Pinkerton's work was done at the lab-scale and did not involve preparation for dosing.

In this work, we present a model study of how to formulate lyophilized nanoparticle dispersions of a weak base of low hydrophobicity through Flash NanoPrecipitation. The API, GW771806X, is a receptor tyrosine kinase inhibitor and has been reported to inhibit angiogenesis [14, 15]. This compound was selected as a model API because its high tendency to crystallize, as well as its relatively low calculated logP (3.92), makes it difficult to deliver. We
outline the steps to formulate the dosable form, from lab-scale screening to optimization using equipment that can readily be used for larger scale production, and finally freeze-drying. This chapter details a systematic approach that begins with the physical chemistry of the API, evaluates techniques for loading the API into nanoparticles, progresses through informed optimization of the nanoparticle formulation by evaluating formulation and process parameters, and ends with the development of dry dosage forms of the API-loaded nanoparticles.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Pamoic acid (PAM) (> 95%) and D-α-tocopherol (AT) (> 97%) were purchased from TCI America (USA). Sodium chloride (≥ 99.5%), (±)-camphor-10-sulfonic acid (CSA) (≥ 98%), D-α-tocopherol succinate (ATS) (semisynthetic), poly(ethylene glycol) (Mₙ~4600 and Mₙ~20,000), and acetonitrile (MeCN) (HPLC grade) were purchased from Sigma-Aldrich (USA). Dimethyl sulfoxide (DMSO) (HPLC grade) and tetrahydrofuran (THF) (HPLC grade) were purchased from Fisher Scientific (USA). Trifluoroacetic acid (TFA) (reagent grade) was purchased from EMD Millipore (USA). GW771806X, sucrose (GSK Comet), D-(+)-trehalose dihydrate (≥ 99%) (Sigma-Aldrich, USA), Tween 80 (EMD Millipore, USA), and Lutrol F68 (BASF, USA) were provided by GlaxoSmithKline (King of Prussia, PA). Poly(D,L-lactide)-block-poly(ethylene glycol) (PLA-b-PEG) (MW: 3.7kD-b-5kD) was generously supplied by Evonik Inc. (USA). Poly(ε-caprolactone) -b-poly (ethylene glycol) (PCL-b-PEG) (MW: 2kD-b-5kD) was synthesized by Dr. Margarita Herrera-Alonso based on the method by Shuai et al [16]. Ultrapure water (MilliQ water) (18 MΩ·cm) was generated from a Barnstead Nanopure purification system.
4.2.2 Solubility Measurements

The solubility of GW771806X with and without co-solutes was determined in a 10 vol% DMSO in MilliQ water mixture. The organic compound(s) was first dissolved in DMSO and then the organic solution was added to MilliQ water to result in 10 vol% DMSO in water. To pure MilliQ water, a small amount of GW771806X powder was added. The mixtures were placed on a rugged rotator (099A RD4512; Glas-Col, USA) overnight. The samples were then filtered through 0.1 µm syringe filters (nylon; Whatman, USA) and analyzed through HPLC.

4.2.3 Nanoparticle Formation

Block copolymer stabilized nanoparticles were produced using two different methods. The first made use of a laboratory-scale confined impinging jets mixer with dilution (CIJM-D) reported by Han et al [17]. Briefly, as an example, 1 mL of the organic stream containing the dissolved API, block copolymer, and possibly co-solutes in DMSO in a disposable plastic syringe (National Scientific Company, USA) was simultaneously manually injected into the mixer against 1 mL of MilliQ water and collected into an 8 mL diluting reservoir of the aqueous phase. The resulting dispersion was 10 vol% DMSO.

The second method made use of a four-jet, or multi-inlet, vortex mixer (MIVM) described by Liu et al [4]. Depending on the amount of organic phase present in the final solvent mixture, different flow rates were used for the different streams, although all at turbulent mixing regimes. Three 50 mL gas tight glass syringes (SGE Analytical Science, Australia) were filled with MilliQ water and one syringe, either glass (25 mL; SGE Analytical Science, Australia) or disposable (20 - 30 mL; AirTite, USA), contained the organic phase. The streams were injected into the mixer using digitally controlled PHD 2000 syringe pumps (Harvard Apparatus, USA). The initial ~5 mL of effluent were discarded and then collection was started. Effluent collection
was stopped prior to stopping the pumps. This ensured that only dispersion produced at steady state operation were collected.

4.2.4 Nanoparticle Dispersion Characterization

4.2.4.1 Particle size distribution measurement

After nanoparticle production and each processing step, the dispersions were characterized for particle size using a Malvern Zetasizer ZS ZEN 3600 (UK). Samples were prepared by diluting the dispersions at least 10-fold in MilliQ water and were contained in disposable 1.5 mL plastic cuvettes (Fisher, USA). All measurements were made with a 633 nm laser at a scattering angle of 173°. Sizes reported are those obtained using the general purpose normal resolution analysis mode of the intensity-weighted distribution. The peak means and span ranges ($\Delta = d_{90} - d_{10}$) are given. Please see Appendix C for a discussion on why this reporting protocol is used. Usually, at least triplicate measurements were made and the metrics given are the averages and standard deviations of at least two measurements.

4.2.4.2 Nanoparticle dispersion composition

API and co-solute concentrations were measured through reverse-phase high pressure liquid chromatography (HPLC). HPLC was conducted on a Hewlett-Packard Agilent LC 1100 equipped with a quaternary pump, diode array detector, and a Gemini C18 stationary phase (5 µm, 100 Å; Phenomenex, USA). Mobile phases used are designated as follows: A - MilliQ water with 0.05 vol% TFA, B - MeCN with 0.05 vol% TFA. API and PAM concentrations were measured using the following conditions: a linear gradient of 95%/5% A/B to 100% B over 2.5 minutes and returning to 95%/5% A/B at 2.7 minutes all at a flow rate of 1.5 mL/min using a column temperature of 55°C. The method run time was 6 minutes with analyte retention times of ~3 and 4.5 minutes for GW771806X and PAM, respectively. The detection wavelength used was
254 nm. The method was calibrated for GW771806X concentrations of $5 \times 10^8 \mu g/mL$ with a linear coefficient of determination, $R^2 = 0.9999$, and for PAM, the method was calibrated for concentrations of $5 \times 10^3 \mu g/mL$ with $R^2 = 0.9942$. For analyzing API concentrations in nanoparticle dispersions, the dispersions were diluted in DMSO at least 20-fold before HPLC analysis. For solubility measurements, the samples were not diluted.

Total solids concentrations (and, indirectly, block copolymer concentrations) were measured through thermogravimetric analysis (TGA). TGA was performed on a Pyris TGA 7HT (PerkinElmer, USA). Prior to each run, the instrument was allowed to cool down to ambient temperature, the sample pan was thoroughly cleaned (rinsed with 1-distilled water/soap, 2-THF, 3-distilled water, 4-ethanol, and then dried in a vacuum oven at 105°C) and the instrument was mass-calibrated at least twice with a 100 mg metal weight standard. Typical runs involved heating from 25°C to 80°C at 5°C/min, holding for 30 minutes, heating from 80°C to 105°C at 5°C/min, and holding for 10 minutes. This method was used only to remove water from the dispersions, such that only the non-volatile nanoparticle components could be weighed. The nanoparticle concentration ($c_{\text{solids}}$) was calculated as follow:

$$c_{\text{solids}} = \frac{m_{\text{final}}}{m_{\text{initial}}} \rho_{\text{water}}$$

(4.1)

Above, $m_{\text{final}}$ and $m_{\text{initial}}$ are the final and initial masses measured by the instrument in each run, where the final mass is that of the nanoparticles and the initial mass is that of the dispersion, and $\rho_{\text{water}}$ is the density of water. It is assumed that the density of the nanoparticle dispersion does not deviate significantly from that of water, as discussed in Appendix H.

Loading efficiency ($LE$) of the API into the nanoparticles was calculated as follows:

$$LE(\%) = \frac{c_{\text{measured}}}{c_{\text{nominal}}} \left( \frac{V_{\text{initial}}}{V_{\text{measured}}} \right) \times 100\%$$

(4.2)
Here, $c_{\text{measured}}$ is the measured concentration through HPLC, $c_{\text{nominal}}$ is the nominal concentration expected if all the solute from the organic phase is recovered, $V_{\text{initial}}$ is the initial volume of dispersion loaded into the dialysis tubing, and $V_{\text{measured}}$ is the volume recovered from the dialysis tubing once dialyzed. The API loading, or the amount of API in the dry nanoparticles by weight, was calculated as follows:

$$\text{loading (wt\%)} = \frac{c_{\text{measured}}}{c_{\text{solids}}} \times 100\%$$

(4.3)

Here, $c_{\text{measured}}$ is the measured concentration through HPLC, and $c_{\text{solids}}$ is the solids concentration measured through TGA. All LE and loading measurements were done on dialyzed dispersions that were filtered.

4.2.4.3 Zeta-potential measurement

Once the dispersion was free of organic solvent, the nanoparticle sample was diluted with sodium chloride to result in a nanoparticle concentration of ~2.5 mg/mL and a salt concentration of ~10 mM. An aliquot of the salt sample was then transferred to a disposable folded capillary cell (Malvern, UK). Zeta-potential measurements were done on a Malvern Zetasizer ZS ZEN 3600 (UK) using the general purpose analysis mode at default instrument settings. Five replicate measurements were made and three were used to obtain the average zeta-potential and deviations that are reported.

4.2.5 Nanoparticle Dispersion Processing

4.2.5.1 Dialysis

Typically, if a dispersion yielded satisfactory results (nanoparticulates present, little or no precipitate visible, and/or stable dispersion within 10-20 minutes) or if the dispersion was to be freeze-dried, the loading efficiency of the solutes had to be quantified when dialyzed, or with the removal of the organic solvent such that the medium was aqueous only. An aliquot of the sample
was loaded into Spectra/Por 1 (MWCO 6-8 kD) dialysis tubing (Spectrum Laboratories, USA). The aliquot was dialyzed against MilliQ water generally at a 1 L to 10 - 25 mL ratio of bath to sample volume. The bath water was refreshed at least hourly for the first five hours and then left overnight. The dialyzed dispersion was then collected from the tubing, the volume change was recorded, and the dispersion was filtered (1 µm, nylon or GF/B; Whatman, USA) prior to further processing.

4.2.5.2 Concentration

To obtain higher API concentrations in the dispersions, the nanoparticles were concentrated using one of three methods after dialysis: dialysis against a concentrated PEG solution, centrifugation filtration, or tangential flow filtration. For dialysis concentration, 60 mL aliquots of the dispersion were loaded into Spectra/Por 1 tubing which were each placed into 1 L of a 40 wt% PEG20kD solution. The solution was stirred on a stir plate. However, due to the high viscosity of the PEG solution, mixing was poor at the tubing interface. Periodically, the solutions were manually agitated to distribute the concentrated water at the exterior tubing interface. After four hours, the aliquots had been concentrated ~20X by volume.

For the centrifugation filtration, 15 mL aliquots of dispersion were loaded into Amicon Ultra-15 centrifugal filter units (MWCO 100kD) (EMD Millipore, USA). The tubes were then centrifuged (Centrifuge 5430R; Eppendorf, USA) for 15 minute intervals at 1500g at 10°C until the retentate had been concentrated ~10X by volume (3-4 intervals); between each interval, the tubes were gently inverted up and down ~20 times in attempt to prevent filter cake formation. The filtrates and retentates from each tube were pooled separately.

For tangential flow filtration, dispersions were concentrated down using a PureTec tangential flow filtration system (SciLog, USA) and a Pellicon XL ultrafiltration cassette with an
Ultracel PLC membrane (300 kD; EMD Millipore, USA). Typically, volumes of > 150 mL were fed at 15 - 30 mL/min in order to maintain a trans-membrane pressure of 20 - 30 psi. This resulted in a permeate flowrate of 5 - 10 mL/min. Dispersions were concentrated 11 - 12X. The cassette was cleaned with HPLC grade water (Sigma-Aldrich, USA) before and after each run and was maintained in 0.1 M sodium hydroxide when not in use.

4.2.5.3 Freeze-drying

In order to obtain a dry nanoparticle powder, the dispersions were freeze-dried in a VirTis Genesis EL 25L pilot scale lyophilizer (SP Scientific, USA). For each run, 1 mL aliquots in 2 mL shell vials (Fisher Scientific, USA) were loaded into the lyophilizer at a shelf temperature of 5°C and were cooled at a -1°C/min ramp down to -55°C and held for 2.5 hours. Primary drying was performed at shelf temperatures of either -38°C or -30°C for 5.5 and 2.3 days, respectively, and secondary drying was done at a shelf temperature of 20°C for 1 - 3 hours. Chamber pressure was maintained at 50 mTorr during drying and the nominal condenser temperature was -85°C. In all cases, the vials containing the lyo-cakes were capped and stored in a standard freezer until reconstitution or exposure to ambient conditions for stability testing.

4.2.5.4 Reconstitution of lyo-cakes

When the lyo-cakes were to be reconstituted, the appearance of the cakes was first noted. Subsequently, the powders were rehydrated with MilliQ water at specified volumes and then mildly manually agitated (~180-210 shakes per minute) for 1 minute. The reconstituted dispersions were then filtered (1 µm, nylon or GF/B; Whatman, USA) and characterized for particle size distributions and API concentrations.

To gauge the degree of redispersibility, the following metrics were calculated and compared. The peak mean ratio, \( S_r/S_i \), is the ratio of the reconstituted peak mean intensity
diameter of the main population to that of the initial dispersion prior to freeze-drying. Similarly, the span range ratio, $\Delta f/\Delta i$, after reconstitution to prior freeze-drying was also considered. The API recovery is the percent of the measured API prior to freeze-drying of the dispersion that was measured after reconstitution and filtration. See Appendix D for more information on how these ratios were calculated and reported.

4.3 RESULTS AND DISCUSSION

4.3.1 Early Screening and Formulation Optimization on a CIJM-D

GW771806X (see Figure 4.1a) is a crystalline free base of ALOGPs 3.92 with a limited water solubility of $0.32\pm0.01$ µg/mL and a solubility of $3.5\pm0.1$ µg/mL in 10 vol% DMSO (Table 4.1).

Table 4.1 Solubilities of the compounds in solvents. Numbers in parentheses describe the stoichiometric amounts of the compounds in the suspensions in terms of molar ratios, except for API/AT which is in terms of mass ratios. BDL = below detectable limits, API = GW771806X.

<table>
<thead>
<tr>
<th>Compound(s)</th>
<th>Solvent</th>
<th>API (µg/mL)</th>
<th>Co-Solute (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>API</td>
<td>Water</td>
<td>0.32 ± 0.01</td>
<td>-</td>
</tr>
<tr>
<td>API</td>
<td></td>
<td>3.5 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>AT</td>
<td></td>
<td>-</td>
<td>9.1 ± 0.1</td>
</tr>
<tr>
<td>ATS</td>
<td></td>
<td>-</td>
<td>135.8 ± 6.3</td>
</tr>
<tr>
<td>PAM</td>
<td></td>
<td>-</td>
<td>142.1 ± 1.6</td>
</tr>
<tr>
<td>API/AT (1/1)</td>
<td>10 vol% DMSO in</td>
<td>6.6 ± 0.0</td>
<td>BDL</td>
</tr>
<tr>
<td>API/ATS (0.8/1)</td>
<td>Water</td>
<td>7.5 ± 0.0</td>
<td>BDL</td>
</tr>
<tr>
<td>API/ATS (1/1)</td>
<td></td>
<td>2.4 ± 0.0</td>
<td>BDL</td>
</tr>
<tr>
<td>API/PAM (1/1)</td>
<td></td>
<td>1.1 ± 0.0</td>
<td>113.0 ± 0.6</td>
</tr>
<tr>
<td>API/PAM (2/1)</td>
<td></td>
<td>7.6 ± 0.1</td>
<td>70.1 ± 0.3</td>
</tr>
</tbody>
</table>

In terms of candidacy for stable nanoparticle formation through FNP, the solubility is low enough that high supersaturation ratios can be achieved, which is favorable for FNP [3, 18]. However, the API has the two problems of a relatively low logP and pH-dependent ionization.
As discussed by Pinkerton et al [13], when crystalline APIs are to be precipitated in nanoparticle form, they are prone to recrystallization during the solvent quality changes associated with mixing, as well as during dialysis. Since GW771806X has a nitrogen on the pyrimidine structure that can be charged, the ionized form will have a lower ALOGPs of 1.57, which correlates with increased water and DMSO/water solubility. As discussed in Chapter 2, higher solubility may lead to decreased loading efficiencies (section 2.5) and will increase the rate of Ostwald ripening of the solute, leading to macroscopic recrystallization (section 2.9.1).

![Chemical structures of core solutes used. a) GW771806X, b) D-α-tocopherol, c) D-α-tocopherol succinate, d) pamoic acid, e) camphor-10-sulfonic acid](image)

**Figure 4.1** Chemical structures of core solutes used. a) GW771806X, b) D-α-tocopherol, c) D-α-tocopherol succinate, d) pamoic acid, e) camphor-10-sulfonic acid

### 4.3.1.1 FNP without a co-solute

When FNP was done on a CIJM-D at a nominal API concentration of 1 mg/mL with 1 mg/mL PCL2kD-β-PEG5kD, macro-precipitates were visible in the very cloudy product. After
filtration (1 µm, nylon; Whatman, USA), the particle size distribution had a peak size of 470 nm that over the course of three days developed three peaks of 248, 36, and 5000+ nm (see trial I in Table 4.2 and trials Ia and Ib in Figure 4.2). The poor size stability and visible precipitate formation suggests dissolution of the API from the polymer stabilized constructs, leading to precipitation of macroscopic crystals through Ostwald ripening and micelle formation from excess polymer on particle surfaces as particles shrink from solute dissolution. Clearly, FNP without modifying the precipitation behavior of GW771806X did not yield acceptable nanoparticles with high API loadings.

![Graph showing particle size distributions for CIJM-D trials for GW771806X.](image)

**Figure 4.2** Particle size distributions of CIJM-D trials for GW771806X. Trial numbers are taken from Table 4.2. Trial Ia refers to the size measurement after FNP, while Trial Ib is a size measurement 3 days after FNP.

### 4.3.1.2 FNP using the solubilization technique

Although, it was not expected to be successful, the next approach for forming stable nanoparticles that was evaluated was solubilization of GW771806X in nanoparticle cores using a co-solute.
Table 4.2 Flash NanoPrecipitation trials attempting to produce nanoparticle dispersions of GW771806X. Particle size distribution data given is representative of the dispersions after dialysis only if LE data is given; otherwise, the size data given was measured after FNP. BCP represents the block copolymer used in the trial where in all cases the hydrophilic block was a PEG5kD and only the hydrophobic block is given.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Mixer</th>
<th>API (mg/mL)</th>
<th>BCP (mg/mL)</th>
<th>Co-Solute</th>
<th>Co-Solute (mg/mL)</th>
<th>Solvent</th>
<th>Solvent (vol%)</th>
<th>Mean Peak Sizes (nm)</th>
<th>Span Range (nm)</th>
<th>LE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>CIJM-D</td>
<td>10</td>
<td>PCL2kD</td>
<td>10.0</td>
<td>-</td>
<td>DMSO</td>
<td>10</td>
<td>470</td>
<td>647</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>CIJM-D</td>
<td>10</td>
<td>PLA3.7kD</td>
<td>20.0</td>
<td>AT</td>
<td>DMSO</td>
<td>10</td>
<td>182, 4756</td>
<td>274</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>CIJM-D</td>
<td>10</td>
<td>PLA3.7kD</td>
<td>22.2</td>
<td>ATS</td>
<td>DMSO</td>
<td>10</td>
<td>136, 4692</td>
<td>242</td>
<td>14</td>
</tr>
<tr>
<td>IV</td>
<td>CIJM-D</td>
<td>10</td>
<td>PLA3.7kD</td>
<td>14.4</td>
<td>PAM</td>
<td>DMSO</td>
<td>10</td>
<td>138, 3210</td>
<td>192</td>
<td>67</td>
</tr>
<tr>
<td>V</td>
<td>CIJM-D</td>
<td>10</td>
<td>PLA3.7kD</td>
<td>14.4</td>
<td>PAM</td>
<td>DMSO</td>
<td>10</td>
<td>93, 4533</td>
<td>102</td>
<td>58</td>
</tr>
<tr>
<td>VI</td>
<td>CIJM-D</td>
<td>10</td>
<td>PLA3.7kD</td>
<td>15.3</td>
<td>CSA</td>
<td>DMSO</td>
<td>10</td>
<td>57, 2157</td>
<td>106</td>
<td>0</td>
</tr>
<tr>
<td>VII</td>
<td>CIJM-D</td>
<td>10</td>
<td>PLA3.7kD</td>
<td>12.2</td>
<td>PAM</td>
<td>DMSO</td>
<td>10</td>
<td>217, 4227</td>
<td>317</td>
<td>85</td>
</tr>
<tr>
<td>VIII</td>
<td>CIJM-D</td>
<td>10</td>
<td>PLA3.7kD</td>
<td>18.9</td>
<td>PAM</td>
<td>DMSO</td>
<td>10</td>
<td>95</td>
<td>95</td>
<td>81</td>
</tr>
<tr>
<td>IX</td>
<td>MIVM</td>
<td>10</td>
<td>PLA3.7kD</td>
<td>14.4</td>
<td>PAM</td>
<td>1:3 DMSO:THF</td>
<td>10</td>
<td>67, 5252</td>
<td>58</td>
<td>51</td>
</tr>
<tr>
<td>X</td>
<td>MIVM</td>
<td>10</td>
<td>PLA3.7kD</td>
<td>14.4</td>
<td>PAM</td>
<td>DMSO</td>
<td>10</td>
<td>95</td>
<td>95</td>
<td>81</td>
</tr>
<tr>
<td>XI</td>
<td>MIVM</td>
<td>6.25</td>
<td>PLA3.7kD</td>
<td>9.0</td>
<td>PAM</td>
<td>DMSO</td>
<td>20</td>
<td>76</td>
<td>68</td>
<td>79</td>
</tr>
<tr>
<td>XII</td>
<td>MIVM</td>
<td>12.5</td>
<td>PLA3.7kD</td>
<td>18.1</td>
<td>PAM</td>
<td>DMSO</td>
<td>10</td>
<td>120</td>
<td>131</td>
<td>78</td>
</tr>
<tr>
<td>XIII</td>
<td>MIVM</td>
<td>20</td>
<td>PLA3.7kD</td>
<td>28.9</td>
<td>PAM</td>
<td>DMSO</td>
<td>10</td>
<td>144</td>
<td>152</td>
<td>77</td>
</tr>
<tr>
<td>XIV</td>
<td>MIVM</td>
<td>15</td>
<td>PLA3.7kD</td>
<td>21.7</td>
<td>PAM</td>
<td>DMSO</td>
<td>10</td>
<td>146</td>
<td>185</td>
<td>70</td>
</tr>
<tr>
<td>XV</td>
<td>MIVM</td>
<td>30</td>
<td>PLA3.7kD</td>
<td>43.3</td>
<td>PAM, AT</td>
<td>DMSO</td>
<td>6.25</td>
<td>266, 5410, 37</td>
<td>380</td>
<td>59</td>
</tr>
<tr>
<td>XVI</td>
<td>MIVM</td>
<td>12.5</td>
<td>PLA3.7kD</td>
<td>37.2</td>
<td>PAM, AT</td>
<td>DMSO</td>
<td>6.25</td>
<td>147</td>
<td>146</td>
<td>100</td>
</tr>
</tbody>
</table>
Therefore, the API was formulated with D-α-tocopherol as the co-solute oil phase, since the United States Food & Drug Administration has labeled it as generally recognized as safe. FNP on a CIJM-D at a nominal API concentration of 1 mg/mL with 1 mg/mL AT and 2 mg/mL PLA3.7kD-b-PEG5kD yielded an opalescent dispersion with visible residues on the vial walls. The particle size distribution had peaks at 182 and 4500+ nm with a span range of 274 nm (trial II in Table 4.2). While the API loading efficiency was not determined, it was later shown that GW771806X has a solubility of less than 4 wt% in AT; therefore, in retrospect, it is not expected that the dispersion would have a considerable amount of solubilized API in nanoparticle form. As shown in Appendix E, the calculated solubility parameters for GW771806X and AT are quite different with a Hansen solubility parameter 3D distance of 7.2. This solubility parameter mismatch would predict poor miscibility between the two compounds. In fact, the solubility of the API in 10 vol% DMSO is increased when AT is introduced, as compared to the API alone in 10 vol% DMSO (Table 4.1), further suggesting the insolubility of GW771806X in AT. Considering that solubility parameter calculations also showed poor miscibility of the API in PCL and PLA homopolymers, dispersion in a hydrophobic polymeric matrix was not explored.

4.3.1.3 FNP using the ion pairing technique

The ion pairing technique was also attempted with GW771806X in hopes of forming nanoparticles with high API loadings. Three different pairing acids were evaluated, all either used in pharmaceutical salt formation [13] or GRAS, with different $pK_a$s: D-α-tocopherol succinate ($pK_a = 5.6$ [19]), pamoic acid ($pK_a = 2.5, 3.1$ [13]), and camphor-10-sulfonic acid ($pK_a = 1.2$ [13]). While the $pK_a$ of GW771806X has not been experimentally measured, the API is capable of forming a salt with phosphate (phosphoric acid $pK_a = 2.2, 7.2, 12.3$ [20]) and thus should be able to interact at least with PAM and CSA; through the use of the demo version of the
Protonation Calculator Plugin for MarvinSketch provided by ChemAxon, the $pK_a$ values for GW771806X were predicted as 5.1 and 2.3 [21]. FNP was performed on a CIJM-D at a nominal API concentration of 1 mg/mL with the acid at a 1:1 normality of acid to base and an equal mass of block copolymer as core-solutes. The particle size distributions are shown in Figure 4.2 and are represented by trials III-VI in Table 4.2.

It was found that ATS could form smaller particles with a tighter size distribution than AT, as the peak size was reduced by 5% to 173 nm and the span range was reduced by 21% to 216 nm. However, the loading efficiency was only 28%. PAM resulted in smaller particles with peak sizes of 136-138 nm and span ranges of 242-192 nm, depending on the block copolymer used. Lastly, CSA resulted in the smallest particles with a peak size of 57 and a tighter span range of 107, but with 0% loading of the API. This is most likely due to CSA forming a water-soluble salt with the API, as was observed for cinnarizine [13]. While this solubilization is interesting, it does not allow for controlled release or targeting that might be possible with a nanoparticle formulation. From these results, it becomes apparent that the ion pairing technique worked better than solubilization for producing API nanoparticles. Pamoic acid, which has low $pK_a$s and a moderate logP (ALOGPs = 4.58), resulted in the highest API loading efficiency (67%). The particles made with PLA-$b$-PEG were stable over 43 hours at ambient conditions, either undialyzed or dialyzed, with only $\leq 2\%$ change in peak size and $\leq 10\%$ drop in span range. While the API/ATS combination was also capable of forming loaded-nanoparticles, the API loading efficiency was 40% less than that of the API/PAM combination.

As Pinkerton showed for the FNP formulation of cinnarizine through ion pairing, a difference of at least 2 pH units between the $pK_a$ values of the acid and base in the dispersion solvent mixture is necessary for ion pair formation [13]. Since the $pK_a$ of GW771806X has not
been experimentally measured, we consider the predicted $pK_a$ of 5.1; since ATS has a $pK_a$ of 5.6, it would not be able to form an ion pair with the API ($pK_a$ difference of -0.5 pH units).

Contrastingly, the $pK_a$'s of PAM are 2.5 and 3.1, resulting in $pK_a$ differences with the API of 2.6 and 2.0, which would be sufficient by Pinkerton's report for the formation of an ion pair. However, Pinkerton also demonstrated that the $pK_a$ values of acids increase and decrease for bases when in an aqueous solvent mixture with a small fraction of organic solvent; this would imply that the $pK_a$ differences for the two cases would decrease, meaning that while ATS could not form an ion pair with GW771806X, PAM possibly could. Solubility measurements for the two acid/base pairs were taken in the dispersion solvent mixture in hopes of finding a correlation with ion pair formation. In referring to Table 4.1, the solubility of the API doubled in the solvent mixture when a stoichiometric amount of PAM was present as compared to no PAM being present (7.6 µg/mL vs. 3.5 µg/mL), while the solubility of PAM was halved in comparison to having no API present (70.1 µg/mL vs. 142.1 µg/mL); the increase in API solubility might be due to deprotonation of PAM, which would drive down the pH and also decrease the solubility of the deprotonated acid. In contrast, both the solubilities of the API and ATS when together decrease in comparison to pure solutions (API: 2.4 µg/mL vs. 3.5 µg/mL; ATS: not detectable vs. 135.8 µg/mL). However, these are equilibrium solubilities, which would indicate that loading efficiencies < 99% are due to kinetic, not equilibrium, phenomena, which is in accordance with the nature of the FNP process. Overall, this analysis suggests that interactions of the two species during precipitation (most likely ionic for API/PAM and hydrophobic for API/ATS) lead to heterogeneous nucleation, resulting in differences in sizes and loading efficiencies of the resulting dispersions.
4.3.1.3.1 Optimizing acid-base stoichiometry

In order to better understand how to optimize loading efficiency of the API when using PAM as a co-solute, nanoparticle dispersions were made with 1:2 and 2:1 acid:base stoichiometries in the starting organic stock solutions. The results are outlined in Table 4.2 (trials VII & VIII). When including the previous 1:1 acid:base result (trial V), there is a clear trend; there is a monotonic increase in peak size, span range and loading efficiency as the amount of PAM is increased. This might be due to the excess of acid groups that are introduced which allow for more of the theorized ion pair to form and decrease the fraction of charged basic species that can participate in recrystallization. In effect, more of the API can be captured in nanoparticle form, thus resulting in larger sizes. Also, in increasing the amount of PAM, the total solids concentration also increases, which was shown in section 2.5 of Chapter 2 to result in larger particle sizes; here, this is observed as a broader distribution or a larger span range. It is expected that the 2:1 acid:base case resulted in nanoparticles with the lowest API loading of the three evaluated stoichiometries, based on the nominal API loadings if 100% loading efficiencies were achieved (API loadings: 1:2 - 41%, 1:1 - 35%, 2:1 - 26%). Thus, only the 1:2 and 1:1 cases were further investigated.

These two nanoparticle formulations were dialyzed against water for 3 days (the water bath was refreshed each day; 5 mL dispersion with < 1 mg/mL API into 1 L water bath), while tracking the ratio of acid to base in the retentate by HPLC; the results are plotted in Figure 4.3. While theoretically the acid:base ratio should be one if the compounds were indeed forming an ion pair, the two nanoparticle formulations reached an average ratio of ~1.2. It may be possible that there is some pamoic acid that is not ion paired in the nanoparticle core, thus increasing the acid:base ratio. Nonetheless, it was obvious that the 1:1 case experienced the least variation in
stoichiometry of the two dispersions. In all subsequent experiments, the acid:base ratio observed was in accordance with this roughly 1:1 finding. Solid state characterization would be necessary to convincingly determine whether this was indeed a true ion pair. However, this is outside of the scope of this work.

![Figure 4.3](image_url)

**Figure 4.3** Acid to base ratio of the retenate of two GW771806X/PAM nanoparticle dispersions that were dialyzed against water, as measured through HPLC. The dispersions differed in the initial acid to base ratio (1:2 or 1:1), prior to dialysis, which is represented by the t=0 timepoint.

### 4.3.1.4 Summary

In summary, using a confined impinging jets mixer, preliminary screening with GW771806X showed that FNP with only a block copolymer could not form acceptable API nanoparticles. Furthermore, solubilization in AT was not successful, since GW771806X is not sufficiently soluble in the oil. However, the ion pairing technique proved successful. Three pairing acids were evaluated: PAM, ATS, and CSA; PAM was shown to form the smallest particles with the highest API loading efficiency because of its hydrophobicity and the larger $pK_a$ difference between the API and PAM. In optimizing the formulation parameters, the ratio of
PAM to API was varied and was shown to reach roughly a 1:1 acid:base, or 1:2 PAM:API (since PAM has two acid groups), molar ratio when provided with a sink for release through dialysis. Of the two block copolymers evaluated, the loading efficiency was the highest for PLA3.7kD-b-PEG5kD, reaching 67%, as opposed to the much lower 14% for PCL2kD-b-PEG5kD. Both polymers have similar logP values for the hydrophobic block at the molecular weights used (PLA3.7kD: 9.3; PCL2kD: 10.6) [22] and a similar 3D distance between the Hansen solubility parameters of 1:2 PAM:API mixture (by mass) and PLA or PCL (PLA: 5.5 MPa^{1/2}; PCL: 5.9 MPa^{1/2}). Most likely, the experimental result depends on the assembly dynamics of the two polymers, such as the relative time scales of polymer aggregation and solute nucleation/growth. For all subsequent studies, 1:2 pamoic acid (2 carboxylate moieties)/GW771806X was used for nanoparticle formation and the block copolymer used was the PEG5kD-b-PLA3.7kD at a 1:1 mass ratio of stabilizer to core.

4.3.2 Translation to a MIVM

While confined impinging jets mixers are scalable and are used industrially, the limitation of these mixers for FNP is that the momenta of the organic and anti-solvent streams must be equal [3, 4, 17]. Although this can be overcome through the dilution step into an aqueous reservoir at the lab-scale and through dilution with an aqueous stream at a mixing point downstream from the mixer at a plant-scale, it is preferable for the mixer effluent to not require dilution, since concentration processes tend to be expensive and time-consuming. An alternative for CIJM-D FNP is the use of a multi-inlet vortex mixer. Han and associates have compared the two mixer types for FNP of β-carotene and shown similar sizes are achieved at comparable mixing conditions, but this simplistic comparison does not consider size distribution spread, particle stability, loading efficiencies, and particle surface characteristics; all these properties are
affected by the mixing and solvent quality change. It is attractive to work with a multi-inlet
t vortex mixer, since the effluent can be tuned to have the desired fraction of organic solvent in the
dispersion solvent mixture. This is important not only to slow down Ostwald ripening
phenomena [23], but also to result in higher supersaturation ratios of the organic solutes during
the mixing/precipitation steps. In addition, the MIVM process is easily scalable from the
laboratory to the plant.

Therefore, the lead nanoparticle formulation from the CIJM-D screening was used as the
starting point for MIVM translation and optimization. In FNP, the precipitation step must occur
under conditions with a characteristic mixing time that is shorter than the time scales for
assembly of the solute(s) and the block copolymer [3], as discussed in section 2.4 of Chapter 2.
There, it was shown that total inlet Reynolds numbers greater than 2000 - 3000 generated
particle sizes that are independent of the mixing conditions; this is the point where micro-mixing
approaches homogeneity in the MIVM. Therefore, all MIVM trials were run at total inlet
Reynolds numbers > 8000, such that excellent micro-mixing was achieved.

The initial experiments involved producing the nominal CIJM-D GW771806X/PAM
nanoparticle formulation on the MIVM. Interestingly, the same nominal nanoparticle
composition yielded a dispersion with a higher loading efficiency (81% vs. 61%) and a smaller
and tighter particle size distribution on the MIVM as opposed to the CIJM-D (peak size: 95 nm
vs. 138 nm; span range: 95 nm vs. 192 nm). This might be attributed to the actual micro-mixing
conditions achieved in each mixer. With the CIJM-D, since the injection of the organic and anti-
solvent streams are manually driven, a constant flowrate is not established, meaning no steady-
state mixing conditions; furthermore, the discontinuous solvent quality change from 1:1 to 1:9
organic:aqueous solvent mixtures when the mixer effluent is diluted in the aqueous reservoir, in
which the mixing is different as well, will result in heterogeneous precipitation conditions. Thus, this would explain the much broader distribution obtained on the CIJM-D. The MIVM, in contrast, experiences steady-state operation and one immediate drop in solvent quality from 100 to 10 vol% organic solvent (instead of 100 to 50 to 10 vol% on the CIJM-D), generally yielding tighter particle size distributions.

### 4.3.3 Process Parameter Optimization on a MIVM

#### 4.3.3.1 Choice of organic solvent

Next, it was important to determine the optimal solvent to use in FNP, since this can affect the kinetics of the precipitation process. Two different solvent systems were evaluated: DMSO and 1:3 DMSO:THF. Although pure THF would have been the preferred comparison to pure DMSO, it could not be used, as neither of the core solutes are soluble in THF; thus, only a small amount of DMSO was introduced into THF, such that the solutes were dissolved. Using flowrates of 130 mL/min water and 14.4 mL/min organic stock, 10 vol% organic solvent in water dispersions were made. Although smaller particles with a tighter distribution were produced with the mixed solvent (peak size: 67 nm vs. 95 nm; span range: 58 nm vs. 95 nm), the loading efficiency was 30% higher for pure DMSO as compared to 1:3 DMSO:THF (51% vs. 81%).

To understand the results, reports from the literature on solvent effects are considered. Pustulka has observed for poly(lactide-co-glycolide)-block-poly(ethylene glycol) (MW: 10kD-5kD) micelles produced through FNP on a CIJM-D that smaller particle sizes were achieved when using acetone as opposed to THF or dimethylformamide, which correlated with the acetone having a higher diffusion coefficient in water [24]. When considering diffusion coefficients, THF has a diffusion coefficient in water at infinite dilution of $1.09 \times 10^{-5} \text{ cm}^2/\text{s}$ [25], while that of
DMSO is 8.85x10^{-6} \text{ cm}^2/\text{s} [26]. Although the experiment was not done in pure THF, a linear combination of the two diffusion coefficients (1.04x10^{-5} \text{ cm}^2/\text{s}) might yield a characteristic value for the mixture. The importance of diffusivities relates to the characteristic mixing time, \( \tau_m \), which for these cases is attributed to precipitation with homogeneous kinetics, as compared to a transport controlled regime [3]. The mixing time can be expressed as follows [5], where \( \lambda_k \) is the Kolmogorov length scale or the distance for an eddy to form in turbulence before viscous effects and microstructure from laminar flow dominate and \( D_{i,j} \) is the diffusion coefficient of i in j.

\[
\tau_m = \frac{\lambda_k^2}{4D_{i,j}}
\]  

(4.4)

The Kolmogorov length scale is a function of the energy dissipation rate, which is largely determined by the dominating aqueous stream; thus, we assume that for both solvent systems, the length scale should be essentially equal. Therefore, the mixing time is inversely proportional to diffusion coefficient of the organic solvent in water, meaning that a faster mixing time will be achieved for 1:3 DMSO:THF than for pure DMSO; by this analysis, smaller particles would be expected to form using the mixed organic solvent system.

In considering another explanation in the literature, Lee et al reported that for nanoprecipitation of PCL-\textit{b}-PEG of varying molecular weights, smaller average particle sizes with broader distributions were produced with acetone as opposed to THF [27]. A comparison was made with the work of Galindo-Rodriguez et al [28], which found that smaller chi interaction parameters of various water-miscible organic solvents in water correlated with smaller particle sizes and broader distributions for nanoprecipitation of Eudragit L 100-55 stabilized by poly(vinyl alcohol). The chi interactions parameters of the solvents in water predict the opposite of the diffusivity argument, since DMSO has a higher affinity for water (\( \chi_{DMSO,water} = 13.0, \chi_{1:3 \text{ THF}:DMSO,water} = 23.2 \)) [29].
Although the diffusivity argument does correlate with the size trend, it is unlikely that such a small difference in the diffusion coefficient of the organic solvent in water would make such an appreciable difference in the FNP results. A more plausible explanation is that the precipitation kinetics could have been different in the two solvent systems. This is particularly the case for the solutes, since ion pair formation is dependent on how easily the solutes can ionize, which will depend on the relative permittivity, $\varepsilon_r$, of the dispersion solvent mixture. Since the relative permittivities of the organic solvents are quite different ($\varepsilon_{\text{THF}} = 7.52$, $\varepsilon_{\text{DMSO}} = 47.24$ [20]), it is expected that the ionization during precipitation will differ in the two cases, leading to different nucleation and growth rates and effectively different nanoparticle sizes and loadings. However, for further optimization, pure DMSO was chosen as the organic solvent, owing to the higher loading efficiency.

4.3.3.2 Fraction of organic solvent in solvent mixture

The next parameter that was optimized was the fraction of organic solvent in the final solvent mixture. As shown in section 2.5 of Chapter 2 for a single core solute, higher fractions of organic solvent in the final solvent mixture yielded smaller peak sizes when total solids concentrations were kept constant; however, since the precipitation process for an ion pair might be very different in comparison to that a single non-ionizable solute, it was important to evaluate the effect of the DMSO fraction for the GW771806X/PAM system. Experiments were done at a nominal API concentration of 1.25 mg/mL (1:2 PAM:API, 1:1 polymer:solutes) to produce API concentrations after dialysis close to 1 mg/mL. By varying the DMSO fraction from 20% to 6.25%, nanoparticle dispersions with the same nominal API concentration were produced. As Table 4.3 shows, increasing the fraction of DMSO in the final solvent mixture yielded smaller particles with a smaller span range while not affecting the API loading efficiency. This result
shows that FNP enables the production of different particle size distributions without changing the nanoparticle composition. It is important to note that while the particle size distributions were changed, the zeta-potential did not change. The zeta-potential measurement suggests that the cores did not have a significant amount of exposed charged groups, further strengthening the argument for ion pair formation between GW771806X and PAM. Since loading efficiency and API loading did not vary amongst the different trials, the formulation with the widest distribution was chosen in continuing forward; this was done in order to evaluate the most challenging cases with regards to freeze-drying, where the wide distribution could result in more heterogeneity in the phenomena that the nanoparticles experience. Therefore, 6.25 vol% DMSO, or 1:15 DMSO:water, was the preferred solvent fraction in the final solvent mixture.

<table>
<thead>
<tr>
<th>Trial</th>
<th>DMSO (vol%)</th>
<th>API Loading Efficiency (%)</th>
<th>API Loading (wt%)</th>
<th>PAM/API (mol/mol)</th>
<th>$\xi$-Potential (mV)</th>
<th>Peak Mean Diameter (nm)</th>
<th>Span Range (nm)</th>
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</thead>
<tbody>
<tr>
<td>XI</td>
<td>20</td>
<td>79</td>
<td>29</td>
<td>0.55</td>
<td>-4.17 ± 5.78</td>
<td>76</td>
<td>68</td>
</tr>
<tr>
<td>XII</td>
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<td>78</td>
<td>30</td>
<td>0.57</td>
<td>-4.79 ± 7.78</td>
<td>120</td>
<td>131</td>
</tr>
<tr>
<td>XIII</td>
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<td>77</td>
<td>29</td>
<td>0.57</td>
<td>-4.65 ± 7.12</td>
<td>144</td>
<td>152</td>
</tr>
</tbody>
</table>

**4.3.3.3 Total solids concentration**

Since the final API concentration in the nanoparticle dispersion after dialysis was still only ~1 mg/mL, which would too low for therapeutic activity for low-potency APIs, increasing the API concentration was investigated. For these experiments, the nanoparticle composition was kept the same, but the total solids concentration was increased from that corresponding to a nominal API concentration of 0.94 mg/mL to that corresponding to 1.88 mg/mL API. The results are presented in Table 4.2 (trials XIII - XV). Essentially, the nominal API concentration of 1.25
mg/mL (trial XIII) yielded the dispersion with the highest loading efficiency and tightest distribution. Although the loading efficiency increased from 70% to 77% when the nominal API concentration was increased from 0.94 mg/mL to 1.25 mg/mL, the peak mean size remained constant. Normally, increasing solute concentration results in larger particle sizes as discussed in section 2.5 of Chapter 2; the reason for the observation with the GW771806X ion pair is unclear. Further increasing the nominal API concentration to 1.88 mg/mL produced a dispersion with a larger, broader size distribution with reduced loading efficiency, which is in agreement with the literature discussed in section 2.5. This phenomena might be attributed to decreasing characteristic precipitation length scales for core solutes, thus reducing the aggregation time scales in comparison to those of the stabilizing block copolymer. Nonetheless, due to the limitations of using high nominal API concentrations, the optimal 1.25 mg/mL GW771806X case was used for future work.

4.3.3.4 FNP using a hybrid technique

Lastly, a hybrid nanoparticle formulation was produced using a "solubilized" ion pair, where GW771806X is loaded in a nanoparticle containing both PAM and AT. In this case, a 1:1 mass ratio of AT to GW771806X/PAM and a slightly higher amount of PAM than previously (1:1.8 PAM:API) was used, while keeping the polymer to solutes mass ratio at 1:1. Here, the peak size and the span range was not drastically different than the base case without the AT (peak size: 147 nm vs 144 nm; span range: 146 nm vs. 152 nm) despite the total solids content in the dispersion more than doubling from the base case. However, the API loading efficiency was increased from 77% to 100%, while resulting in a 1:2.2 PAM:API ratio. It is not obvious how the addition of AT helped to load more of the ion pair. Further investigation would be required to understand the governing phenomena for the hybrid technique. Nonetheless, this finding is
important, since having a liquid-like core can be beneficial for enhanced redispersion of lyophilized nanoparticles [30, 31].

4.3.3.5 Summary

Therefore, to summarize, translation from the CIJM-D to the MIVM was accomplished for the optimal CIJM-D nanoparticle formulation with an improvement to the size distribution and the loading efficiency. Optimization of process parameters involved determining the preferred organic solvent and the fraction of organic solvent in the final dispersion solvent mixture to be used in FNP, as well as the total solids concentration of the nanoparticles in the dispersion. Using pure DMSO in comparison to a 1:3 DMSO:THF mixture as the organic solvent proved to yield the highest loading efficiency. To obtain the tightest size distribution with the highest loading efficiency, a nominal API concentration of 1.25 mg/mL was determine to be optimal. It was also shown that by varying the amount of DMSO in the final FNP solvent mixture the size distribution could be varied without affecting API loading efficiency or zeta-potential. Likewise, D-α-tocopherol could be incorporated into the nanoparticle composition, thereby increasing the loading efficiency without affecting the size distribution. However, in keeping the API loading as high as possible, AT was not included and the nominal API concentration was kept at 1.25 mg/mL.

4.3.4 Concentrating Nanoparticle Dispersions

For cases when concentrated nanoparticle dispersions are necessary to deliver high doses of APIs, a concentration step might be needed. Since the GW771806X nanoparticle dispersion with the smallest tight size distribution was at a nominal API concentration of 1.25 mg/mL, the optimal nanoparticle formulation from the MIVM optimization (trial XIII from Table 4.2) was concentrated by various routes after dialysis.
The first method attempted involved dialyzing the dispersion against a concentrated PEG20kD solution; ideally, the high osmotic pressure of the polymer solution would result in water flux from the dispersion into the polymer phase, thereby concentrating the nanoparticles. However, TGA showed that the mass concentration factor was ~29X, while the volume concentration factor was only ~20X and the API concentration factor was ~11X. This might be due to some of the PEG from the exterior solution having diffused through the tubing (MWCO 6-8 kD) into the dispersion. In addition, macroscopic precipitates were present within the tubing, which might have been API or PAM since their solubilities in the aqueous solution could have increased as PEG diffused into it. This instability in the dispersion during osmotic concentration deemed it necessary to attempt other concentration routes.

The next method evaluated was to centrifuge the dispersions through Amicon centrifugal filter units. While this method resulted in mass concentration factors of ~9X, there was still discrepancy with the volume concentration factor. In general, there was a loss of ~35% of the mass due to particles penetrating the filter membranes into the filtrate, as confirmed through dynamic light scattering and HPLC of the filtrate, and filter cake formation. Overall, the method might be acceptable at the lab scale for relatively small volume volumes, but when scaling-up, the losses would make the method unattractive. Optimization studies investigating parameters such as membrane MWCO, and centrifugation interval durations and accelerations, are recommended if this concentration method is to be used in future work. Refer to section 4.2.5.2 for the experimental protocol used.

Lastly, the final method that was evaluated was tangential flow filtration. Here, dispersions were concentrated ~11-12X without a huge discrepancy amongst the different concentration factors. Rough estimates of the holdup within the tubing pointed to nearly
quantitative recovery of the nanoparticles. Not only was there less material loss in comparison to
the other concentration methods, the process was not lengthy, as concentrating 260 mL of
nanoparticle dispersion to ~20 mL took ~2 hours. This is also the preferred concentration
method, as the process has easier scale-up that the other two methods.

4.3.5 Freeze-Drying & Reconstitution

As has been discussed in Chapters 1-3, it is desirable to convert liquid nanoparticle
dispersions into dry dosage forms to enhance shelf life of the formulation. Thus, in this chapter
we consider freeze-drying the nanoparticle dispersions, as has been done for other formulations
targeted for parenteral administration.

Based on results that are discussed in detail in Chapter 6, it was known that PEG or PEG-
based polymers provide protection of nanoparticles during lyophilization. It was decided to
compare three PEG-based polymers that are used in parenteral biopharmaceutical formulations,
PEG4.6kD, Tween 80, and Lutrol F68, and two sugars, sucrose and trehalose, as protectants for
the GW771806X nanoparticle dispersions. In this study, after the dispersions had been dialyzed
and concentrated though tangential flow filtration, 1 mL aliquots containing 1 mg of API were
formulated with different quantities of the protectant excipients in single or dual combinations.
After freeze-drying, some samples were reconstituted, while others were kept at ambient
conditions for 1 month to assess stability; several metrics for redispersion were measured at
different time points. Table 4.4 details the results of this study. For a reconstitution trial to be
considered acceptable, the peak mean size ratio, $S_f/S_i$, and the span range ratio, $\Delta_f/\Delta_i$, had to be <
1.10, and the API recovery had to be > 80%. For a formulation to be considered stable over the 1
month monitoring period, the percent change between each sampling time point had to be <
|10%|. 

154
Table 4.4 Reconstitution of lyo-cakes containing GW771806X nanoparticle dispersions with 1 mg/mL API. The API/osmolarity value is a ratio of the API concentration at the 4 week time point over the osmolarity contribution of the excipients. Values highlighted in yellow denote size ratios < 1.10 or API recovery > 80%. Trial numbers marked with an asterisk met reconstitution criteria and had a stable lyo-cake (< 10% for any metric) over 4 weeks storage at ambient conditions.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Excipient</th>
<th>Mass Ratio (Excipient 1/NPs)</th>
<th>Excipient 2</th>
<th>Mass Ratio (Excipient 2/NPs)</th>
<th>S/S₀</th>
<th>A/A₀</th>
<th>API Recovery (%)</th>
<th>API / Osmolarity (g/mOsm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.31 ±0.04</td>
<td>1.59 ±0.07</td>
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<td>2</td>
<td>Tween 80</td>
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<td>-</td>
<td>1.56 ±0.09</td>
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<tr>
<td>3</td>
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<td>-</td>
<td>1.38 ±0.05</td>
<td>1.94 ±0.08</td>
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<td>1.47 ±0.10</td>
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<td>-</td>
<td>1.25 ±0.03</td>
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<td>-</td>
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<td>Mass Ratio (Excipient 1/NPs)</td>
<td>Excipient 2</td>
<td>Mass Ratio (Excipient 2/NPs)</td>
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<td>Δf/Δi</td>
<td>API Recovery (%)</td>
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</tbody>
</table>
In examining the results, it is important to consider the following points. Trials exhibiting API recovery < 100% either exhibited residues once reconstituted that could not be sampled during analysis or had redispersed micron-sized aggregates that were filtered off; thus, losses in recovery are a result of particle aggregation. Likewise, peak mean size ratios were all < 2, which is a result of all samples being filtered through 1.0 µm syringe filters prior to analysis. The filtration step was necessary in order to not consider larger micron-size aggregates in API concentration measurements and to facilitate good-quality particle size measurements.

Of the various formulations evaluated, the unprotected nanoparticles and those with Tween 80 alone did not reconstitute well; both of these resulted in completely collapsed lyo-cakes. Since the unprotected nanoparticles did not have a sufficient PEG/water eutectic to prevent intimate contact amongst hydrophobic cores (see Chapter 6), significant particle aggregation was expected. Although the surfactant nature of Tween 80 most likely would provide some steric protection to exposed hydrophobic areas of nanoparticle cores, since Tween 80 is a liquid (melting point: -20.6°C [32]), meltback was expected for a lyo-cake that had a large mass fraction of the liquid. Also, it might be possible for the theoretical ion pair or its components to partition into the Tween 80 liquid phase, which might have led to the formation of unstably-loaded Tween 80 micelles once rehydrated. Of the other two PEG-based polymers considered, PEG4.6kD performed better in reconstitution and stability than Lutrol F68. However, in Chapter 6 it is shown that Pluronic F68 performed better than PEG4.6kD for the reconstitution of the same lyophilized GW771806X/PAM nanoparticle formulation. Although Pluronic and Lutrol F68 are both considered to have the same nominal molecular structure (designated as poloxamer 188), they may in fact have different molecular weight distributions.
and polydispersities, as has been previously reported [33]. Different chain compositions may result in different degrees of protection.

Furthermore, while most trehalose formulations yielded acceptable reconstitution results at the outset, those with Tween 80 did not, which again might be attributed to the liquid nature of the surfactant. The sucrose formulations were generally superior to the trehalose counterparts. Izutsu and co-workers have reported that out of five sugars or sugar alcohol (sucrose, trehalose, maltose, lactose, and mannitol), sucrose was better able to disperse PEG3kD, as observed through the degree of PEG crystallinity in a frozen aqueous solution [34]. This may allow for individual PEG-stabilized particles to be better separated from each other, such that aggregation is minimized in a vitreous sucrose cryo-concentrate; this is in contrast to a trehalose cryo-concentrate, where poorer dispersion of PEG might make particle-rich domains more prevalent, where particle aggregation can be induced through intimate contact.

Of the formulations that yielded acceptable reconstitution, only the lyo-cake formulations with 5:1 PEG4.6kD, 33.3:1 trehalose, and 33.3:1 sucrose/1:1 Lutrol F68 were considered stable over 4 weeks of storage at ambient conditions. While the mechanism of particle aggregation in the lyo-cakes over time was not investigated, it is hypothesized that it may be due to insufficient individual particle separation and/or phase separation leading to particle-rich domains. However, of the formulations that were stable, the lyo-cake with PEG4.6kD had the highest potential for reconstitution with less volume before becoming hypertonic, as displayed by the API/osmolarity ratio from Table 4.4. While trehalose and sucrose may in fact yield acceptable redispersibility of the nanoparticle dispersions, since such large masses in relation to the solid nanoparticles are required, it becomes problematic to keep the dosage form isotonic.
From the results of this study, it was decided to test the limit of how concentrated the best formulations could be reconstituted. Therefore, the nanoparticle formulation used in the previous freeze-drying study was concentrated through tangential flow filtration and samples were prepared with 4, 7, and 10 mg/mL API. For these experiments only the 5:1 PEG4.6kD and the 33.3:1 sucrose/1:1 Lutrol F68 formulations were considered, since trehalose formulations consistently had stability issues. However, in order to counteract the poor steric stabilization of the nanoparticles provided by the PEG from the PLA3.7-PEG5kD (see Chapter 6), the PEG4.6kD formulation was modified to 4:1 PEG4.6kD/1:1 Lutrol F68; similarly, to offset the effects of increasing solids concentrations, the amount of sucrose used was capped at 200 mg/mL for all trials, as this formulation is already hypertonic without dilution and higher sugar concentrations might increase cycle times for complete drying. Furthermore, two different primary drying conditions were evaluated, holding all other steps in the cycle constant: (i) -38°C for 5.5 or (ii) -30°C for 2.3 days. Results are presented in Table 4.5.

These results highlight various findings. For example, reconstitution to less volume (concentration factors higher than 1X) resulted in worse redispersion of the lyo-cakes as seen in the API recovery decrease in comparing B to A and E to D (9-14% decrease). Furthermore, no results were presented for concentration factors of 2.5X for the trials with higher initial API concentrations, as all of these could not be filtered and had large residues after reconstitution. Since these lyo-cakes were immediately analyzed after rehydration and 1 minute of agitation, the results most likely indicate a kinetic effect, as both PEG4.6kD and Lutrol F68 are infinitely miscible with water and sucrose has an aqueous solubility of 2 g/mL.
Table 4.5 Reconstitution of lyo-cakes containing concentrated GW771806X nanoparticle dispersions. Values highlighted in yellow denote size ratios $< 1.10$ or API recovery $> 80\%$. Trial numbers marked with an asterisk met reconstitution criteria. Lyo Conditions represent the primary drying step: (i) -38°C for 5.5 or (ii) -30°C for 2.3 days. The ratios in the Protectant column characterize the ratio of the protectant to nanoparticles by mass. The Filtered column denotes the syringe filter size used prior to analysis. Trials with no standard deviation reported for API recovery or API concentration had no replicates, while all others had duplicates.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Lyo Conditions</th>
<th>Initial API (mg/mL)</th>
<th>Protectant</th>
<th>Filtered (µm)</th>
<th>Conc. Factor</th>
<th>$S_i/S_f$</th>
<th>$A_i/A_f$</th>
<th>API Recovery (%)</th>
<th>API (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>*A</td>
<td>i</td>
<td>4</td>
<td>PEG4.6kD (4:1) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>0.99 ± 0.02</td>
<td>0.89 ± 0.04</td>
<td>89 ± 1</td>
<td>3.54 ± 0.02</td>
</tr>
<tr>
<td>*B</td>
<td>i</td>
<td>2.5X</td>
<td>PEG4.6kD (4:1) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>0.94 ± 0.00</td>
<td>0.80 ± 0.05</td>
<td>88</td>
<td>3.51</td>
</tr>
<tr>
<td>*C</td>
<td>ii</td>
<td>4</td>
<td>PEG4.6kD (4:1) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>0.94 ± 0.00</td>
<td>0.80 ± 0.05</td>
<td>88</td>
<td>3.51</td>
</tr>
<tr>
<td>D</td>
<td>i</td>
<td>1X</td>
<td>Sucrose (200 mg/mL) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>1.12 ± 0.03</td>
<td>0.73 ± 0.04</td>
<td>64 ± 1</td>
<td>2.56 ± 0.05</td>
</tr>
<tr>
<td>E</td>
<td>i</td>
<td>2.5X</td>
<td>Sucrose (200 mg/mL) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>1.20 ± 0.05</td>
<td>0.83 ± 0.07</td>
<td>50 ± 5</td>
<td>5.02 ± 0.53</td>
</tr>
<tr>
<td>F</td>
<td>ii</td>
<td>1X</td>
<td>Sucrose (200 mg/mL) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>0.95 ± 0.03</td>
<td>0.74 ± 0.06</td>
<td>74</td>
<td>2.97</td>
</tr>
<tr>
<td>*G</td>
<td>i</td>
<td>1.0</td>
<td>PEG4.6kD (4:1) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>1.08 ± 0.01</td>
<td>0.99 ± 0.06</td>
<td>82</td>
<td>5.74</td>
</tr>
<tr>
<td>H</td>
<td>i</td>
<td>1X</td>
<td>Sucrose (200 mg/mL) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>1.12 ± 0.06</td>
<td>1.15 ± 0.07</td>
<td>83</td>
<td>5.83</td>
</tr>
<tr>
<td>I</td>
<td>ii</td>
<td>7</td>
<td>Sucrose (200 mg/mL) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>1.95 ± 0.02</td>
<td>1.36 ± 0.08</td>
<td>72</td>
<td>5.02</td>
</tr>
<tr>
<td>J</td>
<td>i</td>
<td>1.0</td>
<td>Sucrose (200 mg/mL) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>3.03 ± 0.27</td>
<td>1.96 ± 0.38</td>
<td>69</td>
<td>4.84</td>
</tr>
<tr>
<td>K</td>
<td>i</td>
<td>1.0</td>
<td>Sucrose (200 mg/mL) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>2.09 ± 0.06</td>
<td>2.08 ± 0.17</td>
<td>69</td>
<td>4.85</td>
</tr>
<tr>
<td>L</td>
<td>ii</td>
<td>5.0</td>
<td>PEG4.6kD (4:1) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>1.81 ± 0.11</td>
<td>1.84 ± 0.22</td>
<td>71</td>
<td>7.13</td>
</tr>
<tr>
<td>M</td>
<td>i</td>
<td>10</td>
<td>PEG4.6kD (4:1) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>1.98 ± 0.12</td>
<td>1.04 ± 0.22</td>
<td>69</td>
<td>6.91</td>
</tr>
<tr>
<td>N</td>
<td>ii</td>
<td>1.0</td>
<td>Sucrose (200 mg/mL) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>0.95 ± 0.00</td>
<td>0.82 ± 0.06</td>
<td>76</td>
<td>7.64</td>
</tr>
<tr>
<td>O</td>
<td>i</td>
<td>1.0</td>
<td>Sucrose (200 mg/mL) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>1.12 ± 0.17</td>
<td>0.99 ± 0.21</td>
<td>71 ± 2</td>
<td>7.06 ± 0.15</td>
</tr>
<tr>
<td>Q</td>
<td>ii</td>
<td>1.0</td>
<td>Sucrose (200 mg/mL) / Lutrol F68 (1:1)</td>
<td>1.0</td>
<td>1X</td>
<td>1.12 ± 0.04</td>
<td>1.21 ± 0.09</td>
<td>73</td>
<td>7.34</td>
</tr>
</tbody>
</table>
However, for a clinical setting, fast and practical reconstitution is necessary; thus, reconstitution must be done manually (low energy input) and cannot be agitated for more than 1 minute for convenience and compliance. In addition, the increase in initial API concentration also yielded poorer reconstitution for PEG4.6kD formulations, as seen with the decreasing API recovery from trials C to I to O. While this behavior was not exhibited by the sucrose formulations (F to L to Q), these trials had API recoveries less than or equal to the corresponding PEG formulations. This might suggest that at more dilute initial concentrations, PEG is better able to disperse individual nanoparticles than sucrose, which is reasonable considering that the stabilizing PEG from the block copolymer is more miscible in PEG than in sucrose.

Also, the effect of the different lyophilization conditions did not appreciably alter the reconstitution results. While there were a few statistically significant differences between the two conditions, nothing was consistent, since in most cases, both trials were not filtered using the same size syringe filter. This result is not surprising for the PEG formulation, since the eutectic temperature of PEG at the molecular weight of interest is > -20°C [35]. For the sucrose formulation, while condition (ii) did have a shelf temperature slightly above the maximally concentrated glass transition temperature of sucrose (-32°C) [36], the endothermic process of ice sublimation might have provided sufficient cooling for the samples to remain at or below the glass transition temperature. Thus, for the PEG formulation, the cycle time could still be optimized further by increasing the primary drying temperature and possibly reducing the cycle time; the sucrose formulation warrants more caution, since the primary drying temperature should not be increased much more, if any.

The freeze-drying trials successfully highlighted a potential candidate for further development. The optimal GW771806X nanoparticle dispersion that was concentrated to 4
mg/mL API (through tangential flow filtration) was formulated with 4:1 PEG4.6kD and 1:1 Lutrol F68 and freeze-dried; the resulting lyo-cake reconstituted well at 1X and 2.5X concentration factors, reaching maximum API concentrations of ~8 mg/mL, and had stable lyo-cakes. This formulation is attractive because no dilution of the reconstituted dispersion is needed to achieve an isotonic dose for parenteral administration, which would be required with any of the sucrose formulations that were evaluated. Although the reconstitution medium necessary for administration of the PEG-based lyo-cake would need to increase the osmolarity of the nanoparticle dispersion (i.e. saline or dextrose solution), this is not problematic. Furthermore, since this formulation utilized a nanoparticle formulation with poor PEG steric stabilization (see Chapter 6) and a wide particle size distribution, it is expected that reconstitution results will be even better when applying the lyo-cake formulation to other nanoparticle formulations with superior PEG stabilization and with tighter size distributions.

4.4 CONCLUSIONS

We have presented a study of how Flash NanoPrecipitation was adapted to the production of lyophilized nanoparticulate formulations of a model hydrophobic weak base, GW771806X. Beginning with preliminary screening at the lab-scale, trials on a confined impinging jets mixer with dilution showed the necessity of using co-solutes to prevent API recrystallization. In evaluating pharmaceutically-accepted acids, it was shown that pamoic acid allowed for the formation of nanoparticle dispersions with the highest API loading efficiencies, particularly when using a PLA3.7kD-PEG5kD stabilizer as opposed to PCL2kD-PEG5kD. Further optimization varying acid and base stoichiometries suggested the formation of an ion pair between GW771806X and pamoic acid, as has been observed for pamoic acid with other hydrophobic weak bases [13]. Upon translation to the multi-inlet vortex mixer and subsequent
optimization, it was shown that nanoparticle dispersions at API concentrations of ~1 mg/mL could be produced with an API loading of 30 wt%. Furthermore, due to the versatility of the Flash NanoPrecipitation process, the particle size distribution could be tuned on the MIVM without affecting the API loading or the zeta-potential. This capability is not commonly found in most nanoparticle synthesis methods, such as solvent evaporation [37] and nano-emulsion [38] techniques, and opens up many possibilities for optimizing formulations for in vivo performance. Finally, through freeze-drying, we were able to reach a ~8 mg/mL dose of GW771806X from a stable polymeric lyo-cake that can be reconstituted easily in a clinical setting.

This model study serves as an example of how the facile nature of FNP can be used to formulate a relatively difficult-to-formulate and hard-to-deliver API in a dosable form for parenteral administration. The presented methodology of rational formulation based on scientific principles involved the careful selection of FNP technique based on the physicochemical properties of the API, the optimization of the nanoparticle formulation variables at the lab-scale screening phase, translation of the optimal formulation from a lab-scale mixer to a larger-scale mixer, optimization of process parameters, and finally freeze-drying. For future endeavors, it is recommended to begin screening and formulation optimization on an MIVM; this will bypass the translation step that can be troublesome, as was shown in Chapter 3 for progesterone. Since the mixing conditions and the solvent quality changes are different in the CIJM-D and the MIVM, the same nanoparticle dispersion results cannot be expected for the same nominal nanoparticle composition. We also envision that next generations of this methodology will make use of the findings presented in this chapter (e.g. choice of organic solvent, how to tune particle size without affecting loading efficiency, effective lyoprotectants, effect of initial nanoparticle concentration when freeze-drying, etc.) for design of experiment studies that more can efficiently
optimize a formulation without needing to cover a variable space that would not yield useful formulations. Although when working on a developmental project that must put forth a pharmaceutical product, optimization must take into account release profiles, since we only worked with a model compound with no final marketable product envisioned, it was irrelevant to present release data. However, it is promising that the use of this FNP-based formulation process has currently reached the stage of pilot scale production for a GlaxoSmithKline proprietary hydrophobic weak base API.

4.5 ACKNOWLEDGEMENTS

We would like to thank Dr. Christopher Brook, Dr. Yan Sun, Samantha Rusk, Christopher Morrison, and Aidan Gilmartin from GlaxoSmithKline for their assistance in procuring materials, setting goals, conducting experiments, and productive discussions for this project. In particular, I thank Christopher Morrison for all his help in understanding and planning the freeze-drying studies. I would also like to thank Prof. George Scherer for granting me access to the Pyris TGA 7HT from the Scherer Lab and Lori Tunstall for training me to use the TGA instrument.

4.6 NOMENCLATURE

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>FNP</td>
<td>Flash NanoPrecipitation</td>
</tr>
<tr>
<td>API</td>
<td>active pharmaceutical ingredient</td>
</tr>
<tr>
<td>BCP</td>
<td>block copolymer</td>
</tr>
<tr>
<td>NPs</td>
<td>nanoparticles</td>
</tr>
<tr>
<td>PAM</td>
<td>pamoic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>AT</td>
<td>D-α-tocopherol</td>
</tr>
<tr>
<td>CSA</td>
<td>(±)-Camphor-10-sulfonic acid</td>
</tr>
<tr>
<td>ATS</td>
<td>D-α-tocopherol succinate</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>PCL</td>
<td>poly(ε-caprolactone)</td>
</tr>
<tr>
<td>PLA</td>
<td>poly(D,L-lactide)</td>
</tr>
<tr>
<td>PXXYkD-b-PEGZkD</td>
<td>poly(XX)-block-poly(ethylene glycol) of MW: YkD-b-ZkD</td>
</tr>
<tr>
<td>MeCN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>CIJM-D</td>
<td>confined impinging jets mixer with dilution</td>
</tr>
<tr>
<td>MIVM</td>
<td>multi-inlet vortex mixer</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>TGA</td>
<td>thermogravimetric analysis</td>
</tr>
<tr>
<td>LE</td>
<td>loading efficiency</td>
</tr>
<tr>
<td>Δ</td>
<td>span range ($Δ = d_{90} - d_{10}$)</td>
</tr>
<tr>
<td>d10</td>
<td>10% of the intensity-weighted size distribution is smaller than this diameter</td>
</tr>
<tr>
<td>d90</td>
<td>90% of the intensity-weighted size distribution is smaller than this diameter</td>
</tr>
<tr>
<td>R²</td>
<td>linear coefficient of determination</td>
</tr>
<tr>
<td>$c_i$</td>
<td>concentration of $i$</td>
</tr>
<tr>
<td>$m_i$</td>
<td>mass of $i$</td>
</tr>
<tr>
<td>$\rho_i$</td>
<td>mass density of $i$</td>
</tr>
</tbody>
</table>
$V_i$ volume of $i$

$S_i/S_i$ redispersibility ratio, or the ratio of peak mean sizes before and after freeze-drying/reconstitution

$\Delta f/\Delta i$ ratio of span ranges before and after freeze-drying/reconstitution

$pK_a$ base-10 logarithm of the acid dissociation constant $K_a$

$\tau_m$ characteristic mixing time

$\lambda_K$ Kolmogorov length scale

$D_{i,j}$ diffusion coefficient of $i$ in $j$

$\chi_{i,j}$ Flory-Huggins interaction parameter for a solute ($i$) in a solvent ($j$)

$\varepsilon_r$ relative permittivity

### 4.7 REFERENCES

1. Torchilin, V.P., *Nanoparticulates As Drug Carriers* 2006: Imperial College Press.


CHAPTER 4  Nanoparticles Loaded with a Weakly Basic API


CHAPTER 5 - PRODUCTION OF PHARMACEUTICAL NANOPARTICLES THROUGH FLASH NANO PRECIPITATION FOR ORAL APPLICATIONS

ABSTRACT

With the prominence of top-down industrial methods, or the use of attrition on bulk drug substances, for the production of pharmaceutical nanoparticle dispersions for oral applications, it is uncommon to see the use of bottom-up, or precipitation-based, methods for production of comparable products. Here, we present the use of Flash NanoPrecipitation, a facile rapid precipitation technique with simple scale-up, to obtain a robust generally recognized as safe formulation applicable to three model active pharmaceutical ingredients, indomethacin, fenofibrate, and cinnarizine. Through careful screening of excipients and optimization of formulation and process parameters, we achieved dispersions with 60 - 100% loading efficiencies and > 10% loadings at concentrations of the active pharmaceutical ingredients > 3 mg/mL. The dispersions had particle sizes of 140 - 200 nm with tight particle size distributions and zeta-potentials > -10 mV. We expect this formulation to be useful for active ingredients with considerable solubilities in tocopherol or poly(ethylene glycol) materials.

5.1 INTRODUCTION

Due to the recent discovery trends of novel active pharmaceutical ingredients (APIs) increasingly being more hydrophobic, and thus water insoluble [1], many new strategies have been employed to formulate drug products. One of the fields that has received much attention in novel drug formulation is that of nanotechnology, especially because API nanoparticulates can improve bioavailability, reduce variability, and increase dissolution rates [2, 3]. Techniques for
developing nano constructs are usually classified under bottom-up (precipitation/crystallization) or top-down (attrition) approaches depending on the way in which nanoparticles are formed [4]. Currently, top-down methods, such as high-pressure homogenization [5] and media milling [6], are preferred in industry because the processes are amenable for scaling down and scaling up for laboratory trials and manufacturing, respectively, as well as the resulting colloidal particles having a high drug loading as compared to typical dispersions produced using bottom-up scheme [7]. However, these methods generally require long processing times and are prone to contamination from metal ions in the case of homogenization or the milling media for media milling [8], both of which can contribute to higher manufacturing costs.

Flash NanoPrecipitation (FNP) is a bottom-up technique, described as a rapid precipitation method with polymer directed assembly resulting in kinetically-frozen particulates; the process is easily scalable [9] and can yield high API loadings [10]. While extensive characterization of the FNP process has been published and is discussed in Chapter 2, publications with regards to specific API formulations are limited. Out of the nine existing reports of FNP-based API formulations, eight [11-18] have been aimed at either parenteral or inhalable administration; only one has been targeted for oral administration [19]. While attention is given to the production of nanoparticle formulations for parenteral administration, particularly because of the prevalence of cancer research, solid oral dosage forms are preferred because of increased patient compliance and the decreased manufacturing costs associated with not requiring sterile production [20]. Of the more than 30 drug products marketed worldwide that are nanoparticulate in nature, at least 12 or more are administered orally [21]. While the FNP parenteral formulations that have been reported might possibly be administered orally, since the material components used are generally recognized as safe (GRAS) by the FDA, the most
obvious problem lies in the block copolymers used. Typically, oral formulations use materials that can be produced at large scales at an effective cost, which is not the case for the parenteral polymers. Therefore, our aim in this study is to present the adaptation of FNP techniques for the formation of API-loaded nanoparticulates that can be used for lower cost oral applications.

We present the formulation of three model APIs that have been widely used in the literature: indomethacin, cinnarizine, fenofibrate. Indomethacin is a non-steroidal anti-inflammatory agent used for rheumatoid arthritis, cinnarizine is an anti-histaminic agent used for motion sickness control, and fenofibrate is an antilipemic agent that reduces cholesterol and triglycerides in the bloodstream. These three APIs are all marketed as many different drug products, although only fenofibrate has been commercially formulated in nanoparticle form (Tricor® and Triglide®). These three APIs have calculated log\(P\) values ranging from 4.2 - 5.2 (refer to Appendix A), which is considered relatively low for candidate solutes to be used in FNP. It was important to not only consider compounds that cannot be charged such as fenofibrate, but also weak acids and bases, since these are generally more challenging to formulate using FNP. As previously discussed in Chapters 2 - 4, solutes with relatively low log\(P\) values will tend to recrystallize and result in nanoparticle dispersions with poor stability, especially if the solute can ionize. While other nanoparticulate production methods do not face difficulties in producing stable nanoparticle dispersions of these APIs, it can be challenging to use FNP for such purposes. Through the incorporation of the GRAS oil, D-\(\alpha\)-tocopherol, or its acidic counterpart, D-\(\alpha\)-tocopherol succinate, we show that all three APIs can be formulated into nanoparticle dispersions, suitable for oral product development, through the same base formulation.
5.2 MATERIALS AND METHODS

5.2.1 Materials

Indomethacin (INDO) (≥99%), fenofibrate (FENO) (≥99%), cinnarizine (CIN), sodium chloride (≥99.5%), D-α-tocopherol succinate (ATS) (semisynthetic), poly(ethylene glycol) (Mn~600) (PEG600), tetrahydrofuran (THF) (HPLC grade), and acetonitrile (MeCN) (HPLC grade) were purchased from Sigma-Aldrich (USA). Methanol (MeOH) (HPLC grade) and acetone (ACS grade) and were purchased from Fisher Scientific (USA). D-α-tocopherol (AT) (>97%) was purchased from TCI America (USA). D-α-tocopheryl poly(ethylene glycol) 1000 succinate (TPGS) (NF grade) was obtained from Eastman (USA). Trifluoroacetic acid (TFA) (reagent grade) was purchased from EMD Millipore (USA). Hydrochloric acid (1.0 N) (HCl) was purchased from Alfa Aeser (USA). Sodium dodecyl sulfate (electrophoresis grade) (SLS) was purchased from JT Baker (USA). Egg lecithin (~60%) was purchased from MP Biomedicals (USA). Hydroxypropyl methylcellulose (Methocl E15 Premium LV) (HPMC) was obtained from Dow Chemical (USA). Ultrapure water (18 MΩ·cm) (MilliQ water) was generated from a Barnstead Nanopure purification system.

5.2.2 Solubility Measurements

The solubility of APIs were measured in AT and in PEG600. Small volumes (< 0.8 mL) of the liquids were charged into 2 mL microcentrifuge tubes (Fisher, USA) and heated to approximately 45°C. The API powders were then measured out into the hot liquids and vortexed (Cole-Parmer Touch Mixer 4721-20) for a while until well mixed. The tubes were then loaded onto a Fisher Vortex Genie 2 and left to shake overnight. The following day, the tubes were removed and left at ambient conditions for at least 1 hour. The samples were then centrifuged
(Centrifuge 5430R; Eppendorf, USA) for 10 minutes at 20,000g at 25°C. The supernatant was then analyzed through HPLC.

5.2.3 Nanoparticle Formation

Amphiphile-stabilized API nanoparticles were produced using two different methods. The first made use of a laboratory-scale confined impinging jets mixer with dilution (CIJM-D) reported by Han et al [25]. Briefly, as an example, 1 mL of the organic stream containing an API, a steric stabilizer, and other possible excipients in THF in a disposable plastic syringe (National Scientific Company, USA) was simultaneously manually injected into the mixer at a syringe jet velocity of ~35 m/s against 1 mL of MilliQ water (or buffer) and collected into an 8 mL diluting reservoir of the aqueous phase. The resulting dispersion was 10 vol% THF.

The second method made use of a four-jet, or multi-inlet, vortex mixer (MIVM) described by Liu et al [22]. Depending on the amount of organic phase present in the final solvent mixture, different flow rates were used for the different streams, although all at turbulent mixing regimes (inlet Reynolds number of > 10,000). Three 100 mL gas tight glass syringes (SGE Analytical Science, Australia) were filled with MilliQ water and one disposable syringe (20 - 30 mL; AirTite, USA) contained the organic phase. The streams were injected into the mixer using digitally controlled PHD 2000 syringe pumps (Harvard Apparatus, USA). The initial ~5 mL of effluent were discarded and then collection was started. Effluent collection was stopped prior to stopping the pumps. This ensured that only dispersion produced at steady state operation were collected.

In order to quantify the loading efficiency of the API, a dispersion was dialyzed to remove organic solvent and thus have an only aqueous medium. An aliquot of the sample was loaded into Spectra/Por 1 (MWCO 6-8 kD) dialysis tubing (Spectrum Laboratories, USA). The
aliquot was dialyzed against MilliQ water generally at a 1 L to 10 mL ratio of bath to sample volume. The bath water was refreshed at least hourly for the first five hours and then left overnight. The dialyzed dispersion was then collected from the tubing and volume change was recorded. All dispersions were filtered (1.0 µm, GF/B; Whatman, USA), analyzed, and stored at T=4°C.

5.2.4 Nanoparticle Dispersion Characterization

5.2.4.1 Particle size distribution measurement

After nanoparticle production and each processing step, the dispersions were characterized for particle size using a Malvern Zetasizer ZS ZEN 3600 (UK). Samples were prepared by diluting the dispersions at least 10-fold in MilliQ water and were contained in disposable 1.5 mL plastic cuvettes (Fisher, USA). All measurements were made with a 633 nm laser at a scattering angle of 173°. Sizes reported are those obtained using the general purpose normal resolution analysis mode of the intensity-weighted distribution. The peak means and span ranges \( \Delta = d_{90} - d_{10} \) are given. Refer to Appendix C for a discussion on why this reporting protocol is used. Usually, at least triplicate measurements were made and the metrics given are the average and standard deviation of at least two measurements.

5.2.4.2 Solute concentration

After removing the organic solvent from the nanoparticle dispersions, API concentrations were measured through reverse-phase high pressure liquid chromatography (HPLC). For analyzing dispersion concentrations, the nanoparticles were diluted in THF at least 20-fold before HPLC analysis. When measuring solubility in AT or PEG600, the samples were diluted further to reach the calibration ranges. HPLC was conducted on a Hewlett-Packard Agilent LC 1100 equipped with a quaternary pump, diode array detector, and a Gemini C18 stationary phase
(5 µm, 100 Å; Phenomenex, USA). Mobile phases used are designated as follows: A - MilliQ water with 0.05 vol% TFA, B - MeCN with 0.05 vol% TFA, C - MeOH, D - MilliQ water.

INDO and CIN concentrations were measured using the following conditions: a linear gradient of 95%/5% A/B to 100% B over 2.5 minutes and returning to 95%/5% A/B at 2.7 minutes all at a flow rate of 1.5 mL/min using a column temperature of 55°C. The method run time was 6 minutes with analyte retention times of ~4 and 3.3 minutes for INDO and CIN, respectively. The detection wavelength used was 254 nm. The method was calibrated for INDO concentrations of 5 - 107 µg/mL with a linear coefficient of determination, $R^2 = 0.9989$, and for PAM, the method was calibrated for concentrations of 5 - 100 µg/mL with $R^2 = 0.9989$.

FENO concentrations were measured using the following conditions: a mobile phase of 90%/10% C/D at a flow rate of 1 mL/min using a column temperature of 30°C. The method run time was 5 minutes with an analyte retention time of ~3.2 minutes. Two detection wavelengths were used, 268 and 289 nm, such that an average concentration could be computed without requiring multiple measurements. The method was calibrated for FENO concentrations of 5 - 107 µg/mL with linear coefficients of determination, $R^2 = 0.9993$, for both wavelengths.

Loading efficiency (LE) of the active solute into the nanoparticles was calculated as follows:

$$LE(\%) = \frac{c_{\text{measured}}}{c_{\text{nominal}}} \left( \frac{V_{\text{initial}}}{V_{\text{measured}}} \right) \times 100\% \quad (5.1)$$

Here, $c_{\text{measured}}$ is the measured concentration through HPLC, $c_{\text{nominal}}$ is the nominal concentration expected if all the solute from the organic phase was recovered, $V_{\text{initial}}$ is the initial volume of dispersion loaded into the dialysis tubing, and $V_{\text{measured}}$ is the volume recovered from the dialysis tubing once dialyzed.
The API loading, or the amount of API in the dry nanoparticles by weight, was determined by analyzing the dry solids. The dispersions were freeze-dried (3 mL aliquots of the dispersion in 5 mL cryogenic vials (VWR, USA)) on a VirTis Benchtop 3.3 manifold lyophilizer (SP Scientific, USA) for 1 day (vacuum pressure < 200 mTorr, condenser temperature < -70°C). A specific mass of the solids was dissolved in a specific volume of THF, diluted, and analyzed by HPLC for API concentration. The loading was calculated as follows:

\[
\text{loading (wt\%) = } \frac{c_{\text{measured}}(x)}{c_{\text{solids}}} \times 100\% \tag{5.2}
\]

Here, \(x\) is the dilution factor and \(c_{\text{solids}}\) is the solids concentration of the lyophilizate dissolved in THF. All LE and loading measurements were done on dialyzed dispersions that were filtered.

**5.2.4.3 pH measurement**

Once the dispersion was free of organic solvent, the pH of the nanoparticle sample was measured using a Fisher Scientific accumet AR20 pH meter (USA). The probe was calibrated against pH 4, 7, and 10 standard buffers (Fisher Scientific, USA) each day before measurements.

**5.2.4.4 Zeta-potential measurement**

Once the dispersion was free of organic solvent, the nanoparticle sample was diluted with sodium chloride to result in a nanoparticle concentration of ~2.5 mg/mL and a salt concentration of ~10 mM. An aliquot of the salt sample was then transferred to a disposable folded capillary cell (Malvern, UK). Zeta-potential measurements were done on a Malvern Zetasizer ZS ZEN 3600 (UK) using the general purpose analysis mode at default instrument settings. Five replicate measurements were made and three were used to obtain the average zeta-potential and deviations that are reported.
5.2.5 Statistical Analysis

Analysis of variance (ANOVA) was performed on factorial studies evaluating the various formulation and process parameters in order to understand the importance of each. General linear modeling was performed using Minitab 15 Statistical Software (USA) with a significance level, $\alpha$, of 0.05.

5.3 RESULTS AND DISCUSSION

5.3.1 Initial Screening - Selection of Component Materials

In attempting to develop a FNP formulation for oral applications, classical approaches of using only the API and a stabilizing polymer yielded poor results with respect to stability and macro-precipitation. It was important to use a technique that can circumvent the problem of API recrystallization, which can result in poor stability and low API loadings. Since the goal of this work is to have a formulation that is applicable to as many non-derivatized APIs as possible, the hydrophobic prodrug [11] and ion-pair formation [17] methods are not desirable. We made use of dispersion or solubilization of the API in a hydrophobic core material, instead, as was used for progesterone [15].

In order to obtain a formulation that would be applicable to all APIs tested, while not requiring an exorbitant number of experiments, it was decided to use INDO as the model API. Indomethacin not only has the lowest ALOGPs of the three APIs, it would also be the most ionized in MilliQ water with unadjusted pH based on its $pK_a$ (see Table 5.1). It was predicted that it should be the most problematic to formulate through FNP; therefore, the hypothesis was that the other two APIs should form acceptable nanoparticle dispersions using the optimal INDO formulation.
In analyzing the results for the screening and all subsequent optimization, the most important criteria was achieving the highest API loading efficiency possible, while maintaining a tight particle size distribution; the peak mean diameter was not of high importance. Oral nanoparticle dispersions are attractive for their submicron size range which provide faster total dissolution rates, as expressed through equation 1.5, which is shown again below.

\[
\frac{dm}{dt}_{tot} = \frac{3Dc_{eq}m_{tot}}{\rho r^2} \left( 1 + \frac{1}{a + b \sqrt{\frac{rU}{v} \left( \frac{v}{D} \right)^{1/3}}} \right) \exp \left( \frac{2\gamma v}{RTr} \right) \tag{1.5}
\]

Here, \( m \) is the mass of an API particle at time \( t \), \( D \) is the diffusion coefficient of the API, \( c_{eq} \) is the saturated equilibrium solubility of the API, \( m_{tot} \) is the total mass dose of the API, \( r \) is the radius of the particle, \( \rho \) is the API density, \( a \) and \( b \) are constants, \( U \) is the fluid velocity, and \( v \) is the fluid kinematic viscosity, \( \gamma \) is the interfacial tension between the solute and fluid, \( v \) is the API molar volume, \( R \) is the universal gas constant, and \( T \) is absolute temperature. Please refer to section 1.1 of Chapter 1 for a derivation of this equation. As mentioned in Chapter 1, equation 1.5 demonstrates that by reducing the particle size, the dissolution rate is increased. This is the primary reason why with NanoCrystal® technology and other representative wet media milling techniques, the target particle size is < 1 µm [26]. Since FNP usually results in peak mean diameters that are also < 1 µm, the size produced was not of high concern.

5.3.1.1 Solubilizing co-solute

As has been shown in Chapters 3 and 4, it is important to perform screening and optimization on various aspects of an FNP formulation. We chose to first assess what core co-solute was to be used in the stabilization technique. As it is important to use materials that are considered safe, three polar oils listed in the United State Pharmacopoeia (-National Formulary)
were investigated as co-solutes for the loading of the APIs into nanoparticles: ethyl oleate, Miglyol 812, and D-α-tocopherol. In order to determine which oil would be optimal for achieving high API loadings, 3D distances of the Hansen solubility parameters of the APIs to the oils, \( r_{i,j} \), were compared. The solubility parameters for M812 were approximated from a 3/2 mixture of caprylic triglyceride/capric triglyceride by volume, which are the main components in the oil according to the manufacturer [23]. Table 5.1 below lists the properties of each API. The solubility parameters clearly show greater affinity for AT over the other two oils for all APIs. Thus, AT was chosen as the co-solute for FNP.

**Table 5.1** Properties of APIs considered in early screening. EtOl = ethyl oleate, M812 = Miglyol 812, AT = D-α-tocopherol. ALOGPs is a calculated logP value, the \( pK_a \) is the acid dissociation constant, and the various \( r_{i,j} \) values are the 3D distances between the Hansen solubility parameters of the API and the respective oil. References are given for literature \( pK_a \) values. Please refer to Appendices C and E for information on calculated logP and solubility parameters, respectively.

<table>
<thead>
<tr>
<th>API</th>
<th>ALOGPs</th>
<th>( pK_a )</th>
<th>( r_{i,EtOl} ) (MPa(^{1/2}))</th>
<th>( r_{i,M812} ) (MPa(^{1/2}))</th>
<th>( r_{i,AT} ) (MPa(^{1/2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin</td>
<td>4.25</td>
<td>4.5 [24]</td>
<td>8.9</td>
<td>8.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Fenofibrate</td>
<td>4.86</td>
<td>-</td>
<td>5.4</td>
<td>4.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Cinnarizine</td>
<td>5.19</td>
<td>7.4 [25]</td>
<td>4.0</td>
<td>3.5</td>
<td>2.9</td>
</tr>
</tbody>
</table>

5.3.1.2 Steric stabilizer

Following the choice of co-solute, the choice of two other components was considered, the polymeric steric stabilizer and a possible ionic surfactant. As with the co-solute, it was important to only evaluate polymeric stabilizers that are safe and commonly used in pharmaceutical formulations. Therefore, the three following materials were tested: Pluronic F127, hydroxypropyl methylcellulose, and D-α-tocopheryl poly(ethylene glycol) 1000 succinate. These three stabilizers are used in top-down approaches for producing nanoparticle dispersions [26] and Pluronic F127 [27] and TPGS [28] have sparingly been used for FNP. Although not a polymer, egg lecithin was also tested because it functions as a surfactant as well. For expedited
results, early screening was done on a lab-scale confined impinging jets mixer with dilution (CIJM-D). Solutions of the API (10 mg/mL), AT (10 mg/mL), and the stabilizer (40 mg/mL) in THF were mixed against water to result in a nominal API concentration of 1 mg/mL. Results are shown in Table 5.2 and are those after dialysis.

<table>
<thead>
<tr>
<th>Steric Stabilizer</th>
<th>Surfactant (wt/wt %)</th>
<th>Peak Mean Diameter (nm)</th>
<th>Micelle Diameter (nm)</th>
<th>Span Range (nm)</th>
<th>API (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pluronic F127</td>
<td>-</td>
<td>dispersion was unstable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPMC</td>
<td>-</td>
<td>290 ± 2</td>
<td>-</td>
<td>300 ± 4</td>
<td>0.05</td>
</tr>
<tr>
<td>TPGS</td>
<td>-</td>
<td>177 ± 2</td>
<td>-</td>
<td>180 ± 2</td>
<td>0.31</td>
</tr>
<tr>
<td>Lecithin</td>
<td>-</td>
<td>323 ± 7</td>
<td>-</td>
<td>360 ± 26</td>
<td>0.05</td>
</tr>
<tr>
<td>TPGS</td>
<td>Lecithin - 20%</td>
<td>246 ± 8</td>
<td>18 ± 0</td>
<td>414 ± 26</td>
<td>0.38</td>
</tr>
<tr>
<td>TPGS</td>
<td>Lecithin - 100%</td>
<td>79 ± 2</td>
<td>-</td>
<td>118 ± 3</td>
<td>0.65</td>
</tr>
<tr>
<td>TPGS</td>
<td>SLS - 20%</td>
<td>175 ± 0</td>
<td>-</td>
<td>140 ± 4</td>
<td>0.28</td>
</tr>
<tr>
<td>TPGS</td>
<td>SLS - 100%</td>
<td>160 ± 11</td>
<td>7 ± 1</td>
<td>220 ± 22</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Pluronic F127, despite being closer in nature to the traditional di-block copolymers used in FNP, was not suitable for FNP; the effluent from the mixer experienced visible macro-precipitation within the first 10 minutes after mixing. As Kumar explained for the case of itraconzole and odanacatib particles stabilized by Pluronic F127, the saturated solubility of the APIs is increased in the presence of the polymer micelles; also, active entrainment of API molecules from particle surfaces into Pluronic F127 micelles occurs as individual polymer chains participate in dynamic equilibrium between dissolved, micelle, and adsorbed phases [27]. The instability caused by the labile Pluronic F127, combined with the increased solubility, aid with recrystallization of the API. Pluronic F127 was re-evaluated in later MIVM studies, but generally showed ~60-80% lower loading efficiencies than TPGS at the same conditions. Although, the
other three stabilizers evaluated did result in dispersions that were stable enough to be put into dialysis, all experienced significant macro-precipitation during dialysis. This resulted in low API loadings as shown by the API concentrations. However, TPGS did result in the highest INDO concentration with the smallest peak mean diameter and span range, as shown in Table 5.2. This might be due to the AT/TPGS combination providing a better sink in which the API can remain, as compared to the other component combinations.

5.3.1.3 Electrostatic stabilizer

With respect to the surfactants evaluated, lecithin and sodium lauryl sulfate were considered. Both materials are used for top-down nanoparticle production [26]. While lecithin did not allow for high API loadings when tested alone as a stabilizer which had been accomplished for cyclosporine A [13, 29], when used as a surfactant in conjunction with TPGS, the INDO concentration increased as compared to the TPGS base case. However, the size distributions also became more polydisperse, as observed by the increases in peak mean diameter and span range at 20% lecithin and by the relative increase in span range at 100% lecithin. On the other hand, the inclusion of SLS did not drastically change the size distributions, but reduced the INDO concentrations from the TPGS base case. In contrast, SLS had previously helped in the stabilization of paclitaxel-loaded particles stabilized by poly(ε-caprolactone)-block-poly(ethylene glycol) (2.9kD-b-5kD) and Pluronic L64. Comparing the two surfactants, egg lecithin is composed of ~80% phosphatidylcholine, which has two fatty acid moieties branching from the choline head group [30]. Contrastingly, SLS has one fatty chain stemming from an anionic sulfate ion. It may be that the cationic moiety of the phosphatidylcholine might have the capability for ion-pair formation with the acidic INDO, which SLS does not. Furthermore, based on molecular weights, lecithin micelles that form are more likely to be less dynamic than those
of SLS, providing better stability to the systems using lecithin as a surfactant. By the same argument, the aggregation kinetics of lecithin and TPGS might be better matched than SLS, allowing for interactions between the stabilizer and surfactant to result in a broader distribution. Figure 5.1 shows particle size distributions for the trials using TPGS with 100% lecithin or SLS, showing the presence of the micelle population for the formulation with SLS.

![Particle size distributions for trials from Table 5.2 using TPGS and 100% lecithin or SLS.](image)

**Figure 5.1** Particle size distributions for trials from Table 5.2 using TPGS and 100% lecithin or SLS.

### 5.3.1.4 Summary

Based on the initial screening, it was decided to use D-α-tocopherol as a hydrophobic co-solute, D-α-tocopheryl poly(ethylene glycol) 1000 succinate as a stabilizer, and egg lecithin as a possible surfactant. Subsequent work involved the optimization of these components, as well as process parameters.

### 5.3.2 Initial Component Optimization

In continuing to the optimization of the components that were chosen from early screening, a translation to a MIVM was made. Since, as discussed in Chapter 4, the MIVM is most likely what would be used in the actual scale-up to plant scale, it was important to be as
close as possible to the final formulation from the start. To optimize the concentrations of the three components (three factors), a $3 \times 2 \times 3$ factorial study was conducted at a nominal INDO concentration of 1 mg/mL in the final dispersion. AT levels of 50%, 100% and 200% with respect to the INDO concentration, TPGS levels of 50% and 200% with respect to combined INDO and AT concentrations, and lecithin concentrations of 20% and 50% with respect to TPGS were evaluated. Although replicate particle size measurements were done for each sample, replicates for each treatment were not done; thus, ANOVA was performed to estimate the importance of only main effects and two-way interactions of each factor. Furthermore, to assess the stability of the different treatments, all samples were stored at $T=4^\circ C$ for 1 week and then filtered (1.0 µm) and analyzed. If the percent change experienced by a sample in peak mean diameter, micelle diameter, span range, and INDO concentration was $| \leq 10\% |$ after 1 week, the trial was considered stable; if only one metric experienced $| > 10\% |$ change and the change was not statistically significant ($p > 0.05$), it was considered as possibly stable; otherwise, the sample was not stable. Table 5.3 lists the trials of the factorial study and Table 5.4 displays the ANOVA results.

The ANOVA results show that the main effects were only important for the peak mean diameter and span range, for which only the TPGS factor was significant. Likewise, the two-way interaction between the AT and TPGS factors was only important for the micelle diameter and the loading efficiency. Figure 5.2 displays the AT/TPGS interaction on the loading efficiency means, where at the 50% TPGS level, increasing the AT level beyond 100% did not significantly increase the loading; this could be due to poor stabilization of the growing cores during FNP resulting in macro-precipitation.
Table 5.3 Factorial study evaluating the importance of the three components at a nominal INDO concentration of 1 mg/mL in the final dispersion with 10 vol% THF. Component concentrations are relative to the following concentrations: AT - INDO, TPGS - combined INDO and AT, lecithin - TPGS. Stability was assessed by determining the percent change of the peak mean diameter (PMD), micelle diameter (MD), span range (Δ), and INDO concentration (API) after 1 week storage at T=4°C. If all metrics experienced | ≤ 10% | change, the trial was considered stable; if only one metric experienced | > 10% | change and the change was not statistically significant (p > 0.05), it is labeled as Y*; otherwise, the trial was not stable.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Components (wt/wt %)</th>
<th>pH</th>
<th>Peak Mean Diameter (nm)</th>
<th>Micelle Diameter (nm)</th>
<th>Span Range (nm)</th>
<th>LE (%)</th>
<th>Stable (Y/N)</th>
<th>Unstable Metric(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 50 0</td>
<td>6.28</td>
<td>139 ± 1</td>
<td>29 ± 4</td>
<td>159 ± 9</td>
<td>9</td>
<td>N</td>
<td>MD</td>
</tr>
<tr>
<td>2</td>
<td>50 50 20</td>
<td>6.02</td>
<td>139 ± 2</td>
<td>26 ± 0</td>
<td>185 ± 9</td>
<td>9</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>50 50 50</td>
<td>5.75</td>
<td>148 ± 5</td>
<td>30 ± 2</td>
<td>201 ± 7</td>
<td>10</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>50 200 0</td>
<td>5.27</td>
<td>182 ± 7</td>
<td>16 ± 1</td>
<td>260 ± 15</td>
<td>22</td>
<td>N</td>
<td>PMD, Δ</td>
</tr>
<tr>
<td>5</td>
<td>50 200 20</td>
<td>5.21</td>
<td>149 ± 15</td>
<td>18 ± 0</td>
<td>119 ± 1</td>
<td>22</td>
<td>N</td>
<td>PMD, Δ</td>
</tr>
<tr>
<td>6</td>
<td>50 200 50</td>
<td>4.98</td>
<td>62 ± 2</td>
<td>-</td>
<td>128 ± 4</td>
<td>36</td>
<td>N</td>
<td>PMD, MD, Δ</td>
</tr>
<tr>
<td>7</td>
<td>100 50 0</td>
<td>6.25</td>
<td>216 ± 7</td>
<td>-</td>
<td>263 ± 19</td>
<td>14</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>100 50 20</td>
<td>5.15</td>
<td>175 ± 0</td>
<td>-</td>
<td>216 ± 5</td>
<td>11</td>
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<td>16 ± 23</td>
<td>217 ± 5</td>
<td>13</td>
<td>Y*</td>
<td>MD</td>
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<td>10</td>
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<td>4.64</td>
<td>87 ± 8</td>
<td>19 ± 1</td>
<td>125 ± 5</td>
<td>33</td>
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<td>-</td>
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<td>11</td>
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<td>119 ± 0</td>
<td>17 ± 0</td>
<td>142 ± 9</td>
<td>35</td>
<td>Y*</td>
<td>PMD</td>
</tr>
<tr>
<td>12</td>
<td>100 200 50</td>
<td>4.60</td>
<td>103 ± 1</td>
<td>22 ± 0</td>
<td>139 ± 3</td>
<td>38</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>200 50 0</td>
<td>5.68</td>
<td>262 ± 10</td>
<td>-</td>
<td>323 ± 24</td>
<td>13</td>
<td>Y*</td>
<td>Δ</td>
</tr>
<tr>
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<td>200 50 20</td>
<td>5.10</td>
<td>248 ± 10</td>
<td>-</td>
<td>311 ± 16</td>
<td>18</td>
<td>Y*</td>
<td>Δ</td>
</tr>
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<td>15</td>
<td>200 50 50</td>
<td>5.00</td>
<td>226 ± 6</td>
<td>-</td>
<td>274 ± 6</td>
<td>19</td>
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<td>-</td>
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<td>16</td>
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<td>4.58</td>
<td>104 ± 1</td>
<td>18 ± 1</td>
<td>151 ± 6</td>
<td>73</td>
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<td>-</td>
</tr>
<tr>
<td>17</td>
<td>200 200 20</td>
<td>4.51</td>
<td>186 ± 12</td>
<td>17 ± 0</td>
<td>196 ± 11</td>
<td>67</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>200 200 50</td>
<td>4.57</td>
<td>124 ± 6</td>
<td>22 ± 0</td>
<td>168 ± 12</td>
<td>55</td>
<td>N</td>
<td>PMD, MD</td>
</tr>
</tbody>
</table>
Table 5.4 ANOVA results for the 3 x 2 x 3 factorial study outlined in Table 5.3. The values listed are the p-values for each term in the general linear model, with $\alpha = 0.05$.

<table>
<thead>
<tr>
<th>Term</th>
<th>Factors (p-value)</th>
<th>Peak Mean Diameter</th>
<th>Micelle Diameter</th>
<th>Span Range</th>
<th>Loading Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT</td>
<td>0.130</td>
<td>0.060</td>
<td>0.222</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>TPGS</td>
<td>0.018</td>
<td>0.098</td>
<td>0.036</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Lecithin</td>
<td>0.452</td>
<td>0.805</td>
<td>0.725</td>
<td>0.938</td>
<td></td>
</tr>
<tr>
<td>AT x TPGS</td>
<td>0.181</td>
<td>0.008</td>
<td>0.270</td>
<td>0.043</td>
<td></td>
</tr>
<tr>
<td>AT x Lecithin</td>
<td>0.784</td>
<td>0.273</td>
<td>0.903</td>
<td>0.691</td>
<td></td>
</tr>
<tr>
<td>TPGS x Lecithin</td>
<td>0.489</td>
<td>0.341</td>
<td>0.960</td>
<td>0.965</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.2 Loading efficiency means as a function of AT and TPGS levels for factorial study displayed in Table 5.3.

Contrastingly, at the 200% TPGS level, while there was no statistical difference between 50% and 100% AT, a further increase to 200% resulted in almost double the loading efficiency. These two results demonstrate that while AT and TPGS were important for loading the API into nanoparticles, it was really the kinetics of TPGS that had the largest impact on the size of the particles. While Table 5.3 shows that treatments with higher amounts of AT did tend to have larger peak mean diameters than those with lesser amounts, this is to be expected because more AT and more INDO were loaded into the cores; it is really the difference in size for samples with 50% and 200% TPGS that were significant. Furthermore, since no terms involving the lecithin
factor were significant, this shows that lecithin did not statistically contribute to any of the metrics.

In terms of stability, few of the samples for trials 1-6 were stable over 1 week, with only the treatments at 50% TPGS with lecithin having produced stable samples. This is might be due to an abundance of TPGS in comparison to the AT, which could possibly have aided in the partitioning of API into micelles, thereby creating instability. Most other samples from trials 7-18 were at least possibly stable, with the exception of 100% AT/50% TPGS/20% lecithin (trial 8) and 200% AT/200% TPGS/50% lecithin (trial 18). These two trials exhibited instability resulting from a change in particle size distributions, which might be attributed to poorly anchored stabilizer and/or surfactant molecules, due to the decreased surface tension.

Considering the importance of the factors evaluated in this study and the loading efficiencies achieved, it was decided to not include lecithin in the formulation and to use AT levels of 100% or more. While it was not desirable to have micelle populations present in the resulting dispersion as this was the most common cause of instability observed, this issue was left to be resolved with further optimization.

5.3.3 Process Parameter Optimization

5.3.3.1 Organic solvent fraction & pH

Considering that in section 5.3.2 the importance of various components in the formulation was evaluated, the next optimization phase investigated process parameters. The organic solvent fraction in the dispersion solvent mixture and the pH of the anti-solvent streams were assessed. The organic solvent fraction, as has been shown in the previous chapters, can affect the growth kinetics involved in FNP and is easily observed in a modulation of particle size distributions. The pH of the anti-solvent stream will determine the pH of the final dispersion,
which can affect the solubility of the precipitating API. Since INDO has a $pK_a$ of 4.5, acidic pH levels should result in the API being mostly protonated, thus reducing water solubility; it is expected that decreased solubility should increase supersaturation levels and possibly increase loading efficiencies.

In the first study, the three best stable trials from Table 5.3 were used as base cases to test 20 vol% THF and a proton concentration in the final dispersion equivalent to a pH of 3. In changing the THF fraction, flow rates were changed to obtain the desired organic fraction while maintaining the inlet Reynolds number constant; this was important for mixing time scales to be much faster than aggregation time scales [22, 31]. The pH of the final dispersion was adjusted by using ~1 mM HCl as the antisolvent streams. A lower final pH was not considered because the manufacturer of the TPGS has shown that the ester linking the AT and PEG1kD from the TPGS will rapidly hydrolyze at low pH levels [32]. The trials for this experiment, including the previous base cases are shown in Table 5.5.

In terms of comparing the effects of each parameter, metric means were compared for each condition. The results are plotted in Figure 5.3 and they show that there was no statistically significant difference in the means as an function of the levels of the two factors ($p > 0.05$). ANOVA on the twelve trials confirmed that neither the main effects or the two-way interaction between THF fraction and dispersion pH were important factors at the levels considered. There was also no consistent trend with respect to stability of the various samples. While it is not clear why these two factors were not significant in the FNP of the system studied, it may be that neither had an important impact on the precipitation kinetics. Since some of the API will remain dissolved in the final dispersion solvent mixture, this will shift the pH downward.
Table 5.5 First process parameter optimization study at a nominal INDO concentration of 1 mg/mL in the final dispersion. Component concentrations are relative to the following concentrations: AT - INDO, TPGS - combined INDO and AT. See Table 5.3 for an explanation on stability.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Components (wt/wt %)</th>
<th>THF (vol%)</th>
<th>Dispersion pH</th>
<th>pH</th>
<th>Peak Mean Diameter (nm)</th>
<th>Micelle Diameter (nm)</th>
<th>Span Range (nm)</th>
<th>LE (%)</th>
<th>Stable (Y/N)</th>
<th>Unstable Metric(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>100</td>
<td>50</td>
<td>10</td>
<td>3</td>
<td>4.92</td>
<td>194 ± 7</td>
<td>230 ± 22</td>
<td>11</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>50</td>
<td>10</td>
<td>7</td>
<td>6.25</td>
<td>216 ± 7</td>
<td>263 ± 19</td>
<td>14</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>50</td>
<td>20</td>
<td>3</td>
<td>5.03</td>
<td>120 ± 3</td>
<td>135 ± 7</td>
<td>13</td>
<td>Y*</td>
<td>Δ</td>
</tr>
<tr>
<td>19</td>
<td>100</td>
<td>50</td>
<td>20</td>
<td>7</td>
<td>5.34</td>
<td>115 ± 4</td>
<td>129 ± 15</td>
<td>15</td>
<td>N</td>
<td>Δ, API</td>
</tr>
<tr>
<td>24</td>
<td>100</td>
<td>200</td>
<td>10</td>
<td>3</td>
<td>4.95</td>
<td>93 ± 2</td>
<td>20 ± 2</td>
<td>32</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>200</td>
<td>10</td>
<td>7</td>
<td>4.64</td>
<td>87 ± 8</td>
<td>19 ± 1</td>
<td>33</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>100</td>
<td>200</td>
<td>20</td>
<td>3</td>
<td>4.93</td>
<td>81 ± 1</td>
<td>16 ± 1</td>
<td>29</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>100</td>
<td>200</td>
<td>20</td>
<td>7</td>
<td>4.10</td>
<td>90 ± 4</td>
<td>16 ± 0</td>
<td>80</td>
<td>Y*</td>
<td>MD</td>
</tr>
<tr>
<td>27</td>
<td>200</td>
<td>200</td>
<td>10</td>
<td>3</td>
<td>4.94</td>
<td>108 ± 3</td>
<td>18 ± 0</td>
<td>132 ± 16</td>
<td>62</td>
<td>N</td>
</tr>
<tr>
<td>16</td>
<td>200</td>
<td>200</td>
<td>10</td>
<td>7</td>
<td>4.58</td>
<td>104 ± 1</td>
<td>18 ± 1</td>
<td>151 ± 6</td>
<td>73</td>
<td>Y</td>
</tr>
<tr>
<td>26</td>
<td>200</td>
<td>200</td>
<td>20</td>
<td>3</td>
<td>4.89</td>
<td>111 ± 4</td>
<td>125 ± 9</td>
<td>72</td>
<td>N</td>
<td>MD</td>
</tr>
<tr>
<td>25</td>
<td>200</td>
<td>200</td>
<td>20</td>
<td>7</td>
<td>5.09</td>
<td>113 ± 0</td>
<td>20 ± 1</td>
<td>128 ± 1</td>
<td>75</td>
<td>Y*</td>
</tr>
</tbody>
</table>
CHAPTER 5

Oral API Nanoparticles

**Figure 5.3** Metric means for factorial study listed in Table 5.5. Each set of four graphs depicts the means as a function of: a) final dispersion pH or b) THF fraction in the final solvent mixture. The means are for the trials based on base cases (BC) 7, 10, and 16 from Table 5.5.

**Table 5.6** Second process parameter optimization study at a nominal INDO concentration of 1 mg/mL in a dispersion with 20 vol% organic solvent. Component concentrations are relative to the following concentrations: AT - INDO, TPGS - combined INDO and AT. See Table 5.3 for an explanation on stability.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Components (wt/wt %)</th>
<th>Organic Solvent</th>
<th>pH</th>
<th>Peak Mean Diameter (nm)</th>
<th>Micelle Diameter (nm)</th>
<th>Span Range (nm)</th>
<th>LE (%)</th>
<th>Stable (Y/N)</th>
<th>Unstable Metric(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>100</td>
<td>200</td>
<td>acetone</td>
<td>4.89</td>
<td>102 ± 0</td>
<td>15 ± 0</td>
<td>122 ± 8</td>
<td>N</td>
<td>PMD, MD, Δ</td>
</tr>
<tr>
<td>22</td>
<td>100</td>
<td>200</td>
<td>THF</td>
<td>4.10</td>
<td>90 ± 4</td>
<td>16 ± 0</td>
<td>125 ± 8</td>
<td>Y*</td>
<td>MD</td>
</tr>
<tr>
<td>32</td>
<td>200</td>
<td>200</td>
<td>acetone</td>
<td>4.57</td>
<td>162 ± 4</td>
<td>16 ± 0</td>
<td>188 ± 7</td>
<td>N</td>
<td>PMD, MD, Δ</td>
</tr>
<tr>
<td>25</td>
<td>200</td>
<td>200</td>
<td>THF</td>
<td>5.09</td>
<td>113 ± 0</td>
<td>20 ± 1</td>
<td>128 ± 1</td>
<td>Y*</td>
<td>MD</td>
</tr>
</tbody>
</table>
The presence of the organic solvent will most likely increase the $pK_a$ of the API, as it has been shown for other weak acids in THF-water mixtures [33]. It might be possible that the pH in all treatments studied was approximately at the $pK_a$ or lower, meaning that the pH adjustment did not contribute much to shifting the pH downward. Further studies are required to fully understand why the two factors are relatively unimportant. For convenience, it was decided to not adjust the pH of the anti-solvent streams and to use 20 vol% THF for all subsequent studies.

### 5.3.3.2 Choice of organic solvent

Finally, the last process parameter optimization study involved the evaluation of another organic solvent instead of THF, namely acetone. From the trial conditions of the previous study, the two treatments with a dispersion pH of 7 and 20 vol% THF with the highest loading efficiency were considered. The same conditions were used, except to switch the THF with acetone. The results are presented in Table 5.6. When using acetone, base case trial 22 resulted in similar particle size metrics, but the loading efficiency decreased drastically from 80% to 35%. Contrastingly, base case trial 25 resulted in a 22% increase in peak mean diameter and a 47% increase in span range when using acetone; however, the loading efficiency increased from 75% to 100%. The difference between the two trials is the level of AT present in the formulation. Although there was not a large change in loading efficiency when increasing the AT level from 100% to 200% when using THF (80% to 75%), it increased roughly three-fold when using acetone. The analysis of Pustulka, where smaller particle sizes were obtained for poly(lactide-co-glycolide)-block-poly(ethylene glycol) (MW: 10kD-b-5kD) micelles when using acetone instead of THF, suggested that organic solvents with higher diffusion coefficients at infinite dilution in water, which mix faster with water, result in smaller particle sizes [34]; however, here the opposite is observed. As explained in section 4.3.3.1 of Chapter 4, it is unlikely that the small
change in the diffusion coefficient by changing organic solvent could explain the resulting
differences from the FNP process. A more plausible explanation is that the precipitation kinetics
are better matched when THF is used, allowing for a more balanced incorporation of the
components, since higher INDO loading efficiencies and smaller sizes are achieved.
Nonetheless, both treatments using acetone resulted in unstable dispersions due to changing
particle size distributions. Therefore, it was decided to continue using THF as the organic solvent
instead of switching to acetone.

5.3.4 Formulation Refinement

Since the process parameter optimization pointed to 20 vol% THF being the most
convenient condition for the organic stream and since the initial component optimization was
done with 10 vol% THF, it was important to refine the optimization. Although it was shown that
there was no significant difference between 10 and 20 vol% THF, the subsequent optimization
used 20 vol% THF to facilitate organic stream preparation. While the initial optimization had
levels of 50%, 100%, and 200% for AT and 50% and 200% TPGS, the lower levels of the factors
did not yield high enough loading efficiencies to be useful; thus, the levels were adjusted to
consider higher AT concentrations and more TPGS concentrations. In refining the optimization,
it was decided to evaluate different levels of 100%, 200%, 300%, and 400% for AT and 100%,
150%, and 200% for TPGS, while yielding a nominal API concentration of 1 mg/mL in a 20
vol% THF dispersion without pH adjustment. The experiments and results are presented in Table
5.7.

These trials yielded particle dispersions with peak mean diameters of 90 - 220 nm and
span ranges of 100 - 230 nm. No micelle populations were observed in these trials.
Table 5.7 Formulation refinement study at a nominal INDO concentration of 1 mg/mL in a dispersion with 20 vol% organic solvent and no pH adjustment. Component concentrations are relative to the following concentrations: AT - INDO, TPGS - combined INDO and AT. See Table 5.3 for an explanation on stability.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Components (wt/wt %)</th>
<th>pH</th>
<th>Peak Mean Diameter (nm)</th>
<th>Micelle Diameter (nm)</th>
<th>Span Range (nm)</th>
<th>LE (%)</th>
<th>Stable (Y/N)</th>
<th>Unstable Metric(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>100 100</td>
<td>4.79</td>
<td>115 ± 4</td>
<td>0</td>
<td>125 ± 4</td>
<td>34</td>
<td>N</td>
<td>PMD, Δ, API</td>
</tr>
<tr>
<td>53</td>
<td>100 150</td>
<td>5.50</td>
<td>106 ± 3</td>
<td>0</td>
<td>103 ± 3</td>
<td>33</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>100 200</td>
<td>4.10</td>
<td>90 ± 4</td>
<td>16 ± 0</td>
<td>125 ± 8</td>
<td>80</td>
<td>Y*</td>
<td>MD</td>
</tr>
<tr>
<td>33</td>
<td>200 100</td>
<td>4.63</td>
<td>166 ± 2</td>
<td>0</td>
<td>163 ± 8</td>
<td>80</td>
<td>N</td>
<td>API</td>
</tr>
<tr>
<td>54</td>
<td>200 150</td>
<td>5.16</td>
<td>147 ± 6</td>
<td>0</td>
<td>149 ± 8</td>
<td>96</td>
<td>Y*</td>
<td>PMD</td>
</tr>
<tr>
<td>25</td>
<td>200 200</td>
<td>5.09</td>
<td>113 ± 0</td>
<td>20 ± 1</td>
<td>128 ± 1</td>
<td>75</td>
<td>Y*</td>
<td>MD</td>
</tr>
<tr>
<td>46</td>
<td>300 100</td>
<td>5.16</td>
<td>193 ± 3</td>
<td>0</td>
<td>222 ± 10</td>
<td>64</td>
<td>Y*</td>
<td>Δ</td>
</tr>
<tr>
<td>47</td>
<td>300 150</td>
<td>5.05</td>
<td>161 ± 2</td>
<td>0</td>
<td>193 ± 3</td>
<td>64</td>
<td>N</td>
<td>Δ</td>
</tr>
<tr>
<td>55</td>
<td>300 200</td>
<td>5.21</td>
<td>140 ± 2</td>
<td>0</td>
<td>137 ± 6</td>
<td>88</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>400 100</td>
<td>5.09</td>
<td>222 ± 3</td>
<td>0</td>
<td>234 ± 6</td>
<td>78</td>
<td>N</td>
<td>Δ</td>
</tr>
<tr>
<td>49</td>
<td>400 150</td>
<td>5.14</td>
<td>173 ± 4</td>
<td>0</td>
<td>202 ± 5</td>
<td>68</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>56</td>
<td>400 200</td>
<td>5.27</td>
<td>137 ± 1</td>
<td>0</td>
<td>135 ± 7</td>
<td>93</td>
<td>Y*</td>
<td>Δ</td>
</tr>
</tbody>
</table>
Excluding the two trials with 100% AT and 100 - 150% TPGS, the loading efficiency was > 60%, reaching 96% for 200% AT and 150% TPGS. In terms of stability, the general trend was that trials with only 100% TPGS were unstable, while all others were at least possibly stable; the only exception was at the 300% AT level, where 100% TPGS resulted in a possibly stable dispersion, while 150% TPGS did not.

In attempting to understand the results, Figure 5.4 shows the trends for the peak mean diameters, span ranges, and loading efficiencies. It is obvious for the peak mean diameter that an increase in AT resulted in larger particle sizes; this is reasonable, since a higher concentration of co-solutes present in the dispersion during FNP will decrease the length scale for growth and lead to larger particles (see discussion in section 2.5 of Chapter 2). While increasing TPGS resulted in at least a modest decrease in size, at the 200% TPGS level, the difference amongst the peak mean diameters for the various AT levels was not drastic. This suggests that although the increase in AT can result in larger particle sizes, if the TPGS concentration is high enough, the effect of the AT increase is mitigated. This usually has not been seen in previous FNP studies, which might be because large di-block copolymers have been used as the stabilizers; here, since the stabilizer is the much smaller TPGS, the diffusion coefficient should be larger, and thus allow for faster growth arrest. Also, there should be high affinity between TPGS and AT, which has not always been the case for classic block copolymers and core solutes. Similar behavior was observed for the span range, with the span range converging to essentially one value at 200% TPGS. Interestingly, there was no clear trend for the loading efficiency. While increasing the TPGS level from 100% to 150% did not significantly increase the loading, increasing from 150% to 200% did result in at least a 20% increase for 100%, 300%, and 400% AT, achieving higher loading efficiencies as AT was increased. However, the 200% AT level did not follow this trend.
and instead had higher loading efficiencies at 100% and 150% TPGS as compared to the other
AT levels and the loading efficiency was the highest at 150% TPGS.

Once again, it was observed that the loading efficiency was similar for all AT levels at 200%
TPGS as with the peak mean diameter and span range.

![Figure 5.4](image.png)

**Figure 5.4** Peak mean diameters (PMD), span ranges ($\Delta$), and loading efficiencies (LE)
for trials from Table 5.7.

The optimum in loading efficiency at 200% AT/150% TPGS, with the 200% AT level
not following the trend of the other AT levels, might suggest the presence of internal phase
separation within nanoparticles. To elucidate more on this observation, the solubility of INDO
was measured in AT and in PEG600; INDO solubility could not be easily measured in PEG1kD,
which is present in the TPGS molecule, because at that molecular weight, PEG is no longer a
liquid, but a paste. The solubility of INDO, FENO, and CIN in AT and in PEG600 are shown in
Table 5.8. INDO actually is not very soluble in AT (~1 wt%) and has very high solubility in PEG600 (~14 wt%). This might mean that a vesicle-type structure might be present in the nanoparticles, with a PEG-rich interior domain into which INDO is loaded. Solid state characterization would be necessary to determine if this is the case.

Table 5.8 Solubility of the three APIs in AT and PEG600 as a weight fraction. Values reported are averages and standard deviations of n=2 measurements.

<table>
<thead>
<tr>
<th></th>
<th>AT</th>
<th>PEG600</th>
</tr>
</thead>
<tbody>
<tr>
<td>INDO</td>
<td>1.1 ± 0.0</td>
<td>13.6 ± 0.0</td>
</tr>
<tr>
<td>FENO</td>
<td>23.9 ± 0.2</td>
<td>8.0 ± 0.1</td>
</tr>
<tr>
<td>CIN</td>
<td>15.9 ± 0.1</td>
<td>1.7 ± 0.0</td>
</tr>
</tbody>
</table>

Overall, the formulation refinement trials showed that the highest loading efficiency was achieved at 200% AT/150% TPGS, while still maintaining a peak mean diameter < 200 nm. This was the optimal formulation achieved from the optimization trials.

5.3.5 Loading Fraction of the API in Nanoparticle Dispersions

For all FNP trial, the pH of the dispersion was measured after dialysis. This data was used to estimate the fraction of the measured INDO after dialysis that was truly loaded within the particle core. From the definition of the acid dissociation constant $K_a$, an expression for the equilibrium INDO solubility at a specific pH can be derived. In equations 5.3-5, $[HA(aq)]$ is the concentration of non-dissociated acid that is soluble in the aqueous solution, $[A^-]$ is the dissociated acid concentration, $[H^+]$ is the proton concentration, and $\log S_w$ is the base-10 logarithm of the equilibrium solubility of the non-dissociated acid. The $\log S_w$ for indomethacin has been reported to be -5.582 [35].

$$c_{eq} = [HA(aq)] + [A^-]$$

$$K_a = \frac{[H^+] [A^-]}{[HA(aq)]}$$
\[ c_{eq} = \left[ HA(aq) \right] \left( 1 + 10^{pK_a - pH} \right) = 10^{\log s_{eq}} \left( 1 + 10^{pK_a - pH} \right) \] (5.5)

Since equation 5.5 is applicable to acid that is in solution, it follows that the HPLC measurement of INDO after dialysis will be a sum of the equilibrium solubility (equation 5.5) and the concentration of precipitated INDO within a nanoparticle core. The fraction of loaded INDO, \( f_{load} \), was determined as the fraction of measured INDO that is not in solution, as shown below.

\[
f_{load} = \left( 1 - \frac{c_{eq}}{c_{measured}} \right) \times 100\% = \frac{1}{c_{measured}} \left[ c_{measured} - 10^{\log s_{eq}} \left( 1 + 10^{pK_a - pH} \right) \right] \times 100\% \] (5.6)

For the 63 trials that were run using INDO, the average loading fraction was 93±18%; however, once 16 outliers were removed through the analysis of the Minitab software (which involves box-plots and the use of interquartile ranges), the average loading fraction was 99±1%. The outliers included trials that made use of lecithin, Pluronic F127, or had no AT in the core. Considering that these outliers are representative of poor nanoparticle dispersions, it is not surprising that the loaded fractions for these cases were rather low. Overall, this calculation shows that essentially all measured INDO is associated with the nanoparticles, and is not in solution, for a satisfactory dispersion.

### 5.3.6 Application to Other APIs

The optimal nanoparticulate formulation achieved for INDO produced a dispersion with a peak mean diameter of 147 nm, span range of 149 nm, API loading efficiency of 96%, and an INDO concentration of 0.8 mg/mL. This dispersion was made using a THF solution with 5 mg/mL API, 10 mg/mL AT, and 22.5 mg/mL TPGS and FNP was performed on a MIVM resulting in 20 vol% THF. Prior to testing this formulation using the two other model APIs, experiments were done attempting to scale-up the API concentration through FNP. While all prior optimization had been done at a nominal API concentration of 1 mg/mL in the final
dispersion, in reality, the API concentrations were lower after dialysis. If this nanoparticulate formulation was to be feasible for subsequent development into a solid oral dosage form, it would be desirable to increase the API concentration in the dispersion. In these studies, the 200% AT/100% TPGS condition was also tested. The results are listed in Table 5.9 and particle size distributions are shown in Figure 5.5.

In increasing the API concentration, while maintaining the same composition, the peak mean diameter and span range decreased for the 200% AT/150% TPGS condition; likewise, the loading efficiency also decreased. While the 200% AT/100% TPGS condition did not exhibit the same monotonic decrease in the particle size metrics as the API concentration increased, the loading efficiency did follow the linear trend. This behavior of reduced loading efficiencies with increased total solids concentrations is the opposite of what was seen with progesterone in Chapter 3 (section 3.3.3); while in both cases the API was loaded into nanoparticles by incorporating AT, it may be that the acidic nature of INDO allows for it to more readily partition out of the lipophilic particle core, especially because it is not very soluble in AT. The decrease in particle size can be explained by the same behavior that was seen in the formulation refinement studies, where an increased concentration of TPGS resulted in decreased particle sizes; although it was shown that increasing AT concentrations also resulted in larger particle sizes, most likely the aggregation kinetics of TPGS are faster than the growth of AT in these cases where the concentrations are much more elevated. Based on the loading efficiencies achieved, only the two trials at a nominal API concentration of 1 mg/mL (base cases 33 and 54) and the trial at a nominal API concentration of 5 mg/mL with 200% AT/150% TPGS (base case 57) were considered acceptable.
Table 5.9 Studies involving the scale-up of API concentration and the application to other APIs with 20 vol% organic solvent and no pH adjustment. Component concentrations are relative to the following concentrations: AT - API, ATS - API, TPGS - combined API and AT/ATS. See Table 5.3 for an explanation on stability.

<table>
<thead>
<tr>
<th>Trial</th>
<th>API</th>
<th>API (mg/mL)</th>
<th>Components (wt/wt %)</th>
<th>pH</th>
<th>Peak Mean Diameter (nm)</th>
<th>Micelle Diameter (nm)</th>
<th>Span Range (nm)</th>
<th>API (mg/mL)</th>
<th>LE (%)</th>
<th>Stable (Y/N)</th>
<th>Unstable Metric(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>INDO</td>
<td>1</td>
<td>200 - 100</td>
<td>4.63</td>
<td>166 ± 2</td>
<td>0</td>
<td>163 ± 8</td>
<td>0.77</td>
<td>80</td>
<td>N</td>
<td>API</td>
</tr>
<tr>
<td>54</td>
<td>INDO</td>
<td>1</td>
<td>200 - 150</td>
<td>5.16</td>
<td>147 ± 6</td>
<td>0</td>
<td>149 ± 8</td>
<td>0.80</td>
<td>96</td>
<td>Y*</td>
<td>PMD</td>
</tr>
<tr>
<td>62</td>
<td>INDO</td>
<td>5</td>
<td>200 - 100</td>
<td>4.98</td>
<td>278 ± 4</td>
<td>0</td>
<td>322 ± 32</td>
<td>1.30</td>
<td>30</td>
<td>N</td>
<td>Δ, API</td>
</tr>
<tr>
<td>57</td>
<td>INDO</td>
<td>5</td>
<td>200 - 150</td>
<td>4.40</td>
<td>136 ± 3</td>
<td>0</td>
<td>146 ± 8</td>
<td>3.20</td>
<td>72</td>
<td>N</td>
<td>MD, Δ</td>
</tr>
<tr>
<td>35</td>
<td>INDO</td>
<td>10</td>
<td>200 - 100</td>
<td>4.34</td>
<td>119 ± 1</td>
<td>0</td>
<td>123 ± 5</td>
<td>2.42</td>
<td>28</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>58</td>
<td>INDO</td>
<td>10</td>
<td>200 - 150</td>
<td>4.48</td>
<td>71 ± 7</td>
<td>13 ± 1</td>
<td>107 ± 1</td>
<td>2.70</td>
<td>26</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>67</td>
<td>FENO</td>
<td>1</td>
<td>200 - 100</td>
<td>7.01</td>
<td>174 ± 4</td>
<td>0</td>
<td>186 ± 11</td>
<td>0.94</td>
<td>107</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>68</td>
<td>FENO</td>
<td>1</td>
<td>200 - 150</td>
<td>8.03</td>
<td>158 ± 5</td>
<td>0</td>
<td>178 ± 16</td>
<td>0.85</td>
<td>83</td>
<td>Y*</td>
<td>Δ</td>
</tr>
<tr>
<td>69</td>
<td>FENO</td>
<td>5</td>
<td>200 - 150</td>
<td>6.67</td>
<td>195 ± 5</td>
<td>0</td>
<td>225 ± 26</td>
<td>3.82</td>
<td>88</td>
<td>Y*</td>
<td>Δ</td>
</tr>
<tr>
<td>64</td>
<td>CIN</td>
<td>1</td>
<td>200 - 100</td>
<td>7.24</td>
<td>159 ± 4</td>
<td>0</td>
<td>156 ± 7</td>
<td>0.40</td>
<td>43</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>65</td>
<td>CIN</td>
<td>1</td>
<td>200 - 150</td>
<td>6.63</td>
<td>144 ± 0</td>
<td>0</td>
<td>145 ± 4</td>
<td>0.34</td>
<td>36</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>66</td>
<td>CIN</td>
<td>5</td>
<td>200 - 150</td>
<td>6.44</td>
<td>188 ± 2</td>
<td>0</td>
<td>217 ± 20</td>
<td>0.78</td>
<td>19</td>
<td>Y*</td>
<td>Δ</td>
</tr>
<tr>
<td>70</td>
<td>CIN</td>
<td>1</td>
<td>- 200 100</td>
<td>6.70</td>
<td>162 ± 2</td>
<td>0</td>
<td>141 ± 2</td>
<td>0.55</td>
<td>48</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>71</td>
<td>CIN</td>
<td>1</td>
<td>- 200 150</td>
<td>6.21</td>
<td>148 ± 1</td>
<td>0</td>
<td>150 ± 1</td>
<td>0.56</td>
<td>56</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>72</td>
<td>CIN</td>
<td>5</td>
<td>- 200 150</td>
<td>5.61</td>
<td>154 ± 0</td>
<td>0</td>
<td>160 ± 5</td>
<td>3.14</td>
<td>62</td>
<td>Y</td>
<td>-</td>
</tr>
</tbody>
</table>
These three formulations were then evaluated using the other model APIs, FENO and CIN. The results are listed in Table 5.9 and displayed in Figure 5.6. All trials yielded dispersions with peak mean diameters < 200 nm, ranging from 136 nm to 195 nm, and no micelle populations. The span ranges were between 141 nm to 225 nm, indicative of the tight distributions. The FENO dispersions consistently achieved high loading efficiencies, which can be attributed to the high solubility of the API in AT (Table 5.8). Although there was not a huge change in size metrics and loading efficiencies for FENO for the three formulations tested, CIN consistently had poor loading efficiencies of 43, 36 and 19% for the different conditions. This was odd, considering the pH values of the dispersions after dialysis were ≤ 7 and since CIN is a weak base, the pH should have been basic if the API did not load well into the particles. Although Table 5.1 shows CIN was predicted to have the highest affinity for AT out of the three model APIs, Table 5.8 shows that the solubility in AT was lower than FENO (16 wt% vs 24 wt%); the solubility in PEG600 is also quite poor (1.7 wt%). This suggests that while INDO and
FENO are sufficiently stabilized in the AT/PEG nano-constructs, CIN is not, resulting in poor loading.

Figure 5.6 Peak mean diameters (PMD), span ranges (Δ), and loading efficiencies (LE) for the trials listed in Table 5.9, where the base cases (BC) of INDO are used to represent the different formulations used. AT and ATS refer to the hydrophobic excipient used.

In hopes of achieving better loadings, the formulation was changed slightly by substituting AT with D-α-tocopherol succinate. The results show that the ATS formulations reduced the size metrics to values comparable to the other APIs; the electrostatic stabilization afforded by ATS might have helped to arrest growth earlier when compared to the steric stabilization of TPGS alone. The loading efficiency also increased to 48, 56 and 62% for the three formulations; while these values are still relatively low, the concentrated formulation resulted in 3.14 mg/mL CIN, which is comparable to the concentration of INDO at the same conditions. In section 4.3.1.3 of Chapter 4, the rule of thumb proposed by Pinkerton et al was
considered [17], where a difference in $pK_a$ values of the base and pairing acid of at least 2 pH units suggests an ion pair can form. The $pK_a$ difference for CIN and ATS is 1.8 in a fully aqueous medium, implying a lower difference in the solvent mixture formed through FNP; therefore, all of the CIN will not form an ion pair. In comparing the 3D distances between Hansen solubility parameters (as shown in Table 5.1) for CIN and ATS in comparison to CIN and AT, the distance is 2.4 instead of 2.9; this indicates that there is better miscibility of CIN in ATS. Therefore, ATS is better suited to favorably interact with CIN than AT, which might explain the enhanced loading efficiencies.

Finally, the API loadings and zeta-potentials for selected trials from the API application study were measured. The experimental values are listed below in Table 5.10.

<table>
<thead>
<tr>
<th>Trial</th>
<th>API</th>
<th>API (mg/mL)</th>
<th>Components (wt/wt %)</th>
<th>API Loading (wt%)</th>
<th>ζ-Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>INDO</td>
<td>1</td>
<td>0.77</td>
<td>200 - 100</td>
<td>8</td>
</tr>
<tr>
<td>57</td>
<td>INDO</td>
<td>5</td>
<td>3.20</td>
<td>200 - 150</td>
<td>11</td>
</tr>
<tr>
<td>67</td>
<td>FENO</td>
<td>1</td>
<td>0.94</td>
<td>200 - 100</td>
<td>19</td>
</tr>
<tr>
<td>69</td>
<td>FENO</td>
<td>5</td>
<td>3.82</td>
<td>200 - 150</td>
<td>14</td>
</tr>
<tr>
<td>64</td>
<td>CIN</td>
<td>1</td>
<td>0.40</td>
<td>200 - 100</td>
<td>10</td>
</tr>
<tr>
<td>66</td>
<td>CIN</td>
<td>5</td>
<td>0.78</td>
<td>200 - 150</td>
<td>3</td>
</tr>
<tr>
<td>70</td>
<td>CIN</td>
<td>1</td>
<td>0.55</td>
<td>- 200 100</td>
<td>13</td>
</tr>
<tr>
<td>72</td>
<td>CIN</td>
<td>5</td>
<td>3.14</td>
<td>- 200 150</td>
<td>12</td>
</tr>
</tbody>
</table>

The concentrated trials (57, 69, 72) yielded API concentrations of > 3.10 mg/mL with the nanoparticles having an API loading of 11 - 14 wt%. The peak mean diameters were < 200 nm, with span ranges < 230 nm, as previously mentioned. The zeta-potentials were ≥ -10 mV for the concentrated trials, with lower values for the INDO and CIN formulations since these had acidic groups present. These formulations were stable for 1 week of storage at T=4°C, with the exception of those with INDO as the API. However, it is possible that the stability is worse at the
lower temperature than at ambient conditions, as was shown in Chapter 3 for progesterone/AT particles (section 3.3.4); considering that the solubility of the APIs in either AT or PEG is probably reduced at lower temperatures, the instability of trials 33 and 62 might be irrelevant. Nonetheless, a possible solution for poor stability might be to proceed to drying immediately after FNP. This will depend on whether a subsequent concentration step is required prior to drying and what drying technique is preferred.

5.4 CONCLUSIONS

We have presented the methodology for achieving an FNP formulation for three APIs, indomethacin, fenofibrate, and cinnarizine, all produced using D-α-tocopherol or D-α-tocopherol succinate and D-α-tocopheryl poly(ethylene glycol) 1000 succinate. Using INDO as a model, initial screening was first done to select these excipient components. Subsequent formulation optimization showed the importance of AT and TPGS for producing acceptable nanoparticle dispersions and proved that the surfactant lecithin did not contribute to the formulations. Process parameter evaluations showed that the amount of THF in the final solvent mixture and the pH of the anti-solvent streams were not important factors at the levels tested, allowing these parameters to be amenable to convenience. Next, the formulation was refined as the process parameters were adjusted, yielding a dispersion of 147 nm in size with 96% loading efficiency of INDO at 0.8 mg/mL API. Although this formulation could not be scaled-up in API concentration much further through FNP, INDO concentrations of ~3.2 mg/mL were obtained. The best formulations were evaluated for the other APIs, which were successfully translated for FENO. The AT formulation performed poorly for CIN, which required substituting ATS for AT to obtain better dispersions. While the GRAS formulations here may not translate perfectly for all APIs, they
serve as good starting points for further optimization of APIs which show sufficient solubility in AT or PEG.

It is important to realize that the nanoparticle formulations presented here would be the first step in the preparation of a solid oral dosage form. Work would be required to identify feasible drying techniques and tablet/capsule formulations. A further concentration step might be desirable as well in order to increase production speed. If a liquid oral dosage form is acceptable, the dispersions should also be further concentrated and the stability at ambient and/or harsher conditions would need to be assessed, possibly requiring the incorporation of other excipients to enhance stability. Furthermore, API dissolution was not presented here because dissolution profiles are important to consider when comparing a dry nanoparticle formulation to the original liquid formulation and to the leading commercial formulation. It would be critical to conduct pharmacokinetic studies to determine how API absorption differs when using an FNP formulation as opposed to other traditional formulations. Our work here emphasizes the point that pharmaceutical nanoparticle dispersions can be produced for oral applications by routes other than wet media milling or homogenization which are commonly used in the industry. This should open up new paths in the future which might be more efficient or cost-effective.

5.5 NOMENCLATURE

API  active pharmaceutical ingredient
FNP  Flash NanoPrecipitation
GRAS generally recognized as safe
INDO indomethacin
FENO fenofibrate
CIN  cinnarizine
ATS  D-α-tocopherol succinate
AT   D-α-tocopherol
SLS  sodium lauryl sulfate
HPMC hydroxypropyl methylcellulose
TPGS D-α-tocopheryl poly(ethylene glycol) 1000 succinate
PEG  poly(ethylene glycol)
THF  tetrahydrofuran
MeCN acetonitrile
MeOH methanol
TFA  trifluoroacetic acid
HCl  hydrochloric acid
CIJM-D confined impinging jets mixer with dilution
MIVM multi-inlet vortex mixer
HPLC high pressure liquid chromatography
ANOVA analysis of variance
$\Delta$ span range ($\Delta = d_{90} - d_{10}$)
$d_{10}$ 10% of the intensity-weighted size distribution is smaller than this diameter
$d_{90}$ 90% of the intensity-weighted size distribution is smaller than this diameter
$R^2$ linear coefficient of determination
$c_i$ concentration of $i$
$V_i$ volume of $i$
x dilution factor
$LE$ 

loading efficiency

$\alpha$ 

significance level for ANOVA

$pK_a$ 

base-10 logarithm of the acid dissociation constant $K_a$

$r_{ij}$ 

distance between the Hansen solubility parameters of two materials $(i,j)$ as defined in a 3D space

$m$ 

mass

$t$ 

time

$D$ 

diffusion coefficient

$c_{eq}$ 

saturated equilibrium solubility

$m_{tot}$ 

total mass dose of an API

$r$ 

radius of a spherical particle

$\rho$ 

mass density

$a, b$ 

constants

$U$ 

fluid velocity

$\nu$ 

fluid kinematic viscosity

$\gamma$ 

interfacial tension between a solute and fluid

$\nu$ 

molar volume

$R$ 

universal gas constant

$T$ 

absolute temperature

$p$ 

probability of achieving a test statistic as extreme as the one observed

$[H^+]$ 

proton concentration

$[A^-]$ 

dissociated acid concentration

$[HA(aq)]$ 

concentration of non-dissociated acid that is soluble
\( \log S_w \) base-10 logarithm of the equilibrium solubility of the non-dissociated acid

\( f_{load} \) fraction of API in a dispersion that is loaded in nanoparticles

5.6 REFERENCES

28. Saad, W., Drug nanoparticle formation via flash nanoprecipitation: conjugation to encapsulate and control the release of paclitaxel, in Chemical Engineering2007, Princeton University.
34. Pustulka, K., *Polymer stabilized nanosuspensions via flash nanoprecipitation: particle formation, structure and freeze drying*, in *Chemical Engineering* 2012, University of Minnesota.

CHAPTER 6 - FREEZE-DRYING: PARAMETERS AFFECTING REDISPERSIBILITY OF LYOPHILIZED PEG-STABILIZED NANOPARTICLES

Note: This chapter contains a section from the publication, "Effervescent Redispersion of Lyophilized Polymeric Nanoparticles. Therapeutic Delivery 4(2), 177-190 (2013)" and is used with permission.

ABSTRACT

While the use of poly(ethylene glycol) (PEG) aids in the stealth properties of pharmaceutical nanoparticulate constructs, it also presents unique challenges when freeze-drying these nanoparticles into a dry powder. While covalently-bound PEG on nanoparticle surfaces can act as a cryoprotectant, it will also crystallize during drying, possibly resulting in particle bridging. Here, we focus on the freeze-drying of PEG-stabilized nanoparticles produced through Flash NanoPrecipitation. Through investigating various nanoparticle formulations, we show that particle aggregation from PEG crystallization can result when particles exhibit dense PEG steric layers on their surfaces. The density of the PEG layer is affected by the relative immiscibility of the blocks of the amphiphilic di-block copolymer. For two block copolymers with poor phase separation, we show that low surface coverage led to inadequate redispersibility, most likely from hydrophobic core contacts leading to aggregation. The use of fluid-like nanoparticle cores can also help to produce better redispersibility of lyophilizates. Effects of initial and reconstituted nanoparticles concentrations are discussed. We compare the use of a salt, sugars, and polymers as protectant excipients and show that PEG-based excipients are better suited as lyoprotectants for PEG-stabilized nanoparticles and contribute less osmolarity to the dispersion than small molecules. Finally, an examination of process parameters highlights the robustness of
formulations using PEG-based excipients. These findings will aid in the rational design of lyophilized PEG-stabilized nanoparticle formulations.

6.1 INTRODUCTION

There has been increasing attention given to nanoparticulate constructs for drug delivery because they provide increased bioavailability, protection for the drug(s) from degradation, prolonged circulation, and in some cases targeted delivery and/or extended release [1-4]. For many of these nanoparticles formulations, the incorporation of stealth elements on particle surfaces is used to increase the circulation time by reducing activation of clearance pathways in vivo [1]. Typical stealth techniques involve coating of the particles with stabilizing polymers used to modify the particle surface. Poly(ethylene glycol) (PEG), also known as poly(ethylene oxide), is often used because of the excellent steric stabilization it provides and its low toxicity [2], as well the ability to avoid opsonization of particles in vivo [3]. In fact, all commercialized stealth drug delivery systems on the market make use of PEG [2]. Although various techniques exist for the production of PEG-stabilized nanoparticles, Flash NanoPrecipitation (FNP) is an attractive method because of its capability to produce dense PEG brush layers [4-6], which can lead to extensive circulation times in vivo [5, 7].

However, one of the aspects of pharmaceutical nanoparticle dispersions that is generally not given much attention is stability. Typical dry dispersions tend to be much more stable than liquid dispersions [8], which are susceptible to sedimentation/creaming, aggregation, macroscopic crystallization and crystal growth through Ostwald ripening [9, 10], conversion from amorphous to crystalline states, and chemical degradation [11]. While dispersions produced through FNP may have excellent short-term stability in liquid form when stored in cool settings
[12, 13], for the development of a commercial pharmaceutical product it is important to have long shelf-life. In order to avoid stability problems associated with liquid dosage forms, it is generally more practical to convert the nanoparticles into a dry dosage form, such as a powder that can be reconstituted. One common technique for achieving dry powders, particularly for parenteral formulations, is freeze-drying, which is the focus of the work presented here.

One of the main challenges in the drying of nanoparticulate dispersions is maintaining particle size, since it is the small nanometer size that contributes to the efficacy of the formulation [14]. For freeze-drying, there are several phenomena that can contribute to particle aggregation. Significant aggregation and the growth of particle size can render the formulation useless as it can no longer be administered intravenously once reconstituted [15]. The three steps of freeze-drying will be considered, highlighting possible causes for aggregation specifically for the case of PEG-stabilized nanoparticle dispersions.

Step 1: Freezing

The first step is freezing. Depending on the formulations that will be frozen, various thermal events will occur, starting with ice formation, most likely at a depressed freezing point. Here, particles can be forced together by mechanical stresses from water crystallization, as the removal of water from the dispersion begins to concentrate the solids in the process known as cryo-concentration [16]. Cryoprotectants are excipients that prevent aggregation during the freezing process. There are two hypothesized mechanisms for how cryoprotectants function: a) glass-forming carbohydrates, such as sugars and polyols, become concentrated as they are excluded by growing ice crystals, reaching the maximally cryo-concentrated point where the cryo-concentrate vitrifies and immobilizes the primary particles; b) primary particles are physically isolated in the cryo-concentrate due to an excess of cryoprotectant(s), whether they
vitrify or not [17, 18]. During cryo-concentration, various phase transitions can occur depending on the cryoprotectants in the formulation. If there are salts present in the initial dispersion, a eutectic point will be reached, resulting in the precipitation of hydrated salts. A eutectic for the hydrated PEG layer on the particle surfaces will also be reached [19-21], which is dependent on the molecular weight of the stabilizing PEG [22]. This crystalline PEG/water eutectic layer on the particle surface will form a protective layer covering the hydrophobic core such aggregation through contacts of hydrophobic surfaces during freezing does not significantly occur. Below this temperature, other excipients may exhibit eutectic formations or glass transitions. With the formation of various phases during freezing determining the structure and distribution of the lyo-cake, particle aggregation may result in later steps.

**Step 2: Primary Drying**

Next, follows primary drying. Once the samples to be freeze-dried have equilibrated with the cooling sink, the temperature is usually raised near the lowest eutectic or glass transition temperature associated with maximally cryo-concentrated excipients and pressure is substantially reduced. During this step, sublimation of ice crystals will proceed, which is quickened by a higher temperature, since the vapor pressure of ice is temperature dependent. Because of the glass transition temperature a system may display, it is important to have precise control of product temperatures during primary drying. If the temperature goes above the solidification point of the maximally cryo-concentrated system, the cryo-concentrate will experience what is termed as "meltback," which is essentially thawing [23]. This process will result in significant loss of volume of the resulting lyo-cake and can result in cake collapse; meltback can decrease the inter-particle distance and increase chances of primary particles aggregating. Thus, it is
important that primary drying be done properly at a temperature where thawing or cake collapse is negligible, such that there is minimal change in cake structure [24].

Step 3: Secondary Drying

Finally, once all the ice has been sublimed, the remaining material will be the hydrated lyo-cake. During secondary drying, the residual water in the lyophilizate will be removed, generally by further increasing the product temperature. As aforementioned, the distribution of the nanoparticles has been established during the freezing step. If only the PEG-stabilized nanoparticles are considered without any excipients, the PEG on particle surfaces can form pure crystals as the water is removed from the eutectic that formed during freezing. Depending on the inter-particle distance, crystallization amongst particles may lead to bridging and poor redispersibility. Lyoprotectants are excipients that protect against aggregation during drying. For PEG, these excipients should effectively function as a "hydrating layer," being able to intercalate with the stabilizing PEG layers of the particles, such that the excipient molecules cannot bridge distinct particles together or crystallize within the PEG layer of a particle such that it cannot be rehydrated once reconstituted. Therefore, if proper lyoprotectants are not incorporated into the formulation, secondary drying can lead to considerable aggregation of PEG-stabilized nanoparticles. If an excipient is present, PEG crystallization can be inhibited if the particles are dispersed in a matrix for which PEG has affinity [25]; however, as we will show, addition of free PEG, which will still crystallize, can help to disperse primary particles such that bridging through stabilizing chain crystallization does not appreciably occur.

While PEG is clearly useful for in vivo performance of pharmaceutical nanoparticles, it presents unique challenges in the freeze-drying process. As discussed above, the phase behavior of PEG can be beneficial during freezing, but can result in poor redispersibility during drying. In
this work, we investigate the effects of various formulation and process parameters in the freeze-drying of FNP-produced PEG-stabilized nanoparticles. In examining formulation parameters, we discuss the importance of characterizing the degree to which PEG stabilizes the particle surfaces and how this surface coverage can affect redispersibility. Likewise, we show that the physical state of particle cores affect the extent of particle aggregation. The concentration of the initial dispersion is also shown to impact the redispersibility of the lyophilizate. A distinction between protectant excipients is also made, in which we highlight the use of PEG-based excipients as effective lyoprotectants for freeze-drying these dispersions. Finally, we evaluate the effects of process parameters, such as cooling rates, drying methods, and the presence of residual organic solvent used in the production of the initial dispersions, on redispersibility. Recommendations on how to rationally approach freeze-drying of PEG-stabilized nanoparticle dispersions are made as final remarks.

Since this is a long chapter, an outline is of the results and discussion section is given below to help guide the reader.

- **6.3.1 Nanoparticle Formulation Parameters**
  - 6.3.1.1 Di-block copolymer stabilizer
  - 6.3.1.2 Physical state of nanoparticle cores
  - 6.3.1.3 Concentration of nanoparticle dispersions
- **6.3.2 Protectant Excipients**
  - 6.3.2.1 Salts
  - 6.3.2.2 Polymers
  - 6.3.2.3 Sugars
- **6.3.3 Process parameters**
  - 6.3.3.1 The presence of organic solvent
  - 6.3.3.2 Cooling rates
  - 6.3.3.3 Drying conditions
6.2 MATERIALS AND METHODS

6.2.1 Materials

Pamoic acid (PAM) (>95%) was purchased from TCI America (USA). Cholecalciferol (D₃) (≥98%), poly(ethylene glycol) (Mₙ≈4600 and Mₙ≈20,000), and acetonitrile (MeCN) (HPLC grade) were purchased from Sigma-Aldrich (USA). GW771806X was provided by GlaxoSmithKline (King of Prussia, PA). Tetrahydrofuran (THF) (HPLC grade), dimethyl sulfoxide (DMSO) (HPLC grade), methanol (MeOH) (HPLC grade), and acetone (ACS grade) were purchased from Fisher Scientific (USA). Trifluoroacetic acid (TFA) (reagent grade) and potassium chloride (≥99%) was purchased from EMD Millipore (USA). Poly(D,L-lactide)-block-poly(ethylene glycol) (PLA-b-PEG) (MW: 3.7kD-b-5kD) was generously supplied by Evonik Inc. (USA). Poly(ε-caprolactone)-block-poly(ethylene glycol) (PCL-b-PEG) (MW: 7kD-b-5kD) and polystyrene-block-poly(ethylene glycol) (PS-b-PEG) (MW: 1.6kD-b-5kD) were purchased from Polymer Source (CAN). Pluronic F68 was supplied by BASF (Germany). Ultrapure water (MilliQ water) (18 MΩ·cm) was generated from a Barnstead Nanopure purification system.

6.2.2 Nanoparticle Formation

Block copolymer stabilized nanoparticles were produced using a multi-inlet vortex mixer (MIVM) described by Liu et al [26]. Depending on the amount of organic phase present in the final solvent mixture, different flow rates were used for the different streams, although all at turbulent mixing regimes. Three 50 mL gas tight glass syringes (SGE Analytical Science, Australia) were filled with MilliQ water and one syringe, either glass (25 mL; SGE Analytical Science, Australia) or disposable (20 - 30 mL; AirTite, USA) contained the organic phase. The organic phase consisted of either: 1) cholecalciferol at 16 mg/mL and PS1.6kD-b-PEG5kD or
PCL7kD-b-PEG5kD at 24 mg/mL dissolved in THF or 2) GW771806X at 20 mg/mL, pamoic acid at 8.9 mg/mL, and PLA3.7kD-b-PEG5kD at 28.9 mg/mL dissolved in DMSO. The streams were injected into the mixer using digitally controlled PHD 2000 syringe pumps (Harvard Apparatus, USA). The following flow rates were used for the two different organic phases: 1) 14.4 mL/min for the THF organic stream and 43.3 mL/min for each of the aqueous streams or 2) 8.7 mL/min for the DMSO organic stream and 43.3 mL/min for each of the aqueous streams. The initial ~5 mL of effluent were discarded and then collection was started. Effluent collection was stopped prior to stopping the pumps. This ensured that only dispersion produced at steady state operation were collected.

In order to quantify the recovery of the API after freeze-drying and reconstitution, it was necessary to determine the solute concentration in the aqueous dispersion prior to freeze-drying. Thus, a dispersion was dialyzed to remove organic solvent and thus have an only aqueous medium. An aliquots of the sample was loaded into Spectra/Por 1 (MWCO 6-8 kD) dialysis tubing (Spectrum Laboratories, USA). The aliquot was dialyzed against MilliQ water generally at a 1 L to 10 mL ratio of bath to sample volume. The bath water was refreshed at least hourly for the first five hours and then left overnight. The dialyzed dispersion was then collected from the tubing, the volume change was recorded, and the dispersion was filtered (1 µm, nylon or GF/B; Whatman, USA) prior to further processing.

6.2.3 Nanoparticle Dispersion Characterization

6.2.3.1 Particle size distribution measurement

After nanoparticle production, dialysis, and reconstitution, the dispersions were characterized for particle size using a Malvern Zetasizer ZS ZEN 3600 (UK). Samples were prepared by diluting the dispersions at least 10-fold in MilliQ water and were contained in
disposable 1.5 mL plastic cuvettes (Fisher, USA). All measurements were made with a 633 nm laser at a scattering angle of 173°. Sizes reported are those obtained using the general purpose normal resolution analysis mode of the intensity-weighted distribution. The peak means and span ranges ($\Delta = d90 - d10$) are given. Refer to Appendix C for a discussion on why this reporting protocol is used. Usually, at least triplicate measurements were made and the metrics are given of at least two measurements that were averaged.

6.2.3.2 Nanoparticle dispersion composition

After removing the organic solvent from the nanoparticle dispersions, solute concentrations were measured through reverse-phase high pressure liquid chromatography (HPLC). Nanoparticle dispersions were diluted in THF or DMSO at least 20-fold before HPLC analysis. HPLC was conducted on a Hewlett-Packard Agilent LC 1100 equipped with a quaternary pump, diode array detector, and a Gemini C18 stationary phase (5 µm, 100 Å; Phenomenex, USA). Mobile phases used are designated as follows: A - MilliQ water with 0.05 vol% TFA, B - MeCN with 0.05 vol% TFA, C - MeOH.

GW771X806X and PAM concentrations were measured using the following conditions: a linear gradient of 95%/5% A/B to 100% B over 2.5 minutes and returning to 95%/5% A/B at 2.7 minutes all at flow rate of 1.5 mL/min using a column temperature of 55°C. The method run time was 6 minutes with analyte retention times of ~3 and 4.5 minutes for GW771806X and PAM, respectively. The detection wavelength used was 254 nm. The method was calibrated for GW771806X concentrations of 5 - 108 µg/mL with a linear coefficient of determination, $R^2 = 0.9999$, and for PAM, the method was calibrated for concentrations of 5 - 103 µg/mL with $R^2 = 0.9942$. 
Cholecalciferol concentrations were measured using the following conditions: 100% C at a flow rate of 2 mL/min using a column temperature of 30°C. The method run time was 5 minutes with an analyte retention time of ~2.6 minutes. The detection wavelength used was 265 nm. The method was calibrated for cholecalciferol concentrations of 5 - 104 µg/mL with a linear coefficient of determination, \( R^2 = 0.9999 \).

Total solids concentrations (and, indirectly, block copolymer concentrations) were measured through thermogravimetric analysis (TGA). TGA was performed on a Pyris TGA 7HT (PerkinElmer, USA). Prior to each run, the instrument was allowed to cool down to ambient temperature, the sample pan was thoroughly cleaned (rinsed with 1-distilled water/soap, 2-THF, 3-distilled water, 4-ethanol, and then dried in a vacuum oven at 105°C) and the instrument was mass-calibrated at least twice with a 100 mg metal weight standard. Typical runs involved heating from 25°C to 80°C at 5°C/min, holding for 30 minutes, heating from 80°C to 105°C at 5°C/min, and holding for 10 minutes. This method was used only to remove water from the dispersions, such that only the non-volatile nanoparticle components could be weighed. The nanoparticle concentration (\( c_{\text{solids}} \)) was calculated as follow:

\[
c_{\text{solids}} = \frac{m_{\text{final}}}{m_{\text{initial}}} \rho_{\text{water}}
\]

Above, \( m_{\text{final}} \) and \( m_{\text{initial}} \) are the final and initial masses measured by the instrument in each run, where the final mass is that of the nanoparticles and the initial mass is that of the dispersion, and \( \rho_{\text{water}} \) is the density of water. It is assumed that the density of the nanoparticle dispersion does not deviate significantly from that of water, as discussed in Appendix H.

Loading efficiency (\( LE \)) of the core solutes into the nanoparticles was calculated as follows:
\[ LE(\%) = \frac{c_{\text{measured}}}{c_{\text{nominal}}} \left( \frac{V_{\text{initial}}}{V_{\text{measured}}} \right) \times 100\% \]  

Here, \( c_{\text{measured}} \) is the measured concentration through HPLC, \( c_{\text{nominal}} \) is the nominal concentration expected if all the solute from the organic phase is recovered, \( V_{\text{initial}} \) is the initial volume of dispersion loaded into the dialysis tubing, and \( V_{\text{measured}} \) is the volume recovered from the dialysis tubing once dialyzed. The solute loading, or the amount of active pharmaceutical ingredient in the dry nanoparticles by weight, was calculated as follows:

\[ \text{loading (wt\%)} = \frac{c_{\text{measured}}}{c_{\text{solids}}} \times 100\% \]

Here, \( c_{\text{measured}} \) is the measured concentration through HPLC, and \( c_{\text{solids}} \) is the solids concentration measured through TGA. All \( LE \) and loading measurements were done on dialyzed dispersions that were filtered.

### 6.2.3.3 Zeta-potential measurement

Once a cholecalciferol dispersion was free of organic solvent, a nanoparticle sample was diluted with potassium chloride to result in a nanoparticle concentration of ~1 mg/mL and a salt concentration of ~3 mM. An aliquot of the salt sample was then transferred to a disposable folded capillary cell (Malvern, UK). Zeta-potential measurements were done on a Malvern Zetasizer ZS ZEN 3600 (UK) using the general purpose analysis mode at default instrument settings. Five replicate measurements were made and three were used to obtain the average zeta-potential and deviations that are reported.

### 6.2.3.4 Differential scanning calorimetry

Thermal events upon freezing were investigated through use of differential scanning calorimetry (DSC). Measurements were performed on a DSC Q2000 (TA Instruments, USA). Typically, at least 35 µL of liquid nanoparticle dispersion was loaded into a hermetic DSC pan.
(TA Instruments, USA) and the following method was used. Samples were equilibrated at -85°C and then heated to 0°C, cooled to -85°C, and heated to 0°C at 10°C/min. Analysis of the calorimetric traces was done using the Universal Analysis 2000 software (TA Instruments, USA).

6.2.4 Nanoparticle Dispersion Processing

6.2.4.1 Freeze-drying

In order to obtain a dry nanoparticle powder, the dispersions were freeze-dried. First, samples of the nanoparticle dispersions were aliquoted into 5 mL cryogenic vials (VWR, USA) or 2 mL microcentrifuge tubes (Fisher, USA). The samples were then frozen through one of the following schemes. The vials/tubes were either submersed in a dry ice/acetone bath for at least twenty minutes OR submersed in a dry ice/brine bath for at least fifteen minutes and then submersed in a dry ice/acetone bath for at least three minutes OR loaded into a CoolCell LX 2mL or 5mL (Biocision, USA) and stored in a New Brunswick Scientific Ultra Low Temperature Freezer Innova U535 (Eppendorf, USA) overnight. Cooling profiles were recorded using a datalogging thermometer (Sper Scientific, USA).

Once frozen, the samples were lyophilized in one of two freeze-dryers (manifold or cabinet). When lyophilized on the cabinet freeze-dryer, the vials/tubes were quickly transferred onto the pre-cooled (< -70°C) shelf of a VirTis Advantage cabinet lyophilizer (SP Scientific, USA). The drying cycle depended on the actual run, but the cycles was generally at least two days long for sample volumes less than 100 mL. When lyophilized in the manifold mode, the vials/tubes were transferred into a pre-cooled (< -70°C) flask, which was then hooked up to either the VirTis Advantage or a VirTis Benchtop 3.3 manifold lyophilizer (SP Scientific, USA). For both modes, a condenser temperature of < -70°C was maintained, as well as a vacuum
pressure < 200 mTorr. In all cases, the vials containing the lyo-cakes were capped and stored in a standard freezer until reconstitution.

### 6.2.4.2 Reconstitution of lyo-cakes

When the lyo-cakes were to be reconstituted, the appearance of the cakes was first noted. Subsequently, the powders were rehydrated with MilliQ water at specified volumes and then mildly manually agitated (~180-210 shakes per minute) for 1 minute. The reconstituted dispersions were then filtered (1 µm, nylon or GF/B; Whatman, USA) and characterized for particle size distributions and API concentrations.

To gauge the degree of redispersibility, the following metrics were calculated and compared. The redispersibility ratio, \( S_f/S_i \), is the ratio of the reconstituted peak mean intensity diameter of the main population to that of the initial dispersion prior to freeze-drying. Similarly, the span range ratio, \( \Delta_f/\Delta_i \), after reconstitution to before freeze-drying was also considered. The API recovery is the percent of the measured API prior to freeze-drying of the dispersion that was measured after reconstitution and filtration. See Appendix D for more information on how these ratios were calculated and reported.

### 6.2.5 Statistical Analysis

Analysis of variance (ANOVA) was performed on factorial studies evaluating the various formulation and process parameters in order to understand the importance of each. General linear modeling was performed using Minitab 15 Statistical Software (USA) with a significance level, \( \alpha \), of 0.05.
6.3 RESULTS AND DISCUSSION

6.3.1 Nanoparticle Formulation Parameters

6.3.1.1 Di-block copolymer stabilizer

In the typical formulation of an active pharmaceutical ingredient (API) using FNP, an amphiphilic di-block copolymers is used to stabilize the nanoparticle dispersions. As discussed in section 2.6.1 of Chapter 2, the choice of the block copolymer is an important formulation parameter, as it can affect the particle size and loading efficiency of the solute, and it usually will determine whether or not it is suitable for *in vivo* applications. We show that the block copolymer can also have a significant impact on the steric stabilization of the nanoparticles. The studies observed two extremes of di-block copolymers: 1) a completely micro-phase separated PS-\(b\)-PEG where essentially all PEG in the outer aqueous phase, and 2) PCL-\(b\)-PEG and PLA-\(b\)-PEG that have a considerable amount of PEG not present in the outer aqueous corona. In the latter case, some of the PEG can be kinetically trapped or dissolved within a hydrophobic domain of the nanoparticles. These two cases resulted in particle aggregation that can be attributed to different causes.

First, we compare the two different extremes. Using an MIVM, nanoparticle systems composed of cholecalciferol, an inactive form of vitamin D with a ALOGPs of 7.98 (see Appendix A for information on ALOGPs), and two different block copolymers, PS1.6kD-\(b\)-PEG5kD and PCL7kD-\(b\)-PEG5kD, were produced. As shown in Table 6.1, although the loading efficiency of the D\(_3\) was higher when using the PS copolymer, most metrics were comparable. This study originally was meant to compare two nanoparticle formulations with similar properties but different PEG content from the block copolymer. The two formulations, while having nominal block copolymer concentrations that were similar, as estimated by subtracting
the HPLC-measured $D_3$ concentration from the TGA-measured nanoparticle concentration, had different PEG contents due to the composition of the block copolymers. Interestingly, when the PEG concentrations were measured using a colorimetric assay that has been reported previously [4, 5], the PS copolymer nanoparticles only had -5% difference as compared to the nominal concentration, while the PCL copolymer nanoparticles had a -49% difference. Furthermore, DSC of the two dispersions revealed a smaller endotherm for the PCL nanoparticles, associated with the melting of the PEG/water eutectic, as shown in Figure 6.1. The endothermic heats revealed that 2.8 times higher energy was released from the thermal event for the PS, as compared to the PCL, formulation, which closely corresponds to the 3.1 times higher PEG concentration, with only a 10% difference between the colorimetric assay and DSC measurements.

<table>
<thead>
<tr>
<th>Table 6.1</th>
<th>Characterization of $D_3$-loaded nanoparticles, stabilized by two different block copolymers.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block Copolymer</td>
<td>PS1.6kD-$b$-PEG5kD</td>
</tr>
<tr>
<td>Initial Size (nm)</td>
<td>116 ± 1</td>
</tr>
<tr>
<td>Span Range (nm)</td>
<td>108 ± 2</td>
</tr>
<tr>
<td>$\zeta$-Potential (mV)</td>
<td>-3.03 ± 9.40</td>
</tr>
<tr>
<td>Loading Efficiency (%)</td>
<td>81 ± 1</td>
</tr>
<tr>
<td>$D_3$ (mg/mL)</td>
<td>1.18 ± 0.02</td>
</tr>
<tr>
<td>Nanoparticles (mg/mL)</td>
<td>3.34</td>
</tr>
<tr>
<td>$D_3$ Loading (%)</td>
<td>35</td>
</tr>
<tr>
<td>PEG Content (%)</td>
<td>49</td>
</tr>
<tr>
<td>PEG-nominal (mg/mL)</td>
<td>1.64</td>
</tr>
<tr>
<td>PEG-measured (mg/mL)</td>
<td>1.56</td>
</tr>
</tbody>
</table>

The discrepancy between the measured PEG concentrations and the estimated nominal PEG concentrations might be explained by the miscibility of the two blocks for each stabilizing di-block copolymer. A common way to determine miscibility between blocks of block copolymers is to consider microphase diagrams, such as those determined by field theory [27];
these diagrams plot the various possible phases as a function of the volume fraction of one block and the product of the number of monomers and the Flory-Huggins interaction parameter.

Beckingham and Register define this product, $\chi N$, as follows [28], where $M_n$ is the number-averaged molecular weight of the block copolymer, $\rho$ is the density of the polymer, $R$ is the universal gas constant, $T$ is absolute temperature, and $\delta_i$ is the solubility parameter of block $i$.

$$\chi N = \frac{M_n}{\rho R T} (\delta_A - \delta_B)^2$$

Equation 6.4 was used to calculate $\chi N$ values for the block copolymer in question by making use of a volume-weighted density from reported density values in the literature for each block and by using Hansen solubility parameters, which are explained and listed in Appendix E. The volume fraction of PEG in the di-block copolymers, $f_A$, was obtained from the molecular volumes used in calculating the solubility parameters. Using the $\chi N$ and $f_A$ values, the mean field phase diagrams for conformationally asymmetric di-block copolymers generated by Matsen and Bates [29] were

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**Figure 6.1** Differential scanning calorimetry of the $D_3$ nanoparticle formulations from Table 6.4 prior to freeze-drying. The inset is a zoomed-in portion. Second heating traces are shown. See the methods section for DSC procedure.
analyzed to determine if the polymer in question would result in a disordered, or miscible, state or if there would be microphase separation. Table 6.2 lists the density and estimated Hansen solubility parameters of PEG5kD, PS1.6kD, PCL7kD, PLA3.7kD, and poly(lactide-co-glycolide) (PLGA7kD) for two different compositions. Table 6.3 lists for several di-block copolymers the volume-weighted density, the volume fraction of PEG, $\chi_N$, and the physical state of the polymer.

Table 6.2 Density and Hansen solubility parameters of polymer blocks used in the di-block copolymers used to stabilize nanoparticle systems. The ratios after the PLGA polymers represent the stoichiometry of lactide to glycolide monomers. The references for the density values are listed. Refer to Appendix E for information on the solubility parameters.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$M_n$ (kg mol$^{-1}$)</th>
<th>$\rho$ (kg/L)</th>
<th>$\delta_d$ (MPa$^{1/2}$)</th>
<th>$\delta_p$ (MPa$^{1/2}$)</th>
<th>$\delta_h$ (MPa$^{1/2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG</td>
<td>5.0</td>
<td>1.064 [31]</td>
<td>17.9</td>
<td>1.3</td>
<td>10.0</td>
</tr>
<tr>
<td>PS</td>
<td>1.6</td>
<td>0.969 [31]</td>
<td>20.5</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>PCL</td>
<td>7.0</td>
<td>1.145 [32]</td>
<td>17.6</td>
<td>0.6</td>
<td>8.4</td>
</tr>
<tr>
<td>PLA</td>
<td>3.8</td>
<td>1.21 [33]</td>
<td>17.6</td>
<td>1.3</td>
<td>12.1</td>
</tr>
<tr>
<td>PLGA (50/50)</td>
<td>7.0</td>
<td>1.34 [34]</td>
<td>18.3</td>
<td>1.1</td>
<td>13.0</td>
</tr>
<tr>
<td>PLGA (75/25)</td>
<td>7.1</td>
<td>1.3 [34]</td>
<td>17.9</td>
<td>1.0</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Table 6.3 Volume-weighted density, PEG volume fraction, and $\chi_N$ values for the di-block copolymers used to stabilize nanoparticle systems. The ratios after the PLGA polymers represent the stoichiometry of lactide to glycolide monomers. None of the copolymers is predicted to be fully miscible at equilibrium, since the $\chi_N$ value of each polymer is above the boundary value and thus is in a microphase separated state in the mean field phase diagram for a conformationally asymmetric di-block copolymer [29].

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$M_n$ (kg mol$^{-1}$)</th>
<th>$\rho$ (kg/L)</th>
<th>$f_A$</th>
<th>$\chi_N$</th>
<th>$\chi_N^{\text{boundary}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS1.6kD-b-PEG5kD</td>
<td>6.6</td>
<td>1.040</td>
<td>0.74</td>
<td>271.2</td>
<td>15.5</td>
</tr>
<tr>
<td>PCL7kD-b-PEG5kD</td>
<td>12.0</td>
<td>1.110</td>
<td>0.44</td>
<td>12.8</td>
<td>11.3</td>
</tr>
<tr>
<td>PLA3.7kD-b-PEG5kD</td>
<td>8.7</td>
<td>1.122</td>
<td>0.60</td>
<td>14.3</td>
<td>11.3</td>
</tr>
<tr>
<td>PLGA7kD(50/50)-b-PEG5kD</td>
<td>12.0</td>
<td>1.210</td>
<td>0.47</td>
<td>38.4</td>
<td>11.0</td>
</tr>
<tr>
<td>PLGA7kD(75/25)-b-PEG5kD</td>
<td>12.0</td>
<td>1.191</td>
<td>0.46</td>
<td>25.7</td>
<td>11.0</td>
</tr>
</tbody>
</table>

Through this calculation, it is shown that PEG5kD has the least affinity for the PS1.6kD block, as represented by the large difference between the calculated $\chi_N$ value and the boundary value; this is consistent with the PEG chains being excluded from the PS phase and forced into
the aqueous phase such that they can be measured. X-ray photoelectron spectroscopy of PS-\textit{b}-PEG block copolymers confirmed that there is virtually complete phase separation between the two blocks [30]. For the PCL7kD-\textit{b}-PEG5kD, both the calculation and the literature point to micro-phase separation for the two species when covalently linked as a di-block copolymer [35]. However, considering that only a fraction of the total PEG was measurable, this suggests that either the resulting morphology of the block copolymer involved domains of PEG that were buried within PCL domains or a non-equilibrium miscible state was formed through FNP; further characterization would be required to verify this. These findings have critical implications not only for freeze-drying purposes, but also for circulation \textit{in vivo}. The lesser degree of PEG steric stabilization afforded by PCL-\textit{b}-PEG5kD block copolymers might account for the observation of D'Addio et al, where poorer circulation rates were achieved for nanoparticles stabilized by various PCL-\textit{b}-PEG5kD for as compared to PS1kD-\textit{b}-PEG3kD [5].

When freeze-drying both nanoparticle dispersions from Table 6.1 on a manifold lyophilizer and reconstituting the resulting lyo-cakes, the results from Table 6.4 were observed.

<table>
<thead>
<tr>
<th>Trial</th>
<th>BCP</th>
<th>Excipient</th>
<th>$S_f/S_i$</th>
<th>$A_f/A_i$</th>
<th>D$_3$ Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>PCL7kD</td>
<td>-</td>
<td>2.35 ± 0.08</td>
<td>2.17 ± 0.25</td>
<td>4 ± 0</td>
</tr>
<tr>
<td>B</td>
<td>PS1.6kD</td>
<td>-</td>
<td>1.25 ± 0.03</td>
<td>1.27 ± 0.07</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>C</td>
<td>PCL7kD</td>
<td>PEG4.6kD (0.64 mg/mL)</td>
<td>3.48 ± 0.13</td>
<td>4.60 ± 0.60</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>D</td>
<td>PCL7kD</td>
<td>PEG4.6kD (10.4 mg/mL)</td>
<td>1.27 ± 0.05</td>
<td>1.30 ± 0.13</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>E</td>
<td>PS1.6kD</td>
<td>PEG4.6kD (10.0 mg/mL)</td>
<td>1.23 ± 0.02</td>
<td>1.16 ± 0.05</td>
<td>103 ± 2</td>
</tr>
</tbody>
</table>

The PCL formulation (trial A) exhibited poor redispersibility, with 96% of the lyo-cake not being recovered after filtration because it either redispersed as micron-sized aggregates or could
not be reconstituted. While the PS copolymer nanoparticles (trial B) did experience limited aggregation, as shown through the ~25% increase in peak mean diameter and span range, the recovery of the core solute was essentially quantitative, indicating intact recovery of the particles. Considering that the addition of enough free PEG4.6kD to compensate for the difference in nominal PEG concentration between trials A and B did not allow for complete recovery for the PCL particles (trial C from Table 6.4), the difference between the two nanoparticle compositions cannot be attributed to PEG content, but rather the distribution of PEG on the surface. The addition of much more free PEG gave comparable results for both formulations (trials D and E), but will be discussed later.

Based on the microphase separation calculation, it is predicted that a PLA3.7kD-b-PEG5kD copolymer would result in microphase separated PEG at equilibrium; therefore, nanoparticles stabilized by this polymer should experience satisfactory redispersibility once lyophilized since the PEG chains should be extended away from the hydrophobic particle surface. However, similar to the PCL7kD, the distance from the miscibility boundary is small. The literature shows that there is compatibility of the two blocks when covalently linked as block copolymers, leading to some miscibility [36]. In fact, GW771806X/pamoic acid nanoparticles stabilized by a PLA3.7kD-b-PEG5kD displayed only a small endotherm associated with the melting of the PEG/water eutectic through DSC when measured at a concentration similar to the D3 nanoparticles (3.25 mg/mL; ~0.97 mg/mL nominal PEG). This is discussed in more detail in section 6.3.2.3, where it is estimated that ~0.08 mg/mL PEG participated in the eutectic formation. Furthermore, the API recovery from the reconstituted freeze-dried GW771806X nanoparticles after filtration (1.0 µm, nylon) was only 5±3%, indicating that most of the nanoparticles aggregated to sizes > 1 µm and were lost in the filter. Considering that PEG5kD
has high affinity for PLA3.7kD and PCL7kD-b-PEG5kD is very close to the miscibility boundary as predicted by microphase separation calculations, it is expected that nanoparticles stabilized by these polymers will not have all the PEG present on the particle surface. This can make preventing particle aggregation during freeze-drying of nanoparticle formulations that use either di-block copolymer challenging.

Next, in further considering the PS-b-PEG block copolymer, which yields particles with the PEG totally excluded to the particle surfaces, we evaluate the effects of surface coverage. The degree of coverage of the nanoparticle core by the stabilizing polymer is generally not something that can easily be measured. While previous studies have characterized the surface coverage of polystyrene latex beads by di-block copolymers [4, 5], nanoparticle dispersions produced through FNP are more complicated systems because the core diameter cannot be measured directly, nor are the particles monodisperse. However, by modifying the method that we have previously reported [6], we can estimate a surface coverage value through the following assumptions. First, the polydisperse nanoparticle dispersion can be represented, on average, by a monodisperse population with a particle size equal to the measured peak mean diameter, as described in Appendix H. Second, the stabilizing PEG layer can be modeled as a uniform brush, which is close to reality from the previous studies [4-6], on a spherical surface. For details on the modified calculation, please refer to Appendix H.

Using this procedure for estimating the surface coverage, we re-examine the results from our work on effervescent redispersion of β-carotene nanoparticle dispersions stabilized by PS-b-PEG produced on a confined impinging jets mixer with dilution (CIJM-D) and freeze-dried on a manifold freeze-dryer [6]. We also consider effervescent redispersion of nanoparticle dispersions of the same composition, but with different core solutes; further details on these nanoparticles
are given in the next section (6.3.1.2). The results are plotted in Figure 6.2 below. In re-examining the data, the samples using PS1.6kD-b-PEG1.8kD and PS1.6kD-b-PEG2.5kD that yielded redispersibility ratios > 6 had poor quality measurements via dynamic light scattering on the Zetasizer. Thus, the large redispersed peak mean diameters that were reported are not necessarily representative of the redispersed particles, since the rest of the data all had acceptable quality for the measurements. These four points are not considered in the analysis and correspond to the points that do not fit with the guiding lines in Figure 6.2 and that do not have error bars. However, the rest of the data shows the same trend: an increase in surface coverage led to an increase in the redispersibility ratio. The trend implies that a nanoparticle surface better covered by PEG induced more aggregation.

![Figure 6.2 Redispersibility ratios of reconstituted lyophilized nanoparticle dispersions consisting of a core solute and a stabilizing PS1.6kD-b-PEG1.8kD, PS1.6kD-b-PEG2.5kD, or PS1.5kD-b-PEG5.3kD. The core solutes were β-carotene (BCT), D-α-tocopherol (AT), PS1.5kD and poly(propylene glycol) (PPG3.5kD). Dispersions were produced on a CIJM-D at a nominal nanoparticle concentration of 2 mg/mL and freeze-dried on a manifold lyophilizer. Samples were freeze-dried with 6 mg/mL sodium bicarbonate and reconstituted with dilute citric acid. The dotted lines are for guiding purposes only.](image-url)
This behavior has been previously been reported by De Jaeghere et al and Zambaux et al for PLA particles stabilized by PLA-b-PEG [37, 38]. In these cases the individual blocks used in the two reports were much larger (PLA51kD-b-PEG2kD, PLA64kD-b-PEG5kD, PLA45kD-b-PEG10kD, PLA45kD-b-PEG20kD) and calculations predict microphase separation; also, the nanoparticles were produced through equilibrium-based synthesis methods such that kinetic trapping of PEG within the particle was not expected. While the aforementioned trend is counterintuitive to the idea that the PEG layer is what helps to keep particle cores from contacting and aggregating, the entire freeze-drying process needs to be properly understood to appreciate the phenomenon. During the freezing step, although particles rejected from the ice front will be pushed into intimate contact through cryo-concentration, the hydrated PEG layer on particle surfaces will from a crystalline eutectic that prevents the contact of hydrophobic cores. This protective layer is the reason why PEG is an excellent cryoprotectant for primary particles. However, once primary drying has removed the ice and secondary drying commences, the water from the PEG/water eutectic will begin to be sublimed. This will cause the PEG to no longer be in a hydrated state, leading to crystallization of the polymer. If individual particles are in intimate contact from the cryo-concentration step, then bridging from PEG crystallization can occur, leading to particle aggregation. De Jaeghere observed that the molecular weight of the PEG, or equivalently the thickness of the PEG layer, did not impact the degree of aggregation [37], as the resulting crystallization bridging is a surface-mediated phenomenon. This is why the data above is presented in terms of PEG chains per surface area instead of mass per surface area.

To test the validity of the hypothesis of PEG crystallization causing particle aggregation during freeze-drying, one of the nanoparticle formulations using polystyrene as the solute from the previous experiments was freeze-dried under the same conditions without any excipients.
Duplicate samples were reconstituted and the particle size was measured at 25°C. Each sample was then heated to 70°C and equilibrated for five minutes on the Zetasizer, proceeding to particle size measurements. It was critical to heat the samples to a temperature above the bulk melting temperature of PS-\(b\)-PEG, which is attributed to the PEG; Polymer Source reports a \(T_m\) of 58°C for PS1.6kD-\(b\)-PEG5kD. The size distributions after each step are presented in Figure 6.3. While the initial reconstitution resulted in an average redispersibility ratio of 2.53±0.14 and an average span range ratio of 3.34±0.45 for the two samples, the heating step allowed for the average redispersibility ratio to drop to 1.40±0.04 and the average span range ratio to drop to 1.32±0.11.

![Figure 6.3](image)

**Figure 6.3** Particle size distributions of reconstituted lyophilized PS nanoparticle samples stabilized by PS1.5kD-\(b\)-PEG5.3kD. Dispersions were produced on a CIJM-D at a nominal nanoparticle concentration of 2 mg/mL with 1.2 mg/mL stabilizer and 0.8 mg/mL PS and freeze-dried on a manifold lyophilizer. Each reconstituted sample was heated and equilibrated at 70°C for five minutes.

However, D'Addio et al had attempted heating of reconstituted lyophilized \(\beta\)-carotene nanoparticles stabilized by PLGA7kD-\(b\)-PEG5kD without successfully observing a reduction in particle size [39]. This polymer is predicted to have strong phase separation (Table 6.3), meaning that most of the PEG should be presented in the aqueous corona. However, in characterizing the
surface coverage provided by various di-block copolymers to polystyrene latex beads, D’Addio also reported blob sizes [5], or the diameter that a PEG chain will cover; although she did not characterize this for PLGA-\(b\)-PEG block copolymers, if PLA is used as a surrogate, a comparison of the blob sizes for PS1.6kD and PLA7kD shows three-fold more coverage for the PS polymer (see equation H.5). Thus, it may be that the PLGA7kD-\(b\)-PEG5kD did not properly cover all hydrophobic surfaces; the observed particle aggregation, may have been due to exposed hydrophobic PLGA shells contacting, which could not have been undone with heat.

Similarly, in further analyzing the results from Tables 6.1 and 6.4, the estimated surface coverage for trials A and B from Table 6.4 are 0.62 and 1.14 PEG chains/nm\(^2\), respectively. These calculations assume that the non-measurable PEG (through the colorimetric assay) was present in the particle cores. Since trial A resulted in poorer redispersibility than B while having less surface coverage, this result would seem to go against the trend in Figure 6.2. However, this comparison is between two block copolymers with different degrees of phase separation, which is not the case in Figure 6.2. Clearly, for the cases where block copolymers do not phase separate well, aggregation cannot be attributed solely to PEG crystallization, as exposed hydrophobic block surfaces can serve as points of aggregation upon contact. Attempting to increase the surface coverage can be useful, such as for the D\(_3\) formulation using the PS1.6kD-\(b\)-PEG5kD instead of the PCL7kD-\(b\)-PEG5kD. Unfortunately, while PS1.6kD-\(b\)-PEG5kD has excellent separation of the two blocks, which allows for aggregation cause by PEG crystallization to be mitigated through heat, the polymer is not biodegradable. Thus, techniques to prevent aggregation, such as the incorporation of protectant excipients which is discussed in section 6.3.2, are necessary for nanoparticle formulations using biocompatible PEG-based polymers.
6.3.1.2 Physical state of nanoparticle cores

Although a typical nanoparticle formulation that maximizes the loading of the API will consist of only the API and a stabilizing polymer, addition of other solutes into the nanoparticle core can modulate the rigidity of the core and can make a drastic difference in the redispersibility of the lyophilized formulation. Previously, nanoparticle dispersions were produced on a CIJM-D with one of four different core solutes, all stabilized by PS1.5kD-b-PEG5.3kD [6]; the nanoparticles were at a nominal concentration of 2 mg/mL, but with varying compositions. The solutes were either a solid or a soft material of either low molecular weight or polymeric, consisting of β-carotene (crystalline solid), D-α-tocopherol (liquid), PS1.5kD (glassy solid) and poly(propylene glycol) (M~3,500) (PPG) (amorphous liquid). In this case, all dispersions were freeze-dried with sodium bicarbonate and reconstituted in dilute citric acid. The results have been plotted as redispersibility versus the mass ratio of block copolymer to solute in the FNP organic stream, as shown in Figure 6.4

Clearly, the nanoparticles with solid cores redispersed to redispersibility ratios that were at least twice as large as those of the particles with liquid cores; the span range ratios followed a similar trend. Figure 6.2 also shows that the guiding line for liquid-core particles is lower than that of the solid-core particles. The reason for why liquid-core nanoparticles, or nano-capsules, had better redispersibility once freeze-dried is not obvious and requires more characterization to properly explain. However, we posit that because nano-capsules are able to deform much more in comparison to solid-core particles, characteristic desiccation and drying forces causing capsules to contact amongst each other will result in lower stresses due to deformation increasing the contact area and thus distributing the forces over a greater area. Contrastingly, solid-core particles cannot deform as much, leading to smaller contact areas and higher stresses. If the
contact sites are locations with exposed cores, then a larger stress will have a higher chance of inducing aggregation. As discussed in the previous section, the redispersibility ratios were lower for nanoparticles with lesser amounts of block copolymer due to the decreased surface coverage by PEG.

The deformable state that a liquid core solute can impart to nanoparticles was useful in the freeze-drying of progesterone dispersions, as discussed in Chapter 3. In those examples, redispersibility ratios very close to one were achieved, even when the original dispersions had nanoparticle concentrations of ~40 mg/mL and made use of a PLA3.7kD-b-PEG5kD block copolymer [40].

![Figure 6.4](image_url)

**Figure 6.4** Redispersibility and span range ratios of reconstituted lyophilized nanoparticle dispersions stabilized by the block copolymer (BCP) PS1.5kD-b-PEG5.3kD. The core solutes are β-carotene (BCT), D-α-tocopherol (AT), PS1.5kD and poly(propylene glycol) (PPG3.5kD).
6.3.1.3 Concentration of nanoparticle dispersions

The maximum achievable nanoparticle concentration for a lyophilized nanoparticle formulation is of great importance, since many times, a high API concentration is required for a treatment to be effective. Two methods for obtaining high nanoparticle concentrations once a lyo-cake has been reconstituted are to either begin with a concentrated nanoparticle dispersion which is freeze-dried and then reconstituted to the original volume OR to start with a dilute nanoparticle dispersion and reconstitute to less than the original volume once freeze-dried. Both methods have limitations, as will be discussed.

When dispersions have high initial nanoparticle concentrations, the density of particles in maximally cryo-concentrated domains will be greater in comparison to initially dilute dispersion [41]. Prior to freezing, individual particles might be in intimate contact with others, which increases the chances of the touching particles to aggregate either through PEG crystallization or hydrophobic contacts as the residual water in the cryo-concentrate is removed during secondary drying. Thus, above a critical threshold concentration that will depend on whether or not a protectant excipient is present and the degree of screening it provides, nanoparticle dispersions may experience significant aggregation. Two examples are presented here to highlight the importance of initial nanoparticle concentrations.

In the aforementioned study on effervescent protectants for freeze-drying [6], CIJM-D-produced β-carotene particles stabilized by PLA3.7kD-b-PEG5kD were evaluated. It was shown that at the initial nominal nanoparticle concentration of 2 mg/mL (1:1 stabilizer to core mass ratio), the resulting lyo-cakes could not be redispersed to small particle sizes without the incorporation of a protectant excipient or exposure to ultrasonication. Moreover, when comparing two different protectant excipients at a 3:1 protectant to nanoparticles mass ratio, it
was found that redispersed particle size increased strongly as the initial nanoparticle concentration was increased when using the sodium bicarbonate protectant. Results are shown in Figure 6.5. In contrast, the dispersions containing Pluronic F68 experienced essentially no aggregation at 1 and 2 mg/mL nanoparticles and only limited aggregation at 3.5 mg/mL. This result highlights the differences in critical threshold concentrations and suggests that Pluronic F68 provides much better screening of individual particles during freeze-drying. The use of Pluronic F68 will be discussed in more detail in section 6.3.2.2.

![Figure 6.5](image)

**Figure 6.5** Redispersibility ratios of reconstituted lyophilized β-carotene dispersions stabilized by PLA3.7kD-b-PEG5.3kD. Dispersions were produced on a CIJM-D and freeze-dried on a manifold lyophilizer with protectant excipients at a 3:1 excipient to nanoparticle mass ratio.

Another example of the effect of initial particle concentration follows, using only Pluronic F68 as a protectant excipient as shown in Table 6.5. MIVM-produced GW771806X/pamoic acid particles stabilized by PLA3.7kD-b-PEG5kD were prepared at ~3.3 and ~33 mg/mL nanoparticles, each with a 3:1 Pluronic F68 to nanoparticles mass ratio. The samples were lyophilized in a cabinet lyophilizer with a shelf temperature of -12°C. After reconstitution to the original volume, the dispersion with a higher initial concentration (trial C) resulted in redispersibility and span range ratios that were ~40% higher than those of the dilute
initial concentration (trial A) and the API recovery was 11% lower. A similar effect was seen in the study presented in section 4.3.5 of Chapter 4, where the same nanoparticle formulation was freeze-dried with a PEG4.6kD/Pluronic F68 mixture and at concentrations of ~14, 24, 34 mg/mL nanoparticles. Although no clear trend could be seen with respect to size metrics since the size was measured after filtration, the API recovery dropped as the concentration was increased. This shows that more particle aggregation can occur as the initial particle concentration prior to freeze-drying is increased.

Table 6.5 Reconstitution of MIVM-produced GW771806X/pamoic acid particles stabilized by PLA3.7kD-b-PEG5kD lyophilized with 3:1 Pluronic F68 to nanoparticles in a cabinet lyophilizer (shelf temperature of -12°C). The concentration factor refers to how much less volume of water was used to rehydrate the lyo-cakes.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Initial Particles (mg/mL)</th>
<th>Concentration Factor</th>
<th>$S_f/S_i$</th>
<th>$A_f/A_i$</th>
<th>API Recovery (%)</th>
<th>API (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.3</td>
<td>1X</td>
<td>1.17 ± 0.03</td>
<td>1.09 ± 0.09</td>
<td>95 ± 3</td>
<td>0.94 ± 0.03</td>
</tr>
<tr>
<td>B</td>
<td>3.3</td>
<td>3X</td>
<td>1.62 ± 0.06</td>
<td>1.56 ± 0.18</td>
<td>78 ± 10</td>
<td>2.33 ± 0.29</td>
</tr>
<tr>
<td>C</td>
<td>33</td>
<td>1X</td>
<td>1.68 ± 0.05</td>
<td>1.53 ± 0.20</td>
<td>84 ± 3</td>
<td>8.67 ± 0.30</td>
</tr>
<tr>
<td>D</td>
<td>33</td>
<td>3X</td>
<td>1.67 ± 0.04</td>
<td>1.53 ± 0.13</td>
<td>65</td>
<td>19.21 ± 0.13</td>
</tr>
</tbody>
</table>

Thus, the initial nanoparticle concentration in the dispersion may be a limiting factor to the API concentration that can be achieved in a reconstituted dispersion with minor particle aggregation. However, this problem can be counteracted by reconstituting to higher concentrations through rehydration with a volume that is less than the initial. Continuing with Table 6.5, when attempting to reconstitute to 3X higher concentrations from samples freeze-dried at the lower initial particle concentration of 3.3 mg/mL (trial B), the redispersibility and span range ratios increased by ~40% and the API recovery dropped 17%. Although the 3X concentration factor meant that the nominal increase in AP concentration should have been 300%, the loss represented by the drop in API recovery resulted in a 248% increase in API concentration. While the redispersibility and span range ratios did not change upon reconstitution...
to a 3X concentration factor at 10 mg/mL (trial D), the API recovery dropped 19% and the API concentration went up by 221%. The same effect of the concentration factor was seen for the previous example of GW771806X/pamoic acid particles (section 4.3.5); this decrease in redispersibility, as mentioned in Chapter 4, might be a kinetic effect attributed to energy input. However, for these two examples, while API recovery decreased when reconstituting to higher concentrations, the API concentration associated with redispersed particles was still increased by a factor greater than 2X. If the lower API recovery is an acceptable compromise, lyophilized formulations can be produced at moderate initial concentrations, freeze-dried, and reconstituted with less water to produce nanoparticle dispersions at concentrations higher than the original synthesis concentrations.

6.3.2 Protectant excipients

As we have shown for various examples of freeze-drying and reconstitution of PEG-stabilized nanoparticles without protectant excipients, redispersibility can be quite limited, especially if the PEG surface coverage is poor. Even when the surface coverage is excellent, bridging from inter-particle PEG crystallization can result in poor redispersibility of lyophilizates. Although heat and intense energy input can help to undo some crystallization bridging for in some cases, this procedure is not feasible in a clinical setting and might not be feasible for heat-sensitive APIs. A common solution to prevent aggregation during freeze-drying is the incorporation of protectant excipients, such as cryoprotectants and lyoprotectants. Three types of protectants are examined here, starting with salts.

6.3.2.1 Salts

Previously we reported the use of sodium bicarbonate for effervescent redispersion of lyophilized nanoparticle dispersions [6]. When using sodium bicarbonate, the salt eutectic point
at -2°C results in salt precipitation during freezing [42, 43]. The scanning electron micrographs from our previous report show that the salt caused the formation of small submicron aggregate clusters of nanoparticles [6]. While the use of the bicarbonate salt prevented the formation of large micron-sized irregular aggregates that did not redisperse well, the formulations using a 3:1 salt to particles mass ratio resulted in redispersibility ratios between 1 and 11 (Figures 6.2, 6.4, and 6.5). Together, the results suggest that the precipitation of the sodium bicarbonate forms mechanical barriers isolating clusters of primary particles and aggregation is induced in these clusters because of intimate contact amongst particles, whether it be attributed to PEG crystallization in the case of particles stabilized by PS1.5kD-b-PEG5.3kD (Figures 6.2, 6.4) or hydrophobic contacts for particles stabilized by PLA3.7kD-b-PEG5kD (Figure 6.5).

In general, few, if any, studies have been done evaluating how well PEG can be dispersed in salt matrices. It would be necessary to investigate how PEG-protected nanoparticles would be distributed when using other salts. However, simple inorganic salts will contribute higher osmolarity than sugars at the same mass concentration. Osmolarity is an indication of osmotic pressure, which is a colligative property, meaning its value depends on the number of entities in solution, $\pi \propto nk_BT$, where $\pi$ is the osmotic pressure, $n$ is the osmolarity or number concentration of species in the dispersion, $k_B$ is the Boltzmann constant, and $T$ is temperature [44]. Considering that the target osmolarity for reconstituted lyophilized powders intended for intravenous administration is that of human blood serum, 289 mOsM [45], using simple salts may be problematic. Thus, next we consider excipients of larger molecular weights: polymers.

### 6.3.2.2 Polymers

In evaluating alternative protectant excipients, the work of Layre et al demonstrated the use of Pluronic F68 as a protectant [46]. Pluronic F68 is a BASF trade name for a grade of
poloxamer 188, which is a tri-block copolymer of PEG-\textit{b}-PPG-\textit{b}-PEG (MW: 3.4kD-\textit{b}-1.7kD-\textit{b}-3.4kD) that is commonly used as a surfactant stabilizer in many nanoparticle synthesis schemes and is generally recognized as safe by the United States Food & Drug Administration. Layre reported good redispersibility of PCL10kD-\textit{b}-PEG2kD stabilized particles when using Pluronic F68 in combination with trehalose. Since this polymer has a molecular weight that is roughly 25X higher than sucrose or trehalose, the osmolarity contribution is also 25X less at an equal mass concentration.

As was shown in Figure 6.5, CIJM-D-produced β-carotene particles stabilized by PLA3.7kD-\textit{b}-PEG5kD were freeze-dried with Pluronic F68 or sodium bicarbonate on a manifold lyophilizer and reconstituted by hand agitation. The samples with Pluronic F68 yielded redispersibility ratios of between 1 and 1.7, while sodium bicarbonate samples had > 500% growth in particle size. Since it is practically infinitely miscible with water at 0°C \cite{43}, this means that as cryo-concentration occurs, the Pluronic F68 will be in solution and can intercalate amongst the PEG chains in the stabilizing layers of the nanoparticles, such that it can function as a lyoprotectant. Also, for particles with poor PEG stabilization, the surfactant can help to cover exposed surfaces. The scanning electron micrograph of lyophilized progesterone-loaded nanoparticles with Pluronic F68 (Figure 3.7d) showed that the primary particles are dispersed in the polymer matrix. Since Pluronic F68 is not limited by a solubility limit or phase separation from the nanoparticles, it can separate primary particles effectively. Therefore, increasing Pluronic F68 concentration above a minimum level for redispersibility does not result in a significant decrease in nanoparticle size.

To test the versatility of Pluronic F68, the redispersibility of lyophilized lipid nanoparticles produced through FNP were also evaluated. PEGlyated lipid nanoparticles loaded
with Luc siRNA were prepared, as explained in Appendix I, and exhibited a particle size of ~90 nm. These nanoparticles were very stable, as the particle size did not fluctuate over a period of four month storage at 4°C and there was no visible precipitate. The lipid nanoparticles were freeze-dried with a 10:1 mass ratio of Pluronic F68 to nanoparticles, which yielded the smallest redispersed particle size of in the mass ratio range of 0:1 to 10:1, on a manifold lyophilizer. When reconstituted to the original concentration, either 1 minute of sonication (20kHz Vibra Cell VC 50 probe-tip ultrasonicator; Sonics & Materials, Inc., USA; 3mm-diameter probe at 25W) or hand agitation resulted in the same redispersed particle size distribution, as shown in Figure 6.6, with comparable redispersibility and span range ratios of 1.72 and 1.96, respectively. A lyophilized formulation that reconstitutes the same, whether a high-energy or low-energy reconstitution method is used, is desirable for a product used in a clinic.

![Figure 6.6](image_url)  
**Figure 6.6** Particle size distributions of reconstituted lyophilized lipid nanoparticle samples containing a 10:1 mass ratio of Pluronic F68 to particles. Dispersions were produced as described in Appendix I and freeze-dried on a manifold lyophilizer. Lyophilized samples were rehydrated and subjected to 1 minute of hand agitation or sonication.
In order to validate the effectiveness of Pluronics as protectant excipients, the GW771806X/PAM nanoparticles with a low concentration of PEG on the surface (section 6.3.1.1) was used as a model system. In this study, duplicate samples were prepared using a dispersion with ~3.3 mg/mL nanoparticles and 0:1, 3:1, 5:1, and 10:1 mass ratio of excipient to particles. The commonly used Pluronics F68 and F127 were compared to trehalose, which is used because of its relatively low hygroscopicity, low chemical reactivity, high glass transition temperature, and no internal hydrogen bonding [47]; the three excipients were also evaluated in dual combinations at 1.5:1.5:1, 2.5:2.5:1, and 5:5:1 mass ratios. Samples were freeze-dried in a cabinet lyophilizer for one day at a shelf temperature of -35°C for primary drying and 18 hours at -1°C for secondary drying. The results after reconstitution and filtration are presented in Figure 6.7. From the results in panels d) through f) (dialyzed samples), it is obvious that the Pluronics were superior in comparison to trehalose, which is discussed in more detail in section 6.3.2.3. There was little difference between the two Pluronics, as they both were able to yield ~100% API recovery at a 3:1 mass ratio with satisfactory particle size distributions. However, it was still not clear whether it was the surfactant nature of the Pluronics or the PEG chains on the poloxamer molecule that was helping to prevent particle aggregation.

Therefore, two more studies were done in order to test the protective capabilities of PEG and Pluronics during freeze-drying of nanoparticles. The first study was done using the same D₃ nanoparticle formulation from section 6.3.1.1. Here, four molecular weights of PEG were used as protectant excipients (2kD, 3.4kD, 4.6kD, and 20kD), as well as three Pluronics (F68, F127 and P104). Nanoparticle samples were prepared with 0:1, 1:1, 3:1, 5:1, and 10:1 excipient to particles by mass and were freeze-dried in a cabinet lyophilizer for 5.5 days at a shelf temperature of -20°C. Results are presented in Figure 6.8.
Figure 6.7 Redispersibility and span range ratios and API recoveries of reconstituted lyophilized GW771806X/PAM nanoparticles stabilized by PLA3.7kD-b-PEG5kD. The nanoparticle dispersion was produced on a MIVM at ~3.3 mg/mL particles and freeze-dried with the various excipients in a cabinet lyophilizer for one day at a shelf temperature of -35°C for primary drying and 18 hours at -1°C for secondary drying. A comparison for non-dialyzed (a - c) and dialyzed (d - f) dispersions are presented.
There was not an appreciable difference between the different molecular weights of PEG, with all resulting in redispersibility ratios of ~1.12, span range ratios of ~1.35, and ~89% API recovery. Contrastingly, the Pluronics were quite different; only the F68 did not result in a significant micelle population, while the F127 had micelles from 3:1 onwards and P104 had micelles from 1:1 onwards. Representative size distributions are shown in Figure 6.9. This is why the redispersibility and span range ratios were generally quite different, with smaller sizes...
span ranges for the smaller P104. This behavior seems to be dependent on the critical micelle concentration (CMC) of the Pluronic. F68 has a CMC at 25°C of ~35 wt% [43], F127's CMC is 0.7% wt/vol and P104's CMC is 0.3% wt/vol [48]. Assuming that the CMC for each of these polymers does not change when present in the nanoparticle dispersion, it is expected that the size distributions corresponding to the samples with 10:1 excipient to nanoparticles would have a peak with only ≤ 33% of the area under the curve if pure Pluronic micelles formed (F68: 0%, F127: 25%, P104: 33%). However, at the 10:1 mass ratio, the micelle peaks corresponded with 7, 85, and 88% of the area under the curve for F68, F127, and P104. This suggests that some fraction of the D3 was stripped out of the original nanoparticles by the surface active Pluronic micelles, resulting in mixed micelle formation. This capability of the Pluronics is enhanced with higher CMCs and is not desirable for nanoparticle formulations with long-lasting API release.

Therefore, only Pluronic F68 is recommended for freeze-drying purposes. However, in comparing the various molecular weight PEGs and Pluronic F68, a clear distinction amongst the excipients could not be made.

**Figure 6.9** Representative particle size distributions of trials from Figure 6.8. The ratios indicate the mass ratio of the Pluronic to nanoparticles.
Since the lyophilized D₃ nanoparticle dispersions tended to reconstitute very well, it was decided to test some of the PEGs and Pluronic F68 using the GW771806X/PAM nanoparticle formulation instead, as this system tended to have poorer redispersibility. For this study, samples were prepared with 3:1 mass ratios of excipient to particles, using PEG4.6kD, PEG20kD, and Pluronic F68. The samples were frozen at different rates, as will be explained in section 6.3.3.2, and then either freeze-thawed or freeze-dried in a cabinet lyophilizer for at least two days on a manifold or at a shelf temperature of -15°C or -40°C. This experiment was used to elucidate whether the excipients are cryo- or lyoprotectants through the comparison of freeze-thawing and freeze-drying; furthermore, testing the various temperature control schemes would allow for cycle time optimization. The results are displayed in Figure 6.10.

First, in comparing the freeze-thawing to the freeze-drying, it becomes apparent that it is during the lyophilization, or the drying step, that particle aggregation occurs. While all freeze-thawed samples achieved, on average, a redispersibility ratio of 0.98, span range ratio of 0.94, and API recovery of 85%, it was only the samples with protectants that achieved comparable API recoveries when freeze-dried. The samples with no protectant achieved ~84% API recovery when freeze-thawed, while freeze-drying resulted in only 26±3% and 6±1% API recoveries, respectively for those frozen in a CoolCell and those in a dry ice/acetone bath and both lyophilized on the manifold. Furthermore, considering that there was little difference in the resulting metrics between the freeze-thawed samples with no protectant and those with protectant, this means that the original particles did not significantly aggregate during the freezing step. During freezing, it is not expected that particles with a protective PEG layer experience significant aggregation, even without excipients, since the PEG from the di-block copolymer will act as a surface anchored cryoprotectant. The results suggest that enough steric
stabilization is present on the particle surfaces to prevent considerable particle aggregation during freezing, which is surprising when taking into account the poor PEG stabilization afforded by the PLA3.7kD-b-PEG5kD. However, considering that the drying process drastically lowered the API recovery of the samples with no protectants, it becomes obvious that the excipients used are lyoprotectants.

**Figure 6.10** Redispersibility and span range ratios and API recoveries of reconstituted lyophilized GW771806X/pamoic acid nanoparticles stabilized by PLA3.7kD-b-PEG5kD. The nanoparticle dispersions were produced on a MIVM at ~3.3 mg/mL particles and prepared with the various polymers at a 3:1 excipient to particle mass ratio. Samples were frozen in a CoolCell LX in a -80°C freezer (CC) or in a dry ice/acetone bath (DIA). The frozen samples were either freeze-thawed (FT) or freeze-dried (FD) in a cabinet lyophillizer for at least 1 day on a manifold (M), at a shelf temperature of -15°C (-15), or at a shelf temperature of -40°C (-40).
Secondly, we are better able to discriminate amongst the three excipients in this study in comparison to the D$_3$ nanoparticle study. In terms of API recoveries, the ranking of the excipients, in order of most protective to least protective, was Pluronic F68, PEG4.6kD, and PEG20kD. Also, the samples with Pluronic generally achieved the lowest redispersibility and span range ratios, while the PEG20kD resulted in the highest size ratios and lowest API recoveries. While these results are not concrete evidence in determining the mechanism by which particle aggregation is prevented, the superior results of the Pluronic F68 (PEG MW: 3.4kD) as compared to the PEG4.6kD suggests that the surfactant nature of the poloxamer may have been beneficial during drying. In section 6.3.1.1, we showed that the original particles had minimal protection provided from the PEG of the PLA3.7kD-b-PEG5kD. Therefore, adsorption of Pluronic F68 onto the particle surface could provide more steric stabilization and increase the total surface density of PEG on the particle surface.

In comparing the two molecular weights of PEG, the 4.6kD PEG was more protective than the 20kD PEG. Although they are chemically equivalent, Ramasubramani has shown that particles stabilized by a di-block copolymer with a 5kD PEG block will phase separate from a PEG20kD solution, while PEG solutions with molecular weights of 4kD and 10kD do not induce phase separation at the same polymer mass concentration [49]. This behavior was attributed to depletion flocculation, or the phenomenon where free polymer in solution causes two particles to aggregate as they approach each other due to the "depletion zone" volumes overlapping, leading to osmotic pressure buildup that pushes the particles together [50]. Seebergh and Berg reported similar observations for polystyrene latexes stabilized by Pluronics L35 and L43, where greater phase separation was observed when introducing free polymer of larger molecular weight [51].
Therefore, it is crucial for PEG protectants to have PEG chains similar or smaller in molecular weight than the stabilizing PEG molecular weight.

Overall, these results point to Pluronic F68 being a better lyoprotectant than PEG4.6kD. However, these studies did not consider the stability of the lyo-cakes. In Chapter 4, it was shown that freeze-dried GW771806X/PAM nanoparticle dispersions that used the pharmaceutical-grade Lutrol F68 instead the standard-grade Pluronic F68, while achieving excellent redispersibility during short storage periods, were not stable over a month of storage at ambient conditions (section 4.3.5). In contrast, while the PEG4.6kD lyo-cakes did not reach as high API recovery as Lutrol F68, the lyo-cake using a 5:1 mass ratio of PEG4.6kD to particles was stable for one month. Thus, in further experiments, only a 1:1 mass ratio of Lutrol F68 to particles was used for extra steric stabilization on the particle surface and a 4:1 mass ratio of PEG4.6kD was used for lyoprotection. Lyo-cakes using this formulation were able to achieve excellent redispersibility. These results suggest that only small amounts of Pluronic F68 should be used, especially if the initial nanoparticle dispersion has poor PEG steric stabilization, and that the bulk of the protectant excipient be a PEG of appropriate molecular weight. In the case where PEG steric stabilization is moderate or excellent, such as that of the D3 nanoparticles, it may be acceptable to use only free PEG. Table 6.4 shows how trials D and E both resulted in quantitative recovery of the particles once reconstituted for both the PS and PCL compositions.

**6.3.2.3 Sugars**

Sugars, such as sucrose, trehalose, lactose, and glucose, are common excipients for freeze-drying, since they can theoretically function as both cryo- and lyoprotectants. During the freeze-drying process, sugars will be rejected from the freezing ice front and concentrate into a viscous domain that will vitrify below a specific glass transition temperature. Depending on how
well PEG can be dispersed in the sugar, the nanoparticle systems will experience different levels of screening. As was shown in section 4.3.5 of Chapter 4, sucrose provides better dispersion for PEG than trehalose and thus allows for a more consistently redispersible lyo-cake; however, there is evidence of some phase separation between PEG and sucrose [52]. Thus, during freezing and primary drying, particles can be immobilized in the vitreous sugar matrix, whether as individual particles or in clusters. Once secondary drying occurs, again depending on how well the sugar can disperse PEG, the sugar molecules can replace the water from the PEG/water eutectic, such that the PEG remains "hydrated". However, PEG does not have high affinity for commonly-used glass-forming sugars [25], meaning that high concentrations of sugars need to be used to efficiently yield a redispersible lyo-cake. In the literature, it is quite common for levels > 5% (wt/vol) of sugar to be used in order to achieve acceptable redispersibility.

Depending on the sugar, the osmolarity contribution of the sugar alone will be roughly equal to (monosaccharide) or half (disaccharide) the blood osmolarity at 5%. This places a severe limit on the maximum achievable API concentration when using formulations using sugars as protectants.

In comparison to the polymeric excipients, trehalose, a vitrifying sugar with a high glass transition temperature, and mannitol, a crystallizing sugar alcohol with a low crystallizing temperature, were also evaluated in several of the aforementioned studies. Trehalose was evaluated in section 6.3.2.2, alongside Pluronics F68 and F127 (see Figure 6.7). Alone, it resulted in poor API recovery at 3:1 and 5:1 mass ratios; while trehalose reached API recoveries comparable to the Pluronics at the 10:1 ratio, the redispersibility and span range ratios were at least 30% higher than those of the Pluronics. The dual combinations of trehalose with a Pluronic showed that trehalose had no meaningful contribution to the redispersibility of the particles. In all cases where trehalose was used at concentrations of ≤ 2% wt/vol, the lyo-cakes experienced
some degree of meltback and complete cake collapse was observed when \( \leq 1\% \) wt/vol was used. Not only does trehalose not disperse PEG very well [17], it also has a relatively low collapse temperature (-30°C) [53]. These two properties are not desirable for a protectant excipient of PEG-stabilized nanoparticles. Mannitol also does not disperse PEG very well, as it has been reported that PEG has no effect on reducing crystallinity of the sugar alcohol or vice versa [54].

The results for the \( D_3 \) and GW771806X/PAM dispersions freeze-dried with trehalose or mannitol are presented in Figure 6.11. In comparison to the lyophilized formulations with no protectant excipient, both trehalose and mannitol resulted in statistically similar API recoveries for both nanoparticle formulations \((p > 0.05)\). Although the mannitol formulation yielded smaller size ratios for the \( D_3 \) nanoparticles \((p < 0.05)\), it was not statistically significant for the GW771806X nanoparticles. Overall, these two saccharides did not provide any meaningful contribution to the formulations at the mass ratio evaluated. Even when lyophilized at a shelf temperature of -35°C (Figure 6.7), the trehalose formulations did not result in drastically different results, with cake collapse occurring as well. While trehalose was shown to be capable of yielding good redispersibility for the GW771806X formulation in Chapter 4, 10% wt/vol was required for a stable lyo-cake that reconstituted to 1 mg/mL AP; this trehalose concentration yields an osmolarity roughly equal to that of blood serum, meaning that higher API concentrations could not be administered using this formulation. This underlines the flexibility that the polymers provide to lyophilized formulations for parenteral applications.

Finally, in further understanding the behavior of the various excipients evaluated, DSC traces are provided in Figure 6.12. Here, the unfrozen GW771806X dispersions from Figures 6.10 and 6.11 were tested. The nominal PEG concentration of the initial dispersion was 1.01 mg/mL.
Figure 6.11 Redispersibility and span range ratios and API recoveries of reconstituted lyophilized nanoparticles with components listed in the legend. The nanoparticle dispersions were produced on a MIVM at ~3.3 mg/mL particles and prepared with the excipients at a 3:1 excipient to particle mass ratio. Samples freeze-dried in a cabinet lyophilizer for at least 1 day at a shelf temperature of -15°C (GW771806X) or -20°C (D3).

The endothermic heat of the PEG/water eutectic melting associated with the nanoparticle formulation with no added excipients was ~19 times lower than that of the D3 dispersion from section 6.3.1.1; using this data, it is estimated that ~0.08 mg/mL PEG was available for forming a eutectic with water on the surface of the GW771806X nanoparticles. The incorporation of the PEG-based excipients increased the magnitude of the melting endotherms of the PEG/water eutectics in the range of -18°C to -13°C, with an increase in the melting temperature as the PEG chain increased (~3.4kD (F68) < 4.6kD < 20kD).
Figure 6.12 Differential scanning calorimetry of the GW771806X/pamoic acid nanoparticle formulations from Figures 6.10 and 6.11 prior to freeze-drying. Second heating traces are shown. See the methods section for DSC procedure.

There was a significant exotherm at -40°C for the Pluronic F68 formulation, which might be attributed to cold crystallization of excess water not associated with PEG/water eutectic [19]. The trehalose had a small glass transition inflection at -31°C, which is consistent with values in the literature [55]; however, there was no observable PEG/water eutectic. Although the DSC trace did not exhibit a collapse event, the lyo-cake did collapse during freeze-drying, suggesting that the product temperature drifted above the glass transition temperature. The mannitol displayed two glass transitions at -35°C and -28°C before a crystallization exotherm at -20°C, which has been reported in the literature [54]. The crystallization of the mannitol most likely resulted in phase separation of clusters of primary particles scattered in the crystalline mannitol, thereby inducing particle aggregation. The PEG/water eutectics that were introduced by the incorporation of the PEG-based excipients form a matrix into which the primary particles that
also have a PEG/water eutectic surface layer can uniformly disperse. Removal of the water from the eutectic will result in reversible PEG crystallization, as shown through the redispersibility of the lyo-cakes, which suggests that the PEG-based excipients prevent hydrophobic contacts amongst the primary particles.

6.3.3 Process parameters

6.3.3.1 The presence of organic solvent

When considering the process for producing lyophilized nanoparticle dispersions, a possibility exists of directly proceeding to freeze-drying after FNP, bypassing the purification step that removes the organic solvent. This might be necessary when working with a nanoparticle system that is not stable through processing steps. An example of this direct freeze-drying was presented in Chapter 3 for the case of progesterone-loaded nanoparticles. However, it is important to understand the effect the organic solvent has on the redispersibility of the resulting lyophilizate. In order to evaluate various process parameters, including the presence of organic solvent, two $2^3$ factorial studies were conducted that tested three two-level factors (presence of organic solvent, cooling rate, and drying conditions) for the two nanoparticle systems that have been discussed previously. The dispersions were prepared at concentrations of 3-3.5 mg/mL nanoparticles and freeze-dried without protectants. The results are given in Table 6.6.

Considering that the GW771806X formulations all yielded poor API recovery, ANOVA was done only on the D$_3$ formulations to understand the importance of each factor. The results of the ANOVA performed using the Minitab software are presented in Table 6.7.

The presence of organic solvent was an important factor, with the main effect term (A in Table 6.7) and the two-way interaction between the presence of organic solvent and the drying
conditions (AC) being significant for all metrics except API recovery; however, since the two-way interaction term was significant, the main effect term is not considered.

Table 6.6 Factorial study testing two nanoparticle systems without protectant excipients.

<table>
<thead>
<tr>
<th>Nanoparticle System</th>
<th>Organic Present? (Y/N)</th>
<th>Freezing Method</th>
<th>Drying Method</th>
<th>$S/S_i$</th>
<th>$A/A_i$</th>
<th>API Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D$_3$ / PS1.6kD-$b$-PEG5kD</td>
<td>Y</td>
<td>Dry</td>
<td>Manifold</td>
<td>1.45 ± 0.06</td>
<td>1.53 ± 0.16</td>
<td>73 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ice/Brine</td>
<td>Shelf (-10°C)</td>
<td>1.40 ± 0.05</td>
<td>1.66 ± 0.15</td>
<td>91 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dry</td>
<td>Manifold</td>
<td>1.49 ± 0.04</td>
<td>1.66 ± 0.13</td>
<td>70 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ice/Acetone</td>
<td>Shelf (-10°C)</td>
<td>1.34 ± 0.04</td>
<td>1.52 ± 0.14</td>
<td>93 ± 1</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>Dry</td>
<td>Manifold</td>
<td>1.52 ± 0.05</td>
<td>1.85 ± 0.11</td>
<td>72 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ice/Brine</td>
<td>Shelf (-10°C)</td>
<td>1.31 ± 0.04</td>
<td>1.56 ± 0.09</td>
<td>91 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dry</td>
<td>Manifold</td>
<td>1.43 ± 0.04</td>
<td>1.71 ± 0.09</td>
<td>70 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ice/Acetone</td>
<td>Shelf (-10°C)</td>
<td>1.33 ± 0.04</td>
<td>1.55 ± 0.13</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>GW771806X/ PAM / PLA3.7kD-$b$-PEG5kD</td>
<td>Y</td>
<td>Dry</td>
<td>Manifold</td>
<td>2.45 ± 0.17</td>
<td>2.59 ± 0.48</td>
<td>7 ± 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ice/Brine</td>
<td>Shelf (-10°C)</td>
<td>1.95 ± 0.14</td>
<td>1.99 ± 0.31</td>
<td>6 ± 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dry</td>
<td>Manifold</td>
<td>2.37 ± 0.20</td>
<td>2.43 ± 0.51</td>
<td>9 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ice/Acetone</td>
<td>Shelf (-10°C)</td>
<td>1.82 ± 0.23</td>
<td>1.99 ± 0.33</td>
<td>5 ± 1</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>Dry</td>
<td>Manifold</td>
<td>1.52 ± 0.08</td>
<td>1.53 ± 0.34</td>
<td>2 ± 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ice/Brine</td>
<td>Shelf (-10°C)</td>
<td>1.71 ± 0.08</td>
<td>1.76 ± 0.36</td>
<td>5 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dry</td>
<td>Manifold</td>
<td>1.59 ± 0.08</td>
<td>1.64 ± 0.32</td>
<td>2 ± 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ice/Acetone</td>
<td>Shelf (-10°C)</td>
<td>1.62 ± 0.07</td>
<td>1.68 ± 0.31</td>
<td>6 ± 1</td>
</tr>
</tbody>
</table>

Table 6.7 ANOVA results for the $2^3$ factorial study outlined in Table 6.6 for the D$_3$ formulations. The values listed are the $p$-values for each term, with $\alpha = 0.05$. Terms: A = presence of organic solvent, B = freezing method, C = drying conditions.

<table>
<thead>
<tr>
<th>Factors (p-value)</th>
<th>$S/S_i$</th>
<th>$A/A_i$</th>
<th>API Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Term</td>
<td>$S/S_i$</td>
<td>$A/A_i$</td>
<td>API Recovery</td>
</tr>
<tr>
<td>A</td>
<td>0.036</td>
<td>0.039</td>
<td>0.366</td>
</tr>
<tr>
<td>B</td>
<td>0.068</td>
<td>0.231</td>
<td>0.304</td>
</tr>
<tr>
<td>C</td>
<td>&lt; 0.000</td>
<td>0.002</td>
<td>&lt; 0.000</td>
</tr>
<tr>
<td>AB</td>
<td>0.435</td>
<td>0.301</td>
<td>0.724</td>
</tr>
<tr>
<td>AC</td>
<td>0.018</td>
<td>0.003</td>
<td>0.572</td>
</tr>
<tr>
<td>BC</td>
<td>0.774</td>
<td>0.323</td>
<td>0.061</td>
</tr>
<tr>
<td>ABC</td>
<td>&lt; 0.000</td>
<td>0.006</td>
<td>0.567</td>
</tr>
</tbody>
</table>
For the D₃ system, samples that were shelf lyophilized exhibited a statistically smaller redispersed size when dialyzed in comparison to those that were non-dialyzed; also, a smaller redispersed span range was obtained for non-dialyzed samples that were manifold lyophilized in comparison to those that were dialyzed. All other treatments corresponding to the AC two-way interaction for the D₃ nanoparticles yielded statistically similar results. The D₃ dispersion was made using tetrahydrofuran, which has a much higher vapor pressure than 1000 mtorr at -10°C, while the GW771806X dispersion was made using dimethyl sulfoxide, which has a vapor pressure of ~750 mtorr at 27.4°C [56]. Clearly, tetrahydrofuran is more volatile and will be more easily removed during lyophilization; considering how lyophilization was done at a shelf temperature of -10°C, dimethyl sulfoxide would still present before complete ice sublimation [57], leading to partial dissolution of the nanoparticles. This might explain why in Table 6.6 the size and span range ratios for the GW771806X formulations were larger for samples that had the organic solvent present, as particle disintegration and aggregate formation could have occurred when only dimethyl sulfoxide, and no water, was left during lyophilization. This was not an issue for samples with tetrahydrofuran present, since it will exist as a solid hydrate at the drying conditions [58], such that it cannot interact with the nanoparticles. The phase behavior of the solvents can thus be used to explain the results observed.

Furthermore, the study presented in section 6.3.2.2 evaluating Pluronics F68 and F127 against trehalose for the GW771806X nanoparticle formulation also investigated the presence of organic solvent. The results from Figure 6.7 show that much worse redispersibility was obtained for the lyo-cakes from non-dialyzed dispersions as compared to the dialyzed dispersions. Without dialysis, adequate redispersibility could only obtained by using moderate levels of both Pluronic F127 and trehalose; at a 2.5:2.5:1 mass ratio of Pluronic F127/trehalose, an API
recovery of 89±2% reached with a redispersibility ratio of 0.97±0.28 and a span range ratio of 1.09±0.41 when using non-dialyzed dispersions. In contrast, for dialyzed dispersions, either Pluronic or any dual combination yielded ≥ 95% API recovery with an average redispersibility ratio of 1.10 and an average span range ratio of 1.04 at mass ratios of 3:1.

6.3.3.2 Cooling rates

During the freezing process, the nucleation and growth of ice crystals will determine the dynamics of cryo-concentration of the solid particles and possible protectants. The rate at which the dispersions are cooled will in turn determine the freezing rate and the rate of phase separation in the system. There is a scarcity of reports in the literature focusing on this phenomenon for nanoparticle dispersions and most reports consider biological materials, such as DNA and proteins. There has been controversy in this literature as to whether slow or fast cooling is desirable for the freezing of dispersions for lyophilization purposes. Slow cooling will induce the formation of large ice crystals from a small number of nuclei, which has been hypothesized to reduce the degree of destabilization due to dehydration at interfaces [59, 60]. Likewise, the formation of larger ice crystals is preferred, because larger channels will form that can speed up sublimation of the ice during primary drying, thus decreasing cycle times; this is why annealing of frozen systems to recrystallize unfrozen water into larger crystals is sometimes done [61, 62]. However, slow freezing can also allow enough time for the kinetic process of phase separation of dispersion components to occur, thereby leading to poorly redispersible lyophilizates [60]. It is also common for freeze-drying of biological material to be performed at fast freezing rates in order to lock the dispersions in a state similar to the initial liquid state, where phase separation does not exist, such that an amorphous state may be possible [63, 64]. With regards to nanoparticle dispersions, Lee has shown that for the freeze-drying of
hydroxypropyl cellulose stabilized crystalline nanosuspensions, faster freezing rates allowed for enhance redispersibility of the resulting lyo-cakes [41, 65]. Overall, it seems that the effect of the cooling rate will depend on the specific formulation that will be freeze-dried.

Thus, in order to understand the importance of the cooling rate on nanoparticle dispersions stabilized by PEG, two studies were conducted. The first has been described in the previous section, which examined three different process parameters. The ANOVA results in Table 6.7 showed that the main and two-way effects associated with the cooling rates were insignificant. However, the two freezing methods utilized both resulted in rapid cooling rates. Figure 6.13 has the cooling profiles for four different freezing methods. Table 6.8 lists the sink temperatures and initial cooling rates of the methods. Since the difference in initial cooling rates was only four-fold for the two dry ice methods used in the factorial study, it was necessary to test freezing methods that had a larger difference in freezing rates.

![Temperature profiles of water frozen through various methods](image.png)

**Figure 6.13** Temperature profiles of water frozen through various methods: held in a CoolCell LX stored in a -80°C freezer, submersed in a dry ice/brine bath, submersed in a dry ice/acetone bath, submersed in liquid nitrogen.
Table 6.8 Properties of the freezing methods presented in Figure 6.13. The initial cooling rate is the average linear drop in temperature prior to freezing for n=2 samples.

<table>
<thead>
<tr>
<th>Freezing Method</th>
<th>Sink Temperature (°C)</th>
<th>Initial Cooling Rate (°C/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoolCell 5mL LX</td>
<td>-80</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Dry Ice/Brine</td>
<td>-20</td>
<td>24.6 ± 9.2</td>
</tr>
<tr>
<td>Dry Ice/Acetone</td>
<td>-78</td>
<td>101.8 ± 17.7</td>
</tr>
<tr>
<td>Liquid Nitrogen</td>
<td>-196</td>
<td>165.2 ± 125.9</td>
</tr>
</tbody>
</table>

The study in section 6.3.2.2 that investigated the use of PEG4.6kD, PEG20kD, and Pluronic F68 for the GW771806X dispersions compared the redispersibilities of lyophilizates frozen in either a CoolCell or in a dry ice/acetone bath. No consistent trend was found among the three reported redispersibility metrics. While the dry ice/acetone bath resulted in smaller redispersibility ratios for formulations with PEG20kD and Pluronic F68, the change was only -8% and -14%, respectively. While statistically significant, the smaller peak mean diameters were not accompanied by statistical changes in API recovery. The 68-fold change in the initial cooling rates between the CoolCell and the dry ice/acetone bath had a negligible effect on redispersibility for the formulations with PEG-based excipients, which is reasonable since phase separation is not expected. For formulations with lyo-cake collapse, such as those with no excipients or those with trehalose, although statistical differences were seen when using different cooling rates, these formulations consistently yielded poor redispersibility and thus, the effects are not particularly important. Overall, the effects of the cooling rate were not significant. DSC of the D₃ nanoparticle dispersion with no protectant excipients was done using cooling rates of 0.5 and 40°C/min to -85°C followed by heating at 0.5°C/min to 0°C. The traces are shown in Figure 6.14. The faster cooling rate of 40°C/min resulted in significant supercooling, as evidenced by the large shoulder of the ice crystallization exotherm reaching to -40°C. Upon heating, the melting of the PEG/water eutectic at the surface of the particle was not preceded by cold crystallization of excess water in either case. This suggests that the kinetics of crystallization of
the hydrated PEG layer are fast enough that excess water is rejected from the crystalline eutectic during rapid freezing. Overall, the freezing rate is not seen to be an important parameter to consider in the cryopreservation of PEG-stabilized nanoparticle dispersions, as shown by the insignificant differences for formulations in Figure 6.10 and by the DSC results in Figure 6.14 which have been discussed.

![Differential scanning calorimetry](image)

**Figure 6.14** Differential scanning calorimetry of the D3/PS1.6kD-b-PEG5kD nanoparticle formulation with no protectant excipient from section 6.3.1.1 prior to freeze-drying. See the methods section for DSC procedure. Exotherms are upward for cooling traces and downward for heating traces.

### 6.3.3.3 Drying conditions

In lyophilization, the control of conditions during drying stages is important. It is commonly known that primary drying should be done at temperatures below or close to the glass transition or eutectic temperature of the cryo-concentrate in order to prevent meltback or collapse of the lyophilizate [24, 60]. In the factorial study presented in section 6.3.3.1, drying conditions were evaluated, comparing lyophilization on a manifold to that done on a shelf at a temperature
of -10°C. The ANOVA revealed that the main effects of this factor were significant for all
metrics; likewise, the two-way interaction between the drying conditions and the presence of
organic solvent were significant for the size metrics. For the D₃ system, regardless of the
presence of organic solvent, lower redispersibility ratios and higher API recoveries were
achieved for treatments with shelf drying. On a manifold lyophilizer, the temperature is
determined by the heat balance between the sublimation of ice/evaporation of volatile organics
and the uptake of heat from the environment through the flask walls. During secondary drying,
the rate of cooling by sublimation may be so low that the lyo-cake may warm. Depending on the
system, glass transition or eutectic temperatures may be exceeded, resulting in meltback and
possible cake collapse. The mobility associated with meltback can cause particle aggregation
during this later stage of drying.

Another point of concern is how there was large variability in redispersibility results for
the D₃ formulations, particularly API recovery. This is shown in Table 6.9. Considering that the
manifold-dried samples from runs I and II were not lyophilized on the same unit, variability in
all three metrics between the two runs might be due to differences in pressure attributed to the
different pumps used, the temperature gradients experienced by the samples, or the batch
volumes. Likewise, for the samples run on the Advantage lyophilizer during runs II and III at
different shelf temperatures, although lyophilized on the same unit with the same vacuum pump,
the batch volumes were quite different; it is possible that more homogeneous heat transfer was
achieved for the smaller batch volume. This is why the ANOVA from Table 6.7 was done only
on a study utilizing one run on one lyophilizer. It is important to keep in mind that different
process conditions can result in differences in redispersed size distributions and API recovery.

While the previous discussion has been focused on formulations that did not include
protectant excipient, we now consider formulations that do include protectants. The study highlighted in Figure 6.10 compared manifold drying to shelf drying at temperatures of -15°C and -40°C. Out of the three redispersibility metrics (i.e. peak mean diameter ratio, span range ratio, and API recovery), only the redispersibility ratio was the most affected by the drying conditions.

Table 6.9 Operating conditions for the various freeze-drying runs of D₃ nanoparticle formulations. All samples were frozen in dry ice/acetone baths.

<table>
<thead>
<tr>
<th>Run</th>
<th>Lyophilizer</th>
<th>Drying Conditions</th>
<th>Batch Volume (mL)</th>
<th>Protectant</th>
<th>$S_f/S_i$</th>
<th>$A_f/A_i$</th>
<th>API Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Benchtop</td>
<td>Manifold</td>
<td>20</td>
<td>None</td>
<td>1.25 ± 0.03</td>
<td>1.27 ± 0.07</td>
<td>97 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PEG4.6kD (3:1)</td>
<td>1.23 ± 0.02</td>
<td>1.16 ± 0.05</td>
<td>103 ± 2</td>
</tr>
<tr>
<td>II</td>
<td>Advantage</td>
<td>Manifold</td>
<td>64</td>
<td>None</td>
<td>1.43 ± 0.04</td>
<td>1.71 ± 0.09</td>
<td>70 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-10°C</td>
<td></td>
<td>None</td>
<td>1.33 ± 0.04</td>
<td>1.53 ± 0.13</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>III</td>
<td>Advantage</td>
<td>-20°C</td>
<td>260</td>
<td>None</td>
<td>1.25 ± 0.03</td>
<td>1.65 ± 0.13</td>
<td>77 ± 4</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PEG4.6kD (3:1)</td>
<td>1.13 ± 0.02</td>
<td>1.25 ± 0.04</td>
<td>94 ± 5</td>
</tr>
</tbody>
</table>

Generally, shelf temperature control resulted in smaller redispersed peak mean diameters than manifold drying. There was not a consistent difference between the two shelf temperatures; however, considering that the PEG/water eutectic melting temperature is in the range of -18°C to -13°C, as shown in Figure 6.14, either shelf temperature should have prevented significant meltback, of which none was observed visually. While drying at temperatures above the PEG/water eutectic will result in PEG crystallization, the mechanism of how the temperature results in larger particle sizes is not clear. Nonetheless, it is clear that the incorporation of PEG-based protectants diminished the effect of drying conditions and it stands in contrast to the strong temperature dependence of sugars such as trehalose. In addition, the variability between runs from Table 6.9 was less when PEG4.6kD was incorporated in the formulation; while there was a statistical change in all metrics when comparing run III to run I ($p < 0.05$), the change was <
|10%| and is thus of small concern. The robustness offer by formulations containing PEG-based excipients under different drying conditions is an important finding for the design and optimization of fast and efficient freeze-drying.

### 6.4 CONCLUSIONS

In this work, we have shown the importance of various formulation and process parameters in the freeze-drying of nanoparticle dispersions stabilized by di-block copolymers with a PEG block, as well as the contribution of protectant excipients. To our knowledge, there has not been any work in the literature that systematically examined the effects of these three key factors. Using two model nanoparticle systems, loaded with either D₃ or GW771806X, the choice of the stabilizing di-block copolymer was shown to determine the degree of PEG that is accessible on the particle surface (section 6.3.1.1). Di-block copolymers with hydrophobic blocks for which PEG has some miscibility (i.e. PCL or PLA) resulted in less measurable PEG in solution and also yielded poor redispersibility for lyophilized nanoparticles (Table 6.4). For PS-\(b\)-PEG, which was shown to have stronger phase separation, a trend was found relating the redispersibility ratio to the surface coverage provided by the PEG stabilizing layer, where greater surface coverage resulted in larger redispersed particle sizes (Figure 6.2). This was attributed to PEG crystallization, possibly resulting in bridging of primary particles; the aggregation could be reduced when applying heat at temperatures above the melting point of PEG (Figure 6.3). Furthermore, the initial concentration at which the particle dispersions were freeze-dried also had a significant impact on the redispersibility of lyo-cakes, where higher concentrations yielded less recovery of the nanoparticles (Figure 6.5, Table 6.5). Likewise, reconstitution to volumes less than the initial resulted in less breakup of the lyo-cake and yielded less API recovery.
In examining the traditional sugar protectants used, the problem of osmolarity is quite apparent for PEG-stabilized dispersions, due to the poor affinity of PEG for sugars. Therefore, in evaluating PEG-based polymeric excipients, the use of Pluronic F68 and free PEG of a molecular weight similar to that of the PEG on the stabilizing di-block copolymer are recommended. Lyophilized nanoparticle formulations using these two excipients were capable of yielding excellent redispersibility ($S_f/S_i < 1.10$) with high recovery of particles (> 85%), which is attributed to the high compatibility between the PEG stabilizing layer of the particles and the excipients (section 6.3.2.2). Furthermore, due to their polymeric nature, the osmolarity contribution of the excipients is minimal. These results, coupled with the lack of a collapse temperature, demonstrate the superiority of the Pluronic F68 and PEG as lyoprotectants over simple sugars.

In investigating the effects of not removing the organic solvent used in the production of the nanoparticle dispersions prior to freeze-drying, it was shown that if the solvent is more volatile than water, it had no effect on the resulting lyo-cake (Tables 6.6, 6.7). The cooling rate used to freeze the dispersions also had no significant effect on reconstitution (Figure 6.10). Also, the drying conditions were important to consider when freeze-drying dispersions with no protectant excipients, as lower product temperature yielded higher reconstituted recoveries (Figure 6.10); however, the incorporation of protectants reduced the effect and temperature control had only a slight effect on the redispersed particle size.

Overall, from the findings of this work, several recommendations are made. For the development of parenteral lyophilized formulations that require high API concentrations upon reconstitution, it is important to not use sugar protectants for freeze-drying PEG-stabilized nanoparticle dispersions. Instead, when using PEG-based excipients, the osmolarity limitation is
much reduced as compared to sugars and the redispersibility is improved. When adapting these PEG-based protectants to freeze-drying practices, it is important to determine the need for Pluronic F68 over free PEG; if the nanoparticle system has a high degree of PEG stabilization, the Pluronic may not necessary, since it can also lead to poor stability of the lyo-cake. However, in the case of poor PEG stabilization, a PEG-based surfactant, preferably with a high CMC, such as Pluronic F68, should be incorporated in order to prevent aggregation through hydrophobic contacts; nonetheless, the bulk of the protectants added should be PEG, as the non-surfactant PEG allows for a more stable lyo-cake. Moreover, caution should be exercised when performing proof of concept studies; nanoparticle dispersions making use of a block copolymer with considerable phase separation that is not biodegradable, such as PS-\(b\)-PEG, should not be assumed to give similar results when the block copolymer is changed to a biodegradable one, such as PCL-\(b\)-PEG or PLA-\(b\)-PEG, which can exist in a state where both blocks are fully miscible. Finally, while process parameters are always important to consider, the use of PEG-based excipients allow for the freeze-drying to be robust in a much wider range of values. This is attractive for development of short cycle times in freeze-drying. Considering how nanoparticulate formulations are becoming more commonplace in today's pharmaceutical horizon, it is important that the proper adjustments be made to traditional developmental platforms, such that rational efficient practices can improve the medical outlook of our society.

6.5 ACKNOWLEDGEMENTS

We would like to thank Dr. Christopher Brook, Dr. Yan Sun, Samantha Rusk, Christopher Morrison, and Aidan Gilmartin from GlaxoSmithKline for their assistance in procuring materials for this project. We also thank Daniel Tien for his help in conducting some
of the experiments using D₃ nanoparticles. I would like to thank Prof. George Scherer for granting me access to the Pyris TGA 7HT from the Scherer Lab, Lori Tunstall for training me to use the TGA instrument, Prof. Rodney Priestley for granting me access to the DSC Q2000 from the Priestley lab, Chuan Zhang for helping me run samples on the DSC, Prof. Jamie Link for allowing me to use his ultra low temperature freezer, and Dr. Suzanne D'Addio for helpful discussions concerning freeze-drying.

### 6.6 NOMENCLATURE

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>FNP</td>
<td>Flash NanoPrecipitation</td>
</tr>
<tr>
<td>API</td>
<td>active pharmaceutical ingredient</td>
</tr>
<tr>
<td>BCP</td>
<td>block copolymer</td>
</tr>
<tr>
<td>CMC</td>
<td>critical micelle concentration</td>
</tr>
<tr>
<td>CC</td>
<td>CoolCell LX</td>
</tr>
<tr>
<td>DIA</td>
<td>dry ice/acetone bath</td>
</tr>
<tr>
<td>FT</td>
<td>freeze-thawed</td>
</tr>
<tr>
<td>FD</td>
<td>freeze-dried</td>
</tr>
<tr>
<td>M</td>
<td>manifold</td>
</tr>
<tr>
<td>PAM</td>
<td>pamoic acid</td>
</tr>
<tr>
<td>D₃</td>
<td>cholecalciferol</td>
</tr>
<tr>
<td>BCT</td>
<td>β-carotene</td>
</tr>
<tr>
<td>AT</td>
<td>D-α-tocopherol</td>
</tr>
<tr>
<td>PPG</td>
<td>poly(propylene glycol)</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PLA</td>
<td>poly(D,L-lactide)</td>
</tr>
<tr>
<td>PCL</td>
<td>poly(ε-caprolactone)</td>
</tr>
<tr>
<td>PS</td>
<td>polystyrene</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactide-co-glycolide)</td>
</tr>
<tr>
<td>PXXYkD-b-PEGZkD</td>
<td>poly(XX)-block-poly(ethylene glycol) of MW: YkD-b-ZkD</td>
</tr>
<tr>
<td>MeCN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>MIVM</td>
<td>multi-inlet vortex mixer</td>
</tr>
<tr>
<td>CIJM-D</td>
<td>confined impinging jets mixer with dilution</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>TGA</td>
<td>thermogravimetric analysis</td>
</tr>
<tr>
<td>DSC</td>
<td>differential scanning calorimetry</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>$\Delta$</td>
<td>span range ($\Delta = d_{90} - d_{10}$)</td>
</tr>
<tr>
<td>$d_{10}$</td>
<td>10% of the intensity-weighted size distribution is smaller than this diameter</td>
</tr>
<tr>
<td>$d_{90}$</td>
<td>90% of the intensity-weighted size distribution is smaller than this diameter</td>
</tr>
<tr>
<td>$R^2$</td>
<td>linear coefficient of determination</td>
</tr>
<tr>
<td>$c_i$</td>
<td>concentration of $i$</td>
</tr>
<tr>
<td>$m_i$</td>
<td>mass of $i$</td>
</tr>
<tr>
<td>$\rho_i$</td>
<td>mass density of $i$</td>
</tr>
</tbody>
</table>
LE  loading efficiency

$V_i$  volume of $i$

$S_f/S_i$  redispersibility ratio, or the ratio of peak mean sizes before and after freeze-drying/reconstitution

$\Delta_f/\Delta_i$  ratio of span ranges before and after freeze-drying/reconstitution

$\alpha$  significance level for ANOVA

$p$  probability of achieving a test statistic as extreme as the one observed

$\chi$  Flory-Huggins interaction parameter

$N$  number of monomers in a polymer

$M_n$  number-averaged molecular weight of a polymer

$R$  universal gas constant

$T$  absolute temperature

$\delta_i$  solubility parameter of block $i$

$f_A$  volume fraction of block $A$ in a di-block copolymer

$\delta_d$  Hansen solubility parameter accounting for dispersion forces

$\delta_p$  Hansen solubility parameter accounting for dipolar interaction

$\delta_h$  Hansen solubility parameter accounting for hydrogen bonding

$\pi$  osmotic pressure

$n$  osmolarity or number concentration of species in the suspension

$k_B$  Boltzmann constant

6.7 REFERENCES


34. PLGA (Poly Lactic co-Glycolic Acid) Uniform Dry Microspheres, I. Polysciences, Editor 2013.


CHAPTER 7 - CONCLUSIONS & FUTURE WORK

7.1 CONCLUSIONS

This dissertation focused on the practical application of nano-formulations of active pharmaceutical ingredients (APIs), highlighting both the production of various nanoparticle dispersions using Flash NanoPrecipitation (FNP) and the use of freeze-drying to yield powders which could be reconstituted back to nanoparticulates. Previous work with FNP has centered on understanding fundamental assembly and phase behavior phenomena or has reported the production of formulations for certain APIs. Here, we have presented scientific studies that assessed the effects of various formulation and process parameters for effective synthesis techniques for at least five different molecules. From this work, we were able to produce robust formulations that could easily be adapted for therapeutic administration. We also have examined the importance of various parameters in the freeze-drying of nano-dispersions, such that future isolation work has a rational basis from which to start.

Chapter 2 was dedicated to reviewing the existing literature that reports the use of FNP for nanoparticle production. Fundamentals on the precipitation of solutes, focusing on classical nucleation and growth theory, as well as the effects of mixing during precipitation, and finally the importance of polymer stabilizers were discussed. With regards to application, a summary on the effect of supersaturation ratios and component concentrations was presented, as well as existing FNP formulation techniques and methods that have been used to convert the resulting liquid nanoparticle dispersions to dry dosage forms. This chapter serves as a basis for understanding what has been done with respect to FNP and the important research areas today, as well as a guide used in the studies presented in this dissertation.
Following in Chapters 3 through 5, case studies were presented for the formulation of progesterone for emergency traumatic brain injury treatment, GW771806X as a model compound for parenteral nanoparticulate administration of ionizable APIs, and indomethacin, fenofibrate, and cinnarizine as model compounds for oral administration of APIs. All of these APIs have relatively low hydrophobicities for FNP candidate compounds, necessitating the use of solubilization or ion pairing techniques for nanoparticle formation. Furthermore, while Chapters 3 and 4 focused on nanoparticle dispersions for parenteral administration, Chapter 5 presented work for oral administration, which has not been a focus for FNP-produced formulations. In these examples, through systematic evaluations of the formulation and process parameters, nanoparticle formulations with high loading efficiencies were produced.

Finally, in Chapter 6, freeze-drying of nanoparticles was studied using the GW771806X dispersions produced in Chapter 4, as well as cholecalciferol dispersions. These studies were aimed at drying nanoparticulate formulations for parenteral administration. The effects of formulations parameters, such as the choice of block copolymer, the physical state of the nanoparticle cores, and nanoparticle concentration, were shown to have a crucial impact on the redispersibility of the dispersions. The role of protectant excipients in modulating the nanoparticle redispersibility was investigated. A comparison of salts, sugars, and polymers showed that PEG-based excipients yielded the lyo-cake formulations with the best redispersibility and the lowest osmolarity, while producing results that were relatively independent of the process parameters used.

Overall, this dissertation was motivated by the lack of systematic experimental designs in the areas of formulation using FNP. Our hope is that the findings with respect to critical experimental parameters and the methodology of the work presented here can be used for further
growth and development of this field, which can accelerate the introduction of new nanoparticle therapeutics for the treatment of various diseases and conditions. We close by providing areas for future research.

7.2 FUTURE WORK

From the work completed for this dissertation, many questions arise that remain to be studied. Below, points of interest are detailed for each chapter.

7.2.1 Flash NanoPrecipitation: Fundamentals & Applications

While this chapter served as a review of FNP literature, several topics were covered pertaining to this dissertation that merit future research. For example, further studies should be done to better understand the effects of supersaturation ratios and/or API concentrations in the three cases presented in section 2.5, especially cases I and II; systematic experiments using several APIs should be done to explore a large range of API concentrations and organic solvent fractions in the solvent mixture to determine the effect of these parameters on particle size distributions and loading efficiencies. This is of significant importance for the optimization of nanoparticle production. In addition, the issue of aggregation zone formation in the MIVM should be addressed; either new designs for the MIVM should be developed or different mixing conditions should be adapted to minimize this phenomenon, in hopes of obtaining dispersions with lower polydispersity. Furthermore, another point of considerable interest would be to systematically evaluate the use of di-block copolymers with different hydrophobic blocks; although some attempt was made in this dissertation to assess different polymers, future experiments should attempt to quantify interactions between the API and the hydrophobic block and probe extreme cases with no and high affinity to determine the effects on the resulting
nanoparticles. Of course, much more research could be proposed with regards to other topics discussed in the chapter, such as targeting, imaging, and liquid dispersion stability, but they are outside of the scope of this dissertation.

### 7.2.2 Progesterone-Loaded Nanoparticles for Emergency TBI Treatment

In section 3.3.6 of Chapter 3, future work was described for obtaining an optimal nanoparticle formulation produced on a multi-inlet vortex mixer. In addition to this, it would be necessary to determine the stability of the dry lyo-cakes that were produced in section 3.3.4. Most likely, the Pluronic F68 that was used as a protectant excipient will result in poor stability for the lyophilizes, as was shown in Chapter 4. Thus, experiments evaluating other polymeric protectants or combinations of other protectants with Pluronic F68, as was shown in Chapter 6 for the PEG4.6kD/Lutrol F68 combination, might discover a formulation with a longer shelf life. Further *in vivo* testing would help to validate the usefulness of this formulation, particularly measurements of progesterone levels in the brain.

### 7.2.3 Production of Lyophilized Nanoparticle Dispersions of a Weakly Basic API

In the study that was presented, no solid state characterization was done. It would be helpful to verify that the GW771806X actually forms an ion pair with pamoic acid ion (through Fourier transform infrared spectroscopy and differential scanning calorimetry) and subsequently determine what fraction of the API loaded in nanoparticles is not in a paired form. Furthermore, it would interesting to compare *in situ* ion pair formation during FNP to FNP using the pre-made ion pair to evaluate which method results in the highest loading efficiency and the smallest/tightest particle size distribution. Also, the lyo-cakes could be investigated in more detail to elucidate the mechanism of particle aggregation during long periods of exposure to ambient conditions.
With regards to the total solids concentration used in FNP, attempting to produce a concentrated nanoparticle dispersion of the same composition as the one chosen for freeze-drying trials, but at the smaller particle size (trial XI from Table 4.2) would merit consideration. The lower supersaturation ratios might allow for less particle growth when increasing the concentration, possibly allowing for a higher initial API concentration. This might make the concentration step through tangential flow filtration unnecessary. Likewise, a comparison between any new formulations and the formulation used for freeze-drying trials (trial XIII from Table 4.2) once lyophilized would be important.

7.2.4 Drug-Loaded Nanoparticles for Oral Applications

It would be useful to conduct solid state characterization on the best formulations for all the APIs to determine the structure of the nanoparticles. In particular, determining whether the indomethacin is dissolved in an internal PEG-rich domain or if it is a dispersed crystalline phase in the D-α-tocopherol domain(s) would help to elucidate why the loading efficiency of trials with the level of 200% D-α-tocopherol did not follow the same trend as trials with the other levels (Figure 5.4). Also, establishing the physical state of each API in the nanoparticles would be interesting for comparing to other traditional FNP formulations that have a core composed of solely the API and use di-block copolymers as stabilizer.

In continuing onto the next steps in development, spray drying, spray granulation, and/or spray coating feasibility assessments should be performed. In particular, the reconstitution of the dry solids in simulated gastric fluids would need to be evaluated, as well as representative dissolution profiles. If quick and complete redispersibility of the particles and dissolution of the API(s) can be demonstrated, then making the drying process more efficient would be the next step, particularly since the low API concentration in the FNP-produced dispersions would most
likely be a limiting step. If possible, *in vivo* pharmacokinetics should be done to determine how differently the FNP formulation is absorbed as compared to formulations produced through top-down approaches.

### 7.2.5 Freeze-Drying of PEG-Stabilized Nanoparticles

While it would be important to test the recommendations set forth for as many nanoparticle formulations as possible to fine-tune the "rules" of freeze-drying, it would also be beneficial to conduct other fundamental studies. Further testing of the dependence of the redispersibility ratio on the surface coverage provided by PEG is necessary, especially for formulations not using sodium bicarbonate; investigating the effect of PEG surface coverage on API recovery would also be important.

Moreover, investigations through solid state characterization techniques that can be used on the frozen dispersions and the cryo-concentrates would be of great interest. Determining the phase behavior of the PEG from the stabilizing di-block copolymer during the whole freeze-drying process would be valuable. While we have shown the formation of the PEG/water eutectic layer during freezing, we do not have any direct knowledge of what occurs during secondary drying that can affect particle aggregation. Using freeze-drying microscopy and X-ray diffraction during secondary drying would help in elucidating what happens. Similarly, cryo-transmission electron microscopy of nanoparticle dispersions with and without protectant excipients could aid in determining the resulting structure that forms in the cryo-concentrates. Also, all future freeze-drying studies should attempt to track the product temperature. While modern lyophilizers are capable of recording product temperatures measured using thermocouples in real-time, the equipment available in the lab does not have this feature. A method would need to be engineered to have a thermocouple immersed in a representative
sample being lyophilized, such that the temperature can be datalogged in real-time. This would give better insight as to the method of failure for poorly redispersible lyophilizates. Finally, in-depth studies on the stability of lyo-cakes is critical, particularly at harsh conditions and for prolonged periods of time.
APPENDIX A - ESTIMATING PARTITION COEFFICIENTS

In order to predict how well a compound will be loaded into nanoparticle form through Flash NanoPrecipitation (FNP), it would be convenient to have information on the precipitation kinetics and the solubility of the compound in various organic/aqueous mixtures. However, this data is not available for most materials. As a substitute for this data, it is common to use a partition coefficient to have some understanding of how soluble the material is in water relative to the hydrophobic core of a nanoparticle. Typically, in pharmaceutical research, this is standardized by considering the partition coefficient between water and octanol. This is reported as a $\log P$, which is the base-10 logarithm of the ratio of concentrations of un-ionized compound between an octanol solution and an aqueous solution.

$$\log P = \log \left( \frac{c_{i,\text{octanol}}}{c_{i,\text{water}}} \right)$$  \hspace{1cm} (A.1)

Although experimental values for the $\log P$ of compounds that have been studied extensively are usually available in the literature, for many polymers and active core solutes, the data has not been reported. Therefore, it is important to have at least a calculated $\log P$ value for such cases and it is important that a consistent comparison be made amongst calculated values only.

Although both D’Addio [1] and Pinkerton [2] have previously reported the use of the MiLogP software developed and provided by Molinspiration [3], an exhaustive comparison by Mannhold et al on over 18 $\log P$ prediction algorithms showed that ALOGPs performed better in predicting the partition coefficients for datasets with over 96,000 compounds than MiLogP [4]. The Virtual Computational Chemistry Laboratory (VCCLab) [5] offers free use of the ALOGPs algorithm [6, 7] and reports other calculated values, such as AC $\log P$, AB/LogP, MiLogP, ALOGP, MLOGP, KowWIN, XLOGP2, XLOGP3, ALOGpS, AC $\log S$, AB/$\log S$, and AB/pKa.
Furthermore, when inputting the compound, VCCLab offers the same JME structure editor used by Molinspiration and it allows for the input of a SMILES representation, CAS number, or a .mol file. The software will also provide values for ion pairs, while MiLogP cannot. Overall, VCCLab is preferred over Molinspiration in terms of accuracy and convenience.

Below are the ALOGPs for several compounds used in the dissertation work. These compounds were first drawn in ChemBioDraw 12.0 (CambridgeSoft, USA), converted into a SMILES representation, and entered into VCCLab. Polymers were drawn by concatenating oligomers until the appropriate molecular weight was obtained.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Formula</th>
<th>MW</th>
<th>ALOGPs</th>
<th>MiLogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(ethylene oxide) 350</td>
<td>C15H32O8</td>
<td>340.47</td>
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<td>Poly(ethylene oxide) 1kD</td>
<td>C45H92O23</td>
<td>1001.37</td>
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<td>-4.54</td>
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<tr>
<td>Poly(ethylene oxide) 2kD</td>
<td>C91H184O46</td>
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<td>Poly(ethylene oxide) 3kD</td>
<td>C135H272O68</td>
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<td>0.52</td>
<td>-6.39</td>
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<td>10.63</td>
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<td>-</td>
<td>11.75</td>
</tr>
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<td>Polystyrene 1.6kD</td>
<td>C120H122</td>
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<td>11.36</td>
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<td>PPG-3 myristyl ether</td>
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<td>272.53</td>
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<td>8.78</td>
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<tr>
<td>Alpha-tocopherol polyethylene glycol 1000 succinate</td>
<td>C78H144O27</td>
<td>1514.22</td>
<td>3.97</td>
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<td>Naproxen</td>
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<td>230.28</td>
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<td>3.38</td>
</tr>
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<td>Camphor-10-sulfonic acid</td>
<td>C10H16O4S</td>
<td>232.33</td>
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<td>-1.30</td>
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<tr>
<td>Indomethacin</td>
<td>C19H16NO4Cl</td>
<td>357.81</td>
<td>4.25</td>
<td>3.99</td>
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<tr>
<td>Pamoic acid</td>
<td>C23H16O6</td>
<td>388.39</td>
<td>4.58</td>
<td>5.52</td>
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<tr>
<td>Pyrene</td>
<td>C16H10</td>
<td>202.26</td>
<td>5.19</td>
<td>4.88</td>
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<tr>
<td>Estradiol</td>
<td>C18H24O2</td>
<td>272.42</td>
<td>3.57</td>
<td>3.43</td>
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### Estimating Partition Coefficients

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Formula</th>
<th>MW</th>
<th>ALOGPs</th>
<th>MiLogP</th>
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<tr>
<td>Progesterone</td>
<td>C21H30O2</td>
<td>314.51</td>
<td>3.58</td>
<td>3.81</td>
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<tr>
<td>Griseofulvin</td>
<td>C17H17O6Cl</td>
<td>352.79</td>
<td>2.71</td>
<td>1.58</td>
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<td>Prednisone</td>
<td>C21H26O5</td>
<td>358.47</td>
<td>2.07</td>
<td>1.41</td>
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<tr>
<td>Fenofibrate</td>
<td>C20H21O4Cl</td>
<td>360.86</td>
<td>4.86</td>
<td>5.54</td>
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<td>Cholecalciferol</td>
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<td>384.71</td>
<td>7.98</td>
<td>7.68</td>
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<td>Beta-carotene</td>
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<td>Solvent yellow 98</td>
<td>C36H45NO2S</td>
<td>555.89</td>
<td>9.81</td>
<td>9.76</td>
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<tr>
<td>Prednisone cosanyl diglycolate</td>
<td>C45H70O9</td>
<td>755.15</td>
<td>7.74</td>
<td>9.45</td>
</tr>
<tr>
<td>Cinnarizine</td>
<td>C26H28N2</td>
<td>368.56</td>
<td>5.19</td>
<td>5.76</td>
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<tr>
<td>GW771806X</td>
<td>C22H24N6O2S</td>
<td>436.59</td>
<td>3.92</td>
<td>3.57</td>
</tr>
</tbody>
</table>

### A.1 REFERENCES


APPENDIX B - MATERIAL & METHODS FOR PRODUCING SOLVENT YELLOW 98 NANOPARTICLES

B.1 MATERIALS

Tetrahydrofuran (THF) (HPLC grade) was purchased from Sigma-Aldrich (USA). Methanol (MeOH) (HPLC grade) was purchased from Fisher Scientific (USA). Poly(ethylene glycol)-b-poly(D,L-lactide) (MW 5000-b-3700) was generously supplied by Evonik Inc. (USA). Solvent yellow 98 (SY98) (hostasol yellow 3G) was obtained from Clariant Corporation (USA). Ultrapure water (MilliQ water) (18 MΩ·cm) was generated from a Barnstead Nanopure purification system.

B.2 SY98 SOLUBILITY MEASUREMENTS

The solubility of SY98 was investigated in MilliQ water, THF, and THF/water mixtures. The compound was first added to THF until saturated and the solution was filtered with a 0.2 µm syringe filter (PTFE; Fisher, USA). Specified volumes of the organic solution were added to MilliQ water to result in 20, 15, 10, and 5 vol% THF in water. To pure MilliQ water, a small amount of SY98 powder was added. The mixtures were placed on a rugged rotator (099A RD4512; Glas-Col, USA) overnight. The samples were then filtered through 0.2 um syringe filters (PVDF; Whatman, USA) and analyzed through HPLC.

B.3 NANOPARTICLE FORMATION

Block copolymer stabilized nanoparticles were produced using a four-jet vortex mixer described by Liu et al [1]. Depending on the final amount of organic phase present in the final
solvent mixture, different flow rates were used for the different streams, although all at turbulent mixing regimes. Three 50 mL gas tight glass syringes (SGE Analytical Science, Australia) were filled with MilliQ water and one disposable syringe (20 - 30 mL; AirTite, USA) contained the organic phase. The streams were injected into the mixer using digitally controlled PHD 2000 syringe pumps (Harvard Apparatus, USA). The initial ~5 mL of effluent were discarded and then collection was started. Effluent collection was stopped prior to stopping the pumps. This ensured that only dispersion produced at steady state operation were collected.

In order to quantify the loading efficiency of SY98, the dispersion was dialyzed to remove organic solvent and thus had only an aqueous medium. An aliquot of the sample was loaded into Spectra/Por 1 (MWCO 6-8 kD) dialysis tubing (Spectrum Laboratories, USA). The aliquot was dialyzed against MilliQ water generally at a 1 L to 10 mL ratio of bath to sample volume. The bath water was refreshed at least hourly for the first five hours and then left overnight. The dialyzed dispersion was then collected from the tubing, the volume change was recorded, and the dispersion was filtered (1 µm, GF/B; Whatman, USA) prior to further processing.

B.4 NANOPARTICLE DISPERSION CHARACTERIZATION

B.4.1 Particle Size Distribution Measurement

After nanoparticle production and dialysis, the dispersions were characterized for particle size using a Malvern Zetasizer ZS ZEN 3600 (UK). Samples were prepared by diluting the dispersions at least 10-fold in MilliQ water and were contained in disposable 1.5 mL plastic cuvettes (Fisher, USA). All measurements were made with a 633 nm laser at a scattering angle of 173°. Sizes reported are those obtained using the general purpose normal resolution analysis.
mode of the intensity-weighted distribution. The peak means and span ranges ($d = d_{90} - d_{10}$) are given. Refer to Appendix C for a discussion on why this reporting protocol is used. Usually, at least triplicate measurements were made on each sample and the metrics given are the average and standard deviation of at least two measurements.

**B.4.2 SY98 Concentration**

SY98 concentrations were measured through reverse-phase high pressure liquid chromatography (HPLC). For analyzing dispersion concentrations, the nanoparticles were diluted in THF at least 20-fold before HPLC analysis. For solubility measurements, the samples were not diluted. HPLC was conducted on a Hewlett-Packard Agilent LC 1100 equipped with a quaternary pump, diode array detector, and a Gemini C18 stationary phase (5 µm, 100 Å; Phenomenex, USA). The mobile phase was MeOH, which was run at 2 mL/min using a column temperature of 50°C. The method run time was 9 minutes with an analyte retention time of ~6.8 minutes. The detection wavelength used was 463 nm. The method was calibrated for analyte concentrations of 5 - 100 µg/mL with a linear coefficient of determination, $R^2 = 0.9995$.

Loading efficiency (LE) of SY98 into the nanoparticles was calculated as follows:

$$LE(\%) = \frac{c_{\text{measured}}}{c_{\text{nominal}}} \left( \frac{V_{\text{initial}}}{V_{\text{measured}}} \right) \times 100\%$$

(B.1)

Here, $c_{\text{measured}}$ is the measured concentration through HPLC, $c_{\text{nominal}}$ is the nominal concentration expected if all the solute from the organic phase is recovered, $V_{\text{initial}}$ is the initial volume of dispersion loaded into the dialysis tubing, and $V_{\text{measured}}$ is the volume recovered from the dialysis tubing once dialyzed.
B.5 CHARACTERIZATION

Solvent yellow 98 is a fluorophore with the chemical structure displayed in Figure B.1a. The absorption (excitation) and emission spectra are presented in Figure B.1b, which shows the maximum absorbance at roughly 450 nm.

![Chemical structure of solvent yellow 98 and fluorescence spectra of SY98](image)

Figure B.1 a) Chemical structure of solvent yellow 98. b) Fluorescence spectra of SY98. This image is taken from reference [2].

Although data for various nanoparticles loaded with SY98 is presented in section 2.4.3 of Chapter 2, representative particle size distributions for two extreme cases are plotted here.

![Particle size distributions of two nanoparticle dispersions loaded with SY98](image)

Figure B.2 Particle size distributions of two nanoparticle dispersions loaded with SY98. The dispersions had different fractions of THF in the dispersion solvent mixture (5 or 20%) and were produced at different total inlet Reynolds numbers on the MIVM. The SY98 loading efficiencies were 95 and 56%, respectively for the 20 and 5% THF dispersions.
B.6 NOMENCLATURE

SY98          solvent yellow 98
THF           tetrahydrofuran
MeOH          methanol
HPLC          high pressure liquid chromatography
LE            loading efficiency
$\Delta$       span range ($\Delta = d_{90} - d_{10}$)
$d_{10}$      10% of the intensity-weighted size distribution is smaller than this diameter
$d_{90}$      90% of the intensity-weighted size distribution is smaller than this diameter
$R^2$         linear coefficient of determination
$c_i$         concentration of $i$
$V_i$         volume of $i$

B.7 REFERENCE

APPENDIX C - REPORTING PARTICLE SIZE DISTRIBUTIONS

The goal of this appendix is to provide rationale for the method of reporting particle size distribution data in this dissertation, since the protocol adopted here has not been used in previous literature. All particle sizes measured and reported in this dissertation were obtained from a Malvern Zetasizer, which uses dynamic light scattering (DLS) to obtain size data. Details on the theory and analysis of DLS can be found elsewhere [1-4]. However, it is important to understand that the instrument measures an intensity autocorrelation function (ACF) from light scattering of particles from the sample. This autocorrelation function is more easily expressed as the normalized electric field, or first order, ACF, $g_1(\tau)$, where $\tau$ is the delay time between measurements. Depending on the nature of the sample being measured, whether monodisperse or heterogeneous, the autocorrelation function is expressed differently.

\begin{equation}
\label{eq:C.1}
   g_1(\tau) = G_0 e^{-\Gamma \tau}
\end{equation}

\begin{equation}
\label{eq:C.2}
   g_1(\tau) = \sum_{i=1}^{m} G(\Gamma_i) e^{-\Gamma_i \tau} = \int_{0}^{\infty} G(\Gamma) e^{-\Gamma \tau} d\Gamma
\end{equation}

Equation C.1 is used for monodisperse systems, where the decay of the ACF versus delay time between measurements can be expressed by a single exponential function with intercept $G_0$ and a decay rate $\Gamma$. For systems that are polydisperse, the normalized first order ACF can be expressed as a sum of multiple exponential decay functions describing each size populations or idealized to the integral of all decay functions over all sizes. The particle size is extracted from these measurements by relating the decay rate $\Gamma$ to the translational diffusion coefficient $D$ and then, through the Stokes-Einstein relation, expressing the particle diameter as a function of the diffusion coefficient.

\begin{equation}
\label{eq:C.3}
   \Gamma = q^2 D
\end{equation}
In equation C.3, \( q \) is the magnitude of the scattering wave vector as defined in equation C.4, where \( n \) is the refractive index of the medium, \( \lambda \) is the incident laser wavelength, and \( \theta \) is the scattering angle or the angle at which the detector is located with respect to the sample cell. In equation C.5, \( k_B \) is Boltzmann's constant, \( \eta \) is the viscosity of the medium, and \( d \) is the hydrodynamic diameter of the particle.

In practice, the Zetasizer makes use of two different analysis modes, such that one can discern the polydispersity of the sample. One of the most commonly used methods is the cumulants analysis [3], which fits the ACF to an average exponential decay function through a Taylor expansion as shown below.

\[
\ln \left[ g_1(\tau) \right] = -\overline{\Gamma} \tau + \frac{\mu_2}{2!} \tau^2 - \frac{\mu_3}{3!} \tau^3 + \ldots
\]  

(C.6)

Here, the first cumulant is an average decay rate, from which the \( z \)-average hydrodynamic diameter, \( <d_z> \), is derived.

\[
\frac{\overline{\Gamma}(q)}{q^2} = \frac{\sum_{i=1}^{\max} I_i D_i}{\sum_{i=1}^{\max} I_i} = \frac{\sum_{i=1}^{\max} K_i N_i M_i^2 P_i B_i D_i}{\sum_{i=1}^{\max} K_i N_i M_i^2 P_i B_i D_i} = \frac{\sum_{i=1}^{\max} N_i M_i^2 D_i}{\sum_{i=1}^{\max} N_i M_i^2} \equiv \langle D_z \rangle 
\]  

(C.7)

\[
\frac{1}{\langle d_z \rangle} \propto \langle D_z \rangle = \frac{\sum_{i=1}^{\max} N_i d_i^6}{\sum_{i=1}^{\max} N_i d_i^5}
\]  

(C.8)

In equation C.7, \( I \) is the scattered intensity magnitude, which is a function of an optical constant \( K \), the number of scattering particles \( N \), the mass of the scattering particles \( M \), the particle form...
factor $P$, and the concentration factor $B$. Here, it is assumed that the sample is homogeneous, such that the optical constant is equal for all particles. Also, the particle form factor is taken as 1 (assuming particles are much smaller than the incident laser wavelength or for measurements extrapolated to a zero scattering angle) and it is assumed that the measurement is done at, or extrapolated to, zero concentration. Since the mass of a spherical particle is proportional to $d^3$, the $z$-average hydrodynamic diameter can be derived from the average diffusion coefficient. Likewise, the second cumulant, or the second order coefficient, is used to determine the polydispersity index, $Q$.

\[ Q = \frac{\mu_2}{\bar{1}^2} \]  

(C.9)

The third cumulant may be used when samples are of high polydispersity, but it is not commonly used. When using this method, care must be to taken so that higher-order terms are negligible and delay times must be short for the Taylor expansion to be valid. While the cumulants method is highly regarded because of its relative robustness with respect to experimental noise, it has been seen from experience that this method is limited when samples have multimodal distributions. Through use of the Seigert relation, equation C.6 can be expressed in terms of the normalized second order ACF, where $\beta$ is a constant [5].

\[ \ln \left[ g_2(\tau) - 1 \right] = \ln \frac{\beta}{2} - 1 \tau + \frac{\mu_2}{2!} \tau^2 - \frac{\mu_3}{3!} \tau^3 + \ldots \]  

(C.10)

In this form, the ACF is taken to have a baseline of 1, which can be problematic experimentally. There are many cases where the right tail end of the ACF is noisy due to larger aggregates in the sample; in these cases, the baseline correction is biased and many times the reported polydispersity index is quite high even though the sample is not truly multimodal. Two examples are shown below in Figure C.1 taken from data presented in the dissertation.
Figure C.1 Normalized first order autocorrelation functions for two nanoparticle dispersions produced through FNP. The data is extracted from the Malvern Zetasizer software.

For these examples, there is an obvious tail before final decay to zero. The tails result in polydispersity indices that are relatively high as seen in Table C.1.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Z-Average (nm)</th>
<th>Polydispersity Index</th>
<th>Peak Mean Intensity Diameters (nm)</th>
<th>d10 (nm)</th>
<th>d50 (nm)</th>
<th>d90 (nm)</th>
<th>Span Range (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>143</td>
<td>0.273</td>
<td>156, 4996</td>
<td>79</td>
<td>140</td>
<td>284</td>
<td>205</td>
</tr>
<tr>
<td>B</td>
<td>36</td>
<td>0.189</td>
<td>33, 5041</td>
<td>23</td>
<td>32</td>
<td>47</td>
<td>24</td>
</tr>
</tbody>
</table>

Generally, a polydispersity index > 0.15 is said to not be representative of tight distributions. Furthermore, for the work presented in this dissertation, it is common for nanoparticle dispersions to have the presence of a second minor size population due to surfactant micelles, such as D-\(\alpha\)-tocopheryl poly(ethylene glycol) 1000 succinate (Chapter 5) or Pluronic/Lutrol F68 (Chapters 3, 4, 6). The polydispersity index would not be useful for such cases and thus, the cumulants method is not an ideal analysis technique for the DLS data.
The Zetasizer also analyzes size data through a deconvolution algorithm using a non-negative least squares fitting. This "distribution analysis" is what the software uses to report the distribution plot for each measurement. The software offers three resolution modes for the distribution analysis: general purpose (normal resolution), narrow multiple modes (high resolution), and protein analysis. The three resolution modes affect the regularization used in the CONTIN algorithm used by the software, which is essentially a measure of the noise in the ACF or the smoothness of the size distribution [6, 7]. While this method can also be prone to the noise in the ACF, as was discussed for the cumulants analysis, it is easier to interpret the spread of the size distribution through this analysis, especially for samples that have multimodal distributions.

In this dissertation, the adopted protocol of reporting particle size distributions is to give the following metrics, which are derived the general purpose distribution analysis: the peak mean intensity diameters (including large micron-sized populations) as a measure of center and the span range as a measure of spread. The span range, \( \Delta \), (equation C.11) is defined as the difference between the \( d_{90} \) and the \( d_{10} \), which are the diameters at which 90% or 10%, respectively, of the intensity-weighted size distribution are smaller or equal. This measure of spread is more biased than the traditional span, as defined below, because for the purposes of the dissertation, it is important to compare absolute size differences instead of normalized differences. The span can still be estimated for symmetric distribution peaks by taking the ratio of the span range to the main peak mean size; the error in assuming that the main peak mean size and the \( d_{50} \) are equal will be roughly \( \leq 10\% \) if there is \( \leq 10\% \) error between the two measures of center.

\[
\Delta = d_{90} - d_{10} \tag{C.11}
\]

\[
\text{Span} = \frac{d_{90} - d_{10}}{d_{50}} \tag{C.12}
\]
Furthermore, in appropriate cases, the plotted size distributions are presented, such that all metrics are consistent with one analysis mode. Previous work has mixed and matched metrics and distributions from both analysis modes, making interpretation and comparisons difficult. Furthermore, for distributions with two distinct populations of comparable intensity, it will be noted that the span range will be broad since it will cover the sizes of both populations; thus, a large span range is easily used to diagnose undesirable broad or multimodal distributions, which has usually been done through the use of the polydispersity index.

Finally, in order to compare the two analysis modes, a few examples are presented below, which give not only the metrics discussed from the two modes, but also the respective distributions. As Thomas has shown, a number-weighted log-normal distribution can be obtained from the z-average and polydispersity index derived from the cumulants analysis [8].

\[ f(d) = \frac{1}{d\sigma_g\sqrt{2\pi}} \exp \left[ -\frac{(\ln d - \alpha)^2}{2\sigma_g^2} \right] \]  
\[ \sigma_g^2 = \ln(1 + Q) \]  
\[ \alpha = \ln \left( \frac{\bar{d}_{ul}}{(1+Q)^5} \right) - 0.5 \ln(1 + Q) \]  

The probability density function for the intensity-weighted distribution is shown below.

\[ f(d) = \frac{d^5}{\sigma_g\sqrt{2\pi}} \exp \left[ -\frac{(\ln d - \alpha)^2}{2\sigma_g^2} \right] \]  
\[ \int_0^\infty \frac{x^5}{\sigma_g\sqrt{2\pi}} \exp \left[ -\frac{(\ln x - \alpha)^2}{2\sigma_g^2} \right] dx \]  

From the examples shown below in Table C.2 and Figure C.2, it is clear that the distribution analysis metrics provide a better representation for the nanoparticle dispersions as compared to the cumulants analysis metrics.
Table C.2 Particle size distribution metrics for four example nanoparticle dispersions comparing metrics derived from the cumulants analysis to those obtained through general purpose distribution analysis.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Z-Average (nm)</th>
<th>Polydispersity Index</th>
<th>Peak Mean Intensity Diameters (nm)</th>
<th>Span Range (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>22</td>
<td>0.397</td>
<td>17, 186</td>
<td>195</td>
</tr>
<tr>
<td>D</td>
<td>33</td>
<td>0.201</td>
<td>36, 4700</td>
<td>40</td>
</tr>
<tr>
<td>E</td>
<td>92</td>
<td>0.222</td>
<td>119</td>
<td>123</td>
</tr>
<tr>
<td>F</td>
<td>142</td>
<td>0.117</td>
<td>162</td>
<td>141</td>
</tr>
</tbody>
</table>

Figure C.2 Particle size distribution for the four example nanoparticle dispersions from Table C.2. Cum = cumulants analysis, Dist = distribution analysis.

For example, the log-normal distribution fails to capture the bimodal nature of trial C. While the log-normal distributions of the other trial are close to those generated from the distribution analysis, the polydispersity indices are quite high and indicative of broad distributions. However, when examining the span ranges of trials D - F, it is shown that all trials had tight distributions. It has been observed that when a large micron-sized peak is reported, the polydispersity index...
tends to be high. This is the case for trial D. Overall, the superior representation by the
distribution analysis metrics seen here was also seen for all work presented in this dissertation.

C.1 NOMENCLATURE

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>ACF</td>
<td>autocorrelation function</td>
</tr>
<tr>
<td>(g_1)</td>
<td>normalized first order (electric field) autocorrelation function</td>
</tr>
<tr>
<td>(\tau)</td>
<td>delay time between measurements</td>
</tr>
<tr>
<td>(G_0)</td>
<td>y-intercept for monodisperse expression describing (g_1)</td>
</tr>
<tr>
<td>(\Gamma)</td>
<td>decay rate for (g_1)</td>
</tr>
<tr>
<td>(D)</td>
<td>translation diffusion coefficient</td>
</tr>
<tr>
<td>(q)</td>
<td>magnitude of a scattering wave vector</td>
</tr>
<tr>
<td>(n)</td>
<td>refractive index of a medium</td>
</tr>
<tr>
<td>(\lambda)</td>
<td>incident laser wavelength</td>
</tr>
<tr>
<td>(\theta)</td>
<td>scattering angle</td>
</tr>
<tr>
<td>(k_B)</td>
<td>Boltzmann's constant</td>
</tr>
<tr>
<td>(\eta)</td>
<td>viscosity of a medium</td>
</tr>
<tr>
<td>(d)</td>
<td>hydrodynamic diameter of a particle</td>
</tr>
<tr>
<td>(\mu_2)</td>
<td>second cumulant</td>
</tr>
<tr>
<td>(\mu_3)</td>
<td>third cumulant</td>
</tr>
<tr>
<td>(I)</td>
<td>scattered intensity magnitude</td>
</tr>
<tr>
<td>(K)</td>
<td>an optical constant</td>
</tr>
<tr>
<td>(N)</td>
<td>number of scattering particles</td>
</tr>
</tbody>
</table>
APPENDIX C  

Reporting Particle Size Distributions

\( M \)  
mass of scattering particles

\( P \)  
particle form factor

\( B \)  
concentration factor

\( d_z \)  
z-average hydrodynamic diameter

\( Q \)  
polydispersity index

\( g_2 \)  
normalized second order (intensity) autocorrelation function

\( \beta \)  
a constant

\( \Delta \)  
span range (\( \Delta = d_{90} - d_{10} \))

\( dN \)  
N% of the intensity-weighted size distribution is smaller than this diameter

\( \sigma_g \)  
standard deviation of a random variable's natural logarithm

\( \alpha \)  
mean of a random variable's natural logarithm

C.2 REFERENCES

APPENDIX D - STATISTICS OF RATIOS

For the analysis performed in this dissertation for reconstitution of lyophilized nanoparticle dispersions, ratios are taken of measurements that consist of an average value with a standard deviation. The ratios are reported in this same manner. Thus, this appendix presents the procedure for properly calculating such statistics.

According to Navidi [1], the propagation of error expression for a nonlinear function $U$ of independent random variables $X_1, X_2, \ldots, X_n$ can be derived through a multivariate linearization. The first-order Taylor series approximation of $U(X_1, X_2, \ldots, X_n)$ around the point $(y_1, y_2, \ldots, y_n)$ with existing partial derivatives is given below.

$$U(X_1, X_2, \ldots, X_n) \approx U(y_1, y_2, \ldots, y_n) + \frac{\partial U}{\partial X_1}(X_1 - y_1) + \frac{\partial U}{\partial X_2}(X_2 - y_2) + \ldots + \frac{\partial U}{\partial X_n}(X_n - y_n) \tag{D.1}$$

Here, each partial derivative is evaluated at the point $(y_1, y_2, \ldots, y_n)$. Equation D.1 can then be rewritten as shown below.

$$U(X_1, X_2, \ldots, X_n) \approx \left[ U(y_1, y_2, \ldots, y_n) - \frac{\partial U}{\partial X_1} y_1 - \frac{\partial U}{\partial X_2} y_2 - \ldots - \frac{\partial U}{\partial X_n} y_n \right]$$

$$+ \left( \frac{\partial U}{\partial X_1} X_1 + \frac{\partial U}{\partial X_2} X_2 + \ldots + \frac{\partial U}{\partial X_n} X_n \right) \tag{D.2}$$

The first term of equation D.2 is a constant and thus does not contribute to the standard deviation of the ratio.

For a linear function of random variable $X$ such as $H(aX + b)$, the mean, $\mu$, and variance, $\sigma^2$, are shown.

$$\mu_H = a \mu_X + b \tag{D.3}$$

$$\sigma_H^2 = a^2 \sigma_X^2 \tag{D.4}$$
Furthermore, for a linear function of independent random variables \(X_1, \ldots, X_n\) such as \(K(c_1X_1 + \ldots + c_nX_n + b)\), the mean and variance are shown.

\[
\mu_K = c_1\mu_{X_1} + \ldots + c_n\mu_{X_n} + b \quad \text{(D.5)}
\]

\[
\sigma_K^2 = c_1^2\sigma_{X_1}^2 + \ldots + c_n^2\sigma_{X_n}^2 \quad \text{(D.6)}
\]

Finally, the mean and standard deviation of the nonlinear function \(U\) from equation D.2 can be ascertained by considering only the second term.

\[
\sigma_U \approx \sqrt{\left(\frac{\partial U}{\partial X_1}\right)^2 \sigma_{X_1}^2 + \left(\frac{\partial U}{\partial X_2}\right)^2 \sigma_{X_2}^2 + \ldots + \left(\frac{\partial U}{\partial X_n}\right)^2 \sigma_{X_n}^2} \quad \text{(D.7)}
\]

Therefore, equation D.7 is applicable for ratios such as \(S_f/S_i\), the ratio of the reconstituted peak mean intensity diameter of the main population to that of the initial dispersion prior to freeze-drying; \(\Delta f/\Delta i\), the ratio of span ranges after reconstitution to prior freeze-drying; or API recovery, the percent of the measured API prior to freeze-drying of the dispersion that was measured after reconstitution and filtration. The mean and standard deviation for the peak mean size ratio are presented below as an example.

\[
\mu_{S_f/S_i} = \frac{\mu_{S_f}}{\mu_{S_i}} \quad \text{(D.8)}
\]

\[
\sigma_{S_f/S_i} \approx \sqrt{\left(\frac{1}{S_i}\right)^2 \sigma_{S_f}^2 + \left(\frac{S_f}{S_i^2}\right)^2 \sigma_{S_i}^2} = \sqrt{\left(\frac{1}{S_i}\sigma_{S_f}\right)^2 + \left(\frac{S_f}{S_i^2}\sigma_{S_i}\right)^2} \quad \text{(D.9)}
\]

### D.1 NOMENCLATURE

- **U, H, K**: functions of random variables
- **\(X_i\)**: a random variable
- **\(y_i\)**: a value of a random variable
\( \mu_i \) mean of a random variable \( i \)

\( \sigma_i^2 \) variance of a random variable \( i \)

\( \sigma_i \) standard deviation of a random variable \( i \)

\( S_j \) peak mean size of dispersion \( (f) \) after reconstitution or \( (i) \) prior to freeze-drying

\( \Delta_j \) span range of dispersion \( (f) \) after reconstitution or \( (i) \) prior to freeze-drying

**D.2 REFERENCE**

APPENDIX E - CALCULATING INTERACTION PARAMETERS

The concept of an interaction parameter is derived from Flory-Huggins theory, which describe the thermodynamics of polymer solutions. A full explanation of the theory can be found elsewhere [1, 2]. The equation describing the Gibbs free energy change from the mixing of polymer solutions is given below, in which the chi interaction parameter is classically encountered.

\[ \Delta G_m = RT \left[ n_A \ln \phi_A + n_B \ln \phi_B + n_A \phi_B \chi_{AB} \right] \]  
(E.1)

Here, \( R \) is the universal gas constant, \( T \) is absolute temperature, \( \phi_i \) is the volume fraction of species \( i \), \( n_i \) is the moles of the species \( i \), and \( \chi_{ij} \) is the interaction parameter. The term containing the interaction parameter is an enthalpic term that accounts for the ease with which the two species will mix; a chi value closer to zero indicates higher affinity between the two species. The interaction parameter is classically defined as follows, where \( \delta_i \) are the Hildebrand solubility parameters of the species and \( v_A \) is the molar volume of A.

\[ \chi_{AB} = \frac{v_A}{RT} \left( \delta_A - \delta_B \right)^2 \]  
(E.2)

The Hildebrand solubility parameter is a measure of the cohesive energy density of a material, as described below, where \( \Delta H_v \) is the heat of vaporization.

\[ \delta_A = \sqrt{\frac{\Delta H_v - RT}{v_A}} \]  
(E.3)

However, this solubility parameter is typically only useful for non-polar substances with no or poor hydrogen bonding. The Hansen solubility parameters are applicable to a larger set of compounds, as three parameters account for dispersion forces (\( \delta_d \)), dipolar interaction (\( \delta_p \)), and hydrogen bonding (\( \delta_h \)). For Hansen solubility parameters, it is common practice to consider
affinity or miscibility of two species by calculating the distance, $r_{ij}$, between the two points determined in a 3D space defined by the individual parameter components.

$$r_{ij} = \sqrt{a(\delta_{d,A} - \delta_{d,B})^2 + b(\delta_{p,A} - \delta_{p,B})^2 + b(\delta_{h,A} - \delta_{h,B})^2}$$ \hspace{1cm} (E.4)

Here, $a$ and $b$ are empirical constants. Typically in the literature, $a = 4$ and $b = 1$ are used which results in a more convenient comparisons [3]. The distance $r_{ij}$ is then compared to a known distance $r_0$ that defines the solubility parameter space for solvents that can dissolve the solute.

In practice, solubility parameters, especially the Hansen parameters, are difficult to measure experimentally and thus it is rare to find literature values for various compounds. In order to have some idea on affinity or miscibility of two species, it is simpler to use group contribution methods to estimate solubility and/or interaction parameters. A common way to estimate Hansen solubility parameters is to use the method of Hoftyzer and van Krevelen, which makes use of the following expressions [4].

$$\delta_d = \frac{\sum F_{d,i}}{v} \hspace{1cm} (E.5)$$

$$\delta_p = \frac{\sqrt{\sum F_{p,i}^2}}{v} \hspace{1cm} (E.6)$$

$$\delta_h = \frac{\sqrt{\sum E_{h,i}}}{v} \hspace{1cm} (E.7)$$

In these expressions, $v$ is the molar volume as calculated from group contributions, $F_{i,j}$ is the molar attraction constant for group $i$ through dispersion ($d$) or dipolar interaction ($p$), and $E_{h,i}$ is the hydrogen bonding energy of group $i$. The Hansen solubility parameters can be collapsed into a 1D parameter, similar to the Hildebrand solubility parameter through the equation below.

$$\delta_{tot} = \sqrt{\delta_d^2 + \delta_p^2 + \delta_h^2} \hspace{1cm} (E.8)$$
For the purposes of this dissertation, inferences regarding affinity between two species is considered through the calculation of the 3D distance using equation E.4 with $a = b = 1$ or through collapsing the solubility parameters as in equation E.8 and comparing the 1D distance obtained from $|\delta_A - \delta_B|$.

Table E.1 lists the volume, molar attraction constants, and the hydrogen bonding energies for various groups; values are taken from Barton [4] and Breitkreutz [5].

<table>
<thead>
<tr>
<th>Group</th>
<th>V (cm$^3$ mol$^{-1}$)</th>
<th>$F_d$ (J$^{1/2}$ cm$^{32}$ mol$^{-1}$)</th>
<th>$F_p$ (J$^{1/2}$ cm$^{32}$ mol$^{-1}$)</th>
<th>$E_h$ (J mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CH3</td>
<td>33.5</td>
<td>420</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-CH2-</td>
<td>16.1</td>
<td>270</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>--CH-</td>
<td>-1</td>
<td>80</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>=CH-</td>
<td>13.5</td>
<td>200</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-C- -</td>
<td>-19.2</td>
<td>-70</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>=C- -</td>
<td>-5.5</td>
<td>70</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>phenyl</td>
<td>71.4</td>
<td>1430</td>
<td>110</td>
<td>0</td>
</tr>
<tr>
<td>phenylene</td>
<td>52.4</td>
<td>1270</td>
<td>110</td>
<td>0</td>
</tr>
<tr>
<td>-O- (not at adjacent C-atoms)</td>
<td>3.8</td>
<td>100</td>
<td>400</td>
<td>3000</td>
</tr>
<tr>
<td>-O- (at adjacent C-atoms)</td>
<td>4.5</td>
<td>120</td>
<td>530</td>
<td>3500</td>
</tr>
<tr>
<td>-OH (not at adjacent C-atoms)</td>
<td>10</td>
<td>210</td>
<td>522</td>
<td>22000</td>
</tr>
<tr>
<td>-OH (at adjacent C-atoms)</td>
<td>13</td>
<td>210</td>
<td>450</td>
<td>20000</td>
</tr>
<tr>
<td>-CO-</td>
<td>10.8</td>
<td>290</td>
<td>770</td>
<td>2000</td>
</tr>
<tr>
<td>-COO-</td>
<td>18</td>
<td>390</td>
<td>490</td>
<td>7000</td>
</tr>
<tr>
<td>HCOO-</td>
<td>32.5</td>
<td>530</td>
<td>420</td>
<td>10000</td>
</tr>
<tr>
<td>HCO-</td>
<td>22.8</td>
<td>400</td>
<td>800</td>
<td>5200</td>
</tr>
<tr>
<td>conjugated double bonds</td>
<td>-2.2</td>
<td>50</td>
<td>37</td>
<td>0</td>
</tr>
<tr>
<td>5(or more)-membered rings</td>
<td>16</td>
<td>190</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-NH2</td>
<td>17.5</td>
<td>300</td>
<td>440</td>
<td>8600</td>
</tr>
<tr>
<td>-NH-</td>
<td>4.5</td>
<td>160</td>
<td>210</td>
<td>3100</td>
</tr>
<tr>
<td>-N- -</td>
<td>-9</td>
<td>20</td>
<td>800</td>
<td>5000</td>
</tr>
<tr>
<td>-C==-N</td>
<td>23.1</td>
<td>430</td>
<td>1100</td>
<td>2500</td>
</tr>
<tr>
<td>=N-</td>
<td>5</td>
<td>20</td>
<td>800</td>
<td>5000</td>
</tr>
<tr>
<td>-S-</td>
<td>12</td>
<td>440</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-SO-</td>
<td>4.3</td>
<td>472</td>
<td>1170</td>
<td>7418</td>
</tr>
<tr>
<td>-SO2-</td>
<td>51</td>
<td>1129</td>
<td>1358</td>
<td>11670</td>
</tr>
<tr>
<td>-Cl</td>
<td>24</td>
<td>450</td>
<td>550</td>
<td>400</td>
</tr>
</tbody>
</table>
### Table E.2 Calculated Hansen solubility parameters for various compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>V (cm$^3$ mol$^{-1}$)</th>
<th>$\delta_d$ (MPa$^{1/2}$)</th>
<th>$\delta_p$ (MPa$^{1/2}$)</th>
<th>$\delta_h$ (MPa$^{1/2}$)</th>
<th>$\delta_{tot}$ (MPa$^{1/2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetrahydrofuran</td>
<td>84.9</td>
<td>16.4</td>
<td>6.2</td>
<td>6.4</td>
<td>18.7</td>
</tr>
<tr>
<td>Dimethylformamide</td>
<td>80.8</td>
<td>15.6</td>
<td>14.0</td>
<td>11.2</td>
<td>23.8</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>71.3</td>
<td>18.4</td>
<td>16.4</td>
<td>10.2</td>
<td>26.7</td>
</tr>
<tr>
<td>Acetone</td>
<td>77.8</td>
<td>14.5</td>
<td>9.9</td>
<td>5.1</td>
<td>18.3</td>
</tr>
<tr>
<td>Ethanol</td>
<td>59.6</td>
<td>15.1</td>
<td>8.7</td>
<td>19.2</td>
<td>25.9</td>
</tr>
<tr>
<td>Poly(ethylene oxide) 350</td>
<td>303.4</td>
<td>17.3</td>
<td>4.9</td>
<td>12.1</td>
<td>21.7</td>
</tr>
<tr>
<td>Poly(ethylene oxide) 600</td>
<td>523.6</td>
<td>17.6</td>
<td>3.7</td>
<td>11.2</td>
<td>21.2</td>
</tr>
<tr>
<td>Poly(ethylene oxide) 5kD</td>
<td>4193.6</td>
<td>17.9</td>
<td>1.3</td>
<td>10.0</td>
<td>20.6</td>
</tr>
<tr>
<td>Poly(ε-caprolactone) 2kD</td>
<td>1790.4</td>
<td>17.6</td>
<td>1.2</td>
<td>8.4</td>
<td>19.5</td>
</tr>
<tr>
<td>Poly(ε-caprolactone) 5kD</td>
<td>4351.4</td>
<td>17.6</td>
<td>0.7</td>
<td>8.4</td>
<td>19.5</td>
</tr>
<tr>
<td>Poly(ε-caprolactone) 7kD</td>
<td>6025.9</td>
<td>17.6</td>
<td>0.6</td>
<td>8.4</td>
<td>19.6</td>
</tr>
<tr>
<td>Poly(ε-caprolactone) 10kD</td>
<td>8685.4</td>
<td>17.6</td>
<td>0.5</td>
<td>8.4</td>
<td>19.6</td>
</tr>
<tr>
<td>Polylactide 3.7kD</td>
<td>2643.1</td>
<td>17.6</td>
<td>1.3</td>
<td>11.7</td>
<td>21.2</td>
</tr>
<tr>
<td>Polylactide 10kD</td>
<td>7036.6</td>
<td>17.6</td>
<td>0.8</td>
<td>11.8</td>
<td>21.2</td>
</tr>
<tr>
<td>Polystyrene 1.6kD</td>
<td>1314.6</td>
<td>20.5</td>
<td>0.3</td>
<td>0.0</td>
<td>20.5</td>
</tr>
<tr>
<td>Polystyrene 3.8kD</td>
<td>3131.1</td>
<td>20.5</td>
<td>0.2</td>
<td>0.0</td>
<td>20.5</td>
</tr>
<tr>
<td>PPG-3 myristyl ether</td>
<td>308.6</td>
<td>16.4</td>
<td>2.3</td>
<td>8.7</td>
<td>18.7</td>
</tr>
<tr>
<td>Ethyl oleate</td>
<td>353.5</td>
<td>16.1</td>
<td>1.4</td>
<td>4.4</td>
<td>16.7</td>
</tr>
<tr>
<td>PPG-11 stearil ether</td>
<td>373.3</td>
<td>16.4</td>
<td>1.9</td>
<td>7.9</td>
<td>18.3</td>
</tr>
<tr>
<td>Caprylic triglyceride</td>
<td>475.5</td>
<td>16.6</td>
<td>0.9</td>
<td>6.6</td>
<td>17.9</td>
</tr>
<tr>
<td>Capric triglyceride</td>
<td>572.1</td>
<td>16.7</td>
<td>0.7</td>
<td>6.1</td>
<td>17.7</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>432.8</td>
<td>17.6</td>
<td>1.6</td>
<td>7.4</td>
<td>19.2</td>
</tr>
<tr>
<td>α-tocopherol succinate</td>
<td>502.5</td>
<td>17.7</td>
<td>1.7</td>
<td>6.4</td>
<td>18.9</td>
</tr>
<tr>
<td>α-tocopherol polyethylene glycol 1000 succinate</td>
<td>1328.9</td>
<td>17.8</td>
<td>2.0</td>
<td>8.4</td>
<td>19.8</td>
</tr>
<tr>
<td>Naproxen</td>
<td>183.0</td>
<td>18.7</td>
<td>3.7</td>
<td>8.6</td>
<td>20.9</td>
</tr>
</tbody>
</table>
Table E.2 lists the molar volumes and Hansen solubility parameters for various materials used in this dissertation.
E.1 NOMENCLATURE

\( \Delta G_m \) Gibbs free energy change of mixing

\( R \) universal gas constant

\( T \) absolute temperature

\( \phi_i \) volume fraction of species \( i \)

\( n_i \) moles of the species \( i \)

\( \chi_{ij} \) Flory-Huggins interaction parameter for a solute \( (i) \) in a solvent \( (j) \)

\( \delta_i \) Hildebrand solubility parameters of species \( i \)

\( v_i \) molar volume of \( i \)

\( \Delta H_v \) heat of vaporization

\( r_{ij}, r_0 \) distance between the Hansen solubility parameters of two materials \( (i,j) \) as defined in a 3D space

\( a, b \) constants

\( \delta_d \) Hansen solubility parameter accounting for dispersion forces

\( \delta_p \) Hansen solubility parameter accounting for dipolar interaction

\( \delta_h \) Hansen solubility parameter accounting for hydrogen bonding

\( F_{i,d}, F_{i,p} \) molar attraction constant for group \( i \) through dispersion \( (d) \) or dipolar interaction \( (p) \)

\( E_{h,i} \) hydrogen bonding energy of group \( i \)

\( \delta_{\text{tot}} \) 1D collapsed Hansen solubility parameter

E.2 REFERENCES


APPENDIX F - SYNTHESIS OF HYDROPHOBIC STEROID PRODRUGS

As discussed in Chapter 2, one of the methods for enhancing the loading efficiency and active ingredient loading of a compound in nanoparticles produced through Flash NanoPrecipitation (FNP) is to produce hydrophobic derivatives. By attaching cleavable moieties that decrease the aqueous solubility and interfere with recrystallization of the parent compound, it is possible to produce nanoparticles loaded with the compound of interest when previously it was not possible. However, another advantage of producing a hydrophobic prodrug is that the release profile can be slowed down; as Ansell et al demonstrated for paclitaxel prodrugs, the circulation half-life of the chemotherapeutic was extended to 24 hours, which was not possible with the parent drug [1].

This appendix presents the synthesis of three steroid prodrugs, two produced using the reaction scheme of Ansell, which were characterized for hydrolysis half-lives, following the work of Dr. Margarita Herrera for estradiol prodrugs (unpublished). Here, the two steroids, estradiol and prednisone, were chosen because they cannot form satisfactory nanoparticle dispersions through FNP without a co-solute and because they have hydroxyl groups that can be used as a point for addition of hydrophobic moieties. While FNP of these compounds was not performed, the results suggest that similar prodrugs produced by Ansell most likely were cleaved by enzymatic routes and not purely by pH-triggered hydrolysis.

F.1 MATERIALS & METHODS

F.1.1 Materials

All solvents used in synthesis were of ACS grade or higher and were purchased from Fisher Scientific (USA). Chloroform-d was purchased from Cambridge Isotope Laboratories.
APPENDIX F

Synthesis of Hydrophobic Steroid Prodrugs

(USA). Estradiol (≥ 98%), prednisone (≥ 98%), 1-eicosanol (98%), 1-docosanol (98%), dodecyl vinyl ether (98%), N,N'-diisopropylcarbodiimide (99%), 4-(dimethylamino)pyridine (> 99%), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (≥ 99%), pyridinium p-toluenesulfonate (95%), triethylamine (≥ 99.5%) were purchased from Sigma-Aldrich (USA). Hydrochloric acid (1.0 N) was purchased from Alfa Aeser (USA). Chromatographic silica gel (60 A, 40-63 µm) was purchased from Sorbent Technologies (USA). Ultrapure water (MilliQ water) (18 MΩ·cm) was generated from a Barnstead Nanopure purification system.

F.1.2 Prodrug Synthesis

F.1.2.1 Steroid prodrugs with diglycolic linkages

For all steps, procedures reported by Ansell et al were followed and are reported elsewhere [1], with notable deviations explained. First, cosanyl and docosanyl diglycolate were prepared from the esterification of diglycolic anhydride with 1-eicosanol and 1-docosanol, respectively. The resultant solids were dried under vacuum and characterized through $^1$H-NMR. In all cases, the product was pure. Next, esterification of the lipid anchors with either estradiol or prednisone was performed. Silica gel flash column chromatography was conducted using a hexane/ethyl acetate/chloroform gradient. The products were dried under vacuum and characterized through $^1$H-NMR.

F.1.2.2 Steroid prodrug with acetal linkage

Following the procedure reported by Herrera-Alonso (unpublished), dodecyl vinyl ether (4 equivalents), prednisone (1 equivalent), and pyridinium p-toluenesulfonate (0.14 equivalent) were dissolved in a 1:10 dichloromethane:tetrahydrofuran mixture at a 100 mg drug scale. The reaction was allowed to proceed at ambient conditions overnight. The reaction mixture was then purified through silica gel flash column chromatography using a hexane/ethyl acetate gradient. The solid product was dried under vacuum and characterized through $^1$H-NMR.
F.1.2.3 $^1$H-NMR characterization

Solid samples were dissolved in chloroform-d at a solids concentration of at least 1 mg/mL and loaded into NMR tubes. Sample spectra were obtained on a 500 MHz Bruker AVANCE III equipped with a cryoprobe and BACS-120.

F.1.3 Characterization of Hydrolysis Rates

Hydrolysis of the steroid prodrugs was characterized in 30 vol% aqueous in tetrahydrofuran mixtures at different initial aqueous pH levels. Typical experiments involved dissolving the prodrug in tetrahydrofuran at a concentration of 1.1 mg/mL and mixing this solution with an aqueous solution at a specified pH. Aliquots (0.5 mL) were prepared and sampled each hour at ambient temperature on a Thermo Fisher Finnigan Surveyor HPLC (Fisher Scientific, USA) equipped with a quaternary pump, a PDA-plus UV/visible detector, and a Gemini C18 stationary phase (5 µm, 100 Å; Phenomenex, USA). Chromatographic conditions were as follows: a linear gradient of 100% MilliQ water at 1 mL/min to 100% methanol at 0.75 mL/min over 23 minutes with the column at ambient temperature. The method run time was 40 minutes with analyte retention times of ~18 and 22 minutes for prednisone and estradiol, respectively, and retention times between 22 - 31 minutes for prodrugs. The detection wavelength used for prednisone and its prodrugs was 212 nm, while 232 nm was used for estradiol and its prodrugs. The method was calibrated for steroid concentrations of 20 - 200 µg/mL with linear coefficient of determination, $R^2 > 0.999$ for all species. Hydrolysis half-lives were estimated by fitting the decay of prodrug to an exponential decay, $exp(-kt)$, where concentrations were converted to molar fractions before fitting.
F.2 RESULTS

F.2.1 Synthesis of Steroid Prodrugs

Since the synthesis procedure reported by Ansell was used for paclitaxel, some noticeable differences were observed when using estradiol and prednisone. For example, the mobile phase gradient used in the flash column chromatography had to be considerably less polar to achieve satisfactory separation of the product from the reactants and side products (hexane/ethyl acetate/chloroform instead of dichloromethane/methanol). Most importantly, when using \( N,N' \)-diisopropylcarbodiimide as the carbodiimide, the product could not be efficiently separated from the hydrophobic urea side product. When switching over to the more hydrophilic \( N-(3\text{-dimethylaminopropyl})-N'\text{-ethylcarbodiimide hydrochloride} \), the urea side product could be removed in the workup, resulting in a pure product after flash column chromatography. When implementing these changes, the yields measured after recovering the pure product were 60% and 51%, respectively for the prednisone and estradiol cosanyl diglycolates. Previously, the synthesis of cosanyl diglycolate resulted in a quantitative yield (step 1) and thus, loss of product is experienced only in the second esterification (2a or 2b) of the two-step reaction scheme (see Figure F.1 below).

![Figure F.1 Synthesis scheme for producing estradiol and prednisone cosanyl diglycolates](image-url)
In addition, a prednisone prodrug linked to a dodecyl alkane through an acetal linkage, 1-(1-(dodecyloxy)ethoxy)prednisone, was synthesized to compare the diglycolic and acetal linkages. Once again, notable differences were observed in comparison to the parent reference. While Herrera-Alonso's synthesis was done using estradiol, when using prednisone, the product could not be isolated by dissolving in hexane, filtering, and extracting with methanol. This resulted in low recovery of the product, since it is not very soluble in hexane. Thus, flash column chromatography was necessary. Nonetheless, a similar yield as that reported by Herrera-Alonso was achieved (69% versus 72%). The scheme is shown below in Figure F.2.

![Synthesis scheme for producing 1-(1-(dodecyloxy)ethoxy)prednisone](image)

**Figure F.2** Synthesis scheme for producing 1-(1-(dodecyloxy)ethoxy)prednisone

The $^1$H-NMR spectra for the four prodrugs, with possible proton assignments, are shown below in Figures F.3-5.
Figure F.3 $^1$H-NMR spectrum of estradiol cosanyl diglycolate
Figure F.4 $^1$H-NMR spectrum of prednisone cosanyl diglycolate
Figure F.5 $^1$H-NMR spectrum of 1-(1-(dodecyloxy)ethoxy)prednisone
F.2.2 Hydrolysis Rates

It was observed for both cosanyl diglycolate prodrugs that significant hydrolysis did not occur for initial aqueous pH levels that were strongly acidic. Figure F.6 shows that hydrolysis was apparent for pH levels of 6 and higher. While the ionic strength was not kept constant in all trials, nor was the pH adjusted with the same acids/bases, it is unlikely that the result could be due solely to the ionic species used. For pH levels of 2, 4, 6, 10, 12, the pH was adjusted with HCl, HCl, nothing, NaHCO\textsubscript{3}/NaOH, NaOH. Half-lives could not calculated with accuracy due to the scatter in the data or too few data points. However, the results suggest that hydrolysis of the diglycolate linkage is preferable under neutral or basic pH.

![Figure F.6](image-url)

**Figure F.6** Hydrolysis of steroid cosanyl diglycolate prodrugs in 30 vol% aqueous in tetrahydrofuran mixtures. The pH levels indicated are those of the aqueous solutions prior to mixing with the organic solutions. PredP = prednisone prodrug, EstP = estradiol prodrug.
In comparison, the acetal linkage was acid labile. As was demonstrated by Herrera-Alonso, the comparable prodrug for estradiol resulted in a half-life of 179 minutes for 0.1 vol% water in tetrahydrofuran at a theoretical proton concentration of $10^{-2}$ M. Obviously, such prodrugs resulted in much quicker hydrolysis than the cosanyl diglycolate ones, as much less water was present. For the 1-(1-(dodecyloxy)ethoxy)prednisone, a hydrolysis half-life of 7 minutes was obtained under the same conditions as the aforementioned estradiol prodrug. The acetal linkage showed the opposite ranking as compared to the diglycolate linkage, where the estradiol prodrug hydrolyzed slower than the prednisone prodrug when using an acetal linkage. However, the estradiol prodrugs did not have the hydrophobic moieties present on the same hydroxyl group and thus, the different ranking may reflect how well the linkages are electron-stabilized.

**F.2.3 Partition Coefficients**

The calculated log$P$ values for the two parent steroids and the three steroid prodrugs reported here, as well as the estradiol prodrug synthesized by Herrera-Alonso are given below in Table F.1. Please refer to Appendix C for more information on the calculation of log$P$ values.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Formula</th>
<th>MW</th>
<th>ALOGPs</th>
<th>MiLogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisone</td>
<td>C21H26O5</td>
<td>358.47</td>
<td>2.07</td>
<td>1.41</td>
</tr>
<tr>
<td>Estradiol</td>
<td>C18H24O2</td>
<td>272.42</td>
<td>3.57</td>
<td>3.43</td>
</tr>
<tr>
<td>Prednisone cosanyl diglycolate</td>
<td>C45H70O9</td>
<td>755.15</td>
<td>7.74</td>
<td>9.45</td>
</tr>
<tr>
<td>Estradiol cosanyl diglycolate</td>
<td>C42H68O6</td>
<td>669.1</td>
<td>9.66</td>
<td>9.73</td>
</tr>
<tr>
<td>1-(1-(dodecyloxy)ethoxy)prednisone</td>
<td>C35H54O6</td>
<td>570.89</td>
<td>6.55</td>
<td>7.84</td>
</tr>
<tr>
<td>17-(1-(dodecyloxy)ethoxy)estradiol</td>
<td>C32H60O3</td>
<td>492.92</td>
<td>9.81</td>
<td>9.05</td>
</tr>
</tbody>
</table>
F.3 FUTURE WORK

It would be useful to repeat the hydrolysis experiment for the diglycolate prodrugs, adjusting pH with the same species and at the same ionic strength.

Furthermore, FNP should be attempted with all prodrugs and hydrolysis rates measured in nanoparticle form.

F.4 REFERENCES

APPENDIX G - ANIMAL RESEARCH PROTOCOL FOR PROGESTERONE

PHARMACOKINETIC STUDY

Note: The following protocol was written by Randy Howard, 1/20/12.

- **Study Title:** Pharmacokinetics of progesterone in rats after single doses of nanoparticles @ 2 mg/kg, i.v. and 10 mg/kg i.m.

- **Study Number:** PG-RPK-12-01

- **Study Date:** 1/26/12 & 1/27/12

- **Purpose:** To estimate PK parameters for progesterone after i.v. and i.m. injection of progesterone nanoparticles containing 2 mg/kg and 10 mg/kg progesterone respectively.

- **Personnel & Facility:** Randy Howard & Deborah Culver; EIDD, Emory University, Atlanta, GA. Experiment done in Emory DAR, Winship Clinic B. BCDMPK Group, EIDD, Atwood 241, Emory University, Atlanta, GA.

- **Materials:** Progesterone nanoparticle powder, (Princeton); sterile PBS; sterile saline (Hospira, NDC 0409-4888-10, lot # 87-153-DK); sterile water (Hospira, NDC 0409-4887-20, lot # 89-517-DK); 18G needles (BD); Li heparin microtainer tubes, 0.5 ml (BD #365971, lot #9328307); Eppendorf microcentrifuge tubes, 1.5ml (Costar 3620, lot #277054-T22799); isoflurane (DAR); Heparin Sodium, Porcine 10K U/ml (Abraxis Pharm Prods, 401807E, lot # 600339); 50% Dextrose Solution (Hospira, NDC 0409-6648-02, lot # 91-412-DK); 23G Luer stub adaptors (Intramedic, Fisher 14-826-19E). Also: blood centrifuge, vortex, ultrasonic bath, Gilson pipettes/tips, eppendorf test tube racks, blue pads, syringes (1, 3 & 5 ml), small curved forceps, small scissors, medium hemostat, wound clip removers, alcohol pads, beakers (100 & 250 ml), clear glass vials (~4-20 ml) for drug.

- **Animals:** 10 jugular-cannulated male SD Rats, 250-300 g (Harlan); ordered 225-249 g.

- **Experimental Design/Methods/Groups:**
  1. **Animals:** Acclimatize the rats for ≥ 2 days after receipt. Weigh rats the day before dosing to calculate dosing volumes. Check cannula patency the morning of dosing. Animals are not fasted. Leave food and water in cages during experiment.

  2. **Vehicle, Drugs & Solutions:**
   - Vehicles: On the day before dosing, make new PBS vehicle; store in refrigerator. A separate dosing vial of vehicle is not required for this experiment.
• Drugs: On the morning of the experiment, make up the dosing solutions as follows. Add 1.15 ml of PBS to each of two plastic vials containing ~11.5 mg of Progesterone each and recap. Shake each vial vigorously up and down for > 1 minute @ ~3-4 shakes/second to reconstitute @ 10 mg/ml. Combine all liquid into one vial. For Group 1 (i.v., 2 mg/kg), vortex, then transfer 0.35 ml from the plastic vial to a new 8 ml glass vial; dilute 1:10 (1 mg/ml) by adding 3.15 ml of PBS and vortexing. Then filter (5 μm) into a new 8 ml glass vial for dosing. Prepare the remainder of the initial 10 mg/ml solution (~ 2 ml) for Group 2 (i.m., 10 mg/kg) by vortexing, then filtering (5 μm) into a new 4 ml glass vial.

• Solutions: One to two days before the experiment, make up the dextrose/heparin, saline/heparin and saline flush solutions. Make 10 ml of 50% Dextrose/500 U/ml heparin solution by adding 0.5 ml of heparin stock (10K U/ml) to 9.5 ml of 50% Dextrose solution and mixing gently; store in refrigerator and dispense in a 1 ml syringe. Make 10 ml of saline/heparin (100 U/ml) by adding 0.1 ml of heparin stock (10K U/ml) to 9.9 ml of saline; store in a refrigerator and dispense in a 1 or 3 ml syringe. Dispense saline in a 3 or 5 ml syringe.

3. Dosing/Groups: Dose nanoparticle progesterone i.v. @ 2 mg/kg, 1 mg/ml & 2 ml/kg (~ 0.6 ml/rat, see Table). First remove the dextrose/heparin plug and replace with 100 μL saline. Then attach drug syringe and infuse over about 30 seconds at ~ 50 μL/3 seconds through the jugular cannula; next flush cannula with ~ 100 μL of saline to wash all drug into blood stream. Finish by filling cannula with 100 μL of dextrose/heparin solution. Sample i.v. rats (0.3 ml/sample) at 8 time points: 0.08, 0.25, 0.5, 1, 2, 4, 6 & 8 hrs. Dose nanoparticle progesterone i.m. into the left thigh of isoflurane anesthetized rats @ 10 mg/kg, 10 mg/ml & 1 ml/kg (~ 0.3 ml/rat) with a 25 G needle. Sample i.m. rats at 10 time points: 0.25, 0.5, 1, 2, 4, 6, 8, 10, 24 & 28 hrs.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>No. of Animals</th>
<th>Test Article¹</th>
<th>Dose Route</th>
<th>Dose Level (mg/kg)</th>
<th>Dose Volume (mL/kg)</th>
<th>Dose Conc. (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>Progesterone</td>
<td>i.v.</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>Progesterone</td>
<td>i.m.</td>
<td>10</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

¹Vehicle is PBS. Progesterone dosage form is a proprietary nanoparticle formulation from the lab of Dr. Robert Prud’homme at Princeton University.

4. Blood Draws: Blood will be obtained from the jugular cannula (0.3 ml) at each time point listed above (see protocol MV-RPK-10-01 for details). Replace blood
volume with 0.4 ml saline and refill cannula with dextrose/heparin lock solution after all time points.

5. Blood Processing: invert Li-Heparin tube with blood gently 3 or 4 times to mix well; then place in a rack in ice water until able to centrifuge (≤ 1 hour). As soon as practical, centrifuge blood at ~ 2000 x g for 10 min in a refrigerated centrifuge to obtain plasma. Then, using a 200 µL pipette, transfer each plasma sample to a labeled 2 ml Eppendorf tube in ice water; freeze as soon as convenient in freezer or on dry ice. Store at -80 °C in BCDMPK prior to analysis. At end of experiment euthanize rats by CO₂ inhalation.

6. Analysis: Analyze both groups for progesterone by LC/MS/MS.
APPENDIX H - ESTIMATING SURFACE PROPERTIES OF PEG-STABILIZED NANOPARTICLES

To estimate the surface properties of stabilizing poly(ethylene glycol) (PEG) layers of nanoparticles produced through Flash NanoPrecipitation, it is important to know the state of the polymer chains. PEG chains on a nanoparticle surface can adopt conformations between two extremes. If the chains are separated on the surface and non-interacting, then their size is that of a random coil in solution. This is called the “mushroom” regime [1]. If the chains are closely packed on the surface, their lateral excluded volume repulsions cause the layer to expand such that its thickness becomes larger than the dimension of a single chain in solution. This is called the “brush” regime [1]. In the brush regime, the density of the chains at the surface is determined by the balance between the stretching energy of the chains in the brush and the energy required to create the unfavorable hydrophobic interface between the aqueous phase and the hydrophobic core of the nanoparticle. The calculations of PEG densities on nanoparticle surfaces have been presented previously by Budijono [2] and Kumar [3] and are briefly summarized below.

The blob size of the PEG chains in a brush regime, \( \xi_{brush} \), is given below.

\[
\xi_{brush} = \left( \frac{4 k_B T N b^{4/3}}{\gamma_{eff} \pi} \right)^{3/10}
\]

(H.1)

In equation H.1, \( k_B \) is the Boltzmann constant, \( T \) is temperature (where \( T = 298 \text{ K} \) is assumed), \( N \) is the number of monomers in the chain, \( b \) is the monomer length, and \( \gamma_{eff} \) is the effective interfacial tension at the hydrophobic block/PEG/water interface. This calculation assumes curvature at the interface can be neglected, which Kumar showed was the case for \( O(10 \text{ nm}) \) brush layers on \( O(100 \text{ nm}) \) nanoparticles. The effective interfacial tension is expressed as a function of the blob size (equation H.2), necessitating the use of iteration to solve for either...
quantity. The interfacial tension between two surfaces (equation H.3) can be estimated by the harmonic mean of the polar ($\gamma_p$) and dispersive ($\gamma_d$) surface tension parameters of the individual materials (equation H.4) [4].

$$\gamma_{eff} = \frac{b^2}{\xi_{\text{brush}}} \gamma_{PS,PEG} + \left(1 - \frac{b^2}{\xi_{\text{brush}}} \right) \gamma_{PS,water} \quad (H.2)$$

$$\gamma_{ij} = \gamma_i + \gamma_j - 4\frac{\gamma_i^d \gamma_j^d}{\gamma_i^d + \gamma_j^d} - 4\frac{\gamma_i^p \gamma_j^p}{\gamma_i^p + \gamma_j^p} \quad (H.3)$$

$$\gamma_i = \gamma_i^d + \gamma_i^p \quad (H.4)$$

The blob size can then be used to determine the number of chains per unit surface area, which is defined as $\sigma$.

$$\sigma_i \equiv \frac{4}{\pi \xi_i^2} \quad (H.5)$$

The thickness of the brush layer, $\delta_{\text{brush}}$, is given in equation H.6.

$$\delta_{\text{brush}} = N \left(\frac{v}{b^3}\right)^{1/3} b^{5/3} \xi_{\text{brush}}^{-2/3} \quad (H.6)$$

Furthermore, as a comparison, the surface area covered by each chain in a mushroom conformation can also be estimated. As done by Auguste and coworkers [5], the blob size of the PEG chains was determined by equation H.7, where $M_{PEG}$ is the molecular weight of the PEG chain.

$$\xi_{\text{mushroom}} = 0.076 M_{PEG}^{1/2} \quad [\text{nm}] \quad (H.7)$$

From the blob size, the number of chains per surface area for the mushroom regime, $\sigma_{\text{mushroom}}$, can be calculated by using equation H.6. For the mushroom regime, the thickness of the polymer layer, $\delta_{\text{mushroom}}$, is equal to the size, $\xi_{\text{mushroom}}$. 
Now to compare to the general theory, an iterative method making use of the experimental data was used to estimate experimental surface properties. For these calculations, the primary peak mean size reported by the Malvern Zetasizer was used, which is in effect the size of the nanoparticle cores plus the thickness of the hydrated PEG layers [6, 7]. Using a mean size is an oversimplification since the nanoparticles exhibit a distribution of sizes. However, attempting to estimate the distribution of surface properties over the size distribution of the nanoparticles would be an embellishment that would imply more precision than the scaling theory or the size measurements warrant. Therefore, the procedure presented assumes that the nanoparticle dispersion size distribution can be represented by the primary peak mean size.

As a starting estimate for the thickness of the PEG layer, both the theoretical mushroom and the brush layer thicknesses were evaluated. Regardless of which conformation is assumed for the chain length, the layer thickness converges to the same value. The diameter of a nanoparticle core was calculated by subtracting twice the assumed PEG layer thickness from the peak mean diameter; using this diameter, the volume of a single core was estimated. Based on the measured mass of core solute and the hydrophobic block from the block copolymer, the total core volume was estimated by assuming a density equal to that of water. While this estimate could be refined, the density of polymers and active pharmaceutical ingredients has not always been reported and when working with small quantities of materials, it is difficult to accurately measure the density. Moreover the density of any of the organic compounds considered in this dissertation are within ±40% of the density of water. Next, by dividing the total core volume by the volume of a single core, the number of cores is calculated. When calculating the number of block copolymer chains present in the system, care must be taken to assume that all of the polymer is on nanoparticle surfaces; if no independent micelle population was observed on the
Zetasizer, the assumption is valid. The number of chains per particle, or the aggregation number, $p$, can be determined as the ratio of total block copolymer chains to total cores and it can then be converted to the number of chains per surface area, $\sigma$, by dividing the chains per particle by the surface area per core. All steps are condensed in the equation below.

$$\sigma_i = \frac{m_{BCP} N_A (d_m - 2\delta_i)}{6M_{BCP} \sum_{j} \frac{m_j}{\rho_j}}$$  \hspace{1cm} (H.9)

In equation H.8, $m_{BCP}$ is the mass of block copolymer, $N_A$ is Avogadro's number, $d_m$ is the peak mean diameter, $\delta_i$ is the layer thickness of iteration $i$, $M_{BCP}$ is the molecular weight of the block copolymer, $\rho_j$ is the density of the core component $j$, and $m_j$ is the mass of core component $j$. For this dissertation, it has been assumed that all $\rho_j$ are equal to the density of water.

Next, depending on the assumed surface geometry, flat plate or spherical surface, the PEG layer thickness is calculated differently. For the case of a brush layer on a flat plate surface, the surface coverage is used to calculate the corresponding layer thickness, through the equation below, which combines equations H.5 and H.6.

$$\delta_{i+1} = N \left( \frac{V}{b^3} \right)^{1/3} b^{5/3} \left( \frac{4}{\pi \sigma_i} \right)^{-1/3}$$  \hspace{1cm} (H.9)

These calculations must be iterated in a self-consistent manner. Thus, through iteration $i$, the layer thickness that was calculated using the surface coverage of iteration $i - 1$ will be used to calculate the surface coverage of iteration $i$ using equation H.8. This surface coverage is then used in equation H.9 to calculate the subsequent layer thickness. This is repeated until the various parameters (e.g. layer thickness, surface coverage, etc.) converge to a constant solution.
For the case of a based a brush conformation on a curved surface, the method outlined by Biver et al is used [8]. Continuing from equation H.8, it is more convenient to convert from the surface coverage $\sigma$ to the aggregation number $p$, which yields the equation below.

$$p_i = \frac{\pi m_{BCP} N_{Av} (d_m - 2\delta_i)^3}{6M_{BCP} \sum_j \rho_j m_j}$$  \hspace{1cm} (H.10)

The PEG layer thickness $\delta$ of the next iteration can then be calculated. The main equation has been recast below in terms of the aggregation number, $p$.

$$\delta = \frac{d_m}{2} \left[ \left( \frac{d_m}{2} \right)^{5/3} - kNb \left( \frac{v}{b^3} \frac{p}{4\pi} \right)^{1/3} \right]^{3/5}$$  \hspace{1cm} (H.11)

Here, $k$ is a constant $O(1)$ and was assumed to be 1 and $v/b^3$ is the excluded volume parameter, which was taken as 1.75 for PEG in water [9]. The layer thickness (equation H.11) is used iteratively to obtain a self-consistent solution for $\delta$ and $p$: the $\delta$ obtained at the end of iteration $i$ is used in iteration $i+1$ to recalculate the volume of a particle core, the number of particles, the aggregation number, and subsequently a new layer thickness. This process is repeated until the layer thickness converges to $\delta_{exp}$, from which the surface coverage can be determined using equation H.12 below.

$$\sigma_{exp} = \frac{4}{\pi \sigma_{min}^2} = \frac{1}{\pi} \left[ \sqrt{p_{exp}^3 - 2} \dfrac{d_m}{2\delta_{exp}} \right]^2$$  \hspace{1cm} (H.12)

Due to curvature, the blobs in the chain will increase in size when moving outward from the core; thus, the minimum blob size, which corresponds to the blob closest to the core, is used to determine the surface coverage at the core surface. The surface coverage can then be converted to mass of polymer per unit area.
Taking the example from Chapter 6 of the cholecalciferol nanoparticle dispersion stabilized by polystyrene-\textit{block}-poly(ethylene glycol) (MW: 1.6kD-\textit{b}-5kD) (Table 6.1), Table H.1 show the estimated results for both the flat plate and spherical surface geometry in comparison to the theoretical mushroom and brush values for a flat plate surface. References for the numerical values for properties used in the calculations can be found elsewhere [10]. While most of the estimated flat plate PEG layer thickness and the surface coverage for both geometries are beyond those of a theoretical brush on a flat plate, there is most likely some discrepancy due to collapsing the size distribution into one mean size. A previous comparison of β-carotene nanoparticles stabilized by PS-\textit{b}-PEG showed that the PEG layers were better exemplified by the brush assumption than that of the mushroom. The result in Table H.1 suggests the same conclusion, strengthening the claim that Flash NanoPrecipitation results in dense PEG layers on the surface of nanoparticles [11].

**Table H.1** Theoretical and estimated surface properties of the cholecalciferol nanoparticle dispersion stabilized by PS1.6kD-\textit{b}-PEG5kD from Chapter 6 (Table 6.1). The nanoparticles had a peak mean diameter of 116 nm and were in suspension at a concentration of 3.34 mg/mL with 35 wt% of the particles composed of cholecalciferol. Mush = mushroom regime, Brush = brush regime, Exp = experimental value.

<table>
<thead>
<tr>
<th>Trial</th>
<th>( \delta ) (nm)</th>
<th>( \sigma ) (chain nm(^2))</th>
<th>( p ) (chain/particle)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flat Plate</td>
<td>Spherical</td>
<td>Flat Plate</td>
</tr>
<tr>
<td></td>
<td>Mush</td>
<td>Brush</td>
<td>Exp</td>
</tr>
<tr>
<td>XI</td>
<td>5.4</td>
<td>19.3</td>
<td>21.7</td>
</tr>
</tbody>
</table>

Furthermore, in comparing the experimentally derived values through the flat plate and spherical surface geometry, it is obvious that there is an appreciable difference between the two cases. Considering that for the case above, the ratio of the PEG layer thickness to the nanoparticle core radius is 0.31, it is obvious that the curvature of the particles cannot be neglected since the two length scales are of the same magnitude. This curvature will allow for the PEG chains to form blobs of different sizes, with smaller blobs at the core surface and larger
blobs at the other end, thus decreasing the layer thickness in comparison to the flat plate case, where all blobs are assumed of the same size. Therefore, it is recommended that the spherical geometry be used when using this calculation, since it is difficult to know the relative scale of the PEG layer thickness in comparison to the nanoparticle core radius \textit{a priori}.

\textbf{H.1 NOMENCLATURE}

\begin{itemize}
  \item \textit{PEG} \quad \text{poly(ethylene glycol)}
  \item \textit{PS} \quad \text{polystyrene}
  \item \textit{PXXYkD-b-PEGZkD} \quad \text{poly(XX)-block-poly(ethylene glycol)} \text{ of MW: YkD-b-ZkD}
  \item \(\xi\) \quad \text{blob size of a polymer chain}
  \item \(k_B\) \quad \text{Boltzmann constant}
  \item \(T\) \quad \text{absolute temperature}
  \item \(N\) \quad \text{number of monomers in a polymer}
  \item \(b\) \quad \text{monomer length}
  \item \(\gamma\) \quad \text{interfacial tension between two surfaces}
  \item \(\sigma\) \quad \text{surface coverage, or the number of polymer chains per surface area, on a surface}
  \item \(\delta\) \quad \text{end-to-end chain length of a polymer, or polymer layer thickness}
  \item \(M\) \quad \text{molecular weight}
  \item \(m_i\) \quad \text{mass of } i
  \item \(N_{Av}\) \quad \text{Avogadro's number}
  \item \(d_m\) \quad \text{peak mean intensity diameter of a nanoparticle}
  \item \(\rho_i\) \quad \text{density of } i
  \item \(k\) \quad \text{a constant}
\end{itemize}
\( v/b^3 \) excluded volume parameter of a polymer

\( p \) aggregation number

### H.2 REFERENCES

APPENDIX I - FLASH NANOPRECIPITATION TECHNIQUES FOR LIPID NANOPARTICLES

As presented by Kumar [1], Flash NanoPrecipitation (FNP) is not limited only to the production of polymeric nanoparticle dispersions, but also for the production of lipid nanoparticles (LNPs). The work presented in this appendix was quickly conducted for the production of fluorescent LNPs loaded with experimental nucleic acids and thus could not be fully characterized. However, since the incorporation of fluorescent dyes into nucleic acid-loaded LNPs has not been reported previously, nor the production of LNPs on a lab-scale confined impinging jets mixer (CIJM), simple procedures and observations are presented as a starting point for any future research. This appendix explains preliminary evaluations for using FNP to produce siRNA-loaded fluorescent lipid nanoparticles through the procedure of Chua [2] on multi-inlet vortex mixers, as well as through a CIJM.

I.1 MATERIALS & METHODS

I.1.1 Materials

Tetrahydrofuran (THF) (HPLC grade), sodium acetate (ACS grade), sodium citrate dihydrate (granular), citric acid (anhydrous), acetic acid (ACS grade), and HyClone phosphate buffered saline (PBS) were purchased from Fisher Scientific (USA). Sodium chloride (≥ 99.5%) was purchased from Sigma-Aldrich (USA). Ethanol (95%, denatured) was purchased from EMD Millipore (USA). The lipids CLinDMA (2-{4\text{-}[(3β)-cholesta-5\text{-}en-3\text{-}yloxy]butoxy}\text{-}N,N-dimethyl3\text{-}[(9Z,12Z)\text{-}octadeca-9,12\text{-}dien-1\text{-}yloxy]propan-1\text{-}amine), cholesterol, and polyethylene glycol2000-dimyristoylglycerol (PEG\text{2000}\text{-}DMG) and luciferase siRNA (Luc siRNA) were used as received from Merck Research Laboratory, West Point. The fluorescent
dye 2,2,10,10-tetraethyl-6,14-bis-(triisopropylsilylthynyl)-1,3,9,11-tetraoxadicyclopenta[b,m]pentacene (EtTP-5) was received from Dr. John Anthony from the University of Kentucky [3]. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Invitrogen (USA). Ultrapure water (MilliQ water) (18 MΩ·cm) was generated from a Barnstead Nanopure purification system.

### I.1.2 Nanoparticle Formation

Lipid nanoparticles were produced using two different methods. The first made use of a either a two-jet vortex mixer (2JVM) or a four-jet vortex mixer (4JVM), depending on whether or not a fluorescent dye was to be incorporated. For example, a brief summary of the procedure reported by Kumar for non-fluorescent lipid nanoparticles is given. The lipids were dissolved in ethanol at a relative molar ratio of 60:38:2 of CLinDMA:cholesterol:PEG2000-DMG at a total lipid concentration of 6.5 mg/mL. An aqueous solution of siRNA at 45 µM in pH 4 acetate buffer was also prepared. Using 25 mL glass syringes (SGE Analytical Science, Australia) driven by a digitally controlled PHD 2000 syringe pump (Harvard Apparatus, USA), the ethanol and aqueous streams were mixed against each other in 2JVM using a flowrate of 28 mL/min for both streams. The initial ~5 mL of effluent were discarded and then collection was started. Effluent collection was stopped prior to stopping the pumps. This ensured that only dispersion produced at steady state operation were collected.

The second method made use of a laboratory-scale confined impinging jets mixer with dilution (CIJM-D) reported by Han et al [4]. Briefly, as an example, 1 mL of the organic stream containing the dissolved lipids and possible a fluorophore in ethanol or THF in a disposable plastic syringe (National Scientific Company, USA) was simultaneously manually injected into the mixer against 1 mL of a buffered siRNA solution and collected into an 2 mL diluting
reservoir of the aqueous phase. The resulting dispersion was 25 vol% organic solvent. In some cases, the procedure was adapted to for use of a CIJM without dilution in a collecting reservoir, resulting in 50 vol% organic solvent.

I.1.3 Particle Size Distribution Measurement

After nanoparticle production and organic solvent removal, the dispersions were characterized for particle size using a Malvern Zetasizer ZS ZEN 3600 (UK). Samples were prepared by diluting the dispersions at least 40-fold in MilliQ water and were contained in disposable 1.5 mL plastic cuvettes (Fisher, USA). All measurements were made with a 633 nm laser at a scattering angle of 173°. Sizes reported are those obtained using the general purpose normal resolution analysis mode of the intensity-weighted distribution. The peak means and span ranges ($\Delta = d_{90} - d_{10}$) are given. Refer to Appendix C for a discussion on why this reporting protocol is used. Usually, at least triplicate measurements were made and the metrics are given the averages and standard deviations of at least two measurements.

I.1.4 Dialysis

Typically, if a dispersion yielded satisfactory results (nanoparticulates present, little or no precipitate visible, and/or stable dispersion within 10-20 minutes), an aliquot of the sample was loaded into Spectra/Por 1 (MWCO 6-8 kD) dialysis tubing (Spectrum Laboratories, USA). The aliquot was dialyzed against PBS or MilliQ water generally at a 1 L to 10 mL ratio of bath to sample volume. The bath was refreshed at least hourly for the first five hours and then left overnight. The dialyzed dispersion was then collected from the tubing and the volume change was recorded.
I.2 RESULTS

Using the legacy conditions (ethanol/acetate buffer) reported by Kumar, non-fluorescent Luc-loaded LNPs were produced on a 2JVM at a nominal solids concentration of 3.6 mg/mL and nominal siRNA concentration of 22 µM. The dispersion had a peak mean diameter of 94 nm once dialyzed against MilliQ water with a span range of 108 nm. Over the course of 103 days of storage at T=4°C, the peak mean diameter and span range experienced less than 10% change. While solids or siRNA concentrations were not measured, the size data suggests that this nanoparticle formulation is very stable. Kumar's formulation yielded a nominal N/P ratio, which is the molar charge ratio of cationic lipid to phosphate groups on siRNA (42 phosphate groups per siRNA molecule), of 3 which has been shown to be optimal for small particle sizes and increased loading efficiency of siRNA [1]. This stoichiometry was kept constant in all subsequent nanoparticle formulations.

In order to incorporate the fluorescent dye EtTP-5 into the nanoparticles, the legacy conditions could not be used since the dye is not soluble in ethanol. Thus, a 4JVM adaptation was used instead based on the work of Chua [2] that made use of THF as the organic solvent and a citrate buffer as the aqueous antisolvent. Resulting in a nominal solids concentration that was four times lower than that of the legacy formulation (siRNA at 5.7 µM), the peak mean particle diameter was 83 nm with a span range of 77 nm. If no Luc siRNA was included in the formulation, the peak mean diameter increased to 103 nm with a span range of 102 nm. Fluorescence measurements (λ<sub>EX</sub> = 460 nm) showed that LNPs with had the signature emission spectrum of EtTP-5 with a slight red shift (~5 nm) and a larger peak at 690 nm as shown in Figure I.1. The dye concentration was estimated as ~4 µg/mL, assuming no quenching when the LNPs were redissolved in THF, based on a calibration curve for the emission of EtTP-5 in
solution \( \lambda_{EX} = 460 \, \text{nm}, \lambda_{EM} = 631 \, \text{nm} \) \( (R^2 = 0.9998) \). Studies are necessary to determine the optimal amount of dye to be included in the nanoparticle formulation, since the nominal dye concentration should have been 13 \( \mu \text{g/mL} \). Lost dye could be accounted for in the dialysis bath, which continually had a greenish tint from dye leaching out. Considering that there was some greenish macro-precipitate after storing the dispersion at 4°C for a few days, it seems that EtTP-5 is not very soluble within the lipid/siRNA construct and prefers to partition out and recrystallize.

![Fluorescence of EtTP-5](image)

**Figure I.1** Fluorescence of EtTP-5 in solution and loaded into LNPs. Excitation was at 460 nm. Data is normalized to the intensity of the maximum fluorescent peak at ~630 nm.

A limitation for using this nanoparticle formulation is the need to use the 4JVM, which cannot be used for the production of very small volumes due to the holdup and waste. Many times, experimental nucleic acids are available at only small masses and thus this production mode is not useful. Therefore, an adaptation using the CIJM-D was developed. While a direct translation of the 4JVM formulation (1 mL THF solution mixed against 1 mL citrate solution collected into 2 mL citrate solution) did not result in the same particle size distribution (peak
mean size: 206 nm; span range: 132 nm), a few adjustments resulted in comparable LNPs that could be produced without a fluorescent dye. By switching back to ethanol as the organic solvent and not diluting the 1:1 ethanol:citrate effluent in a citrate reservoir (CIJM not CIJM-D), non-fluorescent LNPs with a nominal siRNA concentration of 11 µM could be formed with a peak mean size of 72 nm with a span range of 102 nm. Incorporating the cationic fluorescent dye DiI at 25 ug/mL resulted in little change to the particle size distribution (peak mean size: 69 nm; span range: 81 nm). Although the dye loading or concentration was not quantified, there was dye present in the dialysis bath. Again, this indicates that optimization of dye quantities is necessary.

A summary of the presented trials are given below in Table I.1 and in Figure I.2.

I.3 FUTURE WORK

It is necessary for any optimization involving particle size and/or composition modification to consider loading efficiencies of the nucleic acid used, as well as that of the fluorescent dye. It would also be useful to measure total solids concentrations to determine absolute loadings of the two active ingredients.

Furthermore, as mentioned previously, the optimal quantity of fluorescent dye is not yet known. Evaluating how much dye can be loaded into the LNPs without leaching, as well as determining optimal fluorescence, would help in making the nanoparticle formulation useful in biological studies.

I.4 REFERENCES


Table I.1 Flash NanoPrecipitation trials for siRNA-loaded lipid nanoparticles. Nominal concentrations are those in the dispersion after FNP.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Mixer</th>
<th>Organic Solvent</th>
<th>Aqueous Buffer</th>
<th>Dye</th>
<th>Nominal Concentrations</th>
<th>Peak Mean Intensity Diameters (nm)</th>
<th>Span Range (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total Solids (mg/mL)</td>
<td>siRNA (uM)</td>
<td>Dye (ug/mL)</td>
</tr>
<tr>
<td>A</td>
<td>2JVM</td>
<td>Ethanol</td>
<td>Acetate</td>
<td>-</td>
<td>3.6</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>4JVM</td>
<td>THF</td>
<td>Citrate</td>
<td>EtTP-5</td>
<td>0.90</td>
<td>5.7</td>
<td>13</td>
</tr>
<tr>
<td>C</td>
<td>4JVM</td>
<td>THF</td>
<td>Citrate</td>
<td>EtTP-5</td>
<td>0.83</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>D</td>
<td>CIJM-D</td>
<td>THF</td>
<td>Citrate</td>
<td>-</td>
<td>0.89</td>
<td>5.7</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>CIJM</td>
<td>Ethanol</td>
<td>Citrate</td>
<td>DiI</td>
<td>1.7</td>
<td>11</td>
<td>25</td>
</tr>
<tr>
<td>F</td>
<td>CIJM</td>
<td>Ethanol</td>
<td>Citrate</td>
<td>DiI</td>
<td>1.7</td>
<td>11</td>
<td>25</td>
</tr>
</tbody>
</table>

Figure I.2 Particle size distributions of trials presented in Table I.1