Abstract

Nanoparticle carrier systems are gaining importance in the rapidly expanding field of biomedical whole animal imaging where they provide long circulating, real time imaging capability. This thesis presents a new paradigm in imaging whereby long wavelength fluorescent or photoacoustically active contrast agents are embedded in the hydrophobic core of nanocarriers formed by Flash NanoPrecipitation. The long wavelength allows for improved optical penetration depth. Compared to traditional contrast agents where fluorophores are placed on the surface, this allows for improved signal, increased stability, and molecular targeting capabilities. Several types of long wavelength hydrophobic dyes based on acene, cyanine, and bacteriochlorin scaffolds are utilized and animal results obtained for nanocarrier systems used in both fluorescent and photoacoustic imaging modes. Photoacoustic imaging is particularly promising due to its high resolution, excellent penetration depth, and ability to provide real-time functional information.

Fundamental studies in nanoparticle stabilization are also presented for two systems: model alumina nanoparticles and charge stabilized polystyrene nanoparticles. Motivated by the need for stable suspensions of alumina-based nanocrystals for security printing applications, results are presented for the adsorption of various small molecule charged hydrophobes onto the surface of alumina nanoparticles. Results are also presented for the production of charge stabilized polystyrene nanoparticles via Flash NanoPrecipitation, allowing for the independent control of polymer molecular weight and nanoparticle size, which is not possible by traditional emulsion polymerization routes.

Lastly, methods for processing nanoparticle systems are explored. The increasing use of nanoparticle therapeutics in the pharmaceutical industry has necessitated the development of scalable, industrially relevant processing methods. Ultrafiltration is particularly well suited for concentrating and purifying macromolecular suspensions. Processing parameters are defined and optimized for PEGylated nanoparticles, charge stabilized latices, and solutions of albumin. The fouling characteristics are compared and scale-up recommendations made. Finally, a pilot scale spray drying system to produce stable nanocrystalline powders of highly crystalline drugs which cannot be stably formulated by traditional spray drying methods is presented. To accomplish this, a novel mixing device was developed and implemented at pilot scale, demonstrating feasibility beyond the lab scale.
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Table of Contents

Abstract .......................................................................................................................... iii
Acknowledgements ..................................................................................................... iv
Table of Contents ........................................................................................................ v
List of Figures ............................................................................................................... vi
List of Tables ................................................................................................................ xii
Thesis Introduction ...................................................................................................... 1

Section 1 Nanoparticle Imaging Agents
Chapter 1: Introduction to Long Wavelength Optical/Near-IR Biomedical Imaging .............................................. 5
Chapter 2: Biomedical Fluorescence Imaging using Pentacene Dyes ................................................................. 68
Chapter 3: Long Wavelength ICG Nanocarriers for Biomedical Imaging ............................................................. 103
Chapter 4: Nanoparticle Contrast Agents for Photoacoustic Imaging .............................................................. 140

Section 2 Mechanisms of Nanoparticle Stabilization
Chapter 5: Small Molecule Adsorption to Colloidal Alumina ................................................................................. 196
Chapter 6: Flash NanoPrecipitation of Polystyrene Nanoparticles ...................................................................... 233

Section 3 Nanoparticle Processing
Chapter 7: Enhanced Drug Solubility by the Spray Drying and Nanoparticle Assembly Process (SNAP) .......... 268
Chapter 8: Ultrafiltration
Part A ............................................................................................................................. 303
Part B ............................................................................................................................. 330

Appendices
Appendix A .................................................................................................................... 370
Appendix B .................................................................................................................... 378
Appendix C .................................................................................................................... 388
Appendix D .................................................................................................................... 394
Appendix E .................................................................................................................... 402
Appendix F .................................................................................................................... 418
Appendix G .................................................................................................................... 430
Appendix H .................................................................................................................... 436
Appendix I .................................................................................................................... 440
List of Figures

Section 1 – Nanoparticle Imaging Agents

Chapter 1 – Introduction to Long Wavelength Optical/Near IR Biomedical Imaging
Fig. 1 – The electromagnetic spectrum.
Fig. 2 – Absorbance of various tissue and blood components from 200 nm to 10 μm.
Fig. 3 – Jablonski diagrams showing the main energy pathways for different modes of fluorescence.
Fig. 4 – Lung clearance of monodisperse polystyrene particles in rats.
Fig. 5 – Predicted absorption spectra for gold ellipsoids with varying aspect ratios (ARs).
Fig. 6 – Chemical structures for pyrrole, polypyrrole, porphyrin, and phthalocyanine.
Fig. 7 - Increasingly large porphyrin rings have absorption maxima that shift into the NIR (411 – 953 nm).
Fig. 8 – In vivo fluorescent images of targeted QDs in mice.
Fig. 9 - TEM image of oleic acid-trioctylphosphine stabilized NaYF₄:Yb³⁺,Er³⁺ upconverting nanophosphors.
Fig. 10 – Examples of cyanine dyes ICG, Cy5, and IRDye 750 NHS Ester.
Fig. 11 – Examples of squaraines.
Fig. 12 – Examples of BODIPY class dyes.
Fig. 13 – Perylene and an example derivative.
Fig. 14 – Carbon nanotube (CNT) fluorescence emission after uptake into a macrophage-like cell.
Fig. 15 – Atomic force microscope (AFM) images of 30 nm micelles encapsulating hydrophobic ICG complex.
Fig. 16 – Flash NanoPrecipitation schematic.
Fig. 17 – SEM image of poly(lactic-co-glycolic acid)-b-poly(ethylene glycol) protected upconverting nanophosphors (140 nm) with additional 30 nm micelles visible.
Fig. 18 – Novel theranostic agent comprising Au nanoshell and a silica shell doped with SPOI and ICG decorated with anti-HER2 antibodies.

Chapter 2 – Biomedical Fluorescence Imaging using Pentacene Dyes
Fig. 1 – Demonstration of Et-TP5 nanoparticle size control.
Fig. 2 – Molecular structure of Et-TP5 and normalized absorbance and fluorescent emission spectra of Et-TP5 dissolved in THF.
Fig. 3 – Family of pentacene- and hexacene-based fluorescent dyes studied.
Fig. 4 – Size distributions for representative Et-TP5-PS core nanoparticles at various core loadings, fluorescence per particle/mL vs. Et-TP5 core wt. fraction, and I₁/I₂ vs. weight fraction for Et-TP5 nanoparticles cores.
Fig. 5 – Fluorescence per nanocarrier (NC) dependence on NC size at constant dye loading.
Fig. 6 – Comparison of ETTP5 PS core nanoparticles to other fluorescent nanoparticles formed via the FNP process.
Fig. 7 – Cell culture and mouse images of ETTP5 nanoparticles.
Chapter 3 – Long Wavelength ICG Nanocarriers for Biomedical Imaging
Fig. 1 – The optical imaging window.
Fig. 2 – Structure of ICG, tetraoctylammonium chloride, tetradecylammonium chloride, and normalized fluorescence excitation and emission spectra for ICG in DMSO.
Fig. 3 – Fluorescence per nanocarrier vs. ICG complex core loading.
Fig. 4 – Nanocarrier size change following precipitation of ICG-C₈ with different core materials and corresponding changes in ICG complex core loading.
Fig. 5 – Size distributions for ICG-TOAC (ICG-C₈) in situ formed NCs created for size control demonstration, NP sizes vs. total solids concentration and fluorescence intensity per NC vs. core diameter.
Fig. 6 – ICG encapsulation efficiencies as a function of TOAC:ICG ratio.
Fig. 7 – Equilibration time for ICG NC – BSA system.
Fig. 8 – Change in intrinsic ICG fluorescence after adding BSA.

Chapter 4 – Nanoparticle Contrast Agents for Photoacoustic Imaging
Fig. 1 – Simple schematics of pure ultrasound vs. photoacoustic imaging modalities.
Fig. 2 - Penetration depth for optical, PA, and ultrasound imaging techniques.[3]
Fig. 3 – Characteristics of an ideal PA imaging contrast agent compared with broad carbon nanotube (CNT) absorption spectrum.
Fig. 4 - Spectra of LW2, IR122, LDI830 and bacteriochlorin dyes B56, B58, and B146.
Fig. 5 – Spectral characterization of the PAI agent within spleen and kidney regions.
Fig. 6 – Perfusion quantification of PAI agent.
Fig. 7 – Accumulation in subcutaneous tumor and spectral unmixing of PAI agent over 24 hours.
Schematic 1 – Flash NanoPrecipitation.
Schematic 2 – Five steps for optoacoustic probe development.
Fig. 8 – Characterization of LW2 nanocarriers.
Fig. 9 – Performance of LW2 loaded nanocarriers as optoacoustic contrast agents in vitro experiments.
Fig. 10 – Stability and in vitro toxicity of LW2 nanocarriers.
Fig. 11 – Ex vivo study performed on CD-1 mice after subcutaneous LW2 nanocarrier injection.

Section 2 – Mechanisms of Nanoparticle Stabilization

Chapter 5 – Small Molecule Adsorption to Colloidal Alumina
Fig. 1 – Ligand structures and UV-vis absorbance spectra.
Fig. 2 – Dispersion of alumina by two methods and zeta potential vs. pH.
Fig. 3 – Surface schematic of alumina.
Fig. 4 – SEM of AKP50 after dispersion in water.
Fig. 5 – Langmuir isotherm for 2 naphthyl phosphate adsorption (pH adjusted).
Fig. 6 – pH adjusted vs. unadjusted isotherms for 2 naphthyl phosphate adsorption.
Fig. 7 – Adsorption isotherms for 2 naphthyl sulfate and model fits.
Fig. 8 – Adsorption isotherms for triethylene glycol.
Fig. 9 – pH rise upon addition of ligand comparing 2NP, 2NS, and TEG.
Fig. 10 – Isothermal calorimetry results for 2 naphthyl phosphate.
Fig. 11 – Dispersion of alumina with polyacrylic acid size distributions.
Fig. 12 – Scaling of adsorbed polymer brush layer with molecular weight.

Chapter 6 – Flash NanoPrecipitation of Polystyrene Nanoparticles
Fig. 1 - Schematic and actual laboratory setup of the FNP mixing process to generate PS nanoparticles.
Fig. 2 - Representative SEM images and size distributions of PS nanoparticles.
Fig. 3 - Change in PS nanoparticle diameter as a function of PS concentration in Stream 1 of the FNP mixing process for three different PS molecular weights and change in PS nanoparticle diameter as a function of wt% of NaCl in Stream 2 of the FNP mixing process for different PS concentrations in Stream 1.
Fig. 4 - Representative SEM images of PS nanoparticles (M_w = 92 kg/mol) made using varying amounts of NaCl (with respect to PS weight) in Stream 2 and 2 mg/mL PS concentration in Stream 1 of the FNP mixing process.
Fig. 5 - Dependence of zeta potential and size on NaCl concentration for PS (M_w = 92 kg/mol) nanoparticles generated at a polymer concentration of 1 mg/mL in Stream 1 of the FNP process.
Fig. 6 - Interaction potential, W/kT, as a function of distance between two 200 nm diameter PS spheres (solid blue lines) and between a 200 nm sphere and a 3.3 nm unimer (dashed red lines) as calculated from Equations 2-4 at various ionic strengths.
Fig. 7 - Dependence of zeta potential and size on NaCl concentration for PS (M_w = 376 kg/mol) nanoparticles generated at 4.7x10^-4 M (3 wt% with respect to PS) NaCl using a polymer concentration of 10 mg/mL in Stream 1 of the FNP process.

Section 3 – Nanoparticle Processing

Chapter 7 – Enhanced Drug Solubility by the Spray Drying and Nanoparticle Assembly Process (SNAP)
Fig. 1 – API space T_m/T_g (tendency to crystallize) vs. LogP (hydrophobicity).
Fig. 2 – Chemical structures of APIs and excipients used.
Fig. 3 – Solubility data for phenytoin in mixtures of acetone-water and THF-water at room temperature and 37°C.
Fig. 4 – Flash NanoPrecipitation schematic.
Fig. 5 – Process schematics using MIVM mixing geometry and the SNAP coaxial jet mixer design.
Fig. 6 – Powder X-ray diffraction graphs of spray dried dispersions of phenytoin.
Fig. 7 – SEM images of pure bulk phenytoin and spray dried HPMC samples as visual controls.
Fig. 8 – SEM images of PTN-containing spray dried dispersions.
Fig. 9 – Annealing studies on SNAP generated spray dried dispersions.
Fig. 10 – Dissolution and release results for spray dried dispersions of phenytoin.

Chapter 8 - Ultrafiltration

Part A
Fig. 1 – Schematic and flowpath of the KrosFlo TFF Research III ultrafiltration system.
Fig. 2 – Comparison of dead-end and tangential flow filtration.
Fig. 3 - Results of the water permeability test.
Fig. 4 – TMP Optimization for BSA concentrations of 9.59 mg/mL and 65.9 mg/mL.
Fig. 5 - Permeate flow rate and optimization parameter $\xi^*$ vs. BSA concentration.
Fig. 6 - The three phases of concentration and diafiltration.
Fig. 7 – Diafiltration efficiency of sodium salicylate.

Part B
Fig. 1 – Illustration of flow through cake layers comprised of proteins or nanoparticles of different sizes.
Fig. 2 – Size of VE/NR/PEG-PCL NPs over a period of 18 days.
Fig. 3 – Pump calibration tests.
Fig. 4 – Initial water permeability tests on 10kD MWCO mPES membranes in wetted and non-wetted states.
Fig. 5 – Change in PEGylated NP size and PDI (polydispersity index) widths after 1 hr processing at various shear rates and constant solids concentration.
Fig. 6 – TMP optimization experiments for PEGylated nanoparticles.
Fig. 7 – TMP optimization ($Q_P$ vs. TMP and $R_G$ vs. TMP) for concentrations of 6 mg/mL PMMA and 30 mg/mL PMMA at various feed flow rates.
Fig. 8 – Illustration of osmotic pressure rise as a function of concentration for proteins vs. nanoparticles.
Fig. 9 – Diafiltration optimization for PEGylated NPs: Permeate flow rate ($Q_P$) and optimization parameter $\xi^*$ vs. Vitamin E concentration.
Fig. 10 - Permeate flow rate vs. concentration and diafiltration optimization parameter graphs for PMMA.
Fig. 11 – Diafiltration efficiency: THF concentration vs. $V_D$ for the 2 diafiltration experiments done.

APPENDICES

Appendix A
AppA-Fig. 1 – Reaction scheme for methotrexate + 1-octanol.
AppA-Fig. 2 – Proton NMR of MTX-dioctyl ester in DMSO-$d_6$.
AppA-Fig. 3 – $C_{13}$ NMR of MTX-dioctyl ester.
AppA-Fig. 4 – Methotrexate in DMSO UV-vis calibration curve at 388 nm.
AppA-Fig. 5 – DLS size distribution of MTX-dioctyl ester NPs and 3 week stability.

Appendix B
AppB-Fig. 1 – DTPA + 1-hexadecylamine reaction scheme.
AppB-Fig. 2 – $Gd^{3+}$ loading reaction schematic.
AppB-Fig. 3 – Proton NMR of DTPA-(C$_{18}$)$_2$.
AppB-Fig. 4 – Size distributions of DTPA-C$_{18}$ Gd$^{3+}$ nanoparticles.
Appendix C
None

Appendix D
AppD-Fig. 1 – Family of pentacene and hexacene dye structures.
AppD-Fig. 2 – Uptake of ETTP5 nanocarriers into macrophage cells in vitro.
AppD-Fig. 3 – The optical imaging window.
AppD-Fig. 4 – Spectra and intrinsic fluorescence for ETTP5, LD688, and Alexa Fluor 488.
AppD-Fig. 5 – Emission spectra for Et-TP5 excited at 458 and 600 nm.
AppD-Fig. 6 – Size stability of 78% loaded Et-TP5 NCs.
AppD-Fig. 7 – Fluorescence change of Et-TP5 NCs over 10 months.
AppD-Fig. 8 – Fluorescence emission of Et-TP5 in THF vs. toluene.

Appendix E
AppE-Fig. 1 – Demonstration of NC size control – non monotonic behavior.
AppE-Fig. 2 – Covalently modified ICG construct from Persis Science LLC.
AppE-Fig. 3 – Results of NC – BSA incubation experiments.
AppE-Fig. 4 – Formulation of ICG-PDMS nanocarriers and BSA stability.
AppE-Fig. 5 – Comparison between ICG-C12 in situ vs covalent compound.
AppE-Fig. 6 – Proton NMR of ICG-TOAC compound.
AppE-Fig. 7 – Long term size stability of ICG loaded NCs.

Appendix F
AppF-Fig. 1 – Optimization of LW2 NC core loading for fluorescence.
AppF-Fig. 2 – TG analysis of LW2 nanoparticles produced for IBMI collaboration.
AppF-Fig. 3 – Optical characterization of LW2-micelles.
AppF-Fig. 4 – Spectrophotometer analysis of LW2 nanoparticles.
AppF-Fig. 5 – Performance of LW2 loaded NCs as optoacoustic contrast agents.
AppF-Fig. 6 – Stability of LW2 loaded NCs.
AppF-Fig. 7 – Spectrophotometer analysis of ICG as a control dye.
AppF-Fig. 8 – In vitro toxicity of LW2-50 nm after incubation with MDA-MB-231 cells.
AppF-Fig. 9 – Formulation of bacteriochlorin dyes with absorbance spectra.
AppF-Fig. 10 – Size distributions for B58 loaded nanoparticles.
AppF-Fig. 11 – Photostability of B58 loaded nanoparticles vs. free dye.
AppF-Fig. 12 – Size distributions of nanoparticles produced for VisualSonics collaboration.
AppF-Fig. 13 – Photoacoustic signal comparison for 100 nm vs. 50 nm NCs.

Appendix G
AppG-Fig. 1 – DLVO Calculations for TiO2 nanoparticles.
AppG-Fig. 2 – DLS and zeta potential measurements on TiO2 nanoparticles.
AppG-Fig. 3 – Zeta potential and size of PAA coated TiO2.
AppG-Fig. 4 – Derivation of curve fitting equations used for isothermal calorimetry.

Appendix H
AppH-Fig. 1 – PXRD traces of spray dried naproxen formulations by SNAP.
AppH-Fig. 2 – PXRD traces of spray dried phenytoin formulations by SNAP with trehalose rather than HPMC as the excipient.
AppH-Fig. 3 – Solubility data for naproxen acid in THF-water and acetone-water mixtures at room temperature.

Appendix I
AppI-Fig. 1 – Schematic of hollow fiber filtration at membrane surface.
AppI-Fig. 2 – Schematic of gel layer formation.
AppI-Fig. 3 – CSTR model for constant volume diafiltration.
AppI-Fig. 4 – Vitamin E in THF absorbance calibration curve (295 nm).
AppI-Fig. 5 – Nile Red in THF absorbance calibration curve (534.5 nm).
AppI-Fig. 6 – Gel layer reversibility for PMMA latices.
AppI-Fig. 7 – Gel layer reversibility for BSA.
List of Tables

Chapter 1
Table 1 – Absorption or excitation/emission characteristics and chemical/spectroscopic properties of imaging agents.

Chapter 2
Table 1 - Comparison of quenching for various fluorescent nanoparticle systems.

Chapter 3
Table 1 – Summary data for ICG encapsulation efficiency.

Chapter 4
Table 1 – Frequencies and wavelengths for imaging modalities relevant to biomedical imaging.

Chapter 5
Table 1 – Binding characteristics of small molecule and PAA systems.
Table 2 – 2NP Langmuir binding parameters.
Table 3 – Summary of model fits for the 3 ligands
Table 4 – Dispersing alumina with PAA of varying molecular weight.

Chapter 6
None

Chapter 7
Table 1 – Drug and excipient properties.
Table 2 – Key formulation parameters.
Table 3 – Summary of dissolution test data.

Chapter 8
Part A
None

Part B
Table 1 – Comparison of gel resistance for BSA, FNP NPs, and PMMA NPs.

Appendix A
None

Appendix B
AppB-Table 1 – Characteristics of radionuclides.

Appendix C
None
Appendix D
AppD-Table 1 – Comparison of volume vs. surface loaded NCs.
AppD-Table 2 – Comparison of intrinsic fluorescence values for core vs surface loaded NCs.

Appendix E
AppE-Table 1 – Absorbances and extinction coefficients of various ICG constructs.

Appendix F
AppF-Table 1 – Nanoparticle formulations of bacteriochlorin dye B58.

Appendix G
AppG-Table 1 – Ligand pKa data.

Appendix H
AppH-Table 1 – Complete formulation conditions.

Appendix I
None
This thesis is focused on the study and applications of nanoparticle systems, with the broad areas of focus being divided into three sections: nanoparticle applications in biomedical imaging, nanoparticle stabilization, and processing of nanoparticle systems. Motivations for each chapter vary, but in general, the work in this thesis is based on engineering composite nanoparticles formed by the Flash NanoPrecipitation process.

In Section 1, the focus is on the field of biomedical imaging, and the rapidly expanding role of composite, multifunctional nanoparticles in providing high resolution, real time diagnostic information with the potential for effective disease site targeting through the enhanced permeation and retention effect and disease specific targeting using surface conjugated ligands. Chapter 1 is a detailed review of the field of biomedical imaging specifically in the area of optical/near infra-red long wavelength imaging and of the various supramolecular and nanocarrier systems that are revolutionizing the field. Chapters 2-4 are studies into novel nanoparticle imaging systems produced by Flash NanoPrecipitation. Chapter 2 focuses on our initial studies with pentacene based organic dyes that were successfully incorporated into nanoparticles for fluorescence imaging and characterized for size and composition tunability and photostability. In Chapter 3 we successfully incorporated ion paired and covalent variants of the FDA-approved dye indocyanine green (ICG) into nanoparticles for long wavelength fluorescence imaging applications. In Chapter 4, we explored the use of a hydrophobic, hexacene based long wavelength dye termed LW2 for the nascent field of photoacoustic imaging. Preliminary data for several other suitable dyes are also presented.
Section 2 is focused on the subject of nanoparticle stabilization. In Chapter 5, we use alumina nanoparticles as a model system to study the binding energies and isotherms of small molecule hydrophobes and the stabilization effects of polymers. This work was primarily motivated by the need to stabilize security inkjets comprising heavy alumina-like nano-crystals in order to prevent or at least delay settling that could result in blockage of the inkjet head. Binding regimes for charged and uncharged molecules at fixed pH and alumina concentration were characterized and Langmuir or Freundlich/linear isotherms fitted. In Chapter 6, a novel method for producing solely charge-stabilized persulfate initiated polystyrene nanoparticles via Flash NanoPrecipitation is presented in contrast to traditional methods involving emulsion polymerization. This has important implications for researchers that need clean nanoparticle systems for studying glass transition dynamics with independent control over nanoparticle size and polymer molecular weight. More generally, this method can be applied to the production of a wide variety of hydrophobic polymer systems that could include drugs or probe molecules. In this Chapter, DLVO theory is applied to explain the growth mechanism of polymer aggregates into nanoparticles and the eventual endpoint as the repulsive energy barrier grows with increasing size.

Finally, Section 3 explores processing of nanoparticle suspensions, with an eye towards addressing the weaknesses of nanoparticle preparation routes (low concentration, presence of impurities, and short stability window) for industrial implementation. In collaboration with Bend Research, we conducted studies proving the feasibility of formulating highly crystalline drugs with low logP with a combined, sequential, precipitation-spray drying process implemented at
pilot industrial scale. Traditionally, this class of drug has not been formulated successfully as a spray dried dispersion due to aging phenomena that reduce the solubility enhancement of the drug over time and thus the bioavailability. Our approach involved precipitating nanocrystals of drug that were arrested by block copolymers and spray dried before Ostwald ripening rendered large crystal aggregates. In Chapter 8 (Parts A and B), ultrafiltration is explored as a method for concentrating and purifying nanoparticles in a controlled and reproducible fashion. As a process that has traditionally been used in the biotechnology industry, there is very limited literature on process optimization and membrane fouling characteristics of modern engineered composite nanoparticles. We conducted process optimization with three systems: bovine serum albumin, latex nanoparticles, and block copolymer protected and characterized the fouling dynamics on the basis of a hydrodynamic model for cake layer formation. Ultrafiltration was shown to be a viable process for lab scale nanoparticle processing, with direct linear scale up for larger scales.

Though these chapters and sections may seem relatively disparate, they can be thought of as fundamental and applied studies into various aspects of nanoparticle colloid systems, with a common thread being that many of the studies utilize the Flash NanoPrecipitation platform for the production of said nanocarrier systems. The key focus of this thesis is biomedical imaging using these nanocarriers as exogenous contrast agents in absorbance, fluorescence, and photoacoustic imaging modes. The secondary focus of this dissertation is the study of nanoparticle stabilization regimes. The first study focuses on a classical alumina nanoparticle system and the binding curves and energies of various small molecule charged hydrophobes onto the alumina surface while the second focuses on a novel method of producing polymer nanoparticles with Flash Nanoprecipitation solely stabilized by charge repulsion. Lastly, the
tertiary focus is on the processing of nanoparticle suspensions, a field that is of particular importance for industrial applications. Advanced composite nanoparticles often need to be concentrated and purified for final applications. Spray drying was explored as a method to trap fast-growing drug crystals in a solid dosage form at pilot scales. Ultrafiltration was explored as a method to concentrate and purify nanoparticles in a scalable, cost-effective, and reproducible manner using acrylic latices, PEGylated nanoparticles, and proteins as three systems with widely differing characteristics. Taken together, these three sections provide insight into three major areas of nanoparticle colloidal science.
CHAPTER 1
Introduction to Long Wavelength Optical/Near IR Biomedical Imaging


Abstract
The importance of long wavelength and near infra-red (NIR) imaging has dramatically increased due to the desire to perform whole animal and deep tissue imaging. The adoption of NIR imaging is also growing rapidly due to the availability of targeted biological agents for diagnosis and basic medical research that can be imaged in vivo. The wavelength range of 650-1450 nm falls in the region of the spectrum with the lowest absorption in tissue and therefore enables the deepest tissue penetration (several cm). This is the wavelength range we focus on with this review. To operate effectively the imaging agents must both be excited and must emit in this long-wavelength window. We review the agents used both for imaging by absorption, scattering, and excitation (such as fluorescence). Imaging agents comprise both aqueous soluble and insoluble species, both organic and inorganic, and unimolecular and supramolecular constructs. The interest in multi-modal imaging, which involves delivery of actives, targeting, and imaging, requires nanocarriers or supramolecular assemblies. Nanoparticles for diagnostics also have advantages in increasing circulation time and increased imaging brightness relative to single molecule imaging agents. This has led to rapid advances in nanocarriers for long-wavelength, NIR imaging.
Introduction

Broadly, the field of biomedical imaging can be divided into categories based upon the electromagnetic spectrum as shown in Fig. 1: magnetic resonance, optical/near infra-red (NIR), and ionizing radiation (X-rays and gamma rays). Imaging based on ionizing radiation generally refers to the detection of high frequency emissions from radioactive elements such as the gamma ray emitters $^{111}$In or $^{99m}$Tc or the passage of X-rays through the body. The main technologies involved are positron emission tomography (PET)[1-6], single-photon emission computed tomography (SPECT)[7-9], and X-ray computed tomography (CT).[1, 2, 7, 8, 10, 11] Magnetic resonance imaging (MRI) tends to operate on the other end of the spectrum in the MHz frequency range, relying upon contrast agents such as gadolinium or superparamagnetic iron oxide to modify the relaxivity of water molecules to provide soft tissue contrast.[4-6, 12, 13]

The focus of this section is materials for optical and near infrared (NIR) imaging for diagnostic and therapeutic applications in biology and medicine. The important emerging area of whole animal and deep tissue imaging has made long-wavelength and NIR imaging of special significance. While optical and NIR wavelengths range from 400 nm up to 2500 nm, *in vivo* biomedical imaging requires consideration of the so-called 'imaging window', shown in Fig. 2. The figure shows optical absorbance as a function of wavelength and the attenuation arising from various components in a representative tissue sample. The absorbance units are cm$^{-1}$ and, therefore, the inverse of this absorbance represents a characteristic penetration depth. Absorbance by water, melanin, proteins, and hemoglobin (Hb) are high between 200-650 nm, covering essentially the whole visible range. In addition to absorbing light, tissues can also
reflect, refract, and scatter incident photons.[14-16] While this does not create a problem for surface imaging of thin sections or single cell layers, it does point out the problem of imaging through tissue. Within the 200-650 nm range, autofluorescence from tissue also confounds fluorescence measurements[17, 18] by creating high background fluorescence. It is with this realization that much attention has been focused in recent years on the development of absorption imaging agents and fluorophores with absorbance or excitation/emission maxima falling in the region of minimal tissue absorbance/autofluorescence between 650-1450 nm, an ‘imaging window’. Tissues have minimal absorbance in this wavelength range allowing for deep penetration of light (several cm). This enables whole animal imaging with high sensitivity in core organs in real time without the need for dissection. Therefore, we focus on dyes, imaging agents, and imaging nanocarriers in the wavelength range 650-1450 nm. While several excellent reviews cover various aspects of imaging,[19-30] there has not been a review that covers long wavelength and NIR imaging materials adequately.

**Fig. 1** – *The electromagnetic spectrum (adapted from Richards (2001)).*[31]
Fig. 2 – Absorbance of various tissue and blood components from 200 nm to 10 μm. The optical imaging window ranging from 650 – 1450 nm represents the range where tissue penetration is greatest. This wavelength range is the focus of our optical imaging efforts. Adapted from Boulnois (1986)[32] and Susi (1971).[33]
Fig. 3 – Jablonski diagrams showing the main energy pathways for different modes of fluorescence (energy diagrams not drawn to scale). A – single photon Stokes process (i) photon absorption gives excited state, (ii) internal conversion to $S_1$, first singlet excited state, (iii) fluorescence (emission of photon), (iv) nonradiative decay, (v) intersystem crossing to $T_1$ (‘forbidden’ triplet excited state), (vi) phosphorescence, and (vii) nonradiative decay.[24] B – two-photon upconversion (anti-Stokes process). Excited-state absorption (ESA) proceeds via sequential absorption of two photons to give the excited state and a subsequent emission event. In energy transfer upconversion (ETU), an ion directly absorbs one photon while a neighboring ion absorbs another and transfers the energy to the first ion, resulting in upconversion and emission.[22] Reproduced from Lavis and Raines[24] and Haase and Schafer.[22]

While the intrinsic properties of a particular absorber or fluorophore (excitation/emission wavelengths, quantum yield, absorption coefficient, photostability, chemical stability in different environments) play a large role in determining the effectiveness of an imaging agent, the method of delivery can have profound effects as well. Dyes can be delivered as soluble molecules in the bloodstream[34, 35] or encapsulated within nanoparticles that may be polymeric or inorganic in nature.[36-40] The desired nanocarrier size depends strongly on the type of imaging and target organ. Generally, any size is acceptable for GI tract imaging (oral administration) while aerosol targeting to the lungs requires an upper size limit on the order of tens of microns depending on particle density for capture in the deep lung (see Fig. 4).[41, 42] If the carrier is being administered via intravenous injection, particles greater than 1 µm can cause blockage at the
injection site. For broad biodistribution, particles less than ~30 nm in size can penetrate most cells but are cleared relatively rapidly through renal filtration. Nanocarriers from 50-400 nm in size with the appropriate ‘stealth’ surface chemistry provide enhanced circulation time and passive accumulation in tumors due to the enhanced permeation and retention effect (EPR).[43] This non-specific targeting arises from the leaky vasculature that surrounds solid tumors with poor lymphatic drainage, with the result that nanocarriers enter the tumor site but are not rapidly cleared. Dyes can also be used to label antibodies for combined targeting and imaging capabilities.[44] The absorber or fluorophore may itself form nanoscale structures such as clusters, spheres, or rods which can then be used directly or further stabilized by polymers or inorganic agents.[45-47]
Fig. 4 – Lung clearance of monodisperse polystyrene particles in rats (3 µm | 9 µm | 15 µm). Radioactively tagged polystyrene particles, intratracheally administered, were monitored for clearance from the lung. Adapted from Oberdörster (1989) and Snipes (1981).[41, 42]

In this chapter and section, we highlight the development of imaging agents consisting either of pure absorbers, scatterers, or fluorophores that both absorb and re-emit light. Absorbers include traditional highly absorbing materials that attenuate optical radiation. Optical attenuation can also occur by scattering from agents with strong dielectric mismatch with tissue. Detection of scattered light for imaging requires more instrumentally and algorithmically challenging approaches. These rely on more complex interactions with optical radiation including phase, transit time, and polarization information. The reader is directed to excellent reviews of these imaging techniques.[14-16, 48, 49] The agents that produce the dielectric mismatches required
for these imaging techniques are similar to those required for imaging by attenuation, so we do not treat them as distinct imaging materials.

We review traditional organic absorbers and fluorophores, hybrids such as Zn\(^{2+}\)-multiporphyrin complexes,[39] ICG-doped silica-shell Au nanoparticles,[38] and novel inorganic materials such as quantum dots and upconverting phosphors.

While research into novel fluorophores and imaging agents has greatly expanded, the reality is that equally important are the delivery vehicles for these agents. Novel and efficient delivery agents are needed to provide stability, longer circulation time, as well as therapeutic and targeting capabilities, all in one multifunctional package. As such we will conclude this chapter by examining the state of the art in the new field of ‘theranostics’ which combines therapeutics and diagnostics.[50]

**Near-IR Absorption Imaging Agents**

Imaging agents based on absorbance, or optical attenuation, require transmission of light through the entire sample. Even in the “imaging window” wavelengths, the absorption coefficients in Fig. 2 show that this can be challenging. Both organic and inorganic compounds can be used to provide contrast through photon/electromagnetic absorbance. These contrast agents have the advantage of not suffering from quenching effects that are seen with fluorescent agents. Extinction coefficients are relatively insensitive to chemical environments since the attenuation is due to internal electromagnetic structure and not transitions between states.
Gold (Au) nanoparticles exhibit a size- and morphology-dependent surface plasmon resonance absorbance band that is centered at 520 nm for 5 nm particles and shifts into the NIR region for larger spherical clusters or non-spherical morphologies such as nanorods. As seen in Fig. 5, the aspect ratio (AR) of gold nanorods (ellipsoids) strongly affects the plasmon resonance absorption band. It is predicted that an AR of ~3 is required to have absorption at 700 nm. The applicability of Au species for imaging in most cases requires obtaining adequate size in at least one dimension to shift the plasmon frequency to NIR wavelengths. But a problem with application of these larger colloids in vivo is ultimate clearance or toxicity associated with mechanical blockage. The Johnston group has addressed this problem by creating pH-
dependent, polymer-protected, reversible gold nanoclusters from 5 nm Au colloids. These clusters can be tuned to have well defined diameters between 30-100 nm. This size control is primarily achieved through controlling the gold:polymer ratio, where the polymer is a PLA(2k)-\textit{b}-PEG(10k)-\textit{b}-PLA(2k) triblock copolymer that adsorbs weakly onto the gold surface and modifies the energetics of clustering. These clusters have high gold loadings with only 3% polymer by mass of the cluster. Once internalized into endosomes or other structures with low pH, the PLA degrades over time releasing the clusters back to individual Au nanoparticles thus allowing for fast renal clearance.[46, 47]

![Chemical Structures](image)

**Fig. 6** – Adapted chemical structures for \(A \) - pyrrole[53], \(B \) - polypyrrole[54], \(C \) - porphyrin[55], and \(D \) - phthalocyanine (adapted from Sigma-Aldrich Co. LLC, St. Louis MO).

Polypyrroles (PPy) are highly conducting materials due to their highly delocalized electrons which cause them to exhibit strong, broad absorption bands from 800 to ~2500 nm, thus falling into the ‘imaging window’. [56-58] The doped PPys have stronger absorption than the undoped neutral forms. The reader is directed to appropriate reviews for a discussion of the mechanisms responsible for these differences.[59, 60] They display good chemical and thermal stability. While they are most commonly used in organic electronics applications,[26, 57, 58] they are
structurally and chemically related to the phthalocyanines and porphyrins which have already found widespread application as photosensitizers for photodynamic therapy (see Fig. 6).

Bjorklund, et al.[61] published one of the first instances of colloidally stable PPy nanoparticles in the size range 100-200 nm. These particles were composed of pyrrole units polymerized in the presence of methylcellulose.[61] Concurrently, Armes and coworkers produced 100-150 nm monodisperse PPy nanoparticles stabilized with poly(vinyl acetate) (PVA). The stability of these particles depended strongly on the amount of adsorbed PVA.[62] Armes also created larger core-shell PPy particles using PPy coated poly(styrene) (PS) and poly(methyl methacrylate) (PMMA) latices.[63, 64] In the former case, commercially available, charge-stabilized PS latices were coated with poly(vinylpyrrolidone) (PVP) to provide steric rather than charge stabilization. Then, smooth layers of PPy were deposited onto these 1.6-1.8 μm beads, adding less than 20 nm in thickness to the original particles. Colloidal stability was demonstrated for PPy loadings up to 9.9 wt% of the latex mass, beyond which flocculation and instability was observed. Ormond-Prout, et al. deposited PPy onto PMMA latices from 1.2 μm in diameter up to 30 μm with loadings from 3% to 21% by mass. As before, the PPy layer added no more than 20 nm to the particle diameter but had a distinctly globular surface morphology due to the less hydrophobic nature of PMMA compared to PS.[64]

A recent study has shown the feasibility of targeting carboxylated PPy latices and PPy-silica composites with antibodies. These systems were shown to have similar immunoactivity thus opening the way for future targeting studies.[45] In a similar vein, Bousalem, et al. formulated 1.1 μm PS latices coated with PPy and conjugated to human serum albumin (HSA). Their
studies indicated that the surface immobilized HSA retained its immunoactivity against anti-HSA.\[65\] Though PPy would seem to make a good biomedical imaging agent,\[66\] we are not aware of any reports on optical/NIR imaging of PPy-containing imaging agents in an in vivo setting. Though the toxicity of PPy is strongly dependent on the method of polymerization and specific biological setting, many reports have indicated that PPy has minimal negative effects on biological tissues.\[67\] Most of the reports of PPy in animal models focus on their electrical properties rather than the strong optical/NIR absorbance.\[68\]

Taking a different approach, Jang, et al. developed novel water soluble, photofunctional, charged dendrimers with porphyrin or phthalocyanine cores that, when complexed with poly(L-lysine)-b-poly(ethylene glycol) (PLL-b-PEG) or poly(aspartate)-b-poly(ethylene glycol), form charge-neutral micelles on the order of 50 nm in size. These entities were shown to be stable under physiological conditions and are expected to be long-circulating in vivo due to the PEG surface.\[69\] The phthalocyanine core dendrimer (\(\lambda_{\text{abs}} = 685\) nm) proved to have better spectral characteristics for biomedical applications than the porphyrin core (\(\lambda_{\text{abs}} = 560\) nm).\[70\] It is unlikely that porphyrins/phthalocyanines would be used solely for imaging purposes as they are potent producers of reactive oxygen species (ROS) which are cytotoxic to cells. Other approaches involve the synthesis of increasingly large porphyrin rings as Tanaka, et al. have demonstrated (Fig. 7). From the basic porphyrin up to octadecaphyrin, the absorption band shifts from 411 - 953 nm, well into the NIR window.\[71\] Srinivasan, et al. recently introduced bis-metal complexed hexaphyrins with ‘confused’ pyrrolic units which allow for easy complexation of different types of metals. Absorption bands from 325 - 755 nm were observed
for the uncomplexed molecule while the bis-Ni compound had absorption bands from 360 – 1210 nm, well into the NIR range.[72]

![Image of porphyrin structures](image)

**Fig. 7 - Increasingly large porphyrin rings have absorption maxima that shift into the NIR (411 – 953 nm). A – porphyrin, B – dodecaphyrin, C – octadecaphyrin (Ar = C₆F₅). Reproduced from Tanaka, et al.[71]**

**Near-IR Fluorophores**

Fluorescent imaging is the most widely used imaging modality for biomedical research and diagnostics. Fluorophores can be either organic or inorganic and each has its advantages and drawbacks. As fluorescent imagers by definition involve both excitation and emission events (see Fig. 3), it is desirable for *in vivo* applications that the excitation and emission peaks fall into the ‘imaging window’ (650 – 1450 nm) where absorption by tissues and blood is at a minimum. Otherwise, even signals from high quantum yield (>80%) fluorophores will be severely attenuated either from inadequate excitation or absorption of emission.

**Inorganic fluorophores**

Inorganic fluorescent agents fall into the broad categories of quantum dots (QDs) or upconverting phosphors (UCPs).
Quantum dots usually have a core-shell architecture comprising semiconductor materials such as CdSe, CdTe, PbS, or some alloy of those materials, with the composition strongly affecting the excitation and emission characteristics. As most of these materials have well-established toxicities, a shell of ZnS is often added to passivate the surface and improve the quantum yield and stability.[73] Water solubility and additional stability is conferred by coating the QD with amphiphilic copolymers, block copolymers, or other water soluble ligands.[74] QDs usually range in size from 2 to 30 nm (depending on the core size and shell characteristics), with larger cores emitting at longer wavelengths.[73, 74] Theoretical modeling has shown two possible emission bands for in vivo imaging: 700-900 nm and 1200-1600 nm. While most QDs have excitation and emission spectra at shorter wavelengths, Kim, et al. and Park, et al. reported the synthesis and in vivo testing of QDs with ‘imaging window’ wavelengths.[75, 76] QDs have broad excitation bands with narrow emission bands, allowing for multiple-emission agents to be detected at different wavelengths simultaneously with only one excitation source. They are generally bright and photostable with quantum yields from 20% to 60% and do not suffer from the quenching observed for organic fluorophores.[77]
**Fig. 8** — *In vivo* fluorescent images of targeted QDs in mice. *A* — Targeted QDs preferentially accumulate in tumors. *B* — With a single excitation source, QDs tuned to different emission wavelengths can be detected simultaneously. Reproduced from Gao, et al.[77]

Gao and coworkers have demonstrated the feasibility of targeted bioconjugated QD probes for fluorescence diagnostics (see Fig. 8). Their CdSe core, ZnS shell, triblock copolymer protected nanoparticles are capable of 535-630 nm emission profiles and are about 30 nm in diameter. Significantly, they also demonstrated the targeting capabilities of these fluorescent probes by attaching antibodies for the prostate-specific membrane antigen (PSMA). In this way, tumors were identified by active targeting mechanisms. Due to the non-optical window wavelengths, fluorescent light detection within the liver and spleen was very limited.[77]

Kim, et al.[75] and Park, et al.[76] reported QDs with longer wavelength excitation and emission. In the former case, type II QDs were synthesized with an oligomeric phosphine coating to confer water solubility. These QDs had a hydrodynamic diameter of 16 nm with 850 nm emission and a broad excitation band from 800 nm down to 500 nm. The QDs were assessed as real-time surgical aids and were found to accumulate preferentially in sentinel lymph nodes due to their size in both small and large animals (mice and pigs). This lymph node accumulation
is expected to be very useful in the diagnosis and treatment of breast and colon cancers.[75] Park, et al. synthesized 745 nm emitting QDs comprising CuInSe in the core with ZnS as the shell. The average QD diameter was 4 nm with up to 60% quantum yield after ZnS passivation. Acyl chain lipids with PEG units were used as the water transfer agent which increased the hydrodynamic size to 15 nm. In water, the QDs were stable for several days at room temperature. Mouse tail-vein injections showed relatively uniform biodistribution with a clearance half-life of 286 mins.[76] No active targeting approaches were attempted in these studies.

![TEM image of oleic acid-trioctylphosphine stabilized NaYF₄:Yb³⁺,Er³⁺ upconverting nanophosphors. Reproduced from Budijono, et al. (2010).][37]

Upconverting phosphors (UCPs) are a fundamentally different class of inorganic fluorophores that have recently improved to the point where they are now strong alternatives to quantum dots and organic fluorophores.[22] Upconversion is a nonlinear optical process where sequentially absorbed photons are emitted at a shorter wavelength (and thus a higher frequency/energy) thus
making it an anti-Stokes process (Fig. 3). The most efficient upconverting phosphors are hexagonal phase (β) crystals composed of rare earth dopants (lanthanides such as Yb$^{3+}$ and Er$^{3+}$) that provide the fluorescence while the solid state host matrix (NaYF$_4$) optimally positions these ions for energy transfer (Fig. 9). As these fluorescent centers are permanently separated in a solid state matrix, UCPs do not suffer from the same concentration dependent quenching effects as organic fluorophores and do not photobleach.[22] Quantum yields are not defined for upconverting phosphors due to non-linear emission energy scaling with excitation energy.[78] The quadratic dependence of emission means that high illumination power is required for bright emission. Fortunately, the telecommunications industry, with solid state lasers in the wavelength range of 980 nm used for fiber optic communications has made high power very inexpensive in this wavelength range. The excitation of the crystal lattice is quenched by surface defects, therefore, emission intensity scales inversely with crystal size.[78] While 100 nm particles are readily imaged, imaging particles below 30 nm has proven challenging.[78]

Budijono, et al.[37] and Shan, et al.[79] have demonstrated the production of stable, multifunctional, ~200 nm diameter polymer-protected UCP nanoparticles without a photosensitizer (for diagnostic imaging) as well as with a photosensitizer (for photodynamic therapy). [37, 79] These particles have a 980/540,660 nm ex/em profile. The photosensitizer (tetraphenylporphyrin) absorbance profile strongly overlaps with the UCP emission profile at 540 nm to enable reactive oxygen generation by the porphyrins for photodynamic therapy.

Nam, et al. have reported the feasibility of using UCPs as imaging agents in biological systems. Their work involved the synthesis of 40 nm PEG-phospholipid coated UCPs that were taken up
by HeLa cells *in vitro*. At 980 nm laser excitation, the cells did not experience adverse effects and the UCPs were clearly visible at a high concentration within the cells. Cytotoxic effects from the UCPs were negligible and photobleaching was not observed even after 6 hours of continuous tracking.[80]

Coating for the UCP crystal surface is required for biocompatibility and several approaches have been advanced. There are two promising routes to biocompatible surfaces for *in vivo* applications. The first by Hildebrand and Vinegoni involves polyacrylic acid coating followed by amine conjugation of PEG onto the polyacrylic acid coating to make the coated UCP bio-inert.[81, 82] The second route by the Prud’homme group involves block-copolymer-directed assembly with a PEG diblock copolymer to provide a dense PEG surface layer.[37, 79] The directed assembly process is described below in Section IV.

**Organic fluorophores**

Most organic fluorescent agents with NIR excitation/emission profiles fit into one of four categories: cyanines, phthalocyanines/porphyrins/pyrroles (see section IV),[39] squaraines, or BODIPYs.[26] Other categories of dyes with non-NIR wavelengths have been well covered by Lavis and Raines.[24] A wide variety of absorbers and fluorophores have been cataloged and summarized in Table 1.
Table 1 – Absorption or excitation/emission characteristics and chemical/spectroscopic properties of imaging agents where $\lambda_{\text{abs}}$ is/are the absorption maximum/maxima and $\varepsilon$ is the molar extinction coefficient. Average extinction coefficients are given for some of the major classes, in the case the extinction coefficient for a specific compound is not available. Where multiple excitation and emission wavelengths are reported, the values of excitation maxima and emission maxima are given in order.

<table>
<thead>
<tr>
<th>Absorber</th>
<th>$\lambda_{\text{abs}}$ (nm)</th>
<th>$\varepsilon$ (M$^{-1}$ cm$^{-1}$)</th>
<th>Chemical Properties</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gold nanoparticles/nanorods/clusters</td>
<td>Broad 700-900 (size- and aspect ratio- dependent)</td>
<td>See [51, 83]</td>
<td>Thermo and/or pH sensitive coating, clustering</td>
<td>[46, 47, 84]</td>
</tr>
<tr>
<td>QSY 21 (FRET acceptor)</td>
<td>661</td>
<td>$&gt; 90k$</td>
<td>High chemical stability, resistance to photobleaching</td>
<td>[85-87]</td>
</tr>
<tr>
<td>Phthalocyanines/Porphyrins/Polypyrroles</td>
<td>&gt;100k</td>
<td></td>
<td></td>
<td>[26]</td>
</tr>
<tr>
<td>Large cyclic porphyrins</td>
<td>746, 773, 892, 939, 953</td>
<td>88k, 64k, 110k, 100k, 110k</td>
<td>Organic soluble</td>
<td>[71]</td>
</tr>
<tr>
<td>Polypyrroles</td>
<td>Broad 800 – 2500</td>
<td>See [88, 89]</td>
<td>Hydrophobic</td>
<td>[45, 56, 63-65]</td>
</tr>
<tr>
<td>Complexed bis-metal porphyrins</td>
<td>360-1210 depending on chemical modifications</td>
<td>-</td>
<td>Organic soluble</td>
<td>[72]</td>
</tr>
<tr>
<td>Dendrimer phthalocyanines</td>
<td>685 nm</td>
<td>-</td>
<td>Water soluble</td>
<td>[70]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>$\lambda_{\text{EX}}$ (nm)</th>
<th>$\lambda_{\text{EM}}$ (nm)</th>
<th>$\varepsilon$ (M$^{-1}$ cm$^{-1}$)</th>
<th>QY* (%)</th>
<th>Chemical Properties</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Water soluble but aggregates in water, binds to serum proteins. Poor photostability</td>
<td>[34, 35, 38, 40, 90-92]</td>
</tr>
<tr>
<td>Indocyanine Green (ICG)</td>
<td>775</td>
<td>831</td>
<td>113k*</td>
<td>1.3%</td>
<td>Water soluble</td>
<td>[34, 35]</td>
</tr>
<tr>
<td>Cy5</td>
<td>648</td>
<td>666</td>
<td>250k</td>
<td>18%</td>
<td>Water soluble</td>
<td>[19], [85], [24, 93]</td>
</tr>
<tr>
<td>Cy5.5</td>
<td>679</td>
<td>696</td>
<td>250k</td>
<td>24%</td>
<td>Water soluble</td>
<td>[19], [85, 93],</td>
</tr>
<tr>
<td>Dye</td>
<td>M &amp; λ (nm)</td>
<td>Molecular Weight (kDa)</td>
<td>MW (kDa)</td>
<td>Water Soluble</td>
<td>Notes</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>------------</td>
<td>------------------------</td>
<td>----------</td>
<td>---------------</td>
<td>--------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Cy7</td>
<td>745 775</td>
<td>250k</td>
<td>28%</td>
<td>Water soluble</td>
<td>[19], [85], [24, 93, 95, 96]</td>
<td></td>
</tr>
<tr>
<td>DiD</td>
<td>648 669</td>
<td>&gt;125k</td>
<td>33%</td>
<td>Hydrophobic with ionic groups so interfacially active</td>
<td>[85, 97, 98]</td>
<td></td>
</tr>
<tr>
<td>DiR</td>
<td>750 782</td>
<td>&gt;125k</td>
<td>28%</td>
<td>Hydrophobic with ionic side groups so interfacially active</td>
<td>[85, 97, 98]</td>
<td></td>
</tr>
<tr>
<td>IRDye 800CW</td>
<td>778 794</td>
<td>240k-300k†</td>
<td>-</td>
<td>Water soluble</td>
<td>LI-COR Biosciences Inc., Lincoln NE</td>
<td></td>
</tr>
<tr>
<td>IR Dye 750</td>
<td>766 776</td>
<td>260k-330k†</td>
<td>-</td>
<td>Water soluble</td>
<td>LI-COR Biosciences Inc., Lincoln NE</td>
<td></td>
</tr>
<tr>
<td>IR Dye 800RS</td>
<td>770 786</td>
<td>200k-300k†</td>
<td>-</td>
<td>Water soluble nucleic acid label, not salt tolerant</td>
<td>LI-COR Biosciences Inc., Lincoln NE</td>
<td></td>
</tr>
<tr>
<td>IRDye 650</td>
<td>651 668</td>
<td>230k-240k†</td>
<td>-</td>
<td>Water soluble</td>
<td>LI-COR Biosciences Inc., Lincoln NE</td>
<td></td>
</tr>
<tr>
<td>Heptamethine 3H-</td>
<td>782-786</td>
<td>807-814</td>
<td>220k-250k</td>
<td>10-15%</td>
<td>Good photostability</td>
<td>[99]</td>
</tr>
<tr>
<td>Indolenine cyanine dyes</td>
<td>Large Stokes shift cyanine derivatives</td>
<td>Bis(heptamethine cyanine dyes)</td>
<td>Heptamethine dyes with robust C-C bond at center</td>
<td>Alkyl-thioether derivatized cyanine dyes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------------------------------------</td>
<td>--------------------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01% - 1%</td>
<td>602, 617, 783</td>
<td>757, 803</td>
<td>50k, 70k, 200k</td>
<td>47, 38, 17%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>770, 800</td>
<td>785, 811</td>
<td>220k-240k</td>
<td>Water soluble, monofunctional (carboxyl group)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>777-823</td>
<td>812-847</td>
<td>116k-174k</td>
<td>Water soluble, monofunctional (carboxyl group)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Squaraines**

<table>
<thead>
<tr>
<th>Sulfonated squaraine derivatives</th>
<th>100k-300k</th>
<th>8-44%†</th>
<th>Hydrophilic and -phobic derivatives, photostable, conjugation ready</th>
</tr>
</thead>
<tbody>
<tr>
<td>737-780</td>
<td>751-820</td>
<td>~200k</td>
<td>Water soluble, monofunctional (carboxyl group)</td>
</tr>
</tbody>
</table>

**Bis-squaraines with pyrene/thiophene linker**

<table>
<thead>
<tr>
<th>Thiophene: 695, 727</th>
<th>Thiophene: 750, 790</th>
<th>Thiophene: 74k-240k</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrene: 636, 647</td>
<td>Pyrene: 757, 763</td>
<td>Pyrene: 68k-100k</td>
</tr>
<tr>
<td>8.8-10%</td>
<td>0.01% - 1%</td>
<td>Water soluble</td>
</tr>
<tr>
<td>8-74%†</td>
<td>8-74%†</td>
<td>Polar and nonpolar derivatives studied</td>
</tr>
</tbody>
</table>

**Tetralactam encapsulation of squaraines**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrene: 634-738</td>
<td>Pyrene: 634-738</td>
<td>Pyrene: 634-738</td>
</tr>
<tr>
<td>8-74%†</td>
<td>8-74%†</td>
<td>8-74%†</td>
</tr>
</tbody>
</table>

**BODIPYs**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer: 634-738</td>
<td></td>
<td>Polymer: 669-760</td>
<td>Polymer: 669-760</td>
<td></td>
<td>1.1-13%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heteroaryl fused BODIPYs</td>
<td></td>
<td>723, 509-690</td>
<td>738, 517-701</td>
<td>140k-316k</td>
<td>56%, 81-98%</td>
<td>Organic soluble dyes, good photostability</td>
<td>[110, 111]</td>
</tr>
<tr>
<td>BF₂-chelated tetraarylazadipyrromethenes</td>
<td></td>
<td>690-706</td>
<td>714-730</td>
<td>75k-95k</td>
<td>22-30%</td>
<td>Both partial and fully water soluble derivatives</td>
<td>[112, 113]</td>
</tr>
</tbody>
</table>

†: Indicates additional information not specified in the table.
<table>
<thead>
<tr>
<th>Product</th>
<th>Excitation</th>
<th>Emission</th>
<th>Mw (kDa)</th>
<th>Quantum Yield</th>
<th>Solubility</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BODIPY 650/665</strong></td>
<td>664</td>
<td>664</td>
<td>102k</td>
<td>46%</td>
<td>Nonpolar</td>
<td>[85, 114]</td>
</tr>
<tr>
<td><strong>Alexa Fluor 647</strong></td>
<td>650</td>
<td>668</td>
<td>270k</td>
<td>33%</td>
<td>Water soluble</td>
<td>[85, 115]</td>
</tr>
<tr>
<td><strong>Alexa Fluor 660</strong></td>
<td>664</td>
<td>691</td>
<td>130k</td>
<td>37%</td>
<td>Water soluble</td>
<td>[85, 115]</td>
</tr>
<tr>
<td><strong>Alexa Fluor 680</strong></td>
<td>680</td>
<td>704</td>
<td>180k</td>
<td>36%</td>
<td>Water soluble</td>
<td>[85, 115]</td>
</tr>
<tr>
<td><strong>Alexa Fluor 700</strong></td>
<td>694</td>
<td>720</td>
<td>190k</td>
<td>25%</td>
<td>Water soluble</td>
<td>[85, 115]</td>
</tr>
<tr>
<td><strong>Alexa Fluor 750</strong></td>
<td>752</td>
<td>776</td>
<td>240k</td>
<td>12%</td>
<td>Water soluble</td>
<td>[85, 115]</td>
</tr>
<tr>
<td><strong>Alexa Fluor 790</strong></td>
<td>784</td>
<td>814</td>
<td>260k</td>
<td>-</td>
<td>Water soluble</td>
<td>[85, 116]</td>
</tr>
<tr>
<td><strong>Nile Blue</strong></td>
<td>630</td>
<td>660</td>
<td>77k</td>
<td>27%†</td>
<td>Lipid soluble</td>
<td>[85]</td>
</tr>
<tr>
<td><strong>CellMask Deep Red Plasma Membrane Stain</strong></td>
<td>649</td>
<td>666</td>
<td>-</td>
<td>-</td>
<td>Carboxylate, sulfate, aldehyde, amine surfaces available</td>
<td>[85]</td>
</tr>
<tr>
<td><strong>FluoSpheres Dark Red</strong></td>
<td>657</td>
<td>683</td>
<td>-</td>
<td>-</td>
<td>Carboxylate, sulfate, aldehyde, amine surfaces available</td>
<td>[85]</td>
</tr>
<tr>
<td><strong>FluoSpheres Infrared</strong></td>
<td>715</td>
<td>755</td>
<td>-</td>
<td>-</td>
<td>Carboxylate, sulfate, aldehyde, amine surfaces available</td>
<td>[85]</td>
</tr>
<tr>
<td><strong>FocalCheck Double FarRed</strong></td>
<td>669</td>
<td>693</td>
<td>-</td>
<td>-</td>
<td>Water soluble</td>
<td>[85]</td>
</tr>
<tr>
<td><strong>HCS CellMask DeepRed Stain</strong></td>
<td>648</td>
<td>670</td>
<td>-</td>
<td>-</td>
<td>Water soluble</td>
<td>[85]</td>
</tr>
<tr>
<td><strong>HCS NuclearMask DeepRed</strong></td>
<td>Broad 638</td>
<td>Broad 685</td>
<td>-</td>
<td>-</td>
<td>Water soluble</td>
<td>[85]</td>
</tr>
<tr>
<td><strong>LIVE/DEAD Fixable NIR Cell Stain</strong></td>
<td>753</td>
<td>776</td>
<td>-</td>
<td>-</td>
<td>Water soluble</td>
<td>[85]</td>
</tr>
<tr>
<td><strong>MitoTracker DeepRed Stain</strong></td>
<td>640</td>
<td>662</td>
<td>-</td>
<td>-</td>
<td>Mitochondria stain</td>
<td>[85]</td>
</tr>
<tr>
<td><strong>Qnuclear Deep Red Stain</strong></td>
<td>642</td>
<td>656</td>
<td>-</td>
<td>-</td>
<td>Water soluble</td>
<td>[85]</td>
</tr>
<tr>
<td><strong>SYTO 60</strong></td>
<td>649</td>
<td>681</td>
<td>&gt;50k</td>
<td>16%</td>
<td>Water soluble</td>
<td>[85]</td>
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<tr>
<td><strong>SYTOX</strong></td>
<td>641</td>
<td>658</td>
<td>-</td>
<td>-</td>
<td>Water soluble</td>
<td>[85]</td>
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<tr>
<td><strong>TetraSpeck Dark Red Dye</strong></td>
<td>656</td>
<td>684</td>
<td>-</td>
<td>-</td>
<td>Water soluble</td>
<td>[85]</td>
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<td>TO-PRO-3</td>
<td>TOTO-3</td>
<td>Vybrant DyeCycle Ruby</td>
<td>DRAQ5, DRAQ7</td>
<td>X-Sight 650 Nanospheres†</td>
<td>X-Sight 691 Nanospheres†</td>
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<tr>
<td>Code</td>
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<td>643</td>
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<td>650</td>
<td>691</td>
</tr>
<tr>
<td>Code</td>
<td>657</td>
<td>660</td>
<td>Broad 686</td>
<td>Broad 665-800</td>
<td>673</td>
<td>715</td>
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<tr>
<td>Emission Wavelength</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>21k</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Average Photostability</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.3-0.4%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Photostability</td>
<td>Water soluble</td>
<td>Water soluble</td>
<td>Water soluble</td>
<td>Water soluble</td>
<td>Biocompatible, antibody conjugatable</td>
<td>Biocompatible, antibody conjugatable</td>
</tr>
<tr>
<td>Supplier</td>
<td>[85]</td>
<td>[85]</td>
<td>[85]</td>
<td>Biostatus Ltd., Leicestershire, United Kingdom,[118, 119]</td>
<td>Carestream Health, Inc., Rochester, NY</td>
<td>Carestream Health, Inc.</td>
</tr>
<tr>
<td>Material</td>
<td>Excitation Range</td>
<td>Emission Range</td>
<td>Stokes Shift</td>
<td>Quantum Yield</td>
<td>Notes</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------</td>
<td>----------------</td>
<td>--------------</td>
<td>---------------</td>
<td>-------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Quantum dots</td>
<td>Broad visible</td>
<td>555-850</td>
<td>500k-2M</td>
<td>60%</td>
<td>Photostable, nonquenching</td>
<td>[44, 73, 77]</td>
</tr>
<tr>
<td>Zn²⁺-multiporphyrin</td>
<td>Tunable: 410, 500, 690-800</td>
<td>Tunable: 697-817</td>
<td>23k-126k</td>
<td>NR</td>
<td>Depends on side-chains, both hydrophobic</td>
<td>[39]</td>
</tr>
<tr>
<td>IRDye 700DX</td>
<td>680</td>
<td>687</td>
<td>170k</td>
<td>14%</td>
<td>High photostability, water soluble, acid sensitive</td>
<td>LI-COR Biosciences Inc., Lincoln, NE, [24]</td>
</tr>
<tr>
<td>Single wall carbon</td>
<td>Broad 600-800</td>
<td>Broad 950-1300</td>
<td>See [124, 125]</td>
<td>0.01 – 0.1%</td>
<td>Dispersible in water with Pluronics surfactant, very photostable</td>
<td>[125-130]</td>
</tr>
<tr>
<td>nanotubes</td>
<td></td>
<td></td>
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<td></td>
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</table>

*QY = quantum yield

ICG’s extinction coefficient is highly dependent on concentration and solvent. Here we chose 5 mg/L in water.

† solvent polarity dependent

‡ The X-Sight line of fluorescent spheres has been discontinued but has been included here for reference.

NR = Not reported
Cyanines

To date, the only FDA approved long wavelength dye for direct administration in medical diagnostics is indocyanine green.[19, 132] It is one of the dyes in the cyanine family (see Fig. 10), and can be used as a fluorescent agent (see Table 1) or a NIR absorption agent.[133] Cyanines are characterized by two aromatic nitrogen containing heterocycles connected by a polymethine bridge. This length of the polymethine bridge is the key to tuning the excitation/emission profile of the dye. For example, Cy3 (3 methine protons) emits visible light while Cy5 (5 methine protons) and Cy7 (7 methine protons) emit in the far-red and NIR regions.[27] Cyanines tend to be strong absorbers with molar extinction coefficients greater than 200,000 M⁻¹ cm⁻¹ but weak fluorescers with quantum yields from 1 – 18%. However, recent work has yielded positive exceptions to this generalization, as seen in Peng, et al.[100] Extending the polymethine bridge tends to lower quantum yields but shifts emission into the NIR.[19, 26] In addition, it has been found that the addition of the cyclohexenyl moiety at the center of the polymethine bridge as well as complexation with proteins tends to raise the
quantum yield and stability of the dyes.[26, 101] This effect is generally attributed to ‘rigidizing’ the backbone.[102] On the other hand, Chen, et al. substituted various electron donating or withdrawing groups at the N position on the 3H-indolenine rings (rather than modifying the polymethine bridge). They found that electron donating groups offered greater photostability to the molecule while withdrawing groups decreased stability.[99]

Peng, et al.[100] synthesized novel heptamethine cyanine dyes through modification of the central chlorocyclohexenyl group resulting in larger Stokes shifts (>140 nm) and higher fluorescence (several-fold higher quantum yield) compared to commercially available heptamethine cyanine dyes. However, the dyes were significantly blue shifted with lower molar extinctions. Lee, et al. have also developed a novel series of cyanine dyes by reacting the same group to add a carboxyl containing moiety to the central cyclohexenyl group, thus improving water solubility and offering a site for bioconjugation.[103] Their dyes, in contrast, were red-shifted (both absorption and emission) with lower quantum yields.

Fig. 11 – Examples of squaraines (adapted from Umezawa[105] and Nakazumi[107]).

Squaraines
Squaraines are a class of dyes consisting of an oxocyclobutenolate core with aromatic or heterocyclic components at both ends of each molecule (see Fig. 11).[26] They are characterized by high extinction coefficients (10^4-10^5 M\(^{-1}\) cm\(^{-1}\)), quantum yields (>80%), and
However, their planar, highly conjugated architecture results in dyes with poor water solubility. Thus, improving water solubility is an important goal for many researchers in this area. Umezawa, et al. synthesized sulfonated squaraine derivatives with excellent water solubility and emission wavelengths above 800 nm. In addition, they found that adsorption onto BSA proteins greatly increased the fluorescent output, similar to the cyanines. However, the process of sulfonation and the presence of highly polar water reduced the quantum yield to 8% whereas similar nonpolar compounds had quantum yields >40% in organic solvents. Water soluble squaraines were also synthesized by Nakazumi, et al. by forming bis-squaraines separated by pyrene or thiophene linkers and carboxyl end groups. They, too, found that the fluorescence and stability of squaraines improved when bound to HSA or BSA. However, quantum yields were very low, on the order 0.01 to 1% and emission wavelengths were 750-790 nm.

Gassensmith, et al. have taken a different approach to improving water solubility through the encapsulation of squaraines within tetralactam macrocycles. This has the dual effect of preventing aggregation in aqueous solution and protecting the squaraines from chemical attack though they are relatively stable species even without encapsulation. Encapsulated species showed emission bands from 650-700 nm with relatively high quantum yields in nonpolar solvents which decreased in the presence of water or other polar solvents. In vivo imaging was demonstrated by injected squaraine-labeled E. coli and S. aureus into live nude mice and observing the fluorescent emission.
BODIPYs

BODIPY (borondipyrromethene) class dyes were originally discovered in 1968 by Treibs and Kreuzer[134] and have received much attention as agents for biomedical imaging (see Fig. 12). They generally have sharp absorbance and emission profiles and high quantum yields approaching 100%, and have solvent and pH insensitive emission profiles with good stability under physiological conditions.[25] However, they have low extinction coefficients (80,000 M$^{-1}$ cm$^{-1}$) and very few are water soluble.[26] Furthermore, most of them emit below 600 nm, making them unsuitable for deep tissue imaging.[25] Small chemical modifications to the basic BODIPY structure can cause large shifts in the emission profile as well as improving water solubility and the extinction coefficient. Donuru, et al. have taken the approach of synthesizing novel polymeric and copolymeric BODIPY dyes, tuning the emission properties by introducing styryl groups, thus extending the π conjugated system. These hydrophobic polymeric dyes emit from 669 - 760 nm with quantum yields from 1.1 – 13%.[109]

The Suzuki group has taken a variety of approaches, synthesizing a whole range of BODIPY based monomers with a variety of side groups attached to the core BODIPY moiety. The first set of dyes were heteroaryl-fused BODIPYs with tuned emission wavelengths from 583-738 nm and
greatly improved extinction coefficients above 185,000 M$^{-1}$ cm$^{-1}$.[111] In 2009, this strategy was extended to the addition of several types of electron donating moieties as well as the synthesis of asymmetric BODIPY derivatives. The new set of dyes had emission wavelengths as long as 701 nm with high quantum yields (>80%) and extinction coefficients as high as 300,000 M$^{-1}$ cm$^{-1}$.[110] These dyes exhibited excellent photostability but do not appear to be water soluble.

Tasior and O’Shea, on the other hand, have synthesized water soluble and partially water soluble BODIPY based dyes with easily conjugated side groups.[112, 113] The main strategies involved attaching sulfonate or carboxylate terminated alkyl chains or the introduction of quaternary ammonium salts to the molecule to impart charge. These compounds were red shifted into the NIR range with an emission range of 718-730 nm in phosphate buffered saline with bovine serum albumin. Extinction coefficients and quantum yields were lower across the board than the non-water soluble BODIPYs, ranging from 51k-89k M$^{-1}$ cm$^{-1}$ and 22 – 31%, respectively. Nonetheless, these studies represent a step in a positive direction for biomedical imaging.
Perylene Derivatives

Falling somewhat outside the major categories stated above, Quante, et al. synthesized a set of perylenetetracarboxdiimide derivatives with highly extended \( \pi \)-conjugated systems (see Fig. 13).

This class of dyes is generally characterized by excellent chemical, thermal and photostability[121, 135] but has limited solubility in both organic and aqueous solvents and somewhat low extinction coefficients (50,000 M\(^{-1}\) cm\(^{-1}\)). Red-shifting was primarily accomplished by extension of the \( \pi \) system as well as the introduction of halogens to induce bathochromic shifts. The resulting dyes exhibited long wavelength emission in the range 636 – 768 nm and improved organic solubility without a major loss of stability. Quantum yields were not, however, reported.[120] For aqueous applications, Qu, et al. reported the synthesis of water soluble perylenetetracarboxdiimide derivatives by introducing charged groups such as sulfonates. The ionic dyes showed good aqueous solubility and photostability with quantum yields in excess...
of 50%. However, extinction coefficients were low \((10,000 – 30,000 \text{ M}^{-1} \text{ cm}^{-1})\) and emission wavelengths reached a maximum of only 619 nm, outside of the NIR range.\[121\]

Fig. 14 – Carbon nanotube (CNT) fluorescence emission after uptake into a macrophage-like cell (reproduced from Cherukuri, et al.)\[126\]

**Carbon Nanotubes**

A rather unexpected development involves the possibility of using single walled carbon nanotubes (SWCNT) as NIR fluorescence agents. The Weisman lab conducted numerous fundamental as well as biological studies involving these materials.\[126, 128, 129\] The nanotubes in the studies tend to be on the order of nanometers in diameter but 100 nm in length and exhibit a number of excitation bands from 600 – 800 nm and emission bands from 950 – 1300 nm. Their quantum yields were measured by Huang, et al. and found to be 0.01 – 0.1%, orders of magnitude lower than many organic fluorophores. Despite this limitation, however, they report that similar signal to noise ratios were achieved due to correspondingly larger
absorption coefficients.[127] In Cherukuri, et al., pristine hydrophobic SWCNTs 1 nm in diameter and 1μm in length were taken up into phagocytic cells at approximately 1 nanotube per second per cell (Fig. 14). They were found to be photostable after 100 min of tracking and did not cause any cytotoxic effects. However, the nanotubes were stabilized by Pluronics surfactant in order to prevent aggregation in vitro. It is not clear whether that strategy would be efficient in vivo.[126] The use of functionalized nanotubes has been reported by Pantarotto, et al. for the delivery of DNA to HeLa cells. Their 20 nm diameter, 200 nm length nanotubes were functionalized with ammonium terminated oligoethylene glycol chains imparting an overall positive charge and making them water soluble. These nanotubes were complexed with negatively charged plasmid DNA which led to the formation of supramolecular structures such as spheres, toroids or supercoils. The nanotubes were readily taken up into HeLa cells and 5 – 10 fold increases in gene expression were observed compared to DNA treatment alone. Undoubtedly, the cationic charge facilitated nanotube penetration as the delivery mechanism was determined to be passive, not active.[130]

**Fluorescent Proteins**

The development of Green Fluorescent Protein (GFP) revolutionized the study of diseases, cancer in particular, providing insights into real-time growth and metastatic behaviors.[23] In recent years, development of fluorescent proteins has proceeded to the point that researchers now have a variety of proteins as molecular probes spanning the visible spectrum.[136-139] For whole animal imaging applications, bacterial phytochromes provide the best scaffold for NIR excitation and emission wavelengths, as opposed to a GFP-like scaffold.[140] Phytochromes are photosensory receptors that absorb light at far-red and near-infrared wavelengths. According to
Shu, et al., *Rhodopseudomonas palustris* and *Pseudomonas aeruginosa* bacteriophytochromes expressed in *E. coli* have been found to emit photons from 710 to 725 nm. Their engineered proteins derived from *Deinococcus radiodurans* bacteriophytochromes excite at 684 nm and emit at 708 nm with >90k M$^{-1}$ cm$^{-1}$ extinction coefficient and 7% quantum yield.[123] Filonov, et al. have engineered NIR fluorescent proteins from the *Rhodopseudomonas palustris* phytochrome basis. The best variant they produced has maxima at 690 nm (ex) and 713 (em) and an aqueous quantum yield of 5.9%. The photostability of the protein is rather low, exhibiting a 450 s half-life under photobleaching light irradiation. However, it appears to have a higher brightness than the constructs of Shu, et al.[122] Both proteins are reliant on cofactors (such as biliverdin IXα, readily available as an intermediate in mammalian heme metabolism) for maximum emission.[122] Clearly, there is much promise and as yet untapped potential for the development of long wavelength protein fluorophores. The ability of these agents to be inserted into genomic or plasmid DNA sequences to monitor cell metabolism and expression pathways makes them invaluable for biomedical research.

**Future Perspectives: Multifunctional NPs for Diagnostics and Delivery**

While the previous parts focused on the development of fluorophores, we now turn our attention to the means by which fluorophores can be delivered or targeted for maximum efficacy. When the goal is diagnostic or fundamental biology, it is often enough to have a targeting agent (ligand, antibody, etc.) directly attached to the fluorophore or contrast agent. That has driven the effort to synthesize aqueous-soluble fluorophores, as has been mentioned above. However, there are significant advantages for diagnostics and imaging to place the imaging agent in a nanocarrier
format. The larger sizes of nanocarriers increase circulation times, if they are sterically well protected. Increased circulation time translates to better targeting in vivo and in vitro. Most importantly, a single 100 nm particle may carry 15,000 dye molecules, therefore brightness per particle is greatly enhanced over single molecule delivery. The location of the imaging agent in the nanocarrier influences brightness. If the imaging agent is on the surface of the particle, increasing size decreases the brightness per mass of imaging agent since the surface-to-volume ratio decreases with increasing size. Therefore, nanoparticles with dye localized in a hydrophobic core have several advantages: (1) the possibility of increased brightness due to the core volume increasing linearly with mass, (2) a large variety of dyes that cannot be made in hydrophilic form, or which are brighter in hydrophobic form, (3) decreased interactions with the biological environment and less sensitivity of emission to environment, and (4) increased stability against photobleaching or oxidative degradation.

For therapeutic applications, the requirement of multifunctionality can involve delivery of an active agent, targeting, and visualization of delivery. This generally requires the construction of nano-carriers that incorporate these functions. Completely soluble nanocarrier systems include linear polymer chains with grafted pro-drugs, targeting ligands, and imaging agents.[141] Dendrimers, highly branched polymeric structures, have been attractive multifunctional nanocarriers because their high degree of functionality makes multifunctional conjugation possible. However, sequential functionalization of soluble nanocarriers has made precise quantification of the functionalization of each carrier (as opposed to the average composition) difficult. This has been a stumbling block to regulatory approval for some of these materials.
Insoluble nanocarriers have some advantages in more precise control of composition. They also offer particular advantages for NIR imaging in that the hydrophobic cores of nanocarriers enable the encapsulation of hydrophobic imaging agents. The greater rigidity of the environment increases quantum efficiency, decreases photobleaching,[36] and decreases quenching.[36, 142] The insoluble carriers involve either self-assembled micelle structures, or precipitated nanostructures, or carriers made by emulsification and then solvent stripping.

**Silica Nanocarriers**

Benezra, et al. have developed novel nanoparticles that encapsulate fluorescent dyes within an amorphous silica shell that is coated with PEG for increased circulation time as well as $^{124}$I-cRGDY-PEG to allow for multimodal imaging and $\alpha_\text{v}\beta_3$ integrin targeting. The core of the nanoparticle contains the dye Cy5 (see **Table 1**) while the $^{124}$I allows for a second imaging modality through PET. These particles are about 7 nm in diameter and thus very small compared to most liposomes and polymeric nanoparticles. This small size allows for fast renal clearance and wide biodistribution but the presence of PEG and targeting moieties increase circulation time to give a blood half-life of 5.6 hours while excretion analysis showed that ~50% of the particles are eliminated in the first 24 hours in mouse tumor models. Though a longer wavelength fluorescent dye would have improved the penetration depth, Cy5 fluorescence imaging allowed the authors to elucidate sub-millimeter nodal features that PET could not, though PET may be better for deeper penetration (> 5 cm) and quantitation since it has no depth limit. These particles have been given FDA IND approval for clinical trials.[143]
Micellar Nanocarriers

Micellar nanocarriers involve an amphiphilic block copolymer which defines the nanocarrier size, the hydrophobic micelle core where drugs and fluorophores can be captured, and the hydrophilic corona which can confer biocompatibility and targeting.[144, 145]

A particularly interesting imaging agent was developed by Rodriguez, et al. consisting of ICG complexed to quaternary ammonium salts to render them hydrophobic, and enabling encapsulation within 30 nm block copolymer micelles (Fig. 15). This process improved the thermal- and photo-stability of the ICG with a modest red shift in the spectra. Encapsulation within micelles also has the potential to greatly improve in vivo circulation times by preventing plasma protein binding, a key weakness of free ICG. Lastly, this method allows for targeting of the micelles through protein or small molecule conjugation to the hydrophilic block of the copolymer micelles.[40]
Ghoroghchian, et al. have constructed 50 nm – 20 μm size polymersomes built from self-assembled poly(butadiene)-b-poly(ethylene oxide) (PBD-b-PEO) amphiphilic block copolymers. The Zn$^{2+}$-porphyrin fluorophores varied in macrocycle-macrocycle linkage topology, allowing for fine tuning of the emission from 600 – 825 nm. These compounds were stably incorporated into the polymersome membranes, protecting them from chemical attack and potentially improving circulation times in vivo. They were found to be well distributed throughout the membrane without phase separation though modulation of the hydrophobicity of the porphyrin complexes moved the complexes from the hydrophobic interior to the PBD-PEO interface.[39] The quantum yield is expected to be high (> 80%) given the yields of similar compounds discussed earlier.
**Fig. 16** – *Flash NanoPrecipitation* schematic. Rapid mixing of the solvent and nonsolvent streams causes a drop in solvent quality and subsequent precipitation of the hydrophobic solutes. By matching the aggregation ($t_{\text{agg}}$) and nucleation and growth ($t_{\text{ng}}$) time scales (which are both larger than the mixing time scale $t_{\text{mix}}$), homogeneous nucleation kinetics result and polymer stabilized nanoparticles from 30 – 800 nm with narrow size distributions are produced. This scalable method allows for rapid, inexpensive, and versatile encapsulation of various hydrophobic molecules.[146, 147]

**Precipitated Nanoparticle Carriers**

Akbulut, et al. utilized the versatile Flash NanoPrecipitation (FNP) process to encapsulate a variety of fluorophores (pyrene, anthracene, nile red, porphirine) with emissions ranging from 370 to 720 nm. Briefly, this mechanism of nanoparticle formation (**Fig. 16**) involves the intense micromixing of an organic stream containing a dissolved hydrophobic fluorophore and amphiphilic block copolymer such as poly(caprolactone)-$b$-poly(ethylene glycol) (PCL-$b$-PEG).
against a large excess of non-solvent such as water. The resulting drop in solvent quality causes rapid precipitation of the fluorophore while the rapid mixing time ensures uniform growth kinetics. Tuning of the nucleation and growth time scale against the copolymer aggregation time scale ensures that the block copolymer anchors (i.e. adsorbs) to the hydrophobic core surface at the right time to allow nanoparticle production from 30 – 800 nm in size with narrow size distributions.[36, 146] In addition to fluorescent molecules, this process has been applied successfully to encapsulate a variety of drugs, inorganics, and peptides.[37, 142, 148-153] The stealth PEG coating improves circulation time in vivo and provides steric protection against aggregation.[148] The fluorescent nanoparticles in this study were found to be bright with increased photostability over free dye, probably due to molecule immobilization and copolymer protection. The copolymer chains can be conjugated to targeting ligands such as antibodies[150] to provide targeting capabilities. Additionally, Gindy, et al. showed that more than one type of solute can be encapsulated within these nanoparticles cores, thus the possibility of diagnostic and therapeutic functions in one package – theranostics.[151] The main requirement for the fluorophore in this process is that they must be hydrophobic for maximum stability. If they are not, chemical modification is often necessary, as demonstrated by Akbulut, et al.[36, 148]
As mentioned earlier, Budijono, et al. and Shan, et al. have demonstrated the feasibility of encapsulating inorganic upconverting phosphors alongside tetraphenylporphyrin photosensitizer using the Flash NanoPrecipitation process (Fig. 17). The ~200 nm particles produced were stabilized with poly(lactic acid)-b-poly(ethylene oxide) and found to be stable under physiological conditions. More importantly, they demonstrated strong cancer cell killing activity from the production of reactive oxygen species (ROS) under 980 nm illumination. While the UCP emission band is largely absorbed by the porphyrin photosensitizer, fluorescence from the UCPs can be detected for diagnostic purposes making this system multifunctional, though targeting studies have not yet been conducted.[37, 79]
Fig. 18 – Novel theranostic agent. A – schematic of nanoparticles containing a ~70 nm Au nanoshell with a silica shell doped with superparamagnetic iron oxide and ICG and surface-decorated with anti-HER2 antibodies for targeting. B – magnetic resonance imaging of the nanoparticles in vitro (no scale bar provided). C – photothermal ablation capabilities of the theranostic system demonstrated in vitro. D – fluorescence visualization of the nanoparticles after targeted uptake into OVCAR3 cells. Reproduced from Chen, et al.[38]

In addition to UCP-photosensitizer pairing to enable deep tissue ROS generation, another hybrid system involves the pairing of gold structures with NIR emitting fluorophores such as indocyanine green (ICG). As reported by Tam, et al. and previously discussed, gold nanoparticles exhibit a size- and morphology-dependent surface plasmon resonance absorption band centered at 520 nm for 5 nm spherical gold nanoparticles. Increasing the size of these spheres or using nanorods or shells can shift the absorption band into the NIR region. Placing a molecular fluorophore such as ICG directly onto a metal surface generally results in quenching. However, maintaining a few nanometers separation and matching the plasmon resonance of ~120 nm gold nanoshells with the emission band of the ICG, the fluorescence was enhanced by a
factor as high as 50, greatly improving the effective quantum yield.\cite{52, 154} This effect stems primarily from the effect of an additional radiative decay rate introduced by the metal surface:

\[ Q_0 = \frac{\Gamma}{\Gamma + k_{nr}} \]  \hspace{1cm} \textbf{(Eqn. 1)}

\[ Q_m = \frac{\Gamma + \Gamma_m}{\Gamma + \Gamma_m + k_{nr}} \]  \hspace{1cm} \textbf{(Eqn. 2)}

- $Q_0, Q_m$ – original, modified quantum yield
- $\Gamma$ – radiative decay rate
- $\Gamma_m$ – metal induced radiative decay rate
- $k_{nr}$ – nonradiative decay rate

This additional radiative decay $\Gamma_m$ in \textbf{Eq. 2} (compared to \textbf{Eq. 1}) increases the rate of resonance energy transfer and improves the quantum yield.\cite{155} This effect was exploited by Chen, et al. to formulate a multifunctional theranostic system for ovarian cancer diagnosis and treatment (\textbf{Fig. 18}). Briefly, a ~70 nm gold nanoshell is encapsulated in a silica shell which is doped with superparamagnetic iron oxide (SPIO) and ICG. This system benefits from the gold-enhanced fluorescence of ICG, an additional MRI imaging mode using SPIOS, and a gold shell to convert absorbed light to heat for photothermal cancer cell ablation. Finally, anti-HER2 antibodies were chemically conjugated to the nanocomplexes for targeting functionality. These particles were found to be bright and effective in reducing populations of OVCAR3 ovarian cancer cells \textit{in vitro} through the photothermal effect after being taken up via HER2 receptor-mediated uptake.
routes.[38] Though in vivo results were not shown, these theranostic complexes seem very promising as the next generation in pharmaceutical paradigms.

Conclusions

Combining increasingly bright and stable NIR fluorophores with effective delivery systems that enhance the fluorescence, circulation time, and photochemical stability of encapsulated fluorescent molecules is already providing powerful new tools for fundamental biological studies as well as diagnostic applications. With the addition of drug payloads and targeting capabilities, multifunctional ‘theranostic’ agents will be able to provide a complete solution for effective diagnostics and disease therapy. These agents will combine the best of what synthetic organic chemistry, pharmaceutical- and bio-engineering have to offer to create a new paradigm in the biomedical sciences.

Acknowledgments

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CHAPTER 2
Biomedical Fluorescence Imaging using Pentacene Dyes


Abstract

Purpose
In the rapidly expanding field of biomedical imaging, there is a need for nontoxic, photostable, and nonquenching fluorophores for fluorescent imaging. We have successfully encapsulated a new, extremely hydrophobic, pentacene-based fluorescent dye within polymeric nanoparticles (NPs) or nanocarriers (NCs) via the Flash NanoPrecipitation process.

Procedures
Nanoparticles and dye-loaded micelles were formulated by Flash NanoPrecipitation and characterized by dynamic light scattering, fluorescence spectroscopy, UV-vis absorbance spectroscopy, and confocal microscopy.

Results
These fluorescent particles were loaded from less than 1% to 78% by weight core loading and the fluorescence maximum was found to be at 2.3 wt%. The particles were also stably formed at 2.3% core loading from 20 nm up to 250 nm in diameter with per-particle fluorescence scaling linearly with the NC core volume. The major absorption peaks are at 458, 575, and 625 nm; and the major emission peaks at 635 and 695 nm. In solution, the Et-TP5 dye displays a strong concentration-dependent ratio of the emission intensities of the first two emission peaks, whereas in the nanoparticle core the spectrum is independent of concentration over the entire concentration range. A model of the fluorescence quenching was consistent with Förster resonant energy transfer as the cause of the quenching observed for Et-TP5. The Förster radius calculated from the absorption and emission spectra of Et-TP5 is 4.1 nm, whereas the average dye spacing in the particles at the maximum fluorescence is 3.9 nm.

Conclusions
We have successfully encapsulated Et-TP5, a pentacene derivative dye previously only used in light-emitting diode applications, within NCs via the Flash NanoPrecipitation process. The extreme hydrophobicity of the dye keeps it encapsulated within the NC core, its extended pentacene structure gives it relatively long wavelength emission at 695 nm, and the pentacene structure, without oxygen or nitrogen atoms in the core structure, makes it highly resistant to photobleaching. Its bulky side groups minimize self-quenching and localization within the nanoparticle core prevents interaction of the dye with biological surfaces or molecules in diagnostic assays. Loading of dye in the NC core allows 25 times more dye to be delivered than if it were conjugated onto the nanocarrier surface. The utility of the dye for quantifying nanoparticle binding is demonstrated. Studies to extend the wavelength range of these pentacene dyes into the near infra-red are underway.
INTRODUCTION

Fluorescent imaging impacts every field of biomedical science.[1] Nanocarriers (NCs) and nanoparticles (NPs) are of interest as therapeutic delivery agents,[2-5] as targeted imaging agents for diagnosis,[6-10] and in the dual role in therapy and diagnosis as “theranostic” agents.[11-16] The desirable characteristics of a fluorescent imaging agent are brightness, resistance to photobleaching, insensitivity to the microenvironment being targeted, lack of toxicity, and a wavelength range that avoids tissue auto-fluorescence. These characteristics apply to both soluble fluorophores and fluorophores in NC constructs. Most often NCs for therapeutic applications are rendered fluorescent by post-functionalizing the surface of the NC with a fluorescent dye.[17-24] There are two concerns about this functionalization. First, the number of “sites” for surface attachment is far less than the number of sites that are available internally in the NC. For example, a 100 nm PEG protected NC can contain roughly 390,000 molecules with a volume of 1.35 nm$^3$ (i.e. the volume of 840 Dalton organic molecule such as Et-TP5 (2,2,10,10-tetraethyl-6,14-bis-(triisopropylsilyl)ethynyl)-1,3,9,11-tetraoxa-dicyclopenta[b,m]pentacene)); whereas, there will be only 15,000 surface PEG groups onto which the fluorophore could be conjugated.[25] Moreover, not all of these surface sites are available since self-quenching occurs when more than 2-10% of the PEG chains are functionalized.[26] Furthermore, it is known that ligand targeting of NCs occurs with as little as a few percent surface functionalization.[27] Attachment of charged and aromatic fluorophores on the NC surface at these levels may interfere with the desired targeting.
Rather than surface functionalization, it is desirable to have the fluorophore inside the NC, to increase brightness and decrease interference with targeting. Quantum dots and latex spheres with imbibed fluorophores are examples of systems with internal fluorescence. Latex spheres, while good for imaging and diagnostics, are not readily adapted to efficient drug delivery. The potential toxicity of the heavy metals in quantum dots limits their utility to \textit{in vitro} and animal \textit{in vivo} diagnostics.

In this contribution we report on a new family of fluorescent dyes that are uniquely suited for therapeutic and diagnostic applications. Their properties are ideally matched to our block-copolymer-directed nanoparticle formation process, Flash NanoPrecipitation (FNP). They enable single-step formation of NCs with high drug loadings, bright and stable fluorescence and targeting capabilities. The dyes are based on the pentacene fluorophore structure. They were originally synthesized primarily for light-emitting diode applications\cite{28-33} and were designed for resistance to photobleaching, and minimal quenching in the crystalline state. They have not been used previously for biological imaging. Bulky silicon-containing substituents and orthogonally-disposed side groups provide steric hindrance that frustrates close molecular stacking and reduces self-quenching via Förster Resonance Energy Transfer (FRET).\cite{34-37} In addition, they are highly hydrophobic which makes them ideal for encapsulation via Flash NanoPrecipitation (FNP) into the core of the NC.\cite{38-40}

We present the formation of nanocarriers (NCs) and polymeric micelles with pentacene fluorophores via Flash NanoPrecipitation (FNP). The optimization of fluorescence emission per particle is determined by varying the dye concentration in the NC core. We show that the
maximum in fluorescence intensity is predicted from a calculation of the intermolecular distance at which FRET quenching is expected to be significant. Finally, we demonstrate the use of the NC in *in vitro* imaging which was done in our group and published in D’addio, et al.,[27] where the fluorescence of the NCs enables confocal imaging without significant autofluorescence from proteins, and the hydrophobicity of the dye enables quantification by a solvent-stripping technique. We also present preliminary results showing the utility of ETTP5 and the pentacene/hexane family of dyes in whole animal imaging.

**Materials and Methods**

*EXPERIMENTAL REAGENTS*

Et-TP5 was prepared as previously reported.[33] Hydroxyl-terminated poly(styrene) (PS_{1500-OH}) and poly(styrene)-b-poly(ethylene glycol) (PS_{1500-b-PEG_{5000}}) were synthesized as described by Adamson.[41] PS_{1600-b-PEG_{5000}} was obtained from Polymer Source (P13141-SEO, Polymer Source, Montreal, Canada) and used as received. Ultra pure water was obtained from a Barnstead Nanopure unit (Thermo Fisher Scientific, Hampton, NH) giving >17.7 MΩ-cm purity. HPLC grade tetrahydrofuran (THF, T425-4) and HPLC grade toluene (T290-4) were obtained from Fisher Scientific, Hampton, NH and used as received.

*Measurement of I₁/I₂ ratios for Et-TP5 dissolved in toluene*
Et-TP5 was dissolved in toluene at 1 mg/mL and pipetted into a glass cell (101-10-20, Hellma Analytics, Plainview, NY) which was placed into a fluorescence spectrophotometer sample holder (Hitachi F-7000 Fluorescence Spectrophotometer, Hitachi High Technologies, Schaumburg, IL). The slit widths were set to 5 nm and the PMT voltage to 400 V. The fluorescence spectra were taken at both 458 nm and 600 nm excitation to calculate the $I_1/I_2$ ratio. There was no shift in the emission spectrum between the two excitation wavelengths (AppD-Fig. 5) and $I_1/I_2$ ratios were the same as well.

**FNP – PS Core Nanocarriers**

The basis of the FNP process is that the hydrophobic core components (therapeutic agents, neutral fillers, dyes, or hydrophobic colloids) are dissolved in a water-miscible organic solvent. This is rapidly mixed against an aqueous phase in a novel mixing cavity to produce a homogeneous solution on a time scale on the order of 1.5 ms.[42] The rapid micromixing produces a uniform, highly supersaturated solution where the hydrophobic components are at supersaturations as high at 10,000.[3] High nucleation rates and growth by diffusion-limited aggregation[43] enables the formation of nanometer-sized particles at high loadings.[25] The hydrophobic block of the sterically stabilizing polymer adsorbs on the surface of the growing NC, and when sufficient polymer density has been achieved, particle aggregation is arrested. The technique enables the stoichiometric incorporation of multiple drugs, or blends of drugs and imaging agents,[44] with control of particle size over the range of 60 to 400 nm.[2, 38, 44]
There are two mixing geometries that have been developed. A Multi Inlet Vortex Mixer (MIVM)[45] provides for the mixing of several input streams, which enables independent control of the organic and aqueous stream volumes. The control of stream ratios allows the control of supersaturation ratios and greater control of particle stability. This geometry allows scale-up to larger volume production. A Confined Impinging Jet (CIJ)[42, 46, 47] mixer has two inlet streams that must be introduced at equal flow velocities to obtain optimal mixing. The higher organic solvent content in the final stream can produce particle instability. By diluting the exit stream of the CIJ into additional aqueous phase this can often be avoided. An advantage of the CIJ is that a handheld version of the instrument can be used to produce NCs at very small scales.[48]

Et-TP5 powder was dissolved in HPLC grade THF to give final concentrations ranging from 0.01 mg/mL to 8.89 mg/mL (1 wt%). PS$_{1500}$-OH and PS$_{1500}$-$b$-PEG$_{5000}$ were added to give 1 wt% of (dye + PS-OH) and 1 wt% of PS-$b$-PEG. The weight fraction of the dye in the nanoparticle core is calculated as follows:

$$\text{DyeCore Loading} = \frac{[\text{Dye}]}{[\text{Dye}] + [\text{PS} - \text{OH}] + x_{PS} [\text{PS} - \text{PEG}]} \quad \text{Eqn. (1)}$$

where the concentrations are given on a weight basis, and $x_{PS}$ is the mass fraction of the block copolymer that is poly(styrene). The solutions prepared were then rapidly mixed against ultra pure water in a multi-inlet vortex mixer (MIVM) to form nanoparticles.[38, 40] The THF:water flow rate was 12:120 mL/min, a 1:10 mixing ratio.[38, 46] The resulting nanocarrier suspensions were dialyzed against ultra pure water in Spectra-Por regenerated cellulose
membranes (08-670C, Fisher Scientific, Hampton, NH, USA) for 24-48 hours with a minimum of 3 ultrapure water exchanges.

The particles formed are stable (NC size constant for 3 months with some possible minor aggregation after 3 weeks (see AppD-Fig. 6)) with effectively 100% encapsulation of Et-TP5 due to the extreme hydrophobicity of the dye (logP = 9.94 as calculated using MolInspiration[49]). Previous studies have shown that encapsulated dyes are more photostable than the free dye,[6] A loss of about 20% of the original fluorescence was observed after 10 months of storage in darkness at 25°C (AppD-Fig. 7) for NCs core-loaded at 9.4% though at higher loadings there was actually an increase in fluorescence. This is because photodegradation of the dye led to effective loadings closer to the optimum 2.3wt% core loading.

**FNP – Et-TP5 loaded micelles**

We define NCs as having a core phase encapsulated by the amphiphilic block copolymer; whereas polymeric micelles comprise only the amphiphilic block copolymer. The hydrophobic Et-TP5 can be incorporated either in the core phase of a NC, or among the hydrophobic chains in a block copolymer micelle. Et-TP5 was dissolved in THF to give concentrations of 0.01 and 0.1 mg/mL and PS\textsubscript{1600}–b–PEG\textsubscript{5000} was added to give total solids concentrations of 1.8 and 17.6 mg/mL respectively (2.3 wt% Et-TP5 core loading in both cases). Equal volumes of THF (containing dye and PS-b-PEG) and ultra pure water were loaded into disposable syringes (53548-023, VWR International, Radnor, PA) and mixed against each other in a confined impinging jets mixer.[42, 48] The resulting mixed outlet stream was fed into a well-stirred
aqueous reservoir to give an overall THF:water ratio by volume of 1:10. The syringes were manually operated at approximately 0.5 mL/s for each stream giving \( \text{Re} \approx 3600 \).[48] The hydrodynamic radii and fluorescence intensities of the nanoparticles were measured within 3 hours after formation on a Brookhaven dynamic light scattering system (custom built with a 532 nm laser (Brookhaven, Holtsville, NY)), but the particles were stable for months upon storage at 4°C. The Malvern Zetasizer instrument could not be used for size determination because the small size of the micelles combined with the fluorescence of the Et-TP5 excited by the red laser in the Malvern instrument (\( \lambda = 632 \text{ nm} \)) degraded the autocorrelation function.

**HYDRODYNAMIC SIZE MEASUREMENT**

Dialyzed and undialyzed nanocarrier suspensions were diluted with ultra pure water to be optically clear and pipetted into a low volume plastic cuvette (ZEN112, Malvern Instruments, Worcestershire, UK). Particle sizes were measured with a Malvern Zetasizer Nano 3600 instrument (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK). The nanoparticle size was determined from the mean positions of intensity-weighted distribution peaks.

**FLUORESCENCE MEASUREMENT AND ANALYSIS**

All nanoparticle suspensions were diluted to or below 4 μg/mL of total dye in suspension to ensure that the nanoparticle concentration was low enough to minimize light scattering effects on the fluorescence intensity measurements.
Excitation and emission spectra of the nanoparticle suspensions were taken on both undialyzed and dialyzed samples in a glass cell (101-10-20, Hellma Analytics, Plainview, NY, USA) with excitation and emission slit widths of 5 nm and the PMT voltage set to 400 V (Hitachi F-7000 Fluorescence Spectrophotometer, Hitachi High Technologies, Schaumburg, IL, USA). The incident light and detector were arranged at 90°.

In Vivo Experiments (performed at Rutgers University)

Fluorescence imaging of a CD-1 male mouse was performed using an IVIS® 100 small animal imaging system (Caliper Life Sciences, Hopkinton, MA). CY5.5 excitation ($\lambda_{\text{ex}} = 615–665$ nm) and emission ($\lambda_{\text{em}} = 695-770$ nm) filters were used. Identical illumination settings, including exposure time (1 s), binning factor (4), f-stop (2), and fields of view (25 x 25 cm), were used for all image acquisition. Fluorescent and photographic images were acquired and overlaid. The pseudocolor image represents the spatial distribution of photon counts within the organs. Background fluorescence was subtracted prior to analysis. Images were acquired and analyzed using Living Image 2.5 software (Caliper Life Sciences, Hopkinton, MA). The particles, a 3wt% PMMA suspension with ETTP5, were injected as a 100 μL dose via intratracheal instillation (IT). Ketamine and xylazine at doses of 80 mg/kg bodyweight and 12 mg/kg were administered intraperitoneally as anesthetics for the mouse imaging.
RESULTS AND DISCUSSION

Nanoparticle formulation

The NC size is determined by the volume of the hydrophobic phase (core material plus hydrophobic block of the stabilizing polymer) relative to the volume occupied by the hydrophilic portion of the block copolymer and the total concentration of solids. The effect of total solids on particle size is shown in Fig. 1, where particle size varies from 60 nm to 250 nm as the total solids concentration varies from 0.7 mg/ml to 70 mg/ml in the THF stream. In this series, the mass ratio of Et-TP5 : PS-OH : PS-\textit{b}-PEG was held constant at 1 : 36.6 : 37.8 so that Et-TP5 was 2.3 wt\% of the hydrophobic components. This ratio corresponds to 1:1 of (Et-TP5 + PS-OH):(PS-\textit{b}-PEG). At solids concentrations of 190 mg/mL bulk precipitates are formed rather than well formed NCs.
Fig. 1 – Demonstration of Et-TP5 nanoparticle size control (NP diameter (■) and fluorescence per NP (●) vs. total concentration in feed stream). The ratio of ETTP:PS-OH:PS-b-PEG was held constant (Et-TP5 core loading 2.3wt%) while the total material concentration in the THF stream was varied from 0.7 to 77 mg/mL. The rather large variability in fluorescence per nanoparticle (NP) seen at ~75 mg/mL total solids is due to a difference in dye loading between the two samples. Near the optimal dye loading of 2.3wt%, the fluorescence/NP is sensitive to small differences in loading (in this case, 2.3% vs. 2.1% core loading, the latter being lower). However, the NP size is much less sensitive to this difference.

Size is determined by total solids and not by the specific composition of the hydrophobic components. This is demonstrated in Fig. 4(a) where a series of particles are made at a constant total mass of hydrophobic components of 8.9 mg/mL in the THF stream. This corresponds to a ratio of (Et-TP5 + PS-OH):(PS-b-PEG) of 1:1 with a variable Et-TP5 to PS-OH ratio. The fractions of Et-TP5 relative to total hydrophobic content were 0.16, 2.3, 9.4, 28, and 77.5 wt%. All of the particles are within the range 100-120 nm and are narrowly distributed. So the ratio of
fluorophore to core material can be varied over a wide range independent of particle size to optimization of fluorescence emission from the NCs.

**Micelle formation**

Micelles formed by Flash NanoPrecipitation at two different total solids concentrations have sizes which were similar: $d = 18.2$ nm and $d = 22.9$ nm, for 0.1 mg/mL Et-TP5 in THF and 0.01 mg/mL Et-TP5 in THF, respectively. Both samples were made at a 2.3 wt% Et-TP5 concentration, and both give comparable sample fluorescence output at the same total dye concentration. The micelle sizes are determined by the block copolymer hydrophobic block size at these very low core loading values. [46]

**Spectral Characteristics**

**Fig. 2** shows the absorbance and fluorescence spectra of Et-TP5 as well as the molecular structure of the dye. The major absorption peaks are at 458, 575, and 625 nm; and the major emission peaks at 635 and 695 nm. For discussion of the photophysics that follows, the emission intensity at 635 nm will be referred to as $I_1$ while the intensity at 695 nm will be referred to as $I_2$. Fluorescent emission spectra for dissolved Et-TP5 are not strongly dependent on the solvent used based on experiments done with THF and toluene (AppD-Fig. 8). **Fig. 3** shows the family of pentacene and hexacene dyes considered for this and other studies. The variety of steric groups provides varying degrees of protection against $\pi$-stacking and self-quenching. In general, hexacene dyes provide longer wavelengths than pentacene dyes, owing to the longer conjugated backbone. These hexacene dyes will be explored in the next chapters.
Fig. 2 – (a) Molecular structure of Et-TP5 and (b) normalized absorbance (—) and fluorescent emission (— — —) spectra of Et-TP5 dissolved in THF. Absorbance peaks shown: 430, 458, 532, 575, and 625 nm. Emission peaks: 635 and 695 nm. The emission intensity at 635 nm is denoted $I_1$ while the intensity at 695 nm is $I_2$.

Fig. 3 – Family of pentacene- and hexacene-based fluorescent dyes studied. Structure of fluorescent molecule Et-TP5 (MW: 839.3 g/mol) with other similar fluorescent molecules studied: (a) Et-TP5, (b) TSB-SP5, (c) iBu-TP5, (d) Ph-TP5, (e) iBu-TCPH5.

Nanocarrier Fluorescence versus Et-TP5 concentration

Organic fluorescent dyes self-quench by various mechanisms such as FRET[36, 37] or non-fluorescent excimer formation.[35, 50] Et-TP5 has been specifically synthesized to provide a high level of fluorescence intensity through both a high quantum yield (up to 76%)[33] as well as
the inclusion of bulky side groups to inhibit excimer formation and \( \pi \)-stacking of the aromatic rings.

We calculated the per-particle fluorescence intensity for various formulations of Et-TP5 nanoparticles to determine the composition that would give the highest fluorescence when the particles are dispersed, as in a mouse injection. The weight fraction of the dye in the nanoparticle core was calculated according to Eqn. 1. Then, average fluorescence per particle values were calculated as follows:

a. Average nanoparticle core volumes were calculated from average radii obtained from dynamic light scattering data after subtracting the PEG corona thickness (15 nm).\(^{51}\) The core volumes were converted to average core masses by assuming a core density of 1.04 g/cm\(^3\) – the density of bulk polystyrene.\(^{52}\)

b. The average number concentration of nanoparticles (nanoparticles/mL) was calculated by dividing the total mass of materials used to form nanoparticle cores (Dye + PS-OH + PS block) by the average core mass.

c. The raw fluorescence intensities were divided by the number concentration of particles to obtain the average fluorescence per particle.

The per-particle fluorescence intensity varies by an order of magnitude over the range of Et-TP5 concentrations between 0.1 and 75 wt% as shown in Fig. 4(b). The ratio of fluorescence intensities at 638 nm and 697 nm (there is a small red-shift upon nanoparticle encapsulation) is essentially constant over the entire range of Et-TP5 concentrations. The maximum brightness per particle occurs at a core concentration of 2.3 wt% Et-TP5. The fluorescence maximum reflects
the balance between the number of molecules in each nanoparticle core (fluorescence increases with more dye molecules) versus quenching arising from Et-TP5 self-quenching in the nanoparticle core. This fluorescence maximum corresponds to an intermolecular distance between dye molecules of 3.9 nm as shown in the figure. The significance of this distance will be discussed in the calculation of Förster resonant energy transfer in the following section. Since all nanoparticle fluorescence measurements were conducted at 4 μg/mL total dye in suspension or lower (to minimize the influence of nanoparticle light scattering), more lightly loaded formulations necessarily had more particles in suspension relative to the more heavily loaded particles. However, the calculated per particle fluorescence was insensitive to the number of particles in suspension as quenching does not occur between particles. This is because Förster radii range from 2.0-6.0 nm[36] and the nanoparticle PEG steric stabilizing layers keep particles at a spacing larger than ~15 nm.[51]
Fig. 4 – (a) Size distributions for representative Et-TP5-PS core nanoparticles at various core loadings (◆ 0.16 wt% | ▼ 2.3 wt% | ▲ 9.4 wt% | ● 28 wt% | ■ 77.5 wt%). By holding the total hydrophobic content constant at 8.9 mg/ml solutes in THF, the nanoparticle diameters for all Et-TP5 loadings were between 100-120 nm. (b) Fluorescence per particle/mL (AU/(particles/mL)) vs. Et-TP5 core wt. fraction (■ 638 nm emission | ● 697 nm emission). The fluorescence maximum observed at 2.3wt% core loading represents the balance between two opposing effects: increasing the number of dye molecules in the NP core increases the fluorescence per NP but at some point, the dye molecules will be in sufficiently close proximity to promote quenching. (c) $I_1/I_2$ vs. weight fraction for Et-TP5 nanoparticles cores (■), Et-TP5 dissolved in toluene (●), and Et-TP5 crystals (▲). We see that the $I_1/I_2$ ratios depend strongly on the state of the dye. In solution, the intensity ratios show a decrease with increasing dye concentration. In the crystalline state the intensity ratio is 0.16. In the glass PS matrix the dye has a constant value for the intensity ratio, and it is essentially that of the dye in solution at infinite dilution.
Fluorescence Intensity vs. Size

Since 2.3 wt% is the Et-TP5 concentration that produces maximum per-particle fluorescence, a series of NCs were made at this ratio, but over a range of total solids to vary the total particle size. The per-particle fluorescence was calculated and is presented versus hydrophobic core diameter in Fig. 5. The 15 nm thickness of the PEG corona has been subtracted from the measured hydrodynamic diameters, since the fluorescence comes from the Et-TP5 in the hydrophobic core. The per-particle fluorescence intensity scales as: \( I \propto D^{3.1\pm0.1} \). This value, which corresponds to the total fluorescence from a particle depending only on the total volume of the core, indicates that the Et-TP5 is uniformly distributed throughout the core, without significant inhomogeneities arising from particle surface effects, which would not scale with the particle volume.
Fig. 5 – Fluorescence per nanocarrier (NC) dependence on NC size at constant dye loading. Fluorescence per NC scales with the core diameter (the size determined from dynamic light scattering with the 15 nm PEG shell subtracted off)[51] on a log-log plot with a slope of 3.1 – thus the Fluorescence/NC scales with the NC volume.

Spectral $I_1/I_2$ Ratio

The ratio of the fluorescence maxima, $I_1/I_2$, is a sensitive probe of the microenvironment for fluorophores. The $I_1/I_2$ ratio for Et-TP5 in toluene is a strong function of concentration as shown in Fig. 4(c), where it varies from 3.0 to 0.42 over the concentration range $1.2 \times 10^{-5}$ to $1.2 \times 10^{-3}$ wt. fraction Et-TP5. For solid, crystalline Et-TP5, the $I_1/I_2$ ratio is 0.16 as shown in Fig. 4(c) (wt fraction = 1). The data show a strong decrease in the intensity ratio with increasing concentration or decreasing intermolecular distance. The $I_2$ emission likely arises from a more distorted excited state, and increasing aggregation creates a condensed environment preventing the molecule from accessing such a highly distorted configuration.
In sharp contrast, Fig. 4(c) also shows the intensity ratio for Et-TP5 in the solid NC core over the concentration range 0.001 to 0.78 wt. fraction. The intensity ratio is essentially constant, \( I_1/I_2 \sim 3 \), which is the value for the dissolved Et-TP5 at infinite dilution.

**FRET Analysis**

The nature of quenching in the nanoparticle cores can be analyzed from the perspective of Förster Resonant Energy Transfer (FRET). Förster radii range between 2.0-6.0 nm for a wide range of fluorophores and FRET can be used as a spectroscopic ‘ruler’ to measure intermolecular distances.[36] For example, in a protein with two fluorescent domains, one conformation, with large fluorescent domain separation, allows both domains to be fluorescent, but another conformation brings two domains within the Förster radius and leads to fluorescence quenching.[36] Winnik and coworkers used FRET to measure the mixing of labeled polymer chains when PMMA lattices are annealed into a film using time-resolved fluorescence decay measurements.[53] In this way, conformations and distances between molecules or domains can be quantified.

FRET is a radiationless mode of energy transfer from an excited state molecule to a ground state, without the emission and re-absorption of a photon.[34, 36, 37] The rate of such energy transfer is given by:[36]

\[
k_T = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6
\]

**Eqn. (2)**
where:
\( k_T \) = Förster resonant energy transfer rate
\( \tau_D \) = characteristic decay time
\( R_0 \) = Förster radius
\( r \) = intermolecular separation

FRET occurs when there is an overlap between the absorption and emission spectra of the donor and acceptor species (the same molecule in this case). Due to the \( r^{-6} \) dependence of the energy transfer rate on the intermolecular separation, at separations greater than the Förster radius, the FRET energy transfer drops off precipitously. However, within the Förster radius, the energy transfer rate rises sharply. Thus, FRET effects are negligible outside of the Förster radius but significant within it.

The Förster radius is defined as the distance where the energy transfer is 50% efficient and is given by:[36]

\[
R_0^6 = \frac{(9 \ln 10) \kappa^2 Q_D}{128 \pi^3 N n^4} \int_0^\infty F_D(\lambda) \varepsilon(\lambda) \lambda^4 d\lambda
\]

Eqn. (3)

where:
\( \kappa^2 \) = orientation factor (2/3 for randomly oriented molecules)
\( Q_D \) = quantum yield of the dye (50% - see Table 1)
\( N \) = Avogadro’s number
\( n \) = refractive index of the material (poly(styrene): 1.6)[54]
\( F_D = \) fluorescence spectrum of the dye (area normalized to 1)

\( \varepsilon = \) molar extinction spectrum of the dye \([\text{nm}^2 \text{ mol}^{-1}]\) (see Fig. 2(b) - from M\(^{-1}\) cm\(^{-1}\))

\( \lambda = \) wavelength \([\text{nm}]\)

The spectrum for Et-TP5 in Fig. 2(b) shows the region of spectral overlap involved in the integral in Eqn. 3. The calculated Förster radius is given in Table 1.

For the core-shell nanoparticles, we assume a simple cubic lattice to estimate the average intermolecular distance:

\[
R_{ETTP5} = \sqrt[3]{\frac{C_{ETTP5} + C_{PS} + C_{PS-block}}{n_E N_A \rho_{PS}}} \tag{Eqn. (4)}
\]

where:

\( C_i = \) mass concentration of component \( i \) after Flash NanoPrecipitation

\( n_E = \) molar concentration of Et-TP5

\( N_A = \) Avogadro’s Number

\( \rho_{PS} = \) density of bulk polystyrene \((1.04 \text{ g/cm}^3\) \(\) (core density)

Table 1 shows the Förster radius calculated by Eqn. 3 and the experimentally determined concentration at which fluorescence is maximized. The maximum fluorescence per particle is reached at 2.3 wt% core loading of Et-TP5, which corresponds to a lattice spacing between each Et-TP5 molecule of 3.9 nm; very close to the calculated Förster radius of 4.1 nm determined from Eqn. 3. This shows that FRET can account for the quenching behavior of Et-TP5 in the nanoparticle cores. At loadings higher than 2.3 wt%, the intermolecular separation is less than the Förster radius and the fluorescence-per-particle decreases due to nonradiative energy transfer.
between Et-TP5 molecules dissipating the incident energy. At loadings lower than 2.3 wt%, although FRET quenching is reduced, the concentration of fluorophores decreases and so does the per-particle fluorescence. The PS-OH polymer matrix was not included in these calculations as it has negligible spectral overlap with Et-TP5 and thus negligible FRET (according to Eqn. 3).

**Table 1** - Comparison of various fluorescent nanoparticle systems. The Förster radii were calculated from Eqn. (3) and the lattice parameters (intermolecular separation) were calculated from Eqn. (4).

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>R_F (nm)</th>
<th>QY (%)</th>
<th>L_max</th>
<th>a_max (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETTP5</td>
<td>4.10</td>
<td>50[33]</td>
<td>0.023</td>
<td>3.90</td>
</tr>
<tr>
<td>Nile Red (NR)</td>
<td>3.47</td>
<td>70[55]</td>
<td>0.00912</td>
<td>3.82</td>
</tr>
<tr>
<td>Pyrene</td>
<td>2.57</td>
<td>65[56]</td>
<td>0.0201</td>
<td>2.52</td>
</tr>
<tr>
<td>HY3G</td>
<td>2.86</td>
<td>90[57]</td>
<td>0.017</td>
<td>3.74</td>
</tr>
</tbody>
</table>

R_F = Calculated Förster radius  
QY = Quantum yield  
L_max = Core Loading @ maximum per particle fluorescence  
a_max = Cubic lattice parameter @ maximum per particle fluorescence

This analysis also applies to other dyes including nile red and pyrene. We see that the maximum fluorescence lattice parameter matches the calculated Förster radius quite closely, thus explaining the onset of quenching in these nanoparticles on the basis of Förster resonant energy transfer. The optimum loading for these different nanoparticle systems is shown in Fig. 6.

However, in the case of HY3G, the onset of quenching appears to begin at a separation much larger than the calculated Förster radius. This can be explained on the basis of phase separation within the nanoparticle core. As stated in Kumar, et al. (2010), HY3G was found to have an interaction parameter with PS greater than the critical interaction parameter, thus leading to stable microphase separation.[50] On the other hand, it was found that pyrene should be initially well mixed in the nanoparticle PS core on the basis of its interaction parameter. The same is true for ETTP5 and Nile Red as well. Thus, the microphase separated HY3G experiences the onset
of quenching at a lower loading (greater intermolecular separation) than expected due to the HY3G molecules being in closer proximity to each other than the well-mixed assumption predicts.

![Graph showing normalized fluorescence vs. nanoparticle core loading]

**Fig. 6** – Comparison of ETTP5 PS core nanoparticles to other fluorescent nanoparticles formed via the FNP process (▲ ETTP5/PS | ■ Pyrene/PS Core | ● Hostasol Yellow/PS Core | ♦ Nile Red/PS core). All fluorescence intensities were normalized against their respective maxima. The intermolecular separation at which each maximum occurs corresponds closely to the Förster radius for each dye system, except for Hostasol Yellow 3G which experiences microphase separation. Data for pyrene and Hostasol Yellow were extracted from Kumar, et al. (2010).[50]
In Vitro and in vivo examples of Et-TP5 as a Diagnostic Agent

We give as an example the use of Et-TP5 as a fluorescent agent to monitor and quantify NC trafficking. NCs were synthesized with Et-TP5 in the cores and mannose targeting ligands on a fraction of the PEG chains. The goal of the study was to quantify the uptake of NCs by mannose receptors on macrophages, which can be used to target therapeutics to tuberculosis infected macrophages. The details of the study are presented elsewhere.[27] The spectral characteristics of Et-TP5 are ideally suited to confocal imaging since the excitation wavelength is long enough to avoid autofluorescence from proteins. Fig. 7(a) shows the confocal image of the NCs that have been internalized into macrophages after incubation (green). The nuclei of the macrophages have been stained with DAPI dye (red). In addition to imaging, the uptake of NC was quantified by solubilizing the hydrophobic dye encapsulated in the NC core to extract it from the lysed cell debris. The dissolved dye was then quantified by fluorescence spectroscopy. Briefly, the process involved the following steps. After incubating the cells with the NCs, the media was removed and the cells washed 3X with HBSS to remove unassociated NCs. The cells were then lysed in 1 mL of 0.1% (w/v) Triton X-100 (Sigma Aldrich) for 45 min in the dark. The lysates were lyophilized. THF was added to the dried powders to dissolve the NCs which had associated with the cells. The mixture was centrifuged at 7000×g for 10 min to settle the insoluble cellular material. The fluorescence of the transparent supernatant was measured with an excitation wavelength of 485 nm. The peak value in the fluorescence emission spectrum at 632 nm was used to quantify the Et-TP5 mass associated with cells in each well. The extreme hydrophobicity of Et-TP5 makes this quantitative extraction assay possible. ETTP5 loaded PMMA particles were clearly visible (Fig. 7(b)) through lung tissue following intratracheal administration into
mice. They were not, however, visible from the outside of the mouse since the ETTP5 wavelengths are not sufficiently long to fully fall inside the optical imaging window.

**Fig. 7** – Cell culture and mouse images of ETTP5 nanoparticles. **A** - Uptake of ~100 nm Et-TP5 NCs into J774E mouse macrophage cells (red – DAPI nuclear stain, green – Et-TP5 nanoparticles). The NCs are targeted using mannose ligands on the PEG chains. The concentrated green fluorescence indicates internalization into endosomes, and the diffuse green fluorescence shows release of the NCs into the cytosol. Reproduced with permission from the Society of Controlled Release (D’Addio, et al. (2012)).[27] **B** – ETTP5 loaded into poly(methyl methacrylate) latices and administered intratracheally into a mouse (details in the Materials and Methods section). The NPs are clearly visible in the lungs and accumulated there due to the intratracheal route.

**CONCLUSIONS**

We have successfully encapsulated a new, extremely hydrophobic fluorescent pentacene derivative dye within polymeric NCs via the FNP process. The dye, Et-TP5, is a TIPS dioxolane derivative where the bulky side groups minimize π-stacking and fluorescence quenching. Its encapsulation in the core of the nanocarrier (NC), and the dense PEG steric layer on the outside
of the NC, prevents interaction of the dye with biological surfaces and molecules in diagnostic assays or therapeutic delivery. By incorporating the Et-TP5 in the core of a 100 nm NC, more than 25 times more dye can be delivered than if it is conjugated onto the surface of the NC. These fluorescent particles were loaded from less than 1% to 78% by weight Et-TP5 in the NC core and the fluorescence maximum was found to be 2.3% wt. The particles were also stably formed at 2.3% core loading from 20 nm up to 250 nm with per-particle fluorescence scaling linearly with the NP volume. Characterization of the fluorescence spectrum of the dye revealed a phase- and concentration-dependent emission spectrum which allowed us to elucidate details about the microenvironment of the dye within the polystyrene nanoparticle cores. A calculation of the Förster resonance energy transfer distance was consistent with the experimentally observed intermolecular distance where quenching becomes significant.

Other hydrophobic dyes we have used, such as pyrene[58] and nile red,[59] show significant partitioning out of the NC over time and can cause “false positive” readings. The hydrophobicity of Et-TP5 (i.e. logP = 9.94, the log of the ratio of a solubility in octanol vs. water) prevents this partitioning and makes it an attractive new fluorescent marker. This same hydrophobicity makes it necessary to incorporate the dye into NCs during their initial formation by processes such as FNP; post addition of the dye is not feasible. The excitation and emission wavelengths of the dye are just at the edge of being “long wavelength” (~700 nm +) where they would be more suitable for whole animal imaging. The optical attenuation in tissue is shown in Fig. 2 in Chapter 1; moving excitation and emission wavelengths upwards by 50 nm would enable deeper tissue penetration by millimeters to centimeters. Studies to extend the wavelength ranges of these pentacene dyes are underway.
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CHAPTER 3
Long Wavelength ICG Nanocarriers for Biomedical Imaging

Abstract

Indocyanine green (ICG) is a widely used FDA approved fluorophore with complex amphiphilic behavior that has been used to assess cardiac and hepatic function. Its utility in imaging is based on its optical absorption and emission wavelengths, which fall inside the ‘optical imaging window’, the region where deep penetration of light into tissue occurs. A goal of the nanoparticle community has been to incorporate ICG into nanocarriers (NCs) to achieve enhanced circulation time, targeting, and real-time tracking of NCs in vivo.

While previous studies have transferred ICG exogeneously into nanocarriers (NCs), we show that a one-step, block copolymer-directed, rapid precipitation process (Flash NanoPrecipitation (FNP)) creates ICG-loaded NCs with narrow size distributions from 30 to 180 nm. The ICG is stabilized by forming an ion pair with cationic tetraoctylammonium or tetraddodecylammonium chloride, where the ion pair is formed either in situ during the rapid precipitation process, or a pre-formed, organic-soluble ion pair can be introduced into the organic stream during FNP. There is a maximum ICG loading that can be achieved before dye recrystallization out of the NCs. For ICG/polystyrene NCs, 10 wt% core loading can be achieved, while 30 wt% core loading is achieved with tocopherol (vitamin E) cores. However, maximum ICG fluorescence is obtained at 10 wt% ICG core loading, due to Förster energy quenching at higher dye loadings. ICG is amphiphilic, and previous studies have been unable to determine if the ICG is uniformly distributed in the NC, or is localized on the NC interface. We demonstrate that the fluorescence-per-particle scales with core diameter to the third power, showing that FNP enables uniform encapsulation of the ICG throughout the core. To elucidate the mechanism of ICG complex formation and NC loading, the ratio of ammonium counter ion to ICG is varied. Loading efficiencies >80% are achieved by the rapid precipitation, even in the absence of ion pairing, but with 1:1 ion pairing 100% loading efficiency is achieved. Lastly, we present data showing the stability of ICG NCs in the presence of buffers and physiologically relevant amounts of albumin, for which ICG has a very strong binding affinity. While the ICG ion pair is stable in buffer, i.e. the ions in solution do not ion exchange ICG:TOAC ion pair and liberate ICG, in the presence of 4 wt% albumin, the ICG does transfer to the protein in a time scale of <30 minutes.
INTRODUCTION

The field of optical/near infra-red (NIR) imaging has gained importance in recent years as an alternative to radioactive imaging techniques for \textit{in vivo} diagnostics. Though radioactive imaging modalities such as PET, SPECT, and CT currently represent the gold standard in molecular level imaging, optical and NIR methods are rapidly improving to provide high levels of penetration, resolution, and quantification. Contrast agents with excitation and/or emission wavelengths in the range from 700-1450 nm fall into the ‘optical imaging window’, a region where absorbance and autofluorescence from blood and tissue components are minimized (Fig. 1). This allows for deep penetration -- on the order of centimeters.
Desirable characteristics of a fluorescent imaging agent are high quantum yield (for fluorescence), high extinction (for absorbance), resistance to photo-bleaching, insensitivity to the microenvironment being targeted and a lack of toxicity.[2] These characteristics apply to both soluble fluorophores and fluorophores in NCs. NCs are of interest as therapeutic delivery agents[3-6], as targeted imaging agents for diagnosis [1, 7-10] and in the dual role in therapy and diagnosis as “theranostic”agents[11-16]. Most often NCs for therapeutic applications are rendered fluorescent by post-functionalizing the surface of the NC with a fluorescent dye [17-24]. The main concerns with this approach are that 1) far fewer agents can be incorporated on
the surface vs. in the interior before the onset of quenching and 2) the dyes may interfere with targeting ligands placed on the NC surface (ligands that are often present in relatively low amounts). Quantum dots and latex spheres with imbibed fluorophores are examples of systems with internal fluorescence. Latex spheres, while good for imaging and diagnostics, are not readily adapted to efficient drug delivery. The potential toxicity of the heavy metals in quantum dots limits their utility to in vitro and animal in vivo diagnostics. NCs with ‘stealth’ surfaces display long circulation times (\(t_{1/2} > 24\) hours) which enhances accumulation in tumors by the enhanced permeation and retention (EPR) effect and by active targeting moieties conjugated to the surface of the NC.

In particular, the long wavelength imaging agent indocyanine green (ICG, also known as Cardiogreen, see Fig. 2(A)), an FDA approved medical imaging dye, has been implemented by the medical community to assess hepatic and cardiological function in humans. However, free ICG in blood quickly aggregates and complexes with blood proteins, leading to rapid clearance, on the order of minutes, via the mononuclear phagocytic system. Furthermore, free ICG has a low quantum yield of less than 1% in water and has a conjugated backbone that is susceptible to free radical and peroxide attack in water and solvents, which leads to rapid loss of fluorescence as well.

Despite these limitations, ICG is attractive as an imaging agent due to its 771/831 nm absorption/emission wavelengths, which fall into the optical imaging window (Fig. 1), its long history of use in humans, and approval by the FDA. ICG has been incorporated into a wide variety of nanoscale and microscale delivery systems. These range from liposomes...
micelles[16, 32-35], nanocapsules[36, 37], mesocapsules[38], and more complex formulations for dual purpose diagnostics/therapy[12, 39], multimodal imaging[40], systems that can enhance the quantum yield of ICG to several times its natural QY in water[39]. ICG embedded within hydrophobic polymers[41, 42] and mesoporous materials[43] ICG has been incorporated into these agents either as the pure molecule[33], as a complex with a hydrophobic cationic species[34], or conjugated to polymers[35] Ionic complexes of ICG are particularly attractive as they do not require new approval by the FDA for biomedical use and have a much shorter path to clinical use.

Although many types of delivery systems have been studied for the delivery of ICG, several with \textit{in vivo} data for both targeted and non-targeted formulations, there is a notable lack of data on the stability of the delivery systems under physiological conditions. The delivery constructs (such as liposomes or micelles) might be stable, but the ICG may not be stably incorporated into the constructs. ICG’s unique structure, with both hydrophobic and ionic domains, enables incorporation into particles, but also drives strong binding to proteins such as albumin. Therefore, exchange between the particle and serum proteins can be a significant issue for long-term imaging studies. Physical instability is related to imaging stability, since ICG bound to proteins can exhibit significantly higher quantum yields than in particles [29] and therefore, \textit{in vivo} studies intended to track the biodistribution of NCs over time may be confounded by the signal from ICG-protein complexes.

Here, we report the one-step fabrication of block copolymer stabilized composite NCs containing ICG as the fluorophore. The key platform underlying this NCs fabrication is Flash
NanoPrecipitation (FNP), as described by Johnson and Prud’homme (2003)[44]. The flexibility of the process enables co-encapsulation of a variety of drugs[5, 6, 45], pesticides[46], organic imaging agents,[2, 10] nanocrystals,[47] and metal clusters. In the process, dissolved hydrophobic species and an amphiphilic block copolymer in a water-miscible organic stream (often THF) is rapidly mixed against a large excess of anti-solvent (usually water). The key to the process is the specially designed micromixing cavities that create supersaturations as high as 10,000 in 1.5 ms.[6] In this work, we present two ways to form ICG NCs: one consists of pre-forming the hydrophobic ICG-ammonium salt complex (‘premade complex’) and the second consists of forming the ICG-cationic salt hydrophobic complex in situ during nanoprecipitation (‘in situ complex’). We compare these methods of formation and characterize the NCs’ size, fluorescence, and stability. We also provide data on the stability of ICG NCs in the presence of 4wt% bovine serum albumin (BSA) - data currently lacking in the literature.
MATERIALS AND METHODS

Materials
Two forms of indocyanine green (ICG) were used: Cardiogreen, which contains NaI, was obtained from Sigma-Aldrich (I2633) and an iodine free ICG were obtained from Persis Science in Princeton, NJ. The cationic complexation agents tetrabutylammonium iodide (86890), tetrabutylammonium (TBA) chloride (86870), tetraoctylammonium (TOAC) chloride (87991), and tetradodecylammonium (TDDA) chloride (87250) were obtained from Sigma-Aldrich and used without modification. Hydroxyl terminated poly(styrene) and poly(styrene)-b-poly(ethylene glycol) (1.5k-b-5k) were synthesized as previously described.[48] Poly(styrene)-b-poly(ethylene glycol) (1.6k-b-5k) was obtained from Polymer Source (P13141-SEO, Polymer Source, Montreal, Canada). HPLC grade tetrahydrofuran and diethyl ether were obtained from Fisher Scientific (T425-4 and AC615080010 respectively, Fisher Scientific, USA), and ultrapure water was obtained from a Barnstead NanoPure system delivering >17.8 MΩ-cm (hereafter referred to as ‘Milli-Q water’).

Methods

Ion Pairing ICG Complexation Reaction
193.70 mg of iodine-free indocyanine green (Sodium 4-[(1E,3E,5E,7Z(-7)[1,1-dimethyl-3(4-fultonatobutyl)benzo(e)indol-2-ylidene]hepta-1,3,5-trienyl]-1,1-dimethylbenzo(e)indol-3-ium-3-yl]butane-1-sulfonate, formula corresponding to C_{43}H_{47}N_{2}NaO_{6}S_{2}, MW 774.96) was placed in 250 mL of dried diethyl ether in a 1 liter polypropylene container. This mixture was allowed to
mix under nitrogen for 30 minutes. To this mixture was added 251.20 mg of tetraoctylammonium chloride (TOAC), while mixing under nitrogen. While continuously mixing, 250 mL of Milli-Q water was added. The container was sealed with a gasket and lid and placed into a Flacktek DAC400 Speedmixer for 10 minutes using the following settings: 2 min. at 800 RPM, 2 min. at 1200 RPM, 4 min. at 1800 RPM and finally 2 min. at 2000 RPM. The mixture was removed from the Speedmixer and observed to determine that all of the green color (ICG) was removed from the bottom, aqueous layer and dissolved as a dark green color in the top (diethyl ether) layer. The reaction mixture was placed into a 1 liter separation funnel and shaken vigorously for 1 minute. The funnel was allowed to stand for 15 minutes, while occasional spinning of the apparatus was performed to collect all the water droplets. After 5 minutes, the bottom layer was drained and another 250 mL of Milli-Q water was added and the same procedure was repeated three times. The ether solution was stirred by hand and 20 grams of anhydrous magnesium sulfate was added and shaken slightly. The magnesium sulfate was removed by three-fold filtration. The aqueous phase appeared colorless and the ether layer was placed into a 500 mL, single neck round bottom flask. The ether was removed by rotary evaporation. When there was a final solution amount of approximately 10mL to 12 mL, it was transferred to a small 20 mL vial and placed on the rotary evaporator under vacuum at 40°C for about 45 minutes. The resulting crystalline material was dried at room temperature under high vacuum overnight. The material was found to be very insoluble in water. 286.7 mg (94.24% yield) was recovered after vacuum drying. The material was dissolved at 1.0 mg/mL in acetonitrile and run through HPLC on a C18 5 micron column using 35/65 acetonitrile:water at a flow rate of 1.75 ml/min. Only one peak was observed at 2.27 minutes. The DAD, fluorescence and mass indicated the presence of only the ICG-tetraoctylammonium complex with 1:1 molar
pairing having a molecular weight of 1218.86 (name: Tetraoctylammonium 4-[2-[1E,3E,5E,7Z]-7-[1,1-dimethyl-3-(4-sulfonatobutyl)benzo(e)indol-2-ylidene]hepta-1,3,5-triyenyl]-1,1-dimethylbenzo[e]indol-3-iium-3-yl]butane-1-sulfonate, the formula corresponding to C\textsubscript{75}H\textsubscript{115}N\textsubscript{3}O\textsubscript{6}S\textsubscript{2}). This was confirmed by proton (AppE-Fig. 6) and carbon NMR as well as fluorescence using excitation at 785 nm and measuring emission at 800 nm – 1300 nm. Excitation and emission curves were also calculated. The UV-Vis showed a sharp peak at a max of 794 nm and purity by Elemental Analysis of >98%.

**ICG NC formation in situ (fluorescence-per-particle determination)**

The optimal NC loading of ICG for maximum fluorescence on a per-particle basis was determined by formulating particles with different core loadings of ICG. ICG concentrations from 0.3 μg/mL to 8.8 mg/mL in water were mixed against a THF stream containing 1 molar equivalent of tetraoctylammonium chloride (TOAC) and Vitamin E from 1 to 15.5 mg/mL. PS-\textit{b}-PEG was present in the THF stream from 9.3 to 12.8 mg/mL. Total solids ranged from 17.9 to 28.3 mg/mL and NC sizes were in the range from 100-160 nm. ICG complex core loadings were varied between 0.014% up to 78% wt. THF and water streams were mixed in a 1:1 volume ratio in a confined impinging jets mixer (CIJ) and quenched in 9 parts water (overall 1:10 mixing ratio). The resulting NCs were characterized for size by DLS and diluted to multiple concentrations for fluorescence measurements to establish the linearity of fluorescence vs. [NC]. The slope of the fluorescence vs. [NC] or [ICG] line was used as the \textit{intrinsic fluorescence} of that NC system. The system with the highest intrinsic fluorescence (equivalent to \textit{per particle fluorescence}) was the optimally loaded system.
ICG formation in situ (10% loading)

ICG was dissolved in water at 0.80 mg/mL. TOAC was dissolved in THF at 0.52 mg/mL, PS-b-PEG at 9.7 mg/mL, and Vitamin E (VE) at 8.2 mg/mL (so that VE plus TOAC was 1 wt% in the THF feed stream). The aqueous and organic feed streams were rapidly mixed in equal volumes in a confined impinging jets mixer (CIJ) according to Johnson, et al.[44] to form the hydrophobic ICG-TOAC complex and polymer protected NCs via Flash NanoPrecipitation in one step.

In Situ ICG Encapsulation Efficiency - variation of ICG:TOAC ratio

ICG NCs were prepared with varying ratios of ICG:TOAC to test the required amount of TOAC for stable ICG complex and NC formation. TOAC : ICG ratios of 1:10, 1:2, 1:1, 2:1, and 10:1 were tested. Control experiments with no TOAC and no TOAC and no VE were also performed. The VE concentration was held constant at 1 wt% (8.9 mg/mL in THF), [ICG] ranged from 0.3 to 3 mg/mL in the water feed, and [PS-b-PEG] was equal to [ICG]+[TOAC]+[VE] on a weight basis. The THF stream and ICG-in-water stream were rapidly mixed in the CIJ mixer and quenched in a water reservoir with a volume of 9X water in the reservoir relative to the volume of mixed solutions. The final mixing ratio of THF : water = 1:10 in the reservoir. The sizes were measured with DLS. The samples formed were centrifuge filtered with Amicon 100k MWCO filters (UFC210024, EMD Millipore, Billerica, MA) at 7000g for 20 minutes to separate the free ICG from NCs. Free ICG concentrations in the filtrates were measured by optical absorbance (Evolution 300 UV-vis-NIR Spectrophotometer, Thermo Fisher Scientific, Bridgewater, NJ) and compared against initial absorbance values for the initial NC suspension to determine the free ICG %.
ICG NC Size Control Experiments

ICG NCs were prepared with varying amounts of total solids but a constant ratio of all solid components. Total NC solids in the feed ranged from 1 to 100 mg/mL. ICG loading was held constant with 1:1 ICG:TOAC and 10.5% ICG complex core loading. By mass, the NCs were 43% VE, 4% ICG, 3% TOAC, and 50% PS-b-PEG, the same as the optimally fluorescent formulations determined earlier. The feed streams were mixed 1:10 against an excess of Milli-Q water and characterized for size and fluorescence immediately afterwards. The NCs were diluted in water for DLS measurements, and multiple dilutions were measured by fluorescence to establish the linearity of fluorescence vs. [NC].

Determination of NC Size by Dynamic Light Scattering

Approximately 0.1 mL of ICG NC suspension were mixed with > 2 mL of Milli-Q water to dilute the suspension to the point of water clarity in a low volume polystyrene cuvette (ZEN-0112, Malvern Instruments, Boston). The cuvette was placed in a Malvern Zetasizer Nano ZS 3600 instrument (Malvern Instruments, Boston) and the intensity-weighted hydrodynamic size distribution of the NCs was determined by dynamic light scattering. The Zetasizer analysis program in “normal mode” was used for the size determination.

Fluorescence Measurements

NC suspensions were diluted with Milli-Q water by varying amounts such that the curve of fluorescence vs. NC concentration was linear. This was necessary to minimize the effects of NC light scattering on the fluorescence measurements made. The fluorescence per NC was calculated according to the procedure in Pansare, et al.[2] by dividing the fluorescence value by
the average number of NCs in suspension.

**Measuring protein and NC passage through centrifuge filters**

NCs containing 50% by weight VE and 50% PS-b-PEG (1.6k-b-5k) were prepared by Flash NanoPrecipitation at 20 mg/mL total solids in THF. The THF stream was mixed against a 10-fold excess of water and the resulting NCs dialyzed for 24 hours against Milli-Q water. Four test solutions were then prepared: (A) VE NCs in water, (B) 4wt% BSA (602 μM) in 5 mM phosphate at pH 7, (C) 0.073 mg/mL ICG (94.2 μM) in 4 wt% BSA in 5 mM phosphate, and (D) VE NCs in 4 wt% BSA in 5 mM phosphate. Each solution was incubated for approximately 45 minutes at room temperature. Samples were centrifuge filtered with 100k Amicon filters (UFC210024, EMD Millipore, Billerica, MA) and 300k Nanosep Omega filters (OD300C33, Pall Corporation) at 7000g for 20 minutes. Absorbance measurements were taken to quantify the concentrations of BSA (280 nm), VE NCs (295 nm), and ICG (797 nm) before and after centrifuge filtration. Separately, fluorescence measurements were taken on ICG and ICG+BSA mixtures at multiple concentrations in the linear fluorescence regime to establish the intrinsic fluorescence of each system (slope of fluorescence vs. [ICG]).

**ICG NC-BSA Equilibration Time**

The required equilibration time for the ICG NC in BSA solution was determined for the ICG-C₈ premade complex by preparing NCs with 10wt% core loading according to the previously stated protocol with the ICG-C₈ premade complex. The NCs were dialyzed for 24 hours and incubated with 5 mM phosphate and 4 wt% BSA (40 mg/mL) at RT. Fluorescence time points were taken at over 100 minutes.
RESULTS AND DISCUSSION

ICG Structure and Absorbance/Fluorescence Spectrum

The structures of indocyanine green (ICG) and the tetraoctylammonium chloride (TOAC) and tetradodecylammonium chloride (TDDAC) molecules are shown in Fig. 2(A)-(C). ICG possesses an amphiphilic structure with aromatic groups, a conjugated backbone, and charged sulfonate groups. Its overall charge is -1. Thus, it binds to the cationic ammonium salts TOAC and TDDAC, which themselves are known as phase transfer catalysts with strong interfacial activity.[49] We use these compounds instead of the more commonly used butyl (C₄) due to their greater hydrophobicity, which we anticipate would increase NC stability against ICG ion exchange. ICG has a broad excitation spectrum (Fig. 2(D)) with emission at 810-830 nm depending on the ICG concentration, solvent, and NC encapsulation. An excitation wavelength of 772 nm was used for all experiments. These wavelengths fall into the optical imaging window where maximum optical penetration is possible.
Fig. 2 – (A) Structure of ICG, (B) Structure of tetraoctylammonium chloride, (C) structure of tetradodecylammonium chloride, (D) Normalized fluorescence excitation and emission spectra for ICG in DMSO. Excitation wavelength used was 772 nm while the emission peak varied between 810 – 831 nm depending on whether the ICG was free in solution, complexed, or NC-encapsulated.

Maximizing ICG NC intrinsic fluorescence

As explained in Pansare, et al. (2014)[2], loading hydrophobic dyes uniformly within confined volume NC cores results in decreased intermolecular separations as the loading increases.

Although increasing the number of dye molecules per NC initially increases the fluorescence per carrier, once the intermolecular separation drops below the Förster radius, the rate of Förster Resonant Energy Transfer (FRET)[50], a quenching mechanism, rises rapidly, resulting in a decrease in per-particle fluorescence (intrinsic fluorescence). Thus, there is an optimum in the NC dye loading to achieve the maximum brightness per NC. As seen in Fig. 3, the fluorescence maximum for the ICG-TOAC in situ formed system is 10 wt%. The complex core loading is defined as:

\[
ICG \text{ Complex Core Loading} = \frac{[ICG \text{ Complex}]}{[ICG \text{ Complex}]+[Co-exciptent]+[PS-box]} \quad \text{Eqn. (1)}
\]
Where \([X] = \) concentration of component X, the core material is vitamin E, and the PS-block is the 1.6k block of the 1.6k-5k PS-\(b\)-PEG copolymer. Alternatively, the dye loading can be described on the basis of the ICG alone:

\[
\text{ICG Core Loading} = \frac{[\text{ICG}]}{[\text{ICG}]+[\text{Counter-ion}]+[\text{Co-excipient}]+[\text{PS-block}]} \quad \text{Eqn. (2)}
\]

This definition will be more relevant when encapsulation efficiency is studied by changing the ratio of ICG to the counter-ion.
**Fig. 3** – Fluorescence per NC vs. ICG complex core loading. The maximum in fluorescence per NC (highest intrinsic fluorescence) for ~100 nm NCs was found at approximately 10 wt% ICG complex core loading. Subsequent ICG-C₈ complex NCs were formulated with this optimal loading.

**ICG NC Loading Limits**

While studying ICG-C₈ complex stability in VE and PS cores, we observed that at high ICG loadings, a glitter-like precipitate formed in the solutions several hours after NC formation. The precipitates were not NC flocculated particles, but rather an ICG crystal phase. The precipitates could be filtered out with a 5 μm filter and the resulting filtrate contained a single NC population. By measuring the size change and ICG concentration change by absorbance, we determined that each type of NC core had a certain loading threshold above which ICG complex...
precipitates would form and below which no precipitates would form and NCs would be stable for weeks to months (see **Fig 4**).

![Graph A](image1)

**Fig. 4** – *NC size change following precipitation of ICG-C₈ with different core materials and corresponding changes in ICG complex core loading shown at the abscissa: (A) Polystyrene core, (B) No co-solute, (C) Vitamin E core (solid bars = pre-precipitation | striped bars = post-precipitation). The maximum stable loading levels are dependent on the core material.*

In the case of PS cores, NCs loaded with 4.1% and 8.7% core loading ICG-C₈ complex were stable but those loaded at 38% reached a final loading of 9.8% within a few hours. In the case of VE cores, NCs loaded at 32.9%, 58.9%, and 77.7% all reached final effective loadings of 31.6%,
30.5%, and 27.8%. Further formulations with VE below the threshold were all stable. In the case of no co-solute (ICG complex loaded PS-\(b\)-PEG micelles), NCs loaded at 78% fell to a final loading of about 42%. These measurements indicate that the ICG complex is less compatible with polystyrene than with VE. Since 10wt% ICG complex core loading maximizes fluorescence, and since we had experience with VE as a co-excipient for therapeutics in the NC core, we studied only VE-core NCs going forward.

Dialyzed VE core NCs stabilized by PS-\(b\)-PEG were found to be size-stable for several weeks (AppE-Fig. 7) as long as the ICG loading was below the limits shown in Fig. 4. Undialyzed NCs experienced Ostwald ripening.[51]
Size Control and Fluorescence Scaling of ICG NCs

Fig. 5 – (A) Size distributions for ICG-TOAC (ICG-C₈) in situ formed NCs (■ 1 mg/mL | ● 10 mg/mL | ▲ 40 mg/mL | ▼ 70 mg/mL | ▼ 100 mg/mL), (B) NC sizes vs. total solids concentration. (C) Fluorescence intensity per NC vs. core diameter. The fluorescence per NC (intrinsic NC fluorescence) scales linearly with the core diameter to the third power.

Since NC size is an important design parameter, we demonstrate that these ICG NCs could be produced at different sizes at a constant ICG loading (the optimal fluorescence loading
determined earlier). The NC size produced by FNP primarily depends upon the solution concentration.\[2\] We held the ratio of all components constant and varied the total solids from 1 mg/mL to 100 mg/mL in the feed stream. The size distributions (shown in Fig. 5(A)) become slightly broader at larger sizes, in accordance with the mechanism of growth in FNP.\[52\] The size scales roughly linearly with the total solids concentration up to a solids concentration of 70 mg/mL at which point the size plateaus at ~180 nm (Fig. 5(B)). The plateau is most likely due to the competing effects of supersaturation and aggregation. The nucleation rate, $B$, increases with the supersaturation ratio, $S_r$, according to classical nucleation theory by: \[53\]

$$B = K_1 \exp \left( - \frac{16\pi\gamma^3v^2}{3k^3T^3[\ln(S_r)]^2} \right)$$

Eqn. (3)

where $\gamma$ is the interfacial tension, and $v$ is the molecular volume. For a fixed mass of NC solids, a higher nucleation rate, $B$, leads to more nuclei and smaller final particle sizes, since the mass of NC solids is distributed among many more nuclei. However, the overall growth rate is strongly dependent upon the collision frequency between nuclei: $G \propto C^n$, where $G$ is the nucleus/aggregate growth rate and $C$ is the bulk solute concentration. Thus, increasing the total NC solids concentration initially causes larger particles due to the $C^n$ dependence, but at higher concentrations the increased nucleation rate effect dominates and the size plateaus. Although this study was done with liquid VE cores, a similar effect was seen with solid poly(styrene) core NCs.\[2\]

The per-particle fluorescence (Fig. 5(C)) scales linearly with the core volume, i.e. the core
diameter to the 3rd power: \[ \frac{F_L}{N_P} = (5.01 \times 10^{-14})(D^{2.98}), \quad R^2 = 0.98. \] This shows that the ICG is encapsulated uniformly in the core of the NCs, and not just at the core-shell interface, which would have a D^2 scaling. As a result, larger particles are more fluorescent at constant dye loading. We hypothesize that previous studies, which loaded ICG into preformed NCs through the aqueous phase would produce NCs with ICG at the NC interface. However, in those studies fluorescence vs. size was not reported, so the localization of the dye via these other assembly processes cannot be confirmed.
Fig. 6 – ICG encapsulation efficiencies as a function of TOAC:ICG ratio (in situ complex formation): (A) size distributions of particles (■ micelles (0 TOAC, 0 VE) | □ 0 TOAC | ▲ 1:10 TOAC:ICG | ▼ 1:2 TOAC:ICG | ● 1:1 TOAC:ICG | ▶ 2:1 TOAC:ICG | ◆ 10:1 TOAC:ICG) and (B) the ratio of TOAC to ICG was varied from 0 to 10:1 for in situ ICG NC precipitation.
Encapsulation Efficiency and Ion Pairing

Previously, we had successfully created NCs by forming hydrophobic ion pairs of therapeutic drugs using the in situ complexation of anionic and cationic compounds when one was solubilized in the aqueous phase and one in the organic phase.[51, 54] To test the ability to make ICG NCs by a similar ion pairing, the ratio of TOAC to ICG was varied and the degree of ICG encapsulation (Fig. 6(B)) was measured with UV-VIS absorbance. The sizes of the NCs were held constant (Fig. 6(A)) except for the micelle sample with no VE and no TOAC. However, the ICG loading was not kept constant across samples. Since the TOAC:ICG ratio was varied, and in some cases no TOAC was used, the ICG loading (Eqn. (2)) was kept constant rather than the ICG complex loading (Eqn. (1)). In the other experiments in this work where the ICG complex core loading was the optimal 10wt% for fluorescence (with 1:1 ICG:TOAC), the equivalent ICG core loading was 6.5wt%. The results in Fig. 6 are tabulated in Table 1.

Table 1: ICG Encapsulation efficiency, ICG core loading and equivalent complex core loading, and NC diameters for the encapsulation efficiency study where the ICG:TOAC ratio was varied.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Encapsulation Efficiency</th>
<th>ICG Core Loading**</th>
<th>Complex Core Loading†</th>
<th>Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0TOAC/0VE</td>
<td>96.6%</td>
<td>24.8wt%</td>
<td>N/A</td>
<td>25</td>
</tr>
<tr>
<td>0TOAC</td>
<td>94.6%</td>
<td>6.30wt%</td>
<td>N/A</td>
<td>120</td>
</tr>
<tr>
<td>0.1 Eqv. TOAC</td>
<td>89.9%</td>
<td>18.3wt%</td>
<td>3.80%</td>
<td>120</td>
</tr>
<tr>
<td>0.5 Eqv. TOAC</td>
<td>97.4%</td>
<td>12.0wt%</td>
<td>10.3%</td>
<td>113</td>
</tr>
<tr>
<td>1 Eqv. TOAC</td>
<td>99.9%</td>
<td>7.00wt%</td>
<td>11.0%</td>
<td>100</td>
</tr>
<tr>
<td>2 Eqv. TOAC</td>
<td>99.9%</td>
<td>6.90wt%</td>
<td>10.9%</td>
<td>109</td>
</tr>
<tr>
<td>10 Eqv. TOAC</td>
<td>99.7%</td>
<td>2.50wt%</td>
<td>3.90%</td>
<td>100</td>
</tr>
</tbody>
</table>

*The micelle case.
**Defined by Eqn. (2) – takes ICG encapsulation efficiency into account and assumes excess TOAC is not incorporated into the core.
†The complex core loading calculation assumes a 1:1 ICG:TOAC pairing and, thus, the limiting reagent defines the loading.
For 1:1 or higher ratio of TOAC:ICG the cationic counterion drives essentially complete encapsulation of the ICG into the NC core. However, at only 10wt% or less ICG complex core loading, the preponderance of hydrophobic VE and PS-chains in the core still substantially captures the ICG during rapid precipitation. The 0:10, 1:10 and 1:2 ratios of TOAC:ICG yield 94%, 89% and 97% ICG encapsulation, respectively. PS-b-PEG and ICG (no TOAC and no VE) gave encapsulation at 97% in the 25 nm micelle NCs. DeVoiselle, et al. investigated the complexation of ICG in the presence of various surfactants using tensiometric and fluorescence measurements and found that amphiphilic ICG assembles at interfaces.[31] Kircherr, et al. studied the encapsulation of ICG monosodium salt within nonionic surfactant micelles and achieved 90-95% loading efficiency but only 61% within Pluronic F68 micelles due to the higher hydrophilicity of the latter.[33] Similarly, Kim, et al.)[32] achieved encapsulation efficiencies of 39-79% for hydrophobic ICG-tetrabutylammonium salts in Pluronic F127. Rodriguez, et al. formed hydrophobic salts of ICG with tetrabutylammonium iodide (TBA) within poly(styrene)-b-poly(styrene-alt-maleic anhydride) micelles and achieved 87% encapsulation efficiency.[34] The rapid precipitation by FNP yielded higher ICG encapsulation efficiencies than any of the previously reported loading techniques. Since the previous techniques involved introducing the ICG from or through the aqueous phase, the lack of uniform encapsulation into the core may be the reason. The previous studies could not vary NC size, as we can with FNP, and so no comparison of fluorescence intensity with size, or loading efficiency is possible.

We calculated the available interfacial surface area available for ICG adsorption in the case of the polymeric micelles, which from Fig. 6(A) are 25 nm diameter. Based on the projected surface area of an ICG molecule ~0.15 nm² and molecular volume of 5.8 x10⁻² nm³ volume
(calculated with ChemAxon MarvinSketch v6.2.2 Geometry Plugin) [55], a micelle could accommodate $13 \times 10^3$ ICG molecules on its surface and $141 \times 10^3$ ICG molecules in the core. Since the ICG loading in the micelle core is $\sim 25\text{wt\%}$, the core would contain $\sim 35 \times 10^3$ ICG molecules. Therefore, we might expect ICG to be loaded both in the core and at the interface, which might be the reason for the very efficient loading of the micelles, even in the absence of the TOAC counter ion. The surface loading would become less significant for larger NCs.

**ICG NC – BSA Stability**

It is important that the dye remain trapped in the NC core so that the NCs can be effectively tracked without fluorescent signal being confounded with free dye or dye bound to proteins. Kim, et al. [32] reported Pluronic F127 micelles loaded with ICG-tetrabutylammonium (TBA) iodide salt and incubated with 50% serum. A 3-fold increase in fluorescence was seen due to the partitioning of ICG from the micelles. However, we have previously demonstrated that the relatively low CMC of these PPO-PEO copolymers enables them to serve as molecular shuttles of hydrophobic components in the cores of NCs.[6] The block copolymers used in FNP are chosen to have hydrophobic blocks large enough so that they do not partition off of the NC surface; they are kinetically frozen. ICG is known to bind strongly to serum proteins, in particular albumin, globulins, and α-lipoproteins.[28, 56-59] As noted above, Rodriguez et al. tested the release of ICG from NCs only in DI water. This does not address the stability of ICG in NCs in vivo, because in vivo ion exchange of the ICG:TBA and partitioning to serum proteins can occur, as seen by Kim. Our objective was to determine if the ICG:counterion complex was stable in the presence of serum protein, and if the more hydrophobic TOAC might prevent ion exchange and make the NC less susceptible to partitioning to serum proteins.
A NC formed from premade ICG-C₈ complex was incubated at RT with 4wt% BSA and the change in fluorescence was measured over a period of several hours. The change in system fluorescence demonstrates exchange of ICG from the core to serum protein. The system equilibrates within the first 25 minutes (see Fig. 7).

![Fluorescence at 813-818 nm](chart.png)

**Fig. 7** – Equilibration time for ICG NC – BSA system. ICG-C₈ premade NCs were prepared and incubated with 4wt% BSA in buffer. The increased fluorescence is associated with partitioning to serum proteins and occurs within 30 minutes of incubation.
**Fig. 8** – Fluorescence transfer experiments showing the increase in ICG fluorescence upon the addition of BSA to ICG dissolved in 5 mM phosphate buffer. For pure ICG the curve is: $FL = 1.37 \times 10^7 [ICG \text{ (mg/mL)}]$, $R^2 = 0.996$, for ICG+BSA: $FL = 2.36 \times 10^7 [ICG \text{ (mg/mL)}]$, $R^2 = 0.991$.

The fluorescence of ICG in buffer and the fluorescence of ICG in serum protein was measured, and the data shown in **Fig. 8**. The fluorescence is approximately two times higher for ICG bound to protein than for ICG in buffer (the correlation fits to the data are given in the Fig. caption). We can estimate the amount of ICG that has partitioned out of the NC from the data in **Fig. 7** for the initial NC fluorescence and the fluorescence after equilibration with 4 wt% BSA. The initial fluorescence, when the ICG is entirely in the NC, is ~400 (arbitrary units), and after equilibration the fluorescence is ~600. Using the calibration curve in **Fig. 8**, the ICG concentration in the initial NC sample is calculated to be $2.92 \times 10^{-5}$ mg/ml. After equilibration of that amount of
ICG between the original NC phase and the BSA from the final fluorescence measurement and the two calibration curves, it is found that $2.02 \times 10^{-5}$ mg/ml of ICG is associated with BSA—about 70% of the ICG has partitioned out of the NCs. While the ICG is stable in phosphate buffer (see Appendix E), when given the sink of the BSA protein it is exchanged away from the NC. Clearly this would be problematic for quantification in in vivo studies.

CONCLUSIONS

In this work we report the formulation of indocyanine green-based (ICG) NCs, formed either by pre-forming an oil soluble (hydrophobic) ICG-TOAC or ICG-TDDAC ion pair or by in situ forming the ion pair and NC in a one-step process using Flash NanoPrecipitation (FNP). Both methods yield similar NCs in terms of size, stability, optical absorption, and fluorescence for each type of counter ion. Maximum fluorescence-per-particle for the ICG-TOAC complex is 10 wt% core loading, which represents the balance between increasing the concentration of dye in a particle, and avoiding fluorescence quenching due to Förster energy transfer at high concentrations. To elucidate the mechanism of ICG complex formation and NC loading, we varied the ratio of TOAC to ICG and showed that ICG encapsulation efficiencies of 100% were achieved by using a 1:1 or higher ratio of cationic counter ion to ICG. However, the rapid precipitation process produces relatively high encapsulation efficiencies (> 80%) even without stoichiometric counter ion ratios. Limits for the ICG loading of the NCs are presented based on the type of core material used. Above these limits ICG precipitates outside of the NCs into large crystals. VE is found to be a more accommodating core material compared to 1.5k PS. We also
demonstrate the control of NC size between 30 – 180 nm at constant ICG loading and show that the intrinsic fluorescence of the NCs scale linearly with the volume of the NC core.

Lastly we present data showing the photostability of ICG NCs in the presence of physiologically relevant amounts of albumin, for which ICG has a very strong binding affinity. The data on ion exchanging of ICG from NC cores provides important data currently lacking in the literature. The loss of ICG from the NC would still leave a 100 nm NC with $10^4$ ICG molecules in the core – indicating that a bright fluorescence signal would still be achieved. However, the instability must be considered when attempting to relate fluorescence images to NC concentrations in applications such as targeting studies.

**ACKNOWLEDGEMENTS:** We acknowledge financial support from the National Institutes of Health (Award No. 1RO1CA155061-1), and the Stuart M. Essig ’83 and Erin S. Enright ’82 Fund for Innovation in Engineering and Neuroscience.
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CHAPTER 4
Nanoparticle Contrast Agents for Photoacoustic Imaging

Portions of this chapter were presented at the American Chemical Society Colloids Meeting in Philadelphia in 2014.

Abstract

Photoacoustic imaging (or optoacoustic imaging) a new imaging modality combining the best features of optical and ultrasound techniques. In this mode, incident long wavelength laser radiation, in the optical imaging window between 750-1450 nm, excites an in vivo circulating contrast agent that has an absorption peak overlapping with the laser wavelength. The energy dissipated as waste heat from the excitation causes a thermoelastic (pressure) wave to propagate out from the contrast agent, allowing for signal deconvolution with a standard ultrasound receiver. This combination allows for deeper penetration (several cm) compared to pure optical techniques, high resolution, and real-time multiplexed signal tracking without the need for radioactivity. In this chapter we report two studies detailing the development of nanocarrier contrast agents for photoacoustic imaging produced via the Flash NanoPrecipitation process. Compared to other photoacoustic agents such as those based on gold and carbon nanotubes, this process allows for the encapsulation of dyes with sharp absorbance peaks which opens up the possibility of nanocarrier multiplexing. Furthermore, both drugs and dyes can be encapsulated within one nanocarrier package to allow for theranostic applications and nanoparticle size can be tuned to modify biodistribution. Lastly, the nanocarriers are protected by a dense PEG shell which improves circulation time and allows for the attachment of targeting ligands for targeted diagnostics and drug delivery. In collaboration with VisualSonics Inc., these nanocarriers were successfully visualized by photoacoustic imaging methods as some of the brightest contrast agents seen to date. They were shown to be easily unmixed from endogenous hemoglobin background signal and also displayed enhanced accumulation in a murine tumor model with limited general cytotoxicity and improved photostability compared to free dye. In the second study, in collaboration with the Institute for Biological and Medical Imaging in Germany, a framework is presented for evaluating novel photoacoustic contrast agents. Flash NanoPrecipitation is considered especially suitable due to its inherent scalability and ability to flexibly produce long circulating nanocarriers with various payloads and surface chemistries. Multispectral Optoacoustic Tomography is presented as a key technology for the evaluation of these contrast agents. 100 nm and 50 nm nanocarriers were compared in ex vivo and in vivo models. The 100 nm carriers were found to be much more photostable than the 50 nm carriers and displayed lower toxicity and greater biocompatibility in in vitro models. Due to their larger size, the 100 nm carriers also had greater signal output and sensitivity when imaged in phantoms and when subcutaneously injected into mice. These studies prove the utility of Flash NanoPrecipitation for the production of diagnostically relevant nanocarriers that may be produced in a scalable fashion.
INTRODUCTION

Photoacoustic imaging (PAI) is a rapidly emerging biomedical imaging modality that provides functional and molecular information in real-time with deep penetration and high resolution without the need for ionizing radiation, combination not found with other imaging modalities.[1] According to Xu and Wang (2006)[2], the photoacoustic (PA) effect is the physical basis for PA imaging; it refers to the generation of acoustic waves by the absorption of electromagnetic EM energy, such as optical or radio-frequency (rf). In this modality, incident optical or rf radiation is converted to heat by electron excitation and dissipation which results in the generation of heat by dissipation further generating a thermoelastic pressure wave which travels back to the detector at approximately 1.5 mm/us ± 10% (in tissue) with varying time delays depending on the thermal expansion coefficients of the materials involved (Fig. 1). A temperature rise on the order of milliKelvins is common. The maximum initial pressure generated by the absorber is given by:

\[ P_0 = \Gamma F \mu_a \]  

Eqn. (1)

Where \( P_0 \) is the initial pressure generated at the absorber, \( \Gamma \) is the Grüneisen parameter, \( F \) is the light fluence at the absorber, and \( \mu_a \) is the absorption coefficient of the absorber. The pressure wave is attenuated by travel through tissue on the way back to the ultrasound detector. If there are very large acoustic heterogeneities present, pure ultrasound is often used first to map out the physical features of the system under study.
Good images are obtained when three important time scales are correctly tuned relative to each other. The laser pulse time, $\tau_p$ must be lower than the thermal dissipation time $\tau_{th}$ and the stress transmission time $\tau_s$ so that thermoelastic waves are well separated from each other. Of these two conditions, $\tau_s$ is often on the order of 100 ns and is the more stringent condition.

Penetration in tissues is largely a function of absorption and scattering, both of which reduce penetration depth. In vivo, the absorption coefficient in the NIR is $\mu_a = 0.1 - 10$ cm$^{-1}$. However, the effective scattering coefficient is much higher. It is defined as:[2]

$$\mu_s' = \mu_s(1 - g)$$  \hspace{1cm} \textbf{Eqn. (2)}
Where $\mu_s$ is the pure scattering coefficient and $g$ is the anisotropy factor. Compared to a max absorption coefficient of $10 \text{ cm}^{-1}$, the pure scattering coefficient $\mu_s = 100 \text{ cm}^{-1}$ *in vivo* in the NIR range and $g = 0.9$. Thus, scattering of optical/NIR light in tissue can be an order of magnitude higher than absorption.

Pure acoustic imaging such as ultrasound operates in the frequency range of kHz to MHz which allows for deep penetration (dependent on frequency) but has relatively poor contrast and only gives information on mechanical properties and anatomical features. However, ultrasound does have a scattering coefficient 2-3 orders of magnitude lower than optical scattering which allows deep penetration in vivo (*Fig. 2*). Combining ultrasound and optical imaging allows scientists and researchers to obtain high penetration depth (centimeters), high contrast (with the use of appropriate contrast agents), and high resolution over a relatively large volume in real time.

![Fig. 2 - Penetration depth for optical, PA, and ultrasound imaging techniques.[3]](image)

**Table 1 – Frequencies and wavelengths for imaging modalities relevant to biomedical imaging.**

<table>
<thead>
<tr>
<th>EM Radiation</th>
<th>$\nu$ [Hz]</th>
<th>$\lambda$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma ray</td>
<td>$10^{20}$</td>
<td>1 pm</td>
</tr>
<tr>
<td>X-ray</td>
<td>$10^{18}$</td>
<td>100 pm</td>
</tr>
<tr>
<td>Ultraviolet</td>
<td>$10^{16}$</td>
<td>10 nm</td>
</tr>
<tr>
<td>Visible</td>
<td>$10^{14}$</td>
<td>400-700 nm</td>
</tr>
<tr>
<td><strong>Near IR</strong></td>
<td>$10^{14}$</td>
<td><strong>700-2500 nm</strong></td>
</tr>
<tr>
<td>Infra-red</td>
<td>$10^{13}$</td>
<td>10 $\mu$m</td>
</tr>
<tr>
<td>Microwave</td>
<td>$10^{10}$</td>
<td>1 cm</td>
</tr>
<tr>
<td>Radio wave</td>
<td>$10^{5}$</td>
<td>1 km</td>
</tr>
</tbody>
</table>
As a general rule, radiofrequency (rf) excitation is associated with lower resolution but deeper penetration depths compared to optical/NIR excitation (Fig. 2). This is because longer wavelengths (and thus lower frequencies and lower photon energies) are generally associated with lower resolution and deeper penetration on the order of centimeters (within the optical imaging window).

**PA Contrast Agents**

While unaided PA imaging yields detailed chemical information such as oxy/deoxygenated hemoglobin concentrations and oxygen saturation levels, the use of a multifunctional contrast agent greatly opens up the amount and type of information that can be obtained without changing the necessary hardware or image reconstruction algorithms. As implied by the name, contrast agents can ‘light up’ areas of interest far beyond what is possible with native PA imaging.[1]

The ideal contrast agent has a strong, sharp absorbance peak in the optical imaging window (700-1450 nm, shown in Fig. 1 in Chapter 1). An extinction coefficient on the order of $10^5 \text{ M}^{-1}\text{ cm}^{-1}$ or higher is best. In contrast to fluorescence imaging described previously, a low quantum yield is preferred for PA imaging. This can be achieved by using molecules with inherently low quantum yields or packing dye molecules close together in nanocarrier cores to enhance the rate of Förster energy transfer (a form of quenching) and thus increase dissipation by heat. This results in a stronger PA signal. A sharp peak is important because the background photoacoustic signal must be subtracted. The availability of tunable lasers in the range from 680 – 970 nm requires the ideal PA contrast agent maximum absorbance to be in that range.
Based on the characteristics outlined for an ideal PA contrast agent, the list of possible candidates is extensive. However, PA contrast agents reported in the literature can be categorized into those comprising metals that exhibit long wavelength surface plasmon resonance, to dyes with long conjugated π-systems and carbon nanotubes. More specifically, geometries such as 5-7 nm gold nanoparticles[4], spherical gold nanoshells (on a silica substrate)[5], silver nanoplates[6], gold coated carbon nanotubes[7], gold nanorods[8], silica coated gold nanorods[9], gold nanocages (coated on a silver nanocube)[10], iron oxide nanoparticles[11, 12], carbon nanotubes[13, 14], polymeric nanoparticles comprising semiconducting π-conjugated polymers in the core[15], and micelles comprising dyes and photosensitizers such as ICG[16, 17], polypyrroles[18], Prussian blue[19], and NIR830[12] have all been reported. Indeed, several of these reports combine two imaging agents into one nanocarrier package (for example, iron oxide and gold, or gold and ICG). Microbubbles have also been reported in conjunction with gold and iron oxide nanoparticles as microbubbles are potent ultrasound contrast agents[4, 20]. Many of these systems were targeted by antibodies or peptides and have been tested in vitro and in vivo with murine tumor models and, in addition to imaging capabilities, demonstrated disease site accumulation and cancer killing abilities through photothermal ablation (in the case of gold constructs) and release of reactive oxygen species (from photosensitizers).

However, imaging agents based on gold and carbon nanotubes have the serious deficiency of broad absorbance spectra in the optical imaging window (see Fig. 3). This makes background acquisition and subtraction difficult which can make it difficult to obtain a clean separation
between contrast agent and optically active species such as hemoglobin. This is even more problematic for multiplexing applications where sharp, well-separated absorbance peaks are required.

**Fig. 3** – Characteristics of an ideal PA imaging contrast agent (sharp absorbance peak) vs. the broad carbon nanotube (CNT) absorption spectrum shown as the dotted line and on the right. Reproduced with permission by the American Association for the Advancement of Science. [21]
To solve these connected problems in biomedical imaging, we used several different types of dye materials (some of which have never been used in biomedical applications before) in the Flash NanoPrecipitation process\cite{22} to generate dye loaded polymeric nanoparticles or micelles from 20 to 300 nm (Fig. 4 – Nanoparticle/nanocarrier formulation data for bacteriochlorins are shown in Appendix F). With this process, we were able to easily vary dye loading and nanoparticle size independently, as well as introduce sacrificial co-excipients to improve stability and slow photobleaching, and surface targeting moieties to improve disease site accumulation. The dyes selected generally had sharp, well separated peaks in the optical imaging window (especially in
the case of the bacteriochlorins) and were extremely hydrophobic (logP > 9) to ensure stability in physiological conditions. By loading the dyes at high levels in the nanoparticle cores, we were able to achieve concomitantly higher photoacoustic signal due to the increased fluorescence quenching.[23]
Materials and Methods

Materials

LW2 dye (originally known as iBu-TSBSH5, chemical name: 6,15-bis(tri-sec-butylsilylethynyl)-2,3,10,11-bis(diisobutyldioxolanato)hexacene) was obtained as a gift from John Anthony, Department of Chemistry, University of Kentucky. Bacteriochlorins were obtained from NIRvana Life Sciences. The dyes IR122 and LDI830 were obtained from Persis Science, Princeton NJ. Vitamin E (T3251) was obtained from Sigma-Aldrich and used as-is. Polystyrene and PS-b-PEG were obtained from Polymer Source (Morntral, Canada) and used as-is. Tetrahydrofuran (THF) and dimethylsulfoxide (DMSO) were obtained from Fisher Scientific and used as-is. All water used was ultrapure water (>17.8 MΩ-cm) obtained from a Barnstead NanoPure Diamond system.

Methods

Nanoparticle Formulation – VisualSonics Collaboration

LW2 dye and PS$_{1600}$-b-PEG$_{5000}$ were dissolved in THF at 6 mg/mL each and rapidly mixed against 1 part water which was immediately quenched in a water reservoir consisting of 9 parts water. The resulting nanoparticles loaded at 80wt% dye core loading were dialyzed for 24 hours before sodium phosphate was added to make the ionic strength 5 mM. Following this, the nanoparticles were concentrated by a factor of approximately x37 in an AMICON 100k MWCO
centrifuge filter (20 mins each cycle, 7000g). Final nanoparticle solids were estimated at 41 mg/mL. Size and absorbance characterization was performed before and after dialysis.

Size Characterization
Nanoparticles were diluted with water to near water clarity then characterized for diameter by dynamic light scattering (Malvern Zetasizer Nano ZS, Malvern Instruments, UK). Reported sizes are derived from the CONTIN algorithm which gives the intensity weighted size distribution.

Absorbance
Nanoparticles were diluted in THF to dilute and dissolve the nanoparticles to eliminate scattering effects and keep the measured absorbance below 2. Dye concentrations were quantified by a UV-vis absorbance calibration curve for LW2. NP concentrations were determined from the stoichiometry between the dye and total nanoparticle solids.

In vivo Testing
A male, athymic nude mouse with PC3 xenograft (subcutaneous prostate tumor model) was kindly provided by the Sloan Kettering Memorial Cancer Center and was anesthetized and injected with a 100 μL sample of nanoparticles. The mouse was monitored with a VisualSonics VEVO Lanzr Photoacoustic Imaging System (VisualSonics, Toronto, Canada) for 24 hours.
Results and Discussion - Visual Sonics Collaboration

Two types of nanocarriers containing the long wavelength dye LW2 were produced for this study. The first, termed LW2-small, was loaded with LW2 at 2.3wt% core loading, close to the optimal loading for fluorescence (3.8wt%). The second, termed LW2-large, was loaded at 80wt% core loading, which we expected would yield a much stronger photoacoustic signal, based on the higher loading (and therefore greater fluorescence quenching) and large size. The sizes of the two carrier populations are shown in AppF-Fig. 12 where the smaller fluorescent nanocarriers (NCs) are 25 nm in diameter and the larger photoacoustic imaging (PAI) carriers are 110 nm. Both suspensions were concentrated by centrifugal filtration to a final concentration of about 40 mg/mL total solids in suspension with an ionic strength of 5 mM phosphate.

Proof of concept data was obtained in an informal study in collaboration with VisualSonics Inc. (Canada), one of the world’s leading preclinical imaging companies, and a global leader in photoacoustic imaging technology. The PAI agents were compared in phantom *ex vivo* tests to assess photoacoustic signal strength. As expected, the LW2-large NCs (110 nm, 80wt% core loading) were far brighter than the LW2-small NCs (AppF-Fig. 13) and thus all further *in vivo* tests were done on those. The LW2-large PAI agents were first injected into male, athymic nude mice and the PA signal (735 nm absorbance peak) was seen to be clearly distinct from the hemoglobin (Hb) absorbance signal at 900 nm (Fig. 5). The perfusion of these NCs was quantified by identifying the spleen and kidney regions by pure ultrasound, then observing the change in PA signal in those regions as a function of time following tail vein injection of the PAI agent. A rapid rise was seen in the first minutes following injection. Uptake to the kidney was
faster than that for the spleen, but the signals leveled off to equivalent amounts (Fig. 6). A mouse containing a subcutaneous tumor model was also injected by tail vein with the PAI agents and monitored for 24 hours. As seen in Fig. 5, due to the sharp, strong PA signal from the agent which was easily unmixed from the Hb signal by software, we were able to observe passive tumor accumulation over 24 hours by the enhanced permeation and retention effect (Fig. 7).[24] No active targeting moieties such as folic acid were present on the nanoparticles, though it has been shown that hydroxyl-terminated block copolymers (used in this study) do have increased association with and uptake into macrophages compared to methoxy-terminated block copolymers.[25] Thus, the surface properties of the nanocarriers have important implications for cell uptake even when active targeting functionality is not present.
Fig. 5 – Spectral characterization of the PAI agent within spleen and kidney regions. (A) and (B) show the baseline signal from hemoglobin (Hb) around 900 nm. Upon injection of the PAI, (C) and (D) show the influx of the agent with absorbance peak at 735 nm that is clear and well separated from the Hb signal. This allows for efficient spectral unmixing as will be shown in Fig. 7.
Fig. 6 – Perfusion quantification of PAI agent. For the selected spleen and kidney regions shown in (B), there is a rapid rise in the signal attributable to the PAI agent with absorbance at 735 nm following injection of the agent on the order of seconds.
Fig. 7 – Accumulation in subcutaneous tumor (circled in red in (A)) and spectral unmixing of PAI agent over 24 hours. (A) and (B) pure ultrasound images of tumor region. (C) and (D) PA equivalent images to (A) and (B) with PAI agent detected in green, background Hb and HbO\textsubscript{2} in red and blue. (E) Spectrally unmixed image showing accumulation of PAI agent within tumor over 24 hours.
IBMI COLLABORATION

Adapted from Nunes, et al. “Development of Hexacene Loaded Nanoparticles for Optoacoustic Imaging.” In Preparation. (VJP is 2nd author – contributions are specified below).

INTRODUCTION AND MOTIVATION

The introduction of new imaging modalities for pre- and post-operative diagnostics, as well as for real-time intra-operative visualization, will enhance the effectiveness of medical interventions. Optical imaging provides contrast based on the tissue absorption contrast, and therefore has been a fundamental tool for biomedical research [26, 27]. Nevertheless, its capacity for in vivo tissue imaging has been limited by light scattering that restricts application to superficial investigations, even when confocal or multiphoton methods are used [26]. Recent advances in optoacoustic (also termed photoacoustic) imaging now allow imaging at depths and resolutions unprecedented for optical methods[1]. It has emerged as a promising imaging platform that offers new solutions for pre-clinical and clinical imaging [28-30]. Optoacoustic technology bridges the gap between optical imaging, with optical-absorption-generated contrast, and ultrasound imaging, with its ultrasound-based detection system. After illumination by laser light pulses of short duration (10-100ns), a transient photo field is established in the tissue, and photo-absorption leads to thermoelastic expansion of fluid and tissue. This phenomenon leads to the generation of acoustic waves that are detected by acoustic transducers. By detecting the pressure waves emitted by photo excitation, and applying deconvoluting algorithms, visualization of relevant physiology can be achieved. Recent developments include a multispectral and tomographic approach: Multispectral Optoacoustic Tomography (MSOT) Imaging. MSOT is a versatile imaging tool that provides in vivo structural (anatomic) [29-31], functional [30, 32] and molecular [31, 33, 34] information at clinically relevant penetration.
depths, maintaining the high spatial resolution and image contrast, when compared to existing imaging techniques [29, 35]. MSOT relies on near-infrared (NIR) wavelengths, and therefore its detection is minimally affected by optical scattering. Overcoming the limitation imposed by scattering enables studies of cellular processes, a major challenge of post-genome biology [26].

Optoacoustic signals can be achieved based on intrinsic tissue optical properties of endogenous absorbers (e.g. oxyhemoglobin, deoxyhemoglobin, melanin[36, 37]). Contrast can also be generated by exogenous additives -- optoacoustic reporters such as organic dyes, gold nanorods, dendrimers, liposomes-ICG, etc) [38-40]. Gold and other metal-based particles produce strong optoacoustic signals but their use is often limited due to tissue accumulation and toxicity [36, 41-44]. Several soluble organic compounds “grandfathered” by the FDA for other indications have been applied as photoacoustic agents. An example is ICG (and also methylene blue) that has been FDA approved since 1959 and is routinely used in a variety of clinical settings and protocols (ophthalmologic, cardiovascular, etc.)[45]. ICG has notoriously poor in vivo stability (self-aggregation, protein binding) and has rapid blood clearance, which compromises its imaging utility. Advances at the pre-clinical level have been achieved, in particular regarding: enhancement of molecular sensitivity and specificity [39], functional biosensing [46], activated drug release [46, 47], imaging of tumor vasculature and microenvironment [37, 48], and gene delivery [38].

In this emerging and fast growing field, there is a need for new imaging reporter probes to fulfill the potential of optoacoustic imaging. A major gap is in scalable techniques to make biocompatible nanoparticles that flexibly incorporate targeting functionality. Target-specific
moieties in the imaging platform enable functional diagnostics and, in this way, increase the imaging specificity of physiology and pathology events at a molecular level [47].

The scalable technique we use for nanoparticle formation is the block-copolymer-directed rapid precipitation technique, Flash NanoPrecipitation (FNP) (Schematic 1)[49]. FNP is a novel technology for the encapsulation of hydrophobic organic actives (therapeutic or diagnostic molecules) into block-copolymer-stabilized nanoparticles at high concentration with high encapsulation efficiency and is described in detail earlier in this chapter.[50-53]

Schematic 1 - Nanoparticle formation by Flash NanoPrecipitation. The organic active is the long-wavelength hexacene dye iBu-TSBSH5 (referred to here as LW2).

The optoacoustic-active (ie. highly optically absorbing) organic dyes are also novel, in that we employ extremely hydrophobic compounds that reside completely encapsulated in the interior of the nanoparticle. This is in contrast to the more common procedure of attaching optically active
organic molecules to the surface of pre-formed nanoparticles. Acene dye derivatives have proven to be exceptional molecules for organic LED applications, the application for which they were originally developed [54-60]. Recently, we have explored the use of these dyes in fluorescence biomedical imaging.[23] With partition coefficients between water and hydrophobic phases of 1 ppb (logP= 9), these compounds stay with the nanoparticle. Their hydrophobicity makes them ideally suited for encapsulation by FNP. This is in contrast to amphiphilic compounds such as ICG that can partition from nanoparticles to circulating albumin or lipid components. Using the FNP platform, block copolymer nanoparticles are loaded with the long-wavelength hexacene dye 6,15-bis(tri-sec-butyilsilylethynyl)-2,3,10,11-bis(diisobutylidioxolanato)hexacene (referred to here as LW2 whose structure given below).[61] LW2’s narrow absorption peak at 730nm makes it an excellent candidate for optoacoustic imaging, since the narrow peak facilitates subtraction of non-specific tissue response from dye-specific response. Furthermore, the FNP platform enables the production of targeted nanoparticles by using block copolymers with functional groups attached to the terminal ends of some fraction of the block copolymer hydrophilic blocks. For small molecule ligands, such as mannose and RGD peptides, the ligand can be covalently bound to the PEG chain prior to nanoparticle assembly.[51, 62] For larger protein and antibody targeting agents, functionalization can be accomplished after nanoparticle formation.[63] The dense PEG layers on the nanoparticle surface prevents non-specific clearance by the RES.[64]
Schematic 2 - *Five steps for optoacoustic probe development.*

There is no single best optoacoustic agent for all applications, and the selection of the ideal reporter probe is still open for discussion [27]. Therefore, in this work, we present a guide for the screening and evaluating reporter probes for optoacoustic imaging as shown in **Schematic 2**. This guide consists of 5 essential steps that one must take into account when searching for a new class of optoacoustic contrast agents. The evaluation begins with an investigation of the physicochemical and optical characteristics of the new probe (**Step 1**). Then the optoacoustic performance must be evaluated. The next two steps involve the optical and chemical stability (**Step 3**), and biocompatibility (**Step 4**). Finally, the sensitivity in phantoms and *ex vivo* must be demonstrated (**Step 5**). The nanoparticle probes developed in this study are evaluated on the basis of these criteria.
The structure of this study follows the five evaluation criteria presented above. Two nanoparticle formulations were produced, and named as LW2-50nm and LW2-100nm according to their diameters: 50 nm and 100 nm, respectively. Their physicochemical and optical characteristics were determined by UV-NIR spectrophotometric, DLS, TGA, TEM and MSOT studies. Spectrophotometric and phantom studies at different dilutions for each probe were performed to evaluate their optoacoustic performance, stability and photobleaching profile -- all key requirements for an optoacoustic probe. In vitro biocompatibility studies and sensitivity studies after subcutaneous injection in mice were performed to establish their toxicity profile and sensitivity for MSOT detection.

MATERIALS AND METHODS

NOTE: Nanoparticle formulations were completed at Princeton University, while ex and in vivo experiments were done at the Institute for Biological and Medical Imaging (IBMI) in Germany.

Materials and Reagents (Princeton)

LW2 dye was synthesized by John Anthony, Dept. of Chemistry, University of Kentucky according to previously reported procedures.[54, 61] PS_{1600-b-PEG_{5000}} (P13141) was obtained from Polymer Source (Montreal, Canada). HPLC grade THF was obtained from Fisher Scientific (T425), ultrapure water was obtained from a Barnstead Nanopure system delivering > 18.0 MΩ-cm. Sodium phosphate monobasic anhydrous (S3139) were obtained from Sigma Aldrich and used without further modification.
Formulation of LW2 Nanoparticles and Dye Loaded Micelles (Princeton)

Two formulations of LW2-loaded nanoparticles were prepared: LW2-100nm and LW2-50nm. LW2 dye and PS_{1.6k-b-PEG_{5k}} were dissolved in THF at 6 mg/mL each (LW2-100nm) and 0.25 mg/mL each (LW2-50nm) and rapidly mixed against an equivalent volume of water in a confined impinging jets mixer (CIJ)[65] which was quenched with 9 parts water to give an overall mixing ratio of 10:1 and nanoparticles containing about 80% core loading of dye. The nanoparticles were dialyzed against 5 mM phosphate buffer for 24 hours.

A third formulation was also prepared and optimized for fluorescent imaging (LW2-micelles): LW2 dye and PS-b-PEG were dissolved in THF at 0.17 and 17.7 mg/mL respectively and rapidly mixed against an equivalent volume of water in a CIJ and quenched with 9 parts water.

Concentration of LW2 Nanoparticles (Princeton)

LW2-100nm and LW2-50nm were concentrated following dialysis by placing each suspension in a 20 mL Centricon centrifugal filter device (Z368253, Sigma Aldrich) and centrifuging for 20-25 minutes at 7000g. The concentrates were removed and the procedure repeated multiple times to process the entire suspension volume. At the end, the concentrates were collected. Absorbance measurements were taken before and after to determine the factor of concentration for each suspension and subsequent NP concentration.
Characterization of Absorbance (Princeton)

Dye loaded nanoparticles were diluted in THF to dissolve the particles (thus eliminating scattering effects) and keep the measured absorbance below 2 absorbance units. LW2 concentrations were quantified by the absorbance peak at 735 nm. Nanoparticle concentrations were quantified by the stoichiometry between the dye and total solids.

Characterization of Fluorescence (Princeton)

Initial fluorescence measurements were taken on LW2-nanoparticles in a glass cell with a Hitachi F-7000 Fluorometer (5 nm excitation and emission slits, 950 V PMT, 0.5 s response time). The excitation wavelength was 701 nm.

Optimization of LW2 Fluorescence (Princeton)

The optimal nanoparticle loading of LW2 in nanoparticle cores was determined by varying the loading of the dye and performing fluorescence measurements at multiple dilutions to ensure that the fluorescence vs. nanoparticle concentration was linear. The slope of the line was calculated as the intrinsic fluorescence of the nanoparticle system.

TEM Imaging

Samples for TEM were prepared by placing 5 μL of the nanoparticle dispersion on an Ultrathin Carbon Film on a Holey Carbon Support film on 400 mesh copper grid (Ted Pella, Inc., Redding, CA) and drying under ambient conditions. The samples were imaged using a Philips CM100 TEM (Eindhoven, The Netherlands) operated at an accelerating voltage of 100 kV.
Spectroscopy Studies (IBMI)

Five different dilutions of each LW2-nanoparticles were prepared with optical densities ranging from 0.3 to 2.2. The wavelength-dependent absorption spectrum of each stock was then measured from 680 to 900 nm under the UV-vis spectrophotometer. Later the spectrum information was used as an input spectrum for multispectral post-processing of optoacoustic signals.

Multispectral Optoacoustic Tomography (MSOT) (IBMI)

All optoacoustic measurements were performed in a real-time whole-body mouse imaging MSOT system. An earlier version of the system was described previously[66]. Briefly, optical excitation was provided by a Q-switched Nd:YAG laser with a pulse duration of ~10 ns and a repetition rate of 10 Hz and a tunable range of 680-900 nm. Light was homogeneously delivered to the sample using a fiber bundle split into 10 output arms. The emitted ultrasound signal was detected using a 64 element transducer array cylindrically focused and having a central frequency of 5 MHz, allowing acquisition of transverse plane images. The fiber bundle and transducer array were stationary, and the sample could be moved to acquire different imaging planes using a moving stage. Measurements took place in a temperature controlled water bath at 34°C for acoustic coupling, and the samples were kept dry using a thin clear polyethylene membrane attached to the sample holder.

Phantom Studies (IBMI)
Cylindrical phantoms of 2 cm diameter were prepared using a gel made from distilled water containing Agar (Sigma-Aldrich, St. Louis, MO, USA) for jellification (1.3% w/w) and an intralipid 20% emulsion (Sigma-Aldrich, St. Louis, MO, USA) for light diffusion (6% v/v), resulting in a gel presenting a reduced scattering coefficient of $\mu_s \approx 10 \text{ cm}^{-1}$. A cylindrical inclusion containing the sample of approximately 3 mm diameter was put approximately in the middle of the phantom, along with a tube containing classical black ink with a known optical density of around 0.3 at 800 nm for intensity measurement reference.

**MSOT-Phantom acquisition (IBMI)**

Imaging of the phantoms was executed at a single position, located approximately in the middle of the phantom. Data acquisition was performed in the wavelength range 680-900 nm in steps of 5 nm, using 10 averages per wavelength resulting in 1 sec acquisition time per wavelength. For the photobleaching studies, the data was acquired at a single position at peak absorption of LW2-loaded nanoparticles with OD 1.3 (730nm) during continuous illumination for 30 minutes.

Image reconstruction of phantom studies was achieved by a model-based image reconstruction published previously[67, 68]. The algorithm delivers more accurate performance and quantification relative to conventional back-projection algorithms. Following image reconstruction, spectral unmixing was performed using a linear model assuming that the spectra acquired were a linear mix of the LW2-loaded nanoparticles spectrum and a background spectrum. For each pixel in the image, the algorithm fitted
the measured optoacoustic[69] spectrum, normalized for variations in laser energy per wavelength, to the known absorption spectra of the chromophores assumed in the image.

*In-vitro Toxicity studies (MTT assay) (IBMI)*

The cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide (MTT) assay as previously reported.[70] The MDA-MB-231 cell line, a human metastatic breast cancer cell line (HTB-26; American Type Cell Culture Collection, Manassas VA), was grown in 5% CO₂ at 37°C in DMEM (Sigma-Aldrich) containing 10% fetal bovine serum and antibiotics (penicillin and streptomycin, Sigma-Aldrich). Routine culture treatment was conducted twice a week. Cells were harvested and seeded in 96-well plates at 2.4 x 10³ cells per well in a final volume of 100 µl. After overnight incubation for cell adhesion, the medium was removed, and the cells were incubated at 37°C with 100 µl of medium containing LW2-100nm NP (0.1 – 3 mg/ml) or LW2-50nm NP (0.1 – 1.5 mg/ml), 10% dimethyl sulfoxide (DMSO) as a negative control and DMEM as a positive growth control. After 4 h incubation at 37°C, the cells were washed once with 100 µL PBS followed by addition of DMEM. Cells were then allowed to grow for 5 days at 37°C without changing the medium. The cell viability was determined by a colorimetric cell viability assay (Cell Proliferation Kit 1 (MTT), Roche) used according to the manufacturer’s instructions. The absorbance measurement was performed on a microplate reader (Infinite® 200 PRO, Tecan) and was determined at 595 nm, whereby 651 nm was used as a reference wavelength. To quantify cell viability, the ratio of the absorbance of the samples to the absorbance of the non-treated control samples (reference 100%) was calculated.
Animal model & Subcutaneous injection of reporter probes (IBMI)

8-week old female CD-1 mice (Harlan, UK) were injected subcutaneously \textit{ex vivo} with a mixture of matrigel (25 µL) and reporter probe (25 µL) in a total of 50 µL per injected solution. Each reporter probe was injected at 5 different optical densities (OD: 0.3, 0.5, 0.82, 1.3, 2.2) in 5 different locations on the back of the mouse. A solution with the reference ink dye (OD: 0.3) was also injected subcutaneously as a reference control.

\textit{MSOT-ex vivo acquisition (IBMI)}

\textit{Ex vivo} MSOT imaging of the LW2-loaded nanoparticles injected subcutaneously was performed by recording optoacoustic data at multiple excitation wavelengths (700nm, 730nm, 760nm, 770nm, 800nm, 860nm and 900nm) using 30 averaged signals per wavelength, resulting in an acquisition time of 70 s per slice. The wavelengths were selected to resolve the LW2-nanoparticles from the background absorption of the tissue. Multiple slices were acquired at 0.5mm intervals to cover the region where subcutaneous injections were made.

\textit{EpiFluorescent Acquisition (IBMI)}

Following the MSOT experiment, whole-body NIR fluorescence imaging (Epi-fluorescence) was performed to confirm the presence of the different subcutaneous injections of both reporter probes within mouse body. Imaging was performed \textit{ex vivo} using epi-illumination fluorescence with a Leica Z16 macroscopic lens. Fluorescence images were captured at the peak emission wavelength of LW2 dye (around 750 nm).
RESULTS AND DISCUSSION

During the development of new classes of optoacoustic reporter probes, specific attention must be paid to their absorption spectrum. The reporter probe should absorb in the NIR range (700-900 nm), and ideally should have a narrow optoacoustic spectrum profile to facilitate the unmixing step during imaging processing[71] (STEP 1). Another essential criterion for optoacoustic probe development is the stability of the formulation (STEP 3). Optoacoustic contrast probes with high stability over time, as well as strong resistance to photo degradation are favorable, as they could be used throughout different time points and across horizontal time studies, which broadens their preclinical applicability. Importantly, an ideal reporter probe should also enable formulation flexibility in terms of size, imaging agent, and surface functionality. The probe should allow for conjugation to a range of target ligands to allow the design of specific probes for specific biomarkers or pathologic scenarios. All of these features influence the in vivo application and clinical translation of the reporter probe [27]

Step 1 – Physicochemical and optical characterization of LW2-nanoparticles

Figure 8 summarizes optical and physicochemical characterization of the reporter probes. Figure 8A presents a schematic of the LW2 dye loaded into polymeric nanoparticles via Flash NanoPrecipitation. The chemical structure of LW2 dye is shown in Figure 8B, with its excitation/emission spectra shown in Figure 8C. Excitation and emission peaks of LW2 fall in the optimal optical imaging window (700-900 nm), where absorbance by blood and tissue components is reduced, light scattering is reduced and light penetration is maximized. Figure 8D presents spectra of LW2 dye dissolved in THF (LW2-free) versus encapsulated within the nanoparticle core. As observed, there is a
5 nm red-shift when dye is encapsulated, which is often seen for dyes in different hydrophobic environments. At high loadings of dye within the nanoparticle core, the intrinsic fluorescence (fluorescence per particle) of the nanoparticle is reduced due to quenching from Förster Resonant Energy Transfer (FRET) between dye molecules. Maximizing the fluorescence of the nanoparticle requires determining the maximum dye loading just before FRET effects dominate. By varying the dye loading within nanoparticles, but holding the nanoparticle size constant (120 nm), we determined this to be at 3.8wt% core loading. The optimization of the LW2 loading to maximize fluorescence is described in AppF-Fig. 1. For the purposes of photoacoustic imaging, this fluorescence maximum is not crucial as the photoacoustic signal is maximized when incident electromagnetic radiation is entirely converted to heat rather than emitted light. This occurs when quenching is maximized at high dye loading -- for example 80% core loading as we did for the LW2-100nm and LW2-50nm samples (Fig. 8H, Table 2).

Figure 8E shows the results from dynamic light scattering (DLS) for each formulation, confirming their average size of approximately 50 nm and 100 nm for LW2-50nm and LW2-100nm, respectively. Figure 8F and G present TEM images of LW2-100nm nanoparticles. Due to the organic core, the contrast of the micrographs is relatively low, but the nanoparticles are visible as grey ‘fuzzy’ balls. The size as seen by TEM matches well with that reported by DLS (Figure 8E). Figure 8H compares the size, PDI and dye core loading of both LW2-100nm and LW2-50nm.
**Figure 8** - Characterization of the LW2-nanoparticles. (A) Schematic of the LW2-nanoparticles; (B) Schematic of the NIR dye (LW2, chemical name: 6,15-bis(tri-sec-butyldiethynyl)-2,3,10,11-bis(diisobutyldioxolanato)hexacene) used for the formulation of both probes. (C). Fluorescence excitation (■) and emission (●) spectra for LW2 dissolved in THF. (D) Optical absorbance spectra for LW2 in nanoparticle cores (■) and dissolved in THF (●). (E) Dynamic light scattering size distribution of concentrated formulations: LW2-100nm (■) and LW2-50nm (●). (F) Representative TEM micrograph of LW2-100nm. Scale bar: 400 nm. (G) high magnification TEM of dashed square region on F. Scale bar 100nm. (H) Summary of formulation data of size and dye core loading.

**Step 2- Optoacoustic performance of LW2-nanoparticles**

Figure 9 depicts the performance of LW2-nanoparticles as optoacoustic contrast agents using MSOT. Different dilutions of each LW2 nanoparticle were prepared with optical
densities (OD’s) ranging from 0.3 to 2.2 (Figure 9A). Appendix F—Figure 4 presents
the spectrophotometric studies of these dispersions and the spectral information that was
used as an input for multispectral post-processing of optoacoustic signals. As presented in
Figure 9B, each of the diluted solutions of LW2-nanoparticles, together with the internal
OD control were prepared in an agar phantom for MSOT imaging. Appendix F – Figure
5 presents the optoacoustic spectrum measured by MSOT for the different concentrations.
The intensity of the MSOT signal versus the concentration is shown in Figure 9C.
Figure 9D shows the correlation between the normalized optoacoustic spectrum of LW2-
100nm obtained by MSOT (dashed line) and its absorption spectrum obtained by
spectrophotometry (non-dashed line). The same was also performed for LW2-50nm
(Figure 9F), showing the correlation between normalized spectra. Figure 9E and Figure
9G depict, respectively, the MSOT results for LW2-100nm and LW2-50nm after
unmixing of the optoacoustic signal. The intensity of optoacoustic signal (pseudo bright
green color) varies according to the concentration of the solution tested, with increasing
green intensity correlated with increasing OD of the sample. For both formulations tested,
even at low concentrations of OD=0.3, the optoacoustic signal can be detected, revealing
the high sensitivity of MSOT for both LW2 nanoparticles.
Figure 9 - Performance of LW2 loaded Nanoparticles as optoacoustic contrast agents. (A) Relation between optical density and concentration for LW2-100nm (■) and LW2-50nm (●). (B) Schematic of agar Phantom used for MSOT acquisition. (C) Optoacoustic amplitudes of LW2-nanoparticles at 730 nm in an agar phantom as function of MSOT intensity for LW2-100nm (■) and LW2-50nm (●). (D & F) Normalized optoacoustic signal obtained by MSOT and absorbance intensity by spectrophotometer of LW2-100nm (D) and LW2-50nm (F). (E & G) Unmixing of Optoacoustic signal after phantom acquisition by MSOT for LW2-100nm (E) and LW2-50nm (G) at different concentrations.

Steps 3 & 4 - Stability and biocompatibility of LW2-nanoparticles

We investigated the stability and toxicity of the formulations. Figure 10A and Figure 10B depict, respectively, the stability of LW2-100nm and LW2-50nm (OD=1) after incubation at 4°C in the dark for one week. Appendix F – Figure 6 shows the changes in absorption peak (at 730nm) for all the dilutions of each LW2-nanoparticle formulation.
during this period of time. The LW2-100nm is very stable over time, without major changes in its absorption spectrum; however, the LW2-50nm shows a decrease in the intensity of absorption over the 7 days. A photobleaching experiment was conducted by exposing samples to continuous illumination at 730nm for 30 minutes (Figure 10C). The LW2-100nm particle is extremely stable, however the LW2-50nm shows a 60% decrease in MSOT intensity. **Figure 10D** and **Figure 10E** show the absorption spectrum of LW2-100nm and LW2-50nm before and after the experiment. The decrease in MSOT signal, again, tracks with the loss of UV-Vis absorption. It is important to highlight that the scenario tested is unlikely to happen during *in vivo* MSOT imaging, since the dyes are exposed to much lower light intensity levels during an actual *in vivo* experiment. Nevertheless, this experiment allows us to evaluate new probes when compared to the performance of commonly used ones, such as ICG (Appendix F – **Figure 7**). **Figure 10F** and **Figure 10G** presents the *in vitro* toxicity study of LW2-100nm (**Figure 10F**) and LW2-50nm (**Figure 10G**) after incubation with MDA-231 mammalian tumor cells. The results demonstrate the biocompatibility of LW2-100nm and LW2-50nm. Neither LW2-nanoparticles caused toxicity up to 1.5 mg/mL, with more than 85% cell viability.
Figure 10 - Stability and in vitro toxicity of LW2 nanoparticles.  
(A & B) Stability study of LW2-100nm (A) and LW2-50nm (B) in PBS stored in dark during 7 days at 4°C.  
(C) Photobleaching profile acquired by MSOT of LW2-100nm (red line) and LW2-50nm (black line) after 30 minutes of continuous laser illumination at 730nm.  
(D & E) Absorption spectrum by spectrophotometer of LW2-100nm (D) and LW2-50nm (E) acquired before (blue line) and after (red line) photobleaching experiment.  
(F & G) In vitro toxicity study of LW2-100nm (F) and LW2-50nm (G) 48 hours after incubation within MDA MB 231 human breast cancer cell line (n=5).
Step 5- Sensitivity of LW2-nanoparticles—ex vivo proof-of-concept

The last experiment, a proof-of-concept *in vivo* study, demonstrates the applicability of these reporter probes for optoacoustic imaging. The five different dilutions of each LW2-nanoparticle, along with control dye, were injected subcutaneously into the backs of mice. **Figure 11A & B** are color photographs with a schematic representation of each subcutaneous (SC) injection (left panel) and a comparison between planar epi-Fluorescent imaging (middle panel) and planar MSOT imaging (right panel) for LW2-100nm (**Figure 11A**) and LW2-50nm (**Figure 11B**). Epi-fluorescent imaging on **Figure 11A & B** confirm the *ex vivo* MSOT results. The signal by Epi-fluorescence from the lower concentration solutions (SC n°5) for both LW2-nanoparticles was not visible, whereas the signal from the MSOT imaging was. This highlights the better sensitivity of MSOT for the detection of these probes when compared with Epi-fluorescent techniques. An additional advantage of MSOT over Epi-fluorescent techniques is its capability for depth imaging, as shown in **Figure 11C** and **Figure 11D**. In addition to the molecular information, MSOT provides structural and anatomical information. For example, in the plan view, the internal organs, such as kidney, spleen, spinal cord can be identified. **Figure 11C** and **Figure 11D** depicts 2D coronal plan of subcutaneous injection of LW2-100nm (**Figure 11C**) and LW2-50nm (**Figure 11D**), respectively. After spectral unmixing, the LW2-nanoparticles can be identified at the site of injection by a pseudo green color. The intensity of the green color decreases according to the decrease of the concentration (OD’s) of each dilution injected. Overall, the sensitivity of MSOT to LW2-nanoparticles is quite high.
**Figure 11** - Ex vivo study performed on CD-1 mice after subcutaneous injection of different concentrations of LW2-nanoparticles. (A & B Left panel) shows a color photograph of the CD-1 mouse back after SC injection with different concentration of LW2-100nm (A) and LW2-50nm (B). (A & B middle panel) planar Epi-fluorescent of LW2-100nm (A) and LW2-50nm (B). (A & B right panel) planar MSOT of LW2-100nm (A) and LW2-50nm (B). (C & D) coronal section by MSOT after SC injection of LW2-100nm (C) and LW2-50nm (D). Scale bar is 3 mm. Sp, Kd, and SC stands for Spleen, Kidney and Spinal cord.

**DISCUSSION**

Based on the five criteria for evaluating a photoacoustic nanoparticle system, the LW2 nanoparticle system produced by the FNP rapid precipitation process is a highly promising imaging agent. **STEP 1 Nanoparticles:** The highly hydrophobic LW2 dye used for the formulation of this novel class of optoacoustic contrast agents is a part of the acene family of dyes, the most studied being the pentacene and hexacene derivatives [54, 72]. The bulky side
groups reduce π-stacking in the crystalline state, hence reducing the degree of self-quenching. It is fluorescent, which is not crucial for the optoacoustic application, but its absorption maximum at 730 nm puts it in the long-wavelength optical window for optoacoustics. The high loading (~80 wt%) afforded by the rapid precipitation process, FNP, produces particles with high optical contrast. FNP can produce particles from 50 nm to 400 nm in size; here we focus on 50 nm and 100 nm particles (Fig. 8). Our approach for incorporating the imaging agent differs from the standard, where imaging molecules are usually attached to the surface of the nanocarrier. By placing them in the core, a much higher photoacoustic signal can be achieved due to the larger number of dye molecules that can be packed in a 100 nm core compared to the surface (390,000 in core vs. 15,000 on surface). This approach also eliminates potential interference between imaging molecules and targeting agents at the nanoparticle surface.

FNP has been used successfully to produce block-copolymer-protected nanoparticles containing a wide range of actives including drugs, peptides, imaging agents, and inorganic structures.[73-77] The process is scalable from milligram laboratory samples to a thousand kilograms per day in continuous operation.[22, 78] The ability to set nanoparticle composition by setting the concentrations of the inputs enables the production of nanoparticles that deliver both therapeutic compounds and imaging agents, such as LW2, in theranostic applications. Furthermore, the PEG block on the block copolymer stabilizing agent allows for the attachment of targeting ligands to the PEG surface.[25]

**STEP 2 Optoacoustic performance:** The MSOT intensity is essentially linearly correlated with optical density, and optical density is linearly correlated with nanoparticle concentration (Fig. 9).
However, there is a difference in optical density depending on nanoparticle size. The 50 nm particles are 1/3 as optically dense as the 100 nm particles. The reason for this is associated with singlet oxygen degradation of the dye. The smaller particles, with higher surface-to-volume ratios have more dye exposed to degradation. We have observed this phenomena with other fluorescent dyes encapsulated in FNP nanoparticles.[79] For LW2 dye in 30 nm micelles, we observed even faster discoloration (degradation). The lower optical density of the 50 nm nanoparticles is associated with degradation that occurs during normal processing and purification prior to the start of the experiments. A change in color of the LW2 dispersion from green to yellow, indicating initial degradation, was observed during processing of the smaller particles. This is also seen in STEP 3 Stability. Fig. 10A shows a 10% decrease in absorbance for the 100 nm particles stored at 4 °C in the dark for a week. The 50 nm particles show a 25% decrease in absorbance over the same period. Photobleaching is shown in Fig. 10C, where after 30 minutes of continuous illumination (far stronger than would be experienced in an in vivo experiment), the 100 nm particles have decreased 10% in optical density and the 50 nm decreased 60%. We conclude that the greater protection afforded the dye in the cores of larger nanoparticles enhances stability. It may be possible to decrease the instability of smaller particles by co-encapsulating the LW2 with an antioxidant, such as tocopherol (vitamin E), which we have done previously. This would mean reducing the core loading somewhat, but the gain in stability may be significant. For this study the particles were kept in solution during formation, purification, and shipment between the USA and Germany. Putting the nanoparticles into a lyophilized form would decrease singlet oxygen attack. We have shown that these PEG-protected nanoparticles can easily be lyophilized and redispersed back to their original size.[80] This would be the next step in making commercial versions of these optoacoustic nanoparticles.
Fig. 10F&G show that the nanoparticles have minimal toxicity at concentrations as high as 1.5 mg/mL. For incubation with MDA MB 231 human breast cancer cells the 100 nm particles showed no toxicity at 1.5 mg/mL, and the 50 nm particles showed a reduction in cell density of only 15%. In contrast, introducing the dye in DMSO caused cell deaths of 60% - 70% in the two control experiments. By both measures of stability and toxicity the LW2 nanoparticles appear to be viable optoacoustic candidates. There is some preference to the larger particle size, which displays greater storage and photostability.

**STEP 4 Biocompatibility:** Biocompatibility has two components. The first is toxicity, which we have shown is very low for these optoacoustic particles. The complete encapsulation of the very hydrophobic LW2 dye means that the cells see the dense PEG surface layer of the nanoparticle, and not the dye itself. We have shown that the PEG-protected nanoparticles are long circulating[81] and do not produce a complement activation response[64]. Recent work shows that they are mucus-penetrating, so the LW2 nanoparticles may be attractive for studying lung and GI tract nanoparticle delivery and fate. The second component of biocompatibility is long-time biological response. The polymers we have used in this proof-of-concept study are polystyrene-\textit{b}-PEG. The polystyrene block will not biodegrade, and so \textit{in vivo} clearance is unlikely. However, we have shown that equivalent nanoparticles can be made from biodegradable polylactic acid (PLA) or polycaprolactone (PCL) block copolymers.[74, 75, 77] So the transition from the PS-\textit{b}-PEG to PLA-\textit{b}-PEG or PCL-\textit{b}-PEG would be straightforward. The ultimate clearance and long-term effects of the LW2 are unknown and would have to be
evaluated to advance this platform to human subjects. For animal testing, both the PS-\textit{b}-PEG/LW2 or a PLA or PCL-\textit{b}-PEG/LW2 nanoparticle construct will be adequately biocompatible.

Both nanoparticle formulations showed minimal toxicity over a range of concentrations that might be expected \textit{in vivo}. The slight difference in toxicity between the 100 nm and 50 nm particle is interesting. Since they have similar surface chemistry the origin of the difference is uncertain. One possibility is that amount of dye internalized into cells depends on nanoparticle size. Uptake by cells \textit{via} pinocytosis, non-receptor mediated uptake, is reported to occur for nanoparticles less than 60-70 nm in size. It may be that the size difference between 50 nm and 100 nm is significant enough to make a difference in the amount internalized in cells. Further studies are warranted.

\textbf{STEP 5: Optoacoustic sensitivity \textit{in vitro} and \textit{in vivo}.} The performance of LW2 nanoparticles as optoacoustic contrast agents was evaluated by a series of studies performed on agar phantoms that mimic tissue. The optoacoustic signal correlated with the absorption spectrum measured by spectrophotometer, confirming that the optoacoustic signal amplitude is proportional to the amount of optical energy absorbed. In both formulations, a similar imaging pattern was observed, with high intensity of optoacoustic signals that decrease with decreasing NP concentration (i.e. OD’s). For the same mass concentration of the sample, the optoacoustic signal of LW2-100nm was higher (by at least 50\%) than LW2-50nm, which correlated with the higher optical absorbance of the larger particles, when compared at the same dye loading. The performance of both LW2 nanoparticles as optoacoustic contrast agents in agar phantom was quite promising, due to their high optoacoustic amplitude.
MSOT was used to examine the sensitivity of both optoacoustic reporter probes when injected into an animal. This experiment constitutes the proof-of-concept that these formulations could indeed serve as nanocarriers for optoacoustic imaging. After SC injections of the differently diluted samples, 2D planar imaging (epi-fluorescent and MSOT) identified the doses injected. At the lowest concentrations, 48 μg/mL, the MSOT signal was clearly visible, while no signal was observable by epi-florescence. While epi-fluorescent only generates planar 2D images, without depth information, MSOT have, in parallel to the 2D planar images, the capacity to acquire transverse orientation data. This allows deeper tissue imaging.

**CONCLUSIONS – IBMI COLLABORATION**

This study presents a framework for evaluating reporter probes for optoacoustic contrast imaging, and presents a new class of NIR reporter probes based on acene dyes. Using the evaluation criteria, we find that the Flash NanoPrecipitation platform provides a flexible and scalable way to make nanoparticles with variable size (50 nm and 100 nm) that incorporate the hexacene dye LW2 at high concentrations. The strategy behind the selection of the dye is to have extremely hydrophobic dyes that do not partition out of the nanoparticle so that in biological applications the nanoparticle circulation and response is determined solely by the PEG surface coating on the particle, and is independent of the particular hydrophobic NIR dye in the core. The hexacene dye offers a high optical cross-section in the NIR range that makes it appropriate for optoacoustic imaging. The larger nanoparticle is more photostable in storage and under NIR illumination than the smaller nanoparticle. This appears to be due to the larger surface-to-volume ratio as nanoparticle size decreases. Although detailed toxicity studies are
required to establish long-term toxicity of the hexacene dye, the preliminary data and animal experiments indicate that the dye is safe for in vitro and animal studies. The evaluation as an optoacoustic contrast agent and the high sensitivity of MSOT to both formulations offers strong support that these nanoparticles can rapidly progress to become an important imaging agent. Finally, MSOT demonstrates the sensitivity of both optoacoustic reporter probes when injected into an animal. This experiment constitutes the proof-of-concept that these formulations could indeed serve as a molecular carriers for optoacoustic imaging. The high resolution and deep tissue penetration of MSOT can provide unique insights and the use this new class of optoacoustic contrasts agents is expected to contribute to placing MSOT technology on the edge of scientific preclinical and clinical research.

There are additional questions still to be addressed. The smaller particles were adequately “bright” to provide excellent MSOT images even at low concentrations. However, photo-instability would compromise long temporal studies. A detailed understanding of the mechanism of photo-instability would guide strategies to overcome this limitation. One approach may be co-formulation with anti-oxidant compounds. The most important next direction is the incorporation of ligands onto the PEG steric layer to effect targeting. Our experience with creating targeted nanoparticles for therapeutic applications by FNP [25, 79] gives us confidence that this will be an achievable objective.
OVERALL CONCLUSIONS

In these two studies we present the use of photoacoustically active nanocarriers that serve as contrast agents for diagnostic imaging applications. Photoacoustic imaging is particularly attractive as a diagnostic tool as it combines the deep penetration depth of ultrasound imaging with the functional information and resolution afforded by contrast agent-aided long-wavelength optical imaging, where absorption by blood and tissue components is minimized. In collaboration with VisualSonics, Inc. we showed that 120 nm PEGylated nanoparticles containing the hydrophobic hexacene dye LW2 could be efficiently imaged with high sensitivity in a mouse tail vein injection. The sharp absorption peak was easily unmixed from background noise. The nanocarriers were shown to accumulate in a murine tumor model over a 24 hours period. With this initial data, a second formal study was initiated with the Institute for Biological and Medical Imaging in Germany where MSOT was utilized to compare 50 nm and 100 nm LW2-loaded nanocarriers. 100 nm carriers were shown to be very photostable compared to 50 nm carriers and biocompatible with high sensitivity and signal intensity in in vitro and in vivo models. A framework is presented for the evaluation of future photoacoustic contrast agents for biomedical imaging applications. FNP is shown to be particularly suitable due to its scalability, precise control of nanocarrier size, ability to deliver multiple payloads, and easily modified surface chemistry, including targeting agents. Future work includes testing the biodistribution of photoacoustically active nanocarriers of sizes between 20 – 200 nm, multiplexing such nanocarriers and attaching targeting ligands such as folic acid or RGD peptides.
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REFERENCES


CHAPTER 5
Small Molecule Adsorption to Colloidal Alumina

Abstract

Inkjet printing of security features on product packaging using fluorescent inks is increasingly important to prevent the propagation of counterfeit pharmaceuticals. These inks typically take the form of fluorescent dyes or pigments (colloidal systems) with specific absorption and emission bands outside of the visible spectrum. Pigments have the advantage of being resistant to photobleaching but are typically dense colloids and thus require stabilizers to prevent aggregation, sedimentation, and inkjet head clogging and to maintain printing resolution. Here we study the adsorption of small molecules to alumina, a model system for security pigments, using UV-vis absorbance and isothermal calorimetry techniques. AKP50 (α-alumina) is dispersed by two methods. The zeta potential is dependent on the method used but the isoelectric point is not. Essentially complete dispersion is obtained in water at pH 3 (ζ = +35 mV) but modest aggregation occurs at pH 6 (ζ = +25 mV) where the surface charging is less. The adsorption studies focused on 4 types of functional groups: phosphate, sulfate, carboxyl, and hydroxyl (triethylene glycol). The naphthyl group was chosen as a constant hydrophobic group for the charged species. 2-naphthyl phosphate adsorbed most strongly with a plateau at 1.2 molecules/nm², close to the experimentally determined exchange capacity of 1.3 –OH groups/nm² on the alumina surface. At higher concentrations the adsorption becomes weaker and more linear, as it is driven by hydrophobic stacking. Isothermal calorimetry shows the heat of adsorption for the phosphate to be -1.81 ± 0.39 kcal/mol. The 2-naphthyl sulfate shows much weaker binding with a more linear adsorption isotherm. During the adsorption process, a rise in pH is observed that is correlated with the strength of the binding which is actually an exchange process. The triethylene glycol binding was weakest of all, showing no rise in pH during the adsorption as it is driven by hydrogen bonding with chemisorbed water molecules. Poly(acrylic acid) (PAA) was shown to be an effective dispersant for the alumina. An increase in total hydrodynamic diameter of 45 nm was observed going from a 1.8k MW PAA coating to 145k. These results indicate that a polyphosphate block copolymer with a PEG stabilizing block might be optimal for the dispersion of alumina based colloids for security ink jet applications.
Introduction

Product authentication is of special concern in the pharmaceutical industry since counterfeit drugs are estimated to result in an annual loss of $75 billion according to the former FDA Associate Commissioner.[1] Therefore, security markings on the packaging, using covert labeling, are widely used. Ink jet printing of these security features enables information such as batch codes and point of origin to be varied easily. Inks that fluoresce are the most commonly used markers. Fluorescent inks can be based on dyes (soluble organics) or pigments (insoluble colloids); however, pigments are much more stable and resistant to photo bleaching than are dye-based inks.[2] Such pigments consist of crystals that absorb and/or fluoresce at specific wavelengths outside of the visible range (400-700 nm).[3-5] Since one candidate pigment is an alumina-based crystal,[3-5] alumina serves as a model colloid for the study.

The metal-oxide particles have high densities, which makes stabilizing the pigment dispersion against sedimentation a significant challenge. While higher viscosity matrices slow down the rate of settling, it also becomes more difficult to ‘jet’ sufficiently small droplets for high resolution printing purposes. One solution to this problem involves milling pigments to sub-micron particles to reduce the settling rate. Another involves addition of polymer dispersants to provide pigment stabilization by a combination of electrostatic repulsions and steric hindrance.[6] The use of polymer dispersants is particularly attractive as high loadings relative to the pigment can be achieved without a large increase in matrix viscosity.[7] In general, structured polymers containing an anchor segment and a tail segment are used. The use of such structured polymers allows for strong adsorption to the pigment surface (anchor) and sufficient
extension of the polymer into solution to provide steric hindrance (tail).[2] Block copolymers with the structure A-b-(B-co-C) such as poly(methylmethacrylate)-b-(methylmethacrylate-co-methacrylic acid) have been used as well as polyelectrolytes like poly(acrylic acid) and poly(methacrylic acid).[8]

The goal of this study is to understand the binding characteristics of moieties onto alumina with the goal of predicting which polymer blocks might be best as stabilizers for these pigments. There is a large literature on fundamental alumina surface chemistry, and adsorption studies with specific polymers onto alumina, stemming from the importance of alumina in minerals processing and as abrasives. Representative results from this literature are presented in Table 1, where both binding strength and binding enthalpies are shown. As binding constants, enthalpies of adsorption, and max extents of binding all depend on pH, ionic strength, temperature, alumina crystal phase, and chemical structure and functionality of the ligand, comparisons have to be made considering all of these factors.

Data on PAA adsorption is also presented in Table 1. For a polymer such as PAA, binding is strongly driven by entropic and multi-valency. The binding constants for PAA scale with the molecular weight and are orders of magnitude higher than those for small molecule anionic species.
### Table 1: Binding characteristics of small molecule and PAA-alumina systems reported in the literature.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Alumina</th>
<th>T (°C)</th>
<th>pH</th>
<th>Ionic Strength</th>
<th>( K ) (mM(^{-1}))(^†)</th>
<th>( ΔH ) (kcal/mol)</th>
<th>( \Gamma_{\text{max}} ) (molecules/nm(^2))</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium benzoate</td>
<td>α-Al(_2)O(_3)</td>
<td>25</td>
<td>5</td>
<td>0.5 mM</td>
<td>0.74</td>
<td>-3.23</td>
<td>0.172</td>
<td>Das (2005)[9]</td>
</tr>
<tr>
<td>Phthalic Acid</td>
<td>α-Al(_2)O(_3)</td>
<td>25</td>
<td>5</td>
<td>0.5 mM</td>
<td>20.32</td>
<td>-12.7</td>
<td>0.878</td>
<td>Das (2005)[9]</td>
</tr>
<tr>
<td>Phthalate</td>
<td>δ-Al(_2)O(_3)</td>
<td>25</td>
<td>8</td>
<td>20 mM</td>
<td>2.25</td>
<td>-6.91</td>
<td>0.0673</td>
<td>Benoit (1993)[10]</td>
</tr>
<tr>
<td>Benzoate</td>
<td>δ-Al(_2)O(_3)</td>
<td>25</td>
<td>8</td>
<td>20 mM</td>
<td>2.70</td>
<td>-2.49</td>
<td>0.115</td>
<td>Benoit (1993)[10]</td>
</tr>
<tr>
<td>Salicylate</td>
<td>δ-Al(_2)O(_3)</td>
<td>25</td>
<td>8</td>
<td>20 mM</td>
<td>5.13</td>
<td>-5.50</td>
<td>0.180</td>
<td>Benoit (1993)[10]</td>
</tr>
<tr>
<td>C(_8)-benzene sulfonate</td>
<td>α-Al(_2)O(_3)</td>
<td>25</td>
<td>5</td>
<td>0.3% NaCl</td>
<td>2.5</td>
<td>--</td>
<td>4.2</td>
<td>Denoyel (1989)[11]</td>
</tr>
<tr>
<td>C(_8)-benzene sulfonate</td>
<td>α-Al(_2)O(_3)</td>
<td>25</td>
<td>7</td>
<td>0.3% NaCl</td>
<td>1.82</td>
<td>--</td>
<td>3.1</td>
<td>Denoyel (1989)[11]</td>
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<tr>
<td>C(_8)-benzene sulfonate</td>
<td>unknown</td>
<td>50</td>
<td>8.2</td>
<td>30 mM NaCl</td>
<td>--</td>
<td>-11</td>
<td>--</td>
<td>Somasundaran (2000)[12]</td>
</tr>
<tr>
<td>m-xylene sulfonate</td>
<td>90% α/10% γ-Al(_2)O(_3)</td>
<td>43</td>
<td>--*</td>
<td>30 mM NaCl</td>
<td>--</td>
<td>-16</td>
<td>--</td>
<td>Sivakumar (1993)[13]</td>
</tr>
<tr>
<td>p-xylene sulfonate</td>
<td>90% α/10% γ-Al(_2)O(_3)</td>
<td>43</td>
<td>--*</td>
<td>30 mM NaCl</td>
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<td>-26</td>
<td>--</td>
<td>Sivakumar (1993)[13]</td>
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<td>SDS</td>
<td>90% α-Al(_2)O(_3)</td>
<td>25</td>
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<td>0.1M NaCl</td>
<td>2.0</td>
<td>--</td>
<td>4.2</td>
<td>Chandar (1987)[14]</td>
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<tr>
<td>TTAC</td>
<td>90% α-Al(_2)O(_3)</td>
<td>RT</td>
<td>10</td>
<td>30 mM NaCl</td>
<td>0.71</td>
<td>--</td>
<td>1.5</td>
<td>Huang (1996)[15]</td>
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<td>90% α-Al(_2)O(_3)</td>
<td>RT</td>
<td>10</td>
<td>30 mM NaCl</td>
<td>7.7</td>
<td>--</td>
<td>2.4</td>
<td>Fan (1997)[16]</td>
</tr>
<tr>
<td>TTAB</td>
<td>90% α-Al(_2)O(_3)</td>
<td>RT</td>
<td>10</td>
<td>30 mM NaCl</td>
<td>0.77</td>
<td>--</td>
<td>1.6</td>
<td>Fan (1997)</td>
</tr>
<tr>
<td>DTAB</td>
<td>90% α-Al(_2)O(_3)</td>
<td>RT</td>
<td>10</td>
<td>30 mM NaCl</td>
<td>0.077</td>
<td>--</td>
<td>1.0</td>
<td>Fan (1997)</td>
</tr>
<tr>
<td>Maltoside</td>
<td>α-Al(_2)O(_3)</td>
<td>25</td>
<td>7</td>
<td>N/A</td>
<td>7.1</td>
<td>--</td>
<td>3.3</td>
<td>Zhang (1997)[17]</td>
</tr>
<tr>
<td>PAA-2k</td>
<td>γ-Al(_2)O(_3)</td>
<td>25</td>
<td>6</td>
<td>10 mM NaCl</td>
<td>123</td>
<td>+0.62**</td>
<td>1.72</td>
<td>Wisniewska (2009)[18]</td>
</tr>
<tr>
<td>PAA-60k</td>
<td>γ-Al(_2)O(_3)</td>
<td>25</td>
<td>6</td>
<td>10 mM NaCl</td>
<td>3200</td>
<td>+0.89**</td>
<td>0.0602</td>
<td>Wisniewska (2009)</td>
</tr>
<tr>
<td>PAA-240k</td>
<td>γ-Al(_2)O(_3)</td>
<td>25</td>
<td>6</td>
<td>10 mM NaCl</td>
<td>17900</td>
<td>+1.91**</td>
<td>0.0178</td>
<td>Wisniewska (2009)</td>
</tr>
</tbody>
</table>

\( ^†K \) is defined as \( \Gamma_{\text{max}} = \frac{K_c}{1 + K_c} \)

\(^*\)pH not specified, experiments done in triply distilled water.

\(^**\)For polymeric systems such as PAA, the adsorption is largely entropy driven, hence the small positive \( ΔH \).[18] SDS=sodium dodecyl sulfate, C/T/D-TAB=cetyl/tetradecyl/dodecyl-trimethylammonium bromide, maltoside=n-dodecyl β-D maltoside.
Binding and stabilization studies are often conducted under conditions to maximize binding by selecting pH values where the surface charging of the alumina allows strong electrostatic anchoring. However, pH values are constrained for inkjet formulations to avoid damage to the printers, so pH values near neutral are required. In this range alumina surfaces are cationic and the commonly used carboxylic acid functional groups are negatively charged. Increasing the pH increases the negative charge on carboxylic acids, but decreases the positive charge on alumina.

We want to understand binding to alumina of three negatively charged groups (carboxylates, sulfates, and phosphates) and one uncharged group: the neutral ethylene oxide group. In order to separate the specific ionic interactions from non-specific interactions, we employ three naphthyl compounds: 2-naphthyl phosphate (2NP), 2-naphthyl sulfate (2NS), and 2-naphthoic acid (2NA). The naphthyl groups enable UV-vis absorbance detection of adsorbed amounts, and provide equal hydrophobic interactions between each compound and the alumina surface. The compounds are shown in Fig. 1.

Fig. 1 – (A) Structure of 2-naphthyl phosphate \((R = OPO_3^{2-})\), 2-naphthyl sulfate \((R = OSO_3^-)\), and 2-naphthoic acid \((R = COOH)\), (B) Structure of triethylene glycol, (C) UV absorbance spectra for the species 2-naphthyl phosphate \(\bullet\) and 2-naphthyl sulfate \(\circ\). Calibration curves for the two compounds were constructed to allow for quantitative determination of free 2NP \((\lambda = 323 \text{ nm})\) and 2NS \((\lambda = 330.5 \text{ nm})\) concentrations for the construction of adsorption isotherms.
We study the binding of these compounds to alumina colloidal surfaces using UV absorbance measurements for the naphthyl groups, and total organic carbon analysis for the triethylene glycol. The enthalpies of binding are measured using isothermal titration calorimetry. We compare the difference in binding behavior for these anchoring species. Then we present data on binding and stabilization of alumina with poly-carboxylate polymers in the final section.
Materials and Methods

Materials

AKP50 alumina powder was obtained from Sumitomo Corp. and used without modification. 2-naphthyl phosphate sodium salt (N7375), 2-naphthyl sulfate potassium salt (R257915), 2-naphthoic acid (180246), 1.8k and 450k poly(acrylic acid) (323667 and 181285 respectively), potassium chloride (746436), triethylene glycol (90390), hydrochloric acid (1M) (320331), potassium hydroxide (38073), and sodium hydroxide (38215) were obtained from Sigma-Aldrich and used without modification. 6k poly(acrylic acid) (06567-250) and 145k poly(acrylic acid) (no longer available from Polysciences) were obtained from Polysciences, Inc. and used without modification. Ultrapure water (referred to as MilliQ water) was obtained from a Barnstead NanoPure system (Ω > 18.0 MΩ-cm).

Methods

Dispersing alumina

Method 1: (used for all ligand adsorption studies)  Into a 40 mL scintillation vial, 2.00 g of AKP50 was loaded and 20 mL of 10 mM KCl solution in MilliQ water was added. The vial was placed in an ice bath and probe tip sonicated with a ¼” Branson 400W sonifier for 15 minutes at 200 W (50% power). Then the suspension was left to equilibrate overnight (12-16 hours) before being sonicated once more under the same conditions. Then the pH of the suspension was adjusted by adding HCl/KOH to achieve a final pH of 6.00 ± 0.10. The size distribution of the
alumina nanoparticles was obtained by dynamic light scattering. The pH was measured by means of a Fisher Scientific Accumet AR50 dual channel pH/conductivity meter.

Method 1a (Fig. 2A): 2.00 g of AKP50 were suspended in 20 mL milliQ water and sonicated for 30 minutes at 200 W. 20 μL samples were taken and diluted with 10 mL of 100 mM KCl and the pH adjusted between 6 and 11 with HCl/NaOH for size and zeta potential measurements.

Method 1b (Fig. 2C): Two vials each containing 2.00 g of AKP50 in 20 mL milliQ water (10wt% AKP50) were sonicated at 200 W for 30 minutes. Then the pH of one was adjusted to 3.0 and the pH of the other to 10. After equilibration overnight, the size of each was determined by DLS.

Somasundaran Method: (following Chen and Somasundaran[19]) Into a 40 mL vial 1.0 g AKP50 was loaded into 10 mL of 0.03M salt (NaCl) solution. The sample was sonicated at 40 W of power for 1 min (no ice bath). The pH was then adjusted to pH 6.00 ± 0.10, and the suspension was mixed by magnetic stirring for 3 h. The method of Somasundaran provides a less intense ultrasonic dispersion step at a higher ionic strength.

Measuring size

10 μL of 10wt% AKP50 suspension was diluted in pH 6.00 KCl solution in a semi-micro cuvette (ZEN112, Malvern Instruments) so that the final suspension was nearly clear. The cuvette was placed inside a Zetasizer Nano 3600 (Malvern Instruments) and size distribution obtained using Malvern’s ‘high resolution’ curve fitting method.
Measuring zeta potential

10 µL of 10wt% AKP50 suspension was diluted in pH 6.00 10 mM KCl solution to be faintly opaque and the pH then adjusted as desired for the zeta potential measurements. The pH adjusted sample was then loaded into a Malvern disposable zeta cell (DTS1070, Malvern Instruments) and zeta potential obtained using the Malvern Zetasizer Nano 3600. Ionic strengths were kept approximately constant at 10 mM and verified by conductivity measurements on the Zetasizer.

Measurement of adsorbed PAA thicknesses

Samples with adsorbed polyacrylic acid polymer were prepared in the following way:

1) 22 mL of 10 wt% AKP50 in water at pH 5.7 were sonicated at 220 W for 20 mins. At the end of the sonication, the pH was found to be 6.1.

2) 4x5 mL aliquots were distributed among 4 centrifuge tubes containing 1.8k (6.1 mg), 6k (7.1 mg), 145k (6.0 mg), or 450k PAA (6.9 mg) (see Table 4 for alumina loading).

3) The pH was adjusted to 6.

4) Each 5 mL sample was sonicated for 5 mins at 50 W to disperse the AKP50 with polymer.

5) DLS measurements were taken as described above.

SEM Imaging of AKP50
A small amount of the alumina suspension was drop-casted on an SEM stubs with carbon tape. The water was allowed to evaporate overnight. The samples were sputter-coated with iridium (5 nm layer) and imaged on a XL30 FEG-SEM digital scanning microscope.

*Construction of adsorption isotherms by UV-vis or TOC*

Stock solutions of 2NP, 2NS, and triethylene glycol were made in 10 mM KCl at pH 6 at concentrations of 225.2 mg/mL, 30 mg/mL, and 30 mg/mL respectively. 250 μL of each solution were injected into 4 mL of 10 wt% AKP50 (dispersed by Method 1) in 10 mM KCl at pH 6 (in duplicate) and the vials were agitated overnight to allow for binding equilibration. The next day (approx. 12-16 hours later) the pH was checked and one set for each ligand had their pH adjusted back down to pH 6 while another set was left unadjusted. Within 8 hours the vials were then centrifuged at 15,000 g for 15 minutes to pellet the alumina while leaving any unbound ligand in solution. The supernatant solutions were carefully extracted, re-centrifuged, then filtered through a 0.1 μm nylon filter and diluted with 10 mM KCl. The concentration of 2NP or 2NS were quantified by UV-vis absorbance spectroscopy (Evolution 300, Fisher Scientific) at 323 and 330.5 nm, respectively (see Fig. 1). The concentration of triethylene glycol in the supernate was quantified by total organic carbon measurements (see below). Conversion of alumina mass to surface area was accomplished using the specific surface area of the AKP50 of 12 m²/g (manufacturer specified) which was confirmed by Brunauer-Emmett-Teller (BET) measurements.

*Microcalorimetry experiments*
4 mL of AKP50 suspension in 10 mM KCl at pH 6.00 was injected into the sample chamber of a VP-ITC microcalorimeter (Malvern Instruments). 1 mL of 2NP ligand solution in 10 mM KCl at pH 6.00 was loaded into the injector. 15-30 injections of 10-20 μL each were performed and the resulting heat flows measured. Sufficient injections were done to establish an acid-base neutralization heat baseline (following alumina surface saturation) that could be subtracted from the readings. The resulting heats were integrated assuming a single set of sites (single K – adsorption constant) to form a sigmoidal sets of points. The set of points was shifted by subtracting out the heat of acid-base neutralization which occurs at the end of each experiment once the alumina surface is saturated. This results in the sigmoidal curve fit asymptoting to 0 after the alumina surface is saturated.

**Total organic carbon measurements**

Triethylene glycol supernatant solutions were diluted with 10 mM KCl so the expected free ligand concentration would be less than 1000 ppm. Calibration standards were made at 1, 50, 100, 500, and 1000 ppm. Calibration standards and samples were placed in the autosampler of the Shimadzu TOC analyzer (Shimadzu Corp.) and run under NPOC (non-purgeable organic carbon) mode to quantify concentrations of free triethylene glycol.
Results and Discussion

Dispersion of Alumina Nanoparticles

The zeta potential vs. pH for AKP50 using the two different alumina dispersion methods (Method 1a vs. Somasundaran method) are shown in Fig. 2B to verify that our method yields alumina nanoparticles comparable to those in the literature. Both methods yield the same isoelectric point (IEP) at pH ~ 9.2, which corresponds to literature values for α-Al₂O₃.[20, 21] Interestingly, the Somasundaran method yields greater zeta potentials (i.e. higher and lower values) than Method 1a. Whether this is due to some surface erosion caused by the higher energy sonication, or by ion adsorption under the conditions of dissimilar cations and ionic strengths, or by another phenomena is not known. But it does show the importance of complete specification of dispersion conditions.
Fig. 2 – (A) Size distributions of AKP50 alumina dispersed in (■) 30 mM NaCl, pH 4.5 (Somasundaran method) and (●) 10 mM KCl, pH 6 (Method 1a). (B) (■ Somasundaran | ● Method 1a) compares the zeta potentials of AKP50 as a function of pH using the two different methods. The isoelectric points are found to be the same at about pH = 9, in accordance with the literature.[21] (C) Size distributions of AKP50 alumina nanoparticles after sonication in milliQ water (no salt, Method 1b). The pH was adjusted to 3 (▼, diameter = 240 nm) or 10 (▲, diameter = 800 nm) following sonication. Due to the much lower surface charge at pH 10 vs. pH 3, the samples aggregate to a greater degree at pH 10, thus explaining the large hydrodynamic diameter difference. Manufacturer data report the primary particle size to be within 100 – 300 nm (Sumitomo Corp., Japan).

In Fig. 2A, the hydrodynamic size distributions for alumina dispersed by Method 1a vs. that of Somasundaran are approximately the same (close to 1 μm) despite the pH differing by 1.5 units and the ionic strength being slightly higher for the Somasundaran method (30 mM vs. 10 mM).
However, the primary particle size reported by the manufacturer is 100-300 nm. Therefore, the particles are aggregated and this is true for most studies in the literature as well. Generally, however, the particles-particle contacts are few in number and do not take up significant surface area. Thus, especially for studies like this one which focus on small molecule adsorption, the particle aggregation does not negatively affect the results. Un-aggregated particles can be obtained by dispersing the alumina in milliQ water according to Method 1b (Fig. 2C). We see that a particle size of 240 nm is obtainable at pH 3 when the ionic strength is close to 0. However, 10 mM ionic strength (as used in Method 1 and Method 1a) is enough to cause aggregation by screening the repulsive surface charges (Fig. 2A). At pH 10 in water, even though the ionic strength is 0, the surface charges are too weak to prevent aggregation and the size is approximately 800 nm (Fig. 2B and C). It is important to note that dispersing dry alumina in water raises the pH of the medium due to the chemisorption of water molecules at the alumina surface (see Fig. 3). Thus, we generally saw a rise in pH from 6.0 to approximately 7.8 after 15 minutes of sonication at 200 W which had to be re-adjusted back down to 6.0 before ligand binding experiments could be conducted. The time-scale of the pH shift shows that kinetic processes, some with very slow rates, have to be considered in interpreting results.

**Fig. 3** - *Surface schematic of alumina when dry (A) and when hydrated (B). When dry the surface mainly consists of oxide ions which chemisorb water molecules when the alumina is dispersed in water. This results in the presentation of OH\(^-\) which raises the pH of the medium.*
SEM Imaging of AKP50

An SEM digital scanning microscopy image is shown in Fig. 4. The alumina nanoparticles have clustered during the drying process, but the primary particle sizes are in line with dynamic light scattering sizes.

![SEM of AKP50 after dispersion in water.](image)

**Fig. 4** – *SEM of AKP50 after dispersion in water.*

Selection of ligands, UV abs spectra

The ionic ligands studied: 2-naphthyl phosphate (2NP), 2-naphthyl sulfate (2NS), and 2-naphthoic acid (2NA) were chosen because they represent anionic groups that interact with the cationic alumina surface at pH 6.0. The phosphate is doubly charged, the sulfate is a large “soft” anion, and the carboxylate represents carboxylic acid groups, which have been widely used in
polyacrylic and polymethacrylic acid stabilizing polymers. Unfortunately, the carboxyl naphthyl was not sufficiently ionized at pH 6 to be sufficiently water soluble. The hydrophobic interactions dominated and limited solubility. So experiments were only conducted on the 2NP and 2NS. Experiments on polyacrylic acid polymers were conducted and are described below, but are not directly comparable with the naphthyl compound experiments. Linear calibration curves for the two compounds were constructed to allow for quantitative determination of free 2NP and 2NS concentrations for the construction of adsorption isotherms. It was also decided to consider triethylene glycol (shown in Fig. 1) to represent small molecule amphiphiles not studied previously in alumina adsorption studies. This would be a small molecule mimic of polymeric stabilizers like polyethylene glycol (PEG) polymers, which strongly adsorb to alumina surfaces. This provides a comparison of the magnitude of electrostatic binding versus hydrogen bonding interactions.

*Adsorption isotherms for 2NP, 2NS, and TEG*

**Fig. 5** shows the Langmuir isotherm for adsorption of 2-napthyl phosphate (2NP) onto alumina \((T = 25^\circ C, I = 10 \text{ mM KCl}, \text{pH} = 6.0)\) as dispersed by Method 1. The curve displays saturation at approximately 1.2 molecules/nm\(^2\) which matches the experimentally determined maximum exchange capacity of 1.3 –OH groups per nm\(^2\).[23] This would correspond not to charge neutralization, but to a single 2NP associated with each exchangeable site. The plateau does not persist at higher 2NP concentrations, but rather there is a second adsorption isotherm exhibiting weaker binding characteristics. This would be consistent with hydrophobic interactions between 2NP molecules and \(\pi\)-stacking between aromatic groups once the original monolayer of 2NP has been adsorbed. Thus, it corresponds to a multi-layer of weakly adsorbed 2NP molecules.
Fig. 5 – Langmuir isotherm for adsorption of 2 naphthyl phosphate onto alumina ($T = 25^\circ C$, $I = 10$ mM KCl, pH = 6) where the pH was adjusted back down to 6 after ligand addition. The curve displays saturation at approximately 1.2 molecules/nm$^2$ which matches the experimentally determined maximum exchange capacity of 1.3 $\text{OH}$ groups per nm$^2$. [23]
Fig. 6 – Comparing adsorption isotherms for 2NP with pH re-adjustment back down to pH 6 (●) vs. no re-adjustment (■) following introduction of the ligand species. Re-adjustment of the pH to the starting value of 6 results in ~3-fold higher binding of 2NP to the alumina surface.
Table 2 - 2NP Langmuir Binding Parameters

<table>
<thead>
<tr>
<th>Sample pH</th>
<th>$K$ (mM$^{-1}$)</th>
<th>$\Gamma_{\text{max}}$ (molecules/nm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjusted to pH=6</td>
<td>20.4</td>
<td>1.19</td>
</tr>
<tr>
<td>Unadjusted after adsorption</td>
<td>6.74</td>
<td>0.501</td>
</tr>
</tbody>
</table>

Introduction of the 2NP (or any anionic species that is more strongly bound than hydroxyls) results in the exchange of hydroxyls for the 2NP at the nanoparticle surface.[22] We also compared adsorption isotherms for 2NP with pH re-adjustment back down to pH 6 vs. no re-adjustment following introduction of the ligand species (Fig. 6). Re-adjustment of the pH to the starting value of 6 results in ~3-fold higher binding of 2NP to the alumina surface after overnight equilibration. Linearization of the data shown in Fig. 6 allows us to determine the binding constant $K$, and the max extent of binding $\Gamma_{\text{max}}$. These values are shown in Table 2. We see that the binding constant $K$ is ~3 times higher for the pH adjusted set compared to the unadjusted set. The max extent of binding is also more than double that of the unadjusted set. This can be understood on the basis of alumina’s surface charge as a function of pH (Fig. 2).

The adsorption isotherms for 2 napthyl sulfate (2NS) are shown in Fig. 7. There is no clear saturation plateau; however, the adsorption is not linear in the low concentration limit. Comparing the 2NP and 2NS isotherms, it appears that both demonstrate the low affinity increase in adsorbed amount at higher concentrations due to hydrophobic interactions. But the 2NS displays weaker purely electrostatic binding at low concentrations. The result is the lack of a discrete plateau for 2NS binding, compared to the 2NP isotherm. Also, there is not a large difference in binding interaction between samples that had their pH adjusted back down to 6 vs those that did not. This can be explained by the weaker binding interaction resulting in much
fewer 2NS – OH exchange events that would lead to a smaller rise in pH compared to 2NP (see Fig. 9).

Fig. 7 – Adsorption isotherms for 2 naphthyl sulfate (2NS) onto alumina (■ pH unadjusted after binding | ● pH adjusted to 6.0 after binding). (A) No clear saturation plateau is observed due to the much weaker binding interaction between 2NS and alumina as compared to 2NP. We attribute this difference to the weaker electrostatic interactions between 2NS and alumina vs. 2NP and alumina. There is not a significant difference in binding interactions between samples that had their pH adjusted back down (●) to 6 vs those that did not (■). (B) Freundlich curve fits to low concentration region of binding where the binding is more pronounced: \( \Gamma_{\text{adjusted}} = 0.36[2NS]^{0.44} (K' = 0.36, n = 2.3) \) and \( \Gamma_{\text{unadjusted}} = 0.40[2NS]^{0.30} (K' = 0.40, n = 3.4) \). The fits are tabulated in Table 3.

Two types of isotherm models were fitted to the data. Overall, the linear adsorption model fits reasonably well (Eqn. 1) for the both the adjusted (\( R^2 = 0.997 \)) and unadjusted (\( R^2 = 0.973 \)) cases (Fig. 7) but do not capture the curvature in the low concentration region.

\[
\Gamma = K''c \\
\text{Eqn. (1)}
\]

Where

\( \Gamma = \text{surface coverage of ligand} \) (molecules/nm\(^2\))

\( K'' = \text{adsorption constant} \)
\[ c = \text{concentration of free ligand (mM)} \]

Since the low concentration region shown in Fig. 7B has a much higher degree of curvature, a Freundlich model is more appropriate. The Freundlich isotherm is an empirical model that accounts for both monolayer and multilayer adsorption according to the equation \[ \Gamma = K' c^{1/n}. \] [24]

The Freundlich model fits excellently in the low concentration/high curvature region for both the adjusted \((R^2 = 0.994)\) and unadjusted \((R^2 = 0.995)\) cases. The model fits are summarized in Table 3.

The difference in binding strength between the 2NP and 2NS is expected. The 2NP, with two negative charges interacts more strongly than a singly charged species; and the sulfate group with a large ionic radius to delocalize the charge interaction is known to be a weakly bound ion.

The adsorption isotherm for the triethylene glycol (TEG)-alumina system is shown in Fig. 8. As a nonionic molecule, TEG was expected to exhibit much weaker binding to charged alumina at pH 6 than 2NP or 2NS. The key feature here is that the isotherm is clearly linear, as opposed to the isotherms for 2NP and 2NS. This is consistent with previous observations [25-27] that nonionic small molecules exhibit such linearity in adsorption to charged colloidal particles.
Fig. 8 - Adsorption isotherm for the triethylene glycol (TEG)-alumina system with pH adjustment (●) vs. no adjustment post-binding (■). As a nonionic molecule, TEG was expected to exhibit much weaker binding to charged alumina at pH 6 than 2NP or 2NS. The key feature here is that the isotherm is linear, as opposed to the isotherms for 2NP and 2NS. This is consistent with weaker adsorption.

The model fits are summarized in Table 3. The mechanism of TEG adsorption, relative to the anionic 2NP and 2NS adsorption is best seen by considering the pH changes upon addition of the binding ions shown in Fig. 9. For 2NP and 2NS the increases in pH, due to ion exchange of hydroxyls, correspond closely to the amount of ligand adsorbed. The adsorption process is a true ion exchange. In contrast, for TEG adsorption Fig 9 shows no increase in pH upon TEG adsorption. The adsorption is driven by hydrogen bonding rather than electrostatic ion exchange. This has been demonstrated by Saravanan, et al. using a competitive adsorption experiment between ionic and PEG polymers.[28] There is a significant difference in binding between samples that were pH adjusted following TEG addition vs. those that were not adjusted.
Fig. 9 – pH rise upon addition of various ligands to alumina system at pH 6.0 (2NP ■ | 2NS ● | TEG ▲), A: full view, B: zoomed-in view of 2NS and TEG. The Bolus Concentration is the concentration of ligand injected into the alumina system (250 µL bolus for every 4 mL of 10wt% alumina, see Methods section). In accordance with the binding data presented already, the pH rise following ligand corresponds to an ion exchange process for 2NP and 2NS, but not for TEG.

Isothermal Titration Calorimetry (ITC) experiments on 2NP

Once binding curves were constructed for the various ligands, we were able to determine a starting point for what concentrations of alumina and ligand to use for calorimetry experiments. Ultimately, the experiments were carried out with 5 wt% AKP50 and concentrations of 2NP of 0.2, 0.3, and 0.5 mg/mL in the injector following several rounds of trial-and-error. These conditions were chosen to obtain adequate signal and attain saturation with at least 5-10 injections after saturation to obtain the baseline heat of neutralization.
**Fig. 10** – Isothermal calorimetry data for various concentration of 2NP introduced to aqueous dispersed alumina. Raw data in the top row show the heat flows from each injection of ligand and the bottom row shows corresponding curve fits to the integrated heat data for [2NP] injected = 0.2, 0.3, 0.5 mg/mL.

The raw heat data obtained in the top row of **Fig. 10** were integrated and plotted as a function of alumina surface coverage (**Fig. 10** bottom row). A curve fit was applied to each data set assuming a Langmuir adsorption process with a single set of sites (single K value) and the data shifted to subtract the acid-base neutralization enthalpy baseline. The enthalpies of adsorption were all negative, indicating that the reactions were exothermic.[11, 12]
Table 3 – Thermodynamic parameters from ITC curve fits and UV-vis binding curves. In all cases, the data points used are indicated in the Fit column.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$K$</th>
<th>Fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITC Inject 0.2 mg/mL</td>
<td>-2.19 ± 0.15</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>ITC Inject 0.3 mg/mL</td>
<td>-1.41 ± 0.03</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>ITC Inject 0.5 mg/mL</td>
<td>-1.84 ± 0.10</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td><strong>Average:</strong></td>
<td><strong>-1.81 ± 0.39</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV-vis pH Unadjusted</td>
<td></td>
<td>6.74 mM$^{-1}$</td>
<td>Langmuir (Fig. 8)</td>
</tr>
<tr>
<td>UV-vis pH Adjusted</td>
<td></td>
<td>20.4 mM$^{-1}$</td>
<td>Langmuir (Fig. 8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>$n$</th>
<th>$K'$ or $K''$</th>
<th>Fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear pH Unadjusted</td>
<td>1 (defined)</td>
<td>0.19</td>
<td>Full range</td>
</tr>
<tr>
<td>Linear pH Adjusted</td>
<td>1 (defined)</td>
<td>0.22</td>
<td>Full range</td>
</tr>
<tr>
<td>Freundlich pH Unadjusted</td>
<td>3.4</td>
<td>0.40</td>
<td>Low concentration</td>
</tr>
<tr>
<td>Freundlich pH Adjusted</td>
<td>2.3</td>
<td>0.36</td>
<td>Low concentration</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K''$</th>
<th>Fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear fit pH Unadjusted</td>
<td></td>
<td>4.75</td>
</tr>
<tr>
<td>Linear fit pH Adjusted</td>
<td></td>
<td>2.03</td>
</tr>
</tbody>
</table>

$K = \text{mM}^{-1}$ in $\Gamma/\Gamma_{\text{max}} = \frac{Kc}{1+Kc}$

$K' = \text{molecules/mM}^2 \cdot \frac{1}{(\text{mM})^{1/n}}$ in $\Gamma = Kc^{1/n}$

$K'' = \text{molecules/mM} \cdot \frac{1}{\text{mM}}$ in $\Gamma = Kc$

We see in Table 3 that the average enthalpy of binding for 2NP is -1.81 ± 0.39 kcal/mol and from Fig. 10 (bottom row) that the alumina surface is saturated at 0.15-0.2 molecules/nm$^2$. This is lower than the 0.5 molecules/nm$^2$ plateau observed for the unadjusted case in Figs. 5 and 6. This may reflect the differences in time-scales between the experiments. Injections in the ITC experiment were made every few minutes (each experiment runs for 2-3 hours total) while the adsorption isotherms we constructed allowed for equilibration overnight. We observed that the pHs changed over relatively long time frames upon sonication or the addition of ligand. So the slower equilibration may be responsible for the apparently lower binding levels.
Experiments with Poly(Acrylic Acid)

Dispersion with 6k and 450k PAA

Poly(acrylic acids) (PAAs) are commonly used both to disperse and stabilize alumina suspensions as well as to flocculate suspensions depending on the concentration, molecular weight, temperature, ionic strength, and pH.[29] As part of our preliminary experiments with PAA, we wanted to see the effect of small and large MW poly(acrylic acid) (PAA) on the hydrodynamic diameter of alumina nanoparticles. AKP50 was sonicated first at 80 W for 5 minutes (size distribution of bare alumina in Fig. 11A), then PAA (6k or 450k) was added and the dispersion sonicated further at 40 W for 5 mins. This dispersion method was chosen to be gentler than Method 1 and Somasundaran’s method described earlier in order to minimize PAA degradation. Mean hydrodynamic diameters of 226 nm (PAA_{6k}) and 245 nm (PAA_{450k}), respectively, which indicates complete dispersion (Fig. 11B) and in the case of the 6k PAA, the size was slightly smaller than the bare alumina particle dispersed in water at pH 3. PAA rapidly adsorbed to the colloidal particles, and thus aided the dispersion of the particles without the intense sonication used in Method 1. Method 1 was used for all of the small molecule binding experiments shown previously and was outlined in the Methods section. Using small MW PAA allows us to estimate the primary particle size, since the pH of sonicated bare alumina rises to ~ pH 8 upon hydration of previously anhydrous surface groups. This rise in pH reduces the repulsive electrostatic surface charges allowing for aggregation.
Fig. 11 – (A) Size distribution of bare sonicated AKP50 alumina nanoparticles (mean size ~ 280 nm) in water. (B) Size distribution of AKP50 nanoparticles coated with 6k (■) and 450k (●) poly acrylic acid at 1.2-1.4wt% PAA loading. Mean sizes 226 nm and 245 nm respectively.

Hydrodynamic diameters of adsorbed PAA layers

Since dispersion with 6k and 450k PAA produced suspensions with different mean hydrodynamic diameters, we conducted a series of experiments with PAAs of varying molecular weights to test the detection of adsorbed PAA layers on alumina at pH 6.0 by dynamic light scattering at 173° backscatter. The method of dispersion here is slightly different from that used in Fig. 11, as described in the Materials and Methods section. The hydrodynamic sizes are similar however. Fig. 12(A) shows the observed hydrodynamic diameters of polymer-alumina systems as a function of PAA molecular weight. The polymer loading was held constant at about 1.2-1.4w/w% relative to alumina (Table 4). According to Cesarano and Aksay,[8, 30] this level of polymer loading is well into the plateau region for adsorption of poly(methacrylic acid) onto alumina. We see that the primary particle size appears to be close to 218 nm as observed in the case where the small MW 1.8k PAA is adsorbed onto the surface. Increasing the MW of the polymer increases the hydrodynamic diameter of the nanoparticles, as expected, since larger MW polymer chains will have thicker layers.[31, 32] The layer of adsorbed 1.8k PAA is expected to
have a thickness of about 2-3 nm.[31, 33] That would give a primary particle size of about 212-214 nm. The observed total hydrodynamic diameter scales as \( D_h (nm) = 158.7[MW]^{0.0427} \) (R² = 1.00, Fig. 12A), while the brush layer thickness (assuming a 3 nm layer for the 1800 MW PAA) scales as \( \delta (nm) = 0.13[MW]^{0.45} \) (R² = 0.91, Fig. 12B).

In general, the layer thickness (\( \delta \)) of charged polymers to oppositely charged surfaces has no molecular weight dependence in the low ionic strength limit but may scale as \([MW]^{3/5}\) in the high ionic strength regime which is the classical scaling law observed for adsorption of neutral polymers to colloids.[31, 34] Seyrek, et al. found a weaker scaling law of \( \delta \propto [MW]^{0.1} \) for the saturation adsorption of polyelectrolytes onto charged latices at pH 4 with I = 10⁻¹ M.[32] This reflects the domination of attractive electrostatic effects causing the polymer to adopt a ‘flat’ conformation on the latex surface and being relatively resistant to ion-induced charge screening effects. We expect that the higher scaling power we found is due to differences in pH, ionic strength, type of colloid and pH-dependent surface charge, pH-dependent fractional charging of PAA, polydispersity, and kinetic effects between large and small MW polymers. Nonetheless, it is important to note that polyelectrolyte adsorption to charged surfaces may not always obey the 3/5 scaling law as that is derived based only on electrostatics and does not take pH dependent fractional charging of the polyelectrolyte ligand into account.[31]

<table>
<thead>
<tr>
<th>PAA MW</th>
<th>Pigment : Polymer (by mass)</th>
<th>( D_h (nm) \pm \sigma )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1800</td>
<td>1 : 0.012 (1.2%)</td>
<td>218 ± 10.6</td>
</tr>
<tr>
<td>6000</td>
<td>1 : 0.014 (1.4%)</td>
<td>231 ± 1.4</td>
</tr>
<tr>
<td>145000</td>
<td>1 : 0.012 (1.2%)</td>
<td>263.4 ± 2.7</td>
</tr>
</tbody>
</table>

\( D_h \pm \sigma \) is the hydrodynamic diameter ± standard deviation of multiple measurements.
Fig. 12 – (A) Hydrodynamic diameter ($D_h$) of polymer-alumina systems vs. PAA molecular weight plotted on log-log scales. The polymer loading was held constant at about 1.2-1.4w/w% of the alumina, which is above the saturation plateau for acrylic acid polymer adsorption.[30] The primary particle size appears to be close to 218 nm as observed in the case where 1.8k PAA is adsorbed onto the surface. $D_h$ scales with the MW to the 0.0427 power. (B) Layer thickness of adsorbed PAA vs. MW assuming a layer thickness of 3 nm for the 1800 MW PAA, based on literature values. The brush thickness scales with polymer MW to the 0.45 power which is close to the classical 3/5 power.

Conclusions and Future Work

Molecules similar to the ones studied in this work include benzoate (mono-carboxy), phthalate (dicarboxy), salicylate (hydroxy + carboxy), octyl benzene sulfonate, and meta- and para-xylene sulfonates as shown in Table 1. We see first of all that the enthalpy of adsorption of 2NP determined is exothermic and of the same order of magnitude as for the acids found by other calorimetric studies. The studies which looked at sulfonate functionalities either did not have calorimetric data or conducted the studies at elevated temperature (43-50°C). Since $\Delta H$ scales with $T$ through the temperature dependence of the heat capacity,[35] the enthalpies found are correspondingly higher than enthalpies found here at 25°C. $K$ values also match up quite well with binding constant for sodium benzoate at pH 5.[9] 2-naphthyl phosphate displays an almost identical binding constant as phthalic acid which can be explained on the basis of both molecules
having a -2 charge, although phthalic acid would possibly display higher ΔH due to the cooperativity between separate carboxyl groups. Interestingly, we found significantly higher max extents of binding (as determined by Γ_{max}), especially for 2NP. This may be due to our pH readjustment protocol and longer time to equilibration compared to these studies. A more detailed comparison cannot be made as the hydrophobic moieties are very different.

In this study, we explored the binding adsorption of small molecules onto alumina colloids. We add data to the literature on the enthalpy of adsorption of 2NP onto alumina, which comes from the ITC measurements. By selecting molecules that differed only in functional group (2NP vs. 2NS) and a nonionic molecule of similar molecular weight, we were able to determine differences in binding strengths and modes between otherwise similar molecules. The adsorption isotherms of 2NP and 2NS and the TEG show an interesting consistency when the adsorption and pH data are considered together. The divalent 2NP shows strong adsorption with a Langmuir adsorption saturation at 1.2 molecules/nm^2 which corresponds to the reported exchange capacity of alumina. The pH shows a corresponding rise and saturation. The continued rise in 2NP binding is not accompanied by a continued rise in pH because the hydrophobic adsorption is not associated with ionic exchange. Isothermal calorimetry results show that the binding enthalpy for the 2NP interaction is -1.81 ± 0.39 kcal/mol. Both the adsorption and pH increase for 2NS is linear to 120 mM (bolus injection) or 5 mM free 2NS, and is much weaker than the 2NP association. This is consistent with the adsorption in this regime still being based on ion exchange, but the “softer” sulfate group is less strongly bound. For the non-ionic triethylene glycol (TEG), the adsorption is fundamentally different because the adsorption is not accompanied by changes in pH. It is not an ion exchange process but driven by
hydrogen bonding between the ether oxygens and hydroxyls on the alumina surface. The results on zeta potential show that even modest changes in dispersion conditions can make discernible differences in the zeta potentials of the alumina particles although the isoelectric point is unchanged. This study would indicate that the most effective anchoring group for dispersants for alumina surfaces, which represent those of interest for covert ink labeling, would involve phosphate groups, and in light of the polymer adsorption study, polymeric polyphosphate of modest MW (~1000) should be suitable.

Experiments with poly(acrylic acid) (PAA) polymers showed that PAA is an effective dispersant for concentrated alumina nanoparticles, as has been reported previously. Dispersion of the AKP50 alumina at pH 6 resulted in some level of weak aggregation, even with zeta potentials of ~25mV. Complete dispersion could be achieved by increasing the surface potential to ~35 mV or by adsorbing even low MW PAA (1800 MW). Again this would suggest that even lower MW polyphosphate dispersants should be effective. Increasing the MW of the PAA resulted in an adsorbed polymer brush layer that increased the alumina hydrodynamic size from 218 nm to 263 nm with increasing polymer MW from 1.8k to 145k. Increasing size would increase viscosity at high alumina loading which would be a disadvantage in inkjet printing.

The adsorption study would suggest that stabilizing dispersants based on polyphosphates would be helpful in these applications. While these polymers do not appear to be available at this time, ATRP polymerizations have been conducted to produce vinyl phosphate block copolymers[36] and this would be a fruitful area of future research.
Acknowledgements

We would like to acknowledge Honeywell Specialty Materials for financial support of this work and scientific guidance. We would also like to acknowledge Joe Roy-Mayhew of the Ceramic Materials Laboratory at Princeton University for conducting BET measurements on AKP50 and Prof. Peter Jaffé and Hagar El-Bishlawi for help with conducting total organic carbon measurements.
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CHAPTER 6
Flash NanoPrecipitation of Polystyrene Nanoparticles

This work was presented at the American Chemical Society Colloids Meeting in Baltimore in 2012.

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Contributions to this work were divided as follows:
Zhang and Priestley contributions: Figs. 1 – 4
Pansare and Prud’homme contributions - : Figs. 5 – 7

Abstract

Aside from polymerization techniques, polymer nanoparticles can be generated through the displacement of a solvent with a nonsolvent, i.e., nanoprecipitation. In this study, we utilize a facile process termed Flash NanoPrecipitation (FNP) to generate polystyrene (PS) nanoparticles of several different molecular weights. As compared to PS nanoparticles synthesized by surfactant free emulsion polymerization, nanoparticles prepared by FNP show comparable size distributions when the diameter is less than 150 nm. Furthermore, we illustrate that the sizes of PS nanoparticles prepared by FNP can be fine-tuned by changing the polymer and/or electrolyte concentration. The stabilized nanoparticles contain only the radically polymerized polymer chains, which have sulfate anions at the chain termini and no additional external stabilizers.

Calculations of the mechanism of particle formation and stabilization show that the size-dependent electrostatic repulsions between nanoparticles and nanoparticles versus single collapsed polymer chains control assembly and monodispersity. The ability to independently vary polymer molecular weight and nanoparticle size enables fundamental studies of the effect of confinement on polymer dynamics in a way not easily achievable by other techniques.
INTRODUCTION

Polymeric nanoparticles are vital components of biomedical targeting and diagnostic studies.[1-3] Surface functionalization of latex nanoparticles with tightly controlled sizes are used in fluorescent imaging and ligand and antibody targeting.[4-6] These applications rely more on the size and surface chemistry of the polymeric nanoparticle than on its core composition. Other applications of polymeric nanoparticles include fillers in thin films for the improvement of thermomechanical properties[7] and components in photonic and plasmonic structures.[8, 9] In these applications, it is the properties of the nanoparticle core that are of paramount importance. Additionally, polymeric nanoparticles can serve as model systems for examining fundamental property changes due to 3-dimensional confinement.[10-13] Thus from practical and fundamental viewpoints, the ability to concurrently generate neat polymer nanoparticles (i.e., no residual contaminants), as well as independently controlling nanoparticle size and molecular weight of the polymer core, is highly desirable.

Approaches to form polymer nanoparticles can be categorized into three groups: 1) polymerization of monomers (e.g., emulsion, micro-emulsion, mini-emulsion, and interfacial polymerizations),[14-17] 2) emulsification and solvent stripping to form a “pseudo-latex,”[18-20] and 3) nanoprecipitation of a bulk polymer precursor.[14, 17, 21] In typical emulsion polymerization, monomers are combined with an initiator (e.g., ammonium persulfate) and sometimes surfactants (e.g., sodium dodecyl sulfate) in an aqueous medium. The mixture is then allowed to react for several hours to create small emulsions of the polymerizing system. The resulting polymer nanoparticles are monodisperse.[15, 16] A disadvantage of polymerization
techniques is the difficulty of independent control of particle size and molecular weight. That is, both the diameter and the molecular weight increase with increasing extent of polymerization.[16] Moreover, residual components, such as monomers, oligomers, and surfactants, can be difficult to remove from these polymerized nanoparticles and may modify the physical properties of the polymer core, thus frustrating measurements of the effect of confinement on polymer nanoparticle properties. In the emulsion stripping process, a polymer solution is emulsified and then the volatile solvent for the polymer is removed to condense and solidify the particle. These techniques, often called “pseudo-latex” formation processes, enable the formation of sub-micron nanoparticles from polymers that cannot be polymerized by normal free-radical polymerization techniques.[18-20] However, they suffer from the polydispersity that inherently arises from the size distribution of the parent emulsion drops.

An alternative approach to generate polymer nanoparticles is nanoprecipitation, in which nanoparticles are precipitated from polymer chains in solution as a result of displacing a solvent with a non-solvent.[22, 23] In the past decade, nanoprecipitation has been primarily used to form biodegradable polymer nanoparticles for drug delivery purposes including poly(lactic acid) (PLA),[24, 25] poly(lactic-co-glycolic acid) (PLGA),[21, 26] poly(ε-caprolactone) (PCL),[27] and several amphiphilic block copolymers.[28-30] One advantage of nanoprecipitation is that since bulk polymer is used as a precursor, independent control of size and molecular weight should be achievable. Additional advantages include fast processing time, low energy consumption, and high reproducibility.[31, 32] Shortcomings of nanoprecipitation include low mass fraction of nanoparticles in the prepared samples and a broad size distribution for samples with diameters greater than several hundred nanometers.[31]
Whereas nanoprecipitation has been applied almost exclusively to biodegradable polymers (as evidenced by over 80 publications in the last two years), only a handful of examples on other synthetic polymers exist in the literature.[14] Nanoprecipitation of non-biodegradable polymers, such as polystyrene (PS)[32, 33] and poly(methyl methacrylate) (PMMA),[31, 32, 34] have only started to emerge in the past year. This comes as a surprise since the original patent on nanoprecipitation described the process as a method to generate nanoparticles from a wide-range of polymers and not just biodegradable polymers.[22]

Here, we utilized a nanoprecipitation process that achieves rapid solvent displacement by means of novel high intensity mixing geometries. The process has been denoted Flash NanoPrecipitation (FNP). The mixing occurs in a central cavity fed by two incoming solvent streams. The geometry and operation of the device has been previously reported[35] and has been modeled by computational fluid mechanics.[36] Using competitive dye reactions, the mixing time for inlet velocities of ~1 m/s is on the order of 1.5 ms. In our previous papers, syringe pumps were used to drive the fluid flows at predetermined rates, whereas in this study manual injection was used following the adaptation of Macosko and coworkers.[37] Previously, the technique has been used with block copolymer stabilizers to control nanoparticle size, whereas, in this study we use the intrinsic charge of the sulfate-terminated polystyrene chain to produce electrostatic stabilization. As a consequence, sub-150 nm diameter particles of narrow size-distributions can be formed with high reproducibility. In contrast to previous research in the literature which has shown the formation of PS nanoparticles with diameters greater than 200 nm via a more time-consuming dialysis nanoprecipitation technique,[32] this work is the first to
utilize a simple nanoprecipitation technique to form PS nanoparticles of narrow polydispersity with diameters less than 150 nm. We also illustrate the effect of polymer concentration, polymer molecular weight, and electrolyte (NaCl) concentration on the size and size distribution for nanoparticles prepared by FNP. While in this study we produced only ~ 10 mg samples of nanoparticles (an amount sufficient enough to allow for confinement effect studies of the glass transition temperature via differential scanning calorimetry) using manual injection, an advantage of the FNP process is that it is scalable. Using continuous flow in the current geometry would enable production of 3.5 kg/day of material, and currently commercial production of β-carotene nanoparticles are produced by BASF at 1400 kg/day using confined impinging jet technology.
MATERIALS AND METHODS

Material Synthesis

Polystyrene nanoparticles were initially synthesized by surfactant-free emulsion polymerization (SFEP), in accordance with procedures described in detail elsewhere.[38] Briefly, in a typical synthesis, 0.074 g of ammonium persulfate (Acros Organics, 98%) was initially added to 100 mL of MilliQ H₂O in a three-neck flask. The solution was bubbled with N₂ for ~ 20 minutes and then heated to 75 °C. Subsequently, 10 g of styrene (Sigma Aldrich, ≥99%) was combined with 0.05 g of acrylic acid (Sigma Aldrich, 99%), and the mixture was injected into the flask. The solution was then allowed to polymerize under reflux conditions for 20 hr. The addition of a small amount of acrylic acid as a co-monomer provided additional stability to the colloidal suspension. The molecular weight of the synthesized PS was varied by changing the styrene concentration and/or ammonium persulfate concentration in the reactor. After synthesis, the PS nanoparticles were washed twice with MilliQ H₂O through centrifugation and ultimately suspended in water. A portion of the water-suspended sample was subsequently dried and annealed at 150 ºC for 12 hours to form bulk polymer, which was then used as the precursor polymer for Flash NanoPrecipitation.

Fig 1(a) shows a schematic of the FNP process employed to precipitate PS nanoparticles, while Fig 1(b) shows the actual FNP setup employed in the laboratory. A confined impinging jet mixer composed of two separate streams was used. Here, a syringe containing 3 mL of the synthesized bulk PS dissolved in tetrahydrofuran (THF) was placed at the inlet of Stream 1, and a syringe containing 3 mL of pure MilliQ H₂O or a H₂O/NaCl solution was placed at the inlet of
Stream 2. Subsequently, fluid was expressed manually from both syringes at the same rate (~ 3 ml in ~ 4 s), causing the two streams to merge into a mixing stream. The mixing stream was then allowed to flow into a beaker containing 27 mL of MilliQ H₂O under mild stirring. The precipitated PS nanoparticles suspended in an aqueous solution were collected in scintillation vials. THF was subsequently removed through membrane dialysis or air evaporation over the course of 48 hr.

Fig. 1 - (a) Schematic and (b) actual laboratory setup of the FNP mixing process to generate PS nanoparticles.
Characterization

Weight-average molecular weight ($M_w$) and number-average molecular weight ($M_n$) of each polymer sample created from SFEP was determined using gel permeation chromatography (Waters 515 HPLC pump, Eppendorf CH-460 column heaters, Waters 410 differential refractometer, Agilent PLgel mixed B column, THF eluent). Z-average diameters, size distribution curves, and polydispersity indices (PDI) of size distributions of PS nanoparticles suspended in water from SFEP and FNP were determined using dynamic light scattering (DLS) (Malvern Instruments Zetasizer Nano-ZS ZEN 3600). Zeta potentials of nanoparticle samples were obtained using the same instrument (Malvern Instruments Zetasizer Nano-ZS ZEN 3600) in a disposable folded capillary cell. The instrument performs laser Doppler velocimetry to obtain the electrophoretic mobility, which was then converted to a zeta potential via the Henry equation. Images of PS nanoparticles were obtained using scanning electron microscopy (SEM) (FEI XL-30). Prior to imaging, nanoparticles were drop-casted onto carbon tape, allowed to dry in the hood for 12 hours, and then coated with a 5 nm thick iridium layer.
RESULTS AND DISCUSSION

Experimental

The basic premise behind Flash NanoPrecipitation is that rapid micromixing produces solvent exchange between good solvent and anti-solvent conditions in a time scale faster than the aggregation time of the solute. The process is shown schematically in Fig. 1. The solvent stream containing PS dissolved in THF (Stream 1) was rapidly mixed with an incoming water stream (Stream 2), which caused collapse of the hydrophobic polymer chain in the aqueous solvent and subsequent aggregation. The flow rates through the mixer were kept approximately constant at 2 mL/s, which resulted in a jet velocity of ~ 1 m/s through the 0.5 mm diameter orifices and a Reynolds number[35] of ~ 3500. The mixed exit stream was then diluted into a 27 mL water reservoir, which quenched the precipitated nanoparticles. The surfaces of the nanoparticles were negatively charged, as determined from zeta-potential measurements ($\zeta$ ~ -40-50 mV). The inherent negative surface charge, resulting from sulfate-terminated polymer chains from the ammonium persulfate initiator in emulsion polymerization, provided stability to the nanoprecipitated PS particles, which will be discussed below.

The effect of PS concentration in THF on the size of polymer nanoparticles formed via FNP was determined by varying the concentration between 0.5 mg/mL to 10 mg/mL in Stream 1 of the mixer for PS of $M_w = 376$ kg/mol. Figs. 2a-c show representative SEM images of the nanoprecipitated PS particles using concentrations of 2 mg/mL, 5 mg/mL and 10 mg/mL, respectively. It was observed that as the concentration increased, the particle size increased. Additionally, the precipitated particles were fairly monodisperse up until a concentration of 5
mg/mL. At a concentration of 10 mg/mL (which corresponded to a size of ~ 140 nm), the precipitated particles became more polydisperse and exhibited a broader size distribution.

**Figs. 2d-f** show SEM images of PS nanoparticles from SFEP, roughly corresponding to sizes of particles in Figs. 2a-c, respectively. By comparing Fig. 2a to 2d, 2b to 2e, and 2c to 2f, it was observed that for particles with diameters less than 140 nm, *i.e.*, for PS concentrations of 5 mg/mL or less in FNP, the FNP technique generated nanoparticles as monodisperse as particles prepared by SFEP. For larger particles, *i.e.*, for PS concentration of 10 mg/mL in FNP, the sizes of particles from FNP became polydisperse, whereas particles from SFEP remained monodisperse. Thus, one of the major challenges for nanoprecipitation techniques is maintaining low polydispersity for larger particles. A quantitative comparison of size polydispersity between nanoparticles from FNP and SFEP was made by plotting size distribution curves (determined from DLS), as shown in Figs. 2g-i. The solid lines correspond to nanoparticles from FNP, while dashed lines correspond to nanoparticles from SFEP. These size distribution curves, along with corresponding PDI values, provide further evidence that monodispersity, *i.e.*, a narrow size distribution, can be maintained for PS nanoparticles from FNP with diameters less than 140 nm.
Fig. 2 - Representative SEM images of PS nanoparticles ($M_w = 376$ kg/mol) made from PS concentrations of (a) 2 mg/mL, (b) 5 mg/mL, and (c) 10 mg/mL in Stream 1 of the FNP mixing process, with Stream 2 containing pure H$_2$O. SEM images (d)-(f) show similar-sized PS nanoparticles synthesized from SFEP, in comparison to (a)-(c), respectively. Solid lines in (g)-(i) show size distribution curves of PS nanoparticles from FNP in images (a)-(c), respectively, whereas dashed lines in (g)-(i) represent size distribution curves of PS nanoparticles from SFEP in images (d)-(f), respectively.

To examine the effect of polymer molecular weight on nanoparticle size, two additional PS molecular weights ($M_w = 92$ kg/mol and $M_w = 770$ kg/mol) were used in the FNP technique. Note that the molecular weight polydispersity indices (i.e., $M_w/M_n$) for the three samples were all $\sim 3.5$, as determined from GPC. Fig. 3a plots the z-average diameter (determined from DLS) of
PS nanoparticles as a function of PS concentration in Stream 1 of the FNP mixing process for the three different molecular weights. For each molecular weight, the diameter of PS nanoparticles increased linearly with increasing PS concentration in Stream 1, in agreement with the SEM images in Fig. 2. At the same polymer concentration, the particle size increased with increasing molecular weight. This increase in size with molecular weight may be explained by the increased size of the collapsed polymer chain and lower charge density contributed by each chain, resulting in larger particles when these aggregate. From Fig. 3a, it becomes clear that PS nanoparticle size and molecular weight can be controlled independently in a straightforward manner. For example, ~90 nm diameter nanoparticles with molecular weights of 92 kg/mol, 376 kg/mol, and 770 kg/mol can be made using PS concentrations of 10 mg/mL, 5 mg/mL, and 0.5 mg/mL, respectively.

An additional variable in tuning the size of polymer nanoparticles prepared from FNP was explored in this study, i.e., adding NaCl to Stream 2 of the mixer. Fig. 3b shows the change in diameter of PS nanoparticles ($M_w = 92$ kg/mol) for different PS concentrations in Stream 1 as the wt% of NaCl (with respect to PS weight) was varied in Stream 2 from 0 wt% to 50 wt%. As the wt% of NaCl increased, the diameter of the particles increased at each PS concentration. Hence, by changing PS concentration and/or wt% of NaCl, the diameter of PS nanoparticles prepared from FNP can be precisely controlled. Note that similar trends were observed for the other PS molecular weights, i.e., for $M_w = 376$ kg/mol and $M_w = 770$ kg/mol. Clearly, at certain unique values of PS concentration and/or wt% of NaCl for each molecular weight, similar-sized nanoparticles with different molecular weights can be generated from the FNP technique. We
note that for similar-sized nanoparticle prepared with and without NaCl, the width of the size
distribution curves were similar.

**Fig. 3** - (a) Change in PS nanoparticle diameter as a function of PS concentration in Stream 1 of
the FNP mixing process for three different PS molecular weights: 92 kg/mol (●), 376 kg/mol
(■), and 770 kg/mol (▲), with pure H₂O in Stream 2. (b) Change in PS nanoparticle diameter
as a function of wt% of NaCl in Stream 2 of the FNP mixing process for different PS (M_w = 92
kg/mol) concentrations in Stream 1: 0.5 mg/mL (△), 1 mg/mL (○), 2 mg/mL (□), and 5 mg/mL
(◇). Size measurement errors are within the marker size. Solid lines are guides for the eye.
**Fig. 4** shows representative SEM images of PS nanoparticles ($M_w = 92$ kg/mol) made using 0 wt%, 3 wt%, 5 wt%, 10 wt%, 20 wt%, and 50 wt% of NaCl (with respect to PS weight) in Stream 2 and 2 mg/mL PS concentration in Stream 1 of the FNP mixing process. From these images, it is clear that diameters increased with increasing wt% of NaCl, in agreement with the trend observed in **Fig. 3b**. For the particular case, i.e., $M_w = 92$ kg/mol and 2 mg/mL PS concentration, PS nanoparticles appeared to be fairly monodisperse, up until 50 wt% of NaCl (with respect to PS weight).
Fig. 4 - Representative SEM images of PS nanoparticles ($M_w = 92$ kg/mol) made using (a) 0%, (b) 3%, (c) 5%, (d) 10%, (e) 20%, and (f) 50% wt% of NaCl (with respect to PS weight) in Stream 2 and 2 mg/mL PS concentration in Stream 1 of the FNP mixing process.
Mechanism of Assembly

The mechanisms involved in the Flash NanoPrecipitation of high molecular weight polymer are interestingly and subtly different than the mechanisms of controlled assembly of small hydrophobic organic molecules with stabilizing block copolymers, *i.e.*, the normal mode of operation of FNP. The differences are twofold. First, in small molecule nanoparticle formation, the solvent exchange causes supersaturation, but there is an activation barrier to assembly, *i.e.*, a nucleation step. Particles must first grow larger than the critical nuclei size and then the process becomes growth controlled. In systems that are nucleation controlled, increasing nucleation rate decreases the final nanoparticle size because the solute deposits on the larger number of nuclei that are formed. In contrast, if the system is growth controlled, then a higher concentration of solute means that particles grow larger the longer they are in the growth regime, before the stabilizing block copolymer quenches growth. For a 92 kg/mol polymer chain, the collapsed radius is 3.3 nm, as calculated from the mass and density of a single PS chain. This is much larger than a critical nuclei size; therefore, there is no barrier to nucleation. The nanoparticle size is controlled only by micro-mixing to ensure homogeneous polymer distribution in space and by growth kinetics.

The second difference is in the mechanism of stabilization. For traditional FNP, the adsorption of the block copolymer on the nanoparticle surface determines the end of aggregation and freezes the size. In the current process, there is no external stabilizing agent. Each polymer chain, which has been initiated by a sulfate radical, carries with it a unit negative charge. The stronger electrostatic repulsion between two nanoparticles relative to the weaker repulsion between a
nanoparticle and a collapsed polymer chain (unimer) controls growth and stabilization of nanoparticles. The following illustrative calculations demonstrate the essential features of the process and highlight the reason why SFEP produces narrower particle size distributions at larger nanoparticle sizes than does FNP. The measured electrophoretic mobilities of PS nanoparticles produced by FNP from 92 kg/mol PS in varying ionic strength solutions are shown in Fig. 5.

![Graph](image)

**Fig. 5** - Dependence of zeta potential (▲) and size (■) on NaCl concentration for PS (M₆ = 92 kg/mol) nanoparticles generated at a polymer concentration of 1 mg/mL in Stream 1 of the FNP process. The 0.1 M and 0.5 M samples aggregated upon formation, thus their sizes are not shown. The (negative) zeta potential passes through a maximum and decreases (tends towards zero) at higher ionic strength.

Precipitations were conducted at 1 mg/mL PS concentration in the THF stream. As shown, nanoparticle size increased with ionic strength up to $1 \times 10^{-1}$ M at which point the nanoparticle dispersion flocculated upon formation, and stable particles could no longer be produced. The
zeta potential of the nanoparticles was measured to be -39 mV at $10^{-5}$ M and becomes more negative (-55 mV) at $5 \times 10^{-2}$ M. Subsequently, the surface charge diminishes (i.e., trends toward zero) at higher ionic strengths. The increase and then decrease in mobility with ionic strength has been previously reported for similar surfactant free, charge stabilized latexes.[41, 42] This is in contrast to a sharp monotonic decrease in surface potential with ionic strength that would be expected if the PS surface had a fixed density of sulfate groups that determined the surface potential.[43] The origin of the ionic-strength-dependent surface charge is an open debate, but reports of negative potentials on hydrophobic surfaces generally support a mechanism involving the more weakly solvated anions interacting with the predominantly hydrophobic surface and giving a zeta potential that reflects this anionic charging.[42, 44] The surface charge decreases at even higher ionic strength due to conventional electrostatic screening.[42, 43]

The surface charge density ($\sigma$) can be calculated from the Graham equation:[43]

$$\sigma = \sqrt{4\varepsilon\varepsilon_0 kT \left[\langle NaCl \rangle_\infty \left[ \cosh \left( \frac{e\psi_0}{kT} \right) - 1 \right] \right]}^{1/2} \tag{1}$$

where $\varepsilon$ is the dielectric constant, $\varepsilon_0$ is the permittivity of free space (C/(V∙m)), $k$ is the Boltzmann constant (J/K), $T$ is the absolute temperature (K), $\langle NaCl \rangle_\infty$ is the ion number density (#/m$^3$), $e$ is the elementary charge (C), and $\psi_0$ is the surface potential (V). Using the experimentally-determined zeta potential of -41 mV at an ionic strength of $10^{-3}$ M (Fig. 5), the charge density on the nanoparticle surface is calculated to be $3.3 \times 10^{-3}$ C/m$^2$. The charge density on the surface of the single unimer chain with a collapsed radius of 3.3 nm and having one
sulfate group (i.e., one negative charge) at the chain terminus is calculated to be \(1.2 \times 10^{-3} \text{ C/m}^2\).

From the Graham equation, the surface potential of a single unimer would be -16 mV, which is roughly 40% of the value determined for a nanoparticle. The conclusion is that the smaller unimer is somewhat less charged than the nanoparticle as would be expected from the better packing available to the PS chain once it is assembled in nanoparticle form, where the PS chain tail can extend into the nanoparticle core. For the calculations below, we will assume that the potential at the unimer surface is 40% of the value of the nanoparticles surface, since the zeta potential of the unimer is not experimentally accessible.

The interaction energy \(W_{\text{Total}}\) between two 200 nm diameter PS nanoparticles having the experimentally determined surface potentials (Fig. 5) and the energy between a 200 nm nanoparticle and a unimer chain are calculated from the balance of electrostatic repulsions \(W_{\text{Electrostatic}}\) and attractive London van der Waals interactions \(W_{\text{VDW}}\):

\[
W_{\text{Total}} = W_{\text{Electrostatic}} + W_{\text{VDW}} 
\]

\[\text{Eqn. (2)}\]

The electrostatic interactions between two spheres of radii \(R_1\) and \(R_2\) (m) with different surface potentials has been derived by Hogg et al.[45] and Israelachivili:[43]

\[
\frac{W_{\text{Electrostatic}}}{kT} = \frac{2\pi \varepsilon_0 \varepsilon \kappa}{R_1 + R_2} \left[ \frac{-\psi_1 \psi_2}{\kappa} \ln \left( \frac{\kappa D}{2} \right) - \left( \frac{\psi_1^2 + \psi_2^2}{2\kappa} \right) \left( \kappa D - \ln(2 \sinh(\kappa D)) \right) \right]
\]

\[\text{Eqn. (3)}\]
where $\kappa$, the reciprocal Debye length (m$^{-1}$), is directly proportional to the square root of the electrolyte (e.g., NaCl) concentration, $\psi_1$ and $\psi_2$ are the surface potentials of the spheres (V), and $D$ is the sphere separation distance (m). Note that the equation from Hogg has been updated to SI units by adding the factor $4\pi/\varepsilon_0$, where $\varepsilon_0$ is the permittivity of free space. A key point here is the strong size dependence of the electrostatic repulsion between spheres, as shown in Eqn. 3. The attractive van der Waals interaction for two spheres is given by Israelachvili:[43]

$$W_{vdw} = -\frac{A}{6D} \left( \frac{R_1 R_2}{R_1 + R_2} \right)$$  \hspace{1cm} \text{Eqn. (4)}$$

where $A$ is the Hamaker constant (J), which is approximately $10^{-20}$ J.[43]

The total interaction energies, $W_{\text{Total}}$, are shown in Fig. 6. From Fig. 6a, at low ionic strength ($10^{-5}$ M), the much weaker electrostatic repulsions between the unimer polymer chain and nanoparticle cause the potential energy barrier to be only $4kT$, whereas the barrier for nanoparticle-nanoparticle interaction is $105kT$. In $10^{-3}$ M NaCl, the nanoparticle-nanoparticle barrier is still $99kT$, but the unimer-nanoparticle interaction is entirely attractive (Fig. 6b). For $10^{-1}$ M NaCl, the nanoparticle-nanoparticle interactions become attractive (Fig. 6c). The aggregation of single collapsed polymer chains onto either larger nanoparticles or with themselves proceeds at all ionic strengths, but the aggregation of larger nanoparticles amongst themselves is prevented at lower ionic strengths. Therefore, nanoparticles produced at ionic strengths below $10^{-1}$ M would be expected to be stable, while nanoparticles produced at or above $10^{-1}$ M ionic strengths would be expected to be unstable. This is what is observed
experimentally, as shown in Fig. 5. The dependence of nanoparticle size on polymer concentration in the FNP process (as shown in Fig. 3a) can also be explained by considering the interaction potential at low ionic strength in Fig. 6a. The potential barrier is low enough that unimers or small aggregates can continue to add to nanoparticles that are larger than the critical size for electrostatic stability. The growth occurs by continued addition when there is a higher concentration of unimers. The effect of polymer molecular weight on nanoparticle size could be addressed by the model by conducting the calculations with “unimers” with sizes that scale with molecular weight. To demonstrate the physics behind the process, we have selected the unimer size based only on the 92 kg/mol polymer.
Fig. 6 - Interaction potential, W/kT, as a function of distance between two 200 nm diameter PS spheres (solid blue lines) and between a 200 nm sphere and a 3.3 nm unimer (dashed red lines) as calculated from Equations 2-4 at ionic strengths of (a) \(10^{-5}\) M (0.64 wt% of NaCl with respect to PS) with the experimentally measured zeta potential of -39 mV for the 200 nm sphere and -15 mV for the unimer sphere, (b) \(10^{-3}\) M (6 wt% of NaCl with respect to PS) with the experimentally measured zeta potential of -41 mV for the 200 nm sphere and -16 mV for the unimer sphere, and (c) \(10^{-1}\) M (64 wt% of NaCl with respect to PS) with the experimentally measured zeta potential of -20 mV for the 200 nm sphere and -8 mV for the unimer sphere. At all ionic strengths, the repulsive barrier is never high enough to prevent unimer from aggregating with the 200 nm spheres, but the repulsive barrier is large enough at \(10^{-5}\) M and \(10^{-3}\) M to prevent 200 nm sphere from aggregating, thus ensuring colloidal stability. At \(10^{-1}\) M, the potential interaction between 200 nm spheres is entirely attractive and aggregation would be expected as is seen experimentally (Figure 5).

The increase in polydispersity for nanoparticles with increasing size can also be understood in terms of the process of aggregation. In our previous study of the FNP assembly of 3 nm hydrophobic Au colloids, i.e., the same size as the collapsed polymer chain unimers considered here, the size and polydispersity were quantitatively modeled using diffusion limited aggregation.[46] The cutoff time in the previous study was the time scale for polymer stabilization. This mechanism is consistent with the results seen for the current polymer nanoparticles. The lack of sufficient electrostatic stabilization enables particles below a critical electrostatic stabilization size to aggregate. The random aggregation process creates a polydisperse distribution of sizes. However two nanoparticles slightly below the critical size for stability can aggregate to form a stable nanoparticle that is significantly larger than the minimum critical size. The model of random aggregation[46] predicts that the polydispersity of the distribution increases with the number of random aggregation steps; that is, the polydispersity increases with increasing size. In contrast, SFEP involves a growth process where an initial number of nuclei are formed and then growth occurs by single chain addition; the rate of which
is equal for every nucleus in the population. This single chain addition results in a population that is self-sharpening and becomes more monodisperse with increasing size until other destabilizing mechanisms come into play.\[47, 48\] Modeling the concentration dependence of nanoparticle size would involve a population balance approach that would capture both the dynamics of assembly and the electrostatic energy barrier that varies with nanoparticle size in the population.\[49-51\]

The mechanism of electrostatic control of nanoparticle size during FNP assembly is further demonstrated in Fig. 7. Nanoparticles were initially generated from 376 kg/mol PS using 10 mg/ml PS concentration in Stream 1 of the FNP process and 3 wt% of NaCl (with respect to PS weight) in Stream 2 to give a final ionic strength of \(4.7 \times 10^{-4}\) M in the nanoparticle solution. This original sample was subsequently diluted into NaCl solutions to prepare a set of samples over the ionic strength range of \(\sim 10^{-5} - 2\) M. The larger size for the nanoparticles in Fig. 7 relative to Fig. 5 arises because of the higher molecular weight of the PS, as well as a higher concentration of PS in Stream 1 of the FNP process, used in this formulation. Since nanoparticles grow in size until electrostatic repulsions create interaction potential barriers high enough to prevent further aggregation, the nanoparticles are at a critical size in a medium of that ionic strength. When the nanoparticles are placed in a medium of lower ionic strength, the electrostatic repulsions are increased and the nanoparticles are stable and their size is unaffected. However, when the ionic strength is increased from the synthesis conditions of \(4.7 \times 10^{-4}\) M to 0.51 M, electrostatic repulsions are decreased and the nanoparticles aggregate to form clusters of 600 nm. At 1.7 M, the nanoparticles aggregate to 1000 nm. This process of subsequently destabilizing a nanoparticle dispersion that has been stable under low ionic strength is
fundamentally different than the rapid kinetic assembly of nanoparticles under conditions where particle stability is never achieved (Fig. 5, $10^{-1}$ M and above). What is unexpected is that the aggregates that are produced at these higher ionic strengths are not gross precipitates but have a well-defined aggregation size. This phenomenon may be related to the recent discovery of large clusters that can be formed by balancing short ranged attractive forces with longer range cooperative electrostatic forces.[52-54]

![Graph showing dependence of zeta potential and size on NaCl concentration.](image)

**Fig. 7 - Dependence of zeta potential (▲) and size (■) on NaCl concentration for PS ($M_w = 376$ kg/mol) nanoparticles generated at $4.7 \times 10^{-4}$ M (3 wt% with respect to PS) NaCl using a polymer concentration of 10 mg/mL in Stream 1 of the FNP process. The original sample formed at $4.7 \times 10^{-4}$ M NaCl was subsequently diluted into NaCl solutions to prepare a set of samples over the ionic strength range of $\sim 10^{-5}$ - $2$ M. Particle size was unaffected by ionic strength below $10^{-1}$ M, but aggregation was observed above this ionic strength. The (negative) zeta potential increases and then decreases over this range.**

Understanding the mechanism of nanoparticles growth by FNP versus SFEP suggests future research directions to form larger FNP nanoparticles with narrower size distributions. Since unimer addition to monodisperse seeds produces narrow distributions, it may be possible to use
multi-stage FNP mixers to sequentially add unimers to narrowly distribute seed nanoparticles with initial sizes below 150 nm. It should also be possible to produce layered structures with polymers of different compositions or molecular weights using multi-stage mixers. In one sense we have already demonstrated this capability by coating SFEP nanoparticles with an amphiphilic block copolymer.[55]

CONCLUSIONS
In summary, we utilized a facile method termed Flash NanoPrecipitation to successfully generate PS nanoparticles of wide-ranging sizes and different molecular weights. As compared to PS nanoparticles synthesized from surfactant free emulsion polymerization, nanoparticles prepared from FNP showed comparable size-distributions when the diameters of the particles were under 150 nm. Furthermore, we showed that the nanoparticle size can be fine-tuned by changing the PS concentration and/or changing wt% of NaCl in the FNP technique, i.e., an increase in PS concentration and/or wt% of NaCl led to an increase in particle size. The mechanism of nanoparticles growth and stability can be understood by considering the competition between attractive London Van der Waals attraction and electrostatic repulsion. The ability to rapidly produce a range of nanoparticle sizes from a range of polymer precursors with no added stabilizing components or residual impurities will greatly facilitate the studies of polymer dynamics in a 3-dimensionally confined geometry.
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48. The details of the “single chain event” depend on the exact locus of polymerization. If the polymerization occurs in the aqueous phase, then the polymer collapses and diffuses
until it aggregates with an existing nanoparticle. If the locus is inside the nanoparticle with monomer solubilized into the core of the nanoparticle, then polymerization initiates with the entry of a single radical into the nanoparticle core and continues until the next radical enters; the radicals are terminated by recombination. Thus, the polymer mass addition is by a “single chain” or unimer depending on the mechanism. See Ref. 46 for a thorough review of emulsion polymerization mechanisms.


CHAPTER 7
Enhanced drug solubility by the Spray drying and Nanoparticle Assembly Process (SNAP)

Abstract

Spray-dried dispersion (SDD) processing has proven to be a versatile an effective method to produce powders of a wide variety of active pharmaceutical ingredients (APIs). However, one class of compounds that has proven challenging are APIs with strong crystallinity and moderate hydrophobicity. SDD formulations of these compounds frequently encounter problems with long-term stability especially at elevated temperatures or humidities. This arises because the high amorphous API concentration in the SDD matrix can crystallize over time, which results in changes in the dissolution rate and supersaturation profiles in vitro and in vivo. We present a new approach that couples block copolymer-directed precipitation with SDD. Studies with phenytoin and naproxen with supersaturations from 12-140, and calculations of Ostwald ripening rates, show that the nanoparticle formation and mixing must occur at the spray nozzle. A design incorporating the nano-precipitation mixing nozzle inside the spray nozzle enables the formation of particles of phenytoin that maintain supersaturations three times above the equilibrium solubility for at least 20 hours. Powder X-ray diffraction (XRD) shows increased crystallinity and a reduction in crystal size for the phenytoin particles when prepared by nano-precipitation and SDD. These particles also show increased stability under aging conditions. We term the new process the Spray-dried Nanoparticle Assembly Process (SNAP).
INTRODUCTION

Over 40% of new drug candidates have poor water solubility, and hence, poor bioavailability.[1] Many of these drugs have the potential to be safe and efficacious (despite not following the ‘rule of five’),[2] but cannot be effectively delivered. To increase bioavailability various approaches have been pursued. Fahr, et al. 3: and Friesen, et al. [3, 4], reviewed (i) the formation of salts for ionizable compounds, (ii) solutions in solvents, cosolvents, and lipids, (iii) micelle delivery systems, (iv) top down particle fabrication including milling for nanocrystal production, (v) complexation, (vi) prodrug formulations, and (vii) amorphous solids and solid dispersions.

Spray dried solid dispersion (SDD) processing has been an especially effective approach for hydrophobic active pharmaceutical ingredients (APIs) (i.e. APIs with high logP values). The process involves the atomization and rapid drying of hydrophobic API’s solubilized with semi-hydrophobic matrix phases such as hydroxypropylmethylcellulose (HPMC) or hydroxypropylmethylcellulose acetate succinate (HPMCAS). The combination of chemistry and kinetics enables the formation of amorphous, or nearly amorphous, phase APIs, which display high API supersaturations upon dissolution.

However, hydrophobicity is not the only physical/chemical property that determines the success of API formulation by SDD. The propensity for crystallization is also a significant determinant, not necessarily of the ability to spray dry an API, but rather to successfully make a stable API formulation in storage. This property can be parameterized as the ratio of the melting temperature ($T_m$) to the glass transition temperature ($T_g$) of the API: $T_m / T_g$. The processing space for SDD technology is shown in Fig. 1. There are four regions in the SDD processing
phase map. **Region I:** Highly hydrophobic APIs, with logP values greater than 6, process well by SDD technology because the compounds nucleate rapidly, produce nanoparticles in the matrix phase and scavenge soluble API from the matrix during the rapid drying process. The result is a SDD matrix with small nanoparticle (NP) inclusions and low concentrations of kinetically trapped API. The small NP size both increases specific area, which increases the rate of dissolution, and leads to supersaturation by increasing the solubility of the API due to the high curvature of the NP surfaces by the Kelvin effect.[5] The result is a stable API/matrix formulation; coarsening or Ostwald ripening of the API is minimal because the concentration of molecular API in the matrix is low and the NPs are immobile in the matrix. **Region II:** At low hydrophobicity (logP < 6), but low tendency to crystallize ($T_m/T_g < 1.25$) the API is generally more soluble in the matrix polymer, and more completely trapped in an amorphous form. However, these formulations are stable and have little tendency to crystallize over longer time scales. **Region III:** In the intermediate range of crystallinity ($1.25 > T_m/T_g > 1.4$) it is possible to formulate SDD solids with loadings from 35-50 wt%. However, in **Region IV** it has proven difficult to produce stable formulations by SDD. The low hydrophobicity leads to low supersaturations, and low nucleation rates. So the API stays predominantly in an amorphous or solubilized form during solidification. However, the high concentration of API solubilized in the matrix means that diffusion through the matrix results in Ostwald ripening and growth of API particle sizes over longer time scales. The low energy state of the crystal form (inversely related to its high $T_m$) means that a crystal site is a sink for supersaturated API in the matrix phase. Coarsening is accelerated by temperature and moisture, which affects both the matrix and API mobility. The high $T_m$, corresponding to low energy in the crystal state, with resulting low solubility, is distinct from low solubility associated with hydrophobicity.[6] The changes during
aging of both the amount of API dissolved in the matrix and the size of the API crystal domains changes the release profile of the API in the biological application. This variability over time is a major obstacle for the processing of APIs with properties in Region IV by SDD.

![API space: $T_m/T_g$ (tendency to crystallize) vs. LogP (hydrophobicity).](diagram)

Fig. 1 – API space: $T_m/T_g$ (tendency to crystallize) vs. LogP (hydrophobicity). This work is focused on the difficult-to-formulate area where the logP is <5 and the $T_m/T_g$ ratio is high (1.4-1.7). The relatively low logP makes formulation by nanoprecipitation difficult while the high $T_m/T_g$ ratio means the drug has a high propensity to crystallize and phase separate from a spray drying matrix like HPMC after nano-precipitation. However, several drugs fall into this category (phenytoin and naproxen, fenofibrate is near the right edge of Region IV). Adapted from Friesen, et al. (2008).[4]

The rationale for this study is that by pre-forming nano-crystals of APIs in Region IV the amount of API kinetically trapped in amorphous and soluble forms during the rapid SDD drying will be reduced and powder stability will be improved. While the decrease in the amount of amorphous API may reduce the supersaturation upon dissolution, the small nanocrystals enable rapid dissolution by virtue of their high specific area, and retain supersaturation by the Kelvin effect, i.e. curvature at the surface of the nanocrystals.
Therefore, we employ the kinetically-controlled, rapid precipitation process, Flash NanoPrecipitation (FNP),[7, 8] to form nanoprecipitates of phenytoin and naproxen APIs. These nanoprecipitates are then immediately spray dried to make a SDD. The role of the FNP polymeric stabilizer relative to the role of the SDD matrix polymer is shown. Finally, a novel FNP/SDD nozzle design is shown that enables the control of FNP nanoparticle formation and SDD drop formation to make SDD powders displaying stability upon aging and supersaturation during dissolution. The Spray-dried Nanoparticle Assembly Process (SNAP) is successful for the highly crystalline phenytoin, as demonstrated by XRD and dissolution experiments, however, the nanocrystalline structure is not maintained for the very low T_g naproxen, and SDD stability is not improved. The only previous report of anti-solvent precipitation coupled to spray drying is that of Hu, et al. (2011) who processed fenofibrate with SDD matrix polymers.[9] However, fenofibrate, with a logP of 5.6 and T_m/T_g ratio of 1.4, is only at the boundary of Region IV. In this study we consider naproxen and phenytoin, with logPs of 3.3 and 2.47, and T_m/T_g ratios of 1.5 and 1.7, respectively (Table 1 for physical constants of materials, and Fig. 2 for structures).

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW (Da)</th>
<th>T_m (°C)</th>
<th>T_f (°C)</th>
<th>T_m/T_f</th>
<th>logP</th>
<th>Aq. Solubility</th>
<th>D_m (cm^2/s)</th>
<th>γ (N/m)</th>
<th>V_m (Å^3/molec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenytoin (PTN)</td>
<td>252.3</td>
<td>298</td>
<td>71</td>
<td>1.7</td>
<td>2.5</td>
<td>14.7 µg/mL</td>
<td>2.98×10^3</td>
<td>5.43×10^2</td>
<td>223.89</td>
</tr>
<tr>
<td>Naproxen (NXN)</td>
<td>230.3</td>
<td>153</td>
<td>6</td>
<td>1.5</td>
<td>3.3</td>
<td>123 µg/mL</td>
<td>2.92×10^3</td>
<td>4.56×10^2</td>
<td>213.97</td>
</tr>
<tr>
<td>HPMC E3</td>
<td>--</td>
<td>225</td>
<td>140</td>
<td>1.2</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Trehalose</td>
<td>342.3</td>
<td>203</td>
<td>70[10]</td>
<td>1.4</td>
<td>-4.3</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PLA-b-PEG</td>
<td>4.2k-5k</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

*Properties T_m, T_f, and logP are from internal Bend Research data except where references are given.
**Solubility at 22 °C
logP values and V_m were calculated by Molinspiration[11]
γ and D_m were calculated according to Kumar, et al. (2009)[12] and Liu, et al. (2007)[13]
Fig. 2 – Chemical structures of APIs and excipients used. A: phenytoin, B: naproxen acid, C: HPMC (where R = H or CH₃ or CH₃CH(OH)CH₃), D: PLAₙ-b-PEGₘ, E: trehalose.

MATERIALS & METHODS

Materials

Phenytoin (D0894) was obtained from TCI Chemical and used as received. PLA₄.2k-b-PEG₅k (100 DL mPEG 5000) was obtained from Evonik Industries. HPMC E3 (METHOCEL E3 Premium LV) was obtained from Dow Chemical Company and used as received. Tetrahydrofuran (34865) and acetone (270725) were obtained from Sigma Aldrich. Ultrapure water was obtained from a Barnstead E-Pure Ultrapure Water Purification System (18-18.2 MΩ-cm).

Methods

Measurement of solubility
Stock mixtures of acetone/water and THF/water were created at 0%, 25%, 40%, 50%, 70% and 100 v/v% solvent. 10 mL of each mixture were loaded into two 20 mL vials with sufficient phenytoin or naproxen in each vial to ensure saturation with drug. One set of vials was held at 37°C, the other at ambient temperature (~23°C). After overnight stirring the mixtures were centrifuged at 50000g and the supernatant fluids extracted. The supernatant solutions were diluted in either acetone or THF and loaded into 2 mL HPLC vials and the concentration of free drug measured by HPLC on an Agilent 1100 system. HPLC method: Isocratic 57.5% H₂O/42.5% ACN, Total flow rate 1 mL/min, 10uL injection, ~3 min elution time, 30°C column temperature, Waters symmetry C₈ column, 5 μm beads, 3.9 x 150 mm, detection wavelength λ= 242 nm.

Fig. 3 shows that the solubility of phenytoin follows a power law relationship with respect to organic solvent/water for both THF and acetone at RT and at 37°C. The equations are given in Fig. 3. The solubility of phenytoin in THF was found to be about 4.5 times higher compared to phenytoin in acetone. Interestingly, we found that 50vol% and 70vol% THF-in-water systems saturated with phenytoin displayed phase separation into two liquid phases, which made HPLC quantification of dissolved drug impossible, hence the lack of data at those concentrations in Fig. 3. 50vol% and 70vol% THF-in-water systems without any drug present experienced no such phase separation. THF-water phase separation in the absence of drug is reported[14, 15] at higher temperatures. Since our drug formulations never had a final THF vol% higher than 25% it was not an issue.

Data for naproxen solubility is given in Appendix H.
Fig. 3 – Solubility data for phenytoin in mixtures of (A) acetone-water and (B) THF-water at room temperature (■) and 37°C (●). Excluding the pure water data point, the data fit power law relationships: log ([phenytoin]) = 3.39log([THF]) – 1.51, R^2 = 0.98 and log ([phenytoin]) = 2.79log([Acetone]) – 1.11, R^2 = 0.99 (both curves at room temperature). The temperature dependence of the solubility curves is insignificant for THF while there is discernible difference in solubility at RT vs 37°C for phenytoin in acetone (approximately a 2.6% difference in slopes). Phenytoin shows a 4.5x higher solubility in pure THF than pure acetone.

Nanoparticle Formation by FNP

Flash NanoPrecipitation (FNP),[7] is a scalable, continuous process for producing nanoparticles between 20 – 400 nm (see schematic in Fig. 4). In this process, a solvent stream containing dissolved API and amphiphilic block copolymer such as poly(styrene)-b-poly(ethylene glycol) or poly(lactic acid)-b-poly(ethylene glycol) is rapidly mixed against an excess anti-solvent stream (usually water) in a defined mixing geometry such as the multi inlet vortex mixer (MIVM).[16] The attainment of high supersaturations in millisecond time scales[7, 8] causes rapid nucleation and growth. Nanoparticle size is determined by the nucleation and growth rates and the rate of attachment of the hydrophobic block of the block copolymer onto the growing drug precipitates. Polymer assembly arrests further growth and provides steric stabilization. In this way, uniform size distributions are obtained.
Fig. 4 – Flash NanoPrecipitation schematic. In this process, drug and block copolymer are dissolved in a water-miscible organic solvent and rapidly mixed against an antisolvent such as water. By tuning the time scales for nucleation and growth and block copolymer micellization, the growing drug aggregates can be capped and stabilized by the block copolymers which anchor to the hydrophobic surface. This leads to well protected nanoparticles with narrow size distributions. If the times scales are not well matched, large drug aggregates or empty micelles can form instead. Reproduced with permission from Johnson and Prud’homme (2003).[7, 8]

An attempt was made to directly measure the size of nanoparticles produced by FNP in the MIVM mixer. The relatively high solubility and strong crystallinity of both phenytoin and naproxen causes rapid Ostwald ripening after precipitation. For phenytoin and naproxen the nanoparticles grew and settled out before size measurements could be made.

**Inline Precipitation and Spray Drying (SNAP)**

Two nanoparticle formation and spray drying processes were considered: (1) external NP formation followed by spray drying, and (2) the integrated SNAP process, as shown schematically in Fig. 5. The first approach used a multi inlet vortex mixer (MIVM) with the exit stream directed into the liquid inlet of the spray drier. In this process, two syringe pumps
(1000HL, Isco Teledyne, Lincoln, NE) were connected to a multi inlet vortex mixer (see Liu [16] for design specifications). The output from the multi inlet vortex mixer was connected to a two-fluid atomizer spray dry nozzle at the inlet of a Niro spray dryer (PSD-1, manufactured by Niro with modifications by Bend Research).

The SNAP design integrates the mixing nozzle and the spray nozzle to achieve rapid micromixing, API supersaturation, and NP formation in the nozzle where atomization occurs. As shown in Fig. 5, a 100 um ID PEEK HPLC tube was inserted in the spray dry nozzle head and centered by two star nuts. The spray dry nozzle was placed at the inlet of a mini spray dryer (Niro, with modifications by Bend Research). A full list of the spray drying conditions are presented in Appendix H-Table 1. The Niro spray drier was operated with an inlet temperature of 110°C, outlet temperature of 45°C, drying gas flow pressure of 50 mm Hg, and atomization pressure of 20 psi.

Spray dried powders were subjected to a secondary drying step at 40°C and 40% RH overnight.

**SEM Measurements**

Spray dried powder samples were thinly spread on an adhesive coated SEM stage and tapped to remove excess powder. The samples were sputter coated and imaged with a Hitachi S-3400N SEM. SEM images were obtained at up 30,000x magnification.

**Powder X-Ray Diffraction**
Powder samples were scanned on a Bruker D8 Advance from 5 to 40°, at 0.017 s per step using a Cu Kα radiation source (\(\lambda = 1.54 \text{ nm}\)). Only data from 2θ = 5 to 30° are shown.

To estimate the size of the crystalline domains,[17] the peak full width half maxima (FWHM) were extracted from key peaks in the spectrum and analyzed by the Scherrer equation[18] to determine the characteristic crystal size:

\[
D_{hk\ell} = \frac{k\lambda}{B_{hk\ell} \cos \theta}
\]

Eqn. (1)

Here \(D_{hk\ell}\) is the characteristic crystal size for the Miller indices being analyzed, \(K\) is a numerical factor often referred to as a crystalline shape factor (usually 0.9 in the absence of other information), \(\lambda\) is the x-ray wavelength (1.54 nm), \(B_{hk\ell}\) is the FWHM in radians after subtracting instrumental line broadening, and \(\theta\) is the Bragg angle. The Scherrer equation was developed for the ideal case of an infinitely narrow and perfectly parallel, monochromatic X-ray beam incident on a monodisperse powder of cube-shaped crystallites.[19] Important limitations are that the equation becomes inaccurate for crystal domains above 200 nm and that peak line broadening can result from a number of different factors including that inherent to the instrument and crystal defects.[19, 20] We, therefore, present the crystal size as an approximation. The degree of crystallinity was estimated from the crystalline peak area relative to the total peak area of the sample.

**Dissolution and Release Testing - Non-Sink Microcentrifuge Dissolution**

Spray dried powders were weighed into Sorenson tubes (11870, Sorenson BioScience). 1.8 mL intestinal buffered media (Simulated Intestinal Fluid (SIF), BioRelevant, London, UK) were added to each tube to obtain a dispersion concentration of 1000 \(\mu\text{g/mL}\) API with 0.5% SIF in
PBS at pH 6.5 (37°C). Samples were vortexed for one minute, then centrifuged at 15,800g for 1 minute before taking each sample. Samples were taken at 4, 10, 20, 40, 90 and 1200 minutes with re-vortexing after sampling. The supernatant (50µL) was diluted into 250µL solvent (1:1 acetonitrile:water). 250µL of each aliquot were ultracentrifuged at 300,000g for 8 minutes at selected timepoints. Free drug concentrations were determined by HPLC.

Annealing Studies
Samples were placed in a temperature and humidity controlled environment at 40°C and 50% RH after the initial drying sequence. Powder X ray measurements were taken on the samples at 2, 7 and 22 hours to assess degree of aging and crystal growth. Prior to this, the samples were stored at 4°C, well below the Tg of phenytoin and HPMC, but 2°C below the Tg of naproxen.

RESULTS AND DISCUSSION
The spray dried formulation experimental conditions are presented in Table 2 for the samples discussed below, while the entire set of experimental conditions are shown in Appendix H-Table 1. The supersaturation ratio was varied by changing the flow rate ratio of organic and aqueous streams into the precipitation device but keeping the total flow rate constant to maintain similar mixing dynamics across experiments and keeping the mass ratio of block copolymer:API at 1:1 (whenever block copolymer was included) and the ratio of HPMC:API at 1:1.
Table 2: Key Drug Formulations using SNAP

<table>
<thead>
<tr>
<th>Sample</th>
<th>[API]</th>
<th>[BCP]</th>
<th>[HPMC]</th>
<th>THF:water flow ratio</th>
<th>Supersaturation ratio</th>
<th>Drug Loading %</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDD1</td>
<td>100</td>
<td>20.5</td>
<td>-------</td>
<td>1.7:8.3</td>
<td>38.9</td>
<td>50</td>
</tr>
<tr>
<td>SDD2</td>
<td>100</td>
<td>33.3</td>
<td>-------</td>
<td>2.5:7.5</td>
<td>14.8</td>
<td>50</td>
</tr>
<tr>
<td>SDD3</td>
<td>100</td>
<td>25</td>
<td>-------</td>
<td>2:8</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>SNAP1</td>
<td>100</td>
<td>100</td>
<td>33.3</td>
<td>2.5:7.5</td>
<td>14.8</td>
<td>33.3</td>
</tr>
<tr>
<td>SNAP2</td>
<td>100</td>
<td>100</td>
<td>25</td>
<td>2:8</td>
<td>25</td>
<td>33.3</td>
</tr>
</tbody>
</table>

[X] = Concentration of X in THF or water feed stream

Experiments were conducted first with the MIVM where precipitation was conducted externally and then the resulting stream was directed to the Niro spray drier. For neither API was there an indication from the XRD data that the pre-precipitation was effective; the XRD for sequential MIVM followed by SDD, and conventional SDD were similar. We understand this in terms of the rapid ripening that would be expected for APIs with such high solubilities. [13] It was not possible to measure NP size from the MIVM because Ostwald ripening caused gross changes in NP size before measurements could be conducted. From our previous work[13, 21] we can calculate the expected Ostwald ripening time for a 100 nm naproxen or phenytoin nanoparticle from:

\[ t_d \approx \frac{R \gamma r^3}{\nu D c^\infty} \]  \hspace{1cm} \textbf{Eqn. (2)}

Where \( \gamma \) is the interfacial energy, \( \nu \) the molecular volume, \( D \) the diffusion coefficient, and \( c^\infty \) is the solubility in the solvent phase. The constants are given in Table 1. The Ostwald ripening times for naproxen and phenytoin 100 nm NPs would be 0.56 and 0.7 s, respectively, given their solubility in 20% THF. Since the flow time from the MIVM to the spray head on the Niro spray
drier was on the order of 4 seconds, it is expected that the external MIVM particles would not remain \textit{nano} in size and would not provide the desired templating.

Therefore, the impingement mixing tube was placed inside the spray head as shown in Fig. 5, with the objective of decreasing the time between NP precipitation and SDD drying by atomization. An estimate of the time between exiting the mixing nozzle and atomization is 90 ms. This combined spray drying with nanoparticle formation in the spray drying head we term the SNAP design.
Fig. 5 – Process schematics. (A) Process overview using MIVM mixing geometry where the precipitated crystals are fed to the spray dry nozzle. (B) SNAP coaxial jet mixer design where the precipitation occurs just prior to spray drying. (C) The spray nozzle used in process B was a two-fluid atomizer modified by inserting a 0.25 mm ID PEEK tube into the central fluid delivery space. A THF mixture containing the API and block copolymer was delivered through the inner tube while DI water was delivered through the annular space. Key to atomizer schematic in C: (1) atomizing section where the jet is broken up into droplets, (2) star nuts holding the inner tube in place, (3) PEEK tube delivering organic phase.

Using the SNAP process, phenytoin API was processed under three conditions to produce powders for dissolution and XRD testing. First, as-received phenytoin crystals were physically mixed with HPMC. Second, the atomization was done with the phenytoin in the THF stream and HPMC in the aqueous stream in the mixing/spray nozzle (in Fig. 5), but without the block copolymer. The third condition was the phenytoin plus PEG-\textit{b}-PLA block-copolymer in the
THF stream and HPMC in the aqueous stream. This final condition corresponds to our SNAP process where the block copolymer directs and stabilizes nanoparticle formation, and then HPMC forms a matrix phase around the NPs. Phenytoin crystal growth models[22, 23] indicated that the crystal size would reach a size of 100 nm in as little as 100 ms for a supersaturation ratio of 140 (see Appendix H-Table 1). Therefore, if the SNAP block copolymer directed assembly was not effective, then the results from the SNAP and results the convention SDD with external HPMC should be similar.

The phenytoin XRD data are shown in Fig. 6 for 14.8% and 25% phenytoin loading for Fig. 6A and 6B, respectively. The level of loading makes no significant difference on the XRD results. The spectra are displaced vertically for clarity. In each figure (A and B) the bottom trace is the phenytoin powder, as received, showing sharp diffraction peaks between 8 and 37°. The top trace is sample without the stabilizing block copolymer, it shows broad features associated with the HPMC matrix, and very small peaks associated with a minor amount of phenytoin crystals. The middle spectra are the results from the SNAP process with the block copolymer-directed assembly of nanoparticles occurring inside the spray nozzle. The crystal peaks are much more pronounced, indicating the greater level of crystallinity in the SNAP materials: 30-38% crystallinity for SNAP vs. 1-5% crystalline for non-block copolymer containing samples. The FWHM from peaks at 11.4° and 16.5° indicate crystals with sub-200 nm sizes. There is a distinct difference between samples where nanocrystal formation has been achieved and the more amorphous traditional SDD materials.
Fig. 6 – Powder X-ray diffraction graphs of spray dried dispersions of phenytoin with and without block copolymer (A: in order from bottom to top: ▬ phenytoin, ▬ SDD2, ▬ SNAP1, B: in order from bottom to top: ▬ phenytoin, ▬ SDD3, ▬ SNAP2, refer to Table 2). Samples without block copolymer show evidence of line broadening similar to samples with block copolymer but far lower % crystallinity (1-5% crystallinity vs. 30-38% crystallinity for block copolymer containing samples). Samples with block copolymer, on the other hand, show significant crystallinity and line broadening, indicating the presence of nanocrystalline (and thus high energy) phenytoin domains within the spray dried matrix. The broad background peaks are HPMC scattering. Graphs are offset but to scale.
We also utilized the drug naproxen (NXN, properties in Table 1) to see how a drug comparable to phenytoin in terms of $T_m/T_g$ ratio and logP but with very low $T_g$ (6°C) behaved in our SNAP process. We found that varying supersaturation ratio and the presence or absence of block copolymer did not effect on PXRD line broadening compared to pure crystalline naproxen (Appendix H-Fig. 1). This reflects the rapid aging and crystal growth upon formation of naproxen dispersions since the naproxen is highly mobile in the HPMC matrix above its $T_g$ and rapidly crystallizes to macroscopic crystal size at even at room temperature.

We explored the use of trehalose as a matrix former in place of HPMC with the API phenytoin (R4-7, R4-8 in Appendix H-Table 1). We did not observe significant line broadening compared to pure crystalline phenytoin at the single supersaturation ratio studied with or without the presence of block copolymer (Appendix H-Fig. 2). It appears that in addition to the presence of block copolymer being necessary, drug-HPMC interaction is crucial to obtaining nano-crystalline domains.

**SEM Characterization**

Scanning Electron Microscope images (Figs. 7 and 8) were taken on spray dried powders to assess the morphology and size of the spray dried particles and any phenytoin crystals that may be visible on the surface of the spray dried particles. Control images (Fig. 7) were taken on pure phenytoin crystals and HPMC spray dried without drug or block copolymer. Representative images are shown for spray dried powders with and without block copolymer (Fig. 8). The samples with block copolymer show the presence of nano-scale surface crystals, as well as possible crystalline domains intermixed with HPMC. The non-block-copolymer containing
samples show slightly larger crystalline domains but fewer crystals in accordance with the XRD data. Pure HPMC particles show no such crystalline domains. Thus, the presence of the block copolymer appears to help trap nanocrystals.

Fig. 7 – SEM images of pure bulk phenytoin (A and B) and spray dried HPMC samples (C and D) as visual controls. Bulk phenytoin crystals tend to be rectangular in shape while pure spray dried HPMC has either spherical or wrinkled morphologies. Scale bars indicated in images.
Fig. 8 – Samples SDD2 (A-C), SNAP1 (D-F), SDD3 (G-I), and SNAP2 (J-L). Samples SNAP1 and SNAP2 contain the block copolymer PLA-b-PEG while samples SDD2 and SDD3 do not. Samples SDD2 and SDD3 show the presence of sub-micron crystals but the block copolymer-containing samples SNAP1 and SNAP2 show many more such crystals on the surface of spray dried droplets. Scale bars as indicated.
Annealing Studies

The key phenytoin SNAP samples (see Table 2) were annealed at 40°C and 50% RH for a total of 22 hrs with XRD measurements taken at 2, 7, and 22 hrs. As seen in Fig. 9A and B, there is a sharpening of crystal peaks and increase in crystal area in the samples not containing block copolymer, indicating the growth of crystalline domains. Figs. 9A and B are without block copolymer, precipitated at two levels of supersaturation. Supersaturation level does not appreciably alter the XRD. In contrast, the dispersions formed by SNAP (with the block copolymer, Figs. 9C and D) showed much less change in shape or amount of crystallinity over the 22 hour annealing period. Given the broad baseline peaks from the HPMC polymer, we do not believe that quantitative calculations of crystal size from peak width measurements would be meaningful, so they are not reported.
Fig. 9 – Annealing studies on SNAP generated spray dried dispersions (SDDs) for samples SDD1 (A) and SDD2 (B) (A and B have no block copolymer), and SNAP1 (C) and SNAP2 (D) (C and D have PLA-b-PEG block copolymer). In each graph, the bottom-most spectrum is that of pure phenytoin and the curves in ascending order are: \( t = 0 \) (when the samples were made), \( t = 2 \) hrs, \( t = 7 \) hrs, and \( t = 22 \) hrs at 40°C and 50% RH. Peak sharpening can be seen in graphs A and B indicating the growth of crystalline domains over time while such sharpening is not seen in graphs C and D (block copolymer containing samples).

The relative instability of crystal growth with HPMC alone is shown in the aging results.

Crystals grow over time, which is the problem with these high \( T_m/T_g \), low \( \log P \) compounds with traditional SDD processing. The pre-crystal formation by FNP helps to stabilize the formulation.

The hydrophobic methyl groups on HPMC interact with the hydrophobic API. This is important for the success of the SDD process. Exploratory experiments using trehalose, rather than HPMC resulted in unsatisfactory macro-crystallization (Appendix H-Table 1). But the HPMC on its
own is not sufficiently interactive to suppress crystal growth during aging. This distinction in polymer action is noted in the field of polymer stabilizers for waxes in crude oils where ethylene vinyl acetate (EVA) and maleic anhydride copolymers are used. Some polymers are denoted as nucleation inhibitors and some as growth arresters.[24-35] The nucleation inhibitors prevent the formation of the initial crystal nuclei, but once nuclei are formed they are ineffective at stopping the growth of wax crystals. The growth arresters absorb strongly on wax crystal faces and prevent the further growth of crystals. It appears that HPMC is acting as a nucleation inhibitor – keeping most of the API in amorphous form during the initial precipitation and drying process, but it is ineffective on preventing growth during aging. In contrast, the PLA-b-PEG polymers used in the FNP/SNAP process are acting as growth arresters in the final formulation. However, the addition of the PLA-b-PEG polymer on its own is not adequate for these relatively soluble hydrophobic compounds (low logP). In the MIVM experiments, the Ostwald ripening of the precipitates in the initial solution before drying is too rapid. So the block copolymer does not perform as a growth arrester in dilute solution in competition with rapid Ostwald ripening, but it does function as a growth arrester in the “dry” state when solvent is removed. This observation may be useful in designing new polymer additives for SDD with modified polarities of the anchoring block. This would be in accord with the wax crystal modification field where mixtures of growth arrester and nucleation inhibitor polymers are used together. Further studies are warranted.

Dissolution studies:

The dissolution results for the phenytoin produced by SNAP and control samples are shown in Fig. 10 and Table 3. The phenytoin crystals or the physical mixture of phenytoin and HPMC
reached equivalent solution concentrations of 40 μg/mL. The presence of HPMC slowed the dissolution somewhat, so that equilibrium was reached after 40 minutes, whereas for the phenytoin alone equilibrium was reached after 2 minutes.

**Fig. 10** – Dissolution and release results for spray dried dispersions of phenytoin. (A – 1200 mins) and (B – 90 mins). ▲ bulk, crystalline phenytoin | ▼ physical mixture of phenytoin + HPMC (45% API by weight) | ■ Samples SDD1 and SNAP2 respectively (□ without and ■ with block copolymer respectively) | ○ Samples SDD2 and SNAP1 respectively (○ without and ● with block copolymer respectively). Samples SDD1, SDD2, SNAP1, and SNAP2 show enhanced solubility over pure drug and the physical mixture of phenytoin + HPMC. The samples containing block copolymer (SNAP1 and SNAP2) show a lower burst concentration than the non-block copolymer samples, but better sustained concentration over 1200 mins.
Table 3 – Dissolution Test Data. $C_{\text{max}90}$ is the highest API concentration reached within 90 mins, $\text{AUC}_{90}$ is the area under the curve at 90 mins, $\text{Ultra}_{90}$ is the concentration of buoyant drug at 90 mins, $C_{900}$ is the API concentration at 900 mins.

| Sample                  | API Loading | $C_{\text{max}90}$
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<tr>
<td></td>
<td>(μg/mL)</td>
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<tr>
<td>Crystalline Phenytoin</td>
<td>---</td>
<td>40</td>
</tr>
<tr>
<td>Mixture*</td>
<td>45.0%</td>
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<tr>
<td>No block copolymer</td>
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<td></td>
</tr>
<tr>
<td>SDD1</td>
<td>40.4%</td>
<td>130</td>
</tr>
<tr>
<td>SDD2</td>
<td>45.1%</td>
<td>130</td>
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<tr>
<td>With block copolymer</td>
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</tr>
<tr>
<td>SNAP1</td>
<td>28.3%</td>
<td>120</td>
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<tr>
<td>SNAP2</td>
<td>31.0%</td>
<td>120</td>
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$\text{AUC}_{90}$

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<th>(min*μg/mL)</th>
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<tr>
<td>Crystalline Phenytoin</td>
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</tr>
<tr>
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<td>3200</td>
<td>40</td>
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<td>SDD1</td>
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<td>100</td>
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<td>9400</td>
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<tr>
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<td>10100</td>
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<tr>
<td>SNAP2</td>
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$C_{900}$

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<th>(μg/mL)</th>
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<tbody>
<tr>
<td>Crystalline Phenytoin</td>
<td>40</td>
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<tr>
<td>Mixture*</td>
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<tr>
<td>No block copolymer</td>
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<tr>
<td>SDD1</td>
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<tr>
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<tr>
<td>SNAP1</td>
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<tr>
<td>SNAP2</td>
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</table>

*Mixture is a physical mixture of phenytoin and HPMC.

There are distinct differences between phenytoin processed with and without the block copolymer. The samples processed without the block copolymer, which show less crystallinity in the XRD data, show an initial spike or burst release during dissolution. The SNAP processed phenytoin shows a completely flat release from the first measurement time of 2 minutes.

However, the level of supersaturation is three times higher than that of the macroscopic crystal. The Kelvin equation gives the effect of nanoparticle size on solubility:[36]

$$\ln S_r \equiv \ln \frac{C_s}{C_\infty} = \frac{2\gamma M}{\rho RT r}$$

Eqn. (3)

Where $S_r$ is the local supersaturation at the particle surface, $C_s$ is the solute solubility at the particle interface, $C_\infty$ is the bulk solubility, $\gamma$ is the interfacial tension, $M$ is the molecular weight, $\rho$ is the density, $R$ is the gas constant, $T$ temperature, and $r$ is the particle radius. From the physical properties of phenytoin the crystal size that would account for this level of increased solubility would be 20 nm. This is not inconsistent with the XRD data. It is consistent with the observed 10-20 nm nanocrystallite clusters observed by TEM for $\beta$-carotene nanoparticles produced by FNP.[37] The crystal size, apparently, stays essentially constant in the dissolution
assay because the concentration stays constant throughout the 1,200 minute assay. In contrast, the HPMC processed phenytoin (without block copolymer) shows a decrease in concentration in solution over time, which is observed for systems which supersaturate and then re-crystallize as macroscopic crystals during a dissolution experiment.[38] So the SNAP processing with block copolymer aids in both the physical stability during aging and in the retention of supersaturation during dissolution.

Naproxen SDD or SNAP processing, again, showed no effect on the XRD results.

CONCLUSIONS

In this work we have demonstrated the combination of the continuous and scalable processes Flash NanoPrecipitation and spray drying to produce spray dried dispersions containing drug nanocrystals of phenytoin with enhanced physical stability and enhanced bioavailability. Spray dried powders were produced at gram scale thus representing an important step towards the commercial application of this technology and showing that nanoparticles can be processed at pilot scales. To accomplish this, a modified spray drying nozzle was fabricated to allow nanoparticle precipitation in the nozzle tip so that the times between precipitation and atomization were on the order of 100 ms (SNAP). The drug phenytoin was chosen to represent the class of drugs with low logP (2-4) but exhibiting a high tendency to crystallize with $T_m/T_g$ ratios $> 1.5$, a difficult class of drugs to formulate. It was further demonstrated that the presence
of the block copolymer PLA-\textit{b}-PEG was necessary to trap nanocrystals structure to confer enhanced stability upon aging and storage.

Samples precipitated with HPMC alone created more amorphous particles; however, these showed crystallization during aging and not substantially higher supersaturations during dissolution experiments.

Naproxen was also processed by SNAP, but its low $T_g$ (6°C) and concomitant mobility in the HPMC matrix resulted in rapid re-crystallization in the solid state. No improvement in dissolution performance was achieved by SNAP processing.

While this study is the first to demonstrate successful SDD processing for this difficult Regime IV (Fig. 1), there are several questions yet to be addressed. What are the kinetics of diffusion of drugs through the HPMC matrix as a function of humidity, temperature, and the $T_g$ of the drug? Can the kinetics of aging be predicted from these fundamental measurements? Certainly, the diffusion of molecules through polymer glasses and plasticized polymers is now relatively well understood and can be predicted.[39-41] These exploratory experiments were done at constant phenytoin loading, to hold one variable constant. Therefore, the maximum loading of phenytoin tested was 33 wt%; i.e. 1 HPMC : 1 PLA-\textit{b}-PEG : 1 phenytoin. By adjusting ratios what is the maximum loading that can be achieved, while retaining the advantage of a stable formulation? Finally, the analogy between what is known about \textit{nucleation inhibitors} and \textit{growth arresters} in wax crystal control, would suggest that multiple polymers which perform different functions might be useful in SDD processes. And it suggests that studies of the anchoring energies of
polymer functional groups and API crystals might be a fruitful avenue to find new polymers to enhance the performance of SDD processing for difficult-to-process APIs.
REFERENCES


CHAPTER 8 – Ultrafiltration
PART A – Ultrafiltration of Protein Solutions


Abstract

Biology is playing an increasingly important role in the chemical engineering curriculum. We describe a set of experiments we have implemented in our Undergraduate Laboratory course giving students practical insights into membrane separation processes for protein processing. The goal of the lab is to optimize the purification and concentration of a protein by hollow-fiber, tangential-flow ultrafiltration. Experiments guide the students to an appreciation for concentration polarization, i.e. the buildup of a concentrated layer of solute on the membrane surface resulting from opposing processes of convection to the membrane surface and removal from the surface by diffusion and tangential flow. The experiment draws upon concepts the students will have seen in fluid mechanics, mass transfer, and separations courses. Optimization, the process of compromising between opposing phenomena, is demonstrated by having the students design a purification and concentration process that minimizes processing time.
Introduction

Biology is playing an increasingly important role in the chemical engineering curriculum. During the summer of 2010, the Department of Chemical Engineering at Princeton University changed its name to the Department of Chemical and Biological Engineering due to the increasing presence of biological research within the department. Courses such as Quantitative Principles of Cell and Molecular Biology, Separations in Chemical Engineering and Biotechnology, and Metabolic Engineering have become permanent fixtures in the curriculum. Introducing biotechnology concepts into both the classes and the laboratory experience is crucial to prepare our students for careers in the pharmaceutical and biotech industries.

The Chemical Engineering Laboratory course is required for undergraduates in the Department. The course reinforces concepts the students have learned in the classroom with hands-on laboratory experience. In addition, it has a major focus on communication and technical writing. We have recently implemented a new experiment in the course that requires students to design, model, and carry out separation experiments involving ultrafiltration of protein solutions. Here, we show how the ultrafiltration portion of the experiment is carried out and its learning objectives.

Ultrafiltration has traditionally played a role in biotechnology as a means to concentrate and purify proteins. Separation processes such as centrifugation and dialysis are batch processes that are difficult to scale up. With ultrafiltration, large volumes of solutions or suspensions can be concentrated and purified in a few hours with minimal product degradation. There are two modes of operation for membrane separations: concentration and diafiltration, as shown in Fig. 1. In
the *concentration mode* the solute of interest is retained by the membrane (unit 1), solvent passes through the membrane into vessel 6 where the mass is measured to determine transmembrane fluxes. The concentration of the retained solute (in reservoir 3) increases as the volume of the retentate decreases. In the *diafiltration mode*, solvent and a low molecular weight impurity pass through the membrane, the solute of interest is retained, and additional pure solvent is added to the solute volume (from reservoir 4) to keep the solute concentration constant. Diafiltration is a washing or solvent exchange process.

Fig. 1 – *Schematic and flowpath of the KrosFlo TFF Research III ultrafiltration system*. (1): Hollow fiber membrane module, (2): peristaltic pump which defines the feed flow rate $Q_F$ or $Q_{Feed}$, (3): process reservoir containing the solution/suspension to be concentrated or diafiltered, (4): buffer reservoir where pure water or buffer is stored to be delivered to (3), (5): backpressure controller to set the transmembrane pressure, and (6): beaker and weigh scale to measure the mass of permeate collected from which the permeate $Q_{Permeate}$ (or $Q_p$) and retentate $Q_{Retentate}$ ($Q_R$) are determined. The dashed line between (3) and (4) indicates the tube connected to the solvent reservoir (4) for constant volume diafiltration.
Product losses can occur if irreversible gel layers are formed at the membrane surface (membrane fouling) as proteins irreversibly aggregate and denature. However, careful optimization of process parameters can mitigate this risk and product recoveries greater than 90% are achieved in practice.[1, 2] Reversible protein concentration near the membrane surface, called concentration polarization, also affects membrane performance. This is the major concept for the students to understand from this laboratory experiment: how convective flux through the membrane creates a concentrated protein layer near the membrane surface, how this layer limits transmembrane flux, and how tangential flow minimizes concentration polarization. The phenomenon is shown schematically in Fig. 2, where at lower solute concentrations and higher tangential flow rates there is no polarization. At higher concentrations, higher transmembrane fluxes, and lower tangential flow rates, polarization occurs. The concentrated, polarized layer causes a decrease in transmembrane flux due to increased osmotic pressure at the membrane surface, and/or the reduced hydraulic permeability of the gel layer on the membrane surface. For further information on membrane technologies, we recommend three excellent references: Ultrafiltration and Microfiltration Handbook,[3] Basic Principles of Membrane Technology,[4] and Membrane Technology and Applications.[5]
Figure 2 – In dead-end filtration, concentration polarization occurs, and the permeate flux drops at low transmembrane fluxes since the feed flow and permeate flow are the same direction. This problem is mitigated by tangential flow where the feed flow and permeate flow are perpendicular to each other. The tangential shear at the membrane surface sweeps away the polarized layer and minimizes protein concentration at the membrane surface. Much higher transmembrane fluxes can be obtained at equivalent transmembrane pressures. Adapted from Spectrum Labs. [6]

Experiments carried out by the students have the goal of optimizing a process for the concentration and purification of a bovine serum albumin (BSA) solution. The learning objectives for the experiment are:

1) To determine the membrane resistance for solvent flux, and its dependence (or lack thereof) on tangential flow.

2) To determine concentration polarization for protein ultrafiltration by measuring the transmembrane flux as a function of protein concentration, transmembrane pressure and tangential flow rate. To have a physical picture of how these variables interact.

3) To understand the differences between concentration and diafiltration operations.
4) To design an optimized processing scheme to purify and concentrate a protein solution based on an understanding of the phenomena of concentration polarization and the results from the previous experiments.

Separations processes have long been an integral part of the chemical engineering discipline, especially in the areas of membrane separations and chromatography. However, because such technologies are difficult to implement in undergraduate lab courses, there have been relatively few examples of their use.[7] The growing importance of biotechnology and bioseparations has prompted several pedagogical articles on integrating such processes into the undergraduate lab experience.[8-11] Excellent articles on how to instruct students in membrane separations have focused on reverse osmosis[12, 13], ultrafiltration[7, 14, 15], and multi-stage ultrafiltration and modeling.[16] Similarly, articles focusing on affinity[10, 17-19] and ion exchange[20] chromatography are available as well. The ultrafiltration articles tend to focus on hardware design and setup, testing different filter geometries, flow rates as a function of solute concentration and pressure, and modeling to determine mass transfer coefficients. In this experiment we emphasize the fundamental challenge of process engineering, which is optimization of a process. The student must balance the conflicting phenomena of concentration polarization, which is significant at high concentrations and high transmembrane fluxes, with the need to minimize the volume of fluid processed in order to minimize process cycle time. This experiment does an excellent job of highlighting the tradeoffs inherent in optimization. Basic fluid mechanics and mass transfer models are applied to determine operating parameters and key process parameters are also optimized to emphasize the importance of minimal processing time and materials usage.
Materials and Methods

MATERIALS AND EQUIPMENT
We used a KrosFlo Research IIi system (SYR2-U20-01N) with 10 kD hollow fiber membranes (D02-E010-05-N) from Spectrum Labs Inc, Rancho Dominguez, CA. The unit comes with integrated pressure transducers, an electronic balance to measure permeate flow rates, and computer software to capture, analyze, graph, and print data. A schematic of the apparatus is shown in Fig. 1.

Bovine serum albumin (BSA, A2153), sodium phosphate (71640), phosphoric acid (438081), NaOH (72082), and sodium salicylate (S3007) were purchased from Sigma Aldrich and used as received. Denatured ethanol (A407P-4) was purchased from Fisher Scientific. Water was obtained from a Barnstead NanoPure system (Barnstead Corp.) which includes 0.22 μm filtration. UV-vis absorbance measurements were taken on the Evolution 300 spectrophotometer purchased from Thermo Fisher Scientific.

We chose the 10 kD molecular weight cutoff (MWCO) hollow fiber membranes to retain all of the 66.5 kD BSA protein while allowing smaller molecular ‘contaminants’ on the order of 100-500 D to pass through the membrane easily. Other acceptable membranes cutoffs available from Spectrum Labs include 3, 5, and 30 kD MWCO. The fibers are composed of hydrophilic polyethersulfone 0.5 mm in diameter. Hydrophobic polysulfone membranes require higher pressures to filter aqueous solutions and are more prone to protein fouling.
METHODS

Ethanol Wetting and Water Permeability Test

A new hollow fiber membrane unit purchased from Spectrum Labs was first wetted with 40% EtOH solution by circulating 50 mL throughout the membrane for 20-25 minutes at a feed rate of 150 mL/min. Then the system was flushed with water (2 mL water per cm² of membrane area). Since membranes are re-used, this procedure is required only for the first use of the membrane.

To establish the intrinsic membrane permeability, a water permeability test was performed. The tangential flow rate through the hollow fiber module (i.e. the “feed rate”) was set to 100, 200, and 300 mL/min and the transmembrane pressure (TMP) was varied from 10-25 psi using the supplied automatic backpressure controller. Data were recorded directly from the KrosFlo instrument using the supplied software.

Starting BSA Feed Composition

The starting BSA solution consisted of 1 wt% BSA (10 mg/mL) in 5 mM phosphate buffer at pH 7.0. A process volume of 200 mL of BSA solution was used for TMP Optimization and Diafiltration Optimization Experiments. For the diafiltration portion of the experiment, the BSA solution consisted of 1 wt% BSA with 1 mM sodium salicylate in 5 mM phosphate buffer at pH=7.0.

BSA TMP Optimization
TMP optimization was carried out by running the unit in diafiltration mode (with a fresh buffer reservoir connected to the process reservoir). The feed flow rates were set to 300, then 200, then 100 mL/min with the TMP varied from 10 – 25 psi at each flow rate. Following this, the fresh buffer line was disconnected to concentrate the BSA solution from 9.6 to 65.9 mg/mL (6.9 fold concentration). Once the approximate concentration was achieved, the buffer line was reconnected and the flowrates and TMPs repeated once more at this higher concentration. Final BSA concentrations were verified by UV absorbance measurements at 280 nm.

**BSA Concentration Optimization**

The feed flow rate was set at 300, 200, and 100 mL/min and the membrane cleaned between each run (see section on Membrane Cleaning Procedures) to within ~15% of the starting water permeability values. The starting BSA solution was concentrated from 10 mg/mL to approximately 150 mg/mL for each run as measured by UV absorbance measurements at 280 nm.

**BSA Diafiltration**

The BSA solution was first concentrated to an intermediate optimal point as determined by the optimization parameter described in the Results and Discussion section at the previously determined optimal TMP (20 psi). Once the optimal concentration was reached, the buffer line was connected and diafiltration commenced. Approximately 10-15 diavolumes were processed before the buffer line (tube connecting reservoirs (3) and (4) in Fig. 1) was disconnected and concentration resumed until the final desired concentration was achieved. Throughout this process, small (1-2 mL) samples were taken from the permeate line to track the concentration of
sodium salicylate ‘impurity’. Sample concentrations were measured by UV absorbance at 295 nm. Absorbance values were converted to concentration by using Beer’s Law.

**Membrane Cleaning Procedures**

The membrane should be cleaned between experiments to achieve maximal flow rates and reproducibility. Cleaning procedures vary depending on the nature of the suspension filtered prior. First, the membrane should be flushed with pure water (following the 2 mL per cm² membrane area rule), then blown down with air. For protein solutions, we have found dilute bleach (standard Clorox bleach mixed 1:1 with water) provides a sufficiently oxidative environment to degrade and remove proteins from the membrane wall. The dilute bleach (50 mL) should be recirculated at a medium shear rate (5000 – 8000 s⁻¹) for about 20 minutes. The effectiveness of the cleaning is assessed by flushing out the system with water (2 mL/cm²) then running a water permeability test at one of the points previously established (reference crossflow rate and TMP). Achieving permeate water flow rates within 15% of the original values is considered acceptable. Though the membranes are technically disposable, single-use devices, we have routinely used membranes for 4-6 sessions with this cleaning procedure. The replacement frequency is driven by the fact that we force the system into a “failure mode”, i.e. high amounts of fouling to show the limits of operation. If one wants even longer membrane lifetime, then not forcing the system to strong fouling will prolong the membrane life. However, the students learn by finding the limits of operation.
Results and Discussion

Membrane Wetting Protocol

Initially the pores of the membrane are filled with air. The students should be taught about the Laplace pressure across a curved interface, and the contact angle at an air/liquid solid surface. If the membrane is used without the prewetting, at the operating pressures of the hollow filter module approximately 20% of the membrane pores will remain air filled. The pressures are not great enough to displace air from the pores since the water does not adequately wet the membrane surfaces. The apparent water permeability will be reduced. Ethanol or isopropanol does wet the polymer membrane and all air will be displaced. If the students have adequate time they can perform an initial permeability measurement on a new membrane with buffer solution, then wet the membrane and re-measure the water permeability. The students only need to perform alcohol membrane wetting on a brand new membrane. Since membranes are re-used during the course of the lab, not all groups need to perform this.

Water Permeability Test

The water permeability test measures permeate flow rate as a function of TMP (Eqn. 1) and feed flow rate. This allows for the intrinsic resistance of the membrane to be calculated according to Eqn. 2. Since there is no gel layer formation with pure water, the term $R_G$ is equal to 0 and the permeate flow rate should be directly proportional to the TMP as shown in Fig. 3. Since the TMP is independently controlled via the automatic backpressure controller, the feed flow rate does not affect the permeate flux. The students will see this in data they will generate, which will be similar to that shown in Fig. 3. However, the feed flow rate (or crossflow rate) does
affect the filtration of macromolecular solutions since higher crossflow rates result in higher
shear rates (up to approximately 12,000 s\(^{-1}\) at 300 mL/min). The transmembrane pressure driving
force is given by

\[
P_{\text{TMP}} = \frac{P_{\text{feed}} + P_{\text{retentate}}}{2} - P_{\text{permeate}} \quad \text{Eqn. (1)}
\]

and the permeate flux through the membrane, \(J\), defines the intrinsic membrane resistance, \(R_m\),
and the gel layer resistance, \(R_G\) according to D’arcy’s Law:[21]

\[
J_p = \frac{Q_p}{A} = \frac{\text{TMP}}{\mu(R_m + R_G)} \quad \text{Eqn. (2)}
\]

where \(J\) is permeate flux (m/s), \(P_{\text{TMP}}\) is transmembrane pressure (defined in Eqn. (1)), \(\mu\) is fluid
viscosity (Pa\(\cdot\)s), \(R_m\) is the intrinsic membrane resistance (m\(^{-1}\)), and \(R_G\) is gel layer resistance (m\(^{-1}\)).[22] The slope of the linear fit in Fig. 3 gives the value for \(R_m = 2.50 \times 10^8\) m\(^{-1}\). The students
should be asked to justify Eqn. 1 as the single “transmembrane pressure” when there are three
pressures involved in the calculation. For more inquisitive students, they can be directed to the
derivation of the equations for flow in a porous tube,[23] and can then be asked to justify the
linear approximation given in Eqn. 1.
**BSA Trans Membrane Pressure (TMP) Optimization**

During the course of filtration, a decline in permeate flux is observed as the concentration of BSA increases. In general, this flux decline can be attributed to a combination of concentration polarization and fouling. Concentration polarization results in a high osmotic pressure at the membrane surface. This osmotic pressure “subtracts” from the measured hydrostatic pressure difference across the membrane, thereby reducing the solvent flux. Membrane fouling (i.e. membrane surface adsorption, membrane pore plugging, and gel layer formation on the membrane surface) also reduces flux.[24] In general, permeate flux increases linearly with TMP up to some value of the TMP where effects from concentration polarization become significant.[25] At a BSA concentration of 9.59 mg/mL and at feed flow rates above 200 mL/min, the relationship between permeate flow rate and TMP is close to linear, which suggests that there is negligible concentration polarization/fouling (see Fig. 4A). The data show that a
critical TMP (where ‘critical’ is defined as the point where deviation from the linear regime) occurs at a TMP of 17 psi at a feed flow rate of 200 mL/min for the 65.9 mg/mL BSA solution. At a higher feed flow rate of 300 mL/min the critical TMP increases to 20 psi. Higher tangential flow decreases polarization. Note that the permeate flow rates at 9.59 mg/mL are at least twice as high as the permeate flow rates at 65.9 mg/mL, which demonstrates that concentration polarization and membrane fouling are strongly dependent on BSA concentration. The approximate critical TMPs for the six possible combinations of BSA concentration and feed flow rates are plotted in Fig. 4B. Figs. 4C and 4D show the gel resistances, $R_G$, for BSA at 9.6 and 65.9 mg/mL at various feed flow rates. $R_G$ is relatively constant where the permeate flux is linear in the TMP, but rises sharply when the flux plateaus.
Figure 4 – (A) TMP Optimization for BSA concentrations of 9.59 mg/mL (solid symbols) and 65.9 mg/mL (open symbols) at various feed flow rates ($Q_{feed} = \bullet 100 \text{ mL/min} \mid \bullet 200 \text{ mL/min} \mid \bullet 300 \text{ mL/min}$). (B) Summary of TMP Optimization results for various feed flow rates and BSA concentrations ([BSA] = ■ 9.6 mg/mL \mid \bullet 65.9 mg/mL). Gel layer resistance $R_G$ as a function of BSA concentration and feed flow rate for (C) 9.6 mg/mL BSA and (D) 65.9 mg/mL BSA.

The gel layer formed during ultrafiltration may be reversible or irreversible to varying degrees depending on the transmembrane pressure, type of solute, and solute concentration. **Appl-Fig. 7A and B** show the results of a TMP excursion test ramping the TMP up then down to test the reversibility of the gel layer. At 300 mL/min circulation, the absolute flow rates are higher than at 100 mL/min. For the 300 mL/min case, as the TMP is ramped up, the slope of $Q_p$ vs. TMP is almost linear and appears to be plateauing showing that $R_G$ is increasing. As the TMP is ramped
down from the maximum 27.5 psig, the slope is steeper and flow rate values lower showing the presence of a permanently ‘stuck’ gel layer. For the 100 mL/min case, there is a clear plateau on both the ramp up and ramp down phases. During the ramp down, there is a small drop off $Q_p$ but the trend is largely reversible. So the higher circulation rate, by virtue of a higher shear rate, does provide higher $Q_p$. But the shear may also denature the BSA at the gel layer interface causing increased irreversibility compared to lower shear rates.

**BSA Diafiltration Optimization**

Ultrafiltration is used to both concentrate and purify macromolecular solutions or suspensions, thus involving two modes of operation: concentration and diafiltration. In concentration the solute of interest is retained by the membrane and solvent passes through the membrane. In diafiltration, a low molecular weight impurity passes through the membrane, the solute of interest is retained by the membrane, and fresh solution phase is continuously added to the circulating fluid to keep the volume of the ultrafiltered solution constant. The process of continuous diafiltration can be modeled as a CSTR with pure water or buffer as the input. This yields an exponentially decreasing impurity concentration as a function of the number of diavolumes (see Eqn. 6). The process provides an interesting optimization problem to the students: minimize the total processing time while purifying and concentrating a protein solution to some desired level. In one limit, diafiltering before concentration might yield higher flow rates but requires larger fresh buffer volumes to be processed, which requires more time. In the other limit, diafiltering after concentrating reduces the volume of fresh buffer required but the permeate flow rate is slower because at higher concentration of protein, concentration polarization is more significant. The optimal method is a compromise: concentration is
performed first to some intermediate concentration, then diafiltration, then concentration again. The intermediate concentration is determined by the optimization parameter $\xi(t)^*$ (Eqn. 3):[26, 27]

$$\xi(t)^* = C(t)_{BSA} \times J(t)_{perm}$$ \hspace{1cm} \text{Eqn. (3)}$$

Conceptually, this optimization parameter reflects that you want to process at as high a protein concentration as possible and with as high a solvent flux as possible. However going too high in concentration decreases the flux more than linearly in concentration, and decreasing concentration too much requires processing excessive volumes of solvent. The required diafiltration time ($t_{DF}$) and number of diavolumes (i.e. volumes of permeate fluid relative to the fluid volume in the reservoir and flow tubing) ($N(t)$) are given by Equations 4 and 5:

$$t_{DF} = \frac{NV_{process}}{Q_P}$$ \hspace{1cm} \text{Eqn. (4)}$$

$$N(t) = \frac{V_{buffer}(t)}{V_{process}}$$ \hspace{1cm} \text{Eqn. (5)}$$

where $C_{BSA}$ is the bulk BSA concentration at any time $t$, $J(t)_{perm}$ is the permeate flux or flow rate $Q_P$, $t_{DF}$ is the diafiltration time required for a given number of diavolumes (Eqn. 4), $N(t)$ is the number of diavolumes (Eqn. 5), $V_{process}$ is the process volume, and $Q_P$ is the permeate flow rate (constant during diafiltration). The results of the Diafiltration Optimization experiments are shown in Fig. 5. $Q_P$ decays nonlinearly with increasing BSA concentration (Fig. 5A). Higher feed flow rates (and thus higher shear rates) allow for higher permeate fluxes due to reduced
membrane fouling. Plotting the optimization parameter $\xi^*(t)$ against BSA concentration (Fig. 5B) reveals the optimal concentration for beginning diafiltration. At a feed flow rate of 300 mL/min and a TMP of 20 psi, the optimal BSA concentration was determined to be 94 mg/mL by UV absorbance at 280 nm.

Since the software only measures the amount of permeate on the weighing scale, the BSA concentration over time must be calculated from the collected permeate volume by Eqn. 6:

$$[\text{BSA}] = \frac{\text{Mass}_{\text{BSA,initial}}}{V_{\text{initial}} - (V_{\text{permeate}} + V_{\text{holdup}})}$$  \hspace{1cm} \text{Eqn. (6)}$$

Where $V_{\text{permeate}}$ is the volume collected on the scale and $V_{\text{holdup}}$ is the volume held up in the membrane unit, tubing, and permeate exit line.
**BSA Continuous Diafiltration**

The goal of continuous (or constant volume) diafiltration is to remove undesirable permeable solutes (‘impurities’).\cite{28} The concentration and diafiltration process has three phases depicted in Fig. 6: the first concentration phase, the diafiltration phase, and the second concentration phase. During the first concentration step, the BSA solution is concentrated at a TMP of 20 psi and a feed flow rate of 300 mL/min until it reaches the optimal concentration that was determined in the diafiltration optimization experiment. During the diafiltration step, approximately 10-15 diavolumes of fresh buffer are processed to remove the majority of the impurity. Since the buffer enters at the same rate as permeate leaves, the concentration of the feed remains constant. Finally, after sufficient buffer has been processed, the buffer line is disconnected and the solution in the feed reservoir is concentrated to the final desired concentration.
Figure 6 - The three stages of diafiltration: first concentration step (▲), diafiltration step (■), and the second concentration step (●). The diafiltration process is run at a feed flow rate of 300 mL/min and at a TMP of 20 psi.

Modeling the system as a CSTR yields the following:

\[ C(N) = C_0 e^{-TN(t)} \] \hspace{1cm} \text{Eqn. (7)}

\[ T = \frac{C_{\text{impurity, permeate side}}}{C_{\text{impurity, process side}}} \] \hspace{1cm} \text{Eqn. (8)}

Where \( C \) is the concentration of permeable solute (‘impurity’) on the process side, \( C_0 \) is the starting concentration of permeable solute in the process side, \( T \) is the transmission coefficient (Eqn. 8), and \( N(t) \) is the number of diavolumes processed. The transmission coefficient is a measure of how ‘hard’ it is for an impurity to pass from the process side to the permeate side and can be calculated from the slope of the curve in Fig. 7A. Manipulating Eqn. 7 gives the formula for impurity removal:[29]

\[ \text{Removal} \% = 1 - e^{TN} \] \hspace{1cm} \text{Eqn. (9)}
Thus, at 100% transmission, only 4.5 diavolumes are needed achieve 99% impurity removal. At 50% transmission, however, approximately 9.1 diavolumes are needed for the same purity. T values in Fig. 7A are for small molecules not interacting with the solute in the reservoir or the gel layer and thus T ~ 1. Manipulating Eqn. 7 further, the concentration of the impurity leaving the permeate line is given by:

\[
\ln \left( \frac{C(N)_{\text{impurity, permeate}}}{C_{\text{impurity, feed, initial}}} \right) = -TN(t) + \ln T
\]

\textbf{Eqn. (10)}

where \(C_{\text{impurity, permeate}}\) is the impurity concentration in the permeate, \(C_{\text{impurity, feed, initial}}\) is the initial impurity concentration on the process side, T is the transmission coefficient, and N is the number of diavolumes.

**Fig. 7A** shows the control experiment where salicylate is diafiltered out of solution with no BSA present and closely follows the standard exponential decay from Eqn. 7. The transmission coefficient is determined to be T = 1.05 which is within experimental uncertainty to the expected value of 1 for a permeable solute being diafiltered in the absence of interactions. The data in **Fig. 7B** show the concentration of salicylate during diafiltration from a BSA/salicylate mixture. The data do not fit an exponential curve and therefore a single transmission coefficient cannot be derived. The reason for this deviation is that there is a binding interaction between BSA and sodium salicylate that slows the rate of removal of sodium salicylate.[30] As a result, many additional diavolumes are needed to remove the desired fraction of the impurity.[29, 31] It is possible to model the separation when the impurity is binding to the retained solute, but that is
beyond the scope of an undergraduate laboratory experiment. Note that fluorescein can also be used as the impurity. It gives a visual impression of the amount of purification, but it will interact even more strongly with BSA than does the salicylate.

![Graph A](image1)

![Graph B](image2)

**Figure 7** – (A) Concentration of sodium salicylate leaving the permeate line when no BSA is present (control experiment). (B) Concentration of sodium salicylate impurity as it is removed from the BSA solution via diafiltration. The non-linearity shows the binding interaction that occurs between sodium salicylate and BSA.

**Conclusions**

In this study we performed process development for lab scale ultrafiltration of bovine serum albumin solutions, optimizing the various process parameters relevant to the process and characterizing the performance and capabilities of the ultrafiltration equipment. This work was primarily motivated by the development of a new experiment for the Undergraduate Lab course in Chemical and Biological Engineering at Princeton University. In accordance with fluid dynamics and mass transfer models developed, we found that the water permeability of the hollow fiber membrane was linear in the transmembrane pressure. Ultrafiltration of protein solutions at various concentrations, shear rates, and transmembrane pressures showed the
existence of a ‘knee’ where irreversible membrane fouling occurs. The permeate flow rate was found to drop exponentially as a function of protein concentration. Due to the nonlinearity of the curve, a maximum exists in the ultrafiltration optimization parameter for minimizing processing time and volume. This maximum was found to exist at 94 mg/mL BSA which is the intermediate concentration in the process. Experiments were performed to compare the concentration evolution of the small molecule model impurity salicylate in a diafiltration process where significant protein binding was observed that caused deviations from the ideal model. In Part B we examine the ultrafiltration of nanoparticle systems and compare the results against that for proteins.

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REFERENCES


CHAPTER 8 - Ultrafiltration

PART B - Ultrafiltration of Nonionic Core-Shell Nanoparticles and Charge Stabilized Acrylic Latices

Abstract

Ultrafiltration studies were performed on nanoparticle systems formed by Flash NanoPrecipitation and emulsion polymerization for the purposes of concentration-diafiltration process optimization and characterization of membrane fouling. The nanoparticle systems were compared against similar studies done on bovine serum albumin to elucidate the mechanisms of stabilization and membrane fouling based on hydrodynamic models. As the only scalable method for the precise concentration and purification of nanoparticle systems, these studies provide guidance on achieving the desired specifications based on nanoparticle size, geometry and surface chemistry as compared to more traditional protein ultrafiltration routinely employed in the biotechnology industry.
**Introduction and Background**

Concentration and purification of macromolecular species such as proteins, peptides, nanoparticles, liposomes, and microparticles are commonly performed via membrane dialysis, freeze-drying (lyophilization), evaporation under reduced pressure, centrifugation, ultracentrifugation, or ultrafiltration. Indeed, it is often the post-production processing stage where the most time and cost are incurred to bring a raw product up to final specifications. Ultrafiltration often presents the best overall option, in particular, tangential flow, hollow fiber filtration,[1] because it greatly reduces membrane fouling and particle losses compared to traditional dead end filtration systems. Several types of tangential flow configurations are available, the most common being spiral-wound, cassette, and hollow fiber. We chose the hollow fiber system from Spectrum Labs (see Fig. 1 in Part A) as it provides more uniform particle contact with the membrane surface compared to the other configurations, low fouling characteristics (using high shear rates and hydrophilic membranes), disposability, and direct scalability with the membrane surface area. Such membranes can also be cleaned with the unique back-flushing method. The main disadvantages with this type of system are product degradation due to multiple passes through the fiber lumen and generally low pressure limits (30 psig). Tangential flow ultrafiltration scale up has been well validated in the literature.[2-4] Processing times for concentration and diafiltration are generally on the order of hours for liter scale nanoparticle suspensions with concentrations from 1- 10 wt%. By contrast, other methods such as dialysis and lyophilization are awkward to implement at such scales and larger and are far slower, on the order of days to weeks.
In Part B, we focus on the processing of engineered nanoparticles which are an integral component of the exploding field of nanotechnology. Engineered nanoparticles have been made from a wide variety of organic and inorganic materials such as drugs, dyes, polymers, metals, metal oxides, and organometallic compounds. Often the synthesis methods used to prepare such nanoparticles in suspension leave behind surfactants, solvents, and other impurities that much be removed to improve the stability and applicability of the nanoparticles. Additionally, the particles may need to be concentrated up for the final application. Of the several processing methods available for concentration and purification, ultrafiltration is the only one that can achieve both goals in one scalable system.

Compared to the previous studies exploring ultrafiltration as an effective method for concentrating and purifying proteins, the work in this chapter explores the use of this method for processing vitamin E core, PCL-\(b\)-PEG-protected core-shell nanoparticles and charge stabilized poly (methyl methacrylate) (PMMA) latices, the former produced by Flash NanoPrecipitation[5] and the latter produced by the method of emulsion polymerization.

Some key differences exist between the studies in Part B and Part A. For one, the method of stabilization of the proteins vs. nanoparticles. Proteins are largely soluble in buffer, having many ionic and hydrogen bonding interactions with water/buffer molecules. They also have secondary interactions within each protein molecule, and tertiary structure that can be ablated by the process of denaturation. The protein of choice was the well-known bovine serum albumin (BSA) with a molecular weight of 66.5 kDa. It is known that proteins, while being relatively insensitive to shear, can easily be denatured upon adsorption to solid interfaces, such as
membranes.[6, 7] This is driven by the large increase in translational entropy for hydration shell molecules outweighing the entropy penalty from the protein adsorbing to the surface, even when this process is endothermic. The hydrophobic domains within the protein associate with hydrophobic regions of the membrane. This effect is pH dependent, being stronger at pH 7 than at the isoelectric point pH 4.7. Thus, during ultrafiltration, there is the possibility that the BSA adsorbs to the membrane, forming a film layer that is not easily detached, due to the large number of interaction points between the protein and membrane. This membrane fouling can severely reduce permeate flux rates through the membrane.

In the case of nanoparticles, such as those formed by Flash Nanoprecipitation comprising a nonionic core-shell structure or those formed by emulsion polymerization (PMMA latices), if we assume that the structure of the nanoparticles does not change upon membrane adsorption, we would expect very different membrane fouling effects (Fig. 1). PMMA latices comprise approximately 1.12 μC/cm² surface charge which translates to 22500 anionic charges per 320 nm particle with zeta potentials in the range of -40 to -60 mV.[8] Comparatively, nonionic block copolymer protected particles have much lower zeta potentials < 10 mV in magnitude.[9] Of course, the difference in size is also significant. BSA has a Stokes radius of 3.5 nm[10] while the vitamin E nanoparticles formed by FNP were about 90 nm in size and the PMMA latices used here were 320 nm in diameter. Thus, the size difference is also significant in addition to the mechanical and chemical properties.
Fig. 1 – Illustration of flow through cake layers comprised of proteins or nanoparticles of different sizes. By basic geometric arguments, the permeability of the 300 nm nanoparticle cake layer is far greater than that of 6 nm proteins. This of course neglects attractive and repulsive forces due to surface chemistry considerations which are explored in the Discussion section.

In this chapter, we compare the fouling characteristics of PMMA latices compared to core-shell nanoparticles and BSA, the latter two described in previous chapters. We explain these differences on the basis of a physical model first described by Jiao and Sharma[11] that explains the fouling as a balance of forces on individual particles.
Materials & Methods

Materials: Nile red (1188527, International Laboratories USA, unknown purity) (NR) was obtained from International Laboratories USA and used as received. Poly(caprolactone) (PCL\textsubscript{6300}) homopolymer and poly(caprolactone)-b-poly(ethylene glycol) (PCL\textsubscript{7000}-b-PEG\textsubscript{5000}) were synthesized in-house by ring opening polymerization of ε-caprolactone according to Shibasaki, et al.[12] Vitamin E (T3251, >96%) (VE) was obtained from Sigma Aldrich, USA and used as received. Tetrahydrofuran (34865, HPLC grade) (THF) was obtained from Fisher Scientific, USA and used as received. 20-40% EtOH was prepared by mixing denatured EtOH (AK07/0695/E99.86%, Aik Moh Paints and Chemicals Pte. Ltd, Singapore, 99.86% EtOH with 5% MeOH) with ultrapure water. Ultrapure water (hereafter simply referred to as ‘water’) was obtained from a Water Pro PS system (Model 9000705 Water Pro PS, Labconco, USA, R = 18.2 MΩ-cm). Acrylic latices were obtained from Arkema, Inc. (ENCOR) and used without further modification.

METHODS

VE Core Nanoparticle Formulation

New nanoparticles had to be formulated to provide a system with model compounds that could also be characterized by UV-vis absorbance easily. Thus, Vitamin E (VE) was chosen as the core material and Nile Red (NR) as the visual tag with strong absorbance signal at 535 nm. NR, VE, and PCL-PEG were dissolved in THF at 0.1, 8.8, and 8.9 mg/mL respectively. These concentrations were chosen to give ~100 nm particles. These solutions were rapidly mixed
against 10 volume equivalents of water in a multi inlet vortex mixer (MIVM) to form nanoparticles per the procedure in Liu and Prud’homme.[13] Resulting nanoparticle suspensions were dialyzed in 6-8k MWCO dialysis bags (132665, Spectrum Labs, USA) in 2 L of water, refreshed a minimum of 5 times over 24 hours for all experiments except in the diafiltration experiments.

The stability of these systems were studied by measuring the hydrodynamic particle size over time to determine their suitability for ultrafiltration tests. Briefly, 0.1 mL of each nanoparticle suspension were placed in a plastic cuvette (759070D, Brand Scientific, USA) and mixed with 2.5 mL of water. The cuvette was then placed into a Malvern Zetasizer Nano (Zetasizer Nano ZS, Malvern Instruments, UK) and the hydrodynamic radius determined by dynamic light scattering. The optics were arranged at 173° backscatter.

**Pump calibration**

The peristaltic pump provided with the KrosFlo system (SYR2-U20-01N, Spectrum Labs, USA) was used as received. Calibration tests were performed by pumping pure water straight through the pump to the permeate scale and calculating the flow rate using the Spectrum-provided software and mass readings from the permeate scale.

2 L of water were loaded into a 2 L reservoir (ACTO-2PP-01N, Spectrum Labs, USA) and pumped into a 500 mL Erlenmeyer flask that was placed on a weighing scale (ACR2-SC2-01N, Spectrum Labs, USA) accurate to 0.1 g. Mass data, pressures, and pump RPM data were transmitted automatically to a computer with data recording software (KF Comm Version 1.12,
Spectrum Labs, USA). The nominal flow rate was adjusted from 50 mL/min up to 300 mL/min in 50 mL/min increments and the actual flow rate at each point recorded.

**Water permeability tests**

Water permeability tests were performed on 10k MWCO (D02-E010-05-N, Spectrum Labs, USA) and 100k (D02-E100-05-N, Spectrum Labs, USA) MWCO mPES hollow fiber membranes both in a non-wetted and pre-wetted state. This consisted of flowing pure water through the membrane at preset inlet flow rates and trans-membrane pressures and measuring the permeate flow rate.

1.5 L of water were loaded into a 2 L reservoir and recirculated (both retentate and permeate) through the 10k or 100k nonwetted membranes with the flow rate initially at 100 mL/min and ramped up incrementally to 300 mL/min. The TMP was varied between 5-25 psig and the permeate flow rate recorded at each point, giving enough time for pressures and flow rates to stabilize. Then, membranes were wetted by total recirculation of 20% or 30% EtOH solution for approximately 20 minutes. The EtOH was flushed out completely with >2 mL water/cm² membrane area per Spectrum recommendations[14] after wetting. After wetting, the water permeability tests were repeated at the same inlet flow rates and transmembrane pressures.

**Shear resistance tests on VE Core NPs**

Nanoparticles were recirculated through the hollow fiber system at constant concentration for approx. 1 hr to test any changes in size stability and Nile Red loading.
NR+VE core nanoparticles were prepared as described and dialyzed. Approximately 30 mL of nanoparticles were loaded into a 50 mL centrifuge tube with a 3-port dip tube cap with luer-lock fittings (ACBT-050-C1N, Spectrum Labs, USA). The particles were recirculated (retentate + permeate) for approximately 1 hour at 15 psig TMP to simulate expected typical process conditions. The system was then blown-down with air and the nanoparticles collected. The nanoparticle size was then measured by dynamic light scattering. The membrane was then flushed with water (>2 mL/cm²) and cleaned per the procedure in Appendix I between each test.

Trans-membrane pressure optimization

Nanoparticles were diafiltered at low, medium, and high concentrations with variations in the trans-membrane pressure to determine the point at which the permeate flow rate becomes independent of trans-membrane pressure.

Approximately 45 mL of NR+VE core nanoparticles were loaded into a 50 mL centrifuge tube with 3 luer-lock ports. The flow rate was set at the previously determined optimum. The KrosFlo unit was run in diafiltration mode with 1.5 L of water loaded into a 2 L reservoir. The TMP was varied from the minimum set by the pump up to 27.5 psig and the permeate flow rates recorded at each point. Then, the diafiltration line was removed and the nanoparticles were concentrated down to a process reservoir volume (not total process volume) of ~5 mL and the diafiltration line reconnected. The TMP excursions were repeated.

For the high concentration test, approximately 150 mL of NR+VE core nanoparticles were loaded into a 250 mL conical bottom process reservoir with 3 dip tubes with luer lock ports.
(ACBT-250-C1N, Spectrum Labs, USA). The nanoparticles were concentrated down to a process reservoir volume of 4.5 mL and then diafiltered while the TMP was varied up to 21 psig.

PMMA latices were diluted to 6 and 30 mg/mL. 150 mL of suspension at low and high concentration were loaded into a 250 mL conical bottom process reservoir and diafiltered at 100-300 mL/min feed rate and 5-25 psi TMP and permeate fluxes recorded.

Upon completion of the experiments, sizes were taken on the Malvern DLS unit. The NP concentration was also quantified by UV-vis absorbance spectroscopy. Briefly, NPs were diluted in THF by varying amounts in order to dissolve the nanoparticles and remove any background scattering effects. A quartz cuvette (Q104, DKSH, Switzerland) containing pure THF was placed in the sample holder of a Shimadzu UV-vis spectrophotometer (UV-2550, Shimadzu Corporation, Japan) and the baseline recorded (250 – 600 nm). The diluted solutions were then placed in the same quartz cuvette and the spectra recorded. VE and NR concentrations were quantified from calibration curves recorded at 295 and 535 nm respectively.

**Solids concentration optimization**

Nanoparticles were concentrated down as much as possible (the lower volume limit being the holdup volume) and the permeate flow rates recorded at regular intervals to construct the optimization curve.

Approximately 150 mL of VE+NR core nanoparticles were placed in a 250 mL conical bottom process reservoir with 3 dip tubes and luer-lock fittings. The KrosFlo unit was run at the
previously determined optimal feed flow rate and TMP. The permeate flow rates were recorded as the concentration of the nanoparticles proceeded. When air became entrained in the nanoparticle suspension (volume too low), the experiment was stopped. The membrane was then blown down, flushed and cleaned and the nanoparticles characterized by UV-vis spectroscopy and DLS as described previously.

Similarly, 150 mL of PMMA NPs at 30 mg/mL were placed in a 250 mL conical bottom process reservoir and the KrosFlo unit was run at 200 and 300 mL/min feed flow rate at the previously determined optimal TMP and permeate fluxes were recorded.

_Diafiltration efficiency with VE Core NPs_

Nanoparticles were concentrated and diafiltered and the permeate line sampled regularly to determine the degree of THF removal as a function of the number of diafiltration volumes. Two experiments were performed: in Experiment 1, $V_D$ (total diafiltration volumes) = 7.8, in Experiment 2, $V_D = 14.8$. Process parameters were defined in this Chapter, Part A.

Approximately 135 mL of VE+NR core nanoparticles were placed in a 250 mL conical bottom process reservoir with 3 dip tubes and luer lock fittings. Previous experiments were used to determine the optimal feed flow rate, TMP, and solids concentration to begin diafiltration. The KrosFlo unit was run using these parameters. Water was directly injected into the reservoir prior to constant-volume diafiltration to adjust the process volume (and hence the concentration). This is not normally a required step, however. During diafiltration, the process reservoir was held at 20 mL and the permeate line was sampled regularly to track THF concentrations over time. Two
experiments were conducted with different extents of diafiltration. After each experiment, the membrane was blown down, flushed, cleaned and the nanoparticles characterized for size by DLS, concentration by UV-vis spectroscopy, and THF concentration by headspace gas chromatography (HSGC). For HSGC, samples of nanoparticles or permeate were diluted with water if necessary so that the expected THF concentration was in the range of 10 – 1000 ppm (by mass) which was the range of the GC calibration curve previously prepared. 2 mL of each sample were placed into 20 mL GC vials (N9306077, Perkin Elmer, USA) which were placed in the headspace unit (Turbomatrix 16, Perkin Elmer, USA). The vials were equilibrated to 80°C and an aliquot of sample vapor was injected into the column and THF content measured by flame ionization detection (6890N, Agilent Technologies, USA).
Results and Discussion

Nanoparticle (NP) Formulation

Size stability for VE core NR containing NPs is shown in Fig. 2. Samples were stored at 4°C and 25°C and were either dialyzed or undialyzed.

![Diagram showing size stability over time](image)

**Fig. 2** – Size of VE/NR/PEG-PCL NPs over a period of 18 days. Storage conditions: (■ 4°C undialyzed | ○ 25°C undialyzed | ▲ 4°C dialyzed | ▼ 25°C dialyzed). As expected, the best conditions for long term stability required removal of THF and storage at 4°C. NPs that experienced the greatest degree of Ostwald ripening[15] were undialyzed and stored at room temperature (~25°C).

Small precipitates were seen for undialyzed particles at 4°C after 18 days, but the other batches did not show any precipitation or aggregation. Dialyzed particles were relatively stable, the best condition being at 4°C. The lower temperature slows down the rate of Ostwald ripening.[15] Unfortunately, due to Nile Red’s excellent utility as a hydrophobic cell membrane stain,[16] on average we observed about 75% loss of the Nile Red in the nanoparticles to apparent hydrophobic domains within the mPES membranes themselves. This did not affect mass balances significantly since NR only comprised about 0.5% of the nanoparticle mass. No
significant loss of Vitamin E (VE) was observed. The growth in size of the nanoparticles is shown in Fig. 5.

This meant that NR could not be used to quantify NP concentrations using its absorbance peak at 535 nm. Instead, VE was used since it has a strong UV signal at 295 nm.

*KrosFlo Unit Pump Calibration*

![Graph showing pump calibration tests.](image)

**Fig. 3 – Pump calibration tests.** Three tests were done at feed flow rates from 50 to 300 mL/min. The solid black line represents a perfect match between the set point and the measured flow rates. The green line represents the true correlation between the measured and set point flow rates ($Q_{F,\text{true}} = 1.19Q_{F,\text{set}} - 4.49, R^2 = 0.999$).

As Fig. 3 shows, the pump delivers higher-than-set flow rates at any flow rate above 50 mL/min. In fact, the true flow rate deviates linearly from the ideal 1:1 matching. Performing regression analysis on the data set revealed the following function for correlating the set flow rate with the true flow rate:
\[ Q_{F, \text{true}} = 1.19 Q_{F, \text{set}} - 4.49, \ R^2 = 0.999 \]  \hspace{1cm} \text{Eqn. (1)}

\[ J_p = \frac{Q_p}{A} = \frac{\text{TMP}}{\mu(R_M + R_G)} \]  \hspace{1cm} \text{Eqn. (2)}

**KrosFlo Hollow Fiber Membrane Water Permeability Tests**

![Diagram](image)

**Fig. 4** – Initial water permeability tests on 10kD MWCO mPES membranes in wetted and non-wetted states at \( Q_F = 100 \) and 300 mL/min (■ nonwetted 100 mL/min | ● nonwetted 300 mL/min | ▲ wetted 100 mL/min | ▼ wetted 300 mL/min). Membranes wetted with 20-30% EtOH gave approximately double the flow rate of non-wetted membranes. There was essentially no difference between \( Q_p \) at high and low \( Q_F \) for non-wetted membranes. There was a small difference between \( Q_p \) at high and low \( Q_F \) for wetted membranes. The reason for the difference is not clear though it may be related to pore deformation due to the wetting process.

At this point, it is worth introducing a general model for correlating the permeate flux with the TMP, as described by D’arcy’s Law[17]:
Where $Q_P$ is the permeate flow rate, $A$ is the membrane area, $\text{TMP}$ is the transmembrane pressure, $R_M$ and $R_G$ are the membrane and gel layer resistances respectively, and $\mu$ is the viscosity. $R_M$ is a constant for a given membrane while $R_G$ may change over the course of an experiment as the gel/cake layer builds up. We can determine $R_M$ for a given membrane precisely by doing these water permeability tests on a clean, wetted membrane, since $R_G$ will be 0 when filtering pure water:

$$J_P = \left( \frac{1}{\mu R_M} \right) \text{TMP}$$  \hspace{1cm} \text{Eqn. (3)}$$

Thus, the inverse of the slope in Fig. 4 gives the membrane resistance. For a wetted 10kD mPES hollow fiber membrane unit with a 0.5 mm ID fibers, the resistance is $2.50 \times 10^8 \text{ m}^{-1}$. Since $R_M$ is assumed to be constant over the course of an experiment, we can calculate $R_G$ during a filtration experiment since $J_P$, $\text{TMP}$, and $R_M$ are known in Eqn. (2).
**NP Shear Testing on VE core NPs**

![Graphs showing NP size and PDI changes](image)

Fig. 5 – (left) Change in NP size and PDI (polydispersity index) widths after 1 hr processing at various shear rates and constant solids concentration. (Left) There was no change in size at 3800 s⁻¹, 11.5% growth at 7500 s⁻¹, and 45.5% growth at 11400 s⁻¹. (Right) There was essentially no change in PDI width at 3800 s⁻¹, a 21% increase at 7500 s⁻¹, and a 49% increase at 11400 s⁻¹. Legend: t₀ – diagonal lines, t = 1 hr – horizontal lines.

The effects of low (3800 s⁻¹), medium (7500 s⁻¹) and high (11400 s⁻¹) shear rates on VE core nanoparticle size and stability changes were tested (Fig. 5). Generally, for applications with a high potential of fouling, running at a high shear rate (which means a high feed flow rate Q_F) is recommended. The high shear rate improves membrane ‘cleaning’ during processing, improving nanoparticle yields and reducing the incidence of membrane fouling. However, high shear rates also mean more passes through the membrane unit per minute and greater stresses. This can lead to product degradation. Thus, it was important to determine how high the feed flow rate could be set with minimal changes in size and stability. Based on the results in Fig. 5, a feed flow rate of 200 mL/min seemed optimal for subsequent experiments, given that the VE/NR/PEG-PCL nanoparticles experienced an 11.5% growth in size, which was deemed acceptable. In all cases where the size increased, there was also a significant increase in PDI width as well.
The shear rate chosen should depend on the application and the nature of the nanoparticle system. It is important to note that the VE/NR/PEG-PCL nanoparticles have a liquid core, whereas a solid, glassy or crystalline core such as PCL or PS homopolymer would probably be more resistant to deformation and size growth due to shear.
Fig. 6 – (A) graph showing the results of constant volume diafiltration experiments at 3 different solids concentrations ([Solids] = 1.26 (■), 4.5 (●), and 14.8 mg/mL (▲) by UV-vis), (B) graph showing approximate locations of ‘knees’ (points where \( Q_P \) just begins to level off from the top graph) as a function of vitamin E concentration. The relationship is linear (see model). (C) Graph showing how the gel layer resistance \( R_G \) from Eqn. (2) varies with TMP and solids concentration. \( Q_F = 200 \text{ mL/min} \).

Generally, an increase in the TMP via manipulation of an automatic pinch valve results in a higher \( Q_P \) (see Eqn. (2)). However, pressures cannot be increased indefinitely due to the operating constraints of the process equipment (in our case, about 30 psi). Furthermore, \( Q_P \) rises linearly with TMP only up to a certain point (depending on the recirculation rate and product
concentration). Beyond this point, the permeate flux levels off as gel polarization rises, limiting the permeate flux. Mathematically, this means that $R_G$ begins rising once the linear regime is over (Fig. 7C). As seen in Fig. 7A, the location of the ‘knee’ where the linear regime ends depends on the solids concentration (in this case the recirculation/shear rate was held at 200 mL/min). Obviously, gel polarization begins at lower TMPs for higher nanoparticle concentrations.

The ‘knee’ represents an optimal operating point which allows for maximum permeate flux while simultaneously minimizing the amount of gel polarization. TMPs higher than the knee give little additional rise in $Q_P$ while TMPs lower than the knee give low fluxes. Using the concentration of vitamin E (VE) as determined by UV-vis spectroscopy as a proxy for the total solids concentration, we found that the location of the knee is linearly dependent on the VE concentration (and hence the solids concentration)(see Fig. 7B). This allows for easy prediction of the optimal TMP based on the initial and final solids concentrations.

Given that VE comprised 49.5% of the nanoparticle mass, the equation is simply:

$$\text{TMP}_{\text{optimal}} = -0.725[\text{Solids}] + 20.9, \ R^2 = 1.00$$

Eqn. (4)

The optimal strategy for ultrafiltration would involve gradually shifting the TMP down as a NP suspension is concentrated, since the optimal TMP changes with solids concentration. Alternatively, the TMP could be lowered near the end of an experiment since the gel layer is partially reversible. We emphasize that this equation should be used with caution outside of the
[Solids] range tested. A nanoparticle system of a different size would undoubtedly show different gel layer formation characteristics.

**PMMA NPs**

We also investigated the behavior of 320 nm PMMA latices as a model nanoparticle system with very different characteristics from BSA and VE Core – PEG shell NPs. There is a strong linear correlation between permeate flow rate and TMP within the measured TMP range as seen in **Figs. 7A and 7B**. This finding suggests that there is only a minimal rise in osmotic pressure, but a buildup of a gel layer at these concentrations. The interaction between the macromolecular species and the membrane will affect the ultrafiltration process significantly. Therefore, one of the primary reasons for the minimal flux decline is the low affinity between anionic charge stabilized PMMA latices and the hydrophilic polyethersulfone membrane (which takes on a negative charge in aqueous solution).[18] Once the membrane fouling has reached a limiting gel layer concentration, additional gel layer formation by PMMA nanoparticles will begin to cease despite increases in TMP.[19] For this reason, the permeate flow rate continues to increase linearly with each additional unit of TMP. It is important to note that feed flow rate does not have a significant effect on the relationship between permeate flow rate and TMP. For each of these cases, the optimal TMP is greater than 25 psi since no transition to a TMP-independent regime is seen. However, it is not desirable to use a TMP that is higher than 25 psi because the maximum operating pressure of the membrane and the automatic backpressure controller is 30 psi. The osmotic pressure due to PMMA concentration polarization does not continually increase, and this results in a lack of a TMP-independent regime in the PMMA TMP optimization curve. The slope of the curve is lower for 30 mg/mL PMMA than for 6 mg/mL
PMMA as expected since the effects of concentration polarization are higher at higher bulk concentrations.

![Graphs A and B](#)

**Figure 7** – (▲ 100 mL/min | ○ 200 mL/min | ■ 300 mL/min) TMP Optimization ($Q_P$ vs. TMP and $R_G$ vs. TMP) for concentrations of 6 mg/mL PMMA (A & C) and 30 mg/mL PMMA (B & D) at various feed flow rates.

As we did in Part A of this Chapter, we performed as ramp-up-ramp down TMP excursion experiment at 1 wt% solids at 300 mL/min and 100 mL/min circulation rate ([Appendix I – Fig. 6(A) and (B)]). At 300 mL/min, the absolute $Q_P$ were higher than at 100 mL/min as expected. Interestingly, at 300 mL/min, the ramp-up and ramp-down curves are almost identical and linear.
Therefore, the $R_G$ is constant and the gel layer mostly reversible. The larger size of the PMMA latices compared to BSA proteins and strong charge stabilization is likely the cause of this reversibility. At 100 mL/min, the results are less clear. During the ramp-up, the curve $Q_P$ vs. TMP plateaus immediately (increasing $R_G$) and is relatively constant over 10-25 psig TMP. However, on the ramp-down phase, the curve is linear ($R_G$ remains constant). It appears that there may have been a residual gel layer from the previous experiment left on the membrane. An alternative explanation is that during the ramp-up, the gel layer became compressed, resulting in the increase in $R_G$. But on the ramp-down, the gel layer remained irreversibly stuck, likely due to the lower shear rate which has a weaker cleaning effect.

![Diagram](image)

**Fig. 8** – Illustration of osmotic pressure rise as a function of concentration for a protein such as BSA[20] vs. a charge stabilized nanoparticle (PMMA)[21]. Representative values $\Pi_{BSA}(2 \text{ wt\%}) = 10 \text{ kPa}$ whereas $\Pi_{PMMA}(10 \text{ vol\%}) = 1 \text{ kPa}$.

This is clearly visible in Fig. 8 which shows representative curves of osmotic pressure vs. concentration of BSA vs. PMMA nanoparticles. Representative values are $\Pi_{BSA}(2 \text{ wt\%}) = 10 \text{ kPa}$ whereas $\Pi_{PMMA}(10 \text{ vol\%}) = 1 \text{ kPa}$. Thus, the osmotic pressure of BSA rises much faster.
than that of PMMA latices and we see the presence of a ‘knee’ in the QP vs. TMP graph (Fig. 4 in Part A and Fig. 6 in this Part).

**Figs. 7(C) and (D)** show the values of $R_G$ (the gel resistance) that are associated with the TMP optimization curves for different concentrations of PMMA that are shown in **Figs. 7(A) and (B)**.

The most important feature is that the gel resistance $R_G$ is four orders of magnitude higher than the membrane resistance $R_M$ ($10^{12}$ vs. $10^8$ m$^{-1}$) showing that the effects of the gel layer will dominate the permeability of the membrane system. In the case of 6 mg/mL PMMA, the permeate flux rises linearly with TMP – therefore, the gel resistance is constant as seen in **Fig. 7(C)**. For 30 mg/mL PMMA, the permeate flux starts to level off **Fig. 7(B)** which results in a gradual rise in $R_G$ in **Fig. 7(D)** due to the buildup of increased gel resistance. It is possible that $R_G$ would rise in the case of 6 mg/mL PMMA as well at higher transmembrane pressures.
The fouling characteristics of the three systems studied in Parts A and B of this chapter are summarized in Table 1. Since the concentrations, feed flow rates, and transmembrane pressures were in a relatively small range, \( R_G \) values varied by an order of magnitude at most between systems.

The \( R_G \) term in Eqn. 2 of course can represent multiple fouling effects including concentration polarization (and subsequent rise in osmotic pressure), electrostatic repulsions that block permeate flow, and the creation of a cake or gel layer that impedes flow by creating tortuous pathways through the layer and effectively reduce the porosity of the membrane.

Because of this, we see that generally BSA shows the highest gel resistance, due to the osmotic pressure rise as well as denaturation and subsequent mechanical blockage of the membrane. PEG protected nanoparticle display the second highest degree of fouling as measured by \( R_G \) due to the dense PEG brush that surrounds each particle and prevents aggregation by osmotic

---

**Table 1 - Comparison of gel resistance \( R_G \) for BSA, PEG shell NPs, and PMMA NPs**

<table>
<thead>
<tr>
<th>System</th>
<th>Concentration (mg/mL)</th>
<th>( Q_{Feed} ) (mL/min)</th>
<th>TMP Range</th>
<th>( R_G ) Range ( (m^{-1}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>9.6</td>
<td>100</td>
<td>10-25 psi</td>
<td>5.5( \times 10^{12} ) – 9.8( \times 10^{12} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>10-25 psi</td>
<td>5.9( \times 10^{12} ) – 7.9( \times 10^{12} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>13-25 psi</td>
<td>5.9( \times 10^{12} ) – 7.1( \times 10^{12} )</td>
</tr>
<tr>
<td></td>
<td>65.9</td>
<td>100</td>
<td>10-25 psi</td>
<td>1.4( \times 10^{13} ) – 2.6( \times 10^{13} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>10-25 psi</td>
<td>1.2( \times 10^{14} ) – 2.1( \times 10^{14} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>14.2-25 psi</td>
<td>1.5( \times 10^{13} ) – 1.8( \times 10^{13} )</td>
</tr>
<tr>
<td>VE/PS-PEG NPs</td>
<td>1.3</td>
<td>200</td>
<td>10-27.6 psi</td>
<td>4.4( \times 10^{12} ) – 6.6( \times 10^{12} )</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>200</td>
<td>10-27.4 psi</td>
<td>5.9( \times 10^{12} ) – 9.1( \times 10^{12} )</td>
</tr>
<tr>
<td></td>
<td>14.8</td>
<td>200</td>
<td>8.8-21.2 psi</td>
<td>6.0( \times 10^{12} ) – 1.1( \times 10^{13} )</td>
</tr>
<tr>
<td>PMMA NPs</td>
<td>6</td>
<td>100</td>
<td>3-25 psi</td>
<td>2.7( \times 10^{12} ) – 2.9( \times 10^{12} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>7.9-23 psi</td>
<td>2.7( \times 10^{12} ) – 2.9( \times 10^{12} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>14-23 psi</td>
<td>2.7( \times 10^{12} ) – 2.9( \times 10^{12} )</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>200</td>
<td>10-25 psi</td>
<td>1.9( \times 10^{12} ) – 3.1( \times 10^{12} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>15-25 psi</td>
<td>2.4( \times 10^{12} ) – 3.2( \times 10^{12} )</td>
</tr>
</tbody>
</table>
pressure repulsion. Charge stabilized PMMA NPs displayed the lowest $R_G$ values due to strong electrostatic repulsions between nanoparticles (22,500 charges per particle) and the high $T_g$ of PMMA (105°C, data not shown) which prevents permanent coalescence and deformation. Furthermore, the high surface charge on PMMA latices reduces adsorption to the weakly negatively charged polyethersulfone membrane surface. As expected, high feed flow rates reduce the $R_G$ values due to greater hydrodynamic lifting force while higher TMPs increase $R_G$ by increasing the average driving force across the membrane.
KrosFlo Solids Concentration Optimization for Diafiltration

VE Core NPs

Fig. 9 – (A) Permeate flow rate ($Q_p$) and (B) the optimization parameter $\xi^*$ vs. Vitamin E concentration as a proxy for the NP concentration. At a VE concentration of about 10 mg/mL, the TMP controller was turned off, hence the TMP and $Q_p$ dropped then stabilized until the VE concentration reached about 25 mg/mL and air became entrained in the process. Thus, only the data up to 10 mg/mL VE should be used for future experiments. In this case, $[Solids] \approx 2[VE]$.

At this point, previous experiments have guided the selection of the optimal feed flow rate and TMP for 90 nm nanoparticles with VE/PEG-PCL with 0.5wt% NR. The next goal was to optimize the combined ultrafiltration/diafiltration processes to reduce total processing time and water/buffer consumption.
In general, concentrating and purifying a nanoparticle suspension can be done in three schemes:  
1) Concentration first, then diafiltration, 2) Diafiltration first, then concentration, or 3)  
Concentration to some intermediate level, diafiltration, then another final concentration step. As 
seen in Fig. 9A, concentrating nanoparticles results in a gradual, nonlinear decline in \( Q_p \), the 
permeate flow rate. This is due to the phenomena of concentration polarization and gel layer 
formation. During diafiltration, the process volume and nanoparticle concentration are held  
constant, and small molecule impurities are washed out by fresh water/buffer that is pulled into 
the process at the volumetric flow rate of \( Q_p \). The model derivation for this process can be seen 
in Appendix I. The extent of diafiltration is quantified by the number of diavolumes of fresh 
water/buffer processed, \( V_D \), which is the ratio of the volume of fresh water/buffer to the process 
volume. Thus, concentrating a suspension first reduces the process volume allowing for the 
same number of diavolumes processed but with less volume of water/buffer. However, the 
downside to this approach (1) is that \( Q_p \) is also reduced which could lead to longer processing 
times. Due to the nonlinearity of \( Q_p \) vs. [Solids], there exists a maximum in the optimization 
parameter \( \xi^* = Q_p \times [\text{Solids}] \) which allows minimization of the processing time.  

**Fig. 9B** shows the relationship between \( \xi^* \) and [VE]. It is apparent that \( \xi^* \) rises until [VE] 
reaches 9 mg/mL and plateaus until 10 mg/mL. At that point, because the process volume was 
too low, \( \xi^* \) started dropping and air became entrained in the process so the TMP pinch valve was 
opened and the experiment stopped soon after. Thus, 10 mg/mL VE (20 mg/mL solids) was the  
optimum point for the intermediate concentration at which diafiltration should begin.
**PMMA Diafiltration Optimization**

The results of the PMMA Diafiltration Optimization experiment are shown in Figure 10. The data suggest that at a feed flow rate of 300 mL/min and a TMP of 20 psi, the optimal PMMA concentration is above 32 wt% since no maximum is seen. However, there is an aggregation and membrane fouling limitation that must be accounted for. At high concentrations of PMMA (>32wt%), there is significant aggregation and membrane fouling that results in an uncontrolled rise in TMP. Therefore, we do not show data past 32wt% for the experiment at 300 mL/min. This effect was not observed in the 200 mL/min experiment where the TMP was held at 15 psi. This is most likely due to the lower pressure which reduces the degree of aggregation and gel layer formation. The lower shear rate may also reduce any shear induced aggregation. In this case, the optimal concentration for starting diafiltration appears to be over 600 mg/mL. These results are in sharp contrast to the diafiltration optimization results seen in Part A for BSA where a clear maximum was seen, but agrees fairly well with diafiltration optimization done for VE core PCL-\textit{b}-PEG shell NPs. This is because $Q_p$ is linear with the TMP in the case of the acrylic latices.
Figure 10 - Permeate flow rate vs. concentration (A) and diafiltration optimization parameter (B) graphs for PMMA at feed flow rates of 200 (■), and 300 mL/min (●) at 15 and 20 psi respectively.

The results obtained thus far, for TMP and diafiltration concentration optimization, agree with the general principles outlined in the model described by Jiao and Sharma.[11] In essence, membrane fouling occurs due to the deposition of solute onto the membrane surface or growing cake/gel layer. The deposition of particles is controlled by a balance of forces comprising the normal hydrodynamic drag force towards the membrane surface caused by permeate flux and the tangential force from fluid flowing parallel to the membrane surface (removing force).

Assuming that the particles are released by a sliding mechanism, the maximum particle size that gets deposited is proportional to the permeate flux $J_p$. This is because particles larger than the threshold are entrained in the flowing fluid. Therefore, as the cake layer builds up and $Q_p$ is reduced, smaller and smaller particles are deposited until the eventually no more small particles are available in the suspension to deposit and the cake layer reaches equilibrium, as does the permeate flux. We see this behavior demonstrated in all the systems we studied (Fig. 5A in Part A, and Figs. 9A and 10A in Part B) where the permeate flux tends to equilibrium as a function of solute concentration.
Fig. 11 – THF concentration vs. $V_D$ for the 2 diafiltration experiments done (■ Experiment 1, ● Experiment 2). The process volume was 28 mL for Experiment 1 and 27 mL for Experiment 2. Before constant volume diafiltration was started, there were phases of concentration and direct injection of pure water into the process reservoir to prevent the volume from falling too low. Thus, the concentration of THF just prior to beginning constant volume diafiltration was not 84000 ppm but rather closer to 7000-8000 ppm.

As mentioned in the previous section, the optimal intermediate concentration was found to be $[\text{VE}] = 10 \text{ mg/mL}$. Unfortunately, due to the limited starting volume and process holdup, as well as difficulties in maintaining reservoir volumes lower than 20 mL during diafiltration, an intermediate concentration of only 4 mg/mL of VE was achieved. Thus, during the pilot diafiltration experiments, we did not achieve optimal process times and minimal water consumption. Nonetheless, the THF removal and modeling results are still valid.
As seen in Fig. 11, the starting undialyzed NP suspension had a THF concentration of approximately 84000 ppm (by mass). This correlates well with the theoretical concentration of 9vol% from the 1:10 THF:water mixing ratio in the MIVM. Permeate samples were taken during the constant volume diafiltration phase to track the concentration of THF.

It should be noted here that during the experiments, pure water was injected directly into the process reservoir after concentrating and prior to diafiltration. This is known as batch diafiltration though the purpose here was to control the reservoir volume (and hence solids concentration) to achieve the 10 mg/mL VE target. Thus, when constant volume diafiltration began, the THF concentration was not necessarily 84000 ppm. It is unknown how the THF concentration changes during the concentration phase. We can see from Fig. 11 that the model predicts that the initial THF concentration was 7800 ppm in Experiment 1 and 5800 ppm in Experiment 2. This would indicate that the concentration dropped by factors of 10.8 and 14.5 respectively due to the concentration phase and pure water injected in, batch diafiltration-style. Further experiments should be done to determine how much THF is removed by the concentration phase.

The THF concentration drops exponentially once continuous diafiltration begins, as predicted by the model:

\[ \ln[C(t)] = -V_D T + \ln(C_i), \quad R^2 = 0.99 \]

Eqn. (5)

(see Appendix I for derivation)
The slope of the graph (Fig. 11) gives the transmission coefficient, $T$, while the intercept gives the initial THF concentration. We found that in Experiments 1 and 2, the transmission coefficients were 47% and 35% respectively. This means that THF concentrations were higher on the inside of the membrane compared to the outside and that there was a significant gel layer. Nonetheless, the last sample taken was of the NP sample directly. This is important as a low transmission coefficient reduces the diafiltration efficiency. The 720 ppm FDA guideline[22] is satisfied after 6 diafiltration volumes. Ultimately, we were able to achieve 66 ppm THF after $V_D = 14.8$ (Experiment 2). That translated to 400 mL of pure water for that particular experiment with a NP suspension concentrated by a factor of 9.4, a final solids concentration of 16.2 mg/mL and NP yield of 87.5% processed in 62 minutes.

In Fig. 11 it’s clear that Experiment 2 deviates from the standard exponential decay model towards the end. This is most likely due to residual THF trapped in the nanoparticle cores that may be in equilibrium with free THF in solution. To our knowledge, the trapped THF content in nanoparticle cores following free THF removal has not been previously measured in nanoparticles formed by Flash NanoPrecipitation, by headspace GC or any other method. Any assumption that membrane dialysis removes all THF from nanoparticle systems must be questioned in light of these data. Regardless, the diafiltration results do not depend significantly on the composition of the NPs as most of the THF is in the solution and will be removed at the same rate regardless of the NP composition (unless the gel layer is significantly different).
Conclusions and Recommendations

In conclusion, we have demonstrated the utility of ultrafiltration and diafiltration for concentrating and purifying nanoparticles to predefined specifications and explored the membrane fouling characteristics of proteins and nanoparticles stabilized by PEG coronas and charge. We showed that the permeate flow rate $Q_P$ is only a function of TMP for pure water and that the intrinsic membrane resistance was $2.50 \times 10^8 \text{ m}^{-1}$. VE Core block copolymer protected nanoparticles were shown to undergo shear-dependent growth in size and polydispersity though the size distribution remained monomodal. We conducted studies of permeate flux vs. transmembrane pressure for the protein and nanoparticle systems showing that BSA and PS-b-PEG protected NPs formed filter cakes that gave a linear relationship between $Q_P$ and TMP at low TMPs but plateaued and became TMP-independent at high TMP. In contrast, the PMMA NPs did not show such behavior and instead remained linear in $Q_P$ vs. TMP over the TMP range studied. D’arcy’s Law was applied to determine the gel resistance $R_G$ for the 3 systems studied, with BSA having the highest resistance and PMMA latices having the lowest under similar conditions. $R_G$ values varied from $10^{12}$ up to $10^{13} \text{ m}^{-1}$, 4 to 5 orders of magnitude higher than the intrinsic membrane resistance. Thus, the gel resistance dominates the overall membrane resistance. Lastly, process optimization was performed to determine the optimal TMP and intermediate concentration for the combined concentration-diafiltration scheme. The optimization parameter $C^*$ was calculated to determine the optimal intermediate concentration for the start of continuous diafiltration. Diafiltration was performed on BSA and VE core NPs. It was found that a model small molecule impurity sodium salicylate bound strongly to the BSA causing deviations from the diafiltration model which predicted an exponential decrease in
salicylate concentration as a function of the diavolumes. THF removal was studied in the case of the VE core NPs by headspace GC. It was found that at least 6 diavolumes were required to purify these nanoparticles below the 720 ppm THF FDA guideline and that the residual THF trapped in NP cores was about 66 ppm.

Based on the results of this study, we have some recommendations for further work:

- Determine the THF concentration immediately following the first concentration phase prior to the diafiltration phase. It is not clear whether and by how much the concentration phase lowers the THF concentration.
- We have determined $R_g$ as a function of TMP and solids concentration for NPs approximately 90 and 320 nm in diameter stabilized by different mechanisms. We recommend doing the same for NPs of smaller and larger sizes to build a comprehensive model of how the gel layer resistance is a function of all these variables.
- It would be very interesting to obtain a visual representation using SEM of the gel layer following an experiment. It would be interesting to see how open the gel layer is, whether the NPs are irreversibly aggregated, and the approximate thickness of the layer.
- Further experiments should be done on solid core nanoparticles such poly(styrene). In particular, how the shear resistance is different from that observed for VE core NPs.
- The precise mechanism for the shear induced NP growth is unclear. Further experiments should be done on NPs of different composition (solid vs. liquid core) and
sizes to see if these results are reproducible. This could result in a unique way to controllably and reproducibly tune the size of NPs post-production.

Acknowledgements

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REFERENCES


APPENDICES
APPENDIX A

Methotrexate Prodrug and Nanoparticle Formulation

INTRODUCTION
The antifolate drug methotrexate (MTX) is a drug with a long history of use for various diseases (beginning in 1948) including acute leukemia, non-Hodgkin 's lymphoma, osteogenic sarcoma, choriocarcinoma, breast carcinoma, pulmonary and epidermoid carcinoma, and intrathecal chemotherapy. It is also useful in bone marrow transplantation, severe psoriasis, rheumatoid arthritis, dermatomyositis, Wegener's granulomatosis and sarcoidosis[1].

As with many cancer medications, it has relatively low water solubility and thus poor bioavailability. MTX also suffers from resistance mechanisms employed by cancer cells involving the availability of folate receptors and carriers on the cell surface[2].

Mechanism of action
According to Bleyer[1] and Wu[2], MTX’s mechanism of action is quite well understood at the molecular level. The drug is transported into cells by the reduced folate transporter and polyglutamylated to prevent efflux. Methotrexate inhibits dihydrofolate reductase, the enzyme responsible for converting folic acid to reduced folate cofactors. Reduced folates are necessary for the metabolic transfer of 1-carbon units in a variety of biochemical reactions. Those reactions which are of special importance in cellular proliferation are the bio- synthesis of thymidylic acid, the nucleotide specific to DNA, and the biosynthesis of inosinic acid, the precursor of purines necessary for both DNA and RNA synthesis[1]. The drug is highly cell-cycle dependent, acting primarily during DNA synthesis (S-phase). As a result, those tissues
undergoing rapid cellular turnover with a high fraction of the cells in cycle are the most susceptible to the drug’s cytotoxic effects. Adverse effects associated with methotrexate include severe damage to normal cells and organs, narrow therapeutic index, poor selectivity for neoplastic cells, multidrug resistance due to down-regulation of methotrexate polyglutamation, and decreased reduced folate carrier mediated uptake.[2]

Prodrugs

According to D’Souza, a prodrug is a biologically inactive derivative of a parent drug molecule designed to circumvent problems associated with the delivery of the parent drug. Prodrugs usually require the transformation of the prodrug to the drug within the body to elicit therapeutic action[3]. Prodrugs are utilized to overcome various obstacles such as poor drug solubility, the systemic conversion into inactive metabolites, a lack of site specificity or an inefficient cell uptake[4]. Since work in this area has been ongoing for over 50 years, the literature is quite vast. Nonetheless, prodrug systems share certain similarities. Prodrug research in recent years has also focused on the use of nanocarriers systems that exhibit increased circulation time and multifunctionality to allow for simultaneous diagnostic and therapeutic benefits along with passive and active targeting capabilities. Chemotherapeutic drugs have been conjugated to small molecules (either to modify the solubility of the drug[5] or provide targeting and/or additional therapeutic value), peptides, proteins/antibodies, polymers and dendrimers. Various creative strategies have been employed to utilize triggerable linkers between the drug and ‘anchor’ species that allow for drug release upon pH change, optical stimulus (NIR light), magnetic stimulus, and specific enzymatic activity, to name a few[6].
MTX has been incorporated into numerous prodrug and nano-carrier constructs exhibiting various cancer cell-killing efficacies and release characteristics. In particular, dendrimer constructs incorporating MTX in the hydrophobic core or conjugated to the surface have been extensively reported and have shown increased sensitivity and tumor cell killing capabilities in vitro and increased circulation time and reduced tumor volume growth compared to free MTX in vivo[7-9]. MTX has also been conjugated to amino acids[2, 10, 11] and peptides[12] with albumin binding traits which showed similar improvements in therapeutic effects. Other examples include conjugating MTX to lipophilic anchors to improve encapsulation within hydrophobic nanocarriers such as micelles or liposomes[13], an approach we believe is particularly promising given the very long circulation times[14, 15] reported for PEGylated nanocarriers and multifunctionality of such constructs.

To this end, we conjugated MTX to a long chain alcohol as a lipophilic anchor, to reduce the water solubility of MTX and enable high, stable loadings into nanoparticles formed by flash nanoprecipitation. The ester linkage used allowed for degradation in acidic environments, such as that found in an endosome upon uptake by a cell, thus releasing the free drug only upon cell entry. Syntheses were confirmed by NMR spectroscopy and the prodrug was successfully incorporated into nanoparticle cores.

**PROTOCOL AND RESULTS**

*Synthesis*

Several reaction schemes were attempted to generate hydrophobic prodrugs of methotrexate. These include:
1) Coupling vitamin E succinate (VES) to propargylamine, separately reacting 5-azidovaleric acid to sodium azide, then reacting the azide-alkyne functionalities by click chemistry.

2) Directly coupling with VES to MTX by DCC and EDC coupling.

3) Coupling MTX to n-octanol by BF$_3$OEt$_2$

However, these reactions generally gave low yields and/or an abundance of side products. We eventually decided upon the best scheme depicted below:

---

**AppA - Fig. 1 – Reaction scheme for methotrexate + 1-octanol.**

<table>
<thead>
<tr>
<th>material</th>
<th>formula</th>
<th>M. W.</th>
<th>purity, w %</th>
<th>amount, g</th>
<th>millimol</th>
<th>equiv.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td>C$<em>{20}$H$</em>{22}$N$_8$O$_5$</td>
<td>454.44</td>
<td>98</td>
<td>0.050</td>
<td>0.107</td>
<td>1.00</td>
</tr>
<tr>
<td>n-octanol</td>
<td>C$<em>8$H$</em>{18}$O</td>
<td>130.23</td>
<td>99.5</td>
<td>8.25</td>
<td>630</td>
<td>589</td>
</tr>
<tr>
<td>product</td>
<td>C$<em>{36}$H$</em>{54}$N$_8$O$_5$</td>
<td>678.86</td>
<td>100</td>
<td>0.0232</td>
<td>/</td>
<td></td>
</tr>
</tbody>
</table>

Procedure:

1. MTX (Aldrich, A6770, dried by high vacuo overnight) was suspended in a solution of 0.2 mL of concentrated HCl and 10 mL of anhydrous n-octanol (Fluka, 74850, density: 0.825 g/mL). The solution was heated at room temperature for 72 h under Ar in dark.

2. The solution was cooled to room temperature, washed with 10 mL of 5% sodium bicarbonate solution, and rinsed to neutrality with 10 mL of saline and 10 mL of DI water. The organic phase was diluted with 100 mL of CHCl$_3$, dried over Na$_2$SO$_4$, removed of CHCl$_3$ by a rotavap. Then most octanol was removed by a rotavap under high vacuo to give a yellow solid.
3. The mixture was dissolved with ~6 mL CHCl₃ and loaded on a column (11 mL silica gel, 60Å, size: 40-63μm, SorbentTech, 30930M-05).

Blank: 25 mL CHCl₃ + 20 mL CHCl₃/CH₃OH = 20/1

Mixture of octanol (Rᶠ = 0.81 in CHCl₃/CH₃OH = 6/1) + product (Rᶠ = 0.66 in CHCl₃/CH₃OH = 6/1): 10 mL CHCl₃/CH₃OH = 20/1

Product: 10 mL CHCl₃/CH₃OH = 20/1 + 70 mL CHCl₃/CH₃OH = 10/1

The desired factions were combined and dried by a rotavap and high vacuo to give 0.0232 g yellow solid (yield: 32 %). NMR (009H-DMSO, 009C-DMSO) proved the structure.

Second reaction attempt at same scale and conditions: yield 51 %

**AppA - Fig. 2 – (008H) Proton NMR of MTX-dioctyl ester in deuterated DMSO with integrals displayed.**
**Nanoparticle Formulation**

MTX-dioctyl ester was dissolved in DMSO at 20 mg/mL with 20 mg/mL 1.5k-5k PS-\textit{b}-PEG (gift from Douglas Adamson, University of Connecticut). 3 mL of this solution was rapidly mixed against 3 mL of water in a confined impinging jets mixer and quenched with a 27 mL stirred water bath. The nanoparticles were dialyzed for 24 hours to improve stability and analyzed by DLS for sizing. No loss of the characteristic yellow color was observed through the dialysis membrane. The nanoparticles were again sized by DLS after 3 weeks to assess particle stability.

**Methotrexate UV-vis Absorbance Calibration Curve**
**AppA - Fig. 4** – *MTX in DMSO Calibration Curve at 388 nm.*

**AppA - Fig. 5** – *Size distribution of MTX-dioctyl ester nanoparticles at following dialysis (■) and 3 weeks later (●).*
CONCLUSIONS/NEXT STEPS

Hydrophobic MTX prodrugs were successfully synthesized and formulated in nanoparticle cores to form 60 nm nanoparticles that were stable over a period of 3 weeks in water. Further experiments of interest would involve targeting such nanoparticle for specific disease treatments into order to improve delivery of the drug to the disease site, and reduce side effects.

ACKNOWLEDGEMENTS

I would like to thank Dr. Lei Shi for extensive assistance with the synthesis and characterization of the methotrexate conjugate.
APPENDIX B

Cation Chelation for Long Circulating MRI Contrast and Radiotherapeutic Agents

INTRODUCTION

Radiotherapy has been used for many decades in the treatment of numerous diseases, but perhaps most prominently in the treatment of cancer. While initial radiation therapies were relatively unfocused and caused broad and numerous undesirable side effects, more recent technologies such as the gamma knife[16] have proven far more efficacious. These developments mirror the trend in other areas of medicine where more specific and targeted treatments to improve patient outcomes and reduce systemic toxicity and side effects are being explored.

In particular, targeted treatments are a key component of nanomedicine, the engineering of materials at the nanoscale to improve medical outcomes. Nanoparticles or nanocarriers are the next step up from molecular engineering which is really the domain of physical and synthetic chemistry. Some of the more common nanomedical constructs include dendrimers, liposomes, inorganic nanoparticles (gold, silver, silica, iron oxide, etc.), polymeric nanoparticles, and engineered proteins.

Initial efforts in the area of targeted radiotherapy have utilized radionuclides conjugated to proteins (monoclonal antibodies) that have been demonstrated to bind and internalize into specific types of receptors and cells. Two such Abs in commercial use include trastuzumab and rituximab. The proteins chosen have both targeting and therapeutic effects.
The radionuclides used can be categorized by the type of radioemission they produce. The key characteristics are summarized in **AppB-Table 1**. The most popular radionuclides in use today use \( \beta \)-emitters. \( \alpha \) emitters are also in use and possess higher energy deposition over a shorter range than \( \beta \)-emitters. Although Auger emitters can be used, they have extreme cytotoxicity and short range energy deposition that only makes them useful within cell nuclei.

**AppB-Table 1 – Characteristics of various radionuclides used in targeted radiotherapy**

<table>
<thead>
<tr>
<th>Emission Type</th>
<th>Mean path length</th>
<th>Deposition energy</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )</td>
<td>40-100 μm</td>
<td>80 keV/μm</td>
<td>Bi, (^{211})At, (^{225})Ac</td>
</tr>
<tr>
<td>( \beta )</td>
<td>275 μm</td>
<td>0.2 keV/μm</td>
<td>(^{131})I, (^{90})Y, (^{67})Cu</td>
</tr>
<tr>
<td>Auger</td>
<td>1-20 μm</td>
<td>20 keV/μm</td>
<td>(^{67})Ga, (^{123,125})I, (^{195})mPt</td>
</tr>
</tbody>
</table>

Thus, the important factors for radionuclide selection are tumor access, targeting time (vs. half life), and the size of the disease relative to energy deposition length.

The major advantage of radiotherapy over traditional chemotherapy is the extremely general effect of strong radiation upon nucleic acids and cell material. In general, only 1-3 tracks across the nucleus of a cell are enough to cause cell death.[17] Tumor resistance cannot develop to the effects of radionuclides as they can to chemotherapy. Of course, such a powerful effect is a double edged sword and must be controlled well by specific cell targeting, highly stable chelation of the radionuclide to prevent renal or hepato-toxicity during excretion, and correctly choosing the radionuclide so that the energy deposition length is small or equal to the disease length scale and the half-life of the radionuclide is sufficiently long relative to the circulation time to achieve sufficient tumor penetration.

*Linking Chemistry*
The radionuclide must be coupled to the nanocarrier and targeting moiety by chemical means – the most common agent used is the bifunctional chelating agent (BCA). BCAs provide stable chelation along with chemical functionalities to enable covalent bonding to proteins, polymers, or other chemical species. Very strong chelating stability is needed to prevent heavy metal toxicity. The strength of the interaction depends on many factors including the metal coordination number, ionic radius, hard/soft binding characteristics, and reactivity (chelation time vs. half-life vs. stability (binding constant)). In general, cyclic chelating agents (DOTA, NOTA) are better than acyclic types (EDTA, DTPA) though longer loading times are needed for the former.[18]

Nanocarriers
According to Chang, et al.,[19] these radionuclide-Ab constructs suffer from several problems. They include low radioactivity : Ab ratio and short circulation time. The use of liposome or nanoparticles can alleviate these problems by packing many chelated radionuclides into the core or the nanocarrier (volume rather than surface area loading) and conjugating a few Abs per nanocarrier to the surface. This allows for a much more favorable radioactivity : Ab ratio with up to 14 Abs conjugated per liposome and an $^{225}$Ac:Ab ratio of 1:34 and also allows for multivalent interactions between the nanocarrier and cell surface receptors that improve binding strength and internalization.[20] The use of PEGylated liposomes or nanoparticles with well-defined sizes has been demonstrated to greatly improve circulation time[15, 21-23] which allows for tumor retention via a combination of enhanced permeation and retention[24] and active targeting by Abs conjugated to the nanocarrier surface.
Our initial approach followed these guidelines. We synthesized hydrophobic derivatives of the chelating agent DTPA as a proxy for the expensive DOTA molecule. The DTPA-(C\textsubscript{18})\textsubscript{2} construct was loaded into nanoparticle cores then complexed with Gd\textsuperscript{3+} (post-loading) or preloaded with Gd\textsuperscript{3+} (a proxy for the radioactive alpha emitter \textsuperscript{225}Ac\textsuperscript{3+}) and then formulated into nanoparticle cores using Flash NanoPrecipitation. Though Gd\textsuperscript{3+} was chosen as a non-radioactive proxy for \textsuperscript{225}Ac, it does have potential as an MRI contrast agent.

**Materials and Methods**

*Synthesis of hydrophobic chelators*

Due to the high cost of the bifunctional chelator DOTA-N3, we decided to focus our initial experimentation on the chelating agent diethylenetriamine pentaacetic acid (DTPA). DTPA shares many of the same features of DOTA but is acyclic and thus has weaker binding strength to trivalent metal ions.

Initial syntheses attempted to produce DTPA-dioctyl ester, DOTA-VESP (vitamin E propargyl succinate), and DTPA-dihexadecylamide. The DOTA-VESP synthesis yielded no product, and while the DTPA-dioctyl ester and dihexadecylamide constructs were synthesized successfully, they were found to be insufficiently hydrophobic for nanoparticle formulation. Thus, the final synthesis reacted DTPA dianhydride with octadecylamine to produce DTPA-dioctadecylamide.

*Synthesis of Dioctadecylamide of DTPA*
AppB-Fig. 1 – *Reaction scheme for coupling DTPA with 1-hexadecylamine.*

<table>
<thead>
<tr>
<th>material</th>
<th>formula</th>
<th>M. W.</th>
<th>purity, w %</th>
<th>amount, g</th>
<th>millimol</th>
<th>equiv.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTPA dianhydride</td>
<td>C14H19N3O8</td>
<td>357.316</td>
<td>98</td>
<td>1.000</td>
<td>2.74</td>
<td>1.00</td>
</tr>
<tr>
<td>Octadecylamine</td>
<td>C18H39N</td>
<td>269.51</td>
<td>99</td>
<td>1.6426</td>
<td>6.03</td>
<td>2.2</td>
</tr>
<tr>
<td>product</td>
<td>C50H97N5O8</td>
<td>896.33</td>
<td>100</td>
<td>1.4149</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>

1. Octadecylamine (Aldrich, 74750-25G) and diethylenetriaminepentaacetic dianhydride (DTPA dianhydride, Aldrich, 284025) were dissolved in 100 mL of anhydrous CHCl₃. The reaction was refluxed for 36 h at 78 °C under dry Ar.

2. The reaction mixture was cooled to room temperature. The precipitate was filtered, stirred for 10 min in 30 mL hot water (85 °C), and filtered (P5 filter paper, very slow, overnight). The wet solid was mixed with 20 mL water and 20 mL ethanol and filtered again (P5 filter paper, fast). The solid was stirred in 20 mL ethanol overnight and filtered. After drying by high vacuo, 1.4149 g of white solid was achieved as the desired product (Yield: 57 %). NMR (in 1.0 mL CDCl₃ + 1.0 mL CD₃OD + 0.2 mL triethylamine, 007-1H; in CF₃COOD, 007-2H) proved the structure.

3. For further purification, 0.500 g of the product was added to 350 mL boiling ethanol and stirred for 1 h. Cooled to room temperature, the precipitate was filtered and dried by high vacuum to give 0.4488 g white solid (NMR: CF₃COOD, 007-3H).

The resulting DTPA-(C₁₈)₂ construct was then loaded with gadolinium.
Complexation of Gd-DTPA-C<sub>18</sub> Diamide

![Reaction scheme for complexing Gd<sup>3+</sup> with DTPA-(C<sub>18</sub>)_2.]

**AppB-Fig. 2 – Reaction scheme for complexing Gd<sup>3+</sup> with DTPA-(C<sub>18</sub>)<sub>2</sub>.**

<table>
<thead>
<tr>
<th>material</th>
<th>formula</th>
<th>M. W.</th>
<th>purity, w %</th>
<th>amount, g</th>
<th>millimol</th>
<th>equiv.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTPA distearylamide</td>
<td>C50H97N5O8</td>
<td>896.33</td>
<td>100</td>
<td>0.2000</td>
<td>0.223</td>
<td>1.00</td>
</tr>
<tr>
<td>GdCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>GdCl3</td>
<td>263.61</td>
<td>99.9</td>
<td>0.0633</td>
<td>0.240</td>
<td>1.08</td>
</tr>
<tr>
<td>product</td>
<td>C50H94GdN5O8</td>
<td>1050.56</td>
<td>100</td>
<td>0.1631</td>
<td>/</td>
<td></td>
</tr>
</tbody>
</table>

Preparation

1. DTPA distearylamide was suspended in 150 mL of boiling ethanol. A few drops of NH<sub>4</sub>OH solution (NH<sub>3</sub>: 28-30 % wt) was added to the suspension which immediately turned clear. GdCl<sub>3</sub> (Strem Chemicals, 93-6416, anhydrous) dissolved in 1.0 mL DI water was added dropwise under stirring. The reaction was continued for 1 h.

2. After removal of most solvent by a rotavap, the reaction mixture was cooled to 0 °C. The white solid was filtrated, stirred with 15 mL of DI water, and filtrated again. The water washing was repeated twice.

Finally, the solid was dried by high vacuo to give 0.1631 g of white powder (yield: 69 %).

**Gd<sup>3+</sup> Concentration Quantification**
The concentration of free Gd\textsuperscript{3+} was quantified by a colorimetric assay based on xylenol orange.[25] Xylenol orange displays two UV-vis absorbance peaks (433 and 563 nm), the ratio of which changes depending on the degree of Gd\textsuperscript{3+} complexation. The color of xylenol orange also depends on pH, thus the pH must be buffered to get accurate Gd\textsuperscript{3+} concentrations. By creating a calibration curve of the Peak 2: Peak 1 ratios vs. Gd\textsuperscript{3+} concentration, the Gd\textsuperscript{3+} concentration in unknown solutions can be determined.
RESULTS AND DISCUSSION

AppB-Fig. 3 – NMR Spectrum of DTPA-(C\textsubscript{18})\textsubscript{2} with integrals.

We synthesized the desired compounds as shown in AppB-Fig. 3, and formulated both DTPA-C\textsubscript{18} and Gd-DTPA-C\textsubscript{18} into nanoparticles. As seen in AppB-Fig. 4A, PS-\textit{b}-PEG micelles were approximately 30 nm in diameter. In AppB-Figs. 4B and 4C, nanoparticles comprised of DPTA-C\textsubscript{18} and protected with PS-\textit{b}-PEG were found to be relatively unstable over time, containing two populations of micelles and nanoparticles with the micelle population growing over 1 month and 9 days. Pre-loaded Gd\textsuperscript{3+}-DTPA-C\textsubscript{18} containing nanoparticles similarly formed a micelle and nanoparticle population. Following dialysis, the two populations are not
distinguishable by light scattering, but tend towards a single micelle population. Thus, the high polarity of the DTPA and Gd-DTPA complexes do not allow for stable encapsulation.

**CONCLUSIONS**

The results of these studies were largely negative, as it appeared that despite the C\textsubscript{18} chains and theoretical charge neutrality of the Gd\textsuperscript{3+}-DTPA-(C\textsubscript{18})\textsubscript{2} construct, it was not sufficiently soluble in solvents suitable for FNP and the nanoparticles were not sufficiently stable for further study. We
changed the approach to surface loading of Gd-DTPA rather than core loading. This study was undertaken by a senior thesis student and can be found in Ellis, R.A., *Nanoparticle Delivery of Radionuclides for Diagnostic and Therapeutic Applications*, in *Chemical and Biological Engineering*. 2012, Princeton University: Princeton, NJ.
APPENDIX C

Sterilization Procedures for Nanoparticle Animal Studies

(adapted from Kumar, V., PhD Thesis, Princeton University, 2011)

Multi-inlet Vortex Mixer:

1. Rinse/immerse with ethanol (1x)
2. Rinse/immerse with de-ionized water (1x)
3. Rinse/immerse with 40% CIP 100 detergent solution (1x)
4. Rinse/immerse with de-ionized water (3x)
5. Rinse/immerse with 40% CIP-200 detergent solution (1x)
6. Rinse/immerse with de-ionized water (3x)
7. Rinse/immerse with ethanol (2x)

Bio-hood area: sterilize with 70/30 ethanol/water and then with ethanol.

Glass syringes, tubings: soak in 70/30 ethanol/water. Then rinse with ethanol in bio-hood.

Aqueous and organic solutions: filter through 0.2 µm sterile syringe filter with a sterile syringe into a sterile vial in the bio-hood.

Parafilm, dialysis bags (regenerated cellulose): rinse with 70/30 ethanol/water and UV irradiate in the bio-hood.
Autoclave (water, pipet tips): use liquid cycles for 1:9; do not seal caps; secure with autoclave tape/foil; dry items should be autoclaved on gravity cycle; lay pipet-tip boxes on the side.

NP formation procedure

In sterile bio-hood: Load the syringes with solutions and assemble them with the mixer. Guide the outlet tube from mixer into a 50 mL sterile vial (SV50S, Allergy labs) through a sterile needle. A secondary needle should be used to act as a vent and the opening of vent should be connected to a 0.2 µm syringe filter. After NP formation, the sterile vial with NPs solution should be brought inside the hood for further processing.

Dialysis: Dialysis should be done with autoclaved water.

Freeze-drying: Freeze-drying should be done in sterile cryo-vials.
REFERENCES (APPENDICES A-C)


**APPENDIX D**

**CHAPTER 2 – Biomedical Fluorescence Imaging using Pentacene Dyes**

*AppD-Fig. 1 – Family of pentacene- and hexacene-based fluorescent dyes studied. Structure of fluorescent molecule Et-TP5 (MW: 839.3 g/mol) with other similar fluorescent molecules studied: (a) Et-TP5, (b) TSB-SP5, (c) iBu-TP5, (d) Ph-TP5, (e) iBu-TPH5.*

*AppD – Fig. 2 – Uptake of ~100 nm Et-TP5 NCs into J774E mouse macrophage cells (red – DAPI nuclear stain, green – Et-TP5 nanoparticles). The NCs are targeted using mannose ligands on the PEG chains. The concentrated green fluorescence indicates internalization into endosomes, and the diffuse green fluorescence shows release of the NCs into the cytosol. Reproduced with permission from D’Addio, et al. (2012).[1]*
**AppD - Fig. 3** – Absorbance vs. wavelength for various components commonly found in blood and tissues (_water_ | _proteins_ | _Hb_ | _HbO_2_ | _melanin_ | _collagen_). The portion of the spectrum from 650-1450 nm is known as the ‘imaging window’ where the optical absorption of the various substances is at a minimum. The optical absorption coefficient is an inverse length and thus represents a characteristic penetration depth. The maximum penetration depth is achieved in the region of minimal absorbance. Reproduced from Pansare, et al. (2012).[2]

**COMPARISON OF CORE LOADED VS. SURFACE LOADED DYES**

Three nanocarrier formulations were prepared utilizing the dyes ETTP5, LD688 (Exciton Corp., Ohio), and Alexa Fluor 488 (Life Technologies, Inc.). ETTP5 and LD688 were precipitated by Flash NanoPrecipitation in the core of the NCs while AF488 was conjugated to the surface. The formulation details are presented in **AppD-Table 1**. The ETTP5 and LD688 NCs were loaded at the optimum loading for fluorescence. The AF488 NCs were not optimized for fluorescence.

The spectra are shown in **AppD-Fig. 4A-C**.
### Table 1 – Formulation details for the three NC systems studied to compare surface vs. core loading of dyes.

<table>
<thead>
<tr>
<th>Dye:</th>
<th>ETTP5</th>
<th>LD688</th>
<th>AF488</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Dye] (mg/mL)</td>
<td>0.024</td>
<td>0.018</td>
<td>0.058</td>
</tr>
<tr>
<td>[PS] (mg/mL)</td>
<td>0.82</td>
<td>0.81</td>
<td>19</td>
</tr>
<tr>
<td>[PS-b-PEG] (mg/mL)</td>
<td>0.85</td>
<td>0.86</td>
<td>19</td>
</tr>
<tr>
<td>[Total NP solids] (mg/mL)</td>
<td>1.7</td>
<td>1.7</td>
<td>38.2</td>
</tr>
<tr>
<td>Core Loading (%)</td>
<td>2.28</td>
<td>1.77</td>
<td>--</td>
</tr>
<tr>
<td>NP Loading (%)</td>
<td>1.4</td>
<td>1.1</td>
<td>0.15</td>
</tr>
<tr>
<td>NP Size (nm)</td>
<td>100-120</td>
<td>100-120</td>
<td>80</td>
</tr>
</tbody>
</table>

**AppD-Fig. 4** – Excitation and emission spectra for (A) ETTP5, (B) LD688, and (C) Alexa Fluor 488. (D) Fluorescence vs. nanocarrier concentration for AF488 nanoparticles – the slope of the line represents the intrinsic fluorescence of the NC system.
AppD-Table 2 – Intrinsic Fluorescence Intensity (slope of fluorescence vs. [NC]) for the three NC systems studied.

<table>
<thead>
<tr>
<th>Dye:</th>
<th>ETTP5</th>
<th>LD688</th>
<th>AF488</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ&lt;sub&gt;ex/em&lt;/sub&gt;</td>
<td>460/638</td>
<td>488/615</td>
<td>496/521</td>
</tr>
<tr>
<td>NP Loading (mass frxn.)</td>
<td>1.4%</td>
<td>1.1%</td>
<td>0.15%</td>
</tr>
<tr>
<td>NP Size</td>
<td>110</td>
<td>110</td>
<td>80</td>
</tr>
<tr>
<td>Intrinsic FL (NP mass)*</td>
<td>277000</td>
<td>129000</td>
<td>243000</td>
</tr>
<tr>
<td>Intrinsic FL (mol dye)**</td>
<td>1.7×10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>4.3×10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>8.2×10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Slope of fluorescence vs. [NP] (mg/mL) (AppD-Fig. 4D)
**Slope of fluorescence vs. [dye] (M)

These results in AppD-Table 2 show that ETTP5 NCs are more fluorescent than the LD688 and AF488 despite being having longer wavelength emission (normally quantum yield goes down at higher wavelengths). However, the ETTP5 NCs are slightly larger than the AF488 carriers. On the other hand, due to the 90+% quantum yield of the AF488, it is brighter than the other dyes on a dye molar basis. The ETTP5 and LD688 loadings were optimized for maximum fluorescence while the AF488 carriers were not. So it is possible that the AF488 particles could be brighter on a nanoparticle mass basis if the dye loading were increased, mainly due to the high quantum yield of the dye. However at longer wavelengths (which are more relevant for biomedical imaging), the longer emitting Alexa Fluors have lower quantum yield.
**AppD-Fig. 5** – Comparison of emission spectra (normalized) for Et-TP5 excitation wavelengths at 458 and 600 nm.

**AppD-Fig. 6** – Size stability of 78% core loaded Et-TP5 NCs over time (■ pre-dialysis | ◇ post-dialysis | ▲ +3 weeks | ▼ +12 weeks).
**AppD-Fig. 7** – Change in fluorescence over 10 months (solid: starting, dashed: +10 months) for Et-TP5 loaded NCs at various core loadings. A 20% drop was observed for NCs loaded at 9.4% while there was a slight increase in fluorescence at higher loadings, probably because Förster quenching was reduced by photodegradation of the dye (resulting in lower effective loadings closer to the optimum loading).
**AppD-Fig. 8** – *Fluorescence emission of Et-TP5 in THF (■) vs. toluene (●) at 0.05 mg/mL with λ<sub>ex</sub> = 600 nm.*
REFERENCES


MATERIALS AND METHODS

ICG NC-BSA Stability Experiments

The BSA transfer experiments were divided into two parts depending on whether the hydrophobic ICG complex was formed in situ, or was premade (ionic or covalent constructs).

In Situ:

NCs were prepared by Flash NanoPrecipitation with approximately 10 wt% ICG core loading and 20-24 mg/mL total solids loading in the feed streams. TOAC or TDDAC was present in a 1:1 molar ratio or 5:1 excess molar ratio with ICG, VE was present at 1 wt% in THF (8.9 mg/mL), and PS-b-PEG (1.6k-b-5k) was present at 10-11 mg/mL. Three sets of NCs were prepared: ICG-C₈ in situ (where C₈ indicates the tetraoctylammonium counter ion), ICG-C₁₂ in situ (where C₁₂ indicates the tetradoodecylammonium counter ion), and ICG-C₁₂ in situ with 5-fold excess TDDAC. All NCs were dialyzed for 24 hours against MilliQ water before BSA testing.

Premade Ionic Complex:

NCs were prepared by Flash NanoPrecipitation with approximately 10 wt% ICG complex core loading and 20.2 mg/mL total solids in the feed stream. Premade ICG-C₈ or C₁₂ complexes were present at 1.2 mg/mL, VE at 8.9 mg/mL, and PS-b-PEG (1.6k-b-5k) at 10.1 mg/mL in THF. A 2:1 mixture of THF:DMSO was used to dissolve the ICG-C₁₂ premade complex. This did not
affect the solubility of the other components. Two sets of NCs were prepared: ICG-C₈ premade and ICG-C₁₂ premade. All NCs were dialyzed for 24 hours against MilliQ water.

Dialyzed NCs were again analyzed for size by DLS and for ICG and VE concentration by absorbance. The NCs were split into aliquots containing 5 mM sodium phosphate added (pH adjusted to 7 with 1M HCl or NaOH) with or without 4 wt% (40 mg/mL) BSA. Samples were incubated with and without phosphate and/or BSA at RT or 37°C for at least 2 hours (see BSA equilibration time section) before multiple fluorescence measurements were taken at multiple dilutions to ensure the linearity of fluorescence vs. [NC]. The slope of fluorescence vs. [NC] represents the intrinsic fluorescence of the NC or NC+BSA system. Partitioning of the ICG out of the NC cores was therefore measured by the change in intrinsic fluorescence of each NC system upon the addition of BSA.

*Comparison of ICG-TDDAC ion-paired NCs formed in situ vs. premade covalent*

*In situ formation:* ICG-TDDAC (C₁₂) in situ complex NCs were formed by rapidly mixing 1 part aqueous feed stream containing 0.8 mg/mL ICG against 1 part THF feed stream containing 1 molar equivalent of TDDAC, 8.2 mg/mL VE, and 9.7 mg/mL PS-b-PEG into an aqueous quench reservoir containing 9 parts water (overall 1:10 mixing ratio) to give 12% ICG complex core loading. Total NC solids in the feed streams was 19.5 mg/mL.

*Premade formation:* ICG-covalent NCs were formed by rapidly mixing 1 part aqueous feed against 1 part THF feed stream containing 1.5 mg/mL ICG-covalent (0.77 mg/mL ICG), 7.8
mg/mL VE, and 9.4 mg/mL PS-b-PEG into an aqueous quench reservoir containing 9 parts water (overall 1:10 mixing ratio) to give an ICG-covalent core loading of 12.9%. Total NC solids in the feed stream was 17.2 mg/mL.

RESULTS AND DISCUSSION

1. NC Size Control Additional Results

![Graph showing the relationship between NP Diameter (nm) and Total Solids Pre-Mixing (mg/mL).]

**AppE-Fig. 1** - **Demonstration of NC size control by controlling total solids concentration in the THF/water streams while keeping the ratio of the various components constant.** Interestingly, the size plateaus at about 180 nm then decreases with increasing solids concentration. The mechanism for this trend follows the explanation given in the body text regarding the competition between supersaturation and aggregation dependencies on solute concentration.

2. Stability of various ICG NC formulations against BSA

*ICG NC – BSA Stability Studies*
NCs with sizes from 70 nm up to 180 nm were created to assess the stability of 10wt% loaded ICG NCs formed by the *in situ* vs. premade (ionic and covalent) routes, with different core materials (PS vs. VE) in the presence of 4wt% albumin, a physiologically relevant concentration. Unfortunately, the assays to assess ICG partitioning to NCs were problematic. It was not possible to separate NCs from BSA using centrifugation, centrifugal filtration (due to membrane blockage), or salt precipitation. With these particles, we were only able to obtain total fluorescence change upon addition of BSA to various NC systems and were unable to quantify the precise amount of dye that partitioned out in each case. In the main Results and Discussion section, we estimated the amount of ICG lost for the case of the ICG-C₈ premade complex using the fluorescence change over time and a fluorescence calibration curve.

Although the base dye in each system is ICG, the extinction coefficient varies between systems depending on the counter ion ((C₈)₄-NH₄⁺ vs. (C₁₂)₄-NH₄⁺). This is due to the molecular environment and torsional mobility of the ICG, which depends on the counter ion. In this section, we also tested the stability of a novel covalently modified ICG construct provided by Persis Science and depicted in AppE-Fig. 2, termed ICG-covalent. Rather than ion pairing TOAC, hydrophobicity is provided by covalently linking C₈ chains to the tail chains.
The partitioning of ICG out of NC cores to BSA was quantified by calculating the change in intrinsic fluorescence of each system was done by linear regression of fluorescence against absorbance (where absorbance serves as an optical proxy for ICG concentration). Following dialysis, each NC formulation’s ICG absorbance was characterized to establish the initial extinction coefficient, as shown in AppE-Table 1. The somewhat higher values of the extinction coefficients for the premade formulations is probably due to the slightly different stoichiometry between counter ion and ICG for the in situ formed and pre-made ion pair.
**AppE-Table 1** – Absorbances and Extinction Coefficients of various ICG constructs and NCs formed for the ICG NC-BSA transfer experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance Undialyzed</th>
<th>Absorbance Dialyzed</th>
<th>Undialyzed Extinction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICG-C8 in situ</td>
<td>13.1</td>
<td>11.1</td>
<td>$1.40 \times 10^5$</td>
</tr>
<tr>
<td>ICG C8 premade</td>
<td>13.4</td>
<td>11.2</td>
<td>$1.57 \times 10^5$</td>
</tr>
<tr>
<td>ICG-C12 in situ</td>
<td>10.1</td>
<td>8.6</td>
<td>$1.23 \times 10^5$</td>
</tr>
<tr>
<td>ICG-C12 premade</td>
<td>16.8</td>
<td>13.7</td>
<td>$2.31 \times 10^5$</td>
</tr>
<tr>
<td>ICG-covalent PS core</td>
<td>10.5</td>
<td>9.2</td>
<td>$1.22 \times 10^5$</td>
</tr>
<tr>
<td>ICG-covalent VE core</td>
<td>12.0</td>
<td>9.9</td>
<td>$1.40 \times 10^5$</td>
</tr>
</tbody>
</table>

*Extinction coefficients for the premade ionic complexes are calculated on an ICG basis, that is MW = 775 g/mol, rather than a full complex basis. This allows for direct comparisons between the in situ and premade complexes. However, the ICG-covalent has a much larger MW (1271 g/mol) for this extinction calculation.

The change in absorbance going from undialyzed to dialyzed samples is about 15% for all samples. This is attributed to a volume increase during dialysis that is typically about 15% for these types of PEGylated NCs that have significant osmotic pressure. With the data in **AppE-Table 1**, plots of fluorescence vs. absorbance (as a proxy for NC concentration) can be directly compared.

The retention of ICG in NCs in the presence of BSA was studied with several types of ICG constructs in a vitamin E (VE) core: ICG-C$_8$ *in situ* vs. premade and ICG-C$_{12}$ *in situ* vs. premade. We also tested the ICG-covalent compound in both VE and PS cores. The samples were incubated with 4wt% BSA (40 mg/mL) at RT and 37°C to reflect physiological conditions.[1-3] NC concentrations were held constant at ~0.18wt% (1.8 mg/mL) and sizes were on the order of 70-180 nm with 10% ICG complex loading.
AppE-Fig. 3 – Intrinsic fluorescence slopes of various ICG NC and ICG NC – BSA systems (A – ICG-C₈ in situ complex, B – ICG-C₈ premade complex, C – ICG-C₁₂ in situ complex, D – ICG-C₁₂ premade complex, E – ICG-C₁₂ covalent construct - PS core, F – ICG-C₁₂ covalent construct - VE core). Conditions: NCs @ RT, NCs @ 37°C, NCs w/ phosphate @ RT, NCs + BSA @ RT, NCs + BSA @ 37°C. Fluorescence slopes represent the intrinsic fluorescence of each NC or NC + BSA system in the linear regime where fluorescence vs. ICG absorbance is linear (the infinite dilution regime). By taking fluorescence measurements at multiple dilutions, we ensure that measurements are being taken without the influence of NC scattering and secondary absorption-emission events.
Since the fluorescence of ICG is improved upon protein binding due to the stabilization of the molecule,[4] the overall signature of transfer to ICG to protein is an increase in system fluorescence. Also, there is a change in fluorescence wavelength (25 nm redshift) [5] upon binding which was not observed in our studies where the fluorescence did not change significantly.

**AppE-Fig. 3** displays the results obtained from the BSA transfer experiments completed with several different types of ICG constructs. NCs were incubated at RT and 37°C, with phosphate, and with BSA at RT and 37°C to separate the effects of each incubation condition on the fluorescence signal. In general, we see that incubation at 37°C in MilliQ water has the tendency to cause slight degradation of the ICG leading to a loss of signal and addition of phosphate increases the ionic strength of the medium which appears to raise the fluorescence slightly. We see that there is a modest change in fluorescence upon BSA addition for ICG-C₈ in situ (**AppE-Fig. 3(A)**) and a slightly larger change for ICG-C₈ premade complex (**AppE-Fig. 3(B)**) (about 15-18% increase in fluorescence). This is in contrast to the much larger change seen for the ICG-C₁² systems (in situ (**AppE-Fig. 3(C)**, 46% increase) and premade complex (**AppE-Fig. 3(D)**, 45% increase)), a surprising result given that the (C₁₂)₄-NH₄⁺ counter ion was expected to confer much greater hydrophobicity to the complex and therefore better stability. Additionally, we see that the ICG-C₁₂ premade complex has double the intrinsic fluorescence of the ICG-C₁₂ in situ system (98000 vs. 48000 intrinsic fluorescence units). This is in accordance with the extinction coefficient for the ICG-C₁₂ premade complex NCs being almost double that for the ICG-C₁₂ in situ complex (see **AppE-Table 1**).
Results for the ICG-covalent compound indicate that the ICG-covalent compound is relatively stable to the BSA sink (though not much more than the ICG-C_8 compounds) but only in a vitamin E (VE) core, not in a polystyrene (PS) core. In the case of PS (AppE-Fig. 3(E)), the fluorescence signal almost triples upon addition of BSA, mostly likely due to the chemical incompatibility of the ICG-covalent compound and PS, which may be due to the glassy matrix the PS provides. Compared to that, the VE core shows remarkable stability with no change in fluorescence (AppE-Fig. 3(F)) upon BSA addition. This is in agreement with the study on ICG loading limits in various cores in the main Results and Discussion section. This shows that it is not only the inherent hydrophobicity and light stability of the ICG construct that is important, but also the core material used for encapsulation. This study does need to be repeated to establish the magnitude of error, since the fluorescence increases seen here may or may not be significant given the inherent error in the fluorometer measurements.

3. ICG-PDMS NCs

*BSA Testing of PDMS NCs containing ICG-C12 premade complex.*

We formulated ICG NCs with PDMS shells. The aim was to see if the incompatibility of the ICG with the PDMS shell would stabilize the NC against ICG partitioning to albumin. They were stabilized by PDMS_{1.6k-b-PEG_{1.6k}} and contained ICG-C_{12} premade complex in order to more effectively stabilize the ICG within NC cores in the presence of albumin. NCs were formulated with vinyl terminated PDMS of MW 500 (DMS-V05, Gelest Corp.) and 6000 (V21, Gelest Corp.) , hydride PDMS (HMS-991, Gelest Corp.) as a cross linking backbone, and a
platinum catalyst at $10^{-3}$ weight fraction relative to PDMS to cross link the NCs \textit{in situ} (SIP6830.3, Gelest Corp.). The ICG complex and PDMS components as well as PDMS-PEG were dissolved in the THF stream and precipitated to form NCs containing 10wt\% core loading ICG complex. The extent of cross-linking was tested by dissolving 1 part of the dialyzed NCs in 4 parts THF, then removing the THF by rotary evaporation and comparing the resulting size distribution to the original NC size distribution. As seen in \textbf{AppE-Fig. 4(A) and (B)}, the cross-linking was not complete, as the size distribution moved up significantly.

We found that the PDMS NCs did not effectively trap the ICG-C12 premade complex. As seen in \textbf{AppE-Fig. 4(C) and (D)}, large increases in intrinsic fluorescence were seen upon incubation with BSA. The lower intrinsic fluorescence of the 500 Da PDMS (V05) NC system compared to that of the 6000 Da PDMS (V21) system is most likely due to attack of ICG by the Pt catalyst used to cross-link the NCs \textit{in situ}. It is not clear why the V21 NCs incubated with BSA at 37°C has such a low intrinsic fluorescence in (D).
AppE-Fig. 4 – BSA transfer results for ICG-C12 premade complex NCs. (A) and (B) Size distributions of NCs before (▬) and after (▬) THF breakage test. (C) and (D) BSA transfer results showing large increases in intrinsic fluorescence upon the introduction of BSA to the PDMS NC systems.

4. In Situ vs Covalent ICG NCs

Comparison of size, absorbance, and fluorescence of in situ ICG-C_{12} vs. ICG-covalent NCs

VE core NCs comprising the ICG-TDDAC (ICG-C_{12}) complex formed in situ were compared against NCs comprising the covalent ICG compound. The total solids content and ICG loadings were held approximately the same in order to produce NCs with similar sizes, extinction
coefficients, and intrinsic fluorescence values. As seen in AppE-Fig. 5(A), the in situ vs. covalent ICG particles were 60 vs. 70 nm. We also measured the fluorescence of these particles at various NC dilutions as seen in AppE-Fig. 5(B). At high concentrations of the NCs, the fluorescence values are approximately the same due to scattering and absorption. The intrinsic fluorescence (determined at low NC concentration) of the covalent compound was higher (higher slope) than the in situ formed complex NCs. This is in agreement with the fluorescence data in the BSA transfer experiments in AppE-Fig. 3. In AppE-Fig. 5(C) and (D) we compared the extinction coefficients of the two different constructs, which are essentially the same. However, it is apparent that whether the ICG construct is encapsulated in a VE core or dissolved in THF makes a large difference in the observed extinction (>20% increase when in THF). This is likely due to a reduction in the absorption cross-section when encapsulated in NCs and negative deviations from Beer’s Law due to aggregation in the liquid VE core. The level of free ICG in each formulation was effectively 0, as determined by centrifugal filtration of dialyzed NCs, showing that the encapsulation efficiency was 100%, similar to the 1:1 pairing of the ICG-TOAC.
**AppE-Fig. 5** – Comparison between NCs containing ICG-C_{12} formed in situ vs. covalent ICG-C_{12} construct. (A) Size of NCs formed (■ in situ | ● covalent). (B) Fluorescence of in situ (■) and covalent ICG-C_{12} compounds (●) vs. concentration of the complex. (C) and (D) spectra of ICG-C_{12} in situ NCs and ICG-C_{12} covalent NCs in water (■), dissolved in THF (●), and free ICG present in the filtrate from a 100k Amicon centrifuge filter (▲) showing the lack of fluorescence, which demonstrates the complete encapsulation in the NC.
AppE-Fig. 6 – Proton NMR of ICG-TOAC compound formed with peaks identified at the top and integrals at the bottom.
**AppE-Fig. 7** – *Long term size stability of various ICG-complex loaded NCs.*
REFERENCES


Chapter 4 - Nanoparticle Contrast Agents for Photoacoustic Imaging

IBMI COLLABORATION

**AppF-Fig. 1** - Optimization of \( LW_2 \) core loading for maximum fluorescent per particle. The fluorescence per particle is represented by the slope of fluorescence vs. concentration of nanoparticles.
AppF-Fig. 2 - Thermo gravimetric analysis of LW2 nanoparticles. TGA measurements of (A) LW2-100nm and (B) LW2-50nm formulation.
**AppF-Fig. 3** - Optical characterization of LW₂-micelles. (A) Absorbance spectra and (B) MSOT spectra of LW₂-micelles solutions at different concentrations (different optical densities). (C) Normalized absorbance and MSOT spectra of LW₂-micelles. (D) MSOT signal at 730 nm of LW₂-micelles solutions of different optical densities. The black line is a linear fit of the scatter ($R^2 = 0.98$). (E) Summary of physicochemical characteristics of the LW₂-micelles. (F) Photobleaching study of LW₂-micelles: MSOT signal evolution in a LW₂-micelles of OD=1 undergoing successive illuminations at 730 nm.
AppF-Fig. 4 - spectrophotometer analysis of LW2 nanoparticles. Spectrophotometer measurements at five different dilutions for (A) LW2-100nm and (B) LW2-50nm. (C) Relation between Optical density and Concentration for each formulation.

AppF-Fig. 5 - Performance of LW2 loaded Nanoparticles as optoacoustic contrast agents. (A & C) Optoacoustic Spectrum of LW$_2$-100nm (A) and LW$_2$-100nm (C) by MSOT at different concentrations; (B & D) Optoacoustic amplitudes of LW$_2$-nanoparticles at 730nm in an agar phantom as function of optical density for LW$_2$-100nm (B) and LW$_2$-50nm (D).
**AppF-Fig. 6** - Stability of LW2 loaded Nanoparticles. Stability study of LW2-100nm (A) and LW2-50nm (B) in PBS stored in dark during 7 days at 4°C.
AppF-Fig. 7 - Spectrophotometer analysis of ICG. (A) Spectrophotometer measurements at five different dilutions for ICG. (B) Photobleaching profile acquired by MSOT of ICG (Blue line) after 2000 seconds of continuous laser illumination at 800nm. (C) Unmixing of Optoacoustic signal after phantom acquisition by MSOT with different concentrations of ICG.
AppF-Fig. 8 - In vitro toxicity study of LW$_2$-50nm 48 hours after incubation within MDA MB 231 human breast cancer cell line (n=5).
OTHER DYES FORMULATED – BACTERIOCHLORINS

The bacteriochlorin series of dyes was developed in the group of Jonathan Lindsey at NC State University. They consist of a porphyrin core with easily substituted side groups that shift the excitation and emission spectra (AppF-Fig. 9A). These dyes generally have very sharp and well separated absorbance bands making them exceptionally useful as photoacoustic contrast agents (AppF-Fig. 9B).

AppF-Fig. 9 – (A) Structure of bacteriochlorin dyes and common substitutions to shift absorbance bands. (B) Absorbance spectra for bacteriochlorins B56, B58, and B146 from left to right. All have similar extinction coefficients on the order of 94000 M⁻¹ cm⁻¹. Reproduced from Ref. [1] with permission from the Centre National de la Recherche Scientifique (CNRS) and The Royal Society of Chemistry.

Nanoparticle formulations were made with bacteriochlorin B58 provided by NIRvana Life Sciences (North Carolina) with vitamin E (VE) as the co-solute and 1.6k-5k PS-b-PEG as the stabilizer. Formulations are shown in AppF-Table 1. Core loading of the dye was varied from 2.9% up to 56% to test stability. Generally formulations up to 45% core loadings were stable, though secondary peaks were seen below 10 nm (AppF-Fig. 10) from 35-50% loading and possible aggregates (> 6 μm) seen at 50% loading. At 56% loading there is significant
aggregation though it’s worth noting that no VE was used in that sample (AppF-Table 1, Sample 1-2). The peaks in AppF-Fig. 10B below 10 nm are possibly artifacts from absorbance/fluorescence in the sample since aggregates are generally observed in that size range.

For photoacoustic imaging, loadings as high as possible are preferred as a high loading leads to a greater degree of quenching (Förster Resonance Energy Transfer) and therefore greater photoacoustic signal.

**AppF-Table 1 - Nanoparticle Formulations of bacteriochlorin dye B58**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Core Loading (%)</th>
<th>[B58] (mg/mL)</th>
<th>[VE] (mg/mL)</th>
<th>[PS-b-PEG] (mg/mL)</th>
<th>[Total Solids] (mg/mL)</th>
<th>NP Diameter (nm)</th>
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<td>1-1</td>
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<td>Unstable</td>
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**AppF - Fig. 10** – Size distributions for bacteriochlorin B58 loaded nanocarriers as shown in AppF-Table 1. There is a gradual decrease in size as the loading increases.
Lastly, a 16 day photostability study (AppF – Fig. 11) was conducted by placing B58 in THF, formulation F1 (15% loading), and F5 (50% loading) in room light and dark conditions at room temperature. As seen, B58 in THF does not lose any absorbance in the dark but loses 80% of the signal in light conditions. Similarly, the 50% loaded carriers (F5) did not lose any signal in the dark, but did lose 20% signal in the light, showing that the vitamin E core and PS-b-PEG stabilizer improved photostability relative to solvent. For reasons that are not clear, formulation F1 (15% loading) exhibited large aggregates after 16 days and thus showed >60% loss of signal due to the aggregation. However, we would not expect significant loss of signal in the dark had the precipitation not occurred.

**AppF - Fig. 11 – 16 day photostability study of bacteriochlorin B58 in THF solution vs. in nanocarriers at low (F1) and high (F5) loadings.** A: Change in absorbance in light and dark conditions over 16 days. B: Ratio of final to initial absorbance over the 16 day time period in light and dark conditions.
**AppF-Fig. 12** – Size distributions of LW2-small (■) and LW2-large (●) photoacoustic nanocarriers produced for the VisualSonics collaboration study. The mean diameters are 25 and 100 nm respectively.

**AppF-Fig. 13** – Photoacoustic signal comparison of LW2-large vs. LW2-small nanocarriers held in polyethylene tubes.
REFERENCES

APPENDIX G

CHAPTER 5 - Small Molecule Adsorption to Colloidal Alumina

Preliminary Experiments with TiO\textsubscript{2}

Constant potential approximation (applying the Deryagin approx. to the flat plate potential, derived from Israelachvili (2011)[1] and Hogg (1966)[2]):

\[
V_{\text{sphere}} = \frac{2\pi R_1 R_2}{R_1 + R_2} \varepsilon_0 e \left[ -\Psi_1 \Psi_2 \ln \left( \tanh \frac{\kappa d}{2} \right) - \left( \frac{\Psi_1^2 + \Psi_2^2}{2} \right) (\kappa d - \ln(2 \sinh(\kappa d))) \right]
\]

Eqn. (1)

van der Waals interaction[1]:

\[
V_{vdW} = -\frac{A}{6d} \left( \frac{R_1 R_2}{R_1 + R_2} \right)
\]

Eqn. (2)

The net potential:

\[
V_{\text{net}} = V_{\text{electrostatic}} + V_{vdW}
\]

Eqn. (3)

Calculate DLVO interactions for TiO\textsubscript{2} spheres in water with \(\zeta = -55\) mV (experimentally determined) at different ionic strengths.

[AppG - Fig. 1 – DLVO calculations for TiO\textsubscript{2} nanoparticles (\(\zeta = -55\) mV) 50 nm (A), 100 nm (B), and 300 nm (C) in diameter showing interaction potentials normalized against \(kT\). Ionic strengths: \(10^{-4} M\) | \(10^{-3} M\) | \(10^{-2} M\) | \(10^{-1} M\). As a rule of thumb, 30\(kT\) is considered to be the threshold for stability.]
AppG-Fig. 2 – DLS and zeta potential measurements on Dupont R706 (left) and Dupont R900 (right) both at 0.001 wt% TiO2. Isoelectric point is ~4.6 and 3.5 respectively. According to the literature,[3] the IEP of rutile TiO2 is 5.6. Our results suggest that there is silica or aluminosilicate content in these TiO2 samples which would lower the IEP (AlSi IEP[4] = 4.5 and pure silica IEP is 2.2)[5]. Ionic strengths were not controlled. The IEP is not affected by this, but the magnitude of the zeta potential is.

AppG - Fig. 3 – (left) Zeta potential of poly acrylic acid coated TiO2. Zeta potential vs. pH for TiO2-poly(acrylic acid) dispersions in water. (■) 1:1 PAA:TiO2, [TiO2] = 0.00036 wt% and (●) 2:1 PAA:TiO2, [TiO2] = 0.00078 wt%. In this experiment the PAA coated particles are negatively charged until a pH ~2.5. At this point the polymer is no longer ionized (ie. its negative charge is turned off). It then leaves the particle surface and the positive surface charge becomes apparent. (Right) Zeta potential (■) and size (▲) vs. pH for TiO2-PAA in water. [TiO2] = 0.00078 wt%, 2:1 PAA:TiO2. The apparent particle size is “small” at low and neutral pH when the PAA chain is not swollen by electrostatic repulsions. The pKa of PAA is ~4.7. At higher pH the adsorbed chain swells and is more highly charged and gives the particle a thicker polymer layer. The polymer layer apparent thickness at pH=9 is 25 nm.
### AppG-Table 1 - *Ligand pKa data*

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<th>pKa and Source (2)</th>
<th>pKa and Source (3)</th>
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<td>268.11</td>
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<td>5.76 (0.1M, 25°C)</td>
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<td></td>
<td>Purification of Laboratory Chemicals (1-NP disodium)</td>
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<td>[7]</td>
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<td>Sodium 2-naphthalene sulfonate*</td>
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<td>0.27</td>
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</tbody>
</table>

*Sulfonic acids are in general considered very strong acids with >99% ionization. For comparison, the pKa of benzene sulfonic acid is -2.8 (Kirk-Othmer Encyclopedia).

**pKa for the naphthalene sulfonic group, not necessarily 2-naphthalene sulfonate

***Data for ethylene glycol are more readily available: pKa 15.1 (CRC Handbook of Chemistry and Physics (2012)).
**Langmuir Isotherm Calculation**

Definitions:

\[ [\text{Al}]_0 \] – initial concentration of alumina binding sites

\[ [\text{P}]_0 \] – initial concentration of phosphate species

\[ [\text{P}] \] – phosphate concentration after binding event (measured by UV-vis)

\[ [\text{Al}] \] – concentration of unbound alumina sites

\[ [\text{Al-P}] \] – concentration of bound sites

Reaction:

\[ \text{Al} + \text{P} \rightarrow \text{Al-P} \]

Equilibrium constant:

\[ K = \frac{[\text{A}-\text{P}]}{[\text{Al}][\text{P}]} \quad \text{Eqn. (4)} \]

Mass balance:

\[ [\text{Al}]_0 = [\text{Al}] + [\text{Al-P}] \quad \text{Eqn. (5)} \]

\[ [\text{P}]_0 = [\text{P}] + [\text{Al-P}] \quad \text{Eqn. (6)} \]

Rearranging (4):

\[ \Rightarrow [\text{Al-P}] = K[\text{Al}][\text{P}] \quad \text{Eqn. (7)} \]

Substitute (5) in:

\[ \Rightarrow [\text{Al-P}] = K([\text{Al}]_0-[\text{Al-P}])[\text{P}] \]

\[ \Rightarrow K[\text{Al}]_0[\text{P}] - K[\text{Al-P}][\text{P}] = [\text{Al-P}] \]

\[ \Rightarrow K[\text{Al}]_0[\text{P}] = [\text{Al-P}](1+K[\text{P}]) \]

\[ \Rightarrow \frac{[\text{A}-\text{P}]}{[\text{Al}]_0} = \frac{K[\text{P}]}{1 + K[\text{P}]} \quad \text{Eqn. (8)} \]

But \[ [\text{Al-P}] = [\text{P}]_0 - [\text{P}] \]
So,

\[
\frac{[P]_0 - [P]}{[Al]_0} = \frac{K[P]}{1 + K[P]}
\]

Eqn. (9)

Where all quantities except K are directly measureable and K is determined from the plot of

\(([P]_0 - [P])/[Al]_0 \text{ vs. } [P]\).

**Curve Fitting - Single Set of Sites Model Derivation**

\(\Theta = \frac{K[X]}{1 + K[X]} \quad \ldots \ldots \ldots \ldots (1)\)

\(X_i = [X] + n\Theta M_i \quad \ldots \ldots \ldots \ldots (2)\)

\(Q = n\Theta M_i \Delta H V_0 \quad \ldots \ldots \ldots \ldots (3)\)

Combine (1) and (2), solve quadratic for \(\Theta\)
Plug result into (3), get \(Q(n, \Delta H, K)\)

\[\Delta Q_i = Q_i + \frac{\Delta V_i}{V_0} \left[ \frac{Q_i + Q_{i-1}}{2} \right] - Q_{i-1}\]

\(Q_i\) – heat content after \(i^{th}\) injection

**AppG-Fig. 4 – Derivation of curve fitting equations used to fit isothermal calorimetry data.**
REFERENCES


APPENDIX H

CHAPTER 7 - Enhanced drug solubility by the Spray drying and Nanoparticle Assembly Process (SNAP)

**AppH-Fig. 1** – PXRD traces of spray dried naproxen formulations by SNAP. Naproxen dispersions (Pure NXN, Samples R4-1 to R4-6 in ascending order, see **AppH-Table 1**). No significant increase in line width was observed in the spray dried samples vs pure NXN crystals.

**AppH-Fig. 2** – PXRD traces of spray dried phenytoin formulations by SNAP with trehalose rather than HPMC as the excipient (R4-7 and R4-8 in ascending order, see **AppH-Table 1**). No significant increases in line width were observed.
### AppH-Table 1 – Complete formulation conditions

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>[API] (mg/mL)</th>
<th>[BCP]</th>
<th>[HPMC]</th>
<th>THF:water flow ratio</th>
<th>(S_r)</th>
<th>Drug Loading %</th>
<th>Remarks</th>
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<tbody>
<tr>
<td><strong>Round 1</strong></td>
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<tr>
<td>R1-1</td>
<td>100</td>
<td>--</td>
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<td>134</td>
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<td>41 min HOLD*</td>
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<td>7:63</td>
<td>134</td>
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<tr>
<td>R1-4</td>
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<td>5.6</td>
<td>7:63</td>
<td>67</td>
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<td></td>
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<td>Process: MIVM, API: Phenytoin</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>R2-1</td>
<td>10</td>
<td>--</td>
<td>1.11</td>
<td>12:108</td>
<td>13</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>R2-2</td>
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<td>1.11</td>
<td>12:108</td>
<td>13</td>
<td>50</td>
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<tr>
<td>R2-3</td>
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<td>12:108</td>
<td>40</td>
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<td>--</td>
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<td>13</td>
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<td></td>
<td>2.5:7.5</td>
<td>14.8</td>
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<tr>
<td>SNAP2</td>
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<td>2:8</td>
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</tr>
<tr>
<td><strong>Round 4</strong></td>
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<td>20</td>
<td>1.7:8.3</td>
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<td>50</td>
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</table>
API: Phenytoin, Excipient: Trehalose

<table>
<thead>
<tr>
<th>Sample</th>
<th>API (%)</th>
<th>Excipient (%)</th>
<th>Particle Size (μm)</th>
<th>API:Excipient Ratio</th>
<th>Drying Temperature (°C)</th>
<th>Trehalose (%)</th>
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<tr>
<td>R4-3</td>
<td>100</td>
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<td>11.1</td>
<td>1:9</td>
<td>78</td>
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<td>2:8</td>
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<tr>
<td>R4-5</td>
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<td>1.7:8.3</td>
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<td>R4-6</td>
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<td>11.1</td>
<td>1:9</td>
<td>78</td>
<td>33.3</td>
</tr>
</tbody>
</table>

*The sample was held at room temperature for 41 minutes between flash nanoprecipitation and spray drying to observe the effect of Ostwald ripening.
†Dichloromethane was added to the organic stream at 10 mg/mL in an attempt to slow the phenytoin crystal growth.
AppH-Fig. 3 – Solubility data for naproxen acid in THF-water (■) and acetone-water (●) mixtures at room temperature. Curves were linear on log-log plots: THF-water: log(solubility) = 3.39log(%THF) – 4.32, $R^2 = 0.99$, acetone-water: log(solubility) = 2.96log(%acetone) – 3.95, $R^2 = 0.97$. 
APPENDIX I

CHAPTER 8 - Ultrafiltration

Theory

i) Governing Equations

\[
\Delta P = P_F - P_R
\]  \hspace{1cm} \textbf{Eqn. (1)}

App1-Fig. 1 – Schematic of a single hollow fiber with the open circles representing a small molecule, permeable contaminant such as THF, and the black circles representing an impermeable nanoparticle. Gel layer formation is not depicted here. \(P_F, \ P_R, \) and \(P_P\) are the feed, retentate, and permeate pressures respectively.

Tangential flow ultrafiltration is generally used to separate species on the basis of size with pore sizes generally < 1.0 um. This is distinct from reverse osmosis where pore sizes are tight enough to restrict the flow of salts, and microfiltration, where pore size are >1.0 um.

The driving force through the hollow fiber lumens is \(\Delta P\) and is given simply by:

\[
\Delta P = P_F - P_R
\]  \hspace{1cm} \textbf{Eqn. (1)}
Where $P_F$ is the feed pressure and $P_R$ is the retentate pressure.

However, the average driving force across the membrane is given by the transmembrane pressure (TMP):

$$TMP = \frac{P_F + P_R}{2} - P_p \quad \text{------- Eqn. (2)}$$

Where $P_p$ is the permeate pressure (usually 0 psig). The actual flux through the membrane ($J_P$) depends on the TMP, and the resistivity of the membrane and gel layer that forms:

$$J_P = \frac{Q_p}{A} = \frac{TMP}{\mu(R_M + R_G)} \quad \text{------- Eqn. (3)}$$

Where $R_M$ and $R_G$ are the membrane and gel layer resistivities respectively. The gel layer is depicted schematically in AppI-Fig. 2. $R_M$ is generally a constant except at very high pressures where the membrane experiences compression, while $R_G$ can vary highly over the course of an experiment as the solids concentration changes.[1]
**AppI-Fig. 2** – Schematic of the gel layer where \( C_B \) is the bulk nanoparticle concentration and \( C_G \) is the gel layer concentration. \( C_{NP} \) rises from \( C_B \) at the center line to the maximum at \( C_G \) near the membrane wall.

**Diafiltration Model Derivation**

A modified CSTR model is used to model the degree of diafiltration efficiency. The circulation loop is represented by the blue box where the concentration of the permeable species decreases over time but the volume remains constant (constant volume diafiltration). Pure buffer or water is input to the system such that \( Q_D = Q_P \). Because of gel layer formation, the concentration of permeable species within the loop might be different from the concentration in the permeate line, hence \( C(t) \) may
be different from \( C_P(t) \). However, we can define a transmission coefficient \( T \), defined as:

\[
C_P(t) = TC(t) \quad \text{--- Eqn. (4)}
\]

which characterizes how easily the permeable species can leave the system.

Assuming that the system is well mixed and that there are no chemical reactions occurring, we can perform an open system mass balance on the permeable species:

\[
\text{Inflow} - \text{Outflow} = \text{Accumulation}
\]

\[
\Rightarrow Q_DC_0 - Q_PC_P(t) = \frac{d}{dt}(VC(t)) \quad \text{--- Eqn. (5)}
\]

However, \( C_0 = 0 \) and \( C_P(t) = TC(t) \), where \( T \) is the transmission coefficient so the equation simplifies to:

\[
- \frac{TQ_P}{V} = \frac{1}{C(t)} \frac{dC(t)}{dt}
\]

Assuming \( Q_P \) and \( V \) are constants with respect to time, and integrating:

\[
- \frac{TQ_Pt}{V} = \ln[C(t)] + A \quad \text{for } C(t) > 0
\]

Applying the initial condition \( C(t) = C_i \) at \( t = 0 \) yields:

\[
- \frac{TQ_Pt}{V} = \ln \left[ \frac{C(t)}{C_i} \right]
\]

Now, since \( Q_P = Q_D \), \( Q_Pt = Q_Dt = V_D \) (the # of diafiltration volumes)

Therefore,

\[
C(t) = C_i e^{\left[-V_DT\right]} \quad \text{--- Eqn. (6)}
\]

Or, in linearized form:
\[ \ln[C(t)] = -V_DT + \ln(C_i) \quad \text{Eqn. (7)} \]

Practically speaking, this equation can be used to model the concentration of THF over time from GC data, where \( C_i \) is the THF concentration after the initial concentration phase is finished (before diafiltration). The transmission coefficient requires knowledge of the THF concentration in the permeate line and the process volume, which is not always practical to get. Instead, the slope of data can reveal the transmission coefficient.

ii) Scale up

In general, examples from the literature\([1-3]\) show that the most effective and straightforward way to scale up a tangential flow filtration system is by linear scaling. This means that the average flux is calculated at the research scale, the desired industrial scale process volume is defined, and the area at constant flux is calculated. The key equation is simply:

\[ J_p = \frac{Q_P}{A_T} \quad \text{Eqn. (8)} \]

\( J_p \) (permeate flux) is kept constant, while \( Q_P \) (permeate flow rate) and \( A_T \) (total membrane area) are scaled up proportionally. Naturally, this requires keeping the same membrane material, pore size, fiber width, fiber length, and recirculation/shear rate. To increase \( A_T \), the number of fibers can be increased. If the length of each fiber or the fiber width is increased, that will change the pressure drop across the unit. However, this is still OK since the TMP controller can compensate for it by keeping
the TMP constant. We recommend talking to the engineers at Spectrum Labs or Millipore when it becomes clear that the process needs to be scaled up.

Practical Notes

*Hollow fiber preparation*

In general, 3 actions need to be taken when preparing a new hollow fiber membrane: (1) membrane integrity test, (2) pressure hold test, and (3) membrane wetting. (1) and (2) are well described in the official manual and the Hollow Fiber Module Preparation & Instruction Guide (found at [http://www.spectrumlabs.com/filtration/ProcLit.html](http://www.spectrumlabs.com/filtration/ProcLit.html)).

Membrane wetting should be performed as follows:

1. Connect hollow fiber unit to flowpath in total recirculation mode (both retentate and permeate lines connect back to the reservoir).
2. Prepare sufficient 20-40% EtOH to fill the tubing and membrane unit (at least 25 mL in most cases) and load it into the 50 mL reservoir.
3. Ramp up the pump from 10 mL/min up to 100 mL/min to wet the membrane.
4. Recirculate the EtOH solution for 20-25 mins.
5. Once done, flush out the membrane unit with pure water (> 2 mL water/cm² membrane area).
6. Drain the membrane unit completely. It is now ready for baseline water permeability testing and further use.
Flowpath schematic

Detailed flow path schematics can be found in the official manual at http://www.spectrumlabs.com.

Hollow fiber cleaning protocol

The Hollow Fiber Module Preparation & Instruction Guide as well as the official manual describe several methods for cleaning the membrane units. In our experience, there are two methods which work especially well:

(1) Reverse Pump Backflushing

1. After flushing the membrane with pure water and draining, prepare a 50 mL reservoir with 20-40% EtOH (full) and a 250 mL reservoir with 30 mL of 20-40% EtOH.

2. Connect the feed and retentate lines to the 250 mL reservoir and the permeate line to the 50 mL reservoir and run the pump forward at up to 150 mL/min (for a 115 cm², 10kD mPES membrane). The membrane unit and the permeate line should eventually fill up with EtOH and expel all the air in the permeate line.

3. Once all the air is expelled (the 50 mL reservoir may start overflowing), stop the pump and run it in reverse at up to 150 mL/min. Monitor the pressure...
readings carefully as the transducers apparently can only display up to approx. -7.0 psi. If any pressure reading shows **** then reduce the flow rate.

4. The level in the 50 mL reservoir should fall steadily. Stop the pump once the reservoir and permeate line are exhausted.

(2) Direct pump backflushing

A more aggressive (and potentially membrane damaging) cleaning operation is direct pump backflushing. It should be performed after reverse pump backflushing.

1. Prepare 50 mL of 20-40% EtOH in a 50 mL reservoir. Connect this reservoir to the pump and connect the pump to the permeate line but leave one end of the permeate line open.

2. Now, manually fill the membrane unit with 20-40% EtOH with a syringe. It is essential that the membrane unit is completely full of EtOH before starting.

3. Cap all of the ports on the unit and place it in the trilobite holders. Place a collection flask under the bottom feed port.

4. Open the top port, connect the retentate pressure transducer and cap it. Open the top permeate port, connect the permeate pressure transducer and cap it.

5. Now run the pump forward at < 3 mL/min to expel all the air from the permeate line. When the permeate line is full, connect it to the permeate pressure transducer on the membrane unit and quickly open the bottom feed
port. No pressure transducer is necessary on the feed port as the fluid will be draining from there.

6. For a 115 cm$^2$, 10kD mPES membrane, a flow rate of 3 – 3.4 mL/min should give a TMP no higher than 9.2 psi. **To avoid damaging the membrane permanently, the $P_f$ should never be higher than 10 psi!** To be safe, make sure $P_f$ is never higher than 9.2 psi.

7. Once the permeate line is empty, stop the pump and flush the membrane with > 2 mL/cm$^2$ water. Test the water permeability.

UV-vis calibration curves

(1) Vitamin E in THF

![Vitamin E calibration curve in THF: $Abs (295 nm) = 7.55[VE (mg/mL)]$ ($R^2 = 1.00$)]

**Appl-Fig. 4** – Vitamin E calibration curve in THF: $Abs (295 nm) = 7.55[VE (mg/mL)]$ ($R^2 = 1.00$)

(2) Nile Red in THF
AppI-Fig. 5 – Nile Red calibration curve in THF: \( \text{Abs (534.5 nm)} = 123.99[\text{NR (mg/mL)}] + 0.018 \) (\( R^2 = 0.999 \)).

Reversibility of Gel Layers

The reversibility of the gel layers formed was tested by diafiltering 1 wt% PMMA and 1 wt% BSA at 100 mL/min and 300 mL/min feed flow rate from 5 to 25 psi TMP. The results are shown in AppI-Figs. 6 and 7. In these experiments, the diafiltration began at 5 psi and the TMP was increased incrementally up to a maximum around 25-30 psi then incrementally stepped back down to the minimum TMP generated by the action of the pump.
AppI-Fig. 6 – Gel layer reversibility testing for PMMA latices at 1 wt% with feed flow 300 ml/min (A) and 100 ml/min (B) (■ increasing TMP | ▲ decreasing TMP).

We see in AppI-Fig. 6A the change in $Q_P$ as a function of TMP for 1wt% PMMA at 300 mL/min. On the increasing TMP phase, $Q_P$ is linear with TMP as seen in AppI-Fig. 7 in Chapter 8 Part B. We also see that $Q_P$ is linear with TMP with approximately the same slope on the downward TMP phase as well. This indicates that the gel layer formed is reversible over the TMP range studied at 300 mL/min (high shear). In contrast, we see in AppI-Fig. 6B that at 100 mL/min feed rate, $Q_P$ saturates at a relatively low TMP on the upward TMP phase, indicating that the $R_G$ value is increasing. This was unexpected, given that AppI-Fig. 7 in Chapter 8 Part B shows $Q_P$ as linear with TMP over the same TMP range at 100 mL/min feed rate. It could indicate the formation of a cake layer from the previous experiment at 300 mL/min though the membrane was cleaned between experiments at the baseline water flux was within 91% of the starting value. During the course of the 100 mL/min experiment, solids were seen settling on the bottom of the process reservoir at the higher TMPs indicating that aggregates or flocs possibly formed.
On the downward TMP phase, the linear behavior is once again observed showing that the permeability of the cake remained constant.

**BSA**

Similar testing was done with BSA at 1wt% in phosphate buffer at pH 7.0. The results are quite different compared to PMMA latices. Clear linear and TMP-independent regimes are seen at 100 mL/min feed rate (Appl-Fig. 7B) where the TMP-independent regime is dominated by the rise in osmotic pressure that matches the increase in TMP, resulting in no additional flow rate. The curves for the increasing and decreasing TMP phases match quite well, though it is obvious that the formation of an irreversible cake layer decreases $Q_P$ on the decreasing TMP phase by several mL/min. In contrast in Appl-Fig. 7A, flow rates are approximately double, as expected, given the higher shear rate that reduces membrane fouling. We also do not see the TMP-independent regime at this higher shear rate over the range of TMPs studied. We do however observe the flow rates on the decreasing TMP phase to be several mL/min lower than on the increasing phase. Thus, there is an irreversibly bound cake layer that does not become unbound when the TMP is reduced.
AppI-Fig. 7 – Gel reversibility testing for BSA solutions. A – 300 mL/min feed flow rate, B – 100 mL/min (■ increasing TMP phase | ● decreasing TMP phase). The higher shear rate in (A) gives higher $Q_p$. 
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