CALCIUM PHOSPHATE BASED NANOSHELL FOR USE IN BIOMEDICAL APPLICATIONS

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Abstract

by

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There is much interest in the use of nanoparticles as targeted contrast agents, biosensors, targeted drug delivery vehicles, and as artificial oxygen carriers. Nanoparticles of interest consist of liposomes/polymersomes, emulsions, micelles, and silica. Each of these nanoparticles have demonstrated efficacy in at least one of the mentioned areas but none can be effectively used universally. Liposomes, emulsions, and micelles suffer from stability issues in high turbulence environments, polymer based materials require organic solvents for their synthesis that are disadvantageous for biological applications, while silica based materials are not biodegradable. To expand the use of these nanoparticles for other applications requires major synthesis modifications, adding to the time required for implementation.

The objective of this research is to develop, characterize, and demonstrate the applicability of a novel, versatile, calcium phosphate based, core-shell nanocarrier that can be used in the above applications with straightforward modifications. Calcium phosphate is rigid, biocompatible, and can be synthesized in biofriendly conditions. Initially, several novel synthetic methods will be developed and evaluated for reproducible calcium phosphate nanoshell synthesis and a reaction mechanism proposed.
Nanoshells from 5-200 nm are deposited around a 30-200 nm liposome template first (CHAPTER 2) followed by deposition around a 100-350 nm soybean oil and perfluorocarbon emulsions (CHAPTER 3). The ability of the resultant nanoshells to effectively entrap hydrophilic pyranine, and hydrophobic pyrene while protecting them from quenching molecules (H$_2$O$_2$ and Cu$^{2+}$) is verified (CHAPTER 4). The potential for use in targeted delivery applications is demonstrated by development of a covalent attachment method for an anti-FITC IgG antibody and horseradish peroxidase enzyme. The attachment efficiency and residual activity are quantified (CHAPTER 5). The ability of perfluorooctyl bromide cored calcium phosphate nanoshells to carry and deliver oxygen to the body is demonstrated through enzymatic assay methods (CHAPTER 6).

The calcium phosphate nanomaterial developed here shows strong potential to be a universal delivery system. The calcium phosphate shell provides protection, rigidity, stability, and a scaffold for the covalent attachment of proteins and other biomolecules. The ability to change the core hydrophobicity of the nanoshell allows potential entrapment of proteins, dyes, contrast agents, and pharmaceuticals.
DEDICATION

To my wife Stephanie and my son Alexander
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1.1 Overview of this Work

A calcium phosphate based core-shell nanoparticle (nanoshell) intended for use in biomedical applications was developed in this thesis. Desirable features for these nanoparticles include the ability to carry hydrophilic and hydrophobic bioactive molecules, protection of encapsulated molecules from potentially hostile external conditions, and the possibility of delivering the nanoparticles to specific locations in the body via cellular recognition. As will be discussed, particles with these properties are especially relevant for medical imaging, biosensor development, drug/gene delivery, and artificial oxygen carriers. Current nanoparticle based solutions for these applications require development of specific types of particles such as liposomes, emulsions, polymersomes, and porous nanoparticles like hydrogels and calcium phosphates to meet their respective criteria. Each of these nanoparticles requires the development of a new synthesis requiring substantial modifications.

The nanoshell developed in this thesis is a universal particle that can be used in several areas with little modification, and has the following properties:

1) The shells are made of biodegradable components: lipids, oils, phosphate, calcium, and water.

2) The core of the shell is defined by a liposome, lipid emulsified soy-oil nanosized droplet, or lipid emulsified perfluorocarbon nanosized droplet.
3) Synthesis takes place in an aqueous environment at room temperature and at moderate pH (7.5) providing amenable conditions for encapsulation of sensitive biomolecules.

4) The nanoshell size ranges from 30 nm to more than 400 nm by varying the preparation method for liposome or lipid stabilized microemulsions and shell deposition.

5) Nanoshell thicknesses range from less than 5 nm to more than 200 nm.

6) Suspensions of nanoshells are stable for more than 6 months at physiological salt concentrations (140 mM) and concentrated protein solutions (2.5 g/dl).

7) The suspension of particles is optically transparent/translucent

8) The nanoshells are able to encapsulate drugs, fluorophores, aromatics, or their combination.

9) The shell provides rigid structure to the particle and protection of the contents.

10) Have a non-immunogenic surface that can be functionalized with proteins and antibodies through covalent or electrostatic interactions.

11) Can solubilize and release oxygen for potential use as an artificial oxygen carrier (AOC)

Development of the nanoparticles will be accomplished through the novel integrated application of calcium phosphate precipitation chemistry, colloidal self-assembly techniques utilizing amphiphiles, and sol-gel chemistry. Resulting nanoshell suspensions are then subjected to partial characterization for their potential use and efficacy in biomedical applications.
1.2 Current Calcium Based Materials and Applications

1.2.1 Overview

Calcium based ceramic materials \((\text{Ca}_x\text{(PO}_4)_y, \text{CaCO}_3, \text{CaF}_2, \text{CaSO}_4, \text{etc.})\) currently have utility in the biomedical field and novel ones are in development as will be discussed. Calcium ceramics and/or their ions are naturally present in the body (bionative) and thus are both biocompatible and biodegradable. In the body the primary role of calcium phosphates is the construction and maintenance of healthy bones and teeth. Free calcium ions play roles as signaling agents in membrane regulation\(^1\), smooth muscle\(^2\), programmed cell death\(^3\), synaptic transmission\(^4\), and in general cell function\(^5,6\).

As discussed in Section 1.2.2, nanomaterials based on calcium ceramics are nonimmunogenic, biocompatible, biodegradable, bioactive, and can be synthesized in a variety of geometries and are used in many diverse fields ranging from guided tissue regeneration to cellular transfection. These properties provide a firm foundation for the development of new advanced biomaterials containing a calcium phosphate ceramic as a component.

1.2.2 Calcium Phosphate \((\text{Ca}_x\text{(PO}_4)_y)\) Materials

Calcium phosphate can be synthesized in shapes ranging from spherical nanoparticles\(^7\), microparticles\(^8\), high aspect ratio whiskers\(^9\), and filaments\(^10\) to sheets, rods, and needles\(^11\). They are manufactured by precipitation reactions in oil-water microemulsions\(^12-14\), base precipitations\(^15\), sol-gel methods\(^16\), and more exotic methods involving microwave-assisted combustion synthesis\(^17\).
Nano and microparticles of calcium phosphate are specifically used in delivery of antibiotics\textsuperscript{18}, plasmids\textsuperscript{19}, ocular drugs\textsuperscript{20, 21}, proteins\textsuperscript{22}, nucleic acid-based drugs\textsuperscript{23}, as nonviral vectors for gene transfection into cells\textsuperscript{24-26}, and other applications\textsuperscript{27}.

Nanoparticles of calcium phosphate are used as a bioactive component in pastes and injectable cements\textsuperscript{8, 28} used for bone regeneration in periodontics, maxillofacial surgery, and skeletal breaks/defects\textsuperscript{29}. In addition to these fluid cements, rigid combinations of nanoparticles\textsuperscript{30} and nanoparticle/polymer composites\textsuperscript{31} are used as biodegradable/resorbable\textsuperscript{32, 33} scaffolds for the guided regeneration of bone tissue outside of its normal environment and within the bone itself\textsuperscript{34, 35}. Micron sized materials like microneedles coated with porous calcium phosphate and soaked with trehalose are used in transdermal delivery of this molecule\textsuperscript{36}. Alginate–calcium phosphate microspheres are used as enzyme delivery matrices in vivo\textsuperscript{37}. As a coating, calcium phosphate also aids the osseointegration of titanium based orthopedic implants\textsuperscript{38} by acting as a bioactive coating deposited via electrodeposition\textsuperscript{39}, plasma spraying\textsuperscript{40}, sputter coating\textsuperscript{41}, pulsed laser deposition\textsuperscript{42}, peroxide pretreatment and chemical reaction\textsuperscript{43}, and electrochemical reactions\textsuperscript{44}.

1.2.3 Calcium Carbonate (CaCO\textsubscript{3}) and Other Calcium Based Materials

Calcium carbonate is commonly utilized as a dietary supplement of calcium for the body. Nanomaterials composed of calcium carbonates and their composites are biocompatible and function similarly to calcium phosphate in biomedical applications. Engineered microstructured calcium carbonates are used for guided tissue regeneration of periodontal defects\textsuperscript{45, 46} and in facial reconstruction\textsuperscript{47}. Because they are easily dissolved
under acidic conditions to calcium and carbonate ions, they have been used as core-shell nanoparticle templates for silica nanotube growth\textsuperscript{48}, spherical porous silica particles\textsuperscript{49} and hollow titania nanocapsules\textsuperscript{50}. The high compression strength material acts as an extender for tougher and more resilient polymer composites\textsuperscript{51,52}. Due to their porous nature, spherical calcium carbonates find utility in drug delivery by adsorbing hydrophilic drugs and bioactive proteins within the porous matrix and allow a slow delivery of these adsorbed molecules over time\textsuperscript{53}.

Nanoscale calcium sulfate\textsuperscript{54} and calcium fluoride\textsuperscript{55} find limited utility in bone reconstruction\textsuperscript{56} and titania implant coatings\textsuperscript{57,58}.

1.3 The Calcium Phosphate System and Biomineralization

1.3.1 Overview

The precipitation of calcium phosphate is more complicated than many binary crystals as it has many thermodynamically stable crystalline forms as opposed to a simple binary system such as NaCl (solubility product, $K_{sp} = 36$) or AgCl ($K_{sp} = 4 \times 10^{-11}$). In order of increasing stability, the five principle stoichiometrically distinct phases are amorphous calcium phosphate (ACP), dicalcium phosphate dihydrate [CaHPO$_4$$\cdot$2H$_2$O] (DCPD, $K_{sp} = 2.1 \times 10^{-7}$), tricalcium phosphate [Ca$_3$(PO$_4$)$_2$] (TCP, $K_{sp} = 1.15 \times 10^{-29}$), octocalcium phosphate [Ca$_8$(HPO$_4$)$_2$(PO$_4$)$_4$] (OCP, $K_{sp} = 1.25 \times 10^{-47}$), and hydroxyapatite [Ca$_{10}$(PO$_4$)$_6$(OH)$_2$] (HAP, $K_{sp} = 1.8 \times 10^{-58}$)\textsuperscript{59}. The final ratio present in each of these phases can affect the bulk and interfacial chemistry of the final calcium phosphate material.
1.3.2 Calcium Phosphate Crystallization Chemistry

The solubility of a particular calcium phosphate solid phase is dependent primarily on pH, presence of foreign ions, temperature, activity of the ions in solution, and presence of seed crystals\textsuperscript{59, 60}. Most commonly, pH is used to influence the reaction rate by manipulating the phosphate equilibria. Phosphate is triprotic and has four species present in equilibrium with HPO\textsubscript{4}\textsuperscript{2-} and PO\textsubscript{4}\textsuperscript{3-} being the most reactive. The determination of the pH dependent solubility isotherm for the calcium phosphate in an aqueous system at 25° C (Figure 2-21) is discussed in detail in Section 2.4.2.1.

Another important consideration for calcium phosphate crystallization is the formation of nuclei. The thermodynamic driving force for nucleation of calcium phosphate is the reduction of free energy of the system through the formation of a lower energy phase, in this case a crystal. Crystallization can occur either homogeneously or heterogeneously. In homogenous nucleation there is an energy barrier that must be overcome to form a stable nucleus (\(r^*\)) (Figure 1-1) that can not revert back to individual atoms. The Gibbs-Thompson equation (Equation 1-1) explains this effect.

\[
\Delta G_{\text{TOTAL}} = \frac{4}{3} \pi r^3 \Delta G_v + 4 \pi r^2 \gamma \frac{d}{dr} \frac{d\Delta G_{\text{TOTAL}}}{dr} = 4 \pi r^2 \Delta G_v + 8 \pi r \gamma
\]

where,

\[
\Delta G_v = kT \ln(S)
\]

Equation 1-1

Where \(r\) is the radius of the nucleus, \(\Delta G_v\) is the Gibbs volume energy, and \(\gamma\) is the interfacial tension, \(k\) is the Boltzmann constant, \(T\) is the temperature.
Crystallization occurs where the volume free energy decrease ($r^3$ dependence) overcomes the surface energy increase ($r^2$ dependence) and becomes easier at higher supersaturations. The radius where the volume energy dominates and the derivative of the free energy becomes negative is said to be the critical nuclei, defined in Equation 1-2.

$$r^* = \frac{2\gamma}{\Delta G_v}$$

Equation 1-2

where, $r^*$ is the critical nucleus, $\Delta G_v$ is the Gibbs volume energy, and $\gamma$ is the interfacial tension.

Figure 1-1  Graphical visualization of the critical radius of forming nuclei determined by inflection point of the sum of volume free energy and interfacial energy.
Since nucleation effectively requires an activation energy it can be represented by an Arrhenius equation such as the one given below as given by Wankat\textsuperscript{61}:

\[
\frac{dN}{dt} = Ae^{-\frac{16\pi\sigma V_m^2}{3k_B T^3 (\ln S)^3}}
\]

Equation 1-3

Homogeneous nucleation rarely happens due to the difficulty in overcoming this barrier. Nucleation more commonly forms on a surface or a seed as this overcomes the potential energy barrier required for homogenous nucleation. Once crystal growth begins it can be simplified as diffusion limited linear growth given by Wankat\textsuperscript{61} as:

\[
G = k\Delta C = kS
\]

Equation 1-4

where, \(G\) is the linear growth rate, \(k\) is a lumped rate constant, \(S\) is the supersaturation

Nucleation and growth rates are both dependent on the level of supersaturation with growth dominating at low supersaturations and nucleation dominating at high supersaturations. As seen in Figure 1-2, at moderate supersaturations growth is the preferred process, the rapid nucleation causes calcium phosphate to precipitate spontaneously and uncontrollably, resulting in solutions that are difficult to reproduce; therefore these conditions are avoided when attempting controlled growth of the crystal on a surface by utilizing moderate supersaturations.
When forming crystals on a surface, the final crystalline form of a material is not necessarily the one that forms first. This effect is known as the Ostwald rule of stages\textsuperscript{62} which states that the least stable form of a crystal forms first even though the degree of supersaturation or thermodynamic driving force is less than the more stable phases. It is believed that unstable clusters of amorphous calcium phosphate (ACP) act as nucleation sites on which the more stable and often slower growing crystals form\textsuperscript{60}. Very small pieces or layers of calcium phosphate are typically considered amorphous as they lack the long range order needed to be crystalline.

1.3.3 Biomineralization of Calcium Phosphate

Biomineralization is the guided precipitation of a mineral by organic structures within the body. The ability to guide and direct the growth of calcium phosphate with biomolecules such as collagen\textsuperscript{63, 64}, liposomes\textsuperscript{65}, lecithin\textsuperscript{66}, and surfactants\textsuperscript{67} finds great utility in the design and synthesis of organic/inorganic composite nanomaterials based on
calcium phosphate. Many of these growth techniques are mimicked from natural biomineralization. This growth is normally initiated by a local increase in supersaturation caused by electrostatic attraction of calcium or phosphate to the biomolecules surface. Once underway the growth process can also be regulated and directed by proteins or microorganisms resulting in unique functional geometries. Bone is a classic material that is mineralized under the direction of many biomolecules as are teeth. These growth processes are complicated and are not completely understood but they provide insight for synthesizing composite structured nanomaterials.

1.4 Review of Current Core-Shell Nanoparticles (Nanoshells)

1.4.1 Template Synthesis of Core-Shell Nanoparticles (Nanoshells)

A core-shell particle or nanoshell is defined as one containing a rigid shell surrounding a solid and/or liquid core composed of a different material. The dimensions of these particles range from 10 nm to over 1 μm in size making them ideal for injection to the body where the can traverse the capillaries (11 μm in diameter). Their small size and subsequent high surface energy makes nanoshells prone to flocculation but this is tempered by hydration (steric) and columbic (electrostatic) forces which keep them separated. Many core-shell nanoparticles are prepared using either a template as a nucleation site and director for growth, phase separated nanoemulsions and the selective solubility of nanoshell precursors in each phase, or mechanical preparation. Core-shell particles have been prepared from a variety of materials in a variety of configurations such as hollow silica, noble metal (gold, silver, platinum) cored silica.
iron oxide cored silica\textsuperscript{87, 88}, liposome templated silica\textsuperscript{89-92}, hollow polymer\textsuperscript{93, 94}, metal cored polymer\textsuperscript{95, 96}, hollow titania\textsuperscript{97}, and others\textsuperscript{98}.

1.4.2 Uses for Solid Core Nanoparticles

Nanoshell structures with a solid core, usually metallic\textsuperscript{88, 99-106}, or of silica\textsuperscript{107} find utility in medical imaging\textsuperscript{108} but are not good candidates for drug delivery or oxygen transport. The dense solid core prevents encapsulation or solubilization of therapeutic agent. Other than a solid polymer cored particle like a poly-vinyl alcohol (PVA) cored polystyrene (PS) shell\textsuperscript{109}, reported solid cored nanoparticles are not generally composed exclusively of biodegradable components and could suffer from systemic rejection.

1.4.3 Uses for Fluid Core Nanoparticles

Liquid-cored nanoshells like those listed in the following sections are preferred over solid core for drug delivery, oxygen transport, and imaging applications as they can entrap large liquid volumes of liquid solution per shell area. These liquids can range from fluorocarbons, fluorescent dyes, and proteins like horseradish peroxidase or hemoglobin. An advantage of liquid-cored nanoparticles is that immunogenic substances can be delivered in their native environment and protected from the external environment and from the reticuloendothelial system (RES) in vivo.

1.4.3.1 Liposomes

A simple liquid-cored core-shell particle could be constructed using a liposome which is a spherical lipid bilayer surrounding an aqueous compartment. Liposomes can
be composed of phosphatidylcholine\textsuperscript{110}, phosphatidic acid, and other amphiphilic lipids\textsuperscript{111}. Because of their molecular structure, they can self assemble into spherical objects, and are capable of encapsulating great quantities of hydrophilic molecule in their aqueous core and small quantities of hydrophobic material in the lipid bilayer’s tail region. While liposomes alone have been under investigation for use in gene transfection and drug delivery for decades\textsuperscript{112,113}, due to the simple, fragile\textsuperscript{114,115} and primarily aqueous nature of the liposome, it is not an optimum vehicle for transporting species under turbulent conditions, or for hydrophobic species, excluding them as a universal transport vehicle. Because the membranes of cells, bacteria, and viruses are also constructed from lipids, liposomes are subjected to recognition and elimination from the body by the RES\textsuperscript{116} and require modification to a “stealth” liposome which is most commonly done by conjugating PEG to the surface\textsuperscript{117,118} as seen below. This adds an increased layer of complexity to the synthesis.

Figure 1-3 A bare liposome has many of its “antigens” exposed to recognition and consequent action by the immune system. A stealthed or PEGylated liposome hides these antigens through sterically hindering antibodies from approaching the surface
The liquid crystalline nature of the liposomal membrane can result in leaching of the contents of the liposome over time\textsuperscript{119} though this can be alleviated through combinations of high Tc lipids and cholesterol\textsuperscript{111}. They are also susceptible to osmotically driven shrinking/swelling when placed in a hyper/hypotonic environment. Despite these disadvantages liposomes are still being considered for drug delivery\textsuperscript{120, 121} and have been shown to be useful as templates for sturdier core-shell particles with improved mechanical characteristics\textsuperscript{122, 123}.

1.4.3.2 Polymer Nanoshell (Polymerosome)

Polymer nanoshells (polymerosomes) are made from latex\textsuperscript{124}, polystyrene\textsuperscript{125}, polyaniline\textsuperscript{96}, polypyrrole\textsuperscript{126-129}, polymethylmethacrylate\textsuperscript{130, 131}, and others. They are similar in structure and capability to liposomes, some are synthesized on top of liposomal templates\textsuperscript{93, 94} and benefit from an increased amount of protection and versatility through tailoring the chemistry of the shell and its surface. They can encapsulate hydrophilic and hydrophobic substances. Some polymeric materials like polyethylene glycol (PEG), polypropylene glycol (PPG), polystyrene (PS), polyacrylic acid (PAA), polycoproplactone (PC), cellulose acetate (CA), and carboxymethylcellulose (CMC) (list obtained from Sigma) have proven their compatibility with the body and upon degradation are metabolized by the body with no ill effect\textsuperscript{132, 133}, and are thus good candidates for \textit{in vivo} applications. They are also capable of encapsulating hydrophobic drugs for drug release and dispersion\textsuperscript{56, 134} and have functional groups that can potentially be used for surface functionalization.
The potential shortcoming of these particles is the unfriendly conditions required to produce some of these particles, ranging from the need to use organic solvents such as toluene\textsuperscript{135} to high temperatures, organic extractions, and vacuum drying. Many of these preparative steps can damage fragile biomolecules and at present excludes polymersomes as a universal solution to encapsulating bioactive materials. Polymersomes also experience environmentally-triggered leaking of small hydrophilic molecules just like ordinary liposomes\textsuperscript{136}

1.4.3.3 Silica Nanoshell

Silica is a popular component in many solid and core-shell nanoparticles for its chemical and biological inertness and thermal stability\textsuperscript{137, 138}. Its chemistry and interactions with templates and surfaces is well characterized\textsuperscript{139}. Silica crystallization/condensation reactions can be performed in aqueous systems at room temperature to the benefit of biomolecules being incorporated within a particle. The silica surface is charged allowing for colloidal stability in high concentrations and a range of organosiloxane-based crosslinkers are available as a means to conjugate molecules for biochemical functionalization\textsuperscript{140}. Silica resists biodegradation and microbial attack and is dissolved only with HF or strong base. Silica is porous enough for reactants/products to perfuse through the shell and enter/leave the core.

Silica nanoshell applications range from high surface area substrates for platinum, gold, and other metal catalysts\textsuperscript{141-143} and chromatography\textsuperscript{144} to biotechnological applications. Silica is optically transparent\textsuperscript{145} and is a popular choice for core-shell based biosensors and biomarkers that encapsulate fluorescent dyes like FITC\textsuperscript{146}, coumarin.
Silica nanoparticles are also used to protect enzymes\textsuperscript{152}, as a template for many biodegradable polymeric core-shell nanoparticles\textsuperscript{153}, as magnetically separable biocatalysts\textsuperscript{154}, in gene delivery\textsuperscript{155,156}, and other avenues of bionanotechnology reviewed by Tan et al.\textsuperscript{157}. The chemical stability that aids silica in the above functions is also disadvantageous for applications where biodegradability is desired.

1.4.3.4 Emulsion and Micelle Templated Nanoshells

Much of the interest in using nanoparticle carriers is their potential to solubilize hydrophobic molecules and disperse them in an aqueous medium\textsuperscript{158,159}. This is especially relevant for drug delivery where many compounds are hydrophobic, resulting in low solubility and thus bioavailability in the aqueous circulatory system. Two nanosized materials employed to disperse hydrophobes are microemulsions and micelles. A microemulsion is analogous in structure to a liposome but differs by only having a monolayer of lipid or surfactant with the hydrophobic tails immersed in a hydrophobic oil core (Figure 1-4). Emulsions and micelles suffer from many of the same fragility and immunogenic problems as liposomes\textsuperscript{111,160-162}. Silica\textsuperscript{158} and polymers\textsuperscript{163-166} have been successfully deposited around o/w and w/o emulsions providing a rigid shell, though this brings about the same disadvantages as mentioned earlier, and a biodegradable shell would be preferred for in vivo applications\textsuperscript{167}. 


Micelles are perhaps the smallest of self assembled organic structures at approximately 4-5 nm in diameter. In an aqueous environment, micelles are formed when two amphiphiles with large head groups and narrow tails come in proximity and form a sphere with the tails pointing inwards (Figure 1-4). Conversely, in hydrophobic solvents the heads point inward, forming a reverse micelle. Keeping such small particles stable in solution during coating requires special preparations such as low ionic strength or high surface charge of the particles. A major advantage is they can potentially diffuse out of the blood stream into tissues via gaps in the endothelial walls allowing increased tissue access over larger nanoparticles\textsuperscript{168}.

1.5 Biomedical Applications of Calcium Phosphate Nanoshells

There are four areas where calcium phosphate core-shell nanoparticles will potentially be useful: imaging, (1.5.1) biosensors (1.5.2), targeted drug delivery vehicles
(1.5.3), and as artificial oxygen carriers (1.5.4). The challenges and state of the art in these fields are discussed in the following sections.

1.5.1 Medical Imaging
1.5.1.1 Overview

The necessity for increased diagnostic abilities is the driving force behind advancement of medical imaging techniques. Ex-vivo imaging of variations in tissue density within the body is routinely used in the diagnoses of disease and trauma and can be obtained from several contemporary methods. Traditional radiography (X-ray) techniques provided grainy film-based images of different tissue densities. More advanced computed axial tomography (CT) scan provides 3D radiographic images while digital radiography allowed real time high resolution imaging of tissues with similar densities. Each step provided a leap in diagnostic abilities. Modern techniques based upon magnetic resonance (MRI), positron emission (PET), and sound waves (Doppler Ultrasound) have either provided the same or better information, with fewer complications as they do not require high energy radiation. Further details of contemporary imaging techniques can be found elsewhere\textsuperscript{169}. All of these techniques rely on the contrast of an analytical region (tissue, blood vessel, organ etc.) over background to function which is problematic as many tissues have similar densities. Contrast agents are used to improve current imaging by localizing in a specific region of the body and enhance the interaction of that tissue with either radiation or sound waves, providing an improvement in contrast and detection.
1.5.1.2 Engineered Contrast Agents

Current nanoparticle based engineered contrast agents consist of a contrast agent nanoparticle (10-100+ nm) or nanocapsule with a targeting moiety on the surface, such as folate for tumor targeting, which serves to localize the nanoparticle to the analytical region in vivo\(^{170-175}\). Solid core paramagnetic (iron oxide, gadolinium, etc) particles are commonly used as magnetic resonance imaging contrast agents\(^{176-180}\). Streptavidin conjugated iron oxide nanoparticles coupled to a biotynlated monoclonal antibody allow for targeted MRI of breast cancer tumor cells\(^{108}\). Targeted paramagnetic nanoparticles can also be directed with lectins rather than proteins for attachment to the various sugars present on the cellular surface\(^{177,179}\). Perfluorocarbons such as PFOB are versatile contrast agents that can be used in CT-scans as they are radiopaque, in MRI due to the increase in local presence of fluorine atoms, and in ultrasound because of their high density\(^{181}\). Nanoparticles containing ultrasound contrast agents like fluorocarbons\(^{182,183}\) coupled to ligands have demonstrated efficacy in imaging regions with expressed tissue factors induced by angiogenesis within the vasculature\(^{184-186}\) as well as improved imaging of the GI tract\(^{187}\) and the heart and its function\(^{188}\). Radioisotopic nanoparticles of PLGA coupled to \(^{125}\)I or \(^{131}\)I were utilized for whole body imaging of the bone and bone marrow of a mouse\(^{189}\).

1.5.1.3 Spectrophotometric Detection Methods

Alternative nanoparticle based methods under investigation employ spectrophotometric methods of detection. Near IR emitting nanocrystals of CdTe embedded polymeric nanoparticles are developed for in vivo monitoring of drug delivery
processes\textsuperscript{190}. Far infrared radiation can permeate tissue and has been used for imaging particle labeled inflamed arthritic joints\textsuperscript{191}. Other particles have utilized infrared fluorochromes conjugated to targeting molecules (antigens, enzymes, ligands, folate, etc.) and fluorescence optical imaging for microscopic detail \textit{in vivo} to whole body imaging of tumors\textsuperscript{192}. Gold nanoshell conjugates are in early development for scattering based optical imaging techniques\textsuperscript{193-195}. Chemiluminescent techniques can also used in addition to fluorometric techniques. Chemiluminescence can detect ethanol vapors (0.6 $\mu$g/ml) on cataluminescent nanoparticles of ZrO$_2$\textsuperscript{196} or TiO$_2$\textsuperscript{197} \textit{in vitro}.

1.5.1.4 Potential For Calcium Phosphate Nanoshells in Imaging

There are many avenues in which nanoparticles are already improving the efficacy of medical imaging but each application requires a different particle type and synthesis, and not all compositions utilize benign syntheses. A universal biodegradable core-shell particle capable of encapsulating hydrophilic, acoustically sensitive, hydrophobic, or paramagnetic molecules while maintaining the same synthesis and surface chemistry for functionalization for targeting with lectins or proteins would provide a versatile targeted imaging modality not present today. Entrapment of dyes for use as a nanoscale spectrophotometric detector is explored in CHAPTER 4 and the formation of fluorocarbon cored nanoshells that could potentially be used as contrast agents are developed in CHAPTER 3 and CHAPTER 6.

1.5.2 Biosensors

1.5.2.1 Overview
Nanoparticle based biosensors applications range from \( \mu \text{M} \) sensitive glucose sensors for diabetic patients\textsuperscript{198-203}, numerous proteins\textsuperscript{204,205}, DNA mutant detection\textsuperscript{206}, cholesterol oxidase for cholesterol monitoring to 1 \( \mu \text{M} \)\textsuperscript{207}, pesticides\textsuperscript{208}, lactate\textsuperscript{209}, reactive oxygen species (ROS) which are indicators for infection\textsuperscript{210-212}, and neurotoxic organophosphate warfare agents\textsuperscript{213}.

1.5.2.2 Structure of Biosensors

Various nanoparticles are utilized as a component of biosensors including polymers\textsuperscript{204}, metal-polymer composites of gold and chitosan\textsuperscript{199}, silica\textsuperscript{198}, zirconium dioxide\textsuperscript{210} or solid metallic nanoparticles of CdS\textsuperscript{205}, gold\textsuperscript{214,215}, silver\textsuperscript{216}, or palladium\textsuperscript{203}. Noble metal nanoparticles are of interest because their plasmon resonance is dependent on the particle size, shape, composition, and local environment\textsuperscript{217-219}. In a typical biosensor, nanoparticles are coupled to enzymes, commonly ones producing or metabolizing peroxide (peroxidases), and attached to a gold or graphite electrode. The interaction of the enzyme with its substrate causes an electron transfer process and/or a change in weight which can be measured amperometrically\textsuperscript{202, 216, 220, 221}, microgravimetrically\textsuperscript{222}, or photoelectrochemically\textsuperscript{205}. Their sensitivity is beyond sub nanomolar concentrations. These methods typically require reagents as part of the assay reducing the potential for use as on-line sensors but reagentless methods are being explored as well\textsuperscript{223}. This class of composite biosensors has potential for massively parallel high throughput detection\textsuperscript{224}. Silica nanoparticle-enzyme systems are capable of simultaneous detection of cerebral glucose, lactate, L-glutamate, and hypoxanthine\textsuperscript{225}. 
1.5.2.3 Spectrophotometric Assay Methods

Spectrophotometric based biosensors are also potent means of detection for glucose\textsuperscript{226}, proteins\textsuperscript{227, 228}, immunoglobulins\textsuperscript{229}, herbicides\textsuperscript{230}, bacteria\textsuperscript{231}, and general pharmaceutical candidate screening\textsuperscript{232}. This technique relies on labeling the analyte with a fluorescent dye or nanoparticle\textsuperscript{233, 234} by antibody-antigen interactions, avidin-biotin bonds\textsuperscript{206}, or other affinity interactions (TABLE 1-3). Time resolved spectroscopy of labeled europium (III) nanoparticles can improve the detection limit of nucleic acids to the ng/L range\textsuperscript{233, 235}. Fluorescein-isothiocyanate (FITC) embedded into functionalized silica was used to target hepatitis B antigens\textsuperscript{144, 223}. The detection limit for the analyte is usually equal to the detection limit of the fluorophore as there is a 1:1 correlation of the fluorophore to the molecular labels. This situation is optimal when quantization is necessary since the ratio of fluorophore and label to analyte is known; however, when detection is more important such as in the case of pharmaceutical candidate screening or detection of indicator genes or proteins, this is not the best configuration as more fluorescence is required per label. A potential solution is to entrap multiple dyes within a labeled nanoshell yielding higher ratios of dye to label. This was attempted with solid polymeric nanoparticles containing fluorophore and provided 100 times greater protein detection sensitivity over the molecular label, down to 87 fM\textsuperscript{204}. Detection is limited to the number of fluorophores per label and could conceivably be increased further with the use of a fluid core particle loaded with fluorophore. If quenching can be avoided, a fluid environment allows dyes and enzymes that can be affected by attachment or adhesion to function as they would in solution.
It is also possible to forgo the use of dyes as detection agents in favor of enzymes like horseradish peroxidase\textsuperscript{197, 236, 237} or proteins like hemoglobin\textsuperscript{210} that can be encapsulated within and/or coated on an individual shell. Typically these enzymes catalyze a reaction that yields a reactive oxygen species which in turn oxidizes an indicator. The appearance rate of the oxidized indicator can be monitored over time and indicates the interaction of the analyte with the enzyme/label/particle conjugate.

1.5.2.4 Potential For Calcium Phosphate Nanoshells in Biosensors

Biodegradable core-shell particles could provide a universal base particle to execute many of these detection methods, simplifying the synthetic steps required to build biosensors with different entrapped species and surface molecules. Their surfaces can be labeled with one or more targeting molecules either electrostatically or covalently, then immobilized to a surface, as explored in CHAPTER 5. Fluorophores, metals, enzymes, or combinations thereof can be encapsulated within the nanoshells yielding particles with dual detection capabilities. The sensitivity could conceivably be increased further with the use of a fluid core particle loaded with fluorophore since it may be possible to have better control over the quantum efficiency and energy transfer characteristics of the fluorophores. If emission quenching can be avoided, a fluid environment allows dyes to function as they would in solution. Furthermore, core-shell particles allow a combination of properly selected dyes with overlapping emission and excitation wavelengths to be used in a fluorescence resonance energy transfer (FRET) configuration for an increased signal-to-noise ratio\textsuperscript{227, 238, 239}. Encapsulation of fluorophores are explored in CHAPTER 4.
1.5.3  Drug Delivery

1.5.3.1 Overview

The concept of using biodegradable Nano-sized drug carriers for greater drug efficacy and fewer side effects arose over 30 years ago shortly after the introduction of bioresorbable polymeric sutures. The key requirements for an effective targeted drug carrier are retention of the pharmaceutically active ingredient (PAI) in transit to targeted location, evasion from the body’s immune system, accurate targeting of afflicted tissues, and release of the PAI at the targeted location. The delivery of therapeutics to the body is a tremendous hurdle to overcome during drug development and implementation. With few exceptions, traditional methods for delivery like injection, ingestion, transdermal adsorption, and inhalation deliver a dose to the entire body that largely exceeds the amount necessary for treatment. This is exacerbated by the fact that many pharmaceutical active ingredients (PAI) suffer from poor bioavailability and must be introduced in large quantities for treatment to be efficacious. Excessive dosing is a primary contributor to undesirable secondary effects and toxicity issues. Targeted drug delivery aims to solve this problem by delivering the minimum therapeutic dose to a specific tissue in the body. Many promising strategies utilize biodegradable labeled nanoparticle carriers that deliver PAIs to specific tissues in the body. This lower dose results in fewer administrations and can increase the drugs effectiveness by improving patient compliance. Nanoparticles are desirable for this application because their surfaces can be modified with functional groups that assign them specificity for certain
tissues or hide them from the immune system without changing the structure or function of the PAI.\textsuperscript{250}

1.5.3.2 Nanoparticles Used in Drug Delivery

Most nanoparticles used in drug delivery are less than 1 μm in diameter, spherical, and either porous or core-shell type particles. The PAI is typically entrapped within the particle during synthesis or can be uptaken after synthesis.\textsuperscript{251} Nearly any molecule can be encapsulated including therapeutics like the hydrophobic anticancer drugs doxorubicin\textsuperscript{117, 252, 253} daunorubicin\textsuperscript{175} and paclitaxel\textsuperscript{254} as well as hydrophilic dexamethasone phosphate\textsuperscript{133}, ribonuleases\textsuperscript{255}, and proteins\textsuperscript{256}. Entrapped molecules are released over time or upon an external trigger such as pH\textsuperscript{257}, focused magnetic fields\textsuperscript{258}, ultrasound\textsuperscript{259}, or temperature\textsuperscript{260}.

Many materials are used in drug delivery including hydrogels\textsuperscript{261}, lipids/liposomes/micelles\textsuperscript{262-265}, virosomes\textsuperscript{266}, polymers (PLGA\textsuperscript{134, 267}, PMMA\textsuperscript{268}, cyanoacrylates\textsuperscript{269}, and PAA\textsuperscript{270}, biopolymers (collagen\textsuperscript{271} and chitosan\textsuperscript{272}), paramagnetic alginate beads\textsuperscript{273}, colloidal gold\textsuperscript{274, 275}, porous hollow silica\textsuperscript{276}, calcium carbonate\textsuperscript{53}, and core-shell types (liquid filled nanoparticles\textsuperscript{277}, polymer nanocapsules\textsuperscript{136}). Of these, continuous polymers and liposomes have the most investigations.

Most polymeric particles are porous spheroids that entrap PAI in their polymeric matrix and release their contents through diffusion or matrix swelling. PLGA is most common and is representative as it is biocompatible and biodegradable and can encapsulate anticancer agents, antihypertensive agents, immunomodulators, hormones, and macromolecules but suffers from stability problems after preparation.\textsuperscript{267} As was
mentioned before, these materials typically require organic solvents to synthesize which can pose a problem for some biomolecules. Liposomes have long been used as drug delivery vehicles\textsuperscript{278-282} and do not require organic solvents for synthesis making them slightly more biofriendly than polymer nanoparticles, but they suffer from stability and leaking issues due to their thin lipid membrane\textsuperscript{283}. They can encapsulate a similar multitude of both hydrophilic and hydrophobic compounds by encapsulation within their aqueous compartments and lipid membranes\textsuperscript{284, 285}.

Two outstanding challenges in using nanoparticles as drug delivery vehicles is assigning them a target by conjugating them to a targeting moiety as discussed in the next Section 1.5.3.3 and preparing the nanoparticles in a manner to avoid detection and uptake by the reticuloendothelial system (RES) as discussed in Section 1.5.3.4.

1.5.3.3 Targeted Drug Delivery Using Nanoparticles

For nanoparticles or drugs to localize in a specific area within the body they need to be conjugated to a targeting molecule that attaches to specific cells with the receptor. This technique is referred to as receptor mediated delivery\textsuperscript{286}. Nanoparticles have advantages over the direct attachment of drugs to targeting molecules like monoclonal antibodies\textsuperscript{287} as the structure of the drug is not altered and the same targeted particle can house different molecules or combinations of molecules.

Many types of molecules are candidates for targeting labels including carbohydrates\textsuperscript{263}, ligands\textsuperscript{288}, peptides\textsuperscript{289}, antibodies\textsuperscript{255, 290}, selectins\textsuperscript{291}, folate\textsuperscript{120, 170, 171, 173}, and lectins\textsuperscript{263, 292}, oligosaccharides, and glycoconjugates are emerging as versatile targeting molecules because the surface of cells is rich in carbohydrates and attached
glycolipids/glycoproteins\textsuperscript{293}. Folate is the most commonly utilized targeting molecule as many tumors over express the folate receptor and it has been shown that folate functionalized particles localize selectively at the tumor\textsuperscript{171, 294, 295}. Attachment of these molecules to the nanoparticles is accomplished through covalent, electrostatic, or biotynilated conjugation techniques and is reviewed by Herman\textsuperscript{296}.

1.5.3.4 Avoiding the Immune System

All nanoparticles introduced to the body share the problem of having to evade the reticuloendothelial system (RES)\textsuperscript{297}. Upon introduction the particles are targeted by T-cells and engulfed by phagocytes and eliminated through the renal system. Surface properties are to blame and are usually modified to increase the nanoparticle residence time in vivo\textsuperscript{269}. Coating the particle with polyethylene-glycol (PEG) is the most common method of “stealthing” particles\textsuperscript{117, 132, 180, 252, 298}. The long chains of PEG have hydrophilic groups on the end that attract a layer of water resulting in a particle that looks like water to the RES and is ignored (Figure 1-3).

1.5.3.5 Examples of Targeted Drug Delivery Using Nanoparticles

In the past two decades targeted nanoparticles have been investigated for use in targeted chemotherapy\textsuperscript{259, 289, 299, 300}, ocular delivery\textsuperscript{301-303}, oral delivery\textsuperscript{262}, colonic delivery\textsuperscript{261}, pulmonary delivery\textsuperscript{304}, transdermal delivery\textsuperscript{268, 305}, and traversing the blood brain barrier\textsuperscript{298, 306, 307}. Chemotherapy is limited by poor penetration of the anti-cancer agents to the tumor by blood vessel walls, slow diffusion in the interstitial space between vessels and cells and the cell membrane itself\textsuperscript{259}. Bioavailability problems are
exacerbated by systemic toxicity of anti-cancer agents to surrounding tissues, allowing only low systemic doses to be used and negatively affecting the concentration gradients driving the diffusion; therefore, a lower total dose targeted to the tumor region is superior to long term systemic circulation\textsuperscript{132, 252}. The folate receptor is over expressed in a majority of human cancers and folate-receptor targeted nanoparticles are under investigation to deliver larger local doses to tumors\textsuperscript{170, 171, 294}. Once the particles are localized in sufficient concentrations at the tumor the drug can be released by an external trigger such as ultrasound\textsuperscript{259}. Ocular delivery is challenged by the blood-retinal barrier that results in low bioavailability from systemic circulations\textsuperscript{308}. A direct administration method via eye drops is preferred and drops containing slow-releasing polymeric nanoparticles have shown increased ocular residence time over therapeutic drops alone\textsuperscript{297} and also show longer residence in inflamed eyes over normal eyes thereby enhancing the delivery to these tissues\textsuperscript{309}. Oral delivery of proteins, peptides, genes, and vaccines is traditionally difficult due to poor permeability and instability in the gastrointestinal system and this problem can be alleviated by encapsulating these particles in a protective, ligands-targeted, site-specific particulate carrier where they can be delivered to gut-associated tissues or the colon where they can be microbially degraded to release the therapeutic or triggered by an external pH condition\textsuperscript{310, 311}. Nanoparticles are also under investigation for delivery of drugs to the central nervous system (CNS) and brain that are normally impeded by the blood-brain barrier and are reviewed by Lockman et. al\textsuperscript{312}. Nanoparticles can mask the identity of the molecule and allow passage into the brain by receptor mediated endocytosis and provide a metered release of the drug. The permeation can be affected by size and coatings like polysorbate 80 and PEG\textsuperscript{313, 314} as the
surface is more important than the core because it contacts the body fluids and interacts with the immune system\textsuperscript{315}. 

1.5.3.6 Potential for Calcium Phosphate Core-Shell Particles in Drug Delivery

Very few existing drug delivery nanoparticles are core-shell particles\textsuperscript{316-318} and, with the exception of liposomes, very few have aqueous cores. This is likely due to the increased complexity in synthesizing core-shell particles as discussed in Section 1.6.1. The fluid compartment of the shell can also be used to entrap fluorophores, drugs, proteins, genes, and similar in their native aqueous environment. Like their solid counterparts they can be injected or ingested, made of different materials with various surface properties and conjugated to multiple targeting moieties. A calcium phosphate coating can provide structural rigidity to an otherwise fragile liposome or emulsion in addition to providing a tailorable diffusive barrier. Solid calcium phosphate nanoparticles are not known to invoke an immune response and by coating a liposome with this material could provide “stealth” benefits to liposomes without the use of PEG.

1.5.4 Artificial Oxygen Carriers

1.5.4.1 Overview

Artificial oxygen carriers (AOCs) are intended to supplement or replace the donated blood supply in use today for trauma and surgical scenarios. To be an effective oxygen carrier, AOCs must be biocompatible, be able to traverse the vasculature unimpeded, be able to remain in circulation for days/weeks, be able to deliver physiologically relevant doses of oxygen, and remove gaseous metabolic byproducts (CO$_2$, NO, etc). It would be
preferable if the AOC was totally synthetic, non-immunogenic and affordable to produce on a large scale\textsuperscript{319}. Currently, the two principle artificial oxygen carriers are hemoglobin based substitutes\textsuperscript{320-323} and emulsified perfluorocarbons\textsuperscript{181, 324, 325}. Chemical structures and crystalline structures of each of these can be seen in Figure 1-5. Hemoglobin is a natural candidate for an oxygen carrier substitute as it is the native oxygen carrier in the body present within erythrocytes (red blood cells); however, the obstacles in harvesting hemoglobin from red blood cells (human or bovine), stabilizing it against oxidation to methemoglobin which can not release oxygen, and preparing it for use in the body complicate its use and are addressed Section 1.5.4.2. Perfluorocarbons rely on their weak intermolecular forces to allow many times more oxygen and other blood gasses to be dissolved over physiological saline\textsuperscript{326}. The chief drawback with perfluorocarbons is that they are water insoluble and require emulsification before they can be introduced to the aqueous \textit{in vivo} environment where they suffer from low half-lives from degradation and uptake by the immune system\textsuperscript{327}. 
Figure 1-5 Common oxygen transfer chemicals. Chemical structure of perfluorodecalin and perfluorooctyl bromide and a crystal structure of the human hemoglobin protein obtained from the protein data bank (www.rcsb.org)

Even though both of these approaches have shown efficacy in delivering oxygen to the body which is the most critical function in a trauma situation, they are not substitutes for whole blood which provides clotting, regulatory, metabolic, and immune functions in addition to O₂ transport. Currently, there is no way to replace all of these functions and this can lead to problems if inert solutions such as physiological saline are required to bring the artificial oxygen carrier up to a useable volume for blood replacement and can adversely affect clotting if corrective measures are not taken.

1.5.4.2 Hemoglobin Based Artificial Oxygen Carriers

1.5.4.2.1 Hemoglobin
Hemoglobin is an average size protein (64 kDa) that resides within the erythrocytes or red blood cells and is responsible for oxygen and other blood gas circulation in the body. It consists of two pairs of globin (α & β) chains with a Fe atom at the center. The valence of iron is critical in the binding of oxygen molecules to the protein and is moderated by a system of encapsulated enzymes within the erythrocyte without which hemoglobin oxidizes to the ineffective methemoglobin. The structure of hemoglobin allows it to bind oxygen cooperatively meaning oxygen binding increases as oxygen attaches to the protein up to a maximum of four O₂ molecules. This cooperative binding gives rise to the s-shaped sigmoid oxygen binding curve (Figure 1-6) for hemoglobin and is key to hemoglobin’s effectiveness as an oxygen carrier.

Hemoglobin binds 50% of its maximum oxygen (P₅₀) at a relatively low 26 mmHg and is fully saturated at atmospheric pressure.

![Figure 1-6](image)

**Figure 1-6** The sigmoidal binding curve of hemoglobin is critical to its effectiveness as an oxygen carrier. Hemoglobin carries its maximal amount of oxygen at atmospheric pressure and does not benefit from high oxygen partial pressures.
1.5.4.2.2 Pure Hemoglobin as an Artificial Oxygen Carrier

Hemoglobin is harvested from human\textsuperscript{333-335}, porcine\textsuperscript{336, 337}, or bovine\textsuperscript{338, 339} sources. This blood must be collected in a sterile environment, analyzed for potential contagions, typed, and separated, typically by centrifugation, from other blood components like white blood cells and red cell membranes at considerable expense to yield pure hemoglobin. Cell-free tetrameric hemoglobin is ineffective as an \textit{in vivo} oxygen carrier as the tetramer dissociates into toxic dimers in the bloodstream and is rapidly metabolized by the kidneys\textsuperscript{328, 340, 341}. Before free hemoglobin is cleared away, it can also lead to vasoconstriction, hypertension\textsuperscript{342-345}, and nerve damage\textsuperscript{341, 346-348}. In addition to toxicity, the binding of oxygen to free hemoglobin is high and difficult to release which affects the oxygen transport characteristics\textsuperscript{349}. To avoid complications associated with the use of free hemoglobin as a blood substitute, cellular polymerization (Section 1.5.4.2.3), cellular encapsulation (Section 1.5.4.2.4)\textsuperscript{338}, or genetic engineering methods are under investigation as solutions\textsuperscript{328, 350}.

1.5.4.2.3 Acellular Hemoglobin Aggregates as an Artificial Oxygen Carrier

Acellular hemoglobin aggregates are prepared by polymerization of the hemoglobin molecule with PEG\textsuperscript{351} or to itself with glutaraldehyde or o-raffinose\textsuperscript{352-355} to form larger oxygen carriers that, due to their size, can avoid uptake from the kidneys resulting in increased circulation half-life and reduced toxicity\textsuperscript{343-345, 356-359}. Thus, polymerization reduces renal toxicity effects while preserving the oxygen transfer characteristics of hemoglobin. With modifications to the polymerization, acellular
hemoglobins can be made with different oxygen binding coefficients with P50s ranging from the normal 26 mm Hg down to 4 mm Hg to over 34 mm Hg\textsuperscript{360-363} and combinations can be introduced to obtain unique oxygen dissociation profiles.

Three acellular hemoglobin based artificial oxygen carriers are undergoing development, two are from human hemoglobin Polyheme® and Hemolink® while the other is obtained from bovine hemoglobin, Hemopure®. They have shown efficacy in short term treatment of hemorrhagic shock and support of those with anemia\textsuperscript{320}. However, acellular hemoglobins suffer from common disadvantages: vasoconstriction assumed to result from excessive NO sequestering though the mechanism is not completely understood\textsuperscript{328, 364-366}, short circulatory half life that prevents use for long term applications\textsuperscript{367}, and prohibitive cost. One unit of Hemopure® costs $700 to $1000 as opposed to the proven and accepted donor blood at $150 to $200 per unit\textsuperscript{368}. This cost and performance shortcomings are barriers to the acceptance of acellular hemoglobin based substitutes as realistic alternatives to donor blood.

1.5.4.2.4 Encapsulated Hemoglobin as an Artificial Oxygen Carrier

Encapsulation of hemoglobin within a semi-permeable carrier provides two benefits: it allows the encapsulation of other proteins/reducing agents to reduce oxidation of hemoglobin and protection from the reticuloendothelial (RES) system through selection of a carrier surface chemistry providing a longer half life in the bloodstream\textsuperscript{357}. Currently, the encapsulating particle is composed of either pliable liposomes\textsuperscript{369} or polymersomes\textsuperscript{370}. Polymersomes are more rigid but as mentioned before are permeable to small molecules that can potentially deactivate or oxidize encapsulated hemoglobin\textsuperscript{367}. 
Liposomes could be less permeable due to the hydrophobic region within the membrane\textsuperscript{371}; however their liquid crystalline structure leaves them more prone to rupture from high shear forces in the blood stream.

Despite some performance improvements there are still problems. For example, when conjugated to PEG the biocompatibility of encapsulated hemoglobins is improved\textsuperscript{358, 372, 373} and the half-life is one week\textsuperscript{372, 374}. However, the clearance is found to accelerate after repeated injections\textsuperscript{374, 375} and the half-life is still much shorter than red blood cells that circulate for 4 months.

1.5.4.3 Perfluorocarbon Based Artificial Oxygen Carriers

Perfluorocarbons resemble hydrocarbons with hydrogen replaced by fluorine. Single C-F bonds have the highest bond energies of any single C-X bond at 488 KJ/mol resulting in very strong intermolecular forces, weak intramolecular forces, low viscosity, low surface tension, and a chemically and biologically inert molecule that is not metabolized in the bloodstream and are ultimately expelled (unchanged) in the lungs\textsuperscript{376, 377} after a short retention in the macrophage/monocyte system\textsuperscript{328}. Perfluorocarbons are entirely synthetic and can be readily manufactured in large quantities under current good manufacturing practices (cGMP) and are non-immunogenic. A list of relevant chemical properties for common PFCs can be found in TABLE 6-1. This synthetic nature of perfluorocarbons provides production/cost advantages over hemoglobin based substitutes which currently must be harvested as discussed in Section 1.5.4.2.2. The two most commonly used perfluorocarbons are perfluorodecalin\textsuperscript{378} and perfluoroocyt bromide\textsuperscript{326}. Unlike hemoglobin which binds to oxygen cooperatively, fluorocarbons rely on Henry’s
Law to entrap a large volume of blood gases including carbon dioxide. Perfluorocarbon emulsions are prepared at sizes usually less than 200 nm allowing them to deliver oxygen to places where larger particles (red cells are 7 um) can not such as collateral capillaries of an ischemic microcirculation. Emulsions are stable for over a year stored at 2-5°C.

1.5.4.3.1 Solubility of Oxygen in Fluorocarbon Emulsions

Perfluorocarbon emulsions do not transport as much oxygen per weight as blood but have superior oxygen release characteristics compared to hemoglobin as they release nearly 90% of their dissolved oxygen where human hemoglobin releases approximately 25%. Genetically engineered hemoglobin releases as much as 35 %, owing to a modified P₅₀ (Figure 1-7). On a w/w basis perfluorocarbons transport about 1/7th as much as hemoglobin but due to their near complete release characteristics they deliver almost 65% of the oxygen that hemoglobin delivers at atmospheric pressure. The emulsions release their solubilized oxygen into the plasma making it readily available for delivery before hemoglobin releases its bound oxygen, enhancing transport of existing blood-hemoglobin. Perfluorocarbons do not saturate with oxygen like hemoglobin and the dissolved oxygen increases with oxygen partial pressure allowing them to dissolve more O₂ when exposed to pure oxygen. To provide optimal oxygen delivery to the body a patient must breath pure oxygen but this is easily achieved in a surgical or emergency situation. There is evidence that suggests that PFC based emulsions increase plasma oxygen levels which has a positive effect on tissues such as brain, liver, kidney, pancreas, skeletal muscle, etc.
Figure 1-7  A) Recombinant derived hemoglobin can improve oxygen transfer from 24 % to 34 % at atmospheric oxygen partial pressures.  B) Perfluorobenzonitrile (PFOB) emulsions release ~ 40 % of the oxygen stored at atmospheric oxygen pressures. They deliver an equivalent amount of oxygen as blood at ~ 2 x atmospheric oxygen pressure. The efficiency of delivery increases with increasing oxygen partial pressure for fluorocarbons while remaining the same for blood. *Graph adapted from Spahn\textsuperscript{332}.

1.5.4.3.2 Current Perfluorocarbon Emulsion Based Artificial Oxygen Carriers

The use of perfluorocarbons as artificial oxygen carriers is complicated by their low water solubility and low lipophilicity. This low affinity for carbon based lipids, makes emulsification of these chemicals challenging and often requires the use of exotic and occasionally toxic surfactants to properly emulsify the compounds. The first perfluorocarbon based blood substitute Fluosol® used pluronic-f-68 surfactant to emulsify the perfluorocarbon which unfortunately has the potential to cause anaphylaxis\textsuperscript{383}. Fluosol® was removed from the market in the mid 90’s due to adverse side reactions stemming from this surfactant\textsuperscript{387-389}. Second generation formulations of perfluorocarbon based substitutes utilize more tolerable emulsifiers like egg yolk phosphocholine are undergoing clinical trials\textsuperscript{181}. Investigations utilizing fluorinated
phosphocholine as emulsifiers have yielded more stable, less permeant emulsions with improved surface activity, and biological properties\textsuperscript{376, 390}. Perfluoroctylbromide is a newer generation of perfluorocarbons that does exhibit some lipophilicity\textsuperscript{391}. Perfluoron®, a concentrated 60% w/w solution of perfluoroctylbromide is generally well tolerated when the particle size is around 170 nm and the size distribution is narrow\textsuperscript{332, 383, 392}.

Side effects from perfluorocarbon formulations are generally modest and include flu-like symptoms, nausea, headache and light fever\textsuperscript{383, 393-395}. Most of this has to do with the monocyte/macrophage system being activated and can be managed with steroids and cyclooxygenase inhibitors\textsuperscript{328}. However, several 2\textsuperscript{nd} generation perfluorocarbon based oxygen delivery vehicles Oxyfluor® and Oxygent® have been investigated and run into problems with toxicity issues\textsuperscript{368, 396, 397} and short circulation half-lives\textsuperscript{329}. Perfluorocarbon based Perftoran® is approved in Russia for human use but not elsewhere\textsuperscript{398}. Oxycyte® and Pher-O\textsubscript{2}® are undergoing development with applications as artificial oxygen carriers and in preoperative hemodilution\textsuperscript{328}. The current absence of an approved, affordable fluorocarbon based artificial oxygen carrier provides and opportunity to develop a new approach\textsuperscript{399}.

1.5.4.4 Potential For Calcium Phosphate Core-Shell Particles in Oxygen Delivery

An ideal blood substitute should be able to transport oxygen to the body while removing metabolic byproduct gases (CO\textsubscript{2}, NO, etc), stay in circulation for a period of weeks, have manageable side effects, and be shelf stable for months. Rigid core-shell structures could potentially provide these enhancements to the cellular hemoglobin and
perfluorocarbon strategies. Cellular hemoglobin based substitutes could benefit from the exploration of new rigid core-shell nanoparticle that combines the strength of a polymersome with the permeation characteristics of a liposome (Section 1.5.4.2.4). This could be accomplished by deposition of an inorganic material like silica\textsuperscript{400}, an organic polymer\textsuperscript{125}, or calcium phosphate\textsuperscript{401, 402} on a liposome template as discussed in CHAPTER 6. A similar biocompatible rigid core-shell particle would permit the surrounding of a perfluorocarbon emulsion and provide a method to change the surface chemistry. The biological surface activity of the particle exterior plays a pivotal role in the biocompatibility of a perfluorocarbon emulsion or liposome encapsulated hemoglobin\textsuperscript{377, 403}. The shell of the calcium phosphate nanoshell can serve as a scaffold for attachment of stealth molecules like PEG to shield the AOC from the RES and increase circulation time\textsuperscript{338, 358} by circumventing cellular recognition. The military has been interested for decades in a blood substitute that can be kept at room temperature and used under battlefield conditions\textsuperscript{368}. A rigid shell may allow the removal of solvent around the suspension and dry the particles to a powder while retaining the fluid characteristic within the core. The goal is to preserve the hemoglobin functionality and prevent the fluorocarbon from evaporating.
TABLE 1-1
SUMMARY OF ADVANTAGES AND DISADVANTAGES OF VARIOUS ARTIFICIAL OXYGEN CARRIERS AND POTENTIAL FOR CALCULUM PHOSPHATE

<table>
<thead>
<tr>
<th>Substitute</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>Sigmoidal O₂ curve, close to natural blood, full effectiveness at atmospheric O₂ pressure</td>
<td>expensive to obtain, low residence time (1 week), poor stability, oxidation, must be collected, must be encapsulated or polymerized, expensive $700-1000 vs. $150-250 for blood, toxic in free from</td>
</tr>
<tr>
<td>Emulsified Perfluorocarbons</td>
<td>Linear O₂ solubility curve, simple synthesis, completely synthetic, oxygenate the plasma</td>
<td>Linear O₂ sat curve-needs high O₂ pressures to reach maximal efficiency, require emulsification, circulation time (weeks), light symptoms, toxicity not fully known</td>
</tr>
<tr>
<td>Calcium Phosphate (Potential)</td>
<td>Proven to work with fluorocarbons, potential to work as an encapsulant for hemoglobin, powderable</td>
<td>Unproven</td>
</tr>
</tbody>
</table>

1.6 Technical Challenges in Calcium Phosphate Nanoshell Development

The development process of a novel calcium phosphate nanoshell that can be used as either a biosensor (Section 1.5.2), engineered contrast agent (Section 1.5.1), targeted drug delivery vehicle (Section 1.5.3), and as an artificial oxygen carrier (Section 1.5.4) is non-trivial. Development of these particles requires addressing the following synthesis, functionalization, and analysis challenges:

- Synthesis: A novel synthesis must be developed, which involves reproducible preparation of the nanoshell liquid core templates (liposome, microemulsion), reliable coating of the template with calcium phosphate, control over the size and thickness of shells, salt and protein aggregation, and concentrating the shells.
• Functionalization: A protocol for the encapsulation of functional molecules like fluorescent dyes or fluorocarbons, and the attachment of enzymes and antibodies to the shell surface must be established.

• Analysis: Quantitative and well understood analysis methods sensitive to the relatively low concentration of shell suspensions (~1 nM), sub micron size, and heterogeneity of the particles must be validated.

Synthesis of the nanoshells is the most important issue to address in this study since it controls the properties of the final material. Functionalization of the shells is critical in determining the feasibility of using this newly developed material in the biomedical applications covered in Section 1.5. Studying the physical and chemical characteristics of the shells is complicated by both the diminutive size of the nanoshells which affects the type of techniques that can be used for detection, the heterogeneous nature of the suspension complicates analysis of individual shells by bulk techniques and the low concentrations of particles which affects absorbance, fluorescence, chemical, and crystallographic analysis. Solutions to these problems are complex and are addressed in the following sections.

1.6.1 Issues in Synthesis of the Nanoshells

1.6.1.1 Preparation of Templates (Liposomes, Microemulsions)

Convenient templates for the formation of calcium phosphate nanoshells are either microemulsions or liposomes. The goal for template preparation is to obtain a defect free, monodisperse spherical template suspension with narrow size distribution.
that is stable to changes in ionic strength when the inorganic salts are added to the reactant mixture. Liposomal templates normally begin as lyophilized powder that is hydrated to swell the lipid bilayer and eventual budding to form a spherical liquid crystalline lipid bilayer enclosing an aqueous pocket.

Figure 1-8 Process of liposome formation resulting from lipid hydration (taken from Avanti Lipids http://www.avantilipids.com/PreparationOfLiposomes1Big.html).

Microemulsions are produced with the introduction of oil into this lipid suspension and subsequent homogenization through stirring (vortexing), sonication, or high shear
extrusion (Figure 1-9). The resulting suspension in either case are non uniform and not easily reproduced unless attention is paid to the temperature, wt % of lipid, stirring speed and time, sonication time, and adequate resting time.

Figure 1-9  Cartoons illustrating the process of homogenization to form microemulsions, sonication and extrusion. Homogenization relies on the achievement of the lowest energy state of the lipid or lipid-oil system and results in a suspension of emulsions or liposomes when DOPA is used. Sonication reduces a homogenized emulsion to a smaller size by the addition of high frequency pressure waves. Extrusion utilizes shear forces to push a large liposome/emulsion through a small pore and results in a monodisperse emulsion of liposome/emulsions.

1.6.1.1.1  Size Control of Templates (Sizing)

If prepared by simple mixing, liposomes and microemulsions have an irreproducible and wide size distribution. In order to make the suspension more monodisperse size reduction and homogenization methods are used\textsuperscript{111}. The size of the
templates is important in particle transport, loading capacity, and colloidal stability and is influenced by the type of homogenization. Stirring, sonicating, and extrusion are accepted methods for manipulating the size distribution of liposomes and microemulsions. For a given lipid/oil suspension at a constant composition, the outcome of each homogenization method is sensitive to different parameters.

- Stirring effectiveness depends on the time stirred, stirring speed, size of the stir bar, volume and shape of the vessel, and temperature.
- Sonication effectiveness depends on the position of the sample in the sonicating bath and the level of water because this affects the intensity of the pressure waves and sonication geometry. The time of sonication is also important.
- Extrusion through a porous filter produces particles sizes that depend critically on the extrusion filter pore diameter, the number of extrusion passes, the extrusion pressure and temperature.

There is little available theory to determine the parameters required for a certain size a priori. Optimization of these parameters requires a trial and error approach to attain the desired size and is discussed in Section 3.4. Size control of templates and the resulting charge can have an effect on clearance rates from the body with negative charge and sizes around 100-200 nm being optimal.

1.6.1.1.2 Maintaining Template Structural Stability During Synthesis
There are two principle mechanisms by which liposomes and emulsions can reorganize: coalescence/aggregation and bursting from osmotic pressure gradients. Newly formed liposomes/microemulsions with phosphatidic acid headgroups (TABLE 1-2) resist coalescence sterically through the hydration layer present on their exterior in aqueous systems and electrostatically by their pH dependent surface charge resulting from the ionizable protons from the phosphate headgroups.

TABLE 1-2
TABLE OF LIPIDS COMMONLY USED TO FORM LIPOSOMES OBTAINED FROM AVANTI POLAR LIPIDS

<table>
<thead>
<tr>
<th>Lipid Name</th>
<th>Abbreviation</th>
<th>Carbons: Unsaturation</th>
<th>Transition Temp. Tc(°C)</th>
<th>Net Charge at pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-Dilauroyl-sn-Glycero-3-Phosphocholine</td>
<td>DLPC</td>
<td>12:00</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine</td>
<td>DMPC</td>
<td>14:00</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine</td>
<td>DPPC</td>
<td>16:00</td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td>1,2-Distearoyl-sn-Glycero-3-Phosphocholine</td>
<td>DSPC</td>
<td>18:00</td>
<td>55</td>
<td>0</td>
</tr>
<tr>
<td>1,2-Dioleoyl-sn-Glycero-3-Phosphocholine</td>
<td>DOPC</td>
<td>18:01</td>
<td>-20</td>
<td>0</td>
</tr>
<tr>
<td>1,2-Dimyristoyl-sn-Glycero-3-Phosphoethanolamine</td>
<td>DMPE</td>
<td>14:00</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine</td>
<td>DPPE</td>
<td>16:00</td>
<td>63</td>
<td>0</td>
</tr>
<tr>
<td>1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine</td>
<td>DOPE</td>
<td>18:01</td>
<td>-16</td>
<td>0</td>
</tr>
<tr>
<td>1,2-Dimyristoyl-sn-Glycero-3-Phosphate</td>
<td>DMPA.Na</td>
<td>14:00</td>
<td>50</td>
<td>-1.3</td>
</tr>
<tr>
<td>1,2-Dipalmitoyl-sn-Glycero-3-Phosphate</td>
<td>DPPA.Na</td>
<td>16:00</td>
<td>67</td>
<td>-1.3</td>
</tr>
<tr>
<td>1,2-Dioleoyl-sn-Glycero-3-Phosphate</td>
<td>DOPA.Na</td>
<td>18:01</td>
<td>-8</td>
<td>-1.3</td>
</tr>
</tbody>
</table>
Figure 1-10 Structures of commonly used lipids for liposome formation. There are three head groups choline, ethanol amine, and phosphatidic acid as well as four tail types lauryl, myristoyl, palmitoyl, oleyl. DOPA is the most successful for shell synthesis. Structures obtained from Avanti Polar Lipids.
Coalescence can be negatively impacted by an increased salt concentration or a decrease in pH, resulting in a decreased net electrostatic charge on the liposome surface allowing the liposomes to approach close enough to each other to coalesce and reform.

Reorganization of the template prepared in distilled water can occur upon introduction to a reaction solution which includes calcium salts, phosphoric acid, sodium hydroxide and other salts at a different tonicity than the stock. This phenomenon can be understood by considering the internal energy and chemical potential of the system described by:

\[
dU = TdS + SdT - VdP - PdV + \sum_i u_i dn_i + \sum_i n_i du_i \quad \text{where,} \quad u_i = \left( \frac{\partial U}{\partial n_i} \right)_{T,P} + \left( \frac{\partial U}{\partial n_i} \right)_{T,P}
\]

Equation 1-5

and

\[
u_i = u_i^0(T,P) + RT \ln x_i y_i
\]

Equation 1-6

At equilibrium, the energy (U) on either side of the liposome must be equal. Adding liposomes prepared in a dilute solution to a concentrated solution or vice versa introduces an unequal chemical potential (\(\nu\)) gradient across the membrane, which is unstable because the liposome bilayer is impermeable to some ions and small molecules. The system must equilibrate the potential difference, and so for water permeable liposomes
this typically happens by transport of water and ions in and out of the liposome. This leads to either a change in volume or pressure both of which can ultimately lead to a rupture of the original liposome. This can be avoided by minimizing the chemical potential gradient across the membrane by balancing salt concentrations inside and outside of the liposomes during synthesis and coating, which keeps the liposomes stable and avoids rupture and rephasing to other lipid structures like micelles, tubes, multilamellar liposomes, and others.

For cases in which precise chemical potential matching inside and outside of the liposome is difficult to achieve, or for lipids whose structure results in loosely associated liposome bilayers (such as saturated lipids), adding cholesterol to these liposomes is a useful method to enhance the mechanical stability of the lipid bilayer so that it can withstand small fluctuations in osmotic pressure. The cholesterol molecules interact with the hydrophobic tail portion via strong Van der Waals forces and increase the interaction energies between the lipid tails as illustrated below. In contrast to liposomes, microemulsions do not experience the same osmotic pressure swelling problems because the core of an emulsion is immiscible with the exterior aqueous solvent thereby preventing intrusion of water into the emulsion. However their stability is affected by ions which can alter the head group effective charge and association, and by molecules which are somewhat soluble in organic solvents, since these, like cholesterol, become integrated into the bilayer changing it into a complex system whose assembly and packing properties can be quite different from that of the pure lipid.
1.6.1.2 Aggregation of Calcium Phosphate Nanoshells During Synthesis

While precipitation of calcium and phosphate ions onto existing template seeds is thermodynamically favored over homogeneous crystallization, collisions between particles and the speed of the precipitation reaction can lead to irreversible particle aggregation during the reaction (Figure 3-24). This effect increases as the collision frequency (Equation 1-7) given below increases.

\[
Z_{AB} = S \cdot U_R C_A C_B
\]

Equation 1-7

where A (C_A) is the concentration of particle A, B (C_B) the concentration of particle B, \( U_R \) is the relative velocity, and S is the impact cross section of the shell. Not all collisions that occur will result in reaction as the particles must be in contact with each other for a time comparable to the reaction time. This reaction rate increases with concentration of

Figure 1-11  The introduction of cholesterol into a lipid membrane effectively increases tai-tail interactions and results in a more tightly packed and rigid membrane.
calcium (Section 2.4.2.2) and can be mediated by controlling the supersaturation. This leaves the two parameters, supersaturation and collision frequency, to consider when trying to reduce nanoshell aggregation. This is complicated by the fact that experimental determination of the supersaturation (S) and the number of shells (CA, CB) is difficult. Flocking can be reduced by lowering the supersaturation to reduce the reaction rate (Equation 1-3) or by reducing the shell concentration to reduce the collision frequency (Equation 1-7). These modifications require longer reaction times and produce fewer nanoshells per ml requiring an optimization between flock, reaction time, and shell yield.

1.6.2 Issues in Functionalizing the Nanoshells

Functionalization of a particle can be achieved by encapsulating a substance within the shell or attaching a molecule on the outside of the particle. Issues regarding these two methods are described in greater detail below.

1.6.2.1 Loading Calcium Phosphate Nanoshell Templates with Functional Molecules

Nanoshells alone have limited biomedical usefulness, and they need to be enhanced by a functional molecule like a fluorescent dye for tracking, a reactive dye for monitoring, a contrast agent for imaging, an oxygen carrier, a protein or enzyme etc. Maximal loading of a suspension of shells with a functional molecule is limited by the concentration of particles in the suspension, the solubility of the solute in the particle phase(s), the size of the shells, and the ability of the particle to retain the solute. There are various methods used to encapsulate solutes (drugs, fluorophores, biomolecules, etc) into liposomes including freeze thaw\textsuperscript{406, 407}, lipid film hydration\textsuperscript{408, 409}, cross flow injection
method, dehydration rehydration, and others reviewed here. Of these, lipid film hydration is the most direct involving only hydrating a freeze dried lipid film with a solution of the solute to be encapsulated and is used exclusively in this thesis.

1.6.3 Determination of the Concentration and Condition of Encapsulated Solutes via Spectroscopy

The absorbance and fluorescence spectrophotometric techniques provide an accessible and effective way to determine the concentration of species in solutions. The analysis of encapsulated solutes is complicated by the interactions of the analytical technique with the shell itself. This problem is mainly in the form of scattering of the incident or fluorescent light.

Absorbance of light in the UV-visible region occurs from the excitation of outer electrons from a ground to excited state. This occurs on certain functional groups called chromophores that contain valence electrons of low excitation energy (typically n-π, π-π). Since there are many possible excited states available an absorption band is observed that is the superposition of these individual bands. Depending on the type of excitation taking place, solvent polarity can have an effect on the resulting spectra principally due to solvation effects on lone pairs of electrons from n-π transitions.
Absorbance provides reliable determinations of solute concentrations in solutions by virtue of Beer’s Law

\[ A = \varepsilon LC \]

Equation 1-8  Beer’s Law

Where \( A \) is the absorbance from the spectrophotometer, \( \varepsilon \) is the molar absorptivity, \( L \) is the path length of absorbed light, and \( C \) is the molar concentration of the species. However, this method can be complicated by the scattering of the incident light by particles resulting in a measured absorbance higher than expected. For example, in the UV (400
nm or less), light scattering makes absorbance measurements difficult; although, integrating spheres and background subtractions can help to alleviate these issues.

 Fluorescence relies on emitted light rather than absorbed light to ascertain information on the solutes in solution. Fluorescence occurs when a molecule absorbs light and attains an excited state. Molecules normally attain an unstable excited state that lasts $10^{-15}$ to $10^{-9}$ seconds (called the excited lifetime) before relaxing to the lowest energy excited state. From here the molecule can reach its normal unexcited state through emission of a photon with a longer wavelength as some energy lost in the process. This energy is often lost as heat to vibrations with the surrounding solvent and within the molecule itself. A detailed review of fluorescence spectroscopy can be obtained here\textsuperscript{415}.

 Fluorescence spectroscopy is less susceptible to scattering interference than absorbances because the emitted wavelength is generally higher (400+ nm) than the size of the shells and the interaction of emitted light with the sample is less than with absorbance. Stoichiometric analysis via fluorescence is complicated because the quantum yield ($\phi$) can vary unpredictably with temperature, salt concentration, dye concentration, and concentration and composition of solution. This results in a fluorescence intensity that is not linear for all concentrations and is machine dependent and requires calibration.

1.6.3.1 Surface Functionalization

 Surface chemistry influences the stability of the particles in suspension and can give a function within the body such as increased stability\textsuperscript{416} or cellular recognition\textsuperscript{170,417}. 

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The stability of the particles can be improved by addition of PEG\textsuperscript{418} which increases the steric repulsive forces or through the addition of a charged molecule to improve the electrostatic repulsion of the particles. As mentioned in Section 1.5.3 there are multiple methods to modify surfaces that allow a particle to target a specific region or tissue within the body. There are several different chemistries that are commonly used to conjugate molecules (sugars, nucleic acids, and proteins) to particles surfaces. Some of the most common chemistries are listed below in TABLE 1-3 and an extensive review of the field can be found in Bioconjugate Techniques by Hermanson\textsuperscript{296}. For carboxylated surfaces such as those present on calcium phosphate nanoshells the most direct method for attachment is amine carboxylic acid chemistry. For amine-carboxylic acid coupling, the attachment process is complicated by the fact that polymerization of the proteins/antibodies can occur between their carboxyl and amino termini with the same chemistry used to attach them to the carboxylated surface. The active intermediate (\textit{o-}

acylisourea) is short lived and is typically used in conjunction with \textit{N-}

hydroxysuccinimide to increase final conjugation yield. Proteins such as enzymes and antibodies often contain many free amines from their residues in addition to an amino terminus. These amines allow for multiple points of attachment which can cause complications as attachment orientation is important for enzymes and antibodies as their active sites must remain sterically unhindered to react with their substrates and antigens. The specific chemistry and conditions used during carbodiimide attachment determine the attachment efficiency of functional molecules and the percentage of residual activity of antibodies and enzymes and are discussed in CHAPTER 5.
### TABLE 1-3

**COMMON BIOCONJUGATION CHEMISTRIES**

<table>
<thead>
<tr>
<th>Linkage</th>
<th>Crosslinker Used</th>
<th>Bond Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiol-Thiol</td>
<td>dibromobimane, bis-((N-iodoacetyl)piperazinyl)sulfonерhodamine</td>
<td>R—S—S—R</td>
</tr>
<tr>
<td>Thiol-Amine</td>
<td>Succinimidyl trans-4-(maleimidylmethyl)cyclohexane-1-carboxylate</td>
<td>R—S—H—N—R</td>
</tr>
<tr>
<td>Amine-Amine</td>
<td>glutaraldehyde, bis(imido esters), bis(succinimidyl esters), diisocyanates and diacid chlorides</td>
<td>R—H—N—N—R</td>
</tr>
<tr>
<td>Amine-COOH</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC)-N-Hydroxysuccinimide</td>
<td>R—H—C—R</td>
</tr>
</tbody>
</table>

1.6.4 Determination of Oxygen Solubility and Transfer in Nanoparticles

The primary requirement of an oxygen carrier is the particles ability to retain and transport oxygen. This requires special attention due to the heterogeneity of a sample with an oxygen carrying core phase and water as only the continuous phase (water for an emulsion) is directly measurable as demonstrated below.

![Figure 1-13](image) An oxygen probe is in contact only with the continuous (aqueous) phase and not able to directly probe the concentration of the interior of the particles. Existing solutions are to use enzymatic oxygen consumption methods described in CHAPTER 6.
Many calcium phosphate nanoshell samples contain less than 1 wt % of particles that carry less than 10% more oxygen than control samples. Direct measurement of these small amounts of oxygen is not possible with electrochemical probes as they consume an amount of oxygen comparable to what is measured and are not sensitive at very low concentrations of oxygen. Probes that do not consume oxygen during a measurement such as a fiber optic fluorescent probe that monitors the oxygen quenching of ruthenium embedded in silica (FOXY probe-Ocean Optics) can still be used though. Enzymatic glucose assay methods with O₂ as a limiting reagent⁴¹⁹ can be used to determine the O₂ concentration in such a heterogeneous sample by consuming and reporting on all present oxygen within the sample. Hemoglobin is also capable of monitoring the oxygen release characteristics by observing changes in the absorption spectrum as the oxidation state changes⁴²⁰. Methods to determine the oxygen retention and release characteristics are reported in CHAPTER 6.

1.6.5  Assessment of Nanoshell Biocompatibility

Before a novel nanoshell material can be used in vivo it must pass a rigorous series of tests. Biocompatibility for a prolonged implant device in contact with blood such as a circulating nanoparticle is subject to the following tests as determined by the ISO 10993 test matrix and paraphrased from Nelson Labs.

- Cytotoxicity (10993-5): rapid standardized test for detection of harmful extractables to cellular components. The sample is incubated on top a
mammalian cell culture (L-929) cell monolayer covered agar then incubated. Cells are scored for cytopathic effect using 10993-5 standards.

- **Sensitization (10993-10):** determines effects of repeated doses on immune system by exposing skin and potentially exposed organs. Swelling and redness is noted to measure the degree of interaction with body’s immune system.

- **Acute systemic toxicity (10993-11):** determines adverse effects on bodies organs with potential contact of implant. Test is conducted on animals over the period relevant to the device. Accompanied by a histopathology of the animal after that period.

- **Genotoxicity (10993-3):** in-vitro tests designed to detect carcinogens and mutagens. Utilize mammalian cell cultures tests to determine gene mutations, chromosome changes and other gene toxicities. Salmonella typhimurium is often used due to its sensitivity to mutation, Chinese hamster ovary (CHO) cells are used to measure chromosomal aberration.

- **Hemocompatibilty (10993-4):** determines compatibility of device with circulating blood. Tests can be conducted in human, rabbit, or dog blood and range from detection of coagulation abnormalities, osmotic fragility, complement activation, cell lysis/hemoglobin leakage and others covered under ISO-10993 1, 4, and 12.

Detailed explanations for each of these tests can be found within their respective ISO-10993 standards. A battery of tests such as this consists of assays, in vitro cell culture, and live animal studies that can cost over $30,000 and require more than 150 g of
material (Nelson Labs-2005). The material requirements for a nanoshell based material can require a prohibitive number of lab scale reactions which yield only milligram quantities. To reduce both costs and material requirements, some of these tests can be omitted for materials that already use components known to be biocompatible. Other tests can be omitted on a per application basis.

1.7 The Work in this Thesis

This work is focused on the development of a novel bio-nanomaterial that can be used in medical imaging, as a biosensor, for targeted drug delivery, and as a gas transport vehicle in vivo. Calcium phosphate nanoshells aim to improve upon the applications listed in Section (1.5) by providing a nanoscopic vehicle that can carry hydrophilic or hydrophobic materials with good retention, can protect these contents with a rigid-semipermeable shell, can be reproducibly synthesized, are non-toxic, can have variable surface chemistry, can be conjugated to proteins, and could potentially be powderable. All of these criteria can be met with the same versatile nanoshell with little modification of the synthesis.

Much of the investigation is in synthesis development and characterization of size, concentration, thickness, stability, permeability, and morphology of aqueous cored calcium phosphate nanoshells (CHAPTER 2), soy-oil, and fluorocarbon cored calcium phosphate nanoshells (CHAPTER 3) through transmission electron microscopy, atomic force microscopy, spectroscopy, and photon correlation spectroscopy (dynamic light scattering).
The remainder is in the exploration for potential biomedical applications. Imaging and biosensors were explored by encapsulating and characterizing fluorocarbon contrast agents (CHAPTER 4) and the fluorophores: pyranine, pyrene, FITC, sulforhodamine. Determination of the permeability of the shells to peroxide and copper ion was determined from pyranine quenching experiments (CHAPTER 4). Targeted drug delivery and immunoassay potential was investigated by attachment of anti-fluorescein antibodies and horseradish peroxidase to the nanoshell surface through varied bioconjugate chemistries (CHAPTER 5). Their resulting activities were also quantified. Determining the prospect for the nanoshells use in O₂ and metabolic gas transfer was done by encapsulating perfluorodecalin and perfluorooctylbromide and quantifying the oxygen retention and transport characteristics via oxygen electrodes and enzymatic assays (CHAPTER 6).
2.1 Introduction

As discussed in section 1.5 there is an interest in the development of nanoparticles capable of encapsulating hydrophilic materials such as fluorescent dyes, contrast agents, therapeutics, and proteins within them that can be safely used \textit{in vivo}. In this section, several novel methods were developed for the synthesis of liposome templated calcium phosphate nanoshells. Calcium phosphate nanoshells entrapping water soluble fluorophores are discussed in CHAPTER 4. Those fluorescent nanoshells can be used as \textit{in vivo} or \textit{in vitro} sensors when coupled with the protein attachment methods discussed in CHAPTER 5.

The synthetic method design needed to meet the following criteria was reviewed in CHAPTER 1 and is repeated here:

- The quality of the suspension should be reproducible and insensitive to minor fluctuations in template preparation, reagent concentration, temperature, volume, stirring speed, and pressure..

- Nanoshells produced should be monodisperse in aqueous environments with a mean diameter less than 200 nm and size distributions better than \(\pm 20\%\) of the mean.
• The mean diameter and the thickness of the calcium phosphate shell need to be adjustable.

• The shells should be as concentrated as possible while remaining stable at physiological concentrations of salt and protein.

• The product should have a shelf life of at least 6 months at room temperature or refrigerated.

• Components of the synthesis need to be obtainable as cGMP certified compounds and approved for use in potential in vivo applications.

• Product synthesis byproducts should also be biocompatible/biodegradable, and easily removed post synthesis through filtering, centrifugation, size exclusion chromatography, or other suitable separation methods.

• The apparatus for synthesis should not be complicated and be amenable to scale up.

• The synthesis protocol should permit steps to allow for the encapsulation of molecules, and should be mild enough to avoid biomolecule denaturation.

• The product should be analyzable with convenient techniques such as: AFM (for hardness and morphology), TEM (for hollowness and shell thickness), DLS (for size distribution in suspension), Zeta Potential (for surface charge and stability in salt), flow cytometry (for particle quantification), UV-Vis absorption and fluorescence spectroscopy (for monitoring encapsulated molecules).
Developing a synthesis that met all these criteria was the major challenge of this project. This was approached by a combinatorial variation of the conditions for the reaction of calcium and phosphate ions on the surface of liposomes to optimize the synthesis. This method was later extended to shells composed of a microemulsion core in CHAPTER 3.

2.2 Experimental

2.2.1 Materials

Solutions of 0.29 M Calcium nitrate (CaNO₃), 0.29 M calcium acetate (CaAc), 0.29 calcium citrate (CaCit), 1 M calcium chloride (CaCl₂), 0.24 M mono and dibasic potassium phosphate (HK₂PO₄, H₂KPO₄), 0.24 M mono and dibasic sodium phosphate (HNa₂PO₄, H₂NaPO₄), ammonium phosphate ((NH₄)₃PO₄), 1 M phosphoric acid (H₃PO₄), and 1 M sodium hydroxide (NaOH) were obtained from Fisher Scientific and used without further purification to make the indicated stock solutions. Carboxyethylphosphonic acid (CEPA) and cholesterol were obtained from Sigma-Aldrich. The phosphatidic acid lipids DOPA (Figure 2-1), DLPA, DMPA, DPPA, DSPA were obtained as lyophilized powder from Avanti Lipids. Stock solutions of lipids were prepared by adding 25 mg of lipid to 5 ml of water and were used within one week. All water was deionized to 18 MΩ via an E-Pure water filtration system (Millipore). All cellulose acetate (CA) syringe filters, polycarbonate (PC) filters, and extrusion membranes were obtained from Millipore. Snakeskin™ dialysis tubing with 3,500 MWCO (Pierce) and Spectrapore (Pierce) tubing with 60,000, 100,000 MWCO, and 300,000 MWCO were used for removal of unreacted materials. Twenty nm
hydroxyapatite nanocrystals were obtained from Berkeley Advanced Biomaterials and used as a comparison standard.

2.2.2 Methods

2.2.2.1 Liposome Preparation

The liposomal templates for aqueous-cored calcium phosphate nanoshells were formed by the interaction of water with layers of dried lipid which caused swelling and budding of the film into multilamellar (onion-like) liposomes. For this, either dried phosphatidic acid layers formed by either evaporation of chloroform-lipid solution or a lyophilized lipid powder were used with equally good results.

The hydrated multilamellar liposome suspension was not suitable for use as a nanoshell template as its size was not reproducible. To improve the size distribution, the solution was subjected to high shear via mixing, sonication, or extrusion to reorganize the multilamellar liposomes to primarily unilamellar liposomes of defined size. The effectiveness of 1) mixing with a 3/4” magnetic stir bar at 1000 RPM in a 20 ml scintillation vial for 24 hours at room temperature, 2) sonicating by placing 5 ml of lipid suspension below the water line in the center of a Fisher FS-30 bath sonicator filled with one inch of water for 15 minutes to 2 hours, and 3) extrusion through a 25 mm polycarbonate extrusion membrane with 100 nm pore size fitted to a 10 ml thermobarrel LIPEX extruder (Northern Lipids) were evaluated for the formation of nanosized single layer liposomes as follows:
2.2.2.1.1 Mixing

Hydrated lipid solutions were mixed with a 3/4” magnetic stir bar at 1000 RPM in a 2.5 m wide, 20 ml scintillation vial for 24 hours at room temperature to produce liposomes. Mixing produced large liposomes of mean size 427 nm with a standard deviation of 135 nm.

2.2.2.1.2 Sonication

Hydrated lipid solutions were mixed with a 3/4” magnetic stir bar at 1000 RPM for 1 hour in a 20 ml scintillation vial. They were then capped and placed at a 45 degree angle to submerge the liquid containing portion (~ 1 cm) of the vial in the center of a Fisher FS-30 bath sonicator filled with one inch of water. The suspension was sonicated for various lengths of time ranging from 15 minutes to 2 hours until the desired size ranging from (30 nm to 135 nm) is reached. The final temperature was between 45 and 55 C°.
2.2.2.1.3 Extrusion

Hydrated lipid solutions were mixed with 3/4“ magnetic stir bar at 1000 RPM for 1 hour in a 20 ml scintillation vial. A 25 mm polycarbonate extrusion membrane with 100 nm pore size was fitted to a 10 ml thermobarrel LIPEX extruder from Northern Lipids. A 1/4“ x 1/16“ magnetic bar was placed on top of the membrane before the apparatus was closed. The lipid suspension was added to the extruder via Pasteur pipette, which was then capped and placed on top of a magnetic stir plate. The fluid outlet was fed to a retention vial. Industrial grade nitrogen gas (Mitler Gas Supply) was used to drive the fluid through the membrane at 200-800 psi depending on desired flow rate (~ 1-2 ml/min). The suspension was passed through the extruder 11 times to obtain a liposome suspension size distribution comparable to the pore size, or 103 nm +/- 10 nm. Once the extrusion was complete there was a volume loss of about 0.1 ml attributed to filter losses and loss on the inside of the extruder.

2.2.2.2 Synthesis

Three types of synthesis were investigated and are presented in the order they were developed 1) the dropwise synthesis relied on manual titrations of calcium and phosphate salts 2) the one step supersaturation synthesis was performed by simultaneous addition of all ingredients to the reaction 3) the stepwise supersaturation synthesis is a hybrid of the two methods and utilizes the strengths of both methods
2.2.2.2.1 Dropwise Synthesis

Dropwise synthesis is a modified version of the metathesis synthesis. In brief, two 25 ml addition burettes were fused to a 14/20 glass joint and fed to a 4 necked round bottom 150 ml Pyrex flask reactor (Figure 2-2). Nitrogen gas purged the air in the reactor volume and was left on at ~ 100 ml/min to prevent oxygen and CO₂ contamination. The reaction was monitored by an Orion 420A+ pH meter, connected to a custom LabVIEW monitoring program via an RS-232 serial interface to an IBM PC.

To one of the two addition burettes, 10 ml of DI water plus 150 μl of 0.29 M calcium nitrate were added and to the other 10 ml of water plus 105 μl of 0.24 M diammonium phosphate. After purging, the reaction flask was filled with 50 ml of DI water and 2.5 ml of sonicated DOPA liposome solution. Strong ammonia was added under gentle stirring (300 RPM) to the solution dropwise until the pH was 11.5. Once the pH was constant, a 400 μl volume of the 0.29 M calcium nitrate solution was added and allowed to stir for 10 minutes followed by the first 400 μl portion of the ammonium phosphate solution, which stirred for 10 additional minutes. Alternating additions continued in this manner for two more additions then the stirring was increased to 1000 RPM to improve agitation and the alternating additions were spaced from 10 minutes to 2 minutes. Shell suspensions were rested for 3 days to reach equilibrium before any characterization was performed. After the 10th addition the shell thickness was found by TEM analysis to be approximately 3-5 nm.

To verify the effects the liposomes have on calcium phosphate crystallization, a control experiment identical to this one but without the lipid was performed.
2.2.2.2 One Step Supersaturation Synthesis

As will be described later the dropwise synthesis results were difficult to reproduce and an alternative method relying on an improved understanding of the solution supersaturation was developed. To coat the liposomes with calcium phosphate using this method, 50 ml of DI water were mixed with 10 μl of 1 M phosphoric acid and 40 μl of 1 M NaOH (pH ~10). One hundred microliters of 0.1 M CaCl₂ and 150 μl of prepared liposomes were added to this solution within 5 seconds of each other using a volumetric pipette while the suspension was stirred magnetically at 400 RPM at room temperature, typically 25° C. To generate a set of samples with different thicknesses, 50 μl of 0.1 M CEPA solution was pipetted into the stirring solution at 10, 30, 60, 90, 120, 150, and 180 minutes after the reaction began to halt the reaction and stabilize the shell.
suspension. Following CEPA addition, the suspension was allowed to stir for at least 1 day before any post processing or analysis.

Figure 2-3 Schematic of one step supersaturation synthesis. *Hydration* of the lipid film with DI water (or a solution of molecules to be encapsulated (CHAPTER 4)). The lipid film then *swells* and forms a suspension of multilamellar liposomes surrounding the hydration fluid. These multilamellar liposomes must be *sized* to provide a reproducible starting point for the reaction and can be done by extrusion (shown) or sonication. The sized liposomes are then added to a supersaturated synthesis to *initiate* the reaction. In the early stages the calcium and phosphate ions are *localized* around the negatively charged liposome-increasing the local supersaturation around the liposome. The nanoshell begins to *grow* and continues until the shell is *capped* by CEPA thereby halting further growth. The nanoshell suspension is rested for 1 day and then analyzed.

### 2.2.2.2.3 Multistep Supersaturation Synthesis

As will be shown later, the one-step synthesis provided shell thickness control but was cumbersome because the addition of a surface stabilizer (CEPA) had to be timed. To allow for simplified thickness control, an improved method was developed to more accurately control the supersaturation by combining the stepwise and one step supersaturation synthesis methods. Six hundred microliters of 100 nm extruded liposomes were added to a 100 ml Pyrex beaker with 50 ml of DI water and stirred magnetically at 400 RPM at room temperature. Five microliters of 1 M CaCl$_2$ and 1 M
H₃PO₄ adjusted to pH 7 with NaOH were added via a volumetric pipette to the reaction and stirred. After 10 hours, when the solution calcium concentration stabilized (Figure 2-19), the average particulate size distribution was measure using dynamic light scattering size and TEM grid, after which another 5 µl of calcium chloride and pH adjusted phosphoric acid were added to the reaction vessel and stirred for another 10 hours. This process was repeated until the desired shell size was obtained. After the final addition, 200 ul of 0.1 M CEPA were added to the reaction to coat and stabilize the shells.

Figure 2-4  Schematic of the multistep supersaturation synthesis. *Hydration* of the lipid film with DI water (or a solution of molecules to be encapsulated (CHAPTER 4)). The lipid film then *swells* and forms a suspension of multilamellar liposomes surrounding the hydration fluid. These multilamellar liposomes must be *sized* to provide a reproducible starting point for the reaction and can be done by extrusion (shown) or sonication. The sized liposomes are then added to a subsaturated synthesis to *initiate* the reaction. A dose of calcium is added to *supersaturate* the reaction. The ions begin to *localize* around the liposome. *Growth* occurs over a period of hours before another dose of calcium is added. This growth-dosing process is repeated until a shell of desired thickness is obtained and the reaction is *completed.*
2.2.2.3 Post Processing

To reach a higher concentration or to separate unreacted material or unwanted particles dialysis, filtering, or both were employed as follows:

2.2.2.3.1 Dialysis

Ten milliliters of as synthesized shell suspensions were placed in 3,500 MWCO dialysis membranes (Pierce) and immersed in 1 L of DI water or phosphate buffered saline (pH 7.4) to reduce the concentration of excess salts produced from the one step saturation synthesis. Equilibration was allowed to proceed for 24 hours at room temperature with gentle agitation of the dialysate to improve mass transport across the membrane-dialysate interface. Shorter equilibration times were achieved with 300,000 MWCO dialysis membranes (24 hr to 2 hr). The radius of the pores in the dialysis tubing can be approximated by the following equation:

\[ r = \left( \frac{3MW}{4\pi\rho Na} \right)^{1/3} \]

This yields a diameter of ~3 nm for the 3,500 MWCO and 11.5 nm for the 300,000 MWCO membranes. These pore sizes prevent passage of the shell (>30 nm in the smallest case for sonicated liposomes) but allow the transport of small molecules and most proteins (at 300,000 MWCO).

2.2.2.3.2 High Pressure Filtration

Cellulose acetate filters with 50, 400, or 1,200 nm pore size, or polycarbonate filters with 50, 100, or 200 nm pore size were placed in the same 10 ml LIPEX thermobarrel extruder (Northern Lipids) described earlier. The larger pore size filters
were used to retain large calcium phosphate crystals and shell aggregates while passing
small monodisperse shells, while the 50 nm filters were used to retain the shells while
passing smaller crystals and removing water from the shells and effectively concentrating
the suspension. A small ½” magnetic stir bar was placed on the membrane to
continuously prevent clogging of the filter during this process and pressurized with
industrial nitrogen gas at 200 psi or greater.

2.2.2.4 Characterization

2.2.2.4.1 Particle Sizing and Zeta Potential

To measure the particle size distribution and zeta potential of the nanoshell
suspensions, undiluted nanoparticle suspensions were filtered through a 1.2 μm CA filter
to remove dust. A Brookhaven Instruments Corporation (BIC) ZetaPALS Particle Sizer
with a He-Ne laser and a detector angle of 90 degrees was used for particle size
measurements. Each 3 minute scan was averaged 6 times to obtain a standard deviation
of the distribution mean size. Reported average particle diameters were obtained from
unimodal analysis, considering only samples with polydispersity indices less than 0.20.

Zeta potentials were measured by immersing the polycarbonate electrode of the
ZetaPALS machine in a 4 ml 1 cm polyacrylate cuvette with 1.5 ml sampler volume. An
average of 10 runs were recorded as the mean value with standard deviation in mV.

2.2.2.4.2 TEM Microscopy

Carbon coated 300-mesh copper grids with a Formvar support (Ted Pella) were
used for particle visualization using a transmission electron microscope. After the
reaction was completed a 1-2 μl drop of shaken solution was placed in the center of the grid and allowed to air dry. No further staining or grid preparation was used. Either a Hitachi H-600 or a JEOL 100-SX microscope operating at 100 kV was used to obtain images on Kodak 4489 electron microscopy film. The film was developed in Kodak D-19 developer according to manufacturers directions. Energy Dispersive Scattering (EDS) results were obtained from a PGT eXcalibur detector attached to a Hitachi H-8100 TEM at Northwestern’s EPIC facility. X-Ray Diffraction (XRD) ring patterns were obtained via TEM using the Hitachi H-8100.

2.2.2.4.3 AFM Microscopy

Atomic Force Microscope images of the as-synthesized calcium phosphate nanoshells prepared using each of the three syntheses described were obtained using a Multimode IIIa NanoScope (Digital Instruments) operating in tapping mode with OTESPA probes (Digital Instruments) of 15 nm nominal diameter. The same grid used for visualization by TEM was also used for topographic imaging by AFM. TEM grids were fixed to an AFM sample puck (Ted Pella) with double-stick tape. The NanoScope v. 5.12 r3 software bearing analysis tool was used to determine the average height of the particles. There are between 100-1000 particles per square micron.

2.2.2.4.4 Conductivity, Calcium, and pH monitoring

Three methods were used to estimate the total reaction time necessary for all the calcium and phosphate to react: 1) the concentration of free calcium was monitored as a function of time with a Sure-Flow™ calcium sensitive combination electrode (model
9720BN from Orion) calibrated with 0.1 μM 100 μM and calcium standards, 2) the pH was measured using an AccuFET™ pH electrode (Accumet) calibrated using a three point calibration (pH 4, 7, 10). Both calcium concentration and pH were recorded with an AR60 dual channel pH/ISE/conductivity meter (Accumet), 3) conductivity was measured with an Accumet epoxy conductivity meter with a 10 to 2,000 μS range via the AR60 meter, with a one point calibration using DI water. The time dependence of these parameters was recorded with a custom LabVIEW program on an IBM PC.

2.2.2.4.5 Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES)

ICP-OES was performed on a Perkin Elmer Optima 3300 XL ICP-OES machine to determine free calcium in the solution. The machine was calibrated with serial dilutions of a 1000 ppm calcium standard obtained from Spex CertiPerp. To obtain the samples for analysis, 100 μl of a reaction solution was taken and diluted to 10 ml and filtered through a 50 nm polycarbonate filter to remove any particles. The resulting solution contains the calcium that is not part of the shells at a given time. The autosampler injects each sample into the machine and is done in triplicate along with the calibration. These samples were collected over time and produced a calcium concentration versus time graph that agrees well with that obtained from the calcium electrode.
2.3 Results

2.3.1 Transmission Electron Microscopy (TEM)

Typical transmission electron micrographs (TEMs) of the calcium phosphate nanoshells prepared via the dropwise synthesis using sonicated liposomes are shown in Figure 2-5. The shells were 41 nm +/- 9 nm in diameter with a shell approximately 5 nm thick. When no liposomes were added to the inorganic solution the needle-like product (Figure 2-5B-bottom) appeared, very similar in morphology to the standard nanocrystalline hydroxyapatite sample (Figure 2-5B-top). An electron dispersive spectrograph (EDS) of a 1 μm² area of the shell grid revealed that the ratio of phosphate to calcium peaks is 1:2 whereas the ratios in the needle-sample were 1.6:1 Ca:P indicating a match for hydroxyapatite. The 1:2 ratio for the shells suggests an amorphous calcium phosphate structure since it does not match the crystalline ratios of 1:1 (dicalcium phosphate dihydrate), 1.5:1 (tricalcium phosphate), 1.33:1 (octacalcium phosphate), or 1.67:1 (hydroxyapatite)⁶⁰. This conclusion was further supported by absence of crystalline rings in the TEM diffraction pattern seen in Figure 2-6.
Figure 2-5  A) Transmission electron micrographs of calcium phosphate nanoshells prepared by the dropwise synthesis method on sonicated liposomes. B) Control experiments showing synthesis results obtained without addition of liposomes (top) as compared to standard hydroxyapatite crystals (bottom).\textsuperscript{401}

Figure 2-6  Diffraction patterns obtained via TEM for liposome templated nanoshells prepared using dropwise synthesis using sonicated liposomes and commercial hydroxyapatite crystals. Notice the absence of defined diffraction rings in the shell sample indicating the amorphous structure whereas there are well-defined rings in the hydroxyapatite crystal sample.
TEM images of calcium phosphate nanoshells coated with CEPA obtained by the one-step supersaturation method are shown in Figure 2-7. CEPA was used to coat the surface of the nanoshell with carboxyl groups and to add a negative charge to the particles, preventing the suspension from aggregating. Nanoshells obtained via this method were larger with diameters ranging from 120 nm to over 200 nm with uniform shell coverage, in contrast to the ~40 nm diameter particles prepared by sonication. The one step synthesis was found to be superior to the dropwise addition method on the basis of the fact that it allowed the fabrication of shells with various thickness by simply changing the timing of CEPA addition. Nanoshell thicknesses ranging from under 10 nm for a CEPA addition time of 15 minutes after initiation of the reaction to over 40 nm at an addition time of 150 minutes were obtained, as seen in Figure 2-7 A. From Figure 2-7 B I and II it is clear that these particles have a distinct shell and core region that is distinguished by the varying electron density of the two regions in the particle. The solvent contained within these shells appears to have evaporated in part from the heating of the sample by the electron beam and the vacuum present in electron microscopic observation resulting some specimens having lighter cores than others. To ensure clusters seen under TEM were a product of drying and not present in solution, several unfiltered samples were analyzed via DLS and revealed the size distribution to be between 140 and 250 nm indicating no large, micron sized, aggregates (Section 2.3.3).
Figure 2-7 TEM images of shells obtained by one step supersaturation synthesis. A) The effect of CEPA can be seen as a change in average diameter or shell thickness as more reaction time is allowed before CEPA addition. B) Lower magnification image of nanoshells. Inset is a HRTEM of nanoshells prepared with a CEPA time of 15 minutes after reaction onset (II) as well as thicker shells prepared with CEPA time of 150 minutes (I).

Clustering of the shells on the TEM grid is an artifact of grid drying, and occurred due to the local increase in ion concentration as the liquid drop evaporates. These ions shield the negative charge of the particles allowing the Van der Waals attractive forces to overcome the electrostatic and hydrostatic repulsion, resulting in 2-dimensional clustering on the grid. To ensure that these micron size aggregates were the result of drying and not present in solution a filtered sample and a non filtered sample were analyzed by DLS. The maximum mean diameter of the particle distribution did not exceed 500 nm for any sample with or without filtering through a 1.2 μm filter.

2.3.2 Atomic Force Microscopy (AFM)

Atomic force microscopy was used to confirm the rigid spherical morphology of the particles, as well as to confirm the particle sizes against those obtained from DLS.
Figure 2-8A shows a 15 x 15 μm section of a TEM grid with dispersed spherical particles and some aggregates from samples for which CEPA was added 15 minutes after initiation of the reaction. Figure 2-8 B shows a magnified AFM image of one of the particles confirming a spherical shape as well as a textured surface. The pixel height histogram in Figure 2-9 showed a mean particle height of 117 nm +/- 16 nm which is on the same order as that obtained with bulk particle sizing analysis.

Figure 2-8  AFM tapping mode scan of a TEM grid demonstrating the dispersed shells as well as a detailed scan of a single shell showing the spherical shape of the shell.402
Figure 2-9  Bearing analysis histogram of AFM scan in Figure 2-8 A. The average height of those particles is 117 nm +/- 16 nm

Figure 2-10  Height analysis of two individual nanoshells each are approximately 60 nm in height. This is somewhat smaller than average reported in Figure 2-9. The diameter of the shells is approximately 170 nm.
2.3.3 Particle Sizing and Growth Monitoring

Dynamic light scattering (DLS) was used as a bulk sizing method to complement the direct observations from microscopy. Figure 2-11 shows a typical DLS unimodal size distribution curve obtained from the starting liposome suspension, and for the largest product obtained when CEPA was added 2.5 hrs after the initiation of the reaction. To monitor the growth of the shells a reacting suspension of liposomes was placed in a cuvette and the mean diameter measured over time as seen in Figure 2-12. The increase in size occurred over approximately 10 hours after which little change was observed. The particle sizer also provided an instrument count rate proportional to the scattering intensity of the laser beam by the sample. This parameter can indicate an increase in size when the particle number is constant. The scattering intensity was linear with particle concentration as determined by serial dilutions. Over the course of 10 hours, both the size and scattering count rate increased indicating that the liposomes are growing over time. This result was obtained in a solution without mixing. Reaction times for agitated solutions are reduced as will be discussed later (Figure 2-12 vs. Figure 2-16). CEPA is a small bifunctional crosslinker with a carboxyl group and a phosphate end group connected by an ethyl chain as seen below.
Figure 2-11  DLS size distribution histogram of liposomes before reaction takes place (solid line) and the resulting shells after a one step supersaturated reaction (dotted line) show the increase in both size and width of the size distribution after nanoshell formation. The asymmetry of the dotted line suggests that the shell does not grow uniformly around each liposome or that some aggregation has occurred.  

Figure 2-12  DLS mean distribution size taken every fifteen minutes during the reaction to measure the growth of the shell prepared via the one step supersaturation synthesis without stirring over a period of ten hours. After ten hours there was no further growth. After 24 hours the solution size distribution was measured again and the mean size was 164 nm (seen as the 600 minute result).
The organic phosphonate end group permits the reaction of this molecule with the surface of the calcium phosphate nanoshell while the negatively charged (pKa = 3.1) carboxyl groups slow down the continued crystallization of calcium phosphate by sterically hindering incoming ions from attaching to the surface. Since calcium phosphate dissolves at pH below 4 this low pKa ensures the shell will be negatively charged over the relevant pH range for calcium phosphate. The negative charge of the CEPA molecule imparts a negative charge to the nanoshell as shown in Figure 2-14. In addition to providing an electrostatic charge and thus stability to the nanoshell, the COOH group of the CEPA molecule provides a necessary active group for the attachment of aminated molecules such as antibodies and enzymes as discussed in CHAPTER 5.

![Chemical structure of 2-carboxyethylphosphonic acid (CEPA)](image)

Figure 2-13 Chemical structure of 2-carboxyethylphosphonic acid (CEPA)

To verify the attachment of CEPA onto the nanoshells, the change in zeta potential as CEPA is reacted with the suspension can be monitored as seen in Figure 2-14. As synthesized, the zeta potential of the shells is -37 mV and did not increase beyond -55 mV after the DOPA to CEPA ratio reached 1:2. The proposed structure of CEPA-coated nanoshells is schematically represented in Figure 2-15. The very negative zeta potential was not significantly affected by changes in suspension pH from 6.5 to 8.5, varying less than 5 mV. When CEPA was added to a solution of DI water over the same
ranges as Figure 2-14 the apparent zeta potential did not change by more than 3 mV. The small variations may well be the result of machine fluctuations as there were no particles present to monitor mobility.

Figure 2-14 Zeta potential as a function of DOPA to CEPA ratio which effectively measures the increasing negative charge as CEPA is added to the surface of the particles. The plateau reached indicates that CEPA has saturated the surface and no further increase can be achieved.
Figure 2-15 Schematic cutaway view of the resulting nanoshell after the addition of CEPA. The shell consists of an aqueous pocket surrounded by a liposome composed of DOPA lipids. This liposome has calcium phosphate deposited on the outer surface which is coated by CEPA molecules leaving a negatively charged carboxylic acid surface. Placement of potential encapsulated materials is also shown.

The effectiveness of CEPA to “cap” the shells and therefore control the final mean diameter of the shells, was again demonstrated by comparing samples prepared using CEPA additions at 10 and 180 minutes after the reaction was initiated. The suspensions were dialyzed using 3,500 MWCO dialysis tubing for 12 hours against DI water at neutral pH to remove unreacted calcium and phosphate. The final nanoshell diameter as a function of CEPA addition time averaged for 8 reactions is shown in Figure 2-16. As the delay time before CEPA addition was increased, the particle size increased from 120 nm to 180 nm which is within the same range recorded for the unstirred cuvette reaction in Figure 2-12. Sizes in Figure 2-16 are somewhat larger than those shown in Figure 2-12 at the same reaction time because the samples in Figure 2-16 were stirred which probably increases the growth rate.
Figure 2-16  Mean diameter of nanoshells prepared using various CEPA addition times confirming that shells of various thickness can be synthesized by adding CEPA to slow further growth around the shell at various times during the reaction. Each experiment began with the 150 μl liposome template, and 12.5 μl 1 M calcium chloride and 12.5 μl 1 M phosphate salts, and nanoshells were synthesized according to the one step supersaturation method prior to CEPA treatment. Shells up to 200 nm could be produced using this method.

Surface saturation of CEPA was found to occur at a CEPA to DOPA ratio of approximately 2:1 which means approximately one CEPA for every exposed DOPA on the liposome and makes sense if each CEPA occupies the same surface area (d ~ 0.5 nm) as a DOPA head group (d ~ 0.7 nm) as in Figure 2-17. On the surface of a growing calcium phosphate crystal there are incompletely bound phosphates and calciums resulting in regions of various surface charges. Areas with unbound calcium ions contain a positive charge that allows for electrostatic binding of CEPA to the shell as seen below. CEPA addition to the stirring reaction mixture at various times after the start of the initiation of the reaction provided a method to control the shell thickness, within approximately 20 nm but a simpler, more precise method was desired.
On the surface of a growing calcium phosphate crystal there will be places of unbound phosphates and calcium ions. Free calcium allows the binding of the phosphonate portion of CEPA. This cross section of a CEPA coated calcium phosphate shell shows approximately the same number of CEPA molecules as the number of outward phosphate head groups on the liposome (not drawn to scale).

Free Ca$^{2+}$ ions allow CEPA binding.

Figure 2-17  On the surface of a growing calcium phosphate crystal there will be places of unbound phosphates and calcium ions. Free calcium allows the binding of the phosphonate portion of CEPA. This cross section of a CEPA coated calcium phosphate shell shows approximately the same number of CEPA molecules as the number of outward phosphate head groups on the liposome (not drawn to scale).

to allow greater control over the shell thickness for future applications.

The stepwise supersaturation method was developed in order to control the total amount of calcium and phosphate available for the reaction. For this to work, the reagents being added to the reaction were pH adjusted to pH 8 so they did not change the pH when added. If the pH of phosphoric acid is not adjusted (initially it is pH 1) the pH will drop by ~ 2 units and this will effect the equilibrium as discussed in Section 2.4.2.

As shown in Figure 2-18, adding smaller amounts of calcium and phosphate at a time gave increased shell thickness for every 5 µl (half the amount added in the one step supersaturation method) each of calcium and phosphate solutions. The difference in final
nanoshell mean size distribution using this method (120 nm) (Figure 2-18) and the one step supersaturation method (180 nm) (Figure 2-16) example shown is because there are four times as many (600 μl) liposomes present compared to the one step supersaturation synthesis and each act as a nucleation site for calcium phosphate growth resulting in thinner shell overall.

![Graph](image)

**Figure 2-18** The effect of 5 μl aliquots of calcium and phosphate solutions added simultaneously in a stepwise manner every 10 hours. Each dose (5 μl) was half the dose used in the one step supersaturation method. Size distributions in the suspension were measured using DLS just prior to adding the next aliquot.

The nanoshell reaction rate using the one step supersaturation synthesis was monitored via the calcium ion selective electrode and pH meter. As seen from the trend in Figure 2-19 that the calcium concentration as well as the pH decreased gradually over the course of approximately 10 hours (Figure 2-19 and Figure 2-1) with a dependence that agreed well with Figure 2-12. A complementary experiment analyzing 50 nm filtered reaction samples using inductively coupled plasma-optical emission
spectroscopy (ICP-OES) resulted in similar behavior, a steep initial decent and slow continued consumption lasting approximately 6.5 hours (Figure 2-19B). This time to completion is comparable to the 10+ hours determined by Nancollas\textsuperscript{425} when monitoring the calcium concentration over time of a 1 mM calcium phosphate reaction. The reduced time shown below can be attributed to the higher concentration that would accelerate the reaction rate. Some discrepancy in the period it takes to fully grow the calcium phosphate nanoshells suggested by DLS in Figure 2-12 grown by dropwise supersaturation and the approximately 7 hours it took for the calcium concentration to reach equilibrium using the one step supersaturation method as shown in Figure 2-19A,B. This is likely the result of the agitation used with the later reaction.

Figure 2-19  A) pH and calcium ion concentration over time for the one step supersaturation reaction method. The flat profiles obtained in the last two hours of the reaction indicate little change in either Ca\textsuperscript{2+} or OH\textsuperscript{-} concentration signaling the end of the reaction. The final pH was approximately 7.4 which corresponds to the second pK\textsubscript{a} of phosphate and suggests the presence of unreacted phosphate after the reaction. B) ICP-OES data (right) further supports that approximately 10 hours required for the reaction to reach equilibrium.
It is possible that the plateau of calcium concentration seen after 7 hours in Figure 2-19A may not signal the end of the reaction. Ostwald ripening\textsuperscript{62} of the particles can occur which would allow growth by reorganizing the unstable smaller particles in the system onto the larger stable nanoshells without consuming any solution calcium ions. It is probable that there are some metastable amorphous calcium phosphate nuclei present with the shells when the solution reaches thermodynamic equilibrium. Over time the nuclei can reform onto the surface of the larger more stable shells causing an increase in shell diameter without consuming any Ca\textsuperscript{2+} ions from solution. Ostwald ripening is known to occur in calcium phosphate crystallization\textsuperscript{60}.

### 2.3.4 Measurement of Solution Stability

For the shells to be used for practical applications in life sciences they need to resist aggregation while in physiological salt (up to 140 mM) and in the presence of proteins. When 1 ml of a dialyzed nanoshell suspension using any of the three methods and coated with CEPA containing approximately 1 nM (Calculated in Section 4.4.1) shells was introduced to a 0.2 M NaCl solution, the mean size of the nanoshell suspension did not vary by more than 7 \% of the initial value of 124 nm. When added to a 2.5 g/dL, (half of the physiological level) of albumin, the mean distribution size of the suspension decreased to 77 nm due to scattering contributions of the small protein molecules to the unimodal analysis which shifted the mean to a lower value. The zeta potential of as-synthesized nanoshells remaining in 2.5 mM of CaCl\textsubscript{2} was -55 mV but with addition of NaCl the zeta potential fell from -55 mV to -39 mV after 1 hour and to -33 mV after 24 hours. The DLS measured mean sizes of either suspension did not
change significantly over a week, indicating that shells remain dispersed and resisted flocking despite the decrease in zeta potential. Furthermore, several samples of liposome templated nanoshells prepared via the one step supersaturation method were stored at room temperature for 9 months and showed neither structural change when viewed via TEM nor any change in size distribution and were concluded to be shelf stable.

2.4 Discussion

2.4.1 Dropwise Synthesis

As shown in Figure 2-5 it was possible to prepare approximately 35 nm-thick calcium phosphate nanoshells by the dropwise synthesis method (Section 2.2.2.2.1). In the inset of Figure 2-5A was shown a single shell seen as a circular object with an electron dense dark perimeter and less electron dense light core which is created by the additional amount of material the electron beam passes through at the edge of the nanoshell. If there were no liposome templates, then the primary product was calcium phosphate whiskers seen in Figure 2-5B, the same obtained via the metathesis synthesis. This verifies the necessity of liposomes for the formation of a shell structure and exposes an interesting phenomenon of template-driven change in the stability of amorphous calcium phosphates. The composition of the nanoshell material was determined by electron dispersive spectroscopy (EDS) measurements to have a Ca:P ratio of 1:2 which does not match any stoichiometry of crystalline calcium phosphate (Section 1.2.2) and indicates an amorphous material. Amorphous calcium phosphate is not stable alone in solution\textsuperscript{62}; however, it appears to remain stable when deposited on the organic surface of the DOPA liposome. How the curved liposome improves the stability of the amorphous
material is not certain but it is likely that it prevents the calcium and phosphate from reorganizing into a more stable form as it could if not constrained by the curvature of binding to the liposome head groups on the surface.

Dropwise synthesis required inconvenient reaction and preparation conditions that led to difficulty in obtaining reproducible results. This method also had potential complication in future applications and scale-up:

- The pH of 11 required to get the calcium phosphate to condense could denature labile biomolecules intended for future surface attachment or encapsulation thus a more neutral pH would be desired.
- The high pH required the reaction to be nitrogen purged to avoid carbonate formation from the solubilization of atmospheric CO₂.
- The manual burette titration apparatus did not give accurate control over titration volume and timing, varying by as much as +/- 200 μl and +/- 10 seconds, resulting in a product that was difficult to duplicate.
- The liposomes used as nanoshell templates were prepared via sonication which is a convenient method of making unilamellar liposomes but the resulting suspension size was difficult to reproduce, and sonication could also lead to lipid oxidation and degradation of sensitive molecules.
- Ammonia was used for pH control and as the counter ion for the phosphate species and would need to be removed before introduction of the shells in vivo.
- In addition, the assumption was made that the liposomes would behave as rigid seed crystals that would act as a nucleation site for calcium
phosphate growth when in fact they are liquid crystals and are sensitive to the ionic strength gradients present during introduction to the hypertonic inorganic reaction solution. This ionic shock caused the liposomes to deform, shrink, and pinch off daughter liposomes and other random lipid structures that could act as templates which adversely affected the quality of the nanoshell product.

Attempts were made to alleviate some of the problems of the dropwise synthesis such as changing from calcium nitrate to other soluble calcium salts such as calcium acetate, calcium citrate, and calcium chloride. Phosphate salts were changed from ammonium phosphate to other soluble phosphate compounds such as mono and dibasic sodium phosphate and mono and dibasic potassium phosphate salts and phosphoric acid. The salt changes had no effect except that the organic calcium salts produced only large calcium phosphate aggregates and other unrecognizable morphologies on the TEM grid. The presence of sodium, potassium, and chlorine provided no observable improvement to the reaction as confirmed by TEM observations. This suggests that the type of counter ion present does not appreciably affect the reaction. The reaction proceeded faster and more completely at higher pH around 12.5 than it did at pH 9 as would be expected when viewing the calcium phosphate solubility curve (Figure 2-21). When the pH was below 11 there were mixed morphologies including some shells but there was no improvement in reproducibility of the shell suspensions by changing pH at constant concentration of calcium, phosphate, and liposomes as noted by TEM observations and DLS sizing data. At pHs above 11 there were only hydroxyapatite needles present and no visible shells.
The pH effect was a consequence of the decreased solubility of calcium phosphate at high pH and is discussed further in Section 2.4.2.

Several other lipids known to form liposomes with the same 18:1 tail structure but different head groups (phosphocholines, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylserine) (Figure 1-10) were also used to form the liposome templates. Even though liposomes prepared from these lipids appeared to form as determined from DLS observations, nanoshells did not grow on these liposomes. This is most likely due to the absence of a phosphate head group. This was confirmed by the presence of unrecognizable, random, non shell mineralized structures in the TEM.

Liposomes prepared from phosphatidic acid lipids with various tail lengths (12:0, 14:0) were also tried but were not successful and produced unrecognizable, random structures (similar to those obtained from non phosphatidic acid lipids) were seen when observed via TEM. An example of some of these structures is seen below in Figure 2-20. One reason this could occur is due to the non-liposome structure some lipids obtain when below their phase transition temperature ($T_c$) (Figure 1-10). Below those temperatures the liposome membrane is rigid and does not form liposomes. Since DOPA was shown to be an effective template for shell formation, has a low $T_c$ (-8 C), and its preparation is straightforward it was chosen as the lipid for all future synthesis.
Figure 2-20  Representative sample of randomly mineralized structures obtained when using phosphatidic acid lipids other than DOPA as the template for nanoshell synthesis. A) dimyrystoyl-sn-glycero 3 phosphatidic acid and B) dilauryl-sn-glycero-3 phosphatidic acid. Notice the lack of shell structure in A and the similarity of the needle clusters in B to standard hydroxyapatite.

The two variables most affecting reproducibility for the dropwise synthesis were reproducible template preparation and manual titration control. To automate the calcium and phosphate titrations, 2 peristaltic pumps were installed to minimize variations from operator error. The pumps were controlled by power modulating relays driven by a custom LabVIEW program. It was found that the peristaltic pumps could not reproducibly dispense amounts of fluids, and so addition volumes varied by more than 10% which is comparable to that expected from operator error. This is thought to occur from a delay in the motors turning on and off after power was cycled for the pumps. An improved microprocessor controlled syringe pump apparatus based on automating titrations was therefore constructed and developed in CHAPTER 3.
2.4.2 One Step Supersaturation Synthesis

The one step supersaturation method which offers several advantages over the dropwise synthesis.

- All the reactants were added at once eliminating the need for titrations and automation of the synthesis by relying on self assembly.
- Extrusion could be used to prepare the liposomes and improved the reproducibility of liposome templates over sonication. Extrusion allowed highly monodisperse liposome suspensions with average sizes of 50, 100, and 200 nm +/- 10% to be produced by selection of the proper polycarbonate membrane.
- The counter ions of calcium, base, and phosphate used (Ca\(^{2+}\) and Na\(^+\)) are more biofriendly than ammonium and nitrate used previously.
- The reaction was performed at lower pH (8 to 10) than the stepwise addition method.
- Nitrogen purge was unnecessary for this synthesis as it was carried out at lower pHs where calcium carbonate formation was less of a problem.
- The thickness of the shell could be changed by stopping the calcium phosphate reaction on the shell surface by adding CEPA at specific times during the reaction, as before.

The improvements listed above made the one step supersaturation synthesis superior to the dropwise synthetic method for liposome templated calcium phosphate nanoshell preparation. In this method, the process for crystallizing calcium phosphate on
the liposome began with the development of a supersaturated calcium phosphate solution. When the liposomes were added, the growth began instantly and continued until the saturated amount of calcium and phosphate had precipitated and reached an equilibrium condition (Figure 2-19). The growth process could still be interrupted with the addition of CEPA to obtain nanoshells with varying thickness.

2.4.2.1 Determination of a Theoretical Solubility Curve

To determine the appropriate starting conditions for the one step supersaturation synthesis an understanding of calcium phosphate solution behavior was needed. For crystallization to occur at a given pH, temperature, and salt concentration, the calcium phosphate concentration in solution must be supersaturated with respect to the solid phase. The supersaturation was determined from the solubility diagram and was built by plotting the conditions that satisfied $K_{sp} = IP$ where $K_{sp}$ is the solubility product constant and IP is the ion product of the crystalline form. The IP is dependent on ion activities ($f_i$) which were determined using the Debye-Hückel equation (Equation 2-4)$^{59}$. For a general calcium phosphate, $[Ca_vH_w(HPO_4)x(PO_4)yOH_z]$ where v,w,x,y, and z are the number of each ion, the IP is of the form:

$$IP = ([Ca^{2+}]f_{Ca})^v([H^+]f_{H^+})^w([HPO_4]^{2-}f_{HPO_4})^x([PO_4]^{3-}f_{PO_4})^y([OH^-]f_{OH})^z$$

Equation 2-1

The saturation is the difference of dissolved salt from equilibrium and is defined as
\[ S = \frac{IP}{K_{sp}} \]

Equation 2-2

Where S=1 is equilibrium, S > 1 is supersaturated, and S < 1 is subsaturated

Alternatively, supersaturation could be defined as the difference in chemical potential between the liquid and crystalline phases and represents the thermodynamic driving force for crystallization from a liquid to a solid.

\[ S = u_l - u_s = kT \ln \frac{C}{C_{\text{equil}}} \]

Equation 2-3

The solubility curves plotted in Figure 2-21 were calculated for the four phases of calcium phosphate: dicalcium phosphate dihydrate [CaHPO₄•2H₂O] (DCPD, \(K_{sp} = 2.1 \times 10^{-7}\)), tricalcium phosphate [Ca₃(PO₄)₂] (TCP, \(K_{sp} = 1.15 \times 10^{-29}\)), octocalcium phosphate [Ca₈(HPO₄)₂(PO₄)₄] (OCP, \(K_{sp} = 1.25 \times 10^{-47}\)), and hydroxyapatite [Ca₁₀(PO₄)₆(OH₂)₂] (HAP, \(K_{sp} = 1.8 \times 10^{-58}\)) in water in the absence of lipid. Equation 2-1 and Equation 2-2 were solved to yield the theoretical solubility curve as a function of pH and phosphate concentration at 25°C, 200 mOsM, and a PO₄³⁻:Ca²⁺ ratio of 1:1. The pH was determined experimentally to be a more effective control of solubility than temperature.

To solve Equation 2-1 the activities of each ion were calculated. The Debye-Hückel equation seen below was used to determine the activity of each ion and is a good approximation where the solution is dilute (ionic strength is less than 0.01) but does not
account for specific interactions between ions or hydration effects. The equations used were as follows.

\[ \log f_i = \frac{A \cdot z_i^2 I^{0.5}}{1 + (B \cdot \alpha_i I^{0.5})} \]

Equation 2-4

where,

\[ A = \left( \frac{N^2 \varepsilon^2 \sqrt{\pi / 100}}{2.3026R^2} \right) \frac{1}{(DT)^{0.5}} \]

Equation 2-5

and

\[ B = \sqrt{\frac{4\pi \varepsilon^2}{DkT}} \]

Equation 2-6

\[ I = \frac{1}{2} \sum_i m_i z_i^2 \]

Equation 2-7

where \( f_i \) is the activity coefficient of the ion, \( z_i \) is the charge of the ion, \( \alpha_i \) is the effective diameter of the hydrated ion and is listed in TABLE 2-1, and \( I \) is the ionic strength of the solution shown in Equation 2-7 where \( m_i \) is the molal concentration. A and B are dependent on the dielectric constant of water D, permittivity of vacuum \( \varepsilon \), Avogadro's
number, N, the ideal gas constant, R, and temperature. Over our temperature range of 25°C to 37°C A = -0.51 and B = 3.28.

TABLE 2-1

CHARGES AND HYDRATED RADII OF SELECTED IONS

<table>
<thead>
<tr>
<th>Ion</th>
<th>Z</th>
<th>α (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca^{2+}</td>
<td>2</td>
<td>0.6</td>
</tr>
<tr>
<td>PO_4^{3-}</td>
<td>-3</td>
<td>0.4</td>
</tr>
<tr>
<td>HPO_4^{2-}</td>
<td>-2</td>
<td>0.4</td>
</tr>
<tr>
<td>H_2PO_4^-</td>
<td>-1</td>
<td>0.4</td>
</tr>
<tr>
<td>K^+</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td>Na^+</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Cl^-</td>
<td>-1</td>
<td>0.3</td>
</tr>
<tr>
<td>H^+</td>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td>OH^-</td>
<td>-1</td>
<td>0.35</td>
</tr>
</tbody>
</table>

To produce the pH dependent solubility diagram, a pH dependent relation for the ionizable species needed to be used. Triprotic phosphoric acid was selected as its initial concentration is known by the amount of phosphoric acid added. The equilibrium relation Equation 2-8 was used to determine the different ratios of each phosphate species as a function of pH, yielding a pH dependent ion product (IP = f(H^+)) when integrated into Equation 2-1. Using the definitions of each K_a (K_{a1} = 2.2, K_{a2} = 7.2, K_{a3} = 12.2)^59 and the summation of phosphates equation as independent equations, the system is fully defined and one can calculate the concentrations of each phosphate species as a function of pH.

\[
H_3PO_4 \rightleftharpoons K_{a1} \cdot H^+ + H_2PO_4^- \rightleftharpoons K_{a2} \cdot H^+ + HPO_4^{2-} \rightleftharpoons K_{a3} \cdot H^+ + PO_4^{3-}
\]

Equation 2-8
\[
[PO_4] = \frac{[H_3PO_4]K_{a1}K_{a2}K_{a3}}{[H^+]^3}
\]

Equation 2-9

\[
[HPO_4] = \frac{[H_3PO_4]K_{a1}K_{a2}}{[H^+]^2}
\]

Equation 2-10

\[
[H_2PO_4] = \frac{[H_3PO_4]K_{a1}}{[H^+]}
\]

Equation 2-11

\[
[H_3PO_4] = \frac{[PO_4]_{TOTAL}}{1 + \frac{K_{a1}K_{a2}K_{a3}}{[H^+]^3} + \frac{K_{a1}K_{a2}}{[H^+]^2} + \frac{K_{a1}}{[H^+]}}
\]

Equation 2-12

Integrating the relations above with Equation 2-1 and Equation 2-2 yielded a pH dependent solubility curve. The resulting solubility curve agreed well with that determined by Kemenade et al.\textsuperscript{60} and gives us an approximate framework for designing experiments to locate optimum reaction conditions.
From Figure 2-21 and others produced with varying calcium phosphate ratios, phosphate concentrations, and ionic strengths the following relevant conclusions could be drawn about the effect of these parameters on the solubility curve:

- Calcium phosphate forms more readily at higher pH owing to the increased presence of the reactive phosphate $\text{HPO}_4^{2-}$, and $\text{PO}_4^{3-}$.
- Calcium phosphate forms more readily with higher ratios of calcium to phosphate at a given pH.
- Changing the phosphate concentration has little effect on equilibrium due to the buffering of the phosphate system.
Therefore, controlling the pH and concentration of calcium and phosphate should allow sufficient control of the saturation, which in turn would control the final equilibrium in the nanoshell synthesis. The saturation controls both the rate of inorganic nucleation and subsequent growth by increasing the Gibbs free energy of the system as seen in Equation 1-3 and Equation 1-4.

While the plotted lines in the pH dependent solubility curve in Figure 2-21 were a useful guide to the thermodynamic stability of the system, other factors could influence the position of these curves and the subsequent crystallization. Just outside of the solubility curve is the metastable zone where supersaturated calcium phosphate could exist with or without nucleation. Glassware impurities, atmospheric dust, and liposome concentration affect the metastable zone by acting as nuclei.

The specific effects of liposome concentration were difficult to quantify but it is believed the metastable zone narrows with increasing liposome concentration owing to the increased number of effective nuclei. This provides more phosphate head groups to the reaction and increases the surface area available for nucleation.

Temperature, pressure, and tonicity are not as sensitive of reaction parameters for the following reasons: the desired temperature range (20°C to 37°C) for the reaction does not permit an adequate temperature window for effective cooling crystallizations. Furthermore, it has also been shown that calcium phosphate has an inverse temperature dependence on solubility. Remaining within this temperature range prevents complications with future applications involving heat labile biomolecules. Varying pressure to a relevant degree requires a specialized apparatus and with an existing reaction control (pH), would not be cost or energy effective. Changing the tonicity (ionic
strength) to affect the supersaturation is complicated as the effects on solubility are difficult to predict *a priori* and it also requires the tonicity of the liposomes to be matched prior to the reaction.

2.4.2.2 Finding the Optimum Reaction Conditions

An optimal set of initial conditions were discovered experimentally by observing the resulting suspension via TEM and DLS for a range of different reactions. These reactions maintained temperature, reactor volume, pressure, tonicity, stirring speed near constant while changing the following parameters:

- The volume of 6.93 mM DOPA liposome suspension was varied from 50 μl to 1000 μl in 50 ml increments (15 μM-300 μM).
- 1 M phosphoric acid was varied from 5 μl to 20 μl in 2.5 μl increments (0.2-0.8 mM).
- 1 M calcium was varied from 5 μl to 20 μl in 2.5 ml increments (0.2-0.8 mM).
- Sodium hydroxide was varied to yield pH values from 4 to 11 in 0.5 increments.

This yielded the following four-dimensional control parameter matrix:

\[
C = [[\text{DOPA}],[\text{NaOH}],[\text{CaCl}_2],[\text{H}_3\text{PO}_4]]
\]

Equation 2-13
A formal design of experiments method would be ideal here but could not be effectively implanted for the following reasons. A full factorial exploration of these conditions requires nearly 10,000 experiments and was not time practical. A fractional factorial method was not an ideal solution because of the unpredictable synergistic relationship between the variables. For instance when pH was changed it affected the charge on the phosphate headgroup of the liposome resulting in potential reactivity change for the liposomes. This effectively changed the liposome concentration available for reaction in an unpredictable manner by reducing the amount of ionized, reactive phosphate groups. Also, there is no easily quantifiable metric of output such as weight or color and the only way to assess the degree of success of the reaction in terms of what features is subjectively through TEM and through DLS. Theory from section 2.4.2.1 suggests the saturation determines how much calcium and phosphate crystallize as well as the reaction rate and that would be an ideal control parameter; however, there is no reliable way to determine saturation point experimentally for the calcium phosphate system due to the variability of the solubility/metastable limit and multiple crystalline phases. This is further complicated because amorphous calcium phosphate appears to be the phase that forms around the shell (Section 2.3.1) and ACP does not have a defined solubility limit. For these reasons concentration of calcium is a more fitting control parameter. These problems make statistical accounting for the individual effects of the four parameters from a formal design of experiments method a daunting task.

Though rigorous methods could not be effectively implemented, much could still be learned from careful selection of experiments. The first goal was to find the main effect for each variable by varying one while keeping the others constant. The following
were the conclusions drawn from TEM observations when deviating from the following reaction conditions DOPA = 400 μl (0.11 mM), NaOH = 25 μl (PH 7), CaCl₂ = 15 μl (0.6 mM) and H₃PO₄ = 15 μl (0.6 mM).

- As determined by TEM, when DOPA liposome concentration was increased the overall number of particles increased, the number of large 3-D shell clusters increased and the average shell thickness decreases.

- Little change in the thickness and size distribution was observed when changing the phosphoric acid added from 0.2 - 0.8 mM. The measurable effects of increased phosphoric acid were a reduced initial pH and a reduced pH change from reaction due to the buffering from the phosphate system.

- Increasing pH from 4 to 11 increased the concentration of reactive phosphates (HPO₄²⁻ and PO₄³⁻) available therefore increasing the saturation and amount of material crystallized. TEM and DLS results showed the shells were thicker as pH was increased and at very high pHs the solution flocked out, forming large aggregates of shells visible to the naked eye.

- Increasing calcium from 0.2 - 0.8 mM also increased the saturation point and resulted in increased shell thickness and eventual flocking of the shells seen as white flakes at the bottom of the reaction. The high end of the calcium concentrations introduced strange morphologies likely the result of deformation of the liposome membrane by Ca²⁺ and subsequent mineralization.
These observations agreed well with what was expected from theory developed in Section 2.4.2.1 and Section 1.3 and supported the assertion that the reaction was governed by the supersaturation level.

Since it was expected that small deviations would not appreciably affect the size distribution or morphology of the shells, several random initial conditions were chosen for a coarse exploration of the four parameter system. Some regions in the 4-D control parameter matrix (Equation 2-13) could be eliminated from knowledge of the main effect of each parameter. For instance very low calcium and phosphate concentrations coupled with low pH were not likely to produce coated liposomes due to low resulting supersaturation. As before, each experimental condition was evaluated via DLS and visually by TEM. The observations of shell number, shell thickness, shell size, uniformity of coating, shell shape were also subjective and introduced error but are unavoidable. If shells were present their neighboring conditions are explored with more resolution. This process was repeated until no discernable improvement could be made when using TEM.

The most successful set of conditions from approximately 400 experiments were found to be 150 \( \mu \text{l} \) of DOPA (40 \( \mu \text{M} \)), 10 \( \mu \text{l} \) (0.4 mM) each of 1 M CaCl\(_2\) and phosphoric acid with 40 \( \mu \text{l} \) of NaOH in 25 ml of water. These conditions also exhibited good reproducibility from batch to batch. This set of conditions is likely not a global optimum but one of many possible successful synthetic methods.
2.4.3 Stepwise Supersaturation Synthesis

There were two principle drawbacks to the one step supersaturation method:

- Size control was only accurate to +/- 20 nm when using CEPA to stop nanoshell growth.
- Linearly increasing concentrations of DOPA, calcium, phosphate, and NaOH to achieve a higher concentration of shells/ml was not successful (no shells, high floc, high crystals seen via TEM). Scale up of nanoshell concentration to a higher shells/ml would require concentrating a number of reactions and is not desirable.

It was proposed that the low reproducibility of nanoshell thickness achieved in these 2 methods when using CEPA were because CEPA may only hinder further shell growth rather than prevent it completely. CEPA may not fully cloak all the active sites on the nanoshell allowing some growth to occur in the spaces without CEPA as illustrated below.
Figure 2-22 If CEPA does not completely cover the reactive portion of the nanoshell, leaving a ‘bare spot,’ it permits the continued growth of the shell regardless of the CEPA coating.

For future applications in biosensors, drug delivery, and oxygen transport it is preferable to have a concentrated nanoshell suspension that can be stored and diluted as needed rather than a dilute suspension that must be concentrated prior to use. In order to increase the concentration of shells per ml, more liposomes would be required to be introduced into the reaction. If the concentration of calcium and phosphate remained the same there would be less calcium phosphate to coat the shell resulting in reduced thickness. This effect is observed by the different final size of Figure 2-12 and Figure 2-18. This change in thickness can not be simply solved by adding more calcium and phosphate to the reaction as this increases the supersaturation thereby increasing the likelihood of homogeneous nucleation and therefore more side crystallization. Experimentally, raising the saturation to linearly to compensate for an increase in liposome concentration resulted in an increased amount of floc and reduced concentration as determined by DLS.
These shortcomings prompted the development of a further improved synthetic method that expanded the concept of supersaturation control of the reaction. The size control and scalability issues of the one step supersaturation synthesis are addressed by hybridizing it with the dropwise synthesis. This allows a more measured control of supersaturation by adding small amounts of calcium and phosphate, allowing them to grow, then adding another small amount of calcium and phosphate. Due to consumption of calcium phosphate during the interim period between additions, the saturation at the final addition was lower than what it would be if all three additions were added simultaneously. This prevented homogeneous nucleation from outpacing shell growth as it would if the total amount of calcium and phosphate was added simultaneously. Figure 2-18 showed the change in mean shell diameter when the 5 μl of 1 M pH adjusted phosphoric acid and 5 μl of 1 M CaCl₂ is added to 600 μl of DOPA liposomes every 10 hours.

Using this stepwise saturation approach (Figure 2-4), calcium phosphate could be added to vary the final thickness of liposomes and still obtain a more concentrated shell suspension without the need for CEPA as a capping agent. This synthetic method is promising for coating liposomes with calcium phosphate and its theory and method is exploited in CHAPTER 3 where it is used to coat microemulsions.

2.4.4 Proposed Mechanism For Shell Formation

As seen in this chapter, the supersaturation level of ions in solution governs the synthesis of calcium phosphate nanoshells. This can be explained by recalling that the DOPA liposome is a negatively charged colloid owing to the ionizable phosphate head
group of DOPA. When these liposomes are introduced to a subsaturated calcium-phosphate solution there is an electrostatic attraction of Ca\(^{2+}\) to the surface, resulting in a tightly bound Stern layer of calcium ions on the emulsion surface. This behavior is suggested by a decrease in the negative zeta potential from -22 mV to -4 mV when a bare liposome is introduced to a calcium chloride solution. The residual charge from the Stern layer attracts a diffuse layer of phosphate ions around the emulsion. Figure 2-23 graphically represents the increased local concentration of ions resulting from the Stern and diffuse layers. As the reaction is supersaturated, the region around the liposome will be saturated first causing calcium to preferentially form around the particle. Once calcium phosphate grows on the liposome the thin shell acts as a normal calcium phosphate nucleation site for further shell growth.
Figure 2-23  Cartoon depicting the local increase in calcium and phosphate around the negatively charged liposome occurring as a result of the formation of a stern layer of calcium ions and diffuse layer of phosphate ions around the particle. The supersaturation is higher around the particle than the bulk resulting in shell formation selectively around the liposome, forming shells.

2.5 Conclusion

Liposome templated calcium phosphate nanoshells were successfully synthesized by three methods: dropwise synthesis, one step supersaturation synthesis, and stepwise supersaturation synthesis. Dropwise synthesis was successful but the results were difficult to reproduce and modify systematically. The single step supersaturation method improved the reproducibility of the synthesis while adding the ability to control the shell thickness through addition of CEPA to the shell surface at selected times after the initiation of the reaction to stop the reaction. Two parameters that influence the resulting shells formed by the one step supersaturation method were identified.
• Liposome preparation by either sonication or extrusion affected the final suspension size distribution, with extrusion providing the most reproducible suspension of nanoshells.

• Inorganic saturation: Saturation of the calcium phosphate solution seemed to be the primary parameter that affected the final shell suspension and the kinetics of the reaction. It could be increased by either an increase in solution pH or an increase in calcium concentration; however, the supersaturation could not be precisely measured for lack of an experimentally determined saturation line.

The resulting suspensions of calcium phosphate nanoshells took approximately 10 hours to synthesize calcium phosphate nanoshells that were monodisperse and less than 200 nm +/- 10% in diameter as evidenced by AFM pixel height histograms and DLS data. The shells could be visualized individually via TEM, revealing core-shell structures as 2 dimensional rings and AFM which revealed a rigid spherical morphology. CEPA-coated shells produced from the single step supersaturation method were stable in physiological salt and protein solutions owing to the high zeta potential imparted by the CEPA molecule coating. Stepwise supersaturation synthesis was developed as a hybrid of the first two methods and provided all the benefits of the one step synthesis while allowing the production of more concentrated suspensions. The stepwise method appears to be the most promising for future synthesis with its combined benefit of reproducibility and thickness control and is more fully explored as a method to coat microemulsions and control their size without CEPA in Chapter 3.
CHAPTER 3

SYNTHESIS OF HYDROPHOBIC CORED CALCIUM PHOSPHATE NANOSHELLS

3.1 Introduction

The synthesis of calcium phosphate nanoshells for use as carriers of biomolecules will be expanded from the aqueous cored nanoshells discussed in CHAPTER 2 to an oil cored nanoshell in this chapter. These shells can be used to encapsulate various hydrophobic substances as shown in CHAPTER 4 and as a platform for the attachment of antibodies and enzymes as discussed in CHAPTER 5. Motivation for these pursuits was outlined in CHAPTER 1. A synthetic method for encapsulating fluorocarbons was developed that can be used for oxygen transport discussed in CHAPTER 6 and introduced in CHAPTER 1. The approach discussed in this chapter involves changing the shell template from a liposome to a soybean oil microemulsion which allows hydrophobic molecules to be entrapped within the nanoshell core thereby expanding the types of substances that can be encapsulated. This is especially relevant to drug delivery where approximately 60% of new chemical entities (NCEs) discovered are very hydrophobic and pose problems for further development. To prepare high quality materials, the synthetic method was improved to allow better control of the template size and shell thickness. The effect of thickness on transport rate across the shell is presented in CHAPTER 4.
A microemulsion is similar in structure to liposomes but instead of a lipid bilayer encapsulating an aqueous pocket there is a lipid monolayer acting as an emulsifier for an oil droplet as depicted below.

![Figure 3-1](image)

**Figure 3-1** A cartoon depicting the monolayer of DOPA acting as an emulsifier to stabilize the microemulsion

The formation of emulsions in an oil-water solution with DOPA present is a result of free energy minimization of the system and is described in detail by Gregoriadis\(^{111}\) and briefly by the following: Recall that lipids are amphiphiles with a hydrophilic head group and hydrophobic tail group. Reduction in free energy occurs when the alkane tails of a lipid are in proximity to another lipid’s alkane tail or other nonpolar molecule and not surrounded by water (Figure 3-2). Lipid headgroups are more stable when in contact with an aqueous phase; therefore, in an aqueous solution of lipids this attraction forms a bilayer to maximize the tail-tail interactions and maximize the headgroup interaction with water. With DOPA lipids these bilayers eventually turn in on themselves and form liposomes and can be used as templates for biomineralization of calcium phosphate nanoshells as discussed in CHAPTER 2. When oil such as soybean or perfluorocarbon is introduced the stability of system changes as oil is not stable in an aqueous environment and will coalesce to form a single drop so the surface energy of the oil is minimized.
When an emulsifier such as DOPA is present in an oil-water solution it becomes possible to form a stable emulsion as the lipids nonpolar tails are integrated into the nonpolar oil phase which ends up a sphere to reduce surface energy. The result is a DOPA-stabilized oil microemulsion whose presence is indicated by a translucent suspension when DOPA is present as opposed to a 2 phase suspension in its absence.

![Diagram of DOPA Lipids](image)

**Figure 3-2** Formation mechanism of emulsions. DOPA lipids are not stable or soluble in an aqueous environment due to the unfavorable tail-water interactions (A). A more favorable formation is a lipid bilayer which ultimately leads to a liposome if the lipids packing factor is suitable (B). When oil is present (C) the oil forms into small spheres and when agitated in the presence of DOPA can form stable microemulsions with DOPA headgroups (D). The DOPA-stabilized microemulsion forms the scaffold upon which calcium phosphate is deposited to form a shell. Not drawn to scale.

Microemulsions offer several advantages over liposomes:

- They can encapsulate hydrophobic materials at much higher amounts than in liposomes owing to the larger oil core present in an emulsion for solubilization as opposed to the smaller hydrophobic volume of the bilayer of a liposome
• There is increased interaction between the lipid tail and oil core resulting in a more mechanically rigid sphere compared to a liposome\textsuperscript{111}

• Many of the ions present in the reaction volume are only sparingly soluble in oil, greatly reducing the osmotic pressure gradient and subsequent chance of deformation of the structure during nanoshell growth. Though there is a possibility that some cations (Ca\textsuperscript{2+}) can destabilize an anionic lipid layer this is not expected to be an issue at the mM concentrations used\textsuperscript{428}.

• A variety of nonpolar liquids can be used with the same lipid in order to modify the solubility of nonpolar molecules in the nanoshell.

In this chapter, two types of model microemulsions were used as nanoshell templates, soybean oil-cored and fluorocarbon-cored. Soybean-oil was used because it is an FDA approved foodstuff and perfluorodecalin (PFD) and perfluoro-octylbromide (PFOB) are FDA approved fluorocarbons for use in oxygen delivery.

Table 3-1 Physical Properties of Various Nanoshell Core Solvents

<table>
<thead>
<tr>
<th>Property</th>
<th>Soybean Oil\textsuperscript{429, 430}</th>
<th>PFOB\textsuperscript{431}</th>
<th>PFD\textsuperscript{431}</th>
<th>H\textsubscript{2}O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (k) (mm\textsuperscript{2}/s)</td>
<td>38</td>
<td>1.0</td>
<td>2.66</td>
<td>1</td>
</tr>
<tr>
<td>Viscosity (d) (mNs/m\textsuperscript{2})</td>
<td>601</td>
<td>N/A</td>
<td>5.10</td>
<td>1</td>
</tr>
<tr>
<td>Density (g/ml)</td>
<td>0.89-.92</td>
<td>1.98</td>
<td>1.917</td>
<td>1.0</td>
</tr>
<tr>
<td>MW (g/mol)</td>
<td>~300</td>
<td>499</td>
<td>462</td>
<td>18</td>
</tr>
<tr>
<td>Surface Tension (nM/m)</td>
<td>70</td>
<td>18.0</td>
<td>17.6</td>
<td>72</td>
</tr>
<tr>
<td>Boiling Point (C)</td>
<td>&gt;200</td>
<td>143</td>
<td>142</td>
<td>100</td>
</tr>
<tr>
<td>Vapor Pressure @ 37 C (mmHg)</td>
<td>3.4</td>
<td>10.5</td>
<td>14</td>
<td>47</td>
</tr>
</tbody>
</table>
The emulsifier used was the same DOPA phospholipid used to make the liposomes in CHAPTER 2.

The stepwise supersaturation synthesis described in CHAPTER 2 was further improved in this chapter. The synthesis was automated, eliminating the need for manual titrations, reducing human error, increasing the throughput to eight reactions performed simultaneously, and a procedure for controlling the nanoshell size without the use of timed CEPA additions was developed. Three separation methods were explored that concentrated the nanoshells up to 10-fold. A reaction mechanism for calcium phosphate nanoshells was proposed in this chapter and the formation of side products is explored in more detail.

3.2 Experimental

3.2.1 Materials

Calcium chloride (CaCl₂), 85% phosphoric acid (H₃PO₄), and 1 M sodium hydroxide (NaOH) were obtained from Fisher Scientific and used without further preparation to make 0.1 M stock solutions. To obtain pH 7 phosphoric acid, 1 M NaOH was added to a 0.2 M phosphoric acid solution to neutralize the acid and then filled to volume with DI water to yield 5 ml of a 0.1 M solution. Carboxyethylphosphonic acid (CEPA) was obtained from Sigma-Aldrich. The phosphatidic acid lipid DOPA (Figure 2-1), was obtained as lyophilized powder from Avanti lipids and frozen on arrival until used. Soybean oil (Crisco) was purchased from the supermarket and stored at room temperature. Perfluorodecalin (PFD) and perfluoroctyl bromide (PFOB) were obtained from Sigma at >98 % purity. Stock solutions of DOPA stabilized emulsions are prepared
by adding 50 μl PFD or 100 μl PFOB respectively to 5 ml of a 6.93 mM solution of DOPA, stirred rapidly in a 20 ml scintillation vial with a 2/4 " stir bar for 24 hours and used for up to one week or extruded/sonicated as required. All water used in the synthesis was deionized to 18 MΩ using an E-Pure water filtration system (Millipore). Polycarbonate (PC) filters, and extrusion membranes were obtained from Millipore. Spectrapore dialysis tubing with 100,000 MWCO and 300,000 MWCO (Pierce) were used as described. Sephadex chromatography beads (G-25, G-50, G-150) were obtained from Sigma-Aldrich. Centricon YM-100 and YM-50 centrifugal concentrators (Millipore) with 100 kDa MWCO and 50 kDa MWCO respectively were obtained from Fisher and used according to manufacturer’s instructions. Falcon tubes (50 ml) obtained from Fisher were used for other centrifugation steps.

LabVIEW version 6.0 (National Instruments) was used for developing a control front end for the automated reagent titration apparatus controlled via a RS-232 port on an IBM PC. This program was used to add microliter volumes of calcium or phosphate to the reaction vessel using one or a combination of eight Tecan XP-3000 syringe pumps. Each pump was controlled independently and provided feedback on the state of each pump and progress of the reaction. A program outline is contained within the appendix.

3.2.2 Methods

3.2.2.1 Preparation of DOPA-Stabilized Emulsions

Both soybean and fluorocarbon DOPA-stabilized emulsions were prepared by one of three ways: mixing at high speed, sonication in a bath sonicator, or high pressure extrusion through a polycarbonate filter. Each method affected the final size of the
emulsion as seen in TABLE 3-3. The following procedures must be performed above the phase transition temperature \((T_c)\) for the lipid, which for DOPA must be higher than \(T_c = -8 \, ^\circ\text{C}\).

### 3.2.2.1.1 Magnetic Stirring

To test the efficacy of simple mixing, 10 \(\mu\text{l}\) for soybean oil (Crisco) or 10 \(\mu\text{l}\) for PFD or 20 \(\mu\text{l}\) of PFOB, were added to 1 ml of 0.7 mM DOPA to prepare emulsions. These solutions were stirred rapidly in a 20 ml scintillation vial using a ¾” magnetic stir bar for 24 to 48 hours at room temperature. After this period the suspensions were allowed to rest for at least 1 hour before further reaction. The final soybean suspensions were milk white due to light scattering whereas the perfluorocarbon emulsions were translucent. Soybean oil suspensions remain suspended in solution for at least 3 weeks but if the perfluorocarbon suspension rests for more than 48 hours, the emulsion will settle to the bottom due to their high density (~2.0 g/ml) relative to water, but could be resuspended if agitated, as shown by DLS and the photographs in Figure 3-3.
Figure 3-3  A) DLS count rate of PFOB emulsions that settle out due to gravity and resuspend when shaken.  B) The settled emulsion is circled above and C) can be redispersed upon agitation.

3.2.2.1.2  Sonication

To test the efficacy of sonication on the preparation of soybean oil and perfluorocarbon filled microemulsions, 10 µl of soybean oil (Crisco) or 10 µl of PFD or 20 µl of PFOB, were added to 1 ml of 0.7 mM DOPA to prepare emulsions.  These solutions were placed in a 20 ml scintillation vial at a 45 degree angle in the horizontal center of an FS-30 bath sonicator filled with 1- 1½ inches of water (Fisher).  The sonication continued for 45 minutes and the size distribution of the resulting suspension was checked via DLS.  The final temperature was between 45 and 55 C˚.  Soybean emulsions became opalescent/nearly clear with no visible oil floating on the surface, indicating a well dispersed microemulsion.  Fluorocarbon emulsions were translucent and did not have any fluorocarbon film on the top or fluorocarbon drops on the bottom of the vial.  Sonication is considered complete when mean size does not change appreciably (+/- 10 nm) within 10 minutes.  If sonication is not complete the process is repeated and checked at 10 minute intervals.  There was some variability in time required to reach the final size since it depended on the position in the bath, the water level in the bath, and the temperature of the bath.

3.2.2.1.3  Extrusion

To evaluate the efficacy of extrusion on the formation of soybean oil and perfluorocarbon nanoshells, 10 µl of soybean oil (Crisco) or 10 µl of PFD or 20 µl of PFOB, were added to 1 ml of 0.7 mM DOPA to prepare emulsions.  PFOB can be used in
higher amounts as it is more lipophilic. These solutions were mixed for 12 hours following the instructions in Section 2.2.2.1.1. Extrusion was carried out following the method described in Section 2.2.2.1.3. Briefly, a 25 mm polycarbonate extrusion membrane with 200 nm pore size was fitted to a 10 ml thermobarrel LIPEX extruder from Northern Lipids. A 1/4 “ x 1/16 “magnetic bar was placed on top of the membrane before the apparatus was closed. Five ml of the emulsion were added to the extruder which is then capped and placed on top of a spinning (~100 RPM) magnetic stir plate. The fluid outlet was fed to a retention vial. Industrial grade nitrogen gas was used to drive the fluid through the membrane at 200-800 psi depending on desired flowrate (~0.2-1 ml/min). The suspension was extruded 4 times, the extrusion membrane was replaced with another of the same pore size or smaller and the suspension extruded 10 more times. Once the extrusion was complete there was a loss of about 0.2 ml which was attributed to filter and apparatus losses.

3.2.2.2 Synthesis

3.2.2.2.1 Automated Stepwise Addition Synthesis

The basic procedure for automated stepwise addition synthesis of calcium phosphate nanoshells was described in Section 2.2.2.2.3. The idea was improved from the manual and peristaltic pump base synthesis to a more precise and parallelizable method which removed operator error and automated feedstock switching.

Stepwise synthesis of DOPA-stabilized microemulsions coated with calcium phosphate to form nanoshells was performed in two ways, either starting with a phosphate buffered emulsion solution to which calcium was added or a subsaturated
calcium phosphate emulsion suspension to which base was added. The resulting particle morphology and particles size were analyzed as before using TEM (Section 2.2.2.4.2) and DLS (Section 2.2.2.4.1). Each titration required between 15-30 aliquots 10 μl of either 0.1 M NaOH (base titrations) or 0.1 M CaCl₂ (calcium titrations) spaced 30 minutes apart over a period of 7-15 hours. Automation was accomplished via a microtitration system consisting of eight XP-3000 Syringe pumps (Tecan-Cavro) connected in series for use as a microtitration system as shown in Figure 3-4.

Figure 3-4 Automatic stepwise titration apparatus containing 8 reaction stations each with a Tecan XP-3000 syringe pump controlled by a PC and a covered 200 ml Pyrex reaction vessel that is individually magnetically stirred. A) feedstock bottle (either 0.1 M CaCl₂ B) multi-well stirplate C) syringe pumps D) pump inlet to 3-way valve E) pump outlet from 3-way valve.

Each pump consisted of a powered RS-485 communications connector and a microprocessor controlled step motor controlling syringe displacement and a 3 way
valve. The RS-485 interface allowed both power and RS-232 communications to be
delivered from a single communications and power supply. The motor was capable of
3000 steps and could be fitted with 50 μl to 1 ml syringes, allowing for titrations with 20
nL precision. Dispenses of 10 μl were readily achieved with the 14/8 fitted 250 μm
diameter PTFE tubing. The inlet of each pump was connected to the either 0.1 M NaOH
to perform a base titration or 0.1 M CaCl₂ to perform a calcium titration. Outlets were
connected to sealed 200 ml beakers above the water line and rested on the wall of the
vessel to guide the titrant into the suspension. The eight pump bank was programmed
and controlled via an RS-232 interface and controlled via LabVIEW front end developed
in house.

For calcium titration 25 ml of DI water, 600 μl of the emulsion, 200 μl of 0.1 M
pH 7.0 phosphate, and 270 μl of 0.1 M NaOH were added to a 200 ml beaker stirred with
a 1 inch stir bar at ~400 RPM at room temperature resulting in a pH 9.5 solution.
Calcium chloride (0.1 M) was added 10 μl at a time at 30 minute intervals. For base
titration, 25 ml of DI water, 600 μl of the emulsion, 200 μl of 0.1 M CaCl₂ and 200 μl of
0.1 M pH 7.0 were added to a 200 ml beaker with a 1 inch stir bar at ~400 RPM at room
temperature resulting in a pH 7.5 solution. Sodium hydroxide (0.1 M) was added 10 μl at
a time at 30 minute intervals.

3.2.2.2.2 Evaporative Crystallization

Twenty five ml of DI water, 700 μl of the emulsion, 200 μl of 0.1 M CaCl₂ and
200 μl of 0.1 M pH 7.0 were added to a 200 ml beaker with a 1 inch stir bar at ~400 RPM
at room temperature resulting in a pH 7.5 solution. The solution was then allowed to stir
open to air while recording the volume every 12 hours. As the water evaporated from the beaker, the concentration of calcium and phosphate increased and saturated the solution. This induced nucleation of calcium phosphate around the liposomes. Suspension size distributions were taken every 12 hours via DLS. There was some scale formation on the side of the reaction flask that was ignored.

3.2.2.3 Concentration of the Nanoshells

The following three methods centrifugation, centrifugal concentration, and high pressure filtration were used to increase the concentration of microemulsion templated nanoshell materials.

3.2.2.3.1 Centrifugation

As synthesized fluorocarbon shell suspensions were placed in 50 ml Falcon centrifugal tubes and centrifuged at 7500 RPM (6325 x g) for 2 hours in a Sorval T20 tabletop superspeed centrifuge using a model SL-250T rotor. The supernatant was pipetted off and set aside while the bottom 5 ml of fluid containing mostly nanoshell particles was retained. Due to the similarities in density of soybean oil (~0.94 g/ml) and water (~1.0 g/ml) centrifugation was not effective for concentration of soybean oil and aqueous liposome templated nanoshells. The density of fluorocarbon is much higher (~2.0 g/ml). The densities of the particles are taken by adding the weight of the core and the weight of the shell and dividing by the volume. These calculations are described in Section 4.4.1.
3.2.2.3.2 Centrifugal Concentration

Centricon syringe filters were loaded with 10 ml of reaction suspension then spun down at 6500 RPM in a Sorval SL-250T rotor in a Sorval T-20 tabletop centrifuge for 15 minutes and the volume of remaining fluid checked with a graduated cylinder. The process was repeated until the desired volume reduction of the suspension, typically, in the range of 2-12 ml was achieved. Filtration rates varied from sample to sample and generally increased over time due to membrane clogging. The degree of concentration was determined by the ratio of retained volume to initial volume and is qualitatively verified by measuring the scattering intensity of the two samples via DLS (Figure 3-17).

3.2.2.3.3 High Pressure Concentration (HPF)

Polycarbonate filters with 50 nm pore size were placed in a 10 ml LIPEX thermobarrel extruder (Northern Lipids). These filters were used to retain the nanoshells (size > 50 nm) while passing small crystals and water from the shells to concentrate the suspension. A small ½” magnetic stir bar was placed on the membrane to continuously prevent clogging of the filter during pressurization with industrial nitrogen gas at 200 psi or greater. This filtration method is similar to centrifugal concentration in concept but offers the following enhancements: finer control over the filtration rate by changing pressure, wider range of filter materials and sizes available, lower cost ($ 0.60 vs. $ 8.50 per 10 ml sample), membrane surface can be agitated and reduces clogging, scalability based on filter area, and can be run semi-continuously.
3.2.2.4 Analytical

3.2.2.4.1 Particle Sizing and Zeta Potential

Particle sizing and zeta potential measurements were performed as described in Section 2.2.2.4.1. Briefly, nanoshell suspensions were filtered through a 1.2 μm CA filter to remove large dust. A Brookhaven Instruments Corporation (BIC) ZetaPALS particle sizer with a He-Ne laser and a detector angle of 90 degrees was used for particle size measurements. Each 3 minute scan was averaged 3 times to obtain a standard deviation of the distribution mean size. Reported average particle diameters were obtained from unimodal analysis, considering only samples with polydispersity indices less than 0.25.

Zeta potential was measured via immersing the polycarbonate electrode of the ZetaPALS machine in a 4 ml 1 cm poly-acrylate cuvette with 1.5 ml sampler volume. The average of 10 runs was recorded as the mean value with standard deviation in mV. The thickness of the shells was determined by subtracting the particles size from the initial emulsion size and these numbers agreed well with TEM observations.

3.2.2.4.2 TEM Microscopy

Carbon coated 300-mesh copper grids with a Formvar support (Ted Pella) were used for particle visualization using a transmission electron microscope. After the reaction was completed a 1-2 μl drop of shaken suspension was placed in the center of the grid and allowed to dry. No further staining or preparation was used. A JEOL 100-SX microscope operating at 100 kV was used to obtain images on Kodak 4489 electron
film. The film was developed in Kodak D-19 developer according to manufacturers directions.

3.3 Results

The experimental results presented below are structured to provide the following evidence:

- The core solvent of the calcium phosphate nanoshell does not affect the calcium phosphate crystallization
- The existing synthetic methods developed in CHAPTER 2 can be applied to the emulsion templates
- The core of the nanoshell template can be prepared with various sizes through mechanical manipulation (sonication, extrusion) or thermodynamic equilibria by varying the ratio of oil to lipid
- Nanoshell thickness can be modified reproducibly without the use of CEPA (Section 2.3.3) on a variety of nanoshell templates
- The principle process control variable in calcium phosphate nanoshell synthesis is the supersaturation of the solution
- Three synthetic techniques can be used to adjust the supersaturation
  - Calcium titrations
  - Base titrations
  - Evaporative crystallization
- The nanoshells can be concentrated using either centrifugal or ultrafiltration techniques.
These results provide insight about the crystallization behavior, side product formation, and reaction mechanism. They are explored in the discussion.

3.3.1 Effect of Core Solvent on Calcium Phosphate Nanoshell Formation

It was hypothesized that the lipid plays the dominant role in surface behavior of a nanoshell template be it an emulsion or liposome. If this were validated, then one could apply all the information such as synthetic methodology and information regarding final thickness of the shell obtained from one set of templates to any other DOPA stabilized template containing any other solvent. To verify this, nanoshells containing water (liposomes) and emulsions of soybean oil, perfluorodecalin, and perfluoroctylbromide were prepared, and coated with calcium phosphate using an identical synthesis. As seen in Figure 3-5 there was very little observable difference in the shell size, thickness, or appearance. There was also very little difference in the average size of these particles as seen in TABLE 3-2 below. Because of the similarity of the results it was deduced that the DOPA molecule on the perimeter of all these templates governs surface behavior and growth of nanoshell calcium phosphate. A more thorough explanation of this is proposed in the discussion.

<table>
<thead>
<tr>
<th>Nanoshell Template</th>
<th>Mean Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposome Core</td>
<td>136 +/- 16 nm</td>
</tr>
<tr>
<td>Soybean Oil Core</td>
<td>146 +/- 27 nm</td>
</tr>
<tr>
<td>PFD Core</td>
<td>142 +/- 18 nm</td>
</tr>
<tr>
<td>PFOB Core</td>
<td>152 +/- 21 nm</td>
</tr>
</tbody>
</table>
Figure 3-5  Representative TEM series showing that the core solvent had no major effect on the morphology of the nanoshells. Shells were prepared with 400 μl of 100 nm diameter template prepared as discussed in the methods section. The automated stepwise base addition with 15x 10 μl NaOH additions with initial 200 μl of 0.1 M CaCl₂ and 200 μl of 0.1 M H₃PO₄ was used for shell growth. The variations of core darkness are likely due to residual solvent in some cores which did not evaporate in the vacuum of the TEM chamber and is most prevalent in viscous soy oil.
To compare, the one step supersaturation method was used on PFD cored DOPA stabilized emulsions as well (Figure 3-6). The same synthesis with the other core solvents yields equivalent results.

Figure 3-6 Perfluorodecalin cored calcium phosphate nanoshells synthesized via the one step supersaturation method developed in CHAPTER 2 at a pH of 9.5 with 400 μl of DOPA-stabilized PFD microemulsion and 125 μl each of H₃PO₄ and CaCl₂. These shells reported a mean size of 136 nm +/- 24 nm obtained via DLS. This is not significantly different than Figure 3-5C.

3.3.2 Effect of Microemulsion Preparation on Nanoshell Core Size

To demonstrate that the mean size of the nanoshells core could be adjusted, microemulsions with soy or perfluorocarbon (PFC) cores were prepared in different manners according to TABLE 3-3. Sonication produced the smallest particles of the methods but as mentioned in CHAPTER 2 the results were difficult to reproduce. The effects of extrusion membrane diameter on final emulsion size were predictable in that the pores reduced the size of the emulsion to a mean diameter comparable the pore size. There was a negligible difference in the final size of an emulsion obtained with soy oil, PFD, or PFOB and are treated equivalently. Sonication and extrusion are both
mechanical size reduction techniques and the effect on the size of the emulsion is readily understood.

**TABLE 3-3: THE EFFECT OF UNCOATED PFD EMULSION PREPARATION ON FINAL EMULSION SIZE.**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Emulsion Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonicated (1 hr)</td>
<td>56 +/- 26 nm</td>
</tr>
<tr>
<td>Extruded (50 nm)</td>
<td>65 +/- 8 nm</td>
</tr>
<tr>
<td>Extruded (100 nm)</td>
<td>95 +/- 12 nm</td>
</tr>
<tr>
<td>Extruded (200 nm)</td>
<td>190 +/- 24 nm</td>
</tr>
</tbody>
</table>

* Sonicated soy and PFOB emulsions are 52 +/- 31 nm and 62 +/- 18 nm respectively. All DLS measurements were taken after 1 hour of rest from 5 separate solutions. The mixed samples were stirred for 24 hours.

It was also possible to influence the size distribution of the emulsion by altering the ratio of oil to DOPA as seen in Figure 3-7 and TABLE 3-3. As shown in Figure 3-7, at low PFD:DOPA ratios (0-0.025) there was little change in the mean size of the emulsion. As this ratio is increased beyond 0.025-0.03 (25 and 30 μl respectively), emulsions start to appear in greater abundance and dominate the size distribution and as more oil was added, the emulsions size began to swell (Figure 3-7). This observation of a relatively constant size at low solvent:DOPA was similar for PFOB and soy oil (data not shown). A detailed explanation for this behavior is hypothesized in the discussion. TEM was used to verify the DLS results of TABLE 3-3 (Figure 3-8) and to show that the final size of the nanoshell core was affected without affecting the thickness of the shell. Results for PFD are shown.
Figure 3-7  The DLS mean diameter of stirred DOPA emulsions at ratios (0-0.25) of PFD to lipid. This phenomenon was independent of oil type. The equilibrium size of the emulsion after 24 hours is slightly smaller than at the outset.

Figure 3-8  Calcium phosphate nanoshells deposited on fluorocarbon templates prepared via one step supersaturation using 400 μl template 125 μl 0.1 M CaCl₂ and 0.1 M H₃PO₄ and 30 μl of 1 M NaOH. Sonication produced the smallest size, extrusion through a 100 nm filter produced consistently larger shells, and mixing produces the largest shells.
3.3.3 Controlling Shell Thickness by Influencing the Supersaturation

The custom built automated stepwise synthesis apparatus was used to achieve reliable control over the thickness of the shell. This method relies on a manipulation of the solution supersaturation (Section 2.4.2) and could be accomplished by increasing the pH or calcium ion concentration in the suspension over time by adding 10 μl aliquots of CaCl$_2$ (Figure 3-20) or NaOH (Figure 3-21) at set time intervals. This is in contrast to the multistep supersaturation synthesis attempted in Section 2.2.2.2.3 where 50 μl aliquots of both CaCl$_2$ and H$_3$PO$_4$ were added. To determine the optimum time between additions, an experiment was conducted which monitored the growth of the shells during this process for both the CaCl$_2$ and NaOH titrations added at 30 minute intervals. Before the 13$^{\text{th}}$ NaOH addition or the 15$^{\text{th}}$ CaCL$_2$ addition, there was no observable growth. After the 13$^{\text{th}}$ and 15$^{\text{th}}$ additions no more titrant was added and the size observed via DLS changed over the course of 20 minutes and did not change noticeably after 30 minutes as shown in Figure 3-9. Thirty minutes was therefore chosen for the time interval for subsequent additions.

![Graph showing mean diameter over time for NaOH and CaCl$_2$ titrations](image)

- **NaOH Titration**
- **CaCl$_2$ Titration**
Figure 3-9 Determination of time for completion of shell growth after several 10 μl additions of NaOH or CaCl₂. The graph was taken after the 13th titration for the base addition and the 15th addition for the calcium titration.

3.3.3.1 Calcium Titration Synthesis

The onset of nanoshell growth for the calcium titration was affected by the pH of the solution. As seen in Figure 3-10, there was a marked difference in the amount of CaCl₂ required to initiate shell growth as a function of reaction pH. When the pH was approximately 7.5, 55 x 10 μl additions of 0.1 M CaCl₂ are required to initiate shell growth. When pH is increased to 9.5, only 15 x 10 μl of CaCl₂ is required to initiate nanoshell growth. This pH dependent growth onset is discussed in detail later in the discussion. Figure 3-10 verifies the mean diameter of the nanoshells increases proportionately with CaCl₂ titrations once a certain amount is added. This amount is analogous to that necessary to pass the solubility line in Figure 3-20.
Figure 3-10  Mean particle diameter of the nanoshells prepared by extrusion of PFOB emulsions versus the number of 10 μl additions of 0.1 M CaCl₂ to a 1 nM emulsion (700 μl DOPA-PFOB emulsion) containing 270 μl of 0.1 M NaOH resulting in an initial pH of 9.5 (blue diamonds) and to a mixture containing 200 μl of 0.1 M NaOH resulting in pH 7.5 (pink squares). The additions were separated by 30 minutes. The pH was held constant by PBS buffer over the reaction and did not change by more than 0.5. After the 15th addition there was a steady increase in shell diameter as more CaCl₂ was added at pH 9.5. In contrast, the onset of measurable growth occurred after 55 x 10 μl CaCl₂ additions were added when the solution is at pH 7.5.

Figure 3-11 contains representative TEMs of grouped soybean cored calcium phosphate nanoshells synthesized produced by the process described in Figure 3-10 with 22, 25, and 30 x 10 μl additions of 0.1 M calcium chloride are seen in Figure 3-11. Below 15 additions no growth was measurable by DLS. A change in suspension size first appeared with 15 additions of CaCl₂ according to the procedure described in Section 3.2.2.2.1 and the resulting material is shown in Figure 3-11A. The nanoshells were approximately 10 nm thick as measured visually via TEM. The average nanoshell size increased in diameter as the number of 10 μl CaCl₂ additions was increased from 22 to 30 and the typical material produced is shown in Figure 3-11B and C respectively.
Appearance of calcium phosphate needles shown in Figure 3-11D occurred only when the total equivalent amount of CaCl₂ used in Figure 3-11C (300 μl) was added simultaneously as one addition. This synthesis was also conducted with other cores and produced similar results.
Figure 3-11  Transmission electron micrograph series showing typical examples calcium phosphate nanoshells synthesized using the automated stepwise supersaturation synthesis with varying thicknesses obtained by varying the number of 10 μl 0.1M CaCl₂ additions added to a DOPA-soybean oil emulsion prepared as described in Section 3.2.2.2.1. A) 10 nm shells resulting from 22 additions of calcium. B) 50 nm shells resulting from 25 additions of CaCl₂. C) 200 nm shells obtained from 30 additions of CaCl₂, at this thickness it is difficult to see the core as it is a dark center. D) The resulting needles obtained when 30 additions of CaCl₂ is added simultaneously to the reaction.
3.3.3.2 Base Titration Synthesis

Titrating 10 μl aliquots of 0.1 M NaOH could be used as an alternative to the calcium titration described above while achieving similar results. The amount of base needed to be added before measurable nanoshell growth could be observed was affected by the initial calcium concentration of the solution. As seen in Figure 3-12, less NaOH was required to initiate shell growth when more CaCl₂ was initially present. For 200 μl (0.8 mM) of CaCl₂ is initially present approximately 13 x 10 μl additions of 0.1 M NaOH were required before shell growth was measured. The amount of 0.1 M NaOH required to start shell growth was reduced to 10 μl when there is 400 μl (1.6 mM) of CaCl₂.

Figure 3-13 shows representative TEMs of nanoshells resulting from base titration synthesis. As suggested by DLS the thickness of the shells increased with increasing amount of 0.1 M NaOH. Shells with identical cores prepared with the automated stepwise supersaturation synthesis with 15 and 20 x 10 μl additions of 0.1 M NaOH yielded shells of approximately 10 nm and 90 nm respectively as shown in Figure 3-13B and C. The diameter of the nanoshells obtained with the base titration method also increases with increased number of 10 μl NaOH additions resulting from the increased saturation achieved at higher pH. This synthesis was also conducted with other cores and produced similar results.
Figure 3-12  Mean particle diameter of the nanoshells grown on extruded PFOB emulsions versus the number of 10 μl additions of 0.1 M NaOH with either 200 μl (0.8mM) or 400 μl (1.6 mM) of 0.1 M CaCl₂ synthesized using the automated stepwise supersaturation synthesis. The additions were separated by 30 minutes. After the 10th or 13th addition, depending on initial calcium concentration there was a steady increase in particle diameter as more NaOH additions were added.

Figure 3-13  Transmission electron micrograph series showing nanoshell clusters of DOPA-PFD synthesized in various manners.  A.) Calcium phosphate nanoshells produced via the automated stepwise supersaturation synthesis (Section 3.2.2.2) with 15 10 μl NaOH additions are approximately 10 nm thick  B.) Calcium phosphate nanoshells produced via the stepwise supersaturation synthesis with 20 10 μl NaOH additions resulted in nanoshells approximately 90 nm thick.
3.3.3.3 Avoiding Floc Formation

It is important not to shorten the time between additions. When the total amount of calcium or base that was added to a stepwise reaction was added simultaneously rather than over time, side products, particularly flocs (Figure 3-14) and/or crystallites (Figure 3-11D) were produced. It is common for any calcium phosphate nanoshell synthesis to have some flocculation of the shells into irreversible aggregates but this effect is minimized by allowing time for the reaction between additions (applies to additions after the first 13-15). An example of floc resulting from simultaneous addition of 200 ul (20 x 10 μl additions) of 0.1 M NaOH to a fluorocarbon cored template reaction mixture can be seen in Figure 3-14. This TEM shows a small section of a large (>> 10 μm) floc can be seen where one can make out small portions of grouped shells protruding from a much larger opaque section of the floc. This irreversible clustering occurs by a different mechanism than the grouping of shells seen in many of the TEMs which occur as an artifact of grid drying when the salt concentrations approach infinity as the solvent is removed during the final stages of drying. A more thorough explanation on the difference of these effects is covered in the discussion Section 3.4.5.

Fortunately, floc could be separated readily by sedimentation. When a solution containing floc was allowed to settle, and the top portion is analyzed with DLS, for shells produced on templates extruded through a 100 nm filter, the measured size was 156 nm +/- 21 nm and the counts were 764 kcps +/- 121 kcps. When the same solution was agitated or stirred and the size DLS measurement is taken, the machine gives an error
stemming from the excessive light scattering by the large micron sized flocs. Further proof that these flocs are composed of shells is described in Section 4.3.6.

![Image of a piece of floc composed of nanoshells resulting from a simultaneous addition of 200 μl of NaOH. There are portions of the floc that show visible shells. These flocs are visible to the naked eye in the suspension and typically settle out via sedimentation allowing for easy separation. The circle encloses a group of shells protruding from the floc, verifying that flocs are composed of many shells.]

Figure 3-14 Image of a piece of floc composed of nanoshells resulting from a simultaneous addition of 200 μl of NaOH. There are portions of the floc that show visible shells. These flocs are visible to the naked eye in the suspension and typically settle out via sedimentation allowing for easy separation. The circle encloses a group of shells protruding from the floc, verifying that flocs are composed of many shells.

3.3.3.4 Evaporative Crystallization

Calcium phosphate nanoshells can also be synthesized via evaporation crystallization synthesis. Evaporation crystallization develops supersaturation by effectively increasing the concentration of calcium in the suspension and is a slow but effective method for controlling shell thickness. To demonstrate this method, a 25 ml suspension of 700 μl soybean-oil emulsion, 200 μl each of CaCl₂ and buffered H₃PO₄ adjusted to pH 7.5 at room temperature was stirred open to the atmosphere, and after a certain amount of water had evaporated, the suspension size distribution is measured via DLS. Increase in suspension size distribution began after approximately 17 ml of water
had evaporated which took almost 4 days and continues until only 2 ml remain in the beaker at which point stirring stops. Though this method can produce shells, the time required for the synthesis (4d) did not make it an attractive alternative to the base or calcium titration methods.

![Graph showing mean diameter of soybean-oil cored calcium phosphate nanoshells prepared as described in Section 3.2.2.2 measured via DLS increased as water is evaporated from a stirring beaker. This occurs due to the increase in solution supersaturation that occurs slowly over time.](image)

**Figure 3-15** The mean diameter of soybean-oil cored calcium phosphate nanoshells prepared as described in Section 3.2.2.2 measured via DLS increased as water is evaporated from a stirring beaker. This occurs due to the increase in solution supersaturation that occurs slowly over time.

### 3.3.4 Concentrating Nanoshell Suspensions

Centrifugal concentration, centrifugal sedimentation (centrifugation) and high pressure filtration were again tested as methods to increase the concentration of the nanoshells. Centrifugal separations were described in (Section 3.2.2.3.1), centrifugal concentrators in (Section 3.2.2.3.2), and high pressure filtration (HPF) described in Section 3.2.2.3.3. Centrifugation works by increasing the sedimentation force by magnifying gravity but was only effective for nanoshells that were denser than the surrounding water (Figure 3-16A). Centrifugal concentrators contain a membrane with a
specific molecular weight cutoff which solvent readily passes through when spun in a centrifuge (Figure 3-16B). High pressure filtration works by forcing solvent through a membrane with high pressure (Figure 3-16C), similar to the centrifugal concentration but can handle larger volumes.

![Centrifugation](image1.png)  ![Centrifugal Concentrator](image2.png)  ![High Pressure Filtration](image3.png)

Figure 3-16  Various methods of nanoshell concentration that utilize centrifugal forces to accelerate sedimentation (A), or ultrafiltration membranes (B-C).

The retentates and filtrates were measured with the BIC particle sizer and the count rate (scattering intensity) was recorded. Count rate increased linearly with particle concentration when the count rate was below 1 Mcps and could be used to obtain a relative concentration ratio (Figure 3-17) provided both samples counts are below 1 Mcps. Soybean cored and perfluorooctyl bromide nanoshells were each concentrated either by filtration or centrifugation. The resulting change in scattering intensities suspension retained are listed in Table 3-4.
Figure 3-17 Graph showing the linear relation of dynamic light scattering counts as a function of dilution of a ~ 1 nM PFOB-emulsion cored nanoshell suspension. This relationship allows for ratiometric determination of concentration efficiencies by dividing the scattering intensity of the concentrated sample to the initial counts.

For each separation method, 90% of the solvent was removed from the suspension. In centrifugation, 45 ml of the liquid volume (supernatant) were discarded from 50 mls total, for centrifugal concentration 9 of 10 mls were passed through and 1ml was retained, for HPF 9 of 10 mls was passed through the filter whereas 1 ml was retained. Size distributions taken before and after showed less than 10 % difference in particle diameter indicating the particles remained separate after filtration. If a sample read more than 1 Mcps it was diluted until it is below 1 Mcps and then actual counts calculated by multiplying the counts by the dilution ratio. Some material is typically stuck to the filter membrane for HPF and centrifugal concentrator and the scattering before and after agitation is measured.
TABLE 3-4  FILTRATION EFFICIENCIES FOR CENTRIFUGATION, CENTRIFUGAL CONCENTRATION, AND HIGH PRESSURE FILTRATION DETERMINED VIA LIGHT SCATTERING INTENSITY

<table>
<thead>
<tr>
<th></th>
<th>Original (Kcps)</th>
<th>Separated (Kcps)</th>
<th>Factor of Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Soybean Oil</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centrifugation (90%)</td>
<td>456</td>
<td>851</td>
<td>1.87</td>
</tr>
<tr>
<td>50nm HPF (90%)</td>
<td>185</td>
<td>883</td>
<td>4.77</td>
</tr>
<tr>
<td>Agitated HPF (90%)</td>
<td>185</td>
<td>968</td>
<td>5.23</td>
</tr>
<tr>
<td>Concentrator (90 %)</td>
<td>54</td>
<td>497</td>
<td>9.20</td>
</tr>
<tr>
<td>Agitated Concentrator (90%)</td>
<td>54</td>
<td>543</td>
<td>10.06</td>
</tr>
<tr>
<td><strong>PFOB</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centrifugation</td>
<td>147</td>
<td>876</td>
<td>5.96</td>
</tr>
<tr>
<td>50nm HPF (90%)</td>
<td>149</td>
<td>667</td>
<td>4.48</td>
</tr>
<tr>
<td>Agitated HPF (90%)</td>
<td>149</td>
<td>754</td>
<td>5.06</td>
</tr>
<tr>
<td>Concentrator (90 %)</td>
<td>68</td>
<td>603</td>
<td>8.87</td>
</tr>
<tr>
<td>Agitated Concentrator (90%)</td>
<td>68</td>
<td>661</td>
<td>9.72</td>
</tr>
</tbody>
</table>

Note: assumes scattering per particle does not change

3.4  Discussion

3.4.1  Calcium Phosphate Nanoshell Growth is Independent of Core Solvent

From the TEMs in Figure 3-5 it appears there is little observable change in nanoshell formation for identically sized microemulsions prepared with different core solvents or liposomes. This can be understood by considering that the calcium and phosphate ions that make up the eventual shell only interact with the DOPA lipid and have no direct contact with the core solvent. In essence the core is ‘invisible’ to the crystallization reaction, so long as the deposition of inorganic cannot disrupt the stability of the lipid assembly. This hypothesis is illustrated in Figure 3-18.
Figure 3-18  The cross section of membranes of soy oil, liposomes, PFD, and PFOB cored templates show the DOPA lipid is the only portion of the template that is exposed to the exterior solution. Experimental results show calcium and phosphate ions do not appear to interact with the core during crystallization.

Since crystal growth is a surface phenomenon, it is hypothesized that the crystallization chemistry is governed by the DOPA surface not the core and that a set of crystallization conditions will result in the same shell regardless of the core solvent. This is an important assumption as it allows the information and conclusions obtained from different synthesis (one step, automated multistep calcium, and automated multistep base) to be confidently applied to a wide range DOPA stabilized emulsions and liposomes without a rigorous exploration of each system so long as DOPA is the molecule exposed to the surface. It was shown in Section 3.3.1 that this is a suitable assumption for the four core solvents under consideration in this thesis; however, the assumption it would be prudent to verify for each new system.
3.4.2 Mechanism of Microemulsion Size Change Under Various Oil to Lipid Ratios

The ability to change the core size of the nanoshell will influence its capacity to carry any material within its core. The results in TABLE 3-3 show that there are multiple methods available to produce sized microemulsions. Of these sonication and extrusion are readily understood; however, the mechanism of changing size by adjusting the ratio of oil to lipid is more complex. In Figure 3-7 it was observed that the size of the emulsion did not vary until a critical amount of oil was introduced. This can be understood by studying the illustration in Figure 3-19.

Figure 3-19 Figure depicting the hypothesized phenomena responsible for the swelling of the emulsion. At low ratios of oil to liposome the oil may be incorporated into the bilayer. As the ratio is increased, the pocket of oil swells until it becomes large enough to expel a small daughter liposome and transition to a liposome. Further addition of oil to the suspension results in continued growth of the emulsion diameter. The transition oil-liposome to swelling emulsion would happen approximately when the volume of oil equals the interior volume of the liposomes\textsuperscript{432}. In 1 ml of DOPA with 200 nm liposomes there is approximately 30 μl of interior volume and the size change occurs when ~ 30 μl of oil is added.
In absence of any oil, the 1 ml of 7 mM DOPA contained primarily 200 nm liposomes and there is approximately 30 μl of liposome volume as determined by the method proposed in Section 4.4.1. After adding 30 μl of oil to the DOPA solution the size begins to slowly increase as observed in Figure 3-7. It is hypothesized that this occurs due to the transition of a swelled bilayer liposome to a complete emulsion that is enlarge to greater sizes as more oil is added. This method can produce a range of sizes but the recorded mean size distribution is large and takes 24 hours to equilibrate. Of the three methods used to prepare sized DOPA-stabilized emulsions extrusion is used most frequently. Extrusion produces an emulsion with a size distribution near the size of the pores in the filtration membrane and can be done in a few hours with very reproducible results and is the preferred method for template formation.

3.4.3 Controlling the Nanoshell Thickness Through Modification of Supersaturation

A detailed explanation of the calcium phosphate system (Section 2.4.2.1), crystallization concepts (Section 1.3.2) and the methodology used to arrive at the optimum set of reaction conditions (Section 2.4.2.2) were described in previous sections. In this work we showed that the use of a supersaturated solution obtained via:

- Adding calcium slowly to a subsaturated buffered phosphate-emulsion suspension (calcium titration),
- Adding base to a subsaturated CaCl2-phosphate-emulsion suspension (base titration),
- The removal of water by evaporation (evaporative crystallization).
Any of these methods can be effectively used to synthesize calcium phosphate nanoshells of various thicknesses around a DOPA stabilized microemulsion with varying core size regardless of core solvent composition.

3.4.3.1 Calcium Titration Synthetic Method

This method (Section 3.2.2.2.1) adds aliquots of 0.1 M CaCl₂ at a constant pH maintained by the buffering of the phosphate present in solution. A mechanistic rationalization for this process is illustrated in Figure 2-23 and shown graphically in Figure 3-20. The initial sub-saturated state (A) contains emulsions with negatively charged DOPA lipid head groups as well as phosphate at pH 7.5 or 9.5. As CaCl₂ was added stepwise (up arrows) to a concentration above the saturation line (B), supersaturation and growth around the liposome can occur. This initial phase of growth is described in Section 2.4.4. Once the suspension is saturated, growth proceeds, consuming calcium and phosphate (down arrow) for 30 minutes (Figure 3-9) then another CaCl₂ aliquot is added (up arrow). By slowly saturating the solution and allowing the shell time to grow between additions and avoiding the unstable growth region beyond the metastable zone (MSZ) where spontaneous nucleation occurs, heterogeneous growth is favored over homogenous nucleation which allows the shell growth rate to surpass the nucleation of other calcium phosphate species (Section 1.3.2). Through this alternating process (C), the shell forms and grows larger while the core size remains the same (Section 3.3.3.1) suggesting the size change results from increased shell thickness. Upon the last addition, the solution was stirred for 12 hours allowing growth to continue until the concentration reached solid-liquid equilibrium (D). At this point, CEPA can be
added. This titration method differs from the multistep supersaturation method described in Section 2.2.2.2.3 because only CaCl$_2$ is added as opposed to both CaCl$_2$ and H$_3$PO$_4$ and each addition is smaller (10 μl of 0.1 M versus 5 μl of both 1 M solutions).

A benefit of the calcium titration synthesis is that it can be performed at physiological pH (7.4) which is of potential benefit to pH sensitive biomolecules that would be encapsulated within the shells but the degree of reaction completion is not as high as one performed at higher pH owing to the increased level of supersaturation. If required, excess calcium remaining in solution could be removed via dialysis.

![Graphical sketch illustrating the steps taken in the stepwise calcium titration synthesis where i and f denote initial and final states. A) The initial, subsaturated state of the reaction consists of phosphate-emulsion. B) As 10 ml aliquots of 0.1 M CaCl$_2$ are added the solution becomes supersaturated and growth of the calcium phosphate nanoshell begins. C) Thirty minutes of growth was allowed between each addition to allow the growth to finish as evidenced in Figure 3-9. Alternating addition-growth steps were continued to reach a desired thickness and then allowed to grow for 12 hours to let the solution reach solid-liquid equilibrium (D). The pH is held constant throughout by the buffer, the C arrows are shifted to the right to show steps of reaction for clarification.](image-url)
The TEM micrographs in Figure 3-11 visually confirmed that the thickness of the shells could be increased by titrating CaCl₂ to the reaction. Shells in Figure 3-11A had very thin shells that which became moderately thicker in Figure 3-11B and finally become so thick in Figure 3-11C that the electron beam could not penetrate the shell and the core-shell structure could not be resolved. These shells appeared as solid spheres on the grid with a slightly darker center. To confirm that the stepwise addition of calcium was necessary, an equal amount (300 μl) of calcium used in Figure 3-11D was added simultaneously resulting is a suspension of needle shaped crystallites. This is due to the higher supersaturation developed when growth (desaturation) is not allowed to occur between additions resulting in a condition where nucleation is more favorable than growth (Section 1.3) as a result of this higher supersaturation. Although this product is similar to what is obtained without DOPA in Figure 2-5B, it results from a different mechanism. Without DOPA the calcium phosphate can only form solid crystallites whereas in this case the rate of nuclei formation is outpacing crystal growth rate (Figure 1-2). Since shell formation requires preferential crystal growth around the liposome, this reduced rate of nucleation relative to growth results in more crystals and fewer shells.

Observations made via TEM correlate well with those made with DLS results. Figure 3-10 showed increasing CaCl₂ increased the resulting mean diameter of the shells from 10 to 300 nm). The difference in growth rates and onset of measurable particle growth between the different pH values is due to the fact that supersaturation occurs at a higher concentration of calcium at lower pH which can be seen in Figure 2-21. Calcium
titrations work equivalently with fluorocarbon (PFD,PFOB) cored and other templated nanoshells (data not shown).

3.4.3.2 Base Titration Synthetic Method

The base titration synthesis method adds aliquots of 0.1 M NaOH to a subsaturated CaCl₂-phosphate-emulsion suspension. A mechanistic rationalization for this process is illustrated below in Figure 3-21. Titrations in this reaction cross the saturation line in a perpendicular direction to the calcium titrations. The initial sub-saturated state (A) contains emulsions with negatively charged DOPA lipid head groups as well as CaCl₂ and phosphate at pH 7.5. As NaOH is added stepwise to the reaction the phosphate equilibrium is shifted from the protonated species to the deprotonated reactive species and the initial pH increases rapidly at first and then begin to steadily rise until the last addition as seen in Figure 3-20. Once the pH increased sufficiently to saturate the reaction (~pH 8.25) above the saturation line (B) growth of the calcium phosphate nanoshell can occur as indicated by the pH decrease after the base addition. This initial phase of growth is described in Section 2.4.4. Once the suspension is supersaturated, growth proceeds, consuming both calcium and phosphate (down arrow) for 30 minutes (Figure 3-9) then another base aliquot is added. By slowly saturating the solution and allowing the shell time to grow between additions, the unstable growth region beyond the metastable zone (MSZ) where spontaneous nucleation occurs is avoided and heterogeneous growth is favored over homogenous nucleation. This allows the shell growth rate to surpass the nucleation of other calcium phosphate species (Section 1.3.2).
Through this alternating process (C), the shell forms and grows thicker. Upon the last addition, the solution was stirred overnight (D). At this point, CEPA can be added.

Figure 3-21  Graphical sketch illustrating the steps taken in the stepwise NaOH base titration where i and f denote initial and final states. A) The initial, subsaturated state of the reaction consists of calcium-phosphate-emulsion. B) As 10 ml aliquots of 0.1 M NaOH are added the solution pH increases, becomes supersaturated, and growth of the calcium phosphate nanoshell begins. C) Thirty minutes of growth is allowed between each addition to prevent the saturation becoming too high (Figure 3-9). Alternating addition-growth steps are then continued to reach the desired thickness and then the suspension is allowed to grow for overnight to let the solution reach solid-liquid equilibrium (D).
Figure 3-22  A pH versus time trace obtained for a pH titration with 15 x 10 μl NaOH additions. The decrease (0.15 pH units) after the final addition of NaOH can be attributed to absorption of CO₂ from the atmosphere forming carbonic acid.

For a given quantity of calcium and phosphate, there is a higher degree of completion at higher pH owing to the increased supersaturation and decreased solubility of calcium phosphate. A potential disadvantage of the high pHs used in which method is that it could degrade sensitive molecules added for encapsulation within the nanoshell.

The TEM micrographs in Figure 3-13 visually confirm that using this method the thickness of the shells could be increased by 100+ nm.

Shells in Figure 3-6 show that very thin shells that can be produced with the one step supersaturation method developed in CHAPTER 2 verifying that these oil cored templates behave similarly to liposome nanoshell templates. Shells produced via the stepwise base supersaturation method become moderately thicker in Figure 3-13A and become very thick in Figure 3-13B.

Observations made via TEM correlate well with those made with DLS results. Figure 3-13 shows increasing NaOH titrations increases the resulting mean diameter of
the shells. The difference in the amount of NaOH after which measurable growth occurred for the 2 different calcium concentration is because supersaturation occurred at a lower pH when the calcium concentration was increased which can be seen in Figure 2-21. Base titrations worked equivalently with soybean oil and other templated nanoshells (data not shown).

When using the base titration synthesis, the increase in shell thickness (by 77 nm) at 27 x 10 μl (1.08 mM NaOH) additions of NaOH and 200 μl (0.8 mM CaCl₂) of initial CaCl₂ is approximately equal to the thickness change (91 nm) at a composition to 20 x 10 μl (0.8 mM CaCl₂) additions of CaCl₂ with 270 μl (1.08 mM NaOH) initial NaOH in Figure 3-10. These conditions have approximately the same compositions minus what has been consumed during early shell growth and should therefore create a solution at approximately the same supersaturation. The good agreement in shell thickness (within 15 nm) suggests that shell growth depends primarily on supersaturation not necessarily the method used to achieve it.

3.4.3.3 Evaporative Crystallization Synthetic Method

Both the automated calcium and base stepwise supersaturated synthesis require a specialized apparatus to be created and thus can not be used in all laboratories. To address this issue, a simpler synthetic method based on evaporation was explored which follows the same principle of a slow rise to supersaturation followed by controlled growth and is outlined graphically in Figure 3-23. When water was removed through evaporation it increases the concentration and can eventually supersaturate the suspension and cause growth. This is the fundamental principle behind many industrial evaporative
crystallizations\textsuperscript{61}. By controlling the final volume of water evaporated from the solution the final shell thickness could be influenced as seen in Figure 3-15.

While evaporative crystallization was effective in synthesizing calcium phosphate nanoshells with a controlled shell thickness, it took much longer (days vs. hours) than the other available methods and does not result in an improvement (size, thickness, etc.). This lack of improvement does not warrant the extra time required; therefore, the automated stepwise synthesis is the preferred synthetic method.

Figure 3-23 Graphic representation of how evaporation can achieve a supersaturated solution where $i$ and $f$ represent the initial and final states. A solution composed of calcium-phosphate-emulsion is exposed to air and allowed to evaporate. As water evaporates the volume of the suspension is reduced and the concentration increases and so does the pH, eventually saturates causing growth (B). As more water evaporates more growth occurs.

3.4.4 Concentration of nanoshells.

Centrifugal concentration was the most effective at concentrating the nanoshells but is the most labor intensive requiring repeated visual inspection after each short
centrifugation. The total time required for filtration was inconsistent from sample to sample which is likely due to membrane clogging. It is also the most costly method as each concentrator can only be used once before the membrane fouls. Centrifugation was rapid and inexpensive but was only effective for the perfluorooctyl bromide shells due to their high density (~2 g/ml). High pressure filtration was effective for all types of nanoshells, can be monitored visually during the filtration, is cost effective as the filters are less than $1.00, and can easily be scaled up in the future by transitioning to a higher surface area pressure filter by using commonly used solid processing equipment. There are commercially available 1 m filters that provide more than 400 x the area of the filters used in this experiment.

The ability to concentrate of the nanoshells is relevant as current synthetic methods produce shells with less than 0.2 vol %. Direct scale up of the synthesis has proved problematic, producing a great amount of floc and affecting yields. Potential applications for these shells such as biosensors for ELISA type tests (CHAPTER 5) would benefit from an increased concentration and other applications such as oxygen transport require it to be viable (CHAPTER 6). Storage of these shells would also be more efficient if a concentrated solution could be stored and diluted upon use.

3.4.5 Floc Formation Mechanism

The most apparent side product of this reaction is shells with irreversibly clustered or ‘focced’ together. It is hypothesized that the amount of floc present in a reaction increases when both the template concentration and supersaturation are high and
consequently the reaction speed is higher. A possible mechanism for the formation is illustrated in Figure 3-24.

Figure 3-24 Possible explanation of irreversible clustering of shells that results in a floc composed of shells. A-B) shells come in close contact while free calcium ions form solid bridges between the particle (C). The joined shells slow down and allow other shells to repeat the process (D) resulting in a larger cluster (E). The process repeats to produce a large cluster of shells that flocs out of solution (F) as seen in Figure 3-14.

Several mechanisms could explain why nanoshells localize around each other and eventually form floc. If we assume that during growth there is an imbalance of charge and that some shells are negatively charged (excess surface phosphate) while some are positively charged (excess surface calcium) they could be attracted electrostatically. Conversely if there are shells with no surface charge they could be attracted by VDW forces in an effort to reduce their surface energy. Once the shells are close to each other it is possible that free calcium ions can create semi-crystalline ‘bridges’ between the shells. The motion of this larger cluster of shells would slow down which could potentially invite other shells to collide with the cluster owing to its larger cross section. This process could continue in 3 dimensions to produce the large clusters seen in Figure
3-14. The sequestering of shells in these clusters effectively reduced shell yield for a reaction and is further investigated in 4.3.6.

This irreversible floccing occurs by a different mechanism than the clustering or grouping of shells typically seen during drying. Recall when a TEM grid is prepared for observation it is taken directly from the solution and is not post treated so that the entire contents of the reaction can be visualized. There is invariably some Na\(^+\) and Cl\(^-\) counter ions and well as unreacted calcium and phosphate. When the water is removed the concentration of the ions changes from mM to M thereby shielding the repulsive negative charge present of the shells that kept them stable in solution and they group together when drying out. As seen from the TEMs in this chapter, this grouping is 2-dimensional meaning it happens as the shells are confined on the plane of the grid not free in solution where the grouping would likely be 3 dimensional. These 2-dimensional groups/clusters of shells are in sharp contrast to the larger irreversible flocs resulting from high template or solution supersaturations.

3.5 Conclusion

The stepwise supersaturation synthesis developed in CHAPTER 2 was extended to cover the soybean oil, perfluorodecalin (PFD), or perfluorooctyl bromide (PFOB) microemulsion templates in this chapter. The mean diameter of these emulsions could be adjusted from 50 to 400+ nm (Table 3-3) by preparing them via mixing, sonication, or extrusion. An automated apparatus (Figure 3-4) was constructed and programmed with LabVIEW to add 10 \(\mu\)l aliquots of CaCl\(_2\) or NaOH to a subsaturated emulsion suspension to produce nanoshells with thicknesses ranging from 10 nm to over 200 nm around any
DOPA-stabilized microemulsion (Figure 3-11, Figure 3-13, and Figure 6-4). When compared to NaOH, calcium titrations had a larger effect on the size of nanoshells per volume of identically concentrated solution added because they directly affect the saturation whereas NaOH additions indirectly affect the saturation by adjusting the pH (Figure 3-10 and Figure 3-12). The stepwise addition of calcium was critical to shell formation as an large volume of calcium added all at once develops too high of a saturation resulting in many nanocrystallites forming and no shells (Figure 3-11D); similarly, the 30 minute growth period between CaCl\(_2\) additions was important for controlled and reproducible nanoshell growth or else a large number of irreversibly formed shell clusters can form (Figure 3-14). Similar considerations must be made for NaOH titrations.

Though evaporative crystallization did produce shells with controlled thickness in a simplified manner, it was not time effective compared to the presented base and calcium titrations taking approximately 4 days longer. It is conceivable that the rate could be increased under vacuum or nitrogen bubbling to increase the gas/liquid surface area and increase evaporation.

The results from the three methods used to synthesize calcium phosphate nanoshells in this chapter rely on increasing the supersaturation of the nanoshell suspension in order to affect the amount of calcium phosphate crystallized and therefore shell growth. As described in CHAPTER 2 it is proposed that this growth occurs by charged calcium ions forming a Stern layer around the emulsion which generated a local supersaturation allowing calcium phosphate deposition to occur selectively around the emulsion.
Concentration of calcium phosphate nanoshells up to 10-fold was accomplished by centrifugation, centrifugal filtration, or high pressure filtration (TABLE 3-4). The best concentration was obtained using centrifugal concentrators which can concentrate the shells about 10-fold resulting in a maximum estimated particle concentration of approximately 1-2 vol % but the method is slow, expensive due to clogging, and does not scale well since there is a practical limit to the size. High pressure filtration can concentrate about 5-fold, is cheap, and scales well. Centrifugation works as well as high pressure filtration for heavy fluorocarbon cored shells but not well for soybean cored nanoshells.
CHAPTER 4

ENCAPSULATION OF FLUOROPHORES WITHIN CALCIUM PHOSPHATE
NANOSHELLS

4.1 Introduction

A robust nanoshell synthetic method has been developed in the previous 2 chapters. It was concluded that the most effective synthetic method was the automated stepwise addition synthesis (Section 3.2.2.2.1). This method was used to manufacture the various liposome and emulsion cored nanoshells within this chapter. Next, the ability of these two types of nanoshells to encapsulate molecules was addressed. The encapsulation of molecules within the shell bestows functionality to them and is of particular relevance to targeted drug delivery (Section 1.5.2) and biosensors (Section 1.5.2). Pyrene (Figure 4-1B) was used as an encapsulation model for the hydrophobic cored shells while pyranine (Figure 4-1A) was used as the encapsulation model for aqueous cored calcium phosphate nanoshells.
Figure 4-1 Structural and chemical information for the fluorophores: pyranine (A) and pyrene (B). Their representative absorbance-fluorescence spectra as measured in nanoshells are seen below. Pyranine absorbs maximally at 454 nm and emits at 512 nm whereas pyrene absorbs at 337 nm and emits at 373 nm. Pyranine is the model hydrophile and pyrene is the model hydrophobe.
Pyranine is a hydrophilic fluorophore with 3 sulfonic acid groups in addition to one hydroxyl group on a pyrene backbone with a pK$_a$ = 7.3. These ionizable groups lend a pH dependency to the absorbance spectra (Figure 4-22) and consequently pyranine is commonly used as an intracellular pH indicator through ratiometric analysis of its absorbance at 403 and 453 nm$^{434}$ Pyranine monomer emission is quenched by hydrogen peroxide and as a result has been used to monitor peroxide generation within plant cells$^{435,436}$, it can also be quenched with Cu$^{2+}$ as shown in Section 4.3.4. Pyranine is also membrane impermeant meaning that it should not leak out of the DOPA phospholipid liposome or shell making it easy to retain the molecule throughout synthesis. This coupled with its potential application as a pH indicator and peroxide monitor make pyranine an excellent model fluorophore for testing encapsulation of molecules within liposome cored nanoparticles, and the interaction with their environment.

To ensure encapsulation of fluorophores and other substances is not limited to aqueous cored calcium phosphate nanoparticles, hydrophobic pyrene is used as a model encapsulant for soybean microemulsion shells. Pyrene has two unique properties that provide additional information about the encapsulation scenario within the shell, excimer formation and solvent dependent fluorescence spectra.

Pyrene readily forms excited state dimers or excimers$^{437}$ (Figure 4-16) when the molecules are in proximity to each other resulting in a long wavelength emission. This phenomenon, coupled with the pyrene’s ability to easily integrate into a fatty acid bilayer$^{438}$ has been used to monitor membrane integration using fatty acids incorporated with pyrene$^{439,440}$. In addition to the ability to form excimers, the emission spectrum of pyrene is polarity dependent as shown by J.K Thomas et. al.$^{441}$ who concluded the ratio
of the peak at 373 (peak 1) and 384 (peak 3) could indicated if pyrene was in a polar
(3>1) or nonpolar (3<1) environment. This can be observed from the spectra obtained
from Thomas’ classic article comparing n-Hexane and Methanol as solvents seen in
Figure 4-2.

Figure 4-2 Polarity dependent fluorescence spectra measured by JK Thomas\textsuperscript{441}. The
ratio of peak 3 to peak 1 can be used to determine the polarity of pyrene’s solvent.
When the ratio is above unity the solvent is nonpolar and when below unity is polar.
*Image adjusted from original article for clarity.

The use of fluorophores allows spectrophotometric measurement the effectiveness
of molecule encapsulation via fluorescence and absorption spectroscopy. These
techniques were used to verify:

- That organic molecules like these fluorophores could be encapsulated
  within the nanoshell (section 4.3.1, 4.3.2, and 4.3.3)
- How much fluorophore is encapsulated within the nanoshell and how
  efficient is the encapsulation (Section 4.3.1).
• How much if any protection to the optical properties of the dye does the shell provide to the dye from quenching (Section 4.3.4).

• What specific physical and chemical advantages do calcium phosphate shells provide over a free dye or uncoated liposomes
  o Protection from quenchers (Section 4.3.4)
  o Rigidity to survive filtration (Section 4.3.2)
  o Low concentration excimer formation (Section 4.3.2)

• Whether other dyes of commercial interest for fluorescence microscopy be encapsulated within the shell besides pyrene and pyranine (Section 4.3.3).

• What are the most effective/practical methods (dialysis, chromatography, filtration) to remove unencapsulated fluorophore and collect the desired nanoshell product

• What are some demonstrated uses for shells with encapsulated dyes over plain shells (Sections 4.3.5 and 4.3.6)

Results from these investigations presented within this chapter provide a better understanding of the encapsulating microenvironment of calcium phosphate nanoshells and is especially relevant for the selection of appropriate nanoshell for the encapsulation and targeted delivery of proteins and insoluble anticancer agents as discussed in Section 1.5.3. By conjugating targeting molecules (CHAPTER 5) to a shell with a large quantity of encapsulated dye can also be used as biosensors (Section 1.5.2)
4.2 Experimental

4.2.1 Materials

Materials required for aqueous cored nanoshell synthesis are given in Section (2.2.1) and for hydrophobic cored synthesis are given in Section (3.2.1). Pyrene (>98%) and Pyranine (>99%) were obtained as powder from Molecular Probes (Invitrogen). A 0.05 M aqueous solution of pyranine was prepared, covered with foil and kept refrigerated. Pyrene was mixed to make a 50 mg/ml (245 mM) solution with soybean oil (Crisco), covered with foil and left at room temperature. FITC and sulforhodamine were obtained from molecular probes and used to make a 50 mM FITC/soybean oil and 5 mM sulforhodamine/water solutions both solutions are covered and kept at room temperature. Copper chloride (CuCl₂) and hydrogen peroxide were obtained from Fisher and used to make a 4 M solution of CuCl₂ and 30 % aqueous peroxide solution. All water was deionized to 18 MΩ via an E-Pure water filtration system (Millipore). Polycarbonate (PC) filters were obtained from Millipore. Spectrapore tubing with 60,000 MWCO (Pierce) was used as described. Perfector Scientific disposable 4.5 ml, 1 cm cuvettes composed of polymethacrylate were obtained from Fisher and used for both fluorescence and absorbance measurements. Sephadex G-25 chromatography gel was obtained from Sigma and prepared as discussed in Section 4.2.3.3.

4.2.2 Preparation of Fluorophore Encapsulated Calcium Phosphate Nanoshells

The synthesis procedures for aqueous cored and soybean oil- cored calcium phosphate nanoshells were described in CHAPTER 2 and CHAPTER 3. Using those procedures, calcium phosphate nanoshells could be synthesized in sizes from 50 to more
than 400 nm in diameter with a shell from 10 to more than 100 nm thick (Sections 2.3.3 and 3.2.2.4.1). Encapsulating molecules into these nanoshells requires minor modifications to the basic procedure listed below:

- To encapsulate 245 mM pyrene or 20 mM FITC in the DOPA-stabilized soybean oil microemulsion, dye-oil solution was used in lieu of pure soybean oil during template preparation.
- To encapsulate 50 mM pyranine or 5 mM sulforhodamine in a DOPA liposome, the lipid was hydrated with an aqueous dye solution in lieu of DI water during liposome formation.

4.2.3 Methods Used for Separation of Unencapsulated Fluorophores.

Three different methods were used to separate unencapsulated dye molecules and to assess the encapsulation efficiency of the dyes. Each method yielded equivalent results but each had different practical benefits relating to speed, cost, and convenience which are discussed in Section 4.4.2.

4.2.3.1 Dialysis

Five milliliters of as synthesized fluorophore-loaded nanoshell suspensions were placed in 3,000 MWCO Spectrapore dialysis membranes and immersed in 500 ml of DI water to remove excess fluorophore from the nanoshells. Equilibration with the dialysate was allowed to proceed for 24 hours at room temperature with gentle agitation. To ensure complete removal of the free dye from the sample the dialysate was changed the next day but this did not result in a significant difference for pyranine contained within
the shells (Figure 4-4). Equilibration time could be reduced through the use of higher MWCO membranes.

4.2.3.2 High Pressure Ultrafiltration

This procedure was carried out using the apparatus and methods for extrusion described in Section 2.2.2.1.3. Polycarbonate filters with 50 nm pore size were placed in a 10 ml LIPEX thermobarrel extruder (Northern Lipids), and a small ½” magnetic stir bar was placed on the membrane to continuously prevent clogging of the filter during pressurization with industrial grade nitrogen gas at 200 psi or greater. The aqueous-cored shells containing pyranine dye were filtered to 20 % (1ml/5ml) of their original volume and then dye free solution was added to the retained shell suspension to rinse, and repeated 8 times. In the last rinsing step the dye-loaded aqueous-cored nanoshells were resuspended in a smaller volume of DI water (2 ml) than originally to concentrate them 5-fold.

Nanoshells with encapsulated pyrene were also washed using this method and filtered to 20 % of their original 5 ml volume then rinsed with 8 ml of water 8 times. To estimate the encapsulation efficiency, the fraction that comes through the first pass was used to obtain an estimate of the encapsulation efficiency as described in Section 4.3.1.4

4.2.3.3 Column Chromatography

Sephadex G-25 was used to prepare a 1” x 12” column, by washing 8 g of beads in deionized water by gentle magnetic agitation for 5 minutes. The suspension was allowed to settle and the supernatant is decanted off. The washed beads were
resuspended with DI and poured at once into a 1” x 12” glass column attached to a 24”
extender to hold the additional 50 ml of water volume used for elution. The column was
allowed to gravity pack until settled and then the water was drained through the column
to pack the beads. Once settled, approximately 4 ml of rinsed silica sand was placed on
top of the gel to prevent disturbances to the top layer during addition of the sample.

Ten ml of as synthesized dye-shell suspension were loaded gently on top of the
column and the eluent was collected in 50 x 3 ml fractions automatically in disposable
cuvettes for later analysis by UV-Vis. The fractions were also analyzed for size
distribution and scattering intensity via DLS using a Brookhaven ZetaPALS light
scattering machine.

4.2.4 Spectroscopy

4.2.4.1 Absorbance

Absorbance measurements were made by placing the sample in a disposable 4.5
ml polymethacrylate cuvette with a 1 cm path length and a transparency window of 285
nm to 750 nm was used for each sample. A Cary 50 Bio UV-Vis spectrometer was used
to take the absorption spectra from 300 nm to 700 nm for all samples. Single wavelength
measurements were taken for FITC at 490 nm and sulforhodamine 590 nm to determine
their encapsulation efficiencies.

4.2.4.2 Fluorescence

Fluorescence spectra were recorded on an SLM 8100 series fluorescence
spectrometer. This spectrometer was equipped with a mercury arc lamp as the light
source. The fluorescent emission was recorded at a 90 degree angle to incident and was resolved through a monochromator before being recorded via a Hamamatsu photomultiplier tube. A disposable 4.5 ml polymethacrylate cuvette with a 1 cm path length and a transparency window of 285 nm to 750 nm was used for each sample.

Emission spectra were obtained by exciting the dye at the absorbance maximum for each dye. The wavelengths used were 454 nm for the case of pyranine, and 337 nm for the case of pyrene, and recording the emission spectrum over the range of 454 nm to 512 nm, and 337 nm to 600 nm, for each respectively. Time lapse spectra for pyranine were collected by setting the collecting monochromator to either 373 or 512 nm and recording the intensity of fluorescence light emission as a function of time for 10 minutes.

For the experiments that monitored the quenching of pyranine monomer emission with hydrogen peroxide and CuCl$_2$ administered to the suspension. The SLM 8100 fluorimeter was set for 454 nm excitation and recorded 512 nm emission intensity over 20 minutes at 1 second intervals. For hydrogen peroxide, 1 ml of 15% w/w H$_2$O$_2$ was added to 2 ml of the nanoshells (0.55 mM pyranine) after approximately 1 minute resulting in a 187 μM concentration of pyranine in the cuvette and a ten thousand fold molar excess of hydrogen peroxide at 1.25 M. A time trace of the fluorescence intensity over time was obtained as pyranine fluorescence was quenched. One ml of 4 M CuCl$_2$ was added to 1 ml of nanoshells with 150 mM pyranine result in a 75 mM pyranine solution. Copper chloride quenching speed is much faster than peroxide and required adjustment of the recording parameters from 20 minutes at 1 second intervals to 1 minute at 0.05 second intervals.
4.2.4.3 High Resolution TEM (HRTEM)

Sample grids were prepared as discussed in Section 3.2.2.4.2. High resolution TEM was conducted at the University of Chicago on a FEI Tecnai F30 running 62E software with a 30 μm aperture at 300 kV. Micrographs were recorded on a GATA N Ultrascan CCD 4096 x4096 camera.

4.3 Results

The results presented here will confirm that both hydrophilic and hydrophobic molecules can be encapsulated within the nanoshells by using fluorophores, the calcium phosphate coating offers protection to the contents of the nanoshell, and that dye encapsulated shells can provide information that empty shells can not.

4.3.1 Encapsulation of Pyranine in Liposome Cored Calcium Phosphate Nanoshells

The goal here was to determine how much if any pyranine that was added was encapsulated by the nanoshell. It is hypothesized that one of three things can happen: 1) all the pyranine will remain in the liposomes and thus the shells 2) some pyranine will leach out through either the shell or the liposome 3) the liposome will rupture during synthesis and will release all of its encapsulated pyranine leading to no encapsulation.

4.3.1.1 Pyranine’s pH Dependent Absorption Spectra

To determine the concentration of pyranine encapsulated inside the nanoshells absorbance spectroscopy was used. Spectroscopic measurement of pyranine is
complicated by the pH dependent spectra of pyranine due to the ionizable SO$_3^-$ groups shown in Figure 4-1. The series of spectra seen in Figure 4-3 shows that the ratio of peak 403 to peak 453 changes as a function of pH. A pH independent calibration curve can be obtained at the isosbestic point (415 nm) for pyranine and is shown in Figure 4-3.
Figure 4-3 Pyranine's absorbance spectra depends on the pH of the solution between pH 4-10. A pH independent calibration curve can be developed at the isosbestic wavelength (415 nm) and has a molar absorptivity of $9.8 \times 10^3 \text{cm}^{-1}$. To confirm the accuracy of the spectra, the calibration curve at 454 nm in pH 10 solution yields a molar absorptivity ($\varepsilon$) of $2.49 \times 10^4$ which was found to be comparable to $2.4 \times 10^4 \text{cm}^{-1}$ reported by Molecular Probes.
The pH independent calibration curve at 415 nm in Figure 4-3 was used to determine the concentration (μM) of pyranine in solutions and in nanoshells. For nanoshell suspensions, a correction for the scattering of light was subtracted from the absorbance and was approximately 0.08 units at 415 nm. This was determined by recording the absorbance of dye free shells. The pH dependent spectra of pyranine can be used to determine the pH spectrophotometrically and its use will be investigated in Section 4.3.5.

4.3.1.2 Removal of Unencapsulated Pyranine Via Dialysis

In order to determine the amount of pyranine encapsulated within the shells compared to the initial amount of pyranine used, it was necessary to remove unencapsulated pyranine through dialysis, chromatography, or filtration. Using dialysis, suspensions of 5, 7.5, 10, 20, 30, 40 and 50 mM pyranine loaded calcium phosphate nanoshells were put in a 3,000 MWCO dialysis bag and dialyzed at a ratio of 1:100 (10 ml to 1000 ml) against DI water at neutral pH. The concentration of pyranine appearing in the dialysate was measured over time via absorbance spectroscopy. The necessary time needed to remove all the pyranine from the shells or liposome suspension was determined to be 24 hours as seen from a representative experiment in Figure 4-4. After 24 hrs no significant change in pyranine concentration was seen in the dialysate even after replacing the dialysate with fresh DI water. For the purposes of the calculations below, after 1 24 hour period the bag and dialysate pyranine concentrations were assumed to be at equilibrium. The encapsulation efficiency of the shells was determined by assuming that the dialysis bag is at equilibrium with the dialysate at 24 hours and using a mass balance when the initial
concentration of dye in the dialysis bag is known. The procedure for calculating the encapsulation efficiency is shown below.

Figure 4-4  A) One milliliter of liposomes containing 20 mM pyranine, and 20 mM solution pyranine dialyzed against 100 ml of phosphate buffered water with a 3,000 MWCO membrane show similar initial rates of release but deviate at later times. The deviation is due to the retention of the dye within the liposomes. B) Dialysis of pyranine-loaded shells show a similar release kinetic. The data was scaled to account for the dilution of 600 ul of liposomes into 25 ml of reaction solution.
Figure 4-5 Definition of variable used in mass balance for the dialysis setup used to separate unencapsulated dye

Initially for Pyranine:

\[ N_{Total} = N_S + N_L = N_{Bag} \]
\[ N_D = 0 \]
\[ C_S = C_B = C_L \]

After dialysis is complete:

\[ N_{Total} = N_S + N_L + N_D \]
\[ C_D = C_L \]
\[ N_B = C_B \cdot V_B \]
\[ N_D = C_D \cdot V_D \]
\[ N_L = C_L \cdot V_L = C_D \cdot V_L \approx C_D \cdot V_B \]
\[ N_S = N_B - N_L \]
\[ C_S = \frac{N_S}{V_S} \]
\[ \eta = \frac{C_{S_{\text{final}}}}{C_{S_{\text{initial}}}} \]

Equation 4-1

Figure 4-6 Derivation for the encapsulation efficiency (\( \eta \)) for pyranine in nanoshells. \( C_B, C_D \) are the measured quantities before and after dialysis, \( N_{\text{total}}, V_D, V_B \) are known, and \( V_S \) is estimated through the calculation outlined in Section 4.4.1

The two measured quantities were bag concentration \( C_B \) and dialysate concentration \( C_D \) which were determined by absorbance measurements at 415 nm before and after 24 hours of dialysis. Within the volume of the bag \( (V_B) \) there were two phases, calcium phosphate nanoshells with encapsulated pyranine \( (C_S) \) and unencapsulated pyranine \( (C_L) \). Initially there was no pyranine in the dialysate \( (N_D = 0) \) and the concentration of shells was equal to that of the bag \( (C_B) \). After 24 hours of dialysis it was assumed that the bag and dialysate were at equilibrium \( (C_D = C_L) \). With the concentrations of the bag \( (C_B) \) and dialysate \( (C_D) \), the known volumes of the dialysate \( (V_D) \) and liquid \( (V_L) \) (approximated to be the bag), the initial amount of pyranine \( (N_{\text{Total}}) \) present and an estimate for the shell volume \( (V_S) \) the number of moles within the shells, and thus the concentration of pyranine within the shells, could be determined. The ratio of the concentration of pyranine in the nanoshell after dialysis to that initially is termed the encapsulation efficiency \( (\eta) \). Results from this experiment are summarized in TABLE 4-1.
**TABLE 4-1 EXPERIMENTAL RESULTS FOR DETERMINING PYRANINE ENCAPSULATION EFFICIENCY**

<table>
<thead>
<tr>
<th>Loading Concentration (mM)</th>
<th>50</th>
<th>40</th>
<th>30</th>
<th>20</th>
<th>10</th>
<th>7.5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTOTAL (μmoles)</td>
<td>11.6</td>
<td>8.15</td>
<td>5.22</td>
<td>2.72</td>
<td>1.38</td>
<td>1.05</td>
<td>0.32</td>
</tr>
<tr>
<td>CB μM</td>
<td>375</td>
<td>462</td>
<td>267</td>
<td>295</td>
<td>145</td>
<td>122</td>
<td>70.1</td>
</tr>
<tr>
<td>CD μM</td>
<td>13.9</td>
<td>8.98</td>
<td>4.82</td>
<td>1.52</td>
<td>0.48</td>
<td>0.51</td>
<td>0.58</td>
</tr>
<tr>
<td>NB (μmoles)</td>
<td>4.51</td>
<td>3.47</td>
<td>2.68</td>
<td>4.84</td>
<td>1.13</td>
<td>0.86</td>
<td>0.23</td>
</tr>
<tr>
<td>ND (μmoles)</td>
<td>6.95</td>
<td>4.49</td>
<td>2.41</td>
<td>0.76</td>
<td>0.19</td>
<td>0.15</td>
<td>0.09</td>
</tr>
<tr>
<td>VS μL</td>
<td>7.10</td>
<td>7.10</td>
<td>7.10</td>
<td>7.10</td>
<td>7.10</td>
<td>7.10</td>
<td>7.10</td>
</tr>
<tr>
<td>CS mM</td>
<td>22.65</td>
<td>26.98</td>
<td>18.99</td>
<td>16.78</td>
<td>8.32</td>
<td>5.61</td>
<td>0.59</td>
</tr>
<tr>
<td>η</td>
<td>0.45</td>
<td>0.67</td>
<td>0.63</td>
<td>0.84</td>
<td>0.83</td>
<td>0.75</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Encapsulation efficiency was most effective at a loading concentration of 10-20 mM as seen in Figure 4-7.

![Encapsulation Efficiency Graph](image)

**Figure 4-7** Encapsulation efficiency η as a function of pyranine loading concentration. The point at 5 mM is likely an outlier.
Figure 4-8  Resulting concentration of pyranine in the nanoshell as a function of pyranine loading concentration. There is a limit (approximately 25 mM) that can be encapsulated in the shell fraction which is approximately 26,000 pyranines per 100 nm liposome. The volume of the liposomes is calculated in Section 4.4.1.

The curve in Figure 4-8 shows the total concentration of pyranine dye in the nanoshells. To obtain this the number of shells needs to be obtained but is difficult to obtain experimentally. Instead, the number of shells was calculated by making some assumptions regarding the packing arrangement, lamellarity, effective lipid size, and size distribution. The calculation used to obtain this number is discussed in detail in Section 4.4.1 and yields an estimate of a ~ 1 nM nanoshell suspension. A comparison of dialysis to other separation methods can be found in Section 4.4.2

4.3.1.3 Removal of Unencapsulated Pyranine Via Chromatography

Another method for removing unencapsulated dye and obtaining the total concentration of nanoshell entrapped dye, and an estimated internal concentration was column chromatography using Sephadex G-25 column.
This method was investigated in the interest of saving time as dialysis separation took 24 hours whereas column separations could be done in an hour. Ten milliliters of shell suspension with a 10 mM pyranine loading concentration was added to the G-25 column. Fractions of 3 ml were collected in disposable cuvettes for later analysis by UV-Vis spectroscopy and DLS. There were 4 x 3 ml fractions (6 to 9) that contained the majority of the shells as determined by the laser scattering count rate seen in Figure 4-9. The percentage of total shells in these fractions was estimated by taking the sum of the counts in fractions 6 to 9 (185 kcps) and since counts are linear with particle concentration, dividing it by the total (198 kcps) yields ~93% of shells present within fractions 6 to 9. The majority of the free pyranine did not elute until the last 20 ml of 200 ml and was combined with the remaining non-shell fractions. Both this and the shell fractions were analyzed via absorbance to determine the concentration and along with the total volume of the sample, the number of moles in each.
Figure 4-9 Separation of the shells from unencapsulated dye accomplished through elution from a Sephadex G-25 column. The presence of particles in the eluent was monitored via light scattering counts from the BIC Zetaplus analyzer. The concentration of pyranine was determined as described in the text. Fractions 6-9 contained 91% of the particles and retained 81% of the initial moles of pyranine (TABLE 4-2).

The size of the eluted shells was approximately 120 nm +/- 5 nm with polydispersities less than 0.15. There are likely some shells and aggregates in the other fractions but the number is too small to obtain a reliable size distribution as shown with the high polydispersity values in Figure 4-10.
Figure 4-10  Size distribution and polydispersity as a function of fraction obtained from a Sephadex G-25 column. Note the consistent sizes over fractions 6 to 9 that contain the majority of the shells as reported in Figure 4-9. This region coincides with low polydispersities (< 0.2) indicating a more monodisperse size distribution.

Since the unencapsulated pyranine becomes trapped in the pores of the G-25 gel, it is assumed there is no non-encapsulated pyranine present in the shell elution volume. Therefore, the encapsulation efficiency was determined by dividing the number of moles of dye present in the shell containing elution volume (0.028 micromoles) as determined by absorbance spectroscopy and dividing it by the estimated volume of shells which is calculated as described in Section 4.4.1 and is shown to be $\eta \sim 0.81$ in Table 4-2.
TABLE 4-2. EXPERIMENTAL RESULTS FOR COLUMN SEPARATION OF UNENCAPSULATED PYRANINE FROM PYRANINE ENCAPSULATED WITHIN THE NANOSHELLS

<table>
<thead>
<tr>
<th>Loading Concentration of Pyranine (mM)</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Shell Concentration of Pyranine (μM)</td>
<td>6.6</td>
</tr>
<tr>
<td>Moles in Shell Elution (μmoles)</td>
<td>0.028</td>
</tr>
<tr>
<td>Elution volume (ml)</td>
<td>11.04</td>
</tr>
<tr>
<td>Volume of Shells (μL)</td>
<td>3.5</td>
</tr>
<tr>
<td>Concentration in shells (mM)</td>
<td>8.11</td>
</tr>
<tr>
<td>η</td>
<td>0.81</td>
</tr>
</tbody>
</table>

A comparison of column chromatography to other separation methods can be found in Section 4.4.2

4.3.1.4 Removal of Unencapsulated Pyranine Via High Pressure Ultrafiltration

High pressure ultrafiltration was also investigated as a method to separate unencapsulated pyranine from the nanoshells. This technique is attractive because it can be scaled to handle large volumes, is fast, and can remove liquid thereby concentrating the sample. A cartoon illustrating the process is shown in Figure 4-11. It took 8 rinses of water using 8 ml each to remove all the unencapsulated dye from how much nanoshell suspension as shown in Figure 4-12.
Figure 4-11 Cartoon illustrating both the removal of unencapsulated dye and the concentration that occurs as liquid is removed in the final rinse/filter cycle. The initial concentration of the shell $C_s$ and the concentration of the first filtrate can be used to determine the encapsulation efficiency $\eta$.

Figure 4-12 Representative experiment determining the number of 8 ml rinses required to remove the pyranine dye from the 10 ml of as synthesized shells in a high pressure ultrafiltration (50 nm) experiment. The amount of dye in the filtrate is negligible after 8 rinses.
To determine this method’s effectiveness the encapsulation efficiency $\eta$ was determined by measuring the concentration of the sample via absorbance prior to placing it into the filter. The encapsulation efficiency was determined by the following mass balance (notation derived from Figure 4-11).

$$N_I = N_S + N_F$$

$$N_S = C_S V_S$$

$$N_I = C_I V_I$$

$$N_F = C_F V_F$$

where $N_I$, $V_I$, $C_I$ and $V_F$ are known, $C_S$ and $C_F$ are measured, and $V_S$ is calculated according to Section 4.4.1. The table below summarizes the results obtained from 10 ml of 10 mM pyranine loaded shell. Equation 4-1 is used to determine the encapsulation efficiency $\eta$.

**TABLE 4-3. EXPERIMENTAL RESULTS FOR HIGH PRESSURE ULTRAFILTRATION SEPARATION OF UNENCAPSULATED PYRANINE**

<table>
<thead>
<tr>
<th>Loading Concentration (mM)</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_I$ (mM)</td>
<td>0.2719</td>
</tr>
<tr>
<td>$C_F$ (mM)</td>
<td>0.2691</td>
</tr>
<tr>
<td>$C_S - C_F$ (mM)</td>
<td>0.0028</td>
</tr>
<tr>
<td>Moles in Shells</td>
<td>0.0282</td>
</tr>
<tr>
<td>Volume of Shells (μL)</td>
<td>3.57</td>
</tr>
<tr>
<td>$C_S$ (mM)</td>
<td>7.9</td>
</tr>
<tr>
<td>$\eta$</td>
<td>0.79</td>
</tr>
</tbody>
</table>

Note: A comparison of ultrafiltration to other separation methods can be found in Section 4.4.2
4.3.2 Encapsulation of Pyrene in Soybean Oil Cored Calcium Phosphate Nanoshells

To evaluate the encapsulation process for hydrophobic molecules in soybean oil-cored calcium phosphate nanoshell the efficiency of pyrene encapsulation was examined. A calibration curve for pyrene fluorescence emission in soybean oil at an excitation wavelength of 337 nm and emission of 393 nm was shown to be linear from 10 nM to 100 mM in Figure 4-13. To prove that the measured pyrene monomer fluorescence emission arose from dye molecules encapsulated in the nanoshell the pyrene encapsulated nanoshell suspension was introduced to an aqueous environment. Pyrene solubility limit in water is less than 1.5 μg/ml (Figure 4-1). The absorbance of pyrene in 0.05 % w/w of calcium phosphate nanoshells prepared using 50 mg/ml (245 mM) of pyrene/soybean oil has a suspension concentration of pyrene of 60 μM and is shown in Figure 4-14. This is possible because the pyrene was dissolved in high concentrations within the oil microenvironment of the nanoshells.

![Calibration curve of pyrene in soybean oil for concentrations less than 100 μg/ml. The linear calibration curve is taken at 394 nm.](image)

Figure 4-13 Calibration curve of pyrene in soybean oil for concentrations less than 100 μg/ml. The linear calibration curve is taken at 394 nm.
Figure 4-14  Absorbance spectra of a pyrene saturated water solution (6 μM) (circled) and pyrene encapsulated within calcium phosphate nanoshells suspended in aqueous medium (120 μM). The peak height in A is 0.13 and is approximately 21.7 times higher than that in B. Without the presence of the nanoshells it would not be possible to achieve such a high absorbance in aqueous solution.

Determination of the encapsulation efficiency by separating the unencapsulated dye via dialysis (Section 4.3.1.2) and column filtration (Section 4.3.1.3) were also explored for pyranine removal from aqueous cored calcium phosphate nanoparticles. While effective, these methods are not ideal for a potential future where many liters of nanoshells would need to be separated. High pressure filtration is a separation technique that can be used to remove unencapsulated dye and is easily scaled to handle an increased volume and produced equivalent results to dialysis and chromatography. For these reasons, only ultrafiltration will be considered for the removal of unencapsulated pyrene henceforth.

Pyrene encapsulated nanoshells can be separated via high pressure filtration by introducing 10 ml to the Lipex extruder fitted with a 50 nm filter and pressurized to 200 psi with nitrogen to remove 8 ml then rinsed with 8 ml of water 8 times. To determine
the encapsulation efficiency, the fluorescence spectrum was collected from the sample prior to placement in the filter and from the 1st filtrate portion. Fluorescence was used to determine the concentration for 2 reasons: the low UV absorbance of the shells themselves makes obtaining a calibration curve difficult (due to scattering background) and secondly, the concentration was too low in the filtrate to be reproducibly detected. From fluorescence measurements in Figure 4-15 and the calibration curve in Figure 4-14 the before fraction has approximately 6.5 μM pyrene while the first through fraction has 0.19 μM pyrene resulting in an encapsulation efficiency of approximately 96%.

![Fluorescence spectrum](image)

**Figure 4-15** Emission scan of pyrene encapsulated calcium phosphate nanoshells before and after filtration through a 50 nm polycarbonate filter via a high pressure filtration apparatus. Less than 3% of the concentration is seen in the filtrate fraction resulting in an encapsulation efficiency approaching 96%.

The filtration also provided another important piece of evidence for shell formation. If bare emulsions without shells were filtered in the extruder as the above shells are, they passed through because they are ‘flexible’ and can deform. However, the calcium phosphate coating on the nanoshells provides a mechanical rigidity, that and
resisted deformation and being pushed through the pores in the filter. This enhanced mechanical strength would become a specific benefit of having a calcium phosphate coating to protect the contents of the emulsion in future applications.

The shell microenvironment also offers a unique opportunity to form excimers from a solution concentration of pyrene well below where excimers typically can form. This provides a greater effective Stokes shift from excitation to emission than would normally be available at the saturation concentration of pyrene in water and would be useful in reducing signal to noise when using bandpass filters for fluorescent detection. Pyrene concentration within each of the shells was approximately 245 mM, high enough for excimer formation, but the solution concentration is approximately 4 μM. Since more than 95% of the pyrene was located within less than 0.5% of the volume of the solution the proximity of the encapsulated pyrene molecules is very close and excimer formation can occur. The spectra in Figure 4-16 compare three solutions of equivalent solution pyrene concentration (~4 μM). As can be seen there is a significant difference in excimer emission when pyrene is encapsulated within shells as opposed soybean oil solution. The ratio of peak 3 to peak 1 was higher than unity for both cases further suggesting that the pyrene is indeed encapsulated within the nonpolar environment of the shells. When pyrene was in an aqueous environment there was no excimer formation and the ratio of peak 1 to 3 was less than one, confirming a polar solvent. A similar result was obtained by Wang⁴⁴² while encapsulating pyrene within toluene cored silica nanoshells.
Figure 4-16  Fluorescence spectra comparing emission spectra of 0.05 mg/ml Pyrene in oil, 1 vol % calcium phosphate nanoshells with 50 mg/ml soybean cores, and pyrene saturated water excited at 337 nm. Excimer emission was only present when pyrene is encapsulated within the shell microenvironment.

4.3.3 Encapsulation of Other Fluorescent Dyes (FITC and Sulforhodamine)

To verify the successful encapsulation experienced with pyranine and pyrene is not an isolated case, brief experiments were conducted to verify encapsulation was possible with other fluorophores. Fluorescein iso-thiocyanate (FITC) was used as another sample hydrophobe and sulforhodamine 101 was used as another model hydrophile.
Encapsulation efficiencies were obtained using dialysis for convenience since speed or concentration was not important in this case. Dialysis was performed as described in Section 4.3.1.2 with the exception that a 300,000 MWCO dialysis bag was used and the equilibration time was shifted to 10 hours. The table below summarizes the results. The success of encapsulating these dyes lends evidence that many substances of varying hydrophobicity can be encapsulated and retained with a calcium phosphate nanoshell.
TABLE 4-4. ENCAPSULATION EFFICIENCIES OF FITC WITHIN OIL CORED SHELLS AND SULFORHODAMINE 101 IN AQUEOUS CORED SHELLS.

<table>
<thead>
<tr>
<th>Loading Concentration (mM)</th>
<th>FITC</th>
<th>Sulforhodamine 101</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final Concentration in Shells (mM)</td>
<td>39.3</td>
<td>3.77</td>
</tr>
<tr>
<td>η</td>
<td>0.78</td>
<td>0.75</td>
</tr>
</tbody>
</table>

4.3.4 Diffusion of Fluorescent Quenchers (H₂O₂ and CuCl₂) into the Shell

One function of the calcium phosphate nanoshell coating is to protect the contents within the shell from outside influence. To verify the nanoshell prepared around aqueous-cored liposomes offers protection of the encapsulated contents the fluorescent quenchers hydrogen peroxide (H₂O₂), and copper chloride (CuCl₂) were introduced to the suspension and the rate at which they quenched the intensity of the pyranine monomer emission at 513 nm was monitored via fluorescence. First, a calibration curve of pyranine fluorescence intensity (cps) vs. pyranine concentration (µM) was developed. As seen in Figure 4-18 the fluorescence intensity was linear with pyranine concentration from 10 µM to 300 µM.
Figure 4-18  Calibration curve for pyranine using fluorescence at 512 nm. Fluorescence is linear over a pyranine concentration range of 10 to 300 μM.

![Calibration curve for pyranine using fluorescence at 512 nm](image)

Figure 4-19  Calculated concentration of 1.25 M peroxide entering the shells quenching pyranine emission contained within liposomes and 35 nm thick calcium phosphate nanoshells. At this concentration the peroxide diffusion through the liposome and the quenching reaction is very fast compared to that for the nanoshell suspension. Considering the linear region (A), the diffusion coefficient for peroxide in shells is estimated to be $6.5 \times 10^{-13}$ cm$^2$/s. The method used to transform the raw intensity versus time profiles is discussed in Section (4.4.4.1).
Figure 4-20  Calculated concentration of peroxide entering the nanoshells versus time for 0.1 M 10 nm thick pyranine shells with various amounts of peroxide. The initial rates of peroxide quenching are assumed to be linear over region A and used to determine the diffusion coefficients. For 0.1 ml and 0.25 ml peroxide added to ~ 1 nM nanoshells the diffusion rates were $7.5 \times 10^{-12}$ cm$^2$/s and $D = 6.5 \times 10^{-12}$ cm$^2$/s respectively.

In Figure 4-19 is shown the results obtained by mixing 2 ml of ~6.7 nM, 35 nm-thick shells with 1 mM encapsulated pyranine in a 4 ml fluorescence cuvette with 1 ml of 15% hydrogen peroxide. The raw fluorescence time traces in this section were converted to concentration time traces by using the calibration function and then subtracting that curve from the initial concentration as described in Section (4.4.4.1). The calculated concentration of pyrene left unquenched, as a function of time for solution, liposomes and nanoshells was compared. Loss in the pyranine emission was immediate for solution pyranine and pyranine encapsulated liposomes, as seen in Figure 4-19. In contrast for the nanoshells this quenching took place over more than 20 minutes. From Figure 4-19 the rate of quenching of 0.012 μM/s was estimated from the slope of the graph at 150 s to 250 s (region B in Figure 4-19) averaged from two separate sets of shells. This value is converted to mole/s by multiplying by volume of liquid in the cuvette (3 ml) By dividing
by the estimated surface area of the shells (0.016 m²), the flux in mol/s/m² the resulting
diffusion constant is about 6.5 \times 10^{-13} \text{ cm}^2/\text{s}. A detailed calculation and list of
assumptions used to obtain the diffusion constant is discussed in 4.4.4.

A supporting experiment to investigate the effect of shell thickness on the
diffusion constant was conducted with thinner (10 nm) shells of various concentrations of
peroxide. This experiment was performed in the same manner as above, only with 1 ml
of shell suspension rather than 2 ml resulting in a 94 \mu M pyranine suspension.
Concentration versus time traces for this experiment are shown in Figure 4-20. The
resulting diffusion constants for these 10 nm shells are very similar to each other under
various peroxide concentrations as expected (7.5 \times 10^{-12} \text{ cm}^2/\text{s} and D= 6.5 \times 10^{-12} \text{ cm}^2/\text{s} for
0.1 ml and 0.25 ml respectively) but are an order of magnitude higher than the one
obtained for the thicker, 35 nm thick shells. An explanation for this difference is
proposed in Section 4.4.4.

In addition to peroxide, diffusion of Cu^{2+} ions into the shell was also used as a
quencher to investigate the effect size and charge has on diffusion through the shell. At
concentration of 2 M, copper chloride quenches 75 \mu M pyranine very rapidly in solution
or in liposomes as seen in Figure 4-21. In contrast for pyranine-loaded 35 nm thick
calcium phosphate nanoshells (75 \mu M), it took approximately 15 seconds for the
encapsulated pyranine to be quenched by half. The initial rate obtained as before was
used to approximate the diffusion constant of copper through the calcium phosphate
nanoshell as 7.9 \times 10^{-10} \text{ cm}^2/\text{s} which is four orders faster than peroxide diffusion.
Thinner (10 nm) shells could not be used as the rate of quenching was too fast to measure
an initial rate.
Figure 4-21 The concentration of copper entering the shell versus time trace obtained from 2 M CuCl₂ quenching the fluorescence of 0.1 M pyranine encapsulated within 35 nm thick shells. At this concentration the Cu²⁺ ion diffusion through the liposome is much faster compared to the diffusion rate of the shell. The diffusion constant for Cu²⁺ through the shell was estimated to be \( D = 7.9 \times 10^{-10} \).

4.3.5 Ratiometric Measurement of pH using Pyranine

Pyranine is commonly used as a fluorescent pH indicator in cellular biology\(^\text{443}\) and can be used to detect the pH of the shell microenvironment spectrophotometrically as seen by the pH dependent absorption spectra below. This is possible through monitoring the ratio of the absorption at 453 nm and 403 nm as seen in Figure 4-22.
Figure 4-22 The pH dependent absorption spectrum of pyranine lends itself for use as a spectrophotometric pH indicator by measuring the ratio of 453 nm and 403 nm wavelengths.

A suspension of 35 nm thick shells with 1 mM encapsulated pyranine was placed in a beaker and adjusted with 0.1 M NaOH to change the pH while being monitored by a pH probe. These additions were spaced 30 minutes apart and then analyzed with an absorbance spectrometer and the ratio of 403 nm to 453 nm was taken to obtain the pH. There was little difference in the pH obtained by the pH meter and the pH obtained via absorbance and would allow pyranine to act as a ratiometric pH indicator while encapsulated within the shell. Acting as a nanoparticle based pH sensor gives the calcium phosphate nanoshell some functionality.
Figure 4-23  There is little difference in the pH of the solution measured by an Accumet pH meter and the pH obtained by spectrophotometric analysis at a ratio of 403 to 453 nm of a shell suspension with encapsulated pyranine. The spectra is obtained after 30 minutes of equilibration time after each change in pH.

4.3.6  Investigation of Floc Composition

The presence of dyes within the shells allows verification that the flocs present in some reactions are indeed collections of shells and not solid crystallites. Flocs are large collections of shells formed as an end-product of shell formation. In Section 4.3.2 it was shown that 96% of pyrene present in a shell solution was present within the shells themselves. This high encapsulation efficiency also permits the qualitative measurement of the degree which the shells floc by measuring the pyrene fluorescence spectra when the suspension is settled and after it is agitated. This concept is illustrated in Figure 4-24.
Figure 4-24 Cartoon illustrating the particle distribution of settled and suspended floc, and how it can be deleted by fluorescence. When the cuvette with floc was settled the fluorescence intensity was lowered as a result of less interaction of the incident light beam with the particles. When the cuvette is shaken to resuspend the particles the intensity increases to its original well mixed value.

Figure 4-25 Fluorescence spectra of 12 μM pyrene encapsulated soybean cored calcium phosphate nanoshells prepared with 20 (A) additions and 30 (B) additions of CaCl₂. The sample in A had no floc present and the shells remain suspended while the sample in B has some floc present causing some material to settle to the bottom.
Fluorescence traces shown in Figure 4-25 shows the pyrene fluorescence spectra of a suspension of shells with floc and without floc. Figure 4-25A shows fluorescence spectra of calcium phosphate nanoshells with soybean oil/DOPA cores prepared with 20 additions of calcium at pH 9.5. This solution contained very little flocc and was settled for 3 days before measuring the fluorescence of the top portion (Settled). The suspension was shaken to resuspend any settled particles and measured again (Shaken). There is little change between the settled and agitated samples. Figure 4-25B shows fluorescence spectra of calcium phosphate nanoshells with soybean oil/DOPA cores prepared with 30 additions of calcium at pH 9.5. There is a significant portion of flocc in this reaction that visibly settles to the bottom of the beaker. The suspension is allowed to settle for 3 days when there was visible floc on the bottom before measuring the fluorescence spectrum of the reactions top portion (settled). The suspension was then shaken to resuspend the settled particles and measured again (shaken). This increase suggests that a majority of the pyrene and thus shells are contained within the flocc on the bottom.

In Section 4.3.2 it was shown that fluorescence intensity would be considered linear over 10 nM to 100 mM, so assuming no change in the chemical environment for the dye, the ratio of the fluorescence intensity at 394 nm before (0.18) and after agitation (0.84) can be used to approximate the percentage of shells that settled. If the shaken pyrene emission intensity is assumed to represent the full concentration of dye containing shells and the settled intensity is assumed to represent only the suspended fraction of the shells then the percentage of settled shells can described by Equation 4-2. This reveals that only 20 percent of the shells prepared with the 30 addition synthesis are free shells while the others are lost to floc.
\[ Floc = 1 - \frac{\text{Suspended}}{\text{Settled}} = 1 - \frac{0.18}{0.84} = 79\% \]

Equation 4-2  Relation to determine the percentage of floc in a solution

4.4 Discussion

4.4.1 Calculation of Number and Volume of Nanoshells

Determining the encapsulation efficiency of pyranine and other dyes requires an estimate of the number of nanoshells within a specific volume of suspension. In practice, this is a difficult number to obtain. It is possible to estimate how many shells through a calculation based on the following assumptions illustrated in Figure 4-26.

- The lipids have perfectly spherical headgroups 0.7 nm in diameter
- The lipids pack in a hexagonal pattern (3 lipids/hexagon) to form the curved lipid bilayer. Each hexagon is related to the diameter of the lipid headgroup (d) by the following relation:

\[ Hexagon = 6 \cdot \frac{1}{2} d \cdot \sqrt{d^2 - \frac{d^2}{2}} \]

- Each liposome is composed of a continuous bilayer and is unilamellar (composed of one lipid bilayer).
- The shells are monodisperse and only composed of one diameter.
With these assumptions, and straightforward geometry calculations, the number of lipids per μm² of bilayer is $4.7 \times 10^6$ which is close to the accepted estimate of $5 \times 10^6$ lipid molecules in a bilayer\textsuperscript{444} lending validity to the assumptions. Through stoichiometry we can determine the concentration of liposome particles for a 25 ml reaction volume with 700 μl of 6.93 mM lipid solution extruded through a 100 nm membrane to be approximately 1 nM with an internal volume of 10 μl. This concentration is on the same order as flow-cytometry observations which determined the concentration to be 0.4 nM\textsuperscript{445}. The difference can be attributed to multilamellarity, polydispersity, or sample loss prior to measurement. Using the calculated internal volume and the number of moles left within the shell portion after separation via dialysis, column chromatography, or high pressure filtration we can calculate the concentration within each liposome or emulsion.

4.4.2 Encapsulation of Pyranine and Comparison of Separation Methods

Three methods were explored for their effectiveness in removing unencapsulated pyranine. In all of these separation methods, there was experimental error to be expected;
however, the concentrations determined from theses methods were high enough (mM) to safely conclude that much of the pyranine remains encapsulated within the shells. If the shell were to rupture the encapsulated concentration would be equivalent to the reaction concentration approximately 500 μM or about 100 x lower. The high retention is likely due to the membrane impermeant properties of pyranine keeping pyranine from leaching out during synthesis.

From the dialysis data obtained in Section 4.3.1.2 it was determined that there was an optimum loading concentration to achieve the maximum encapsulation efficiency of approximately 10 mM for pyranine as seen in Figure 4-7. This is a consequence of what appears to be a limit to the amount of pyranine that can be encapsulated within the shell portion of a suspension of approximately 27 mM as shown by the plateau in Figure 4-8. This limit is likely due to a spatial restriction on the number of pyranine molecules that could fit within the volume of the liposome, approximately 26,000 per 100 nm liposome or about one pyranine in each 20 nm³ of shell volume.

Column chromatography and high pressure ultrafiltration were also performed on a 10 mM pyranine loaded shell suspension to verify the effectiveness of different separation methods. These methods resulted in approximately the same encapsulation efficiency (~0.8) for pyranine in the nanoshells and could be assumed equivalent with respect to effectiveness. There were some practical differences to these methods regarding speed and convenience that affected their selection as a separation method and these are summarized below.
TABLE 4-5. COMPARISON OF THE PROS AND CONS OF THE VARIOUS SEPARATION METHODS USED TO DETERMINE THE ENCAPSULATION EFFICIENCY

<table>
<thead>
<tr>
<th>Method</th>
<th>$\eta$</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialysis</td>
<td>0.83</td>
<td>Convenient</td>
<td>Slow, some dilution,</td>
</tr>
<tr>
<td>Chromatography</td>
<td>0.81</td>
<td>Fastest, one pass separation, easy calculation</td>
<td>Dilutes the sample, particle loss, laborious sample acquisition, small amounts at a time</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>0.79</td>
<td>Quick, can concentrate particles</td>
<td>Laborious to separate, filter can foul, expensive equipment required, few relevant filter sizes</td>
</tr>
</tbody>
</table>

Dialysis was the most convenient method and required little attention until it was complete; however, it took many hours to perform the separation and diluted the sample about 10-20% in the process. Column chromatography was a quick method that separated the pyranine in one pass as the pyranine molecules moved slowly through the column. Column chromatography could only separate small amounts of sample at a time, diluted the sample, and resulted in particle losses (how much-from dye percent). Chromatography was used when speed was the most important property and collecting the entire sample is not critical. Ultrafiltration was the only method of the three that could concentrate the shells which becomes important in later chapters. It was also quicker when compared to dialysis but laborious to remove all dye, requiring 8 filtrations; however, only one pass is required to determine the encapsulation efficiency. The
filtration membranes could foul and there are only two available polycarbonate filter sizes that can retain nanoshells (50 and 100 nm).

Ultrafiltration’s ability to concentrate the nanoshells makes it very desirable where speed and convenience are not the utmost concerns. This method is also easily scaled with larger filter areas and filtration volumes. The efficacy and ability to concentrate the nanoshells make ultrafiltration the method of choice for separations for the majority of this work presented in this thesis.

4.4.3 Encapsulation of Pyrene, FITC, and Sulforhodamine

Pyrene was encapsulated within oil cored calcium phosphate nanoshells to verify that hydrophobic cored calcium phosphate nanoshells could also be utilized as effective carriers of dyes/substances. The low solubility of pyrene in water forced pyrene to remain in the microenvironment of the shell, resulting in a suspension with a much higher pyrene concentration than could be achieved in an aqueous solution without the shells (Figure 4-14). The fluorescence spectra obtained after one pass of ultrafiltration verified that pyrene is indeed present within the shell (Figure 4-15). The confined volume of the shell also permitted the formation of pyrene excimers within the shell at suspension concentrations lower than required for excimer formation when dissolved in oil alone (Figure 4-16).

Encapsulation of fluorophores was not limited to pyranine or pyrene as FITC and sulforhodamine 101 have been encapsulated with encapsulation efficiencies of approximately 0.75. The success of the encapsulation method described in this chapter is extendable to many other types of molecules.
4.4.4 Diffusion of quenchers into shell

4.4.4.1 Conversion of time traces to concentration traces

Before the resulting time traces of fluorescent intensity could be used to determine the diffusion coefficient they was converted to concentration of pyranine versus time graphs by using the calibration curve in Figure 4-18 and by subtracting that number by the initial concentration of pyranine in the sample. One mole of peroxide quenches one mole of pyranine and the disappearance of one can be attributed to the appearance of another.

Figure 4-27  Pyranine fluorescent intensity as a function of time when 1.25 M of H2O2 was added to 2 ml of ~ 1 nM nanoshells containing 20 mM pyranine (A) is converted to a concentration versus time curve(B) through the calibration function in Figure 4-18 and by subsequent subtraction of the trace from the initial concentration of pyranine.
4.4.4.1 Calculating the Diffusion Coefficient for the Calcium Phosphate Nanoshell

The quenching of pyranine depends on a five part process: 1) diffusion of the quencher to the surface of the shell, 2) diffusion of the quencher through the calcium phosphate shell, 3) diffusion through the liposome, 4) diffusion of the quencher into the core of the shell and 5) reaction of the quencher with the pyranine. This process is illustrated graphically in Figure 4-28.

Figure 4-28 Cartoon illustrating the steps a quenching molecule takes before it is able to quench pyranine. The rate of transport across the shell is the rate limiting step for this system as proven below.
The steady state rate of pyranine quenching \((Q)\) can be described using Fick's first law of diffusion by the following relation at steady state:

\[
\frac{d\text{Pyranine}}{dt} = Q_B = Q_S = Q_L = Q_C = R_Q
\]

Using Fick's first law,

\[
\frac{Q}{A} = J = D \frac{dC}{dx}
\]

Yields,

\[
\frac{d\text{Pyranine}}{dt} = D_B A_B \frac{C_B - C_{BS}}{\Delta x_B} = D_S A_S \frac{C_{BS} - C_{SL}}{\Delta x_S} = D_L A_L \frac{C_{SL} - C_{LC}}{\Delta x_L} = D_C A_C \frac{C_{LC} - C_C}{\Delta x_C} = R_Q
\]

Simplifying

\[
\frac{d\text{Pyranine}}{dt} = R_Q = \frac{C_B - C_C}{\frac{\Delta x_B}{D_B A_B} + \frac{\Delta x_S}{D_S A_S} + \frac{\Delta x_L}{D_L A_L} + \frac{\Delta x_C}{D_C A_C}}
\]

Equation 4-3  Steady state flux of quenchers into the nanoshell.
Where $Q_B, A_B, \Delta x_B, Q_S, A_S, \Delta x_S, Q_L, A_L, \Delta x_L, Q_C, A_C, \Delta x_C$, are transport rates (Q), areas of transport (A) and distances of transport ($\Delta x$) for the bulk, shell, lipid, and core respectively. The transport per area (flux) is defined as $J$ and the rate of quenching is defined as $R_Q$. The measured variable in this system is the rate of pyranine quenching ($R_Q$) and we wish to obtain the diffusion constant of the calcium phosphate nanoshell ($D_S$). In order to simplify the analysis the following assumptions were made in order to reduce Equation 4-3 to something more manageable:

1. The peroxide concentration was in gross excess and did not change appreciably and was taken to be constant ($C_B - C_C = C_{\text{peroxide}}$)
2. 1 mole of peroxide totally quenched 1 mole of pyranine and is irreversible
3. Steady state was reached in the first several seconds and the flux for the time of interest is constant and linear
4. The quenching reaction was much faster than any of the transport rates
5. The flux of peroxide through the liposome was significantly greater than through the calcium phosphate nanoshell ($Q_L \gg Q_S$; $D_L \gg D_S$).
6. The observed decrease in fluorescent intensity was due solely to pyranine encapsulated within the shell (omit any pyranine quenching on outside of shell).
7. The shell was assumed to be stationary with respect to the quencher
8. The quenching of pyranine began immediately once the quencher enters the core of the shell ($Q_C \gg Q_D$; $D_C \gg D_S$)
9. The curvature of the shell was ignored and the system was approximated as planar (all areas are equal to the shell surface area).
These assumptions were validated as follows: (1) is acceptable because peroxide is in 10 thousand fold molar excess of pyranine and not expected to change appreciably in the external solution over the time range considered (first 40 seconds), (2) quenching of pyranine by peroxide is by degradative attack via the peroxy free radical and is irreversible, (3) flux of peroxide at early times can be considered linear as regression analysis of these times results in an $R^2$ greater than 0.98 (Figure 4-19 to Figure 4-21), (4) quenching of pyranine in solution occurred almost instantaneously at the concentrations of interest (100 $\mu$M pyranine and 1.25 M peroxide) as seen in Figure 4-19, (5) similarly, the rate of peroxide diffusion through the liposome could be neglected as seen in the rapid quenching of pyranine encapsulated within the liposome in Figure 4-19, and based on the work of others who have shown peroxide is known to penetrate membranes readily\cite{446}, (6) remaining pyranine on the outside of the shell would be quenched much faster than that on the interior of the shell and could be ignored as shown in Figure 4-19, (7) the diffusion constant of the shell through the fluid is on the order of $2 \times 10^{-8}$ or 100 times slower than diffusion of the quencher molecules, (8) there is very little distance between the pyranine molecules within the shell less than 2.5, and (9) the size and the thickness of the shell are on the order of nanometers and areas won’t change appreciably over the distances involved. These assumptions result in the following reduced equation.

\[
\frac{d\text{Pyranine}}{dt} = R_0 = A_S \frac{C_{\text{Peroxide}}}{\frac{\Delta x_B}{D_B} + \frac{\Delta x_S}{D_S} + \frac{\Delta x_C}{D_C}}
\]
The rates of bulk and core diffusion were estimated with the Stokes-Einstein equation. Where \( k \) is the Boltzman’s constant (1.3807 \times 10^{-23} \text{ Nm/K}), \( T \) is the absolute temperature (298 K), \( r \) is the radius of the particle (m), \( \mu \) is the viscosity of water (1 cP = 0.001 Ns/m²)

\[
D = \frac{kT}{6\pi r\mu}
\]

Equation 4-4 Stokes Einstein Equation

For hydrogen peroxide (\( r \sim 0.3 \text{ nm} \)) the bulk diffusion constant speed is \( 7.3 \times 10^{-6} \text{ cm}^2/\text{s} \) and for copper ion (\( r \sim 0.15 \text{ nm} \)) the bulk diffusion constant is \( 1.5 \times 10^{-5} \text{ cm}^2/\text{s} \). In work with organic cored silica nanoshells Wang showed that the viscosity of the fluid in the core of the shell increases by a factor of \( 10^{442} \) due to the reduced mobility of the fluid in confined spaces. This increase in viscosity would affect the diffusion speeds of peroxide and copper to \( 7.3 \times 10^{-7} \text{ cm}^2/\text{s} \) and \( 1.5 \times 10^{-6} \text{ cm}^2/\text{s} \) respectively. For now, assume these rates are much greater than the flux through the shell (this will be verified shortly) and we are left with the reaction rate limited by the transport across the shell seen below.

\[
\frac{d\text{Pyranine}}{dt} = R_Q = A_s \frac{C_{\text{Peroxide}}}{\Delta x_S} = A_s D_s \frac{C_{\text{Peroxide}}}{\Delta x_S}
\]

\[
D_s = \frac{R_Q \Delta x_S}{A_s C_{\text{Peroxide}}}
\]
This means that the rate of pyranine quenching is directly related to the flux of quencher within the shell. Through fluorescence spectroscopy, the rate of pyranine quenching can be measured, $D_{\text{S}}$ is the shell thickness, and $C_{\text{Peroxide}}$ is the initial concentration of $\text{H}_2\text{O}_2$ (1.5 M) and diffusion constant for the calcium phosphate shell can be determined. The calculated diffusion coefficients are seen in TABLE 4-6. It can be seen that these coefficients are much lower than the estimated bulk and core diffusion verifying our initial assumption that the quenching of pyranine is limited by the flux of quencher through the shell.

<table>
<thead>
<tr>
<th>Shell Thickness</th>
<th>Quencher</th>
<th>D (cm$^2$/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 nm</td>
<td>Cu$^{2+}$</td>
<td>$7.9 \times 10^{-10}$</td>
</tr>
<tr>
<td>35 nm</td>
<td>$\text{H}_2\text{O}_2$</td>
<td>$6.5 \times 10^{-13}$</td>
</tr>
<tr>
<td>10 nm</td>
<td>$\text{H}_2\text{O}_2$</td>
<td>$7.0 \times 10^{-12}$</td>
</tr>
</tbody>
</table>

From the results above an interesting result arises revealing the diffusion coefficient of the calcium phosphate material is thickness dependent. This can be explained by one or a combination of the following effects:

**Effect 1: Knudsen diffusion: not fully developed pore structure**

If there are pores present in the calcium phosphate material that are on the same order as the diffusing solutes then the diffusion can be defined as Knudsen type diffusion. The means the diffusion coefficient is dependent on the porosity of the shell ($\varepsilon$) and the turtuosity of the shell ($\tau$) by the relation:
$$D_k = D \frac{\varepsilon}{\tau}$$

Equation 4-5 Knudsen Diffusion

The cartoon below proposes a mechanism for increased tortuosity as the shells grows. As the shell grows, cracks, fissures, and dislocations in the calcium phosphate may become more interconnected and increase the effective distance that a solute must travel.

Figure 4-29 Cartoon illustrating possible increased tortuosity of a shell as it grows thicker.
Effect 2 Incomplete coating of the liposome by the shell.

A second possibility is that when a shell is very thin, it does not completely cover the surface of the liposome and effectively increases the porosity of the shell as illustrated below.

Figure 4-30 Pores are possibly present in thin shells as formation begins. As shells become thicker they could cover the pore locations and increase the difficulty for solutes to diffuse through the shell.

If the shell deposits on the liposome irregularly it is probable that when the shell is very thin there will be locations of the shell that are not completely covered, providing pores that accelerate the diffusion of solutes through the shell. This hypothesis is supported by the spotted appearance of the following HRTEM image of a 15 nm thick shell in Figure 4-31. The speckled appearance of the core may be due to local deposits of calcium phosphate on the liposome. There is also a portion of the shell that is noticeably thicker than the remainder of the shell indicating that the shell may not deposit uniformly around the liposome. This could introduce pores, or areas of low shell density that would affect the diffusion coefficient compared to a thicker shell.
Figure 4-31  HRTEM image of a 15 nm shell. The spotted appearance of the core may be due to incomplete coverage of the calcium phosphate on the liposome.

The TEM series below in Figure 4-32 shows the change in calcium phosphate shell appearance as it is exposed to the electron beam under high vacuum (1 x 10^-5 torr). It is hypothesized that the change in shell morphology are due to the evolution of water trapped within the crystal lattice, something which is possible for both amorphous calcium phosphate (ACP) and dicalcium phosphate dihydrate (DCPD) materials. Diffraction evidence and nonstoichiometric results presented in Section 2.3.1 confirm that the calcium phosphate nanoshell is amorphous. It can be seen in the upper right frame of Figure 4-32B-ii that there is a denser outer shell that begins approximately 30-50 nm from the template boundary with a less dense region in between. This observation indicates that there may be a two stage shell deposition process. The first stage is unorganized covering of the template by amorphous calcium phosphate and results in the lighter region. After this amorphous coating is on the shell, a denser, possibly more organized coating crystallizes around what would resemble a seed crystal. This process
is analogous to Ostwald ripening where a more ordered form precipitates on a less stable nucleus and is well known to occur for calcium phosphate. These two regions would explain the difference in diffusion constants obtained with different shell thicknesses.

Electron dispersive spectroscopy (EDS) or other chemical/crystallographic information can not be obtained for the various states of this sample as the transient states captured last only long enough to expose a film. The EDS measurements require exposing the sample for minutes per measurement and it would not be possible to obtain chemical or crystallographic information for the various stages of this process.
Figure 4-32  The long term exposure from the electron beam to a nanoshell causes the fluid trapped within the shell to evaporate and the inorganic coating to reorganize. A) The shell in the upper right hand corner is completely opaque when first seen and starts to lighten after several seconds of exposure. B) These are pictures taken after approximately 3, 6, 9, 12 seconds of exposure each and reveal the evolution of fluid from the core as seen by the increased light (less electron dense) area within the particle due to the heat and high vacuum within the TEM column.

The evidence presented here shows that the calcium phosphate provides a transport barrier to quenching molecules. The protection of an encapsulated dye against external quenchers like H₂O₂ is improved significantly over a bare liposome even in the presence of molar concentrations of quencher. Small quenchers like Cu²⁺ can still permeate readily. Diffusion also becomes more difficult as the shell becomes thicker owing to a potential increase in turtuosity or a decrease in porosity.
4.4.5 Detection of Solution pH and Monitoring of Shell Location

The pH dependent spectrum (Figure 4-22) of pyranine allows it to be used as a spectrophotometric pH detector of the interior pH of the shell. The small differences between solution pH obtained via an electrode and those of the shell interior obtained with absorbance indicate that H⁺ ions equilibrate across the shell (Figure 4-23). Pyrene’s presence in oil core-shells allowed the verification of the hypothesis that flocs are composed mainly of nanoshells. This provides a method to approximate the percentage of floc in a sample through linear correlation of dye intensity of a settled and suspended sample containing floc as shown in Section 4.3.6.

4.5 Conclusion

The goal of this chapter was to verify that organic molecules can be encapsulated within either aqueous or hydrophobic cored calcium phosphate nanoparticles. This was done by:

- Encapsulating pyranine within a liposome cored calcium phosphate nanoshell (synthesized in Section 2.2.2.2).
- Demonstrating the encapsulated pyranine was retained throughout the shell formation process.
- Measuring the encapsulation efficiency by dialysis, column chromatography, and high pressure ultrafiltration.
- Showing that pyranine can be used to ratiometrically determine the pH of the solution with good accuracy.
• Pyrene was encapsulated within the soybean cored calcium phosphate nanoshells (synthesized in Section 3.2.2.2) resulting in a higher suspension pyrene concentration than can be achieved in water alone.

• More than 95% of the pyrene remained within the shells after shell formation.

• Pyrene formed excimers within an aqueous shell suspension but not in oil or water solutions.

• Copper and H₂O₂ diffusion into the shell to quench pyranine and emission was hindered by the presence of the shell as compared to a liposome.

• The quenching rate could be used to determine the flux and diffusion coefficient of quenchers across the shell.

• FITC and sulforhodamine fluorophores were also be encapsulated with analogous methods and results as pyranine and pyrene.

These results prove that fluorophores could be encapsulated with efficiencies of at least 75%, the shell provided protection from quenching ions, and the shells could potentially perform useful functions in the biosensor arena as pH sensors or fluorescent markers.
5.1 Introduction

In previous chapters the synthesis of calcium phosphate nanoshells with liposome (CHAPTER 2) and emulsion (CHAPTER 3) cores were developed and characterized. The nanoshells ability to encapsulate both hydrophilic and hydrophobic substances was investigated via the fluorescent models pyranine and pyrene (CHAPTER 4). The goal for this chapter is to explore chemical bioconjugate methods to attach proteins, specifically antibodies and enzymes, to the surface of the nanoshell.

A protein is a long polymer of amino acid molecules joined by peptide bonds (R-NH-CO-R’) that through electrostatic, hydrophobic, and Van der Waals interactions fold to a very specific, functional shape. As a result of peptide bonding the end of a protein contains an amino terminus (NH$_3^+$) and a carboxyl (COO$^-$) terminus. In addition to the amino and carboxyl termini of a protein, there are several other residues on the protein itself which contain carboxyl or amine groups that can be used to conjugate functional molecules. An excellent resource for many different bioconjugate methods is Bioconjugate Techniques by Hermanson.

A specific type of protein that is of interest for targeted drug delivery (Section 1.5.3), and biosensor applications (Section 1.5.2) is an antibody due to its ability to bind an antigen with high specificity. A detailed review of antibodies and antibody antigen interactions can be found in texts such as Immunology by Kuby. The most common type of
antibody is immunoglobulin G (IgG) and is a Y shaped protein composed of several smaller polypeptides linked by disulfide bonds and is illustrated in Figure 5-1. The body of the IgG contains many receptors for immune cells and is responsible for much of the biological activity whereas each Fab arm is responsible for binding one antigen. Immunoglobulin G is approximately 11.5 nm long and has a mass of 150 kDaltons.448

Figure 5-1 General immunoglobulin G (antibody) structure. An antibody is composed of several smaller polypeptides linked by a series of sulfide bridges. The Fab fragment is responsible for antigen binding and gives the antibody its specificity. The Fab fragment is connected to the Fc fragment by a flexible connection of disulfide bonds. The Fc fragment is the backbone of the antibody and contains many amino acid residues (lysine, aspartagine, glutamine, and arganine all contain amines) that give it biological activity and allow for sites to conjugate the antibody to the shell through EDAC-S-NHS chemistry (Figure 5-4).

Antibodies are produced as part of an animal’s immune response when a foreign molecule is introduced with IgG being produced in the highest amounts. The small site on an antigen which “attracts” the antibody is called an epitope and usually consists of a specific 3 dimensional structure formed by 1-6 sugars or 5-8 amino acid residues447 resulting in very specific binding between the antibody and antigen. The binding region of Fab fragments on the antibody is folded in a way which conforms to a specific antigen
or series of antigens. Antibodies are either monoclonal which bind one specific antigen or polyclonal that can bind several. Antibody-antigen binding is reversible and can be defined for monoclonal antibodies as:

\[
K_{AB} = \frac{[\text{AntibodyAntigen}]}{[\text{Antibody}][\text{Antigen}]}
\]

The binding coefficients can range from \(10^5\) mol\(^{-1}\) to above \(10^{12}\) mol\(^{-1}\)\(^{447}\). Binding coefficients are not defined for polyclonal antibodies such as the one used in this chapter as they interact with more than one epitope.

Fluorescent dyes or enzymes are commonly conjugated to Fc fragment if IgG so the antibodies can be detected via spectrophotometry either directly (dyes) or through generation of a fluorescent reporter (enzyme). This principle is the basis for the enzyme linked immunosorbent assays (ELISA) seen below that can detect levels of antigen in the pg per ml range. Horseradish Peroxidase (HRP) is a small enzyme approximately 40,000 Daltons in size and is commonly used in ELISA tests where it is attached to the antibody and acts as a reporter to the antibodies presence (Figure 5-2). Resorufin is generated when peroxide reacts in a mole for mole amount with Amplex Red. Typically, the limit of detection is more important than the concentration of enzyme. The current detection limit of HRP is on the order of \(1 \times 10^{-11}\) M. A typical ELSA assay can take up to an hour for the enzyme to produce measurable quantities of a fluorescent reporter; therefore, there is a need for ELISA assays that can be done in shorter times without sacrificing detection limits.
Figure 5-2 A cartoon illustrating the steps in an enzyme-linked immunosorbent assay (ELISA) that confirms the presence of an antibody. Step 1: a solution containing antibodies (IgG) is introduced to an antigen covered microarray plate and if able, antibodies will adsorb onto the antigens. The plate is then rinsed to remove unbound antibody. In step 2, an anti-antibody with HRP attached to it is added to the solution and attaches to the remaining antibodies that are adsorbed on the antigen coated surface. After a final rinse, in step 3 peroxide and amplex red are added and the production of resorufin can be monitored if there are HRP-antibodies present via fluorescence spectroscopy.

By attaching antibodies to the surface of a calcium phosphate nanoshell loaded with a high concentration of fluorescent dyes it is possible that the specificity of the antibody will allow the nanoshells to interact with a specific antigen, be it in a microwell plate for immunoassays or within the body for targeted delivery of drugs or imaging agents (Section 1.5). The presence of the shell as opposed to single dyes or enzymes makes the conjugate larger and can carry far more dyes per antibody which can potentially improve detection limits by 1,000 fold in immunoassays by improving the dye to antibody ratio. The resulting protein coated, dye filled, calcium phosphate nanoshell resembles the cartoon below.
Figure 5-3  Cartoon illustrating the hypothetical structure of an antibody loaded, dye filled calcium phosphate nanoshell.

The model antibody chosen for this study was anti-fluoroscein rabbit IgG fraction-Alexa Fluor 594 conjugate (AB-594C) which is a polyclonal antibody that quenches the fluorescence of fluorescein and other similarly structured fluorophores. This antibody is chosen for its robust analytical characteristics. It contains 4 Alexa Fluor 594 dyes per antibody allowing for spectrophotometric quantization of antibody concentration. Furthermore, the antibody quenches the fluorescence of fluorescein allowing its activity to be assessed via spectrophotometrically via the quenching of fluorescein. This allows both the attachment efficiency and residual activity to be measured with fluorescence spectroscopy.

In order to verify the attachment method developed in this chapter works for other types of protein, the enzyme horseradish peroxidase was also attached and its residual activity before and after attachment was measured. Horseradish peroxidase is a standard protein model and was chosen for its ubiquitous presence in enzyme linked immunosorbent assays (ELISA), readily available assay kits, and low cost.

The chemistry utilized to conjugate the proteins to the shell surface is based upon carbodiimide chemistry\textsuperscript{296} that results in a peptide bond between an amine and a carboxyl...
Since the calcium phosphate nanoshells are coated with carboxyl groups resulting from their treatment with CEPA (Section 2.3.3), this chemistry is acceptable for conjugation of proteins to the shell by targeting the aminated residues lysine, aspargine, glutamine, and argine as well as the amino terminus of a protein. There are two variations of the carbodiimide chemistry seen in Figure 5-4, one involving a less stable o-acylisourea intermediate and the other involving an NHS ester intermediate providing improved stability through addition of sulfo-n-hydroxysuccinimide (S-NHS).

Figure 5-4 Carbodiimide chemistry begins when 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) is introduced to a solution of carboxylated shells. The resulting o-acylisourea intermediate is unstable in water and the protein is usually added soon after with the shells to ensure the intermediate is still active. A resulting peptide bond binds the protein to the surface of the shell. Alternatively, sulfo-n-hydroxysuccinimide can be added simultaneously with the EDAC to stabilize the intermediate prior to introduction of the protein and can improve attachment efficiency more than 20% as seen below.
This chapter focuses primarily on development of a protein attachment method and experimentally evaluating the degree of attachment and residual activity of the attached proteins. Design goals for the protein coated shells were as follows:

- Protein must be attached to the shell and withstand separation of the unattached antibody
- Protein must remain functionally active after attachment
- Unattached protein must be easily separable from attached protein.
- The synthesis should be straightforward and not negatively affect the shell structure, the encapsulated contents, or the protein.

Successful synthesis of protein coated calcium phosphate nanoshell and demonstration of the potential functionality would demonstrate the potential of these particles in targeted drug delivery and immunoassays.

5.2 Experimental

5.2.1 Materials

Materials required for the synthesis of pyrene encapsulated soybean-oil microemulsions are listed in CHAPTER 4. The antibody AB-594C (anti-fluorescein/Oregon Green®, rabbit IgG fraction, Alexa Fluor® 594 conjugate) was obtained as a liquid from Molecular Probes and refrigerated upon receipt and used as is within 6 months. A solution of 100 nM fluorescein (Molecular Probes) was prepared to monitor the activity of AB-594C. Immunopure horseradish peroxidase (HRP) was obtained from Pierce and kept dried and frozen. Solutions of 1 mg/ml HRP were prepared in phosphate buffer prior to conjugation to the shells. An Amplex Red based HRP assay kit (A22188)
used to monitor HRP activity was obtained from Molecular Probes. Attachment chemicals 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (EDAC) and sulfo-n-hydroxysuccinimide (SNHS) were obtained from Molecular Probes and kept frozen until needed to make 0.05 M solutions. A ten fold (10x) concentrated phosphate buffered saline (PBS) solution at pH 7.4 is prepared by adding 7 g K$_2$HPO$_4$ (40 mM), 1.4 g KH$_2$PO$_4$ (10 mM) and 85 g of NaCl (1.45 M) to 1 L of DI water to yield a 1.45 M buffer at pH 7.4.

Figure 5-5 Schematic representation of the anti-fluoroscein/Oregon Green®, rabbit IgG fraction, Alexa Fluor® 594 conjugate. As fluoroscein (the antigen) binds to the active sites the fluorescence is quenched. Alexa Fluor 594 has a peak absorption of 594 nm and a peak emission of 614 nm.
5.2.2 Methods

5.2.2.1 Synthesis of Protein Coated Shells

The synthesis of calcium phosphate shells with various core sizes and shell thicknesses was described in CHAPTER 2 and CHAPTER 3. The encapsulation of pyrene within the nanoshells was explored in CHAPTER 4. These were used exclusively as the templates for protein attachment. Two milliliters of as prepared pyrene loaded shells prepared according to the automated stepwise synthesis (Section 3.2.2.2.1) were placed in a disposable cuvette with a 3/16” magnetic micro stir bar (Fisher). The shells were coated with 40 μl of 0.1 M CEPA (Figure 2-15) and stirred for 20 minutes. After the CEPA was attached, 10 μl of EDAC and 20 μl of SNHS were added to the shells to activate the carboxyl groups of CEPA and prepare them for attachment of the proteins as shown in Figure 5-4. The solution was then incubated for twenty minutes, then 3 μl of the 1 mg/ml AB-594C or 10 ul 1 mg/ml HRP was added and reacted for 1 hour before analysis. When SNHS was not used, the protein was added after 1 minute because the intermediate is shorter lived. To determine the attachment efficiency and activity of the proteins, unattached protein was separated via the 10 ml Lipex extruder (Northern Lipids) equipped with a 50 nm polycarbonate membrane at 200 psi. The protein coated shells were diluted to 5 ml and placed in the extruder and 3 ml was filtered out and kept as the filtrate fraction, 6 more washes of 5 ml of PBS were found to be sufficient to remove all detectable unattached protein.
5.2.2.2 Microarray Analysis

A Synergy HT (Bio-Tek SIAFRT) 96-well plate microarray reader was used to determine the fluorescein quenching capability of the AB-594C antibody before and after the attachment chemistry as well as the presence of the Alexa Fluor 594 dyes conjugated to the protein. Additionally, the microarray was used to monitor the activity of HRP through production of fluorescent resorufin from amplex red over time. For FITC assays, 50 μl of 400 nM fluorescein in 4x PBS (Section 5.2.1) and either 50, 100 or 150 μl of shells from before and after filtration were added along with water to yield 200 μl of a 100 nM fluorescein and shell suspension in 1x PBS solution. The microarray spectrometer used an excitation filter of 485/20 nm (peak/bandpass) and an emission filter of 528/20 nm at a machine sensitivity of 25 to record the FITC fluorescence. Filters of 590/20 nm and 645/40 nm were used to record the fluorescence of the Alex Fluor 594 dyes on the protein (4 per protein).

Horseradish peroxidase catalyzes the reduction of H2O2 to water and requires a molecule that acts as a hydrogen donor (Figure 5-14). The Amplex Red assay kit was used as described to monitor the activity of the horseradish peroxidase (HRP). In brief, 154 μg of Amplex Red was dissolved in 60 μl DMSO to make the stock solution and a 20 mM H2O2 solution was prepared by dissolving 22.7 μl of 3 % H2O2 solution into 977 μl of PBS buffer. A “working solution” of 200 μl of H2O2 solution and 50 μl of Amplex Red was filled to 5 ml with PBS resulted in a 2 M Amplex Red/ 0.1 % H2O2 and was enough for 100 assays. For the assay, 100 μl of HRP conjugate shells was added to the wells of a 96 well plate and just prior to measurement, 50 μl of working solution was added and the recording was initiated immediately. The reactions remain buffered at pH
so there was no HRP activity change resulting from a pH shift. The microarray spectrometer was set to read the fluorescence intensity at 5 minute intervals for 12 hours from 530/25 nm excitation and 590/35 nm emission filters at a PMT sensitivity setting of 25.

5.2.2.3 Fluorescence

Fluorescence emission of the attached Alexa Fluor 594 dyes was performed with the SLM 8100 spectrofluoromter in a similar manner as described in (4.2.4.2). The presence of antibody was monitored by setting the emission to 594 and reading from 600 to 650 nm which corresponds to the emission peak of Alexa Fluor was 614 nm. The fluorimeter was used to calibrate the concentration of the protein.
5.3 Results

There were two principle goals for this chapter: 1) Verify that the protein attachment chemistry was compatible with calcium phosphate nanoshells and what percent of the protein could be attached to the nanoshell surface and 2) quantify the percentage of remaining protein activity after the attachment of the protein to the surface.

Once the protein was attached to the nanoshell, the resulting fraction was analyzed fluorometrically for the presence of the Alex Fluor 594 dye or the activity of the HRP enzyme. This fraction is referred to as the “before” fraction and is illustrated in Figure 5-6. After measurement, a small amount of solution was filtered through a high pressure filtration apparatus on the first pass and is referred to as the “through” fraction (Figure 5-6). This fraction contains antibodies alone in a solution and is representative of the solution phase of all fractions.

Figure 5-6 Determination of attachment efficiency can be done via a straightforward mass balance on a filtered sample of shells. Concentrations are measured fluorometrically and the volumes are measure before and after the filtration and account for dead volume in the filtration apparatus (~800 μl)
Through these two measurements and a calibration curve (Figure 5-11) the concentration of antibody could be determined in each fraction and along with the amount of protein lost to filtration, it was possible to determine the molar fraction of antibody attached to the shell through the following mass balance.

\[
N_B = N_R + N_R + N_F
\]

\[
V_B C_B = V_R C_R + V_T C_T + N_F
\]

\[
N_B = N_{Shells} + N_{Liquid}
\]

*Dividing by the volume \((V_B)\) to obtain measurable variables*

\[
C_B = C_{Shells_B} + C_{Liquid}
\]

\[
C_{Liquid} = C_T + N_F
\]

\[
C_{Shells_B} = C_B - C_T - N_F
\]

\[
N_{Shells_B} = C_{Shells_B} V_B
\]

\[
N_{Shells_B} = N_{Shells} = N_{Shells}
\]

\[
Attached = \frac{N_{Shells}}{N_{Total}}
\]

Equation 5-1  Mass balance on the antibody after filtering
Stated simply, the total moles of protein in the system (NB) referred to as “before” is equal to the sum of the moles in the retained fraction (NR), the filter (NF), and the “through” fraction (or filtrate) (NT). There are two components in the “before” fraction that contain protein, the shells (NShells) and the surrounding liquid (NLiquid). The concentration in this fraction (CB) could be decoupled into a concentration due to the shells CShells and CLiquid by dividing by the total volume (VB). It was assumed (and confirmed experimentally in Figure 5-7) that the filtration did not pass any shells so the concentration in the “through” fraction (CT) was equal to the concentration in the liquid of the original fraction (CLiquid). The antibodies attached to the shells were not present in this fraction. Therefore the concentration contribution due to the shells in the “before” fraction can be determined from the measurable parameters CT, NF, and CB. The lack of particles in the “through” fraction was further verified by the lack of scattering counts (less than 10 kcps) and consequently particles in the through fraction when measured with dynamic light scattering. By multiplying by the volume of the before fraction (VB) the number of moles attached to the shells can then be obtained. The ratio of protein attached to the shells to the total number of protein in solution is called the attachment efficiency. This method was used to determine the attachment efficiencies of antibody throughout the results section.
Figure 5-7  Emission spectra of antibody coated, pyrene encapsulated calcium phosphate nanoshells that are passed through a 50 nm PC filter. As can be seen there is very little pyrene emission in the through fraction indicating effective filtration of the fluid and retention of the particles.

5.3.1 Control experiments

To determine how much protein was lost due to the structure of the filter ($N_F$), two filter materials were compared, one composed of cellulose acetate (CA) and one of polycarbonate (PC) the same used in extrusions. Solutions of (8.5 nM) antibody were prefiltred through a 0.45 μm filter to remove dust and then filtered through each of the filters via the Lipex extruder. From the graphs in Figure 5-8 it can be seen that approximately 5 % of the antibody was retained in the filter. This number ($N_F$) was used to more accurately determine the concentration of the “through” fraction by subtracting this value.
5.3.2 Attachment Chemistry

The primary components necessary for attaching proteins to the nanoshell are as follows:

- CEPA coated shells that provide a carboxylated surface for the proteins to attach to.
- EDAC must be present to activate the carboxyl groups to an o-acylisourea active intermediate.
- Protein that contains exposed amino acid residues available to react with the activated carboxyl group to form a covalent peptide bond.

To ensure that the attachment chemistry was working as expected, two experiments were conducted to compare the attachment efficiency when CEPA coated nanoshells were replaced with uncoated ones and when the attachment chemicals were not present when CEPA coated shells were used. As seen in Figure 5-9, both CEPA coated nanoshells and...
the attachment chemicals were needed for protein conjugation. Without CEPA there are no sites on the shell with the necessary carboxyl groups needed for binding and without the attachment chemicals, there is no possibility for forming the active intermediates required for covalent linkage.

![Graph](image)

**Figure 5-9** Emission spectra of antibody and shells reacted with the absence of either CEPA coated shells or absence of the EDAC and SNHS attachment chemicals. Nearly all of the antibody applied was recovered in the filtrate indicating that these molecules are required for antibody binding.

5.3.3 Antibody Attachment Efficiency

It was clear from the above study that both the attachment chemicals and CEPA coated shells were required in order to expect conjugation of the protein to the nanoshell surface. As discussed in the introduction, the o-acylisourea active intermediate is not stable in aqueous systems but this stability can be enhanced through the addition of SNHS to stabilize to intermediate to an NHS ester. However, in Figure 5-10 it is shown that there is a measurable decrease in the fluorescence intensity from the Alexa Fluor 594 dyes attached to the proteins in the “through” fractions when using SNHS. Possible explanations for this phenomenon are addressed in the discussion.
Figure 5-10  A) When SNHS is included with EDAC for the antibody attachment the attachment efficiency was approximately 70%.  B) When SNHS is excluded the attachment efficiency was reduced to approximately 50%.  These fluorescence spectra were taken after filtering 1 ml of solution through a PC filter leaving 4 ml behind.

To obtain the attachment efficiencies of protein to the surface of the shell, a calibration curve of AB-594C was obtained for fluorescence intensity versus AB-594C concentration from the SLM 8100 fluorometer and is seen in Figure 5-11.  The intensities from Figure 5-10 at 614 nm were converted to an antibody concentration with the calibration function and by using the mass balance (Equation 5-1) were used to determine the encapsulation efficiency.  Alternatively the ratios of the “through” and “before” peak intensities can be used with the following relation owing to the linear dependence of concentration on fluorescence intensity (I) and the negligible loss of material in the filter.

\[ \text{Attached} \% = 1 - \frac{I_{\text{through}}}{I_{\text{before}}} \]

Equation 5-2 Ratiometric method to determine the attachment efficiency
Figure 5-11  A) The calibration curve of fluorescence intensity versus AB-594C concentration allows the determination of the concentrations of antibody within the various fractions. B) The Alex Fluor e594 excitation and emission spectra* shows a peak absorbance at 594 nm and a peak emission at 614 nm. Alexa Fluor has an extinction coefficient of (73,000 cm\(^{-1}\))* From Molecular Probes

The following table summarizes the retention results obtained using this procedure. Attachment efficiencies over 70 % were achieved with this conjugation method when SNHS was used to stabilize the intermediate and almost 50% without SNHS. When CEPA is not added but EDAC/NHS is used, the attachment is 11.2 % and when CEPA is present but no EDAC/NHS is used only 5.8 % is attached. Under these conditions, with approximately 1 nM of nanoshells there were approximately 6 antibodies for every nanoshell. A typical IgG immunoglobulin is approximately 12.5 nm\(^{448}\) in diameter and if we assume that each requires 100 nm\(^{2}\) of surface area to bind them on a 100 nm shell with approximately 50,000 nm\(^{2}\) of surface area there is room for a maximum of 500 antibodies which means the prepared nanoshell surface was about 1 % saturated with antibody.
TABLE 5-1: ANTIBODY ATTACHMENT EXPERIMENTAL SUMMARY. BOTH CEPA AND EDAC-SNHS NEED TO BE PRESENT FOR OPTIMAL ATTACHMENT. ALL SAMPLES PREPARED ACCORDING TO SECTION 5.2.2.1 EXCEPT THE SUBTRACTION OF THE NOTED CHEMICAL(S).

<table>
<thead>
<tr>
<th>Case</th>
<th>CEPA</th>
<th>EDAC</th>
<th>SNHS</th>
<th>Before (cps @ 615 nm)</th>
<th>Through (cps @ 615 nm)</th>
<th>Attach Eff %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>260</td>
<td>245</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>242</td>
<td>215</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>807</td>
<td>414</td>
<td>49</td>
</tr>
<tr>
<td>4</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>803</td>
<td>211</td>
<td>74</td>
</tr>
</tbody>
</table>

5.3.4 Antibody activity

In the previous section it was confirmed that antibodies could be securely attached to the nanoshell surface. In this section the effects of attachment on antibody activity were investigated. The ability of the AB-594C antibody to quench the fluorescence emission of fluorescein at 10 μM was confirmed prior to attachment on the nanoshells to determine the number of fluorescein molecules that each antibody quenches. From the line in Figure 5-12A it can be seen that 2 μM of fluorescein was quenched for every 1 μM of AB-594C antibody because each antibody has 2 active sites at the end of each FaB fragment capable of quenching a fluorescein molecule (Figure 5-5).
Figure 5-12 A) Addition of the anti-fluoroscein antibody to a 5 μM fluoroscein solution shows a 1:2 molar ratio of antibody to fluoroscein quenching verifying that each antibody quenches 2 fluorosceins. B) Sensitivity of the microarray to fluoroscein fluorescence is linear from 100 μM until the sensitivity limit of approximately 10 pM.

This expected 2:1 relation of antibody to fluoroscein can be used to determine a percentage of antibodies that are still active after being attached to the nanoshell. A microarray plate reader was used to measure intensity of fluoroscein fluorescence of 100 nM as 50, 100, and 150 uL of ~1 nM suspension of shells (containing 8.5 nM AB-594C attached via EDAC, S-NHS chemistry) was added to a buffered solution of 100 nM fluorescein. These shells were rinsed in the 50 nm PC filter 4 times to remove free antibody to ensure that all measured loss in fluorescence emission was due to the antibody-shell suspension. The first rinse was collected and its ability to quench fluorescein was determined. The experiment was performed twice with different batches of shells and the results averaged.
In Figure 5-13, it is seen from the slope of each curve that each antibody quenches approximately 1.6 molecules of fluorescein when attached to the shells. The free, unattached antibodies which passed through the filter retain a slope equal to that of the free antibody measured in Figure 5-12A. This suggests that the loss of activity is not a chemical denaturation as it should have affected the unbound antibody as well. This amounts to a reduction of approximately 20 % of the antibody activity as a consequence of attachment to the shell. Possible explanations for this reduction in activity are described in the discussion.

Figure 5-13  A) The retained and rinsed portion of the shell from antibodies attached to the shells quenches 20 % less fluorescein than free antibody per mol of antibody present. B) Antibody that passes through the filter maintains its ability to quench 2 fluorescein molecules.
5.3.5 Horseradish Peroxidase (HRP) Activity.

To determine whether the attachment chemistry could also be used with enzymes, horseradish peroxidase enzyme was attached to the shell using the procedure described above and the activity of the enzymes measured by monitoring the production of resorufin, a fluorescent compound that is produced when Amplex Red acts as a hydrogen donor during the peroxidase catalyzed reduction of H₂O₂ to H₂O (Figure 5-14).

![Figure 5-14](image)

Figure 5-14 In the presence of HRP during the reduction of H₂O₂ to H₂O, Amplex Red acts as the hydrogen donor and reacts in a 1:1 ratio with peroxide thereby changing from a nonfluorescent molecule to fluorescent resorufin which can be detected spectrofluorometrically in a microarray at an emission wavelength of 587 nm when excited using 563 nm light. At this wavelength the molecule’s extinction coefficient is 54,000(449) cm⁻¹.

The HRP enzyme was attached to the shell with the same EDAC-SNHS chemistry used to attach the antibody to the shell described in the previous section. The EDAC/NHS attachment chemistry is not selective to different types of amines, it chooses the most available one.
Figure 5-15  Crystalline structure of recombinant horseradish peroxidase C1A. Structure obtained from the protein data bank (www.rcsb.org). There are over one hundred aminated residues on the body of HRP providing multiple locations for attachment.

After attachment the shells were filtered in the same way to yield the “before” and “through” fractions that are subsequently analyzed with the Amplex Red assay. As seen with the antibody, if the EDAC-S-NHS chemicals were not used then there was no attachment of horseradish peroxidase to the shell as evidenced by the lack of activity in the through fraction in Figure 5-16A.
Figure 5-16 The attachment of HRP to the outside of the shell was demonstrated through an Amplex Red assay of shells with ~ 25 pM HRP. A) Very little resorufin was generated in the “through” fraction of the shells when EDAC-SNHS are added suggesting that nearly all the enzyme used attached to the shell. B) When no EDAC-SNHS was added to the shells prior to the HRP no attachment occurred as seen by the same production rate of resorufin in the right graph.

It has been shown by Held\textsuperscript{449} that the initial rates of the HRP are approximately linear with HRP enzyme concentrations following a standard spectrophotometric protocol. This relationship allows us to use a ratiometric method to estimate an attachment efficiency of HRP to the nanoshells when the initial concentration of HRP is known (Equation 5-2). In Figure 5-17A the calibration curve of resorufin fluorescence is shown and Figure 5-17B the early time data points of Figure 5-16 representing the “before” and “through” fractions of HRP conjugated shells seen in Figure 5-16 converted to concentration units via the calibration.
Figure 5-17  A) The fluorometrically obtained calibration curve for resorufin is linear.  B) Using this curve the fluorescence intensity as a function of time shown in Figure 5-16 was converted to concentration of resorufin. The initial rates of resorufin production in a 2M amplex red solution from the “before” fraction (23 mM/min) and “through” fraction (51 μM/min) show that nearly all the HRP is attached to the shells. If all enzymes are assumed to be active and the activity for each enzyme is independent of nature of the fractions this indicates that only 0.2 % of the HRP protein is not attached to the shells, or was denatured.

The initial rate of resorufin formation of the through fraction was about 0.2 % (5.1 x 10^{-5} / 2.2 x 10^{-2}) of the initial rate of resorufin formation from the before fraction indicating that effectively all the HRP is attached to the shells and remained active. This high attachment efficiency allows us to assume that all the HRP activity measured in the shell fraction was due to HRP that is physically bound to the shells.

Comparing the resorufin produced at different concentrations of HRP (25 pM, 12.5 pM) after 100 minutes for bound and free HRP reveals that the enzymatic activity for bound enzyme shows a nearly 10 fold decrease in enzymatic activity upon attachment to the shell. Possible explanations for this are addressed in the discussion.
Figure 5-18  Comparison of the loss of HRP activity when bound to the shell surface. The reaction takes place at pH 7.4 with ~ 1 nM shells with 25 pM and 12.5 pM of either bound or free HRP with 2 M of Amplex Red. The amount of resorufin produced at 100 minutes from the amplex red reduces nearly 90% when bound to the shell surface.

Since quantification of HRP concentrations with the Amplex Red assay kit relies on the initial rates of reaction\textsuperscript{449} and the rate of reaction was modified during attachment to the nanoshell, and also an accurate quantization of actual enzyme concentration was not possible. Fortunately, for immunoassays detection limit is more important than quantization and as seen in Figure 5-19 the detection limit for HRP is approximately 1 pM (or about 2 fmol of HRP in a 200 ul well) which is on the same order as free other HRP based assays (1 x 10-13 M\textsuperscript{450}). Whereas the detection limit of HRP attached to the shells was similar, the time to detection was approximately 10 times longer owing to the decreased activity.
Figure 5-19 The detection limit of the HRP enzyme is approximately 10 pM. This corresponds to a shell concentration of 0.1 pM of calcium phosphate nanoshells. There are approximately 100 enzymes per shell. The curve at low concentration is due to nonlinear response of the detector at low fluorescent intensities.

Since all the enzyme was attached to the nanoshell, approximately 100 HRP enzymes attached to the nanoshell surface. The number and area of shells is estimated in Section 4.4.1. HRP is a smaller protein (40,000 kDa) than the AB-594C protein (150,000 kDa) and likely requires about a third of the space as the antibody or an estimated 30 nm². This leaves room for nearly 1,500 HRP enzymes per shell and a surface approximately 7 % coated with HRP. This is in contrast to AB-594C which left ~ 5 copies per shell for less than 0.1% coating of antibody.

5.4 Discussion

The covalent attachment of antibodies and enzymes to the shells with peptide bonds was accomplished through the use of carbodiimide chemistry. It was verified that CEPA coated shells as well as the EDAC chemicals were the minimum requirements to achieve attachment. It was also observed that the inclusion of SNHS to the conjugation
chemistry improves the attachment efficiency for antibodies by 20%. This improvement in attachment efficiency could be attributed to the longer lived NHS-ester intermediate (6+ hours) over the unstable o-acylisourea (1-2 min) intermediate. Once it was realized that the SNHS improved the conjugation efficiency of proteins to the shell, this method was used exclusively for further conjugations of antibody and enzymes.

A potential explanation for the low attachment of antibodies could be polymerization of the proteins to each other. Recall that the protein is added shortly after the EDAC when SNHS is not used and that could allow exposed carboxyl groups on the antibody to become activated by unreacted EDAC and allow for conjugation-polymerization to another antibody. The improved efficiency of HRP attachment may be due to the increased number of aminated residues present on the body of HRP, allowing for multiple bindings per enzyme. The difference in efficiencies from these two proteins indicates that attachment should be assessed on a per protein basis.

The 20% reduction in antibody activity after attachment can be understood as unfavorable attachment geometry. The presences of multiple amines groups on the Fc portion of the antibody provide several potential locations for the antibody to attach to and can potentially lead to undesired alignments of the antibodies on the surface. Below is a structure of human IgG which is structurally similar to the rabbit IgG. Notice the different residues on the Fc fragment, many of these can take part in the conjugation which is not selective.
Figure 5-20  Structure of human immunoglobulin showing the numerous chemically active sites. Some of these residues (circled) contain amines and can react with the carboxyl groups on the shell\textsuperscript{451}.

As illustrated in Figure 5-21 there are a range of attachment configurations that could occur depending on which residues are conjugated to the shell. Fortunately the amino termini of the antibody is sterically hidden and generally not available for binding\textsuperscript{447} and this prevents case C; therefore, it is likely due to orientation B that causes the approximately 20\% of antibody activity is lost after attachment.
Figure 5-21  Possible orientations of antibodies on the surface of the nanoshell. A: both Fab fragments are available for antigen binding and no activity is lost. B: on Fab fragment is close to the shell and may sterically prevent an antigen from coming into contact with that arm resulting in a loss of half of the activity. C: an antibody that attaches by its Fab arms will have both antigen binding sites blocked resulting in complete loss of activity. It is likely that a combination of these effects is the cause for reduced antibody activity once attached to the shell.

Figure 5-22  Possible problems when aminating a shell surface with a diamine. A) if the diamine is long enough it is possible that both amines will attach to the carboxylated surface of the shell. B) The possibility exists that an amine can attach to other shells and form a large floc of covalently linked nanoshells. C) The desired effect of aminating an entire surface to covalently attach carboxyl groups is unlikely given the possibility of side reactions.
Solving this alignment problem would require more control over the attachment geometry and is difficult when there are many potential conjugation targets. A potential solution to this issue is to aminate the surface of the shells (convert the COOH to NH₃), then to attach the antibodies to the shell via the carboxyl termini at the end of the Fc fragment of the antibody (Figure 5-1) leaving the nanoshell resembling Figure 5-3. This would require the amination of the CEPA surface with a diamine (ethyl diamine or aminated PEG for example). However this approach poses several problems: 1) aminated surfaces are not as bio-friendly as carboxylated surfaces and illicit a greater immune response, and 2) it is possible to conjugate both ends of the amine to the shell (Figure 5-22A) or to attach shells to each other (Figure 5-22B) with this chemistry as it is not selective. Furthermore, there is a possibility that reversal in the surface charge could negatively affect the stability of the particles. This amination approach was not explored due to the expected problems but would be a suitable topic for a future investigation.
The HRP enzymes also lost about 90% activity when attached to the as seen in Figure 5-18. In addition to the possibility that the enzymes attach in unfavorable orientations much like the antibodies (Figure 5-21) enzymes can also lose net activity because of the reduced mobility they have in solution now that they are attached to the larger nanoshells. Recall Equation 1-7 that reaction speed depends on, among other things, collision frequency:

\[ Z_{AB} = S \cdot U_r C_A C_B \]

It was determined from the Stokes-Einstein equation in Section 4.4 that the diffusion coefficient for nanoshells was approximately 100 times slower than free protein. This would result in a relative velocity \( (U_r) \) of about half. This reduction in relative velocity alone would result in a 50% decrease of apparent activity without the enzyme itself changing conformation. The remaining 40% of activity could be lost due to steric hindrance and unfavorable orientations as described in Figure 5-21.

5.5 Conclusion

The feasibility of attaching antibodies to the surface of calcium phosphate nanoshells was demonstrated using AB-594C, a dye-labeled antibody which quenches the fluorescence of fluorescein. This antibody was attached with an efficiency of nearly 70% when using SNHS to stabilize the o-acylisourea intermediate of EDAC whereas without SNHS the attachment efficiency was approximately 50%. Using EDAC-SNHS chemistry, horseradish peroxidase was also attached to the nanoshells at nearly 100%
efficiency. There was a loss of activity of both antibodies and enzymes when attached to the shell. The antibody lost approximately 20 % of its activity while the enzyme lost nearly 90 %. It was hypothesized that this was due to unfavorable orientations of the antibody which obscured some of the Fab fragments or denaturation of some of the enzyme on the shell. Additionally for enzymes, the reduced diffusion speed of nanoshell-attached enzyme could also explain the reduced activity.

The successful attachment of proteins to dye entrapped calcium phosphate nanoshells provides promise for improving the detection limit and speed of immunosorbent assays.
CHAPTER 6

FLUOROCARBON EMULSION CALCIUM PHOSPHATE NANOSHELLS AS
OXYGEN TRANSPORT VEHICLES

6.1 Introduction

In this chapter the potential for using fluorocarbon emulsion derived nanoshells as oxygen delivery vehicles was examined. The motivation for the development of these artificial oxygen carriers was discussed in detail Section 1.5.4. In order for a substance to be used as an effective artificial oxygen carrier (AOCs), the following minimum criteria should be met.

1. The AOC must be able to carry physiologically relevant doses of oxygen
2. The AOC must be able to release the oxygen to its surroundings
3. The AOCs must be nontoxic, biocompatible, biodegradable
4. The AOC must be able to be manufactured using current good manufacturing practice (cGMP) and be sterilizable
5. The AOCs need to be stable and not aggregate under conditions present in the blood stream (170 mM salt, and protein)
6. The synthesis and product should be well defined and potentially be able to produce shells in physiologically relevant quantities (~30% v/v)
The fluorocarbon microemulsion calcium phosphate nanoshells developed within this thesis are good candidates for an AOC as point numbers 3, 4, 5, and 6 have been demonstrated in earlier sections of this work. It was shown in CHAPTER 2 and CHAPTER 3 that the nanoshells can be manufactured sterily from biodegradable components currently available as cGMP (current good manufacturing compounds). Since the size is below 200 nm they can also be sterile filtered, typical bacteria are greater than 1 μm. The synthesis is well defined and can produce shells of various core size and thickness (Section 3.3) which affects the transport of hydrogen peroxide and copper ion across the shell as shown in Section 4.3.4. In Section 2.3.4, the shells were introduced to a physiological salt solution along with protein and due to their high zeta potential (~ -50 mV) did not aggregate, suggesting that the shells would be stable in the blood stream where high salt and protein concentration are the primary causes of colloidal aggregation. Furthermore, work by S. Schmidt showed the nanoshells were biocompatible with HFOB 1.19 osteoblast cell cultures and demonstrated no cytotoxicity. These properties of the nanoshells meet the necessary material criteria for an AOC. The functional criteria, particularly the ability of the shells to carry (1) and deliver (2) oxygen was investigated in this project. As mentioned in the introduction, currently investigated artificial oxygen carriers (AOCs) are either hemoglobin based (Section 1.5.4.2) or fluorocarbon based (Section 1.5.4.3).
The principle difference between the two methods is in the mechanism in which they transport and deliver oxygen. As mentioned in Section 1.5.4, hemoglobin binds oxygen cooperatively owing to the nature of the hemoglobin molecule and is saturated with oxygen. For fluorocarbons Oxygen solubility is linearly dependent on pressure and is governed by Henry’s law.

\[ P = kC \]

This linear dependence allows fluorocarbons to carry more oxygen if a patient is breathing pure oxygen as opposed to air. This ability to solubilize large amounts of gases is due to the low intermolecular interactions of fluorocarbons.

The practical benefits of fluorocarbon emulsions such PFOB based Perflubron\textsuperscript{396} (Figure 6-1) is that they can deliver much greater amounts of oxygen when used under higher oxygen pressure where as hemoglobin cannot. Additionally the degree of delivery
of hemoglobin is lower 25% than perfluorocarbons 90% when delivering to an oxygen
depleted tissue at a venous partial O₂ pressure of 5.3 kPA although perfluorocarbons
require more oxygen to deliver the same amount. The viscosity of fluorocarbon
emulsions decreases rapidly with fluorocarbon concentration and at 30% w/w the
viscosity is less than human blood⁴⁵². More information on the comparison of
hemoglobin and perfluorocarbon based substitutes can be found in Section 1.5.4.

In this work we used the encapsulated fluorocarbon microemulsions inside
calcium phosphate nanoshell particles developed in CHAPTER 3 as the starting point.
Two commonly used fluorocarbons for oxygen transport were used: perfluorodecalin and
perfluorooctyl bromide.

TABLE 6-1  OXYGEN CARRYING CAPACITIES OF FLUOROCARBONS⁴³¹

<table>
<thead>
<tr>
<th></th>
<th>Water</th>
<th>PFOB</th>
<th>PFD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Density (g/ml)</strong></td>
<td>1</td>
<td>1.92</td>
<td>1.95</td>
</tr>
<tr>
<td><strong>Vapor Pressure (mmHg)</strong></td>
<td>47</td>
<td>10.5</td>
<td>14</td>
</tr>
<tr>
<td><strong>Surface Tension (dyn/cm)</strong></td>
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<td>18</td>
<td>15</td>
</tr>
<tr>
<td><strong>O₂ Solubility (ml/L)</strong></td>
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<td>530</td>
<td>490</td>
</tr>
<tr>
<td><strong>CO₂ Solubility (ml/L)</strong></td>
<td>570</td>
<td>2100</td>
<td>1400</td>
</tr>
<tr>
<td><strong>Boiling Point (C)</strong></td>
<td>100</td>
<td>144</td>
<td>142</td>
</tr>
</tbody>
</table>

Of these, perfluorooctyl bromide is generally considered superior to perfluorodecalin as it
is capable of carrying 8 % more oxygen than perfluorodecalin and is more lipophilic¹⁸⁷,
³²⁹, resulting in easier emulsion formation¹⁸⁶. The oxygen carrying capability of PFOB-
cored calcium phosphate nanoshells was examined using an enzymatic detection method
based on a glucose assay. Transport properties of the PFOB-nanoshells were investigated
by monitoring the change in oxygen concentration over time when subjected to exposure
of nitrogen gas.
6.2 Experimental

6.2.1 Materials

Materials required for the synthesis of calcium phosphate shells with perfluorocarbon shells are listed in Section 3.2. A Glucose Assay Kit GAG020 (Sigma-Aldrich) was used to determine the oxygen concentration of the perfluorocarbon emulsion and used as described below.

6.2.2 Methods

6.2.2.1 Synthesis of Perfluorocarbon Cored Calcium Phosphate Nanoshell

The synthesis and properties of the perfluoroctyl bromide shells were described in detail in Section 3.2.2.2.1.

6.2.2.2 Concentration of Nanoshell Suspension

Nanoshell concentration was done using 50 ml Falcon® tubes (Fisher) spun at 10,000 RPM in a Sorval T20 tabletop superspeed centrifuge using a model SL-250T rotor spun at 11,250 x g. The bottom 5 ml (which contained mainly shells) was collected and was measured via DLS to determine the degree of concentration as described in Figure 3-17. The degree of concentration was determined to be approximately a factor of 20. Other concentrations methods were discussed in Section 3.2.2.3.
6.2.2.3 Measurement of Dissolved Oxygen

Two methods of determining dissolved oxygen were used in this study. The oxygen electrode is simple to use and can provide kinetic information but suffers from accuracy at low oxygen concentrations. The enzymatic method provides more accurate results compared to the electrode but only provides endpoint information.

6.2.2.3.1 Oxygen Probe

Dissolved oxygen in the PFOB-nanoshe ll suspension was measured with an Accumet Self-Stirring BOD Probe (Fisher) connected to an Accumet AR60 Benchtop meter (Fisher). A custom LabView program was developed to record time traces of dissolved oxygen and temperature. The probe was calibrated with air-saturated water (8.37 mg/L @ 25° C) and is refilled and recalibrated approximately each week.

6.2.2.3.2 Enzymatic Method for Determining Oxygen in Shell Suspension

To determine the total amount of oxygen contained within both the aqueous and emulsion/shell phase of a suspension a glucose assay kit was used and the method modified to detect O2 in the presence of excess glucose, as opposed to glucose in the presence of excess O2. In excess glucose, the reaction continued until it has consumed all the O2 present in the solution and shells. This glucose limited modification was described by Friere419 for the purpose of determining oxygen concentrations in PFOB emulsions. Briefly, glucose is oxidized to gluconic acid and H2O2 by glucose oxidase. The peroxide reacts with o-dianisidine to form a colored intermediate that can be used to determine oxygen concentration when glucose is in excess.
The kit was used as follows: 200 μl of refrigerated glucose assay solution (o-dianisidine, glucose oxidase, peroxidase) were added to a 10 ml test tube, covered with a septum and evacuated to a pressure of approximately 2 torr within 5 minutes using a rotary vacuum pump, purged with nitrogen for 1 minute, then re-evacuated to 2 torr and kept at 37°C in a water bath prior to injection of the sample.

Figure 6-2  Glucose assay kit reaction pathway modified to determine oxygen concentration in a fluorocarbon emulsion/shell suspension adapted from Friere^419

Figure 6-3  Experimental apparatus used to vacuum the assay solution and keep the reaction environment anoxic until a measured amount of perfluorocarbon emulsion/shells is added. This limited amount of oxygen present where glucose is in excess allows the correlation of absorbance of reduced o-anisidine to oxygen content.
Separately, 200 μl of the suspension to be analyzed was added to 10 μl of 100 mg/ml aqueous glucose solution and kept at 37°C with a water bath. The suspension was injected to the evacuated glucose assay solution and was reacted for exactly 30 minutes. After the reaction, 200 ml of 12 N H₂SO₄ is added to stop the reaction, one ml of DI water was added and the absorbance taken at 540 nm and compared against a calibration curve.

A calibration curve of oxygen concentration versus absorbance was created by adding 10 μl of 100 mg/ml glucose and 0, 25, 50, 75, 100, and 150 μl of DI water at constant temperature 37°C to the evacuated glucose assay solution. These volumes of water have a known O₂ concentration at a given temperature (6.9 mg/ml @ 37°C) The reaction was allowed to progress for 30 minutes at 37°C and stopped via the addition of 200 μl 12 N H₂SO₄. Each solution was brought to a final volume of 1410 μl with an appropriate amount of DI water at neutral pH and the absorbance is measured at 540 nm.

6.3 Results

6.3.1 Synthesis of PFOB Cored Calcium Phosphate Nanoshells

Calcium phosphate nanoshells produced from DOPA-stabilized perfluorooctylbromide (PFOB) microemulsions extruded a 100 nm polycarbonate membrane using the automated stepwise synthesis described in Section 3.2.2.2.1. A typical TEM image of these is shown in Figure 6-4A. A higher magnification picture (B) of the same grid shows the thin 10 nm shell obtained by adding 15 x 10 μl additions of CaCl₂ to a pH 9.5 suspension. This visually measured thickness is approximately equal to the mean size obtained by DLS of 135 nm +/- 16 nm.
Figure 6-4 Calcium phosphate coated nanoshells prepared using extruded DOPA-stabilized PFOB microemulsions prepared with 15 x 10 μl additions of CaCl₂ at pH 9.5, described in Section 3.2.2.2.1. The shell wall is approximately 10 nm.

6.3.2 Verifying Oxygen Carrying Capability

The measurement of the amount of oxygen present within the nanoshell requires calculation as the oxygen content of the perfluorooctylbromide core itself cannot be probed.
6.3.2.1 Estimating Total Oxygen Content Within the Suspension

An approximation of how much O$_2$ should be present in the system can be calculated given the solubilities of oxygen in the aqueous phase and PFOB phase at a given partial pressure of surrounding oxygen. Where, $C_x^{\text{sat}}$ is the equilibrium concentration at a specific percentage of oxygen partial pressure of gas. At a gas composition 20% O$_2$-80% N$_2$ at 1 ATM and 25° C water has a $C^{\text{sat}}$ of 215 $\mu$mol/L of O$_2$ where PFOB has a $C^{\text{sat}}$ of 3816 $\mu$mol/L of O$_2$ (TABLE 6-1). The graph below (Figure 6-6) is a plot of Equation 6-1 and shows how oxygen concentration changes as a function of PFOB vol % and estimates the increase on oxygen concentration that should be expected experimentally.

\[
A_{\text{aqueous phase}} (C_{\text{water}}, V_{\text{water}}) \quad \text{Fluorocarbon phase} (C_{\text{PFC}}, V_{\text{PFC}})
\]

Figure 6-5 Diagram of the separate phases present within a fluorocarbon cored calcium phosphate nanoshell suspension. If it is assumed that oxygen is only present within the perfluorocarbon (PFC) phase and the water then the total amount of oxygen present is the sum of the concentration of both phases (Equation 6-1).

\[
n_{O_2,\text{total}} = n_{O_2,\text{PFC}} + n_{O_2,\text{Water}}
\]

\[
C_{O_2,\text{Total}} V_{\text{Total}} = C_{\text{water}}^{\text{sat}} V_{\text{water}} + C_{\text{PFC}}^{\text{sat}} V_{\text{PFC}}
\]

Equation 6-1 Total oxygen concentration in perfluorocarbon emulsion
Figure 6-6  Graph showing how oxygen concentration in a PFOB emulsion changes with increasing PFOB vol %. Current generation Perflubron is a 30% PFOB uncoated emulsion and contains approximately 4 times as much O₂ as water at atmospheric conditions.

6.3.2.2 Concentration of Nanoshells

Since PFOB emulsions are usually synthesized to approximately 0.5 vol % which results in only an 8 % difference in oxygen carrying capacity of the suspension, too little a difference to be reliably measured (Equation 6-1). The nanoshell suspension was concentrated using centrifugal concentration as discussed in Section 3.3.4 taking advantage of the fact that PFOB cored shells are twice as dense (1.92 g/L) as their surrounding medium (water). The shells were concentrated by a factor of 20 to approximately 10 vol %

6.3.2.3 Oxygen Electrode Measurements for Oxygen Content

The oxygen probe was used to measure the oxygen content in the nanoshell suspension by monitoring the decrease in dissolved O₂ as dry N₂ gas was passed over the sample at a constant rate. A sample data set is seen in Figure 6-7.
Figure 6-7 Profiles of O$_2$ concentration versus time for suspensions of various compositions. The probe is not sensitive below 1.5 mg/L.

Unfortunately, the probe available did not take stable readings below 1.5 mg/L and the complete profile and consequently the complete integral could not be resolved. From this data the initial rate of deoxygenation of the suspension was evaluated and used to estimate the rate of oxygen transfer from an emulsion compared to the nanoshell in Section 6.3.3.

6.3.2.4 Enzymatic Consumption method

The enzymatic method relies on the consumption of all oxygen in the solution and shells by an enzymatic reaction (Figure 6-2) that produces a colored compound oxidized o-dianisidine whose absorbance 540 nm can be correlated with the amount of oxygen present (Figure 6-8). The nanoshells were first concentrated by centrifugation to 10 % v/v as confirmed by DLS and are then diluted serially to obtain a graph of oxygen concentration versus vol % encapsulated PFOB (Figure 6-9).
Figure 6-8  A) Calibration curve of absorbance versus volume of glucose assay solution are done to ensure the kit works as described. B) Calibration curve in the presence of limited oxygen for use in determining oxygen concentrations of the perfluorocarbon cored nanoshells.

Figure 6-9  Oxygen concentrations determined by the enzymatic assay method when the calcium phosphate coated PFOB shells and uncoated PFOB emulsions are exposed to 20% O₂/80 % N₂ (Air). The concentration of O₂ is obtained from the absorbance of the assay solution and compared to the calibration plot in Figure 6-8. The predicted line is obtained from Figure 6-6.
In Figure 6-9 it is clear that the amount of oxygen contained within the shell suspensions increased linearly with perfluorooctyl bromide concentration with a slope that agrees well with expectations when using Equation 6-1. When liposome or soybean cored nanoshells were used, their oxygen content is not different than that of water, verifying that the oxygen carrying capabilities are the result of the PFOB entrapped within them.

6.3.3 Verifying Release of Oxygen from the Nanoshells

To quantify the release kinetics of the solubilized oxygen from the nanoshell suspensions a 3-necked 250 ml round bottom Pyrex flask containing shells, uncoated emulsions, or water was fitted with an oxygen electrode connected to an Accumet AR-60 meter, a nitrogen gas inlet and a vent. Inside the flask the suspension to be analyzed was placed along with a ½” stir bar. The suspension was stirred very gently at ~ 400 RPM so as not to visually disturb the top layer of liquid in an effort to maintain constant mass transfer area. This stirring provided some convection within the fluid to aid transport through the liquid without disturbing the constant surface area of the air-water interface (determined visually).
Figure 6-10  Experimental apparatus to determine oxygen concentration through evaporation of O₂ from water and aqueous fluorocarbon shell solutions. Solutions were bubbled with an O₂/N₂ mix until the probe was stable. The flask was purged with N₂ for 5 minutes before introduction of the sample.

The initial traces obtained by the oxygen probe are seen in Figure 6-11. The rates were subtracted from the initial O₂ concentration 25 mg/L to plot the amount of O₂ purged from the suspension over time. To verify that the 10 nm calcium phosphate nanoshell has a negligible impact on the release rate of oxygen, a bare PFOB-DOPA emulsion is compared to a 10 nm thick PFOB cored calcium phosphate nanoshell similar to those shown in Figure 6-4.
There was a negligible difference in transport rates of oxygen between nanoshells and DOPA stabilized PFOB emulsions of equivalent size. Using the method described in Section 4.4.4 we can estimate a diffusion coefficient of $O_2$ through the nanoshell to be approximately $1.2 \times 10^{-12}$ cm$^2$/s. The method used to determine this value is covered in the discussion.

Figure 6-11  A) Plot of initial rates of oxygen leaving the suspension for water, emulsions, shells.  B) Column plot of initial rates of A obtained from linear regression analysis.
6.3.4 Demonstration of Drying and Resuspendability

One of the potential benefits of the calcium phosphate coating present on the perfluorocarbon emulsion is the ability of the nanoshells to be dried and resuspended while retaining the perfluorocarbon core within the shell. To demonstrate this a concentrated, dialyzed suspension of perfluorocarbon shells was allowed to evaporate and dry to a powder. The size distribution of the suspension was measured before evaporation and after resuspension with DI water. The size was 138 nm +/- 14 nm with 1.1 Mcps and upon reconstitution was measured to be 148 +/- 36 nm with 78 kcps after settling meaning at best 10% of the shells survived the drying-resuspension process. Measurements of the O₂ concentration of the shells after they were resuspended did not show an increase over water.

Figure 6-12 TEM of shells that have been dried and resuspended. Many of the shells have formed clusters though few remain free.
6.4 Discussion

The prime criteria for an AOC is based is its ability to transport and release oxygen. Although the use of an oxygen probe to quantify the oxygen content in the heterogeneous PFOB shell/emulsion system proved limited in its ability to determine the oxygen content of a heterogeneous system, the enzymatic method showed good agreement with expectations.

To estimate the diffusion coefficient of oxygen across the nanoshell, the following assumptions illustrated below (Figure 6-13) were made to simplify the system.
Figure 6-13  Simplified path oxygen takes when exiting the shell and leaving the water interface into the nitrogen stream.

1. Steady state at early times ($Q_C = Q_S = Q_L = Q_I$)
2. Concentration changes in each phase little during the first few seconds
3. Concentration at PFOB-shell interface equals PFOB oxygen concentration
4. Concentration at liquid-shell interface equals liquid concentration
5. Curvature of the shell is ignored
6. Shell assumed to be rate limiting step

The steady state mass balance (where all fluxes are equivalent) for the system described in Figure 3-17 can provide a good estimate for the diffusion constant of the calcium phosphate nanoshell.
For the shell + emulsion + liquid case

\[ \frac{dO_2}{dt} = Q_C = Q_S = Q_L = Q_S \]

Substituting Fick’s law into the above

\[ \frac{Q}{A} = J = D \frac{dC}{dx} \]

Yields,

\[ \frac{dO_2}{dt} = D_C A_C \frac{C_C - C_{CS}}{\Delta x_C} = D_S A_S \frac{C_{CS} - C_{SL}}{\Delta x_S} = D_L A_L \frac{C_{SL} - C_{LS}}{\Delta x_L} = D_I A_I \frac{C_{IL} - C_{IG}}{\Delta x_I} \]

Considering only the shell transport

\[ \frac{dO_2}{dt} = Q_S \]

\[ \frac{dO_2}{dt} = D_S A_S \frac{C_{CS} - C_{SL}}{\Delta x_S} \]

Solving for \( D_S \) yields

\[ D_S = \frac{1}{A_S} \frac{dO_2}{dt} \frac{\Delta x_S}{C_{CS} - C_{SL}} \]
The area of the nanoshell surface is estimated via the method outlined in Section 4.4.1 and is taken to be 1000 cm² (for ~1 nM of 110 nm shells). The O₂ concentration inside the nanoshells at 60% O₂/40% N₂ was taken to be the concentration of oxygen of PFOB (0.78 μmol/ml) and the concentration at the shell liquid interface was taken to be the concentration of oxygen of water (14 μmol/ml). The shell thickness was 10 nm and the rate as measured in (Figure 6-11) was 16 nmol/s. Substitution of these values into the above equation yielded a diffusion coefficient of 1.2 x 10⁻¹² cm²/s and is comparable to the diffusion obtained for the structurally similar hydrogen peroxide through the shell determined by pyranine quenching 7 x 10⁻¹² cm²/s (Section 4.3.4).

The resuspended shell appeared to lack oxygen carrying capability but it could not be determined if this was due to the loss of shells during the resuspension process or the evaporation of the fluorocarbon out of the shell. The concept of resuspending the shells was demonstrated but further development is necessary for powdering to be a viable storage method for the PFOB emulsion cored calcium phosphate nanoshells.

6.5 Conclusion

In this chapter both the oxygen transport and release characteristics of calcium phosphate nanoshell PFOB emulsions were verified and do not differ appreciably from a bare emulsion. This is promising, as calcium phosphate nanoshells could potentially offer several advantages over bare PFOB emulsion AOCs such as an increased mechanical resilience to filtration, with comparable uptake capacity and release characteristics.
CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

The principle goals of this thesis were to develop a synthesis for biocompatible/biodegradable core-shell nanoparticles based on biomineralization of calcium phosphate around amphiphiles that could potentially be used as a universal delivery device for fluorescent biosensors, targeted drug delivery vehicles, and as an artificial oxygen carrier. To demonstrate the potential of this novel material the following criteria needed to be met:

1) The nanoshells need to be synthesized by a robust and reproducible method that allows the use of different core solvents, core sizes, and shell thickness.
2) The nanoshells need to be able to encapsulate both hydrophobic and hydrophilic molecules
3) The nanoshells should provide the necessary scaffold for targeting molecules like antibodies to be attached
4) Perfluorooctyl bromide cored nanoshells need to be able to solubilize and release oxygen comparable to a bare emulsion.

In CHAPTER 2 and CHAPTER 3 it was determined that the synthesis of calcium phosphate nanoshells relies on control of the supersaturation of the reaction. It was
hypothesized that the local supersaturation around the negatively charged liposome is higher than in the bulk, providing a preferential location for shell growth. The negatively charged phosphatidic acid headgroup of 1,2 dioleoyl-sn-glycero-3-phosphatidic acid (DOPA) and its low Tc (-8°C) make it an ideal lipid for calcium phosphate deposition on liposomes or microemulsions at room temperature. Preparing microemulsion templates instead of liposomes required mixing the DOPA lipids with soybean oil, perfluorodecalin (PFD), or perfluorooctylbromide (PFOB) prior to sizing instead of water. The type of core solvent was shown not to greatly affect the formation of the nanoshell. Compared to mixing and sonication, extrusion of the templates provided superior speed and reproducibility when sizing templates with 50, 100, and 200 nm mean diameters. Of the methods investigated for nanoshell synthesis, the most effective method was the automated stepwise synthesis when compared to the dropwise method, one step supersaturation method, evaporative crystallization, and the stepwise supersaturation method. The principle behind the automated stepwise synthesis is to achieve a supersaturation sufficient for calcium phosphate growth but moderate enough to avoid homogenous nucleation by titrating 10 μl aliquots of 0.1 M NaOH or 10 μl aliquots of 0.1 M CaCl₂ into a subsaturated solution of either liposomes or microemulsion templates every 30 minutes utilizing an in-house developed automated microtitration system. The number and time-spacing of these titrations is important to final shell quality and was directly proportional to the final shell thickness as determined by comparing TEM and DLS of the shells to the uncoated templates. The resulting calcium phosphate nanoshells can range in core size from 40 to 200+ nm and shells from 5 nm to more than 200 nm resulting in nanoshells over 400 nm in diameter with a standard deviation of less than 15
% as determined by AFM, TEM, and DLS. The effectiveness of concentrating the dilute nanomolar nanoshell suspensions with centrifugation, centrifugal concentration, and high pressure filtration was investigated. It was concluded that high pressure filtration worked the best overall for all suspension compositions with centrifugation working only for fluorocarbon cored nanoshells due to their high density. Zeta potential measurements reveal that the surface of the nanoparticle is negatively charged at -20 mV which can be increased to -50 mV when the shells are coated with a carboxyethyl phosphonic acid (CEPA) which effectively coats the shells in carboxylic acid groups. These CEPA coated shells were shown to be stable in up to 200 mM NaCl solutions and 2.5 g/dL of bovine serum albumin protein by DLS measurements. The results suggest that the nanoshells would not clot when in contact with blood. After synthesis, the nanoshells are stable in suspension for more than 6 months, they retain their rigid structure when dried on a TEM grid and subjected electron radiation and vacuum, and can survive high pressure filtration where as bare liposomes and emulsions can not, providing a specific benefit over these types of nanoscopic materials.

In CHAPTER 4 the feasibility of calcium phosphate nanoshells to act as carriers/protectors of encapsulated molecules was demonstrated. The pyranine and pyrene fluorophores were used as chemical models for the encapsulation of both hydrophilic and hydrophobic molecules respectively. Three methods: dialysis, column chromatography, and high pressure filtration were used to separate the unencapsulated molecules and each performed equally well. Pyranine was encapsulated within DOPA liposomes at approximately 75 % efficiency and resulted in nanoshells with a maximum internal pyranine concentration of approximately 30 mM. Once pyranine was
encapsulated it could be used to determine the pH of the core of the shell through ratiometric absorbance measurements at 403 and 453 nm. Core and solution pH did not differ appreciably and confirms that H\(^+\) ions can move freely across the shell. The measured initial rate of pyranine emission quenching occurring as H\(_2\)O\(_2\) or Cu\(^{2+}\) entered the shell was used to determine the diffusion coefficient (D) of the nanoshell for each of these substances. Copper ion diffused much faster (7.9 x 10\(^{-10}\) cm\(^2\)/s) than hydrogen peroxide (6.5 x 10\(^{-13}\) cm\(^2\)/s) owing to it smaller size. It was also determined that the diffusion coefficient was dependent on the thickness of the shell varying by an order of magnitude for 35 nm shells (6.5 x 10\(^{-13}\) cm\(^2\)/s) versus 10 nm shells (7.0 x 10\(^{-12}\) cm\(^2\)/s). It was hypothesized that this is the result of a reduction in pore size as well as a more tortuous diffusion path that develops as the shell increases in thickness and was supported by electron microscopy observations. Pyrene was encapsulated within soybean oil cored nanoshells with approximately 97% efficiency and resulted in a 245 mM core concentration and a suspension concentration of only 0.12 mM. The high local concentration resulted in an excimer peak that would not have been achieved in a 0.12 mM solution without the local high concentrations provided by the nanoshells. In addition to pyranine and pyrene, FITC and sulforhodamine were also encapsulated at 78% and 75% efficiencies resulting in 39 mM and 3.7 mM core concentrations respectively. The successful encapsulation of hydrophiles and hydrophobes demonstrates the possibility of the nanoshells to solubilize and deliver many similar substances.

To demonstrate the possibility of using the nanoshells as a targeted delivery vehicle for drugs or contrast agents, the attachment of antibodies and enzymes were explored in CHAPTER 5. The model antibody used is itself conjugated to 4 Alexa Fluor
594 fluorophores and has active sites capable of quenching fluorescein. Horseradish peroxidase is used as a model enzyme for its ubiquitous presence in immunoassay chemistry. The carboxylated surface of the nanoshell provided by the CEPA coating provided a method to attach aminated molecules such as proteins to the surface with carbodiimide chemistry. When using ethyl diaminocarbodiimide (EDAC) and sulfo-n-hydroxysuccinimide (SNHS) to conjugate the carboxyls from the shell to free amines on the protein, a zero-length covalent peptide bonds is formed. This chemistry resulted in an attachment efficiency for antibodies of ~70% and ~99% for horseradish peroxidase (HRP). Upon attachment to the shell the antibodies lost about 20 % of their ability to quench FITC where as the enzymes lost nearly 90 % of their activity. The successful attachment of proteins to the outside of the calcium phosphate nanoshells demonstrates their potential for use as targeted delivery vehicles.

In CHAPTER 6 the concept of oxygen delivery was investigated using perfluorooctyl bromide (PFOB) cored calcium phosphate nanoshells. Through the use of a modified enzymatic based glucose assay, it was determined that the PFOB cored nanoshells held an amount of oxygen comparable to a bare emulsion. An oxygen electrode was used to measure the initial rate of oxygen transport out of the solution and was not found to differ appreciably from a bare emulsion. PFOB cored nanoshells possess the same oxygen solubilization and delivery characteristics as the uncoated PFOB emulsions but contain the added benefit of a rigid calcium phosphate nanoshell that allows the emulsion to be powdered and rehydrated. These nanoshells meet the necessary criteria for an artificial oxygen carrier.
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