ENCAPSULATION OF PHOSPHORYLATED CHEMOTHERAPEUTICS IN CALCIUM PHOSPHOSILICATE NANOPARTICLES AND THE TRANSLATIONAL PERSPECTIVE TO NANOPARTICLE DRUG DELIVERY

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The dissertation of Welley S. Loc was reviewed and approved* by the following:

James H. Adair  
Professor of Materials Science and Engineering,  
Biomedical Engineering, and Pharmacology  
Dissertation Co-Advisor  
Co-Chair of Committee

Thomas Mallouk  
Head, Department of Chemistry, Evan Pugh Professor of Chemistry,  
Biochemistry and Molecular Biology, and Physics  
Dissertation Co-Advisor  
Co-Chair of Committee

David Boehr  
Professor of Chemistry

Peter J. Butler  
Professor and Associate Dean for Education in the College of Engineering

*Signatures are on file in the Graduate School
ABSTRACT

Cancer retains negative associations to chemotherapy even as treatments evolve. Alopecia, neuropathy, nail loss, and invasive surgical procedures, can arguably make a more detrimental experience than having the disease at early stages. On the other hand, patients cannot benefit from the best treatment outcome when symptoms surface later, so early detection is the key to maximize chances of survival. However, patients are often present at a certain stage of cancer when tumors are detectable by current imaging capabilities. This is the case for those with pancreatic ductal adenocarcinoma (PDAC). Being the fourth most lethal type of all cancers with a ≤10% 5-year survival, there is no treatment to effectively manage the condition.

The motive of this dissertation emphasizes the possibilities and importance of enhancing the efficacy of existing medicine for pancreatic cancer. The common antimetabolite, 5-fluorouracil (5-FU), is amongst the most effective drug agents when first discovered in 1957 and is still part of the modern treatment regimen. Because 5-FU disrupts DNA and RNA replication, there are many toxic side effects from drug metabolism, so the ability to target cancer is a pivotal characteristic that 5-FU and other first generation chemotherapeutics lack. Advances in nanotechnology has opened a new era in this area by offering meticulous ways to engineer nanoparticles to encapsulate and deliver drugs to specific sites. Given the known mechanisms of 5-FU in cells from years of basic research and clinical trials, drug reformulation is a valuable alternative to the conventional route of drug discovery.

Calcium phosphosilicate nanoparticles (CPSNPs) offer an effective drug delivery platform that is bioresorbable with a narrow particle size distribution to enable penetration of dense fibrotic tumors such as PDAC. Developed by the Adair, Kester, and Matters teams, both gastrin (T. Morgan, 2009 and E. Altinoglu, 2010 dissertations) and aptamer-modified (X. Tang, 2016 dissertation) CPSNPs doped with indocyanine green (ICG) dye have demonstrated accumulation
and specific targeting at the pancreas of orthotopically transplanted BxPC-3 and PANC-1 athymic mice cancer models. The reports herein are a continuation of these investigations that focus on the therapeutic capabilities of CPSNPs. This involves the significance of active phosphorylated agents on drug load optimization in CPSNPs through adsorption-mediated encapsulation. These active metabolites include fluorodeoxyuridine monophosphate (FdUMP) for 5-FU and gemcitabine monophosphate (GemMP; dFdCMP) for gemcitabine (Gem; dFdC). Conversion from the prodrugs (biologically inactive 5-FU and gemcitabine) to the active forms is an enzyme-catalyzed event in cells. Without targeting abilities, majority of 5-FU and gemcitabine become catabolized before the agents can carry out mechanisms of action.

Concepts behind adsorption-mediated encapsulation based on zeta potential evaluations and the role of potential-determining ions (hydroxyls, protons, and phosphates) from solution to the surface of calcium phosphate were examined. The relationship between adsorption and speciation diagrams of the different ions was also interpreted. These experiments were conducted to show that compounds with phosphate and other charged groups can adsorb to calcium phosphate, which was indicated by shifts in surface polarity. Brushite was synthesized and subjected to various concentrations of potassium hydroxide (KOH), hydrochloric acid (HCl), sodium citrate (Cit), adenosine triphosphate (ATP), 5-FU, and FdUMP, to analyze trends in the zeta potential curve vs. pH. The isoelectric point (IEP) of brushite in water at room temperature was pH ~7 and the addition of OH\(^{-}\) and H\(^{+}\) ions influenced the solubility products of calcium phosphate at the solid-solution interface. At pH >7, the zeta potential was negative due to the net anionic species produced. The opposite effect was observed at pH <7. Both Cit and ATP were found to robustly adsorb to calcium phosphate and shifted the zeta potential to increasingly negative values at increasing concentrations. The effects of adsorption by FdUMP were not as profound as Cit and ATP even though FdUMP was expected to make the zeta potential more negative. The presence of FdUMP shifted the isoelectric point of the calcium phosphate and the event was not present with 5-FU.
Results on ATP and FdUMP raised implications that a higher uptake of phosphorylated compounds can be achieved with CPSNPs by taking advantage of the affinity between calcium ions on calcium phosphate and the phosphate group on the compound.

The non-phosphorylated drug analogs were not well encapsulated into CPSNPs, suggesting the phosphate modification is essential for effective encapsulation. In vitro proliferation assays, cell cycle analyses and/or thymidylate synthase inhibition assays verified that CPSNP-encapsulated phospho-drugs retained biological activity. Analysis of orthotopic tumors from mice treated systemically with tumor-targeted FdUMP-CPSNPs confirmed the in vivo uptake of these particles by PDAC tumor cells and release of active drug cargos intracellularly.

In Chapter 5, a novel synthesis of gold nanoparticles using sodium thiophosphate (TP) was introduced in the development of gold-calcium phosphosilicate (Au-CPS) core-shell nanoparticles for theranostic applications (combined imaging and therapeutic capabilities). Thiophosphates were the highlight of this work to induce gold core encapsulation through calcium ion immobilization at the gold surface to seed CPS growth. The kinetics of the Au-TP reduction was studied with the Johnson-Mehl-Avrami-Kolmogorov (JMAK) model and analyzed alongside with the gold particle number concentration as reactions progressed for 60 min. The reaction was conducted at various molar ratios of chloroauric acid to TP at room temperature. The values for the Avrami reaction rate constant, \( k \), implied that TP was significant for inducing the supersaturation of gold-thiophosphate monomers into nuclei. The Avrami parameter, \( n \), suggested a diffusion controlled mechanism. With additional excess thiophosphate, both \( k \) and particle concentration increased during the nucleation period (within 10 min). Overall particle sizes were smaller as TP concentration increased and the nanoparticles absorb within 500-600 nm in the surface plasmon resonance (SPR) band region. While the JMAK model has limitations such that the constants can only be interpreted within the Avrami definitions, the study offered insights into possible mechanisms and ways to control
colloidal stability and size by comparing the reaction at different reactant ratios. The first example of Au-CPS gold cores was also presented with suggestions for future refinement work.

Lastly, this entire work is described within the context of translational science in Chapter 6. To reach an understanding of the drug discovery cycle and the challenges is important for those conducting research to impact global healthcare. In STEM research, the need to generate data on the fundamental sciences can often overshadow the purpose to provide applicable solutions to improve patient outcome. Here, encountered experimental scenarios for nanoparticles were presented and discussed to identify components that are critical for advancing these benchtop findings into clinical trials.
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Chapter 1

Background and Significance

(Sections of this chapter are adapted from an in-preparation manuscript by Loc, W.S; Linton, S.S.; Wilczynski, Z.R.; Matters, G.L.; McGovern, C.O.; Fox, T.; Gigliotti, C.; Tang, X.; Tabakovic, A.; Martin, J.A.; Clawson, G.A.; Smith, J.P.; Butler, P.J.; Kester, M.; Adair, J.H. Effective Encapsulation and Biological Activity of Phosphorylated Chemotherapeutics in Calcium Phosphosilicate Nanoparticles for the Treatment of Pancreatic Cancer.)

This chapter gives an overview of research that has been previously completed on calcium phosphosilicate nanoparticles (CPSNPs) as well as current work on drug encapsulation and optimization through the use of phosphorylated agents. Pertinent background for the investigation of thiophosphate reduction of gold and encapsulation to create Au-CPS core-shell hybrid nanoparticles is also summarized.

1.1 Calcium phosphate nanoparticles (CPSNPs) before and now

1.1.1 CPSNPs and significance of phosphorylated chemotherapeutics

Applications of nanoparticle (NP) delivery systems to medical oncology include the use of NPs for encapsulated therapeutic drug delivery as well as for non-invasive tumor imaging. Due to their small size and surface chemistry, NPs are ideal platforms to penetrate poorly vascularized and fibrotic tumors.\(^1\) NP-based delivery platforms encompass a wide range of materials including liposomes,\(^2,4\) dendrimers,\(^5,6\) gold NPs,\(^7\) and quantum dots.\(^8,9\) The design and composition of NPs are critical to ensure optimal drug loading and protection of the cargo until release is triggered.
Furthermore, a NP platform should have both a reliable clearance mechanism that removes NPs that do not accumulate in the target tissue and a sufficient half-life to produce a therapeutic effect.\textsuperscript{10}

Amorphous calcium phosphosilicate nanoparticles (CPSNPs) have been used to deliver a diverse range of therapeutic\textsuperscript{11-13} and imaging agents\textsuperscript{12,14-17} to tumor cells. CPSNPs are composed of bioreorable materials, \textsuperscript{10,18} and bioactive agents (such as chemotherapeutic drugs) are encapsulated within the CPSNP matrix to be protected from metabolic breakdown and clearance. The attachment of methoxypolyethylene glycol (mPEG) to the surface of CPSNPs mitigates particle agglomeration and non-specific interactions and facilitates bioconjugation of tumor targeting agents, such as peptides, antibodies or aptamers, to the NP surface.\textsuperscript{13-14,16-17} Once taken up by cells, CPSNP dissolution is triggered by the low pH of late-stage endosomes and the change in osmotic pressure ruptures the endosome to release active agents into the cytosol.\textsuperscript{12,17}

In this work, the therapeutic drugs considered are pyrimidine compounds. These are a specific group of chemotherapeutics, or prodrugs, that need the phosphorylation pathway in cells to become pharmacologically active agents. This group includes 5-fluorouracil (5-FU), gemcitabine (Gem), capecitabine (Cap), arabinosylcytosine (Ara-C), and decitabine (Dec). The compounds are also known as antimetabolites in which the mechanism of action to eradicate cells is to block normal DNA and RNA synthesis for cell division and repair. Being similar in structure to the common pyrimidine-based nucleic acids or deoxyribonucleosides such as uracil and deoxycytidine, respectively, the compounds can be incorporated into key enzymes or in DNA/RNA strands to induce cell death. The diagram in Figure 1-1 summarizes the key pathways for drug metabolism.
Figure 1-1. Metabolism of 5-fluorouracil (5-FU), gemcitabine (Gem), capecitabine (Cap), arabinosylcytosine (Ara-C), and decitabine (Dec), leading to RNA or DNA damage in a cell. The active phosphorylated metabolites are in red. Enzymes are specified in blue (DPD, dihydropyrimidine dehydrogenase; CDA, deoxycytidine deaminase; CDK, deoxycytidine kinase; TS, thymidylate synthase; TK, thymidine kinase; OPRT, orotate phosphoribosyltransferase; TP, thymidylate phosphorylate).

While these are deadly mechanisms to prevent the proliferation of rapidly dividing cancer cells, at least 80% of the administered 5-FU is catabolized to the inactive form by dihydropyrimidine dehydrogenases (DPD) in the liver (similarly for capecitabine when converted to 5-FU), ~90% of Gem becomes inactivated intracellularly by deoxy/cytidine deaminases,\(^{19,20}\) ~70-80% of the administered Ara-C is excreted in urine as the inactive form by pyrimidine nucleoside deaminases,\(^{21}\) and less than 1% of the administered decitabine is recovered in urine, which is suggestive of total body clearance via metabolism by deaminases.\(^{22}\)

Treatments by intravenous delivery with short half-lives can be administered by continuous infusion to maximize the therapeutic benefits. The side effects are consequently the result of these
rigorous protocols and from the breakdown of accumulated chemotherapeutics in healthy tissue. The use of CPSNPs to target and concentrate the agents in cancer cells will not only minimize the side effects, but also protect agents from drug inactivation pathways by delivering the phosphorylated active metabolites directly. Phosphorylated agents are compatible with the calcium phosphate platform in CPSNPs, fundamentally, because calcium binds to phosphates. The discussions on effective drug encapsulation is further gauged in Chapter 3 and applied in Chapter 4.

1.1.2 Double reverse-micelle synthesis and encapsulation mechanism insights

The synthesis of CPSNPs is carried out in reverse micelles with cyclohexane as the outer bulk oil phase and nonionic surfactants, Igepal CO-520, to form reaction vesicles around water nanodroplets. The isotropic water/surfactant/oil system (that is not limited to ionic surfactants), creates thermodynamically stable microemulsions which have been used to precipitate many types of semiconductor, metallic, and oxide NPs and more. The advantages of reverse micelles enable adaptation of numerous water-soluble inorganic NP reactions to a confined space for size and shape control, and to maintain colloidal stability of the growing NPs by surfactant adsorption. The NP size in micelles is approximated by the inner water pool radii \(R\), the [water]/[surfactant] mole ratio and the micelle size can be characterized via dynamic light scattering (DLS).

The steps to produce CPSNPs are illustrated in **Figure 1-2** for a double reverse micelle system. The amounts of the aqueous precursors that contain calcium, phosphate, and silicate ions, are restricted to maintain an \(R\) value of 4. Starting with surfactants dispersed in cyclohexane (A), solubilized reactants form the aqueous pools of two micelle suspensions (B). The reaction that promotes nucleation and growth is initiated when the two suspensions mix to exchange core contents through collision, coalescence, and fragmentation of the micelles (C). NP growth (D) is
quenched by sodium citrate that robustly binds to available calcium ion sites to cease calcium phosphate formation. The citrate ligand also serves as an electrostatic stabilizer to prevent agglomeration of NPs after the micelles erupt by a low dielectric solvent such as ethanol (E). Doping agents are added on either the calcium or phosphate-containing microemulsion at step (B). Agents that are calcium ion-competing are typically added to the phosphate side.

**Figure 1-2.** Illustration of the double-reverse micelle synthesis starting with (A) 29 vol% Igepal CO-520 surfactant dispersed in cyclohexane. In (B), the addition of calcium chloride forms the first microemulsion while sodium hydrogen phosphate and metasilicate form the second. Water, drug, or dye, agents are typically added to the phosphate-containing side for CPSNPs. The water pool radius in the micelle and can be adjusted by the [water]/[surfactant] mole ratio. Micellar exchange through fusion, coalescence, and fragmentation, of micelles in (C) occurs after the two microemulsions mix. Nucleation and growth in (D) results in the precipitation of calcium phosphosilicate nanoparticles that are electrostatically dispersed with a layer of citrate in (E). High-angle annular dark field imaging (HAADF) of an osmium-stained CPSNP next to (D) reveal 1-3 nm sub-particles and is highly suggestive of an agglomerative-growth mechanism that is known to occur for Stöber silica.
The mechanism to CPSNP formation is currently under investigation. Preliminary findings\(^a\) (see Appendix G for synthesis and characterization particulars) suggest an agglomerative-growth process where therapeutic or dye molecules adsorb to primary nuclei between steps (C) and (D) and become encapsulated within the matrix as growth proceeds. Primary CPSNP nucleation in (C) produces 1-3 nm nuclei that aggregate in step (D) to undergo secondary growth into the final 40-60 nm CPSNPs. This agglomerative-growth model\(^{25}\) is well-known in Stöber silica NPs\(^{26-28}\) and is speculated to be observed in the calcium phosphate system for the first time. The implications of this mechanism is true encapsulation in which the doping agents are entrapped and protected within the CPSNP matrix and the only mechanism of release is by dissolving the matrix. Whether doping agents can bind to the calcium phosphate precursors for uptake is our main interest for encapsulating phosphorylated molecules.

The use of adsorption to enhance the loading efficiency of agents have been reported with Stöber NPs and organic dyes. The Stöber method involves the hydrolysis and condensation of tetraethylorthosilicate (TEOS) in ethanol with aqueous ammonium hydroxide as a catalyst to produce silica spheres.\(^{26}\) There are several ways to achieve encapsulation for organic dyes: (1) utilize non-covalent interactions between the agent and silica precursors, (2) covalent bonding of the dye to the silica precursor, (3) solubilize the dye in a reverse microemulsion with the silica precursor, and (4) have the dye adsorb to a selectively dissolvable substrate (e.g., gold NPs)\(^{29}\) and entrap the substrate in a silica shell with the method by Liz-Marzan et al.\(^{30}\) The incorporation of organic dyes into Stöber silica has been found to be significantly dependent on the adsorption forces between doping agents and silica starting materials.\(^{31}\) The first method yields poor encapsulation

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efficiencies and gradual dye leakage from the silica matrix, so encapsulation by covalent interactions or in microemulsions are generally the preferred methods.

1.1.3 Packed-bed vdW-HPLC laundering, colloidal stability in complex liquids, and surface modification

Surfactants offer a wide range of synthetic routes for various types of NPs. One of the major challenges in microemulsion synthesis, however, is effective washing of the surfactants without compromising the NP surface integrity for colloidal stability. In drug delivery, surfactants are a source for cell toxicity and is a concentration dependent effect.\textsuperscript{32} The van der Waals high performance liquid chromatography (vdW-HPLC) method to wash and disperse NPs was first developed by Adair and coworkers for SiO\textsubscript{2}-based nanocomposites that was then modified for CPSNPs.\textsuperscript{33-34} Washing by differential centrifugation was also recently presented in Tabakovic’s work\textsuperscript{34} with mixed solvents for sedimentation and re-dispersion of CPSNPs. The advantage of using the packed-bed vdW-HPLC is the ability to manipulate surface interactions to maximize collection of low density NPs are not easily sedimented by centrifugation. The packed-bed method to control deposition and the release of NPs from the stationary phase is illustrated in Figure 1-3.
Figure 1-3. Illustration of packed-bed vdW-HPLC washing of CPSNPs with UV-Vis monitoring of Igepal CO-520 at 276 nm vs. time. The column glass beads were pre-conditioned in neat ethanol adjusted with 1M KOH to an operational pH 7. In (A), the erupted micelle suspension that contains nanoparticles in cyclohexane and ethanol are deposited onto the stationary phase (glass beads). The mobile solvent is switched to neat ethanol in (B) to wash out the nonionic surfactants as CPSNPs remain attached to the beads from the minimized electrostatic repulsive forces in a low dielectric constant solvent. In (C), 70/30 ethanol/water (v/v) is introduced and CPSNPs recover negative zeta potential of the same surface polarity as the glass beads. This generates enough repulsive electrostatic force that release CPSNPs from the column.

In step (A), the erupted micelle suspension is loaded onto the packed-bed column containing glass beads that were pre-conditioned in neat ethanol (adjusted to an operational pH 7 with 1M KOH). The suspension contains a mixture of NPs, 2 % water, 25 % cyclohexane, 10 % surfactant, and 63 % ethanol (v/v). At maximum UV-Vis absorption at 276 nm, the surfactants and cyclohexane elute from the column as the NPs deposit onto the spheres. In (B), remaining surfactant is washed from the column with ethanol as CPSNP retention on the stationary phase is
preserved, and in (C), CPSNPs are collected and dispersed in 70/30 ethanol/water (v/v) at an operational pH 7.

In water, NPs form an electrical double layer (Figure 1-4) via surface group dissociation, adsorption of ionic species or surfactants from solution, and isomorphic substitution, mechanisms that generate surface charge.\textsuperscript{34-35} At the edge of the Stern plane, known as the slip plane, a liquid boundary divides surface ions traveling with the particle and ions that interact with the surface from the diffuse layer. This boundary is quantified as the zeta potential (\(\xi\)-potential) and is a metric for colloidal stability. Also referred as the electrostatic potential, the values are generated from electrophoresis where an electric field is applied and particle velocity is measured. Stability of a suspension is indicated by the sign (+/−) and magnitude of the potential. Particles that hold more positive or negative surface charge will travel at higher velocities, which translate to a larger potential magnitude than particles with minimal to no charge. Particles in the latter scenario are more likely to agglomerate from the lack of repulsive interactions.

\textbf{Figure 1-4.} Illustration of the electrical double layer consisting of the diffuse and Stern layers generated by a particle and surrounding counter-ions when suspended in water. Redrawn from Ref. \textsuperscript{36}.
The total particle interaction energy \( (V_T) \) is a sum of attractive van der Waals \( (V_A) \) and repulsive electrostatic forces \( (V_R) \) and this can be manipulated by changes in solvent pH or the dielectric constant \( (\varepsilon) \) to induce deposition and detachment of NPs from a surface for washing applications. In the case of the packed-bed column, attractive and repulsive forces in complex non-aqueous systems are governed by the zeta potential magnitude of CPSNPs. The three favorable solvent conditions, summarized by Tabakovic,\(^{34}\) have relatively low (Figure 1-3A; cyclohexane/Igepal CO-520/ethanol/water mixture; \( \varepsilon = 18.37 \)), intermediate (Figure 1-3B; ethanol; \( \varepsilon = 24.5 \)), and high (Figure 1-3C; 70/30 ethanol/water; \( \varepsilon = 30.76 \)), dielectric constants. These are responsible for the respective deposition, retention, and elution, mechanisms of CPSNPs with glass beads. Transition between the three solvent stages results in CPSNPs with increasingly negative zeta potential values (or repulsive forces) from ~0 mV during deposition, +2 mV during washing, and up to -50 mV during NP collection. CPSNP dispersion in 70/30 ethanol/water is achieved with surface citrate ligands acting as electrostatic barriers.

Sodium citrate with terminal carboxyl groups is a common dispersant for colloids. The citrate layer also provides an active surface for NPs to participate in covalent interactions for surface conjugation of PEGs and receptor targets. The most straightforward and common route to obtain these modifications is through the use of carbodiimides to activate the carboxyl groups for primary amine coupling.\(^{37}\) The optional use of N-hydroxysulfosuccinimide (Sulfo-NHS) stabilizes an o-acylisourea intermediate to form an amine-reactive sulfo-NHS ester to improve the coupling efficiency.\(^{38}\) Figure 1-5 outlines the coupling reactions that have been used by our group to yield CPSNPs with methoxy, gastrin, and aptamer, surface group terminations.
Figure 1-5. Surface modification via carbodiimide (EDC; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) reaction chemistry to activate CPSNP carboxyl termination groups to form primary amine linkages with PEG. The addition of sulfo-NHS is optional, but can be used to improve the coupling efficiency. The PEGylated CPSNPs were conjugated to the following targets: amine-terminated aptamer to carboxyl-PEG-CPSNP, cysteine-terminated gastrin to maleimide-PEG-CPSNP, and methoxy-PEG-CPSNP for no target (controls).

The aptamer oligo (see Appendix H for AP1153 aptamer particulars) and gastrin peptide sequences were customized to target pancreatic cancer receptors. Figure 1-6 compares the targeting studies completed in vivo before and now on ICG-doped CPSNPs injected (tail vein) into athymic nude mice models with BxPC-3 and PANC-1 human pancreatic cancer cells that were xenografted orthotopically into the pancreas.
Figure 1-6. Comparison of *in vivo* targeting with indocyanine green-doped (ICG) CPSNPs from earlier work by Barth et al.\(^\text{17}\) on gastrin-10 (G10) and latest work by Clawson et al.\(^\text{b}\) on gastrin-16 (G16) and the aptamer targets. In (A), athymic nude mice models with orthotopic BxPC-3 human pancreatic cancer were imaged 24 h post-tail vein injection of ICG-doped CPSNPs bioconjugated with (i) no target (ii) G10, and (iii) pentagastrin. G10 promoted off-target binding of the nanoparticles in the gastrointestinal tract and the brain. The corresponding excised organs shown in (B) highlight nanoparticle uptake in the pancreas for all three injections as well as off-target binding in the brain for G10 nanoparticles. In (C), 18 h post-injection images are shown of the same mice models, but with PANC-1 human pancreatic cancer, that received ICG-doped CPSNPs bioconjugated with (i) no target, (ii) G16, and (iii) aptamers. While CPSNPs were not detected in the brain, G16 nanoparticles were bound to the pancreas, stomach, and intestines, where CCKB receptors are in abundance. Aptamer nanoparticles showed specific uptake in the pancreas/tumor without off-target effects. The excised organs after 24 h are in (D) for mice treated with G16 nanoparticles and in (E) for the aptamer nanoparticles. Hepatic clearance of CPSNPs resulted in relatively significant accumulation in the liver, kidneys, stomach, heart, and intestines (all but the tumor-bearing pancreas). Images adapted from Ref. \(^\text{17}\) and animal models for the G16 and aptamer targets courtesy of G.L. Matters group from Penn State Hershey Biochemistry and Molecular Biology Department.

In the past, *in vivo* in athymic mice targeting with gastrin-10-modified CPSNPs showed uptake in the pancreas (A-ii).\(^\text{17}\) Off-target staining was also significant in other organs (mainly the stomach) where endogenous cholecystokinin-B (CCKB) receptors for gastrin are expressed.
Additionally, G10 facilitated crossing of the blood brain barrier (B-ii). A longer peptide, gastrin-16, was used to minimize NP uptake in the brain, but still promoted off-target binding in the gastrointestinal tract (C-ii).\textsuperscript{39,b} Gastrin was also found to stimulate tumor cell proliferation, which is an effect not observed in the recent aptamer target development led by Clawson et al. (C-iii).\textsuperscript{b}

The aptamer CPSNPs accumulated mainly in the pancreas where the PANC-1 tumor manifested before ICG cleared via hepatic clearance (D and E showing clearance for both C-ii and C-iii cases, respectively).

1.1.4 Seeding secondary nucleation and growth of calcium phosphosilicate (CPS) on gold core nanoparticles

There are three reasons why the encapsulation of gold NPs in calcium phosphosilicate (CPS) is of interest to this research. First, as briefly mentioned in Section 1.1.1, gold NP accumulation is a concerning phenomenon because little is known about the toxicity mechanisms. It is hypothesized that encasing gold with CPS will mitigate these effects so that Au-CPS can circulate through the same CPSNP clearance pathways. Second, we want to stretch the applications of phosphorylated agent encapsulation to solid surfaces. That is, chloroauric acid is directly reduced by sodium thiophosphate into colloidal gold with ideal surface groups to immobilize calcium ions and seed CPS nucleation and growth. And third, the use of CPS allows for simultaneous

encapsulation and protection of phosphorylated drug agents within the shell matrix to create a theranostic platform. The proposed NP design is presented in Figure 1-7.

![Illustration depicting the formation of a Au-CPS core-shell hybrid nanoparticle with drug agents encapsulated within the CPS matrix. The Au core is precipitated by reduction with sodium thiophosphate (the surface group net charge may vary depending on the pH and presence of dissolved ion species). The core-shell nanoparticle is a multi-functional platform with therapeutic and imaging capabilities. A citrate-functionalized surface allows colloidal stability through electrosteric interactions and for conjugation of PEGs and targets.](image)

Figure 1-7. Illustration depicting the formation of a Au-CPS core-shell hybrid nanoparticle with drug agents encapsulated within the CPS matrix. The Au core is precipitated by reduction with sodium thiophosphate (the surface group net charge may vary depending on the pH and presence of dissolved ion species). The core-shell nanoparticle is a multi-functional platform with therapeutic and imaging capabilities. A citrate-functionalized surface allows colloidal stability through electrosteric interactions and for conjugation of PEGs and targets.

Mechanisms behind the wet chemical reduction of gold by thiophosphate is first explored to evaluate the final surface functionality, colloidal stability, and methods for size control. The earliest foundations to colloidal synthesis was laid out by Michael Faraday in 1857 when he postulated that the transparent red solution from chloroaurate reduction with white phosphorus was caused by colloidal gold.\(^{40}\) His work on gold sols was ground-breaking as he revealed size-dependent optical properties of metallic particles without ever physically observing the materials at the nanoscale. Many models were developed in subsequent years to aid the mechanistic interpretations of metal NP formation since Faraday’s discovery (see Table 1-1).\(^{41-42}\) Over a century later, our understanding of the mechanisms has been broken down to two events: nucleation and growth. But even today, characterization of nucleation events is limited by the size where sub-5 nm nuclei made of several hundred atoms are too large to be treated as single atoms and too small to
apply bulk thermodynamic theories, probed by light scattering, and by most electron microscopy, methods.\textsuperscript{43} The timescale at which the nucleation occurs is also typically brief in nature for NPs. Regardless, it is incredible that modern nanoscience has managed to yield a variety of semiconductor and metallic materials with such precision on size and morphology based on deductions from a sequence of observable events.

Since Faraday, Wilhelm Ostwald introduced the concept of Ostwald ripening in which NP growth is explained by the dissolution of smaller particles that re-deposit on larger particles.\textsuperscript{44} With the invention of the immersion ultramicroscope in 1910s by Richard Zsigmondy,\textsuperscript{45} observations of colloids with diameters below the wavelength of visible light (<400 nm) led to the birth of the classical nucleation theory\textsuperscript{46} and subsequent LaMer burst model.\textsuperscript{47} The combined Lifshitz-Slyozov-Wagner (LSW) model was the first to mathematically demonstrate Ostwald ripening as a diffusion-limited process. This remains as one of the most predominant models for NP growth along with LaMer nucleation.
Table 1-1. Summary of nucleation and growth models for nanoparticles with later key additions and modifications.

<table>
<thead>
<tr>
<th>Parent model</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classical nucleation</td>
<td>The foundations of homogenous nucleation stem from the thermodynamic</td>
</tr>
<tr>
<td>(1878, 1935)</td>
<td>postulations by Gibbs on changes of total free energy, $\Delta G$. Kinetic</td>
</tr>
<tr>
<td></td>
<td>models by Volmer and Weber (later improved by Becker and Döring),</td>
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<tr>
<td></td>
<td>involved the phase transformation of supersaturated water vapor to a</td>
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<tr>
<td></td>
<td>liquid. The process produced short-lived condensation nuclei that</td>
</tr>
<tr>
<td></td>
<td>evaporated and reformed continuously until stable droplets were formed</td>
</tr>
<tr>
<td></td>
<td>by coagulation or in a highly supersaturated vapor environment. A stable</td>
</tr>
<tr>
<td></td>
<td>droplet only formed when additions of the parent phase to the nucleus</td>
</tr>
<tr>
<td></td>
<td>reached a critical size to grow the new phase.</td>
</tr>
<tr>
<td>LaMer burst nucleation and</td>
<td>$LaMer$ burst nucleation and growth (1950) – distinctly separates</td>
</tr>
<tr>
<td>growth (1950)</td>
<td>nucleation and growth as two distinct stages from studies on homogenous</td>
</tr>
<tr>
<td></td>
<td>nucleation and precipitation of sulfur sols. The concepts extended to NP</td>
</tr>
<tr>
<td></td>
<td>applications.</td>
</tr>
<tr>
<td>Heterogeneous nucleation</td>
<td>$Heterogeneous$ nucleation (1952) – nucleation occurs more readily than</td>
</tr>
<tr>
<td>(1952)</td>
<td>homogeneous nucleation at an existing surface or interface. The energy</td>
</tr>
<tr>
<td></td>
<td>barrier for nucleation is now influenced by distortions of the deposited</td>
</tr>
<tr>
<td></td>
<td>nuclei, which changes the volume free energy, and the creation of a new</td>
</tr>
<tr>
<td></td>
<td>solid-solid interface, which changes the interfacial free energy.</td>
</tr>
<tr>
<td>Ostwald ripening</td>
<td>Growth is a diffusion-limited process, dominated by surface energies of</td>
</tr>
<tr>
<td>(1900)</td>
<td>suspended NPs. There exists a chemical potential gradient between atoms</td>
</tr>
<tr>
<td></td>
<td>in the interfacial region and surrounding bulk phase. Based on the Gibbs-</td>
</tr>
<tr>
<td></td>
<td>Thomson equations, smaller particles have</td>
</tr>
<tr>
<td>Lifshitz-Slyozov-Wagner</td>
<td>$Lifshitz$-Slyozov-Wagner (LSW, 1961) – first mathematical models for</td>
</tr>
<tr>
<td>(1961)</td>
<td>Ostwald ripening that described diffusion as the slowest step in growth.</td>
</tr>
<tr>
<td>Finke-Watzky (1997)</td>
<td>$Finke$-Watzky (1997) – proposed that nucleation is the slow step and</td>
</tr>
<tr>
<td></td>
<td>occurs simultaneously with autocatalytic growth.</td>
</tr>
</tbody>
</table>

Ref. 46, Classical 47-48, LaMer 49 50, Ostwald 51-52, LSW 53
<table>
<thead>
<tr>
<th><strong>Increasingly higher chemical potential, which lead to solid dissolution.</strong> The solutes re-deposit and grow on larger particles. Particle “coarsening” via mass transport or diffusion-limited processes can also be explained by this theory.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oriented attachment</strong> (HREM* observations from early 1990s via sintering; term coined later by Penn and Banfield) – preceding growth, particles preferentially attach to one another at crystallographic surfaces that are similar in dimension to reduce surface energy.</td>
</tr>
<tr>
<td><strong>Coalescence/aggregation/coagulation</strong> (HREM observations from early 1990s via sintering) – preceding growth and unlike oriented attachment, particles join at dissimilar crystallographic planes in a random-like manner.</td>
</tr>
<tr>
<td><strong>Intraparticle ripening</strong> (2000) – monomer diffusion is driven by differences in facet energies on a NP. The monomers preferentially grow on low energy facets while high energy facets dissolve away. This yields particles of various shapes.</td>
</tr>
<tr>
<td><strong>Digestive ripening</strong> (2005) – the opposite process of Ostwald ripening where larger particles are consumed to grow smaller particles.</td>
</tr>
<tr>
<td>54-55</td>
</tr>
<tr>
<td>54, 56</td>
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<tr>
<td>57</td>
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<td>58</td>
</tr>
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</table>
The two types of nucleation are homogenous and heterogeneous. The first kinetic models for the classical theory are for homogenous nucleation from the condensation of supersaturated water vapor. To explain within the context of this work on NP precipitation, we can consider the scenario where a stationary solid phase forms within the liquid parent phase by the packing of atoms. Each solid nucleus is assumed to be spherical with a radius, \( r \). This spontaneous transformation event only occurs when Gibbs free energy, \( \Delta G \), is negative. Changes in the free energy are contributed by (1) the latent heat of crystallization released from bond formation and (2) the energy needed to create a new surface. Thus, total \( \Delta G \) is the sum of the following energies,

\[
\Delta G = \frac{4}{3} \pi r^3 \Delta G_v + 4\pi r^2 \gamma
\]

From contribution (1), the overall volume free energy is the product of \( \Delta G_v \) and the spherical volume, \( \frac{4}{3} \pi r^3 \). By convention, \( \Delta G_v \) is negative. From contribution (2), the new surface that sets the solid-liquid phase boundary has a positive surface free energy, \( \gamma \), and is multiplied by the spherical surface area, \( 4\pi r^2 \). Therefore, the \( \Delta G_v \) parameter predominates for cases of large nuclei and the \( \gamma \) parameter predominates for smaller nuclei. These free energy parameters are normally plotted as a function of nucleus radius as shown in Figure 1-8.
The nucleus is required to reach a critical radius, $r^*$, before growth can take place. The critical free energy, $\Delta G^*$, is also an activation energy barrier. Below $r^*$ or $\Delta G^*$, the nucleus redissolves and reforms until enough building blocks join to create a stable nucleus. The critical radius and $\Delta G^*$ are derived from Equation 1.1 with respect to $r$,

$$
\frac{d\Delta G}{dr} = 0 = \frac{4}{3} \pi \Delta G_v (3r^2) + 4\pi\gamma (2r)
$$

When we solve for $r$ in Equation 1.2, assuming $r = r^*$,

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

Further, $\Delta G^*$ is obtained by substituting Equation 1.3 into 1.1,

$$
\Delta G^* = \frac{16\pi\gamma^2}{3(\Delta G_v)^2}
$$

The activation barrier is related to the critical supersaturation ratio, $s^*$. Supersaturation, $s$, is the ratio of the solute concentration to solubility. Via the Gibbs-Kelvin equation, the volume free energy is the following,
(1.5) \[ \Delta G_v = \frac{mkT}{v} \ln s = \frac{4y}{2r} \]

With this, **Equation 1.4** can be rewritten as,

(1.6) \[ \Delta G^* = \frac{16\pi y^3v^2}{3(mkT \ln s)^2} \]

The terms \( m \) is the number of ions in a neutral molecule, \( T \) is temperature, \( k \) is the Boltzmann constant \((1.3807 \times 10^{-23} \text{ J/K})\), and \( v \) is the molecular volume. The rate of nucleation, \( N \) (nuclei per volume/s), is derived from the law of mass action,

(1.7) \[ \frac{dn^*}{dt} = N = A \exp\left(-\frac{\Delta G^*}{kT}\right) = A \exp\left(-\frac{16\pi y^3v^2}{3k^3T^3(m \ln s)^2}\right) \]

where \( n^* \) is the number of nuclei of the solid phase formed over time, \( t \). When \( N \) is plotted as a function of \( s^* \), we can see that nucleation rate is a slow process below \( s^* \) and fast above \( s^* \) (**Figure 1-9**).

![Figure 1-9. Rate of homogenous nucleation, \( N \), as a function of supersaturation ratio. Redrawn from Ref. 43.](image)

Unlike a homogeneous system (e.g., synthesis of wholly CPSNPs), heterogeneous nucleation occurs more readily on an existing surface or interface, and so, \( \Delta G^* \) is considerably lower than homogenous nucleation. The matter of seeding CPS growth on a gold NP is a heterogeneous nucleation process where the material being crystallized is different from the seed.
In the ideal scenario, the terminal thiophosphate groups on gold NPs provide sites to nucleate a solution of calcium phosphate with silicate. The challenge is to identify a balance of the CPS precursor concentration in solution to the gold NPs so that homogenous nucleation of calcium phosphate does not dominate. While the primary interest is to synthesize and study the gold NP, examples of this are given and discussed later sections of Chapter 5.

1.2 Objectives

The main objectives presented in this research involve developing therapeutic and theranostic solutions for pancreatic cancer using CPSNPs as a delivery platform. In particular, the use of phosphate chemistry is of interest for optimizing active drug uptake in the NPs and for encapsulating solid gold in a calcium phosphate matrix.

- Chapter 1 is a summary of the previous work on CPSNPs (the reverse micelle synthesis, vdW-HPLC laundering, and targeted delivery of ICG) and latest developments on drug encapsulation and targeting. The history and concepts of classical particle nucleation and growth are also covered.

- Chapter 2 is a literature review manuscript on the biology and genetics of pancreatic cancer, conventional treatment protocols, and potential next-generation medicine or methodologies for early detection, prevention, and disease management. Emphasis is placed on NP avenues such as CPSNPs.

- Chapter 3 evaluates the adsorption behavior of phosphorylated compounds on calcium phosphate as a proof-of-concept for encapsulation. The adsorption of citrate, ATP, FdUMP, and 5-FU are compared. The effects of potential-determining ions (phosphates, protons, and hydroxyls) on the surface polarity of calcium phosphate are examined by the zeta potential as a function of pH.
• Chapter 4 applies the work from Chapter 3. The encapsulation efficiencies of pyrimidine-based cancer drug analogs 5-FU, FUdR, and Gem, and their phosphorylated counterparts, FdUMP and GemMP, are compared. *In vitro* analyses on human pancreatic cancer cell proliferation are used to confirm the potency of the encapsulated dose relative to an unencapsulated dose of FdUMP. The toxic effects of the FdUMP-doped CPSNPs are relatively spared on healthy cells. The biological activity of encapsulated FdUMP, or the ability to inhibit thymidylate synthase, is retained and verified *in vitro* and then *in vivo* with aptamer-1153 to target PANC-1.

• Chapter 5 covers the homogenous synthesis of gold NPs by chloroauric acid and sodium thiophosphate in water. The reduction mechanism is modeled with the JMAK equation to understand particle growth and size over time at various chloroauric acid to thiophosphate molar ratios. Different starting concentrations of CPS precursors (1, 1/10, 1/100 of the standard CPSNP concentrations) are then used to promote CPS shell growth on the synthesized gold seed NPs.

• Chapter 6 is a discussion of the Adair group research in the context of translational science and where the work fits within the conventional scheme. Experimental scenarios are given to highlight important points for NP formulations in the pre-clinical stage.

• Chapter 7 summarizes findings from this dissertation and provides suggestions for future work.

Additionally, there are eight appendices that supplement Chapters 3-6.

• Appendices A to C provide all the relevant sample IDs from the drug encapsulation work, and information on the materials for reproducing the results.
• Appendix D outlines the method used for particle size analysis with TEM images.

• Appendix E contains the protocol developed for quantifying endotoxins in water for a surface science laboratory. A detailed log of endotoxin levels in selected buildings on campus is included.

• Appendix F contains the formulation sheet for CPSNP synthesis and wavelength settings for vdW-HPLC washing.

• Appendix G presents a method developed to stain CPSNPs with osmium for high-contrast TEM imaging to study growth/encapsulation mechanisms.

• Appendix H presents the aptamer target properties that is currently in use for in vivo mice model targeting of human pancreatic cancer with CPSNPs.
Chapter 2

Pancreatic cancer: pathogenesis, prevention, diagnosis, treatment, and evidence-based medicine.

(This chapter has been published as an invited review in Loc, W.S.; Smith, J.P.; Matters, G.; Kester, M.; Adair, J.H. *World Journal of Gastroenterology*. 2014, 20 (40), 14717–25 and is reproduced and updated here with permission from the Baishideng Publishing Group, Inc for academic use only.)

In the absence of effective treatment options, achieving early diagnosis in patients that are at risk of developing pancreatic ductal adenocarcinoma (PDAC) is a priority in this research area. This chapter is an overview of conventional strategies that are used to manage pancreatic cancer and recent discoveries on the role of early onset symptoms leading to tumor progression. Investigations include pancreatic type 3c diabetes, selected biomarkers and pathways related to PanIN lesions, drug resistance, and advances in nanomedicine, which can provide future solutions for early detection and therapies.

2.1 Introduction

Pancreatic cancer is responsible for over 40,000 deaths every year in the United States, representing about 3% of the newly diagnosed cancer cases. However, pancreatic cancer is the fourth most common cause of cancer related death in the US, predominantly affecting patients ages 60-80.\(^{50-63}\) Pancreatic ductal adenocarcinoma (PDAC) constitutes up to 95% of pancreatic malignancies.\(^{63}\) Due to poor prognosis and delayed treatment, survival rate during the first year of diagnosis is 20% at most and 6% by the fifth year.\(^{61}\)

PDAC is highly resistant to standard chemotherapeutics with extensive fibrosis that further prevents drug penetration. While early detection is possible, only 10-15% of patients are diagnosed
early enough for surgical resection to be offered.\textsuperscript{64-65} Over 90% of patients are diagnosed with PDAC in the advanced stages.\textsuperscript{63}

Efforts over the years have been focused on translational science research, particularly nanomedical avenues, to create novel drug delivery approaches and to understand the early developmental stages of pancreatic cancer. Stem cell signaling pathways or genetic markers, for instance, have been used to develop targets for personalized medicine and to reduce the side effects of common drugs. Nanomaterials can also be engineered as multi-functional vehicles for both imaging and therapeutic applications. Such discoveries promise better alternatives to chemotherapy and radiation by providing versatile platforms that are tailored to perform at optimal efficacy.

### 2.1.1 Histopathology of pancreatic cancer

Tumors are classified as invasive ductal carcinoma (IDC), intraductal papillary mucinous neoplasm (IPMN), neuroendocrine tumors (NET), or islet cell tumors.\textsuperscript{66} Invasive ductal carcinoma is referred to as pancreatic ductal adenocarcinoma (PDAC). Pancreatic intraepithelial neoplasia (PanIN) lesions are suspected as precursors of PDAC coinciding with multiple successions of genetic mutations.\textsuperscript{67} These mutations may be provoked by inflammatory stimulus such as alcohol abuse or metabolic syndrome.\textsuperscript{68} PanIN lesions are categorized as type 1A, 1B, 2, or 3, from minimum to severe expansion of immature cells at the ductal epithelium (Figure 2-1).
Figure 2-1. An example of PanIN development to PDAC. Based on the grade of dysplasia or abnormal cell growth, PanIN lesions are classified as Types 1A/B, 2, 3, and PDAC as the latest stage. Inflammatory stimuli trigger the activation of pancreatic stellate cells (PaSC), principle progenitors of stroma fibrosis, in surrounding acinus cells. Inflammatory cells (monocytes, T-cells, neutrophils, mast cells, and macrophages) gather and release interleukin ligands that activate genes to promote PanIN development in susceptible tissue with oncogenic mutations such as the KRAS. Figure adapted and redrawn with permissions from Macmillan Publishers Ltd and Elsevier from Refs. 68 and 69.

Genetic defects that follow PanIN-2 and PanIN-3, involve the dysfunction of one or more tumor suppressor genes that result in aberrant signaling pathways driving pancreatic cancer 60, 67, 70, 9, 11

2.1.2 Symptoms associated with PDAC

Early PanIN lesions are asymptomatic for up to years and symptoms do not manifest until the advanced stages. 71 The range of symptoms present are not pathognomonic features to pancreatic cancer. Traditional diagnoses highlight notable symptoms, including obstructive jaundice, abdominal and back pain, weight loss, anorexia, dyspepsia, gallbladder enlargement, migratory
thrombosis (Trousseau syndrome), subcutaneous fat necrosis (panniculitus), and hyperglycemia.

Carcinoma at the head of the pancreas is often detected when tumors compress the bile duct, resulting in obstructive jaundice in about 75% of subjects. Nausea, vomiting, lethargy and weight loss may result from change of appetite, bowel habits, and cancer cachexia. While PDAC can cause abdominal and back pain, it is common for patients to have painless jaundice that leave symptoms undetected. Tumors that invade the superior mesenteric vessels or splenic vein can result in hemorrhage from varices.

Collective evidence suggests that type 3c diabetes is diabetes caused by chronic pancreatitis or is the result of a paraneoplastic phenomenon from pancreatic cancer. Although further studies are to be conducted to distinguish cancer-induced diabetes from traditional exocrine pancreatic diseases, type 3c is acknowledged as a distinct type of diabetes mellitus by the American Diabetes Association. Type 3c precedes most cancer-specific symptoms by several months or years before tumors become radiologically detectable. New-onset diabetes may also increase the likelihood of pancreatic cancer by 5-8 times, with approximately 1% of patients developing the cancer within three years. Progressive and unintentional weight reduction is associated with type 3c. Unlike type 2 that is associated with weight gain and obesity, patients with type 3c continue to lose weight as glycemic control worsens with cancer advancement (Figure 2-2A). Weight loss is an early event of type 3c that is attributed to either cachexia or loss of adipose tissue. Cachexia is a chronic physical wasting and malnutrition disease that results in more than 10% body weight loss in late cancer stages. Before cachexia, weight loss is induced by adipose tissue inflammation from interactions with pancreatic cancer (Figure 2-2B). Inflammation in adipose tissue contributes to peripheral insulin resistance by altering adipocyte secretion and propagate pathogenic processes similar to type 2 diabetes.
About 90% of the hormonal secretion from adipose tissue macrophages is comprised of inflammatory cytokines. The accumulation of inflammatory cytokines triggers abnormal adipocyte secretion and reduced hepatic insulin sensitivity. This reaction leads to an increase of leptin levels (related to the loss of appetite) and decrease in adiponectin. Leptin and adiponectin are primary precursors to insulin resistance in type 2 diabetes, which can be regulated by limiting glucose intake and weight gain. Quite the contrary, dietary restrictions such as for carbohydrates have minimal effect on pancreatic cancer-induced diabetes. Weight loss symptoms and diabetes persist until the tumors are resected. A potential mediator of the cancer-associated diabetes is the over-expression of a pluripotent hormone adrenomedullin that mediates insulin resistance through the interaction of adrenomedullin receptors on beta-cells. An increase in endogenous expression of adrenomedullin results in beta-cell dysfunction which inhibits insulin secretion in the plasma and tumors.
Figure 2-2. Development of type 3c diabetes preceding pancreatic cancer. (A) A timeline comparison of weight-loss to cancer-specific symptoms and (B) a schematic representation of the cause for progressive weight reduction and insulin resistance. Adipose tissue inflammation triggers an alteration of adipocyte secretion and propagates pathogenic processes similar to type 2 diabetes, eventually leading to cachexia. Figures redrawn with permission from Ref. 75.

The relationship between diabetes and pancreatic cancer has been studied since the early 1830s, but the biological significance of type 3c diabetes in relation to pancreatic cancer had not been acknowledged until recently. 81 Early identification of type 3c could potentially lead to timely diagnosis and treatment of patients with pancreatic cancer up to years before tumors appear radiologically. However, distinction between type 2 and type 3c diabetes requires an adept level of awareness and expertise to prevent underdiagnoses or misdiagnoses of the latter. Severe weight loss is also intimately associated with a variety of cancers several months before death. 82 Thus, the
collective effects of type 3c is pivotal knowledge for distinguishing pancreatic cancer from the diverse array of malignancies.

2.2 Diagnostic tests and biomarkers

To date, a definitive test for early pancreatic cancer does not exist. Routine radiographic tests or endoscopic ultrasound screening is recommended for individuals with a family history of pancreatic cancer, chronic pancreatitis, precancerous lesions, or new-onset diabetes. Serological markers such CA19-9, MIC-1, CEA, beta-HCG, and CA72-4 have also been of interest but lack sufficient sensitivity and specificity for early detection.

Despite the vast similarities between all cancers, there is continuous discovery of genetic markers that are more distinctly related to pancreatic cancer cell phenotypes. The role of mucin-1 (MUC-1) in malignant cells was first reported to upregulate multi-drug resistance (MDR) genes such as ABCC1, ABCC3, ABCC5 and ABCB1. MUC-1 is a transmembrane glycoprotein that lines the apical surface of epithelial cells to shield against infectious pathogens. Overexpression of MUC-1 is found in patients with common cancers that include pancreatic, breast, ovarian, and thyroid cancers. There are implications that MUC-1 overexpression is enabled by the phosphatidylinositol 3'-kinase/Akt (PI3K/Akt) signaling pathway, a pathway associated with chemotherapeutic drug resistance in other cancers.

2.3 Genetic mutations associated with pancreatic cancer

There are at least 25 altered genes related to cancer pathways (e.g. cell adhesion, apoptosis, and replication) and only a handful have been identified for pancreatic cancer.
mutations are found in up to 10% of those with PDAC. A germline variant of the cholecystokinin-B gene has been identified in over 35% of patients with PDAC and predicts both risk and survival.\textsuperscript{86} Activated Kirsten-Ras (KRAS) oncogene is harbored in >95% of pancreatic cancer tumors and is critical in cell proliferation and apoptotic resistance to hostile microenvironments (in the presence of anti-cancer agents).\textsuperscript{60} Activation of the KRAS oncogene releases Ras proteins that initiate mitogen-activated protein (MAPK) cascades.\textsuperscript{95-97} MAPK participates in critical cellular events, including cell division, response to surroundings, movement, and cell death. Mutated KRAS is a medically accepted driver gene for pancreatic cancer. KRAS mutations can propagate a series of ongoing cellular signal transduction processes leading to uncontrollable proliferation and architectural abnormalities such as the replacement of acinar tissue by ductal lesions.

Mutated KRAS are known to reduce tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) sensitivity.\textsuperscript{98} Abnormalities are likely to occur at codon 12 (G12D), involving a point mutation of one glycine to aspartic acid (G12D), or glycine to valine (G12V). TRAIL is a transmembrane protein that can be proteolytically cleaved from the cell surface to mediate apoptosis and anti-tumor activities.\textsuperscript{98} KRAS mutations can trigger an enrichment of a cytokine receptor, osteoprotegerin (OPG), which directly inhibits TRAIL solubility and induce apoptosis.\textsuperscript{96-99} Interestingly, increased OPG and TRAIL levels are also found in subjects with type 2 diabetes mellitus,\textsuperscript{100} but connections to type 3c diabetes have not been implicated. Inhibitors that target oncogenic KRAS have not yet been developed, but remain an active area of investigation.

\subsection*{2.4 Signaling pathways activated in pancreatic cancer}

Oncogenes depend on various signaling pathways to initiate tumor formation. Since most attempts to inhibit oncogenes such as KRAS have failed, there is a shift in interest towards other critical pathways in targeted therapy development.\textsuperscript{101} The Notch pathway, for instance, exerts
influence on homeostasis during embryonic development in multicellular organisms and is important for the growth of the pancreas. Healthy pancreatic cells undergo three stages of growth: (1) endoderm formation, (2) pancreatic morphogenesis, and (3) beta cell differentiation to endocrine and exocrine cells. The loss of Notch signaling in the pancreas results in premature differentiation of endocrine and exocrine cells. Therefore, this pathway is essential for determining the fate of functioning pancreatic cells in epithelial and non-epithelial tissues, but contradictions persist in literature as to whether the pathway is an inhibitor or promoter for tumor progression.

Lateral inhibition mechanisms of the Notch pathway involve a group of receptors (Notch1, Notch2, Notch3, and Notch4), targets, and ligand key components that contribute uniquely to PanIN progression. For example, it has been demonstrated that deletion of the Notch1 receptor accelerates PanIN lesion development and lowers the median survival of Pdx1-CreERT2;LSL-KrasG12D, Pdx1-Cre;KrasG12D, and Ptf1a-Cre;KrasG12D mouse models. The loss of the Notch2 receptor in Ptf1a mouse models, however, halts lesion progression and increases the chance of survival. Tumor inhibition was also reported in several studies where the up-regulation of Hes1 from activated Notch pathway suppresses the expression of p57, which prevents progenitors from undergoing premature differentiation and uncontrollable proliferation. Similarly, tumor suppression was achieved in zebrafish by forcing exocrine pancreatic precursors through Notch signaling to inhibit acinar cell differentiation without affecting healthy adult cells. Ongoing investigations on type 2 diabetes imply that the Notch pathway is responsible for insulin resistance in pancreatic cells (from the expression or inactivation of Hes1 gene, Rbp-Jk protein ligand, and Ngn3 gene). The function of the Notch pathway during PDAC development is dependent on the targeted receptor and the genes expressed. Thus, a target for the Notch signaling pathway may offer a viable therapeutic route for pancreatic cancer, but in general such therapies must be exercised.
with caution since a wide variety of cells rely on ligand-dependent pathways for growth and survival.

2.5 Treatment

Treatment for late-stage PDAC patients are limited to chemotherapy and radiation. Conventional chemotherapeutic agents are ineffective against PDAC for several reasons among which include fibrosis. PDAC tumors are highly fibrous and poorly vascularized, prohibiting adequate penetration by chemotherapeutic agents. The heterogeneous nature of cancer cells and tissue hypoxia is also associated with drug resistance, often requiring higher drug dosages during treatment and increased toxicity such as peripheral neuropathy, bone marrow toxicity, and cardiotoxicity. Gemcitabine is considered as the gold standard for advanced PDAC, but only affords survival up to six months. Survival on gemcitabine is typically improved in conjunction with other agents.

Capecitabine and 5-fluorouracil are common antimetabolites in clinical trials as a standard single-drug treatment. These drug agents have been used along with platinum-based agents and others such as leucovorin, exactecan, and irinotecan. Radiation therapy is recommended in conjunction as an adjuvant and a chemosensitizer. Clinical trials that administer combined drug therapy such as FOLFIRINOX (5-fluorouracil with leucovorin, irinotecan, and oxaliplatin) have shown greater efficacy for metastatic cancer, but with profound limitations due to systemic toxicity and neurotoxicity. More recently, survival from PDAC has been marginally improved by using a combination of protein-bound nab-Paclitaxel and gemcitabine.

Alternatively, surgery is offered when the absence of metastasis is verified by PET, MRI, and enhanced CT scans. The Whipple operation is performed at the head of the pancreas given that
the superior mesenteric vessels are not affected.\textsuperscript{65,123} The Pylorus-preserving Whipple operation removes the first section of the duodenum and/or a part of the stomach. Adjuvant chemotherapy and radiation therapy typically follow resection to decrease relapse rates. However, preoperative chemotherapy and radiation therapy can restage tumors that disqualify patients to surgical resection. Surgery assures the longest survival, but the majority of candidates are not eligible for resection because of late discovery.\textsuperscript{124-125} This further justifies the need for early detection screening that is not yet available in practice.

2.6 Future directions based on nanomedicine

Given the grim statistics and negative effects of drug treatment, it is no surprise that novel cancer therapies are in demand. Personalized medicine research on small molecules such as proteins and nucleic acids, and inorganic platforms such as composite NPs and polymers, are a few of the many well-established fields. The ability to simultaneously seek, treat, and track tumors is slowly emerging from the basic nanoscience toward clinical deployment to treat pancreatic cancer. Adair et al. reviewed the selection criteria for drug delivery strategies based on several nanomaterial platforms.\textsuperscript{10} The selection criteria for nanomaterial drug delivery systems are summarized in Tables 2-1 and 2-2. Strategies developed by nanotechnology can lead to advantages in early detection via bio-imaging and specific targeting of cancer cell receptors. Drug delivery procedures can be improved to overcome drug resistance without causing serious off-target toxicity.
Table 2-1. Desired characteristics for a nanoparticle drug-delivery platform*

<table>
<thead>
<tr>
<th>Desired characteristic</th>
<th>Comments</th>
</tr>
</thead>
</table>
| Biocompatibility: inherently non-toxic material and degradation products | The initial material selection should be based on non-toxic materials that can be cleared or reabsorbed with an aim towards human health care.  
There is not a particular size that seems most efficacious, particularly based on *in vivo* studies although there have been reports that sub-100nm provides optimal fibrotic pancreatic tumor penetration. About 10-200nm is a range of particle diameters that have proven most effective for a wide variety of delivery systems. Also of note is the debate around the influence of particle shape.  
The active agent must be encapsulated within the nanoparticle vehicle. Surface decoration (*i.e.*, adsorption) will often be effective *in vitro* but falls short for *in vivo* studies because of the reticuloendoplasmic systems *in vivo*.  
The nanoparticle vehicle and surface functionalization must resist agglomeration for the solution pH values, ionic strength, macromolecular interactions, and temperature encountered in the physiological environment.  
The nanoparticle vehicle must have a ready clearance mechanism to avoid the cumulative and/or systemic effects of the drug-laden particles.  
Resistance to agglomeration and other effects that remove the nanoparticle-encapsulated drug from the patient must be avoided to promote long circulation times in the circulatory system for as many of the nanoparticles to find and sequester in the cancer cells as possible.  
There should be a trigger mechanism such as the acidic pH within the tumor or during endosome maturation designed into the nanoparticle platform to ensure the release of the encapsulated drug into the targeted tissue.  
The nanoparticle platform should be capable of surface bioconjugation to target molecules for the specific cancer to provide the greatest uptake with the lesions and fewest least side effects with healthy tissue. |

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Table 2-2. Comparison of nanoparticle drug delivery systems*

<table>
<thead>
<tr>
<th>Material</th>
<th>Size (nm)</th>
<th>Active agent</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biodegradable Polymers</td>
<td>10-100</td>
<td>Plasmid DNA, proteins, peptides, low MW organic compounds</td>
<td>Sustained localized drug delivery for weeks</td>
<td>Exocytosis of undissolved nanoparticles. Fixed functionality after synthesis may require new synthetic pathways for alternate surface functionalities</td>
</tr>
<tr>
<td>Ceramic</td>
<td>&lt;100</td>
<td>Proteins, DNA, chemotherapeutic agents, high MW organic compounds</td>
<td>Easily prepared, water dispersible, stable in biological environments</td>
<td>Toxicity of materials, exocytosis of undissolved nanoparticles, time consuming synthesis, surface decoration instead of encapsulation</td>
</tr>
<tr>
<td>Metals</td>
<td>&lt;50</td>
<td>Proteins, DNA, chemotherapeutic agents</td>
<td>Small particles present a large surface area for surface decoration delivery</td>
<td>Toxicity of materials, exocytosis of undissolved nanoparticles, time consuming synthesis, surface decoration instead of encapsulation</td>
</tr>
<tr>
<td>Polymeric Micelles</td>
<td>&lt;100</td>
<td>Proteins, DNA, chemotherapeutic agents</td>
<td>Suitable for water-insoluble drugs due to hydrophobic core</td>
<td>Toxicity of materials, fixed functionality after synthesis</td>
</tr>
<tr>
<td>Dendrimers</td>
<td>&lt;10</td>
<td>Chemotherapeutic agents, antibacterial, antiviral agents, DNA, high MW organic compounds</td>
<td>Suitable for hydrophobic or hydrophilic drugs</td>
<td>May use toxic materials, time consuming synthesis, fixed functionality after synthesis may require new synthetic pathways for alternate surface functionalities</td>
</tr>
<tr>
<td>Liposomes</td>
<td>50-100</td>
<td>Chemotherapeutic agents, proteins, DNA</td>
<td>Reduced systemic toxicity,</td>
<td>Fixed functionality after synthesis, some leakage of encapsulated agent,</td>
</tr>
<tr>
<td>Calcium phosphates or phosphosilicates</td>
<td>20-60</td>
<td>Chemotherapeutic agents, RNA, high and low MW organic compounds, imaging agents</td>
<td>Simple preparation, suitable for hydrophilic or hydrophobic drugs, colloidal stability in physiological environments, pH-dependent dissolution results in intracellular delivery of drugs, composed of bio-resorbable material</td>
<td>Encapsulated materials limited to solubility in water or organic solvent</td>
</tr>
</tbody>
</table>

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One of the most promising nanomedical approaches reported in recent years is based on a novel material system, calcium phosphosilicate nanoparticles (CPSNPs), in which encapsulated imaging or drug agents, can be delivered in a targeted manner to a variety of cancers including pancreatic cancer.\textsuperscript{10-12, 14, 16, 129-130} For example, Barth et al. have demonstrated that a FDA-approved near infra-red fluorophore, indocyanine green (ICG), also known as Cardio-Green\textsuperscript{TM}, when encapsulated in the CPSNPs, can be used as a theranostic (e.g., a combined diagnostic and therapeutic) agent for a variety of cancers based on a new cancer diagnosis and treatment strategy designated as photo-immuno nanotherapy (PINT).\textsuperscript{13} PINT results resurrection of the immune response of the host animal, permitting the immune system to fight the cancer directly. In an earlier report, Barth et al. also demonstrated that gastrin-10 can be used for targeted delivery of ICG-encapsulated CPSNPs in vivo based on an orthotopic graft of a human pancreatic cancer in the athymic murine model.\textsuperscript{17} The trigger to release the chemotherapeutic agent is inherent dissolution of the CPSNPs in either the acidic local pH in the fluid surrounding many solid tumor types or, after endosomal uptake of the drug-laden CPSNPs into cancer cells, the decreased pH associated with maturation of endosome to endo-lysosomes. Targeting permits the PINT to be used for efficacious uptake in solid tumors and, in an unprecedented fashion, for non-solid tumor cells such as chronic myeloid leukemia.\textsuperscript{13, 16-17}

The ability to create and combine targets with nanoparticle vehicles offers versatile strategies that can be applied to any type of cancer research. Targets that include growth factor inhibitors (e.g., opioid growth factors)\textsuperscript{131-132} introduce promising directions towards cancer management despite limitations. Novel biomarkers like Plectin-1 (Plec1)\textsuperscript{133} have been found to be useful in the early detection of small pre-invasive PanIN III lesions and metastases. Such biomarkers provide an advantage in early detection when they are over-expressed in specific organs. It was shown that Plec1 can also be used to safely distinguish PDAC from benign conditions, which is more effective than cross-sectional abdominal and invasive endoscopic
imaging techniques. Thus, the combination of early detection with more efficacious delivery and effective treatment promised by nanomedical approaches is emerging as a viable alternative for managing pancreatic cancer. While a cure for PDAC is out of reach, research in the next decade will inevitably introduce better prevention and prognosis modalities to diagnose and improve chances of survival.
Chapter 3

Surface titration of calcium phosphate by ions in solution: a zeta potential evaluation for specific adsorption-mediated drug encapsulation

3.1 Introduction

Interactions at calcium phosphate surfaces in aqueous conditions can be described by trends in the electrochemical potential relative to pH.\textsuperscript{134} In the case of calcium phosphosilicate nanoparticles (CPSNPs), applications of this knowledge can improve agent uptake based on the surface ability to adsorb compounds from solution. A way to probe these solid-solution interactions is by surface titration combined with zeta potential measurements. The zeta potential is known as the slipping plane in the electrical double layer model, a liquid layer boundary within the diffuse region that divides ions that travel with the particle and ions interacting in the diffuse layer.\textsuperscript{135} The magnitude of the measured zeta potential is an indication of colloidal stability from net charge repulsion at the surface. Acid and base additions from surface titration will cause an accumulation of positive and negative species that subsequently increase or decrease the potential magnitude, respectively. Therefore, the zeta potential of suspended solids depends on the pH. The pH where the net charge is neutralized is called the isoelectric point (IEP). Not to confuse with the point of zero charge (PZC), the ISE is used when there are other adsorbed ions besides the potential-determining ions, H\textsuperscript{+} and OH\textsuperscript{-}.\textsuperscript{136} Potential-determining ions are found to be critical species that adsorb and change the surface potential via chemical reactions. The IEP and PZC values are generally equal when only H\textsuperscript{+} and OH\textsuperscript{-} are involved in adsorption.

Changes in interfacial properties, such as charge magnitude and the IEP, reveal fundamental equilibrium reactions that govern the solubility products of calcium phosphate and ions that take part in the reactions.\textsuperscript{137} Discussions in this chapter refer to surface reactions that occur
on brushite, a calcium phosphate derivative\textsuperscript{138-139} that is used as a surface model in zeta potential evaluations for the groundwork of CPSNP encapsulation. The encapsulation of water soluble agents in double reverse micelle synthesis of CPSNPs have not been studied previously in terms of surface adsorption. Without this information, there is no metric to explain why the encapsulation of one dye or drug agent is more successful than another. The titration of brushite with different compounds will allow identification of key functional groups that are involved in adsorption. This opens possibilities for functionalization of existing compounds to enhance adsorption on specific surfaces.

The hydrolysis products of calcium phosphate, mainly hydroxyapatite, in water have been well established by Rootare et al.\textsuperscript{140} and Somasundaran.\textsuperscript{136-137, 141} Brushite is crystallized at room temperature and titrated with hydrochloric acid (HCl), potassium hydroxide (KOH), citrate (cit) adenosine triphosphate (ATP), 5-fluorouracil (5-FU), and fluorodeoxyuridine monophosphate (FdUMP), to observe changes in the IEP and zeta potential magnitude. The surface behavior is further analyzed based on literature, basic ion speciation, and possible reactions, that occur within a pH range. In particular, the role of phosphates in CPSNP encapsulation is the primary interest in this evaluation for the encapsulation of chemotherapeutic drugs such as 5-FU that are enzymatically phosphorylated in cells to inhibit cellular function.\textsuperscript{115} Successful encapsulation of phosphorylated derivatives will enable direct delivery of active metabolites for a more facile and potent cytotoxic effect in tumors without having to undergo additional conversion pathways.

The methods presented covers a conditioning procedure that can be applied to various adsorbates within the framework of CPSNPs. By combining titration and zeta potential determinations, adsorption can be quantitatively assessed through ion-exchange interactions, changes in equilibrium, and dissociation/stability constants associated with those reactions. Thus, the zeta potential is not only useful as a quality control measure for colloidal stability, but also for understanding the association of terminal functional groups with other charged species.
3.2 Materials and methods

3.2.1 Materials

Calcium chloride (CaCl₂, ≥99%) and sodium hydrogen phosphate (Na₂HPO₄, ≥99%), 5-fluorouracil (5-FU, ≥99%), 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP, ~85-91%), adenosine 5'-triphosphate disodium salt hydrate (ATP, 99%), and sodium citrate dihydrate (Cit, ≥99%), were purchased from Sigma-Aldrich and used as is. Solution pH was adjusted with potassium hydroxide (KOH) from J.T. Baker and hydrochloric acid (HCl) from Fisher Scientific.

3.2.2 Methods

Water from High-Q 200PT-SYS (RO/IX2) purification system was degassed with argon and then filtered with a 0.2 μm cellulose acetate membrane on the same day. The conductivity of degassed purified water was 2-6 μS/cm. An equimolar amount of filtered aqueous calcium chloride and sodium hydrogen phosphate was capped airtight and stirred at ~200 rpm for at least 20 h under inert atmosphere. The resultant white precipitate was washed twice by centrifugation (4000 rpm for 5 minutes) with copious amounts of water to isolate the pellet, which was then re-dispersed in water for immediate use. This suspension was diluted to a solids loading of 0.2/100 g/mL for surface titration with citrate, ATP, 5-FU, and FdUMP.

The initial pH of the brushite was recorded after allowing the solids to settle. Following the addition of citrate, ATP, 5-FU, or FdUMP, the suspensions were conditioned overnight with continuous mixing. The final adsorbate concentrations and solids loading of brushite are listed in the Table 3-1, as an example, for citrate titration.
Table 3-1. Tabulated solids loading of synthetic brushite and citrate concentrations* for surface titration.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Component</th>
<th>Initial conc.</th>
<th>Volume</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (pH ~6)</td>
<td>Brushite</td>
<td>0.3/100 g/mL</td>
<td>5 mL</td>
<td>0.2/100 g/mL</td>
</tr>
<tr>
<td>B (pH ~7)</td>
<td>Citrate</td>
<td>0 M</td>
<td>0 mL</td>
<td>0 M</td>
</tr>
<tr>
<td>C (pH ~7.5)</td>
<td>Degassed H₂O</td>
<td></td>
<td>2.5 mL</td>
<td></td>
</tr>
<tr>
<td>D (pH ~8)</td>
<td>Total Volume</td>
<td></td>
<td>7.5 mL</td>
<td></td>
</tr>
<tr>
<td>A (pH ~6)</td>
<td>Brushite</td>
<td>0.3/100 g/mL</td>
<td>5 mL</td>
<td>0.2/100 g/mL</td>
</tr>
<tr>
<td>B (pH ~7)</td>
<td>Citrate</td>
<td>0.001 M</td>
<td>0.075 mL</td>
<td>1E-05 M</td>
</tr>
<tr>
<td>C (pH ~7.5)</td>
<td>Degassed H₂O</td>
<td></td>
<td>2.425 mL</td>
<td></td>
</tr>
<tr>
<td>D (pH ~8)</td>
<td>Total Volume</td>
<td></td>
<td>7.5 mL</td>
<td></td>
</tr>
<tr>
<td>A (pH ~6)</td>
<td>Brushite</td>
<td>0.3/100 g/mL</td>
<td>5 mL</td>
<td>0.2/100 g/mL</td>
</tr>
<tr>
<td>B (pH ~7)</td>
<td>Citrate</td>
<td>0.001 M</td>
<td>0.375 mL</td>
<td>5E-05 M</td>
</tr>
<tr>
<td>C (pH ~7.5)</td>
<td>Degassed H₂O</td>
<td></td>
<td>2.125 mL</td>
<td></td>
</tr>
<tr>
<td>D (pH ~8)</td>
<td>Total Volume</td>
<td></td>
<td>7.5 mL</td>
<td></td>
</tr>
<tr>
<td>A (pH ~6)</td>
<td>Brushite</td>
<td>0.3/100 g/mL</td>
<td>5 mL</td>
<td>0.2/100 g/mL</td>
</tr>
<tr>
<td>B (pH ~7)</td>
<td>Citrate</td>
<td>0.001 M</td>
<td>0.750 mL</td>
<td>1E-04 M</td>
</tr>
<tr>
<td>C (pH ~7.5)</td>
<td>Degassed H₂O</td>
<td></td>
<td>1.75 mL</td>
<td></td>
</tr>
<tr>
<td>D (pH ~8)</td>
<td>Total Volume</td>
<td></td>
<td>7.5 mL</td>
<td></td>
</tr>
<tr>
<td>A (pH ~6)</td>
<td>Brushite</td>
<td>0.3/100 g/mL</td>
<td>5 mL</td>
<td>0.2/100 g/mL</td>
</tr>
<tr>
<td>B (pH ~7)</td>
<td>Citrate</td>
<td>0.1 M</td>
<td>0.750 mL</td>
<td>1E-03 M</td>
</tr>
<tr>
<td>C (pH ~7.5)</td>
<td>Degassed H₂O</td>
<td></td>
<td>1.75 mL</td>
<td></td>
</tr>
<tr>
<td>D (pH ~8)</td>
<td>Total Volume</td>
<td></td>
<td>7.5 mL</td>
<td></td>
</tr>
</tbody>
</table>

*final concentrations adjusted to desired range

The pH was remeasured and samples A to E were adjusted with minimum 0.1M KOH or HCl to create a pH range approximately between 6 and 11. The suspensions were equilibrated again overnight before measuring the final pH and zeta potential. The zeta potential was acquired with the Brookhaven ZetaPlus software v. 3.23 (Holtsville, NY) with aqueous parameters. Averages were from a total of five measurements with 95% confidence intervals.

Powder X-ray diffraction (Cu Kα = 1.54059 Å, PANalytical Empyrean) was employed to obtain the XRD pattern of the calcium phosphate pellet, which was isolated and dried for 48 h before the solids were transferred over a zero background sample holder. TEM was performed on the FEI Tecnai G² Spirit BioTWIN TEM at 120 kV (Materials Characterization Lab, Pennsylvania...
State University). The aqueous suspension was diluted and a drop was transferred to a copper grid and left to dry for 48 h.

3.3 Results and discussion

3.3.1 Characterization of synthetic brushite and roles of carbonates and potential-determining ions, H⁺/OH⁻, on the surface behavior of calcium phosphates

Indexed peaks from the XRD pattern of the dried pellet in Figure 3-1 corresponds to a mixture of crystalline brushite for the (020), (-121) and (-112) reflections (PDF # 00-011-0293; lattice constants = 5.83 Å, 15.21 Å, 6.26 Å) with other calcium phosphate derivatives. The Miller indices of (002), (211), (112), (202), (310), (222), and (213), also belongs to hydroxyapatite crystallites, Ca₁₀(PO₄)₆(OH)₂. Based on the relative peak intensity of (020), the composition is mainly brushite. The physical properties of the calcium phosphate pellet were powdery white with sheet and needle morphologies based on TEM in the figure inset.
Figure 3-1. Powder X-ray diffraction (PANalytical Empyrean) pattern confirms the characteristic reflections for brushite, (020) especially, in a mixture of calcium phosphates. This includes hydroxyapatite, which has peaks corresponding to (020), (211), (112), (202), (310), (222), and (213), Miller indices.\textsuperscript{142} TEM (inset) shows that the morphology of the crystals appears as folded sheets and needles.

Synthetic brushite, CaHPO\textsubscript{4} \cdot 2H\textsubscript{2}O; Ca/P = 1.00, was used as a model surface for zeta potential evaluations because the stoichiometry is similar to hydroxyapatite (Ca/P = 1.67) and the amorphous material (Ca\textsubscript{x}H\textsubscript{y}(PO\textsubscript{4})\textsubscript{z} \cdot nH\textsubscript{2}O, n = 3-4.5; 15-20\% \cdot H\textsubscript{2}O; Ca/P = 1.0-2.2) as a model for CPSNPs.\textsuperscript{143} The hydrolysis of brushite in alkaline conditions is regularly used for the conversion to hydroxyapatite with excess calcium to drive the compositional increase of Ca/P.\textsuperscript{144} Similar crystalline products can also be obtained from amorphous calcium phosphate.\textsuperscript{145} Brushite is a relatively stable form of calcium phosphate up to pH 7.5-8 at room temperature, but readily converts to hydroxyapatite at high temperatures because brushite is thermodynamically unstable in nature.

The initial pH of the brushite suspensions were maintained below 7.5. For suspended calcium phosphates, the foremost step is to remove dissolved carbonates (CO\textsubscript{3}\textsuperscript{-}) that can react with calcium chloride to precipitate calcium carbonate (CaCO\textsubscript{3}). This practice applies to the CPSNP
synthesis for the same reasons. Dissolved atmospheric CO$_2$ is in chemical equilibrium with carbonic acid (H$_2$CO$_3$), which dissociates into bicarbonate (HCO$_3^-$) and carbonate depending on the pH. The diagram for the dissociation of carbonic acid as a function of pH in Figure 3-2 was used to identify the major species in solution. This plot was generated with the known dissociation constants, $K_{a1} = 4.45 \times 10^{-7}$ and $K_{a2} = 4.69 \times 10^{-11}$. At pH 7.5, majority of the ion species is bicarbonate (~93%). Carbonate is present at a bare minimum of ~1.4% and drastically increases from pH 8.0. The total ion species is expected to be 50/50 bicarbonate and carbonate at pH 10.33. The amount of dissolved carbonate from CO$_2$ in the atmosphere is more than what is needed to convert calcium phosphate to calcium carbonate. The pH of purified water, not degassed, was measured to be about pH 5.4-5.6. For a CO$_2$ partial pressure of $3.5 \times 10^{-4}$ atm above water at 25°C, this pH range corresponds to about $1.2 \times 10^{-5}$ M of dissolved CO$_2$. Therefore, degassing water is an important procedure in calcium phosphate precipitation to reduce carbonate content.

![Figure 3-2](image.png)

**Figure 3-2.** Speciation of carbonic acid by percentage of H$_2$CO$_3$, HCO$_3^-$, and CO$_3^{2-}$, as a function of pH at 25°C. The dissociation constants, $K_{a1} = 4.45 \times 10^{-7}$ and $K_{a2} = 4.69 \times 10^{-11}$, correspond to log $K$ values of 6.35 (50/50 H$_2$CO$_3$/HCO$_3^-$) and 10.33 (50/50 HCO$_3^-$/CO$_3^{2-}$), respectively.
Brushite suspensions are kept below pH 7.5 (dotted black line) to reduce the potential precipitation of calcium carbonate at the solid-solution interface.

The most stable polymorph of calcium carbonate is calcite, which Somasundaran and Agar reported to have an IEP, or zero point of charge (ZPC) in their experimental context, of pH 8-9.5. The effects on the IEP of apatite in calcite supernatant, or calcite in apatite supernatant, was also discovered by Amankonah and Somasundaran. They showed that apatite and calcite surfaces can take on the characteristics of the other. The conversion of apatite is due to the precipitation of calcium carbonate at the surface, vice-versa, and is implied by the direction of IEP shift. The chemical conversion between apatite and calcite relies on the amount of dissolved phosphate or carbonate at a specific solution pH. The reaction that occurs is described by the following:

$$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2(s) + 10\text{CO}_3^{2-} \rightleftharpoons 10\text{CaCO}_3(s) + 6\text{PO}_4^{2-} + 2\text{OH}^-$$

The zeta potential curve in Figure 3-3 represents the surface potential behavior of brushite produced for the work in this chapter. Brushite isolated at 4 h, 11 h, and 72 h, possessed IEPs of approximately pH 6.8 (black), 7.1 (red), and 7.3 (blue), respectively. The IEP is mainly between pH 7.0 and 7.5, and agrees with literature values for synthetic apatites (pH ~7.0-7.4). We can gather that carbonate content was a minor factor because the IEPs fall within the range of calcium phosphates and not basic pH range for calcites. However, inconsistencies in apatite IEP is still possible from insufficient equilibration time of raw brushite. Rootare et al. identified variations in the solubility product of hydroxyapatite reported in literature that is due to the failure to achieve equilibrium (varying 18 h to 30 d time points for some cases) in the mixture. Somasundaran later conducted a zeta potential study on the changes of synthetic apatite surfaces over time. In KNO₃ solutions (an electrolyte that does not contribute to changes in the IEP), it was found that apatite initially has an IEP of pH 4, but then shifts to pH 6 with time before reaching equilibrium. The ZPC, when $\text{H}^+$/OH⁻ are the potential-determining ions in the system, was about pH 7. The ZPC
is also indicated by the minimum solubility of apatite at pH 7 (verified by measuring the calcium and phosphate ion concentrations) which reflects an equal amount of H⁺/OH⁻ species consumed and produced during solid surface-solution equilibrium. Below pH 7, experimental brushite dissolves and the cloudy white precipitate suspension becomes transparent. The expected behavior of brushite synthesized for 11-72 h (and conditioned overnight with the selected ions) was observed, so the times were sufficient for these experiments to ascertain the role of selected ions on surface behavior.

**Figure 3-3.** The zeta potential curves of synthetic brushite in water and titrated with minimum 0.1 M KOH or HCl to a pH range between 6 and 8.5. Brushite isolated at 4 h, 11 h, and 72 h, were found to have ISEs of pH 6.8 (black, WSL2-83), 7.1 (red, WSL4-34), and 7.3 (blue, WSL1-105), respectively. The error bars represent 95% confidence intervals for a total of 5 measurements. The measurements agree with literature values for the ISE of apatites (pH 7.0-7.4) in aqueous conditions with H⁺/OH⁻ potential-determining ions. The conditioning time of calcium phosphate mixtures have a role in shifting the ISE for zeta potential determinations.¹³⁶
Trends in the zeta potential of brushite before and after the IEP can be explained in terms of the potential-determining ions for calcium phosphate in water. In other words, added ions that significantly influence the interfacial properties of a solid are determined by changes in the IEP and zeta potential magnitude and direction. This requires knowledge of the solubility products of calcium phosphate hydrolysis to understand the behavior of the surface species when equilibrium is disrupted. It was believed for some time that hydroxyapatite did not have constant products because of incomplete equilibrium across various studies, but Rootare et al.\textsuperscript{140} was the first to solve this phenomenon for hydroxyapatite and the hydration products at the solid-solution interface (which was later modified and updated by Chander and Fuerstenau).\textsuperscript{152}

\begin{align*}
(3.2) & \quad \text{Ca}_{10}(PO_4)_{6}(OH)_2(s) + 6H_2O \rightleftharpoons 4[\text{Ca}_2(\text{HPO}_4)(\text{OH})_2] + 2\text{Ca}^{2+} + 2\text{HPO}_4^{2-} \\
(3.3) & \quad \text{Ca}_2(\text{HPO}_4)(\text{OH})_2 \rightleftharpoons 2\text{Ca}^{2+} + \text{HPO}_4^{2-} + 2\text{OH}^- \\

\text{Hydration of hydroxyapatite results in the hydrolysis of terminal phosphate ions at the surface into Ca}_2(\text{HPO}_4)(\text{OH})_2 \text{complexes. Somasundaran took the postulation further and summarized the subsequent hydrolysis products from the second reaction,}^{136,153}
\end{align*}

\begin{align*}
(3.4) & \quad \text{Ca}^{2+} + \text{OH}^- \rightleftharpoons \text{CaOH}^+ \quad K = 10^{1.4} \\
(3.5) & \quad \text{CaOH}^+ + \text{OH}^- \rightleftharpoons \text{Ca(OH)}_2(aq) \quad K = 10^{1.37} \\
(3.6) & \quad \text{Ca(OH)}_2(aq) \rightleftharpoons \text{Ca(OH)}_2(s) \quad K = 10^{2.45} \\
(3.7) & \quad \text{H}_3\text{PO}_4 \rightleftharpoons \text{H}^+ + \text{H}_2\text{PO}_4^- \quad K = 10^{2.15} \\
(3.8) & \quad \text{H}_2\text{PO}_4^- \rightleftharpoons \text{H}^+ + \text{HPO}_4^{2-} \quad K = 10^{7.2} \\
(3.9) & \quad \text{HPO}_4^{2-} \rightleftharpoons \text{H}^+ + \text{PO}_4^{3-} \quad K = 10^{12.3} \\
(3.10) & \quad \text{Ca}^{2+} + \text{HPO}_4^{2-} \rightleftharpoons \text{CaHPO}_4 \quad K = 10^{2.7} \\
(3.11) & \quad \text{Ca}^{2+} + \text{H}_2\text{PO}_4^- \rightleftharpoons \text{CaH}_2\text{PO}_4^+ \quad K = 10^{1.08}
\end{align*}

At pH above the IEP, the equilibrium for Reactions 3.4 to 3.9 shift to the right to produce net negative ions that decrease the zeta potential. The equilibrium is reversed at acidic pH below
the IEP to produce excess positive ions that increase the zeta potential. Of the different charged species explored for adsorption, hydrogen, hydroxyl, phosphate, and fluorides, were found to be potential-determining.\textsuperscript{141} As mentioned, H\textsuperscript{+}/OH\textsuperscript{−} additions governs the direction of the equilibrium at the surface, which ultimately affects the net charge. Phosphate, added in the form of potassium dihydrogen phosphate, was found to shift the IEP to the acidic region at increasing concentrations and made the zeta potential more negative. A similar trend was observed for fluorides, but the effect on decreasing the zeta potential was less prominent compared to phosphates.\textsuperscript{141} And quite the contrary, the effects by calcium ions from calcium nitrate was found to be not potential-determining even though calcium made the zeta potential more positive at pH below and above the IEP. The trend is comparable to potassium nitrate because the curves only shifted to more positive values along the same IEP with more calcium. The fact that calcium ion is not considered as potential-determining does not exclude it as an adsorbing ion. At pH <7, calcium ion influence at the surface is minimal because conditions are below the solubility limit of hydroxyapatite. The ion, however, adsorbs to hydroxyapatite at basic pH when terminal phosphate groups are deprotonated (see speciation of phosphates in Section 3.3.3).\textsuperscript{154} Harding et al. believes that Ca\textsuperscript{2+} displaces two protons for every two phosphate groups it binds to.\textsuperscript{154} The protons are then neutralized by 2OH\textsuperscript{−} to maintain constant pH. Accumulation of calcium at these sites effectively leads to an increased zeta potential. Thus, the evaluation of interfacial properties and the role of ion adsorption by zeta potential, as the whole, can be verified by adjusting the species of hydroxyapatite written in the form of $X_{10}(PO_4)_6(Z)_2$ ($X = \text{Ca}^{2+}$, Ba\textsuperscript{2+}, or Sr\textsuperscript{2+}; Z = OH\textsuperscript{−}, or F\textsuperscript{−}).\textsuperscript{137}

In summary, brushite was synthesized by the combination of calcium chloride and phosphate at room temperature and exhibit surface behavior expected for hydroxyapatite in water with H\textsuperscript{+} and OH\textsuperscript{−} titration. The IEP was mainly between pH 7.0 and pH 7.5, with slight variations from different conditioning times. The potential-determining ions, H\textsuperscript{+} and OH\textsuperscript{−}, were verified based on studies by Somasundaran and Rootare and their coworkers on the hydrolysis species of
hydroxyapatite. At pH >7, the gradual dissolution of synthetic brushite was witnessed and acidic conditions drove the equilibrium towards excess positively charged products, resulting in positive zeta potential values. The opposite effect occurred at pH < 7 for negative zeta potential values. Further, atmospheric CO$_2$ was recognized as an important factor in skewing the IEP by the precipitation of calcium carbonate on hydroxyapatite, but was effectively removed from the water source and did not play a major role in these experiments.

3.3.2 Adsorption of citrate on calcium phosphates

Interaction mechanisms between citrate (cit) and hydroxyapatite surfaces are not well understood, but it is clear that citrate has the tendency to chelate with calcium ions in bulk solution.$^{155}$ There are several studies on the citrate and hydroxyapatite interactions with different conclusions. One involves the exchange of one citrate ion from solution to the surface for 1.5 calcium and 2 phosphate ions from the (100) crystal surface to solution.$^{156}$ Another presents convincing evidence on the ion exchange between citrate and phosphate followed by the binding of citrate at calcium sites.$^{157}$ Discussion for the latter is based on the stability constants for the species involved with hydroxyapatite and is adapted in Table 3-2.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Log $K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$ + Hcit$^{2-}$ ⇋ Ca(Hcit)$^0$</td>
<td>2.09</td>
</tr>
<tr>
<td>Ca$^{2+}$ + cit$^{3-}$ ⇋ Ca(cit)$^-$</td>
<td>3.53</td>
</tr>
<tr>
<td>Ca$^{2+}$ + HPO$_4^{2-}$ ⇋ Ca(HPO$_4$)$^0$</td>
<td>2.66</td>
</tr>
<tr>
<td>Ca$^{2+}$ + H$_2$PO$_4$ ⇋ Ca(H$_2$PO$_4$)$^+$</td>
<td>1.41</td>
</tr>
<tr>
<td>H$_2$PO$_4$ + H$^+$ ⇋ H$_3$PO$_4$</td>
<td>2.15</td>
</tr>
<tr>
<td>HPO$_4^{2-}$ + H$^+$ ⇋ H$_2$PO$_4$</td>
<td>7.21</td>
</tr>
<tr>
<td>PO$_4^{3-}$ + H$^+$ ⇋ HPO$_4^{2-}$</td>
<td>12.35</td>
</tr>
</tbody>
</table>
Reactions to form calcium phosphate, such as for CPSNPs, can be quenched and stabilized with citrate, because of its higher affinity for calcium to form stable complexes than for calcium to phosphate, or citrate to phosphate. The ion species involved is governed by the pH and at pH 7-7.5, the major phosphate ions are $\text{H}_2\text{PO}_4^-\text{ and HPO}_4^{2-}$, which reflects the phosphate species present at the hydroxyapatite surface. Therefore, the stability constants, $\log K$, for surface calcium phosphate reactions are expected to be 2.66 and 1.41. The constant for calcium and $\text{cit}^{3-}$ is 3.53 and is higher than calcium with phosphates. Majority of the citrate are in the form of $\text{cit}^{3-}$ (80-93%) from pH 7-7.5 according Figure 3-4 with decreasing amounts of $\text{Hcit}^{2-}$ (20-7%). Therefore, the likelihood of ion exchange between solution $\text{cit}^{3-}$ and surface $\text{H}_2\text{PO}_4^-$ or $\text{HPO}_4^{2-}$ is plausible in this context.

![Figure 3-4](image)

**Figure 3-4.** Speciation of citrate by percentage of $\text{H}_3\text{Cit}$, $\text{H}_2\text{Cit}^-$, $\text{H}_2\text{Cit}^{2-}$, and $\text{Cit}^{3-}$, as a function of pH at 25°C. The plot is generated based on dissociation constants, $K_a1 = 7.4 \times 10^{-4}$, $K_a2 = 1.7 \times 10^{-5}$, and $K_a3 = 4.0 \times 10^{-7}$.146
Following the exchange, calcium sites are exposed, allowing different ways for citrate ions to orient along the surface. There are three $-\text{COO}^-$ groups per citrate molecule that become deprotonated in increasingly alkaline conditions for interactions at the calcium-rich surface. Referring to the proposed model by López-Macipe et al.\textsuperscript{157}, cit$^{3-}$ can weakly interact in a bidentate manner between two calcium ions given the smaller physical size of citrate relative to the calcium-calcium ion distance. Calcium-Hcit$^{2-}$ interactions are also possible at lower pH and, in fact, may form stronger monodentate or bidentate interactions than cit$^{3-}$ with one calcium ion. This reasoning supports their findings on citrate adsorption, with more adsorbed at pH 6 (4% H$_2$cit$^+$, 69% Hcit$^{2-}$, 27% cit$^{3-}$) than at pH 8 (<1% H$_2$cit$^+$, 2% Hcit$^{2-}$, 98% cit$^{3-}$) per unit surface area. However, chelating interactions that lead to stable bonds are not likely because cit$^{3-}$ and Hcit$^{2-}$ adsorption seem to be reversible. Modern advances in imaging by atomic force microscopy (AFM) revealed that only one terminal carboxylic group is actually involved in adsorption with one calcium ion.\textsuperscript{158} Hydrogen bonding at free carboxylate and hydroxyl groups promote self-assembly at the surface, which may be the reason for the reversible interactions and citrate desorption.

Citrate adsorption decreases the zeta potential of calcium phosphates and is shown in Figure 3-5 as a function of pH. The IEP of synthetic brushite was about pH 7.2. With increasing concentration of citrate from $10^{-5}$ M to $10^{-3}$ M, the surface potential becomes more negative at both acidic and basic pH values.
Figure 3-5. Zeta potential curves of brushite between pH ~6 and 11 and titrated with citrate (final concentrations, 10^{-5}, 5 \times 10^{-5}, 10^{-4}, and 10^{-3} M). The isoelectric point for the synthesized brushite was approximately pH 7.2. An increase of citrate concentration decreases the overall zeta potential of brushite and imply −COO\(^-\) adsorption at the surface across the pH range studied.

Specific adsorption is driven by the −COO\(^-\) groups on citrate and this zeta potential behavior is indicative of changes in the interfacial properties of the brushite. The increasingly negative zeta potential at higher citrate concentrations expresses the excess negative charge accumulated and is the basis for maintaining colloidal stability by electrostatic and electrosteric dispersion. The final zeta potential for CPSNPs are typically ~20-40 mV when conditioned in 7.5 \times 10^{-3} M citrate. The net surface charge allows for re-dispersibility between 70/30 ethanol/water and buffered saline solutions under the Derjaguin, Verwey, Landau and Overbeek (DLVO) theory.\(^{159}\) Other uses of citrate in the calcium phosphate system is for modifying nanoparticle morphology through adjustments of citrate:calcium molar ratio.\(^{160}\)
Therapeutic applications of this chemistry is used to create drugs for kidney stone (calcium phosphate or calcium oxalate) recurrence.\textsuperscript{161} Brushite stones are a subset of calcium phosphate-type stones.\textsuperscript{162} There are conflicting conclusions within this area of research on whether citrate medications (e.g., Urocit\textsuperscript{®}-K, potassium citrate) can reduce calcium stone supersaturation in the urinary tract. Although citrate is calcium ion-binding and can inhibit calcium phosphate nucleation on a fundamental level,\textsuperscript{163} citrate therapies can also increase urine pH to induce crystallization to more calcium stones.\textsuperscript{164-165}

Thus, surface interactions of calcium phosphate are highly pH-dependent events. Basic equilibrium reactions elucidate ion species present and likely products at specific pH conditions. Citrate, with three carboxylate and two hydroxyl groups, adsorb to calcium phosphate at surface calcium sites with additional van der Waals interactions. The increase of citrate concentration drastically decreased the zeta potential of synthetic brushite, which is evidence of a net negative surface charge from specific adsorption. Zeta potential evaluations of adsorption gives information on surface functionalization and dissolution that is not only applicable in colloid chemistry, but also in pH-sensitive biological situations such as kidney stone disease.

3.3.3 Adsorption of phosphates on synthetic brushite to verify adsorption-mediated encapsulation for phosphorylated chemotherapeutics

Phosphate-containing inorganic compounds have biological significance in producing free energy in cells via ATP hydrolysis. In DNA and RNA, phosphates make up the backbone that joins single nucleotides. For some drugs, phosphorylation is the key mechanism for converting compounds into active metabolites to inhibit cellular functions. Gem and 5-FU utilize enzyme-catalyzed phosphorylation in cells to become monophosphate compounds. Because these are pyrimidine derivatives, the compounds can bind to thymidylate synthase to inhibit thymidine
production and disrupt DNA/RNA synthesis. Effective drug encapsulation in CPSNPs rely on phosphates to induce calcium-binding interactions. To verify these interactions for the proof-of-concept of adsorption-mediated encapsulation in calcium phosphates, phosphate ions were probed for adsorption on brushite with the same method to study citrates. ATP, 5-FU, and FdUMP, were used specifically to demonstrate adsorption with and without the phosphate group. The speciation of phosphates to $H_3PO_4$, $H_2PO_4^-$, $HPO_4^{2-}$, and $PO_4^{3-}$, at 25°C is shown in **Figure 3-6**. Around the pH of interest, pH 7-7.4, the majority of ions is $HPO_4^{2-}$ (~61%) and $H_2PO_4^-$ (~39%).

**Figure 3-6.** Speciation of phosphate at 25°C by percentage of $H_3PO_4$, $H_2PO_4^-$, $HPO_4^{2-}$, and $PO_4^{3-}$, as a function of pH. The plot was generated with the dissociation constants, $K_{a1} = 7.1 \times 10^{-3}$, $K_{a2} = 6.2 \times 10^{-8}$, and $K_{a3} = 4.5 \times 10^{-13}$.146

ATP is a molecule with three phosphate groups joined together by phosphaanhydride bonds. Around neutral pH, cleavage of the terminal phosphate group relieves charge repulsion. Although hydrolysis to adenosine diphosphate (ADP) + $HPO_4^{2-}$ + $H^+$ is an exergonic process ($\Delta G^{\circ} = -30.5$ kJ/mol), an enzyme is needed to overcome an activation energy barrier from MgATP$^{2-}$ stabilization in cells.166 Magnesium ions shield the negative charge and protect ATP from...
undergoing spontaneous hydrolysis in cellular environments. In unbuffered water or at extreme pH, ATP is not stable and hydrolyzes to ADP by the nucleophilic attack by water molecules at the electropositive phosphorus. With either ATP or ADP in solution, adsorption on brushite is expected to decrease the zeta potential by deprotonated phosphate groups. This was evident with increasing ATP concentration in Figure 3-7A generating highly negative zeta potential values over the pH and concentration range at magnitudes similarly observed for citrate. Isoelectric points for the curves and final adsorbate concentrations are separately listed in Table 3-3. At 10^{-3} M ATP, the zeta potential increased. It is possible that while calcium ions bind to ATP to form stable Ca-ATP metal-ligand complexes (log $K = 3.6$), dissolving the brushite to release more Ca^{2+}, the release of free phosphates from ATP hydrolysis contribute to unstable zeta potential measurements. The error bars for 10^{-3} M ATP cover a wide range of values, that include both negative and positive zeta potentials that is reflect these species.
Figure 3-7. Synthetic brushite conditioned with various concentrations of (A) ATP, (B) 5-FU, and (C) FdUMP. Error bars represent averages of five measurements with 95% confidence intervals. With increasing ATP concentrations $10^{-4} - 5 \times 10^{-4}$ M, the zeta potential decreased to more negative values. At $10^{-3}$ M, the zeta potential increased with large error, which is indicative of brushite dissolution from the formation of the Ca-ATP complexes and the release of free phosphates from ATP hydrolysis at extreme pH. There were no significant changes to the zeta potential and IEP (pH 7-7.2) of brushite treated with 5-FU. FdUMP adsorption shifted the IEP of brushite although the effects are not as prominent at ATP. The implications of this result is that phosphate groups are important for adsorption on calcium phosphates.

Table 3-2. Estimated isoelectric points of zeta potential curves as at various ATP, 5-FU and FdUMP concentrations

<table>
<thead>
<tr>
<th>ATP conc. (M)</th>
<th>IEP</th>
<th>5-FU conc. (M)</th>
<th>IEP</th>
<th>FdUMP conc. (M)</th>
<th>IEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.2</td>
<td>0</td>
<td>7.1</td>
<td>0</td>
<td>7.2</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>n/a</td>
<td>$10^{-5}$</td>
<td>7.0</td>
<td>$10^{-5}$</td>
<td>6.8</td>
</tr>
<tr>
<td>$3 \times 10^{-4}$</td>
<td>n/a</td>
<td>$5 \times 10^{-5}$</td>
<td>7.1</td>
<td>$10^{-4}$</td>
<td>6.9</td>
</tr>
<tr>
<td>$5 \times 10^{-4}$</td>
<td>n/a</td>
<td>$10^{-3}$</td>
<td>7.2</td>
<td>$8 \times 10^{-4}$</td>
<td>7.0</td>
</tr>
</tbody>
</table>

*not applicable, zeta potential shifted to more negative values with increasing ATP concentrations.
As shown by Somasundaran, calcium phosphates have a Nernstian surface with $\text{Ca}^{2+}$, $\text{H}_3\text{PO}_4^{3-}$ and $\text{OH}^-$ as potential determining ions. Since phosphates are weak acids, phosphates and, of course, hydroxide concentrations are both highly dependent on solution pH, then an evaluation of zeta potential (usually equated to the Stern Potential, the electric potential at the shear plane near the particle surface), is often performed as a function of solution pH. If there is a shift in isoelectric point, with concentration of a molecule such as ATP, 5-FU, and FdUMP, this is strong evidence that calcium sites are covalently binding to phosphate groups, if present on the molecule. For example, a strong Ca-ligand such as citrate produces a high negative zeta potential on the calcium phosphosilicate nanoparticles as shown by Morgan et al. and in Section 3.3.2. In fact, citrate functionalization is used to control dispersion during synthesis of the CPSNPs and provide carboxylate groups for subsequent bioconjugation.

The pH at which the zeta potential polarity changes is defined as the isoelectric point. For the calcium phosphate family of materials including hydroxyapatite and brushite, the IEP is between pH 7.0 and pH 7.5. The 5-FU did not display significant shift in the isoelectric point of the brushite in the Figure 3-7B with the IEPs occurring within a narrow range of pH 7.0 and pH 7.2 for the four different concentrations. For FdUMP, there is a more gradual shift in IEP from pH 7.2 (no FdUMP) to pH 6.8 ($10^{-5}$ M) and then a gradual increase to pH 7.0 ($8 \times 10^{-4}$ M FdUMP), indicating that FdUMP specifically adsorbs, but with a significantly smaller adsorption coefficient than ATP. This may be due to the fact that ATP has more phosphate groups than FdUMP. Ca-ATP complexes have a higher stability constant than the Ca-ADP complex, so the number of phosphates may play a role in various degrees of adsorption. To quantitatively assess adsorption, future analysis should be carried out to obtain the specific surface area of the loaded brushite by Brunauer-Emmett-Teller (BET) testing and the amount of un-adsorbed species in the supernatant by mass spectrometry.
Given that there is an observed effect by FdUMP adsorption and not 5-FU, it was concluded that adsorption-mediated encapsulation of FdUMP in CPSNPs should be more successful than 5-FU. Results showed that ATP/ADP robustly adsorbed to brushite based on the significant decrease of zeta potential values. Phosphate groups are critical for driving adsorption with implications that this work can be applied to other phosphorylated compounds within the framework of calcium phosphates.

### 3.4 Conclusions

In summary, changes that occur at the calcium phosphate surface is a pH-dependent process. Brushite was synthesized and evaluated based on early zeta potential work on hydroxyapatites. Synthetic brushite was formed at room temperature and had an IEP of pH 7-7.5 in water. The effects of protons and hydroxyl ions in solution were surface potential-determining and greatly influenced the equilibrium products of calcium phosphate hydrolysis at the solid-solution interface. At basic pH, the zeta potential became increasingly negative as more negative species were produced. At acidic pH, the equilibrium was driven to the opposite direction to release excess positive species.

The adsorption of citrate, ATP, 5-FU, and FdUMP, were also probed on brushite and it was found that citrate and ATP robustly adsorbed to the surface, specifically to calcium, and decreased the zeta potential. FdUMP shifted the IEP of brushite although not as prominent as ATP. On the other hand, 5-FU did not induce significant changes to the ISE or zeta potential because of the absence of phosphate groups. Results from this study established the groundwork for adsorption-mediated encapsulation of phosphorylated compounds in CPSNPs.
3.5 Acknowledgements

Chris Gigliotti is acknowledged for his assistance on the citrate and 5-FU adsorption experiments.
Chapter 4

The role of phosphorylated chemotherapeutics in encapsulation efficiency of active metabolite FdUMP vs. 5-FU and dFdC vs. dFdCMPc

(Sections of this chapter are adapted from an in-preparation manuscript by Loc, W.S; Linton, S.S.; Wilczynski, Z.R.; Matters, G.L.; McGovern, C.O.; Fox, T.; Gigliotti, C.; Tang, X.; Tabakovic, A.; Martin, J.A.; Clawson, G.A.; Smith, J.P.; Butler, P.J.; Kester, M.; Adair, J.H. Effective Encapsulation and Biological Activity of Phosphorylated Chemotherapeutics in Calcium Phosphosilicate Nanoparticles for the Treatment of Pancreatic Cancer.)

4.1 Introduction

Treating pancreatic cancer with standard chemotherapeutic agents is challenging. Most pancreatic adenocarcinoma (PDAC) patients respond poorly to chemotherapeutic drugs, and even new drug combinations have demonstrated only a modest improvement in patient survival.122 This lack of efficacy has been attributed in part to inadequate drug delivery and metabolic drug inactivation.169 Two chemotherapeutics commonly used to treat PDAC, 5-FU and gemcitabine (dFdC), act by blocking key enzymes in nucleotide synthesis. 5-FU is metabolized to 5-fluorodeoxyuridine monophosphate (FdUMP) which, in the presence of 5,10-methylenetetrahydrofolate (CH₂THF), irreversibly inhibits thymidylate synthase (TS).170 TS inhibition results in nucleotide pool imbalances, impaired DNA synthesis, and a reduction in DNA repair.114 Similarly, the prodrug dFdC is phosphorylated intracellularly by deoxycytidine kinase to form dFdCMP, dFdCDP and dFdCTP.171 Gemcitabine has multiple modes of action; gemcitabine diphosphate (dFdCDP) inhibits ribonucleotide reductase, which is responsible for producing the deoxynucleotides required for DNA synthesis and repair. This favors incorporation of the

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triphosphate form (dFdCTP) into DNA, resulting in stalled DNA replication forks and apoptosis.  

While both 5-FU and dFdC are activated within tumor cells by conversion to phosphorylated metabolites, direct delivery of phosphorylated metabolites is limited by cell impermeability, lipid insolubility and precipitation.

Systemic administration of many standard chemotherapeutics is limited by the need for high dosing regimens due to metabolic inactivation and rapid clearance. For example, *in vivo* studies have shown that less than 20% of the 5-FU becomes activated to FdUMP while more than 80% of 5-FU is converted to the inactive 5-FU metabolite 5-fluorodihydrouracil by the liver enzyme dihydropyrimidine dehydrogenase (DPD). Up to 10% of patients have low DPD activity due to heterozygosity for single nucleotide polymorphism (SNP rs3918290; also known as DPYD*2A). These patients have reduced 5-FU clearance and increased risk for 5-FU induced toxicities. Likewise, dFdC can be inactivated by cytidine deaminase and rapidly cleared from the body. Both dFdC and 5-FU are transported into tumor cell by nucleoside transporter systems, including human equilibrative nucleoside transporters, which have low affinity for FdUMP or dFdCMP. Tumor targeted, NP-based delivery of phosphorylated drug metabolites could avoid drug inactivation processes and enhance drug uptake by tumor cells. This study compares the encapsulation efficiency of both phosphorylated and non-phosphorylated forms of 5-FU and dFdC and outlines a novel approach to deliver these chemotherapeutics directly to pancreatic tumors using targeted CPSNPs.
4.2 Materials and methods

4.2.1 Materials

Igepal CO-520 (Rhodia Chemical Co.), neat ethanol (Koptec) and cyclohexane (EMD Millipore). 5-FU, FdUMP, calcium chloride dihydrate, sodium hydrogen phosphate, sodium metasilicate, sodium citrate dihydrate, ATP and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich. FUdR, dFdC, and dFdCMP were either from Tocris or Toronto Research Chemicals. N-hydrosulfosuccimide (Sulfo-NHS) was purchased from Thermo Scientific. Methoxy-polyethylene glycol-amine tether (2kD) was purchased from JenKem Technology. Aqueous solutions were filtered through 0.2 μm cellulose acetate syringe filters before use. Deionized distilled water was obtained from a High-Q 200PT-SYS (RO/IX2) purification system and purged with pre-purified argon. Purified water was tested for endotoxins (≤0.050 EU/mL) using a Charles River Limulus Amebocyte Lysate Endochrome kit. All solution pH measurements, including ethanol-water mixtures, were conducted with a HACH ISFET meter calibrated against aqueous standards. Non-aqueous pH values are designated as “working pH”.

4.2.2 Nanoparticle preparation and laundering

CPSNPs were prepared by a reverse-micelle water-in-oil (Igepal CO-520/cyclohexane/water) microemulsion method and purified by van der Waals HPLC. Microemulsion A that contained 0.650 ml of 10 mM CaCl\(_2\)·H\(_2\)O\(_{aq}\) in 14.06 ml of 29 vol% Igepal CO-520/cyclohexane and microemulsion B that contained 0.065 ml of 60 mM Na\(_2\)HPO\(_4\)\(_{aq}\) /0.065 ml of 8.2 mM Na\(_2\)SiO\(_3\)\(_{aq}\)/0.520 ml of purified water in 14.06 ml of 29 vol% Igepal CO-520/cyclohexane were separately equilibrated for 15 min with constant stirring at ~200 rpm.
For drug-loaded CPSNPs, purified water was substituted by an equal volume of drug agent to maintain the micelle aqueous pool size (R=4 and is determined by the molar ratio of water to surfactant). The starting concentration of the 5-FU agent was 6.3 mM, 100 mM for ATP:5-FU, 9.2 mM for FdUMP, 2.5 mM for dFdC and dFdCMP. Microemulsions A and B were combined and mixed for 2 min. The reaction was quenched with the addition of 0.225 ml of 10 mM citrate followed by 15 min equilibration. The CPSNPs were released from the micelles with 50 ml of ethanol (working pH 7-9). The HPLC stationary phase consisted of solid glass microspheres (Spheriglass A-Glass 1922, Potters Industries) soaked in purified water for 48 hr and rinsed with 1 mM HCl and 1 mM NaOH with water in between before use. The stationary phase was wet-packed in ethanol into a 5 cm long x 3/8" OD, 1/4" ID polycarbonate tube (McMaster-Carr). The CPSNP suspension was loaded onto the HPLC column at a flow rate of 2.5-3ml/min and was monitored by UV-Vis absorption (λ_Igepal CO-520=276 nm, λ_5-FU=266 nm, λ_FdUMP=269 nm, λ_FUdR, dFdCMP=270 nm, λ_dFdC=262 nm, λ_ATP=260 nm). Surfactants and excess reagents were eluted for 20-30 min with neat ethanol (working pH 7-9) and subsequent CPSNP fractions were collected in 70/30 ethanol/water (v/v; working pH 7-9). The suspension was laundered by HPLC (Waters) and CPSNP fractions were eluted as described in Chapter 1.1.3 and Appendix F.

4.2.3 PEGylation of nanoparticles

Citrate-functionalized (Cit) CPSNPs were filtered (0.2 μm regenerated cellulose) and preheated to 50°C with stirring at 550 rpm. For every 10 ml of Cit-CPSNPs, 1 ml of EDC (1 mg/ml) was added drop-wise to the particle suspension. After 5 min, 1 ml of Sulfo-NHS (15 mg/ml) and 1 ml of the 2 kDa mPEG-amine tether (6 mg/ml) were added drop-wise and the reaction proceeded for 15 h. Alternatively, Sulfo-NHS can be excluded to minimize acid digestion of the NPs and mPEG additions were increased to 1.11 ml (10 mg/ml) with 1.11 ml of EDC (1 mg/ml). PEGylated
CPSNPs are purified via filtration for 2-3 min at 5000 g (Amicon Ultra-15 30 kDa). The NPs were reconstituted in sterile phosphate buffered saline (PBS; Cellgro 21-031-CV). Bioconjugation of the aptamer is outlined in Clawson et al.179

4.2.4 Characterization of nanoparticles

PEGylation was verified with the Brookhaven Instruments zeta potential analyzer in ZetaPlus software v. 3.23 (Holtsville, NY). Suspensions were diluted in 70/30 ethanol/water (working pH 7-9). The solvent parameters for this mixture were 1.363, 2.025 cP, and 30.23 for the refractive index, viscosity and dielectric constant, respectively. 180 Measurements were an average of n=10. Particles were imaged on the FEI Tecnai G² Spirit BioTWIN TEM (MCL, Pennsylvania State University) at 120kV. Selected CPSNP samples were diluted in 70/30 ethanol/water (v/v; working pH 7-9) and a drop was transferred onto a copper grid. The images were processed and quantified with Image J (NIH) and the data was transferred to Origin (OriginLab) for fitting to obtain the lognormal mean (\( \bar{z} \)) and lognormal standard deviation (\( \sigma_z \)) for each size distribution (n=379 and n=334 for mPEG-FdUMP-CPSNPs and mPEG-dFdCMP-CPSNPs, respectively).

4.2.5 Encapsulated drug quantification by LC-MS/MS

Particles were diluted in 10% methanol with 0.1% formic acid and 5-CU (Sigma Aldrich) was spiked in as an internal standard. Chromatography was done on a 2.1 mm x 10 cm HSS T3 or C18 CSH column (Waters) at 40°C. For FdUMP, mobile phase A was water with 5 mM ammonium acetate and B was methanol. The flow rate was 0.5 ml/min and the chromatography consisted of holding at 7.5% B for 1 min, increasing to 95% B over 0.5 min, holding at 95% B for 0.5 min before equilibration to starting conditions. For dFdC and dFdCMP, the flow rate was 0.4 ml/min
and the chromatography consisted of holding at 95% A (water with 0.1% formic acid) for 1 min increasing to 95% B (methanol with 0.1% formic acid) over 0.5 min, holding at 95% B for 0.4 min before equilibration to starting conditions. Eluate was analyzed by an inline Waters TQ-S mass spectrometer. The capillary was set at 1.0 kV, source temperature at 150°C, desolvation temperature at 600°C, cone gas at 150 l/h, and desolvation gas flow at 1200 l/h. Multiple reaction monitoring was used to detect 5-CU (145 > 42), FdUMP (325 > 195), and FUdR (245.2 > 155) in negative ion mode and dFdC (264 > 112.1) and dFdCMP (344.1 > 112.1) in positive ion mode. Drug concentrations were determined using TargetLynx version 4.1 (Waters) using an external calibration curve with 1/x weighting.

4.2.6 Cell proliferation

BxPC-3 and PANC-1 were obtained from ATCC. PANC-1 cells were cultured in Dulbecco’s modified Eagle medium with 10% FBS and BxPC-3 cells in RPMI 1640 with 10% FBS (Invitrogen). PANC-1 and BxPC-3 cells were seeded onto 96-well plates at 5,000 cells/well. After 24 hr, treatment with vehicle (1x sterile PBS without calcium and magnesium), free drug, empty CPSNPs or phospho-drug-loaded CPSNPs were initiated. Viable cell determinations were made after 72 hr using an alamarBlue® assay (Life Technologies). Relative proliferation for all treatment groups was normalized to vehicle controls.

4.2.7 Thymidylate Synthase Immunoblotting

PANC-1 and BxPC-3 cells were seeded onto 6-well dishes and incubated for 24 hr in one of the following treatment groups: no treatment, PBS vehicle, free FdUMP, mPEG-FdUMP-CPSNPs, and empty mPEG-CPSNPs. Lysates were collected by aspirating the media, washing with 1x PBS, and
adding RIPA buffer containing Complete Mini protease cocktail (Roche). Protein concentration was determined by microBCA assay (Thermo Scientific) and 20 μg of total protein separated by gel electrophoresis. After transfer to HyBond ECL and blocking for 1 hr in 5% BSA, blots were probed overnight with anti-TS antibody (#9045 Cell Signaling Technology, 1:1000) or beta-actin antibody (#A2228; Sigma, 1:10,000). Membranes were washed, incubated with secondary antibody coupled to horseradish peroxidase (Amersham), and visualized using an enhanced chemiluminescent substrate (Pierce). Quantitation of scanned blots was done using Image-J software (NIH). After normalizing to β-actin, % active TS is the amount of uncomplexed TS divided by the amount of total TS (active TS and TS-FdUMP ternary complex).

4.2.8 Cell Cycle Analysis

PANC-1 cells were treated with vehicle, free FdUMP, mPEG-FdUMP-CPSNPs or an equal volume of mPEG-CPSNPs, or left untreated for 72 hr. Cells were fixed in 75% ethanol overnight and immediately prior to analysis treated with 1 μg/mL RNase A and 50 μg/mL propidium iodide. Cellular DNA content was determined using a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed with Cellquest (Verity Software).

4.2.9 In vivo assessment of aptamer-targeted FdUMP-CPSNP uptake by PDAC tumor cells.

All animal protocols were approved by the Penn State Hershey IACUC committee. Orthotopic PANC-1 tumors were established in male, athymic (nu/nu) mice (Charles River). Pancreata were injected with 5x10⁶ cells and treatment with CSPNPs was initiated at one week post-surgery. Treatment groups (n= 4-5 mice per group, with two experimental replicates) included empty mPEG-CPSNPs, mPEG-FdUMP-CPSNPS without the addition of targeting agents to the NP
surface, mPEG-FdUMP-CPSNPs surface bioconjugated with gastrin 16 peptide, and mPEG-FdUMP-CPSNPs surface bioconjugation with the CCKBR aptamer AP1153. CPSNPs were administered at a FdUMP dose of 100 μg/kg, or an equal volume of empty CPSNPs, twice weekly via tail vein injection. After six weeks of treatment, tumor proteins were extracted and thymidylate synthase immunoblots were performed as described above.

4.2.10 Statistical Analysis

Results were expressed as means ± standard error. Student t-tests were used to evaluate statistical significance with a p < 0.05 considered to be significant. To calculate EC₅₀ ± 95% CI, nonlinear regression analysis was performed to generate the curve of best fit for the data according to a Sigmoidal regression using a 4-parameter logistic curve calibration \[Y = Yo + \left(\frac{a}{1+((X/Xo)^b)}\right)\] in SigmaPlot 12 (Systat, Inc.).

4.3 Results

4.3.1 CPSNP Characterization

Preparation of mPEG-terminated CPSNPs used a reverse micelle approach and resulted in CPSNPs with mean diameters <100 nm; a range that is optimal for NP penetration into fibrotic pancreatic tumors. The lognormal mean of the size distribution for mPEG-FdUMP-CPSNPs was 8 nm (σₓ=0.22) and 10 nm (σₓ=0.39) for mPEG-dFdCMP-CPSNPs (Figures 4-1A-C).
Figure 4-1. TEM micrograph of mPEG-FdUMP-CPSNPs (A), the lognormal size distribution of mPEG-FdUMP-CPSNPs (B) and mPEG-dFdCMP-CPSNPs (C).

CPSNPs were surface functionalized with citrate (Cit) and methoxy-terminated PEG tether, which improves colloidal stability. The zeta potential was screened to verify changes in surface functionalization (Figure 4-2). The mean values for Cit-CPSNPs, Cit-FdUMP-CPSNPs and Cit-dFdCMP-CPSNPs were $-37\pm 6$ mV, $-44\pm 5$ mV and $-28\pm 7$ mV, respectively. After PEGylation, the uncharged methoxy group termination on the CPSNP surface exhibits a zeta potential of nearly zero, so mPEG-CPSNPs, mPEG-FdUMP-CPSNPs and mPEG-dFdCMP-CPSNPs, displayed mean values of $-1\pm 5$ mV, $-4\pm 6$ mV and $1\pm 9$ mV, respectively.
4.3.2 Encapsulation efficiency of chemotherapeutics into CPSNPs.

We hypothesized that chemotherapeutic drugs such as 5FU or dFdC would be less effectively encapsulated into CPSNPs than their bioactive nucleotide analogs FdUMP and dFdCMP (Figure 4-3). To address this hypothesis, the encapsulation efficiencies (EE) for 5-FU, ATP:5-FU complex, F UdR (5-fluorodeoxyuridine), FdUMP, dFdC, and dFdCMP (Table 4-1) were experimentally determined. The EE is defined by the relationship,

\[ {\text{mol}}\% \text{ EE} = \frac{m_f}{m_i} \times 100 \]

where \( m_i \) was the total drug content in moles and \( m_f \) was the moles encapsulated as assessed by LC-MS/MS. CPSNP encapsulation of 5-FU was only 4.1 (±1.8) \( \times 10^{-7} \text{ M} \), or 0.11 (±0.04) mol% EE.
(Table 4-1), and extending micellar exchange times failed to improve 5-FU encapsulation. FUdR, the deoxynucleoside analog of 5-FU, was incorporated in CPSNPs even less effectively than 5-FU, with an EE of <0.01 mol% and 2.8 (±1.2) ×10⁻⁸ M encapsulated drug. A triple microemulsion method was then implemented to create a core-shell CPSNP, which consisted of a calcium phosphosilicate (CPS) core layered with both 5-FU and ATP and an additional CPS shell. However, the weak hydrogen bonds between 5-FU and ATP were compromised by the high ionic strength in the micelles, and encapsulation efficiency of 5-FU was only marginally improved, with an average EE and 95% CI of 1.8 (±2.5) ×10⁻⁶ M.

Figure 4-3. Structures of 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (FUdR), 5-fluorodeoxyuridine monophosphate (FdUMP), 2′,2′-difluorodeoxycytidine (dFdC, or gemcitabine), 2′,2′-difluorodeoxycytidine monophosphate (dFdCMP, or gemcitabine monophosphate), and the adenosine triphosphate:5-fluorouracil complex (ATP:5-FU).
Table 4-1. Encapsulated concentrations and encapsulation efficiency (EE) for 5-FU, ATP:5-FU, FUdR, FdUMP, dFdC or dFdCMP in citrate-functionalized (Cit) CPSNPs.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Drug Concentration (M)*</th>
<th>EE (mol%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cit-5-FU-CPSNP</td>
<td>4.1 (±1.8) x 10^-7</td>
<td>0.11 (±0.04)</td>
</tr>
<tr>
<td>Cit-ATP:5-FU-CPSNP</td>
<td>1.8 (±2.5) x 10^-6</td>
<td>0.36 (±0.39)</td>
</tr>
<tr>
<td>Cit-FUdR-CPSNP</td>
<td>2.8 (±1.2) x 10^-8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cit-FdUMP-CPSNP</td>
<td>3.0 (±1.4) x 10^-4</td>
<td>41 (±16)</td>
</tr>
<tr>
<td>Cit-dFdC-CPSNP</td>
<td>1.9 (±0.6) x 10^-9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cit-dFdCMP-CPSNP</td>
<td>2.3 (±0.4) x 10^-5</td>
<td>22 (±6)</td>
</tr>
</tbody>
</table>

* Concentration and encapsulation efficiency (EE) are expressed as the mean±95% CI of n=4-6 experimental replicates.

In contrast to the low encapsulation efficiencies of 5-FU and FUdR, FdUMP was reproducibly encapsulated into CPSNPs at 3.0 (±1.4) x 10^-4 M and an EE of 41 (±16) mol%—several orders of magnitude higher than 5-FU or FUdR encapsulation (Table 4-1). The increased EE for FdUMP versus FUdR suggests that encapsulation is enhanced by the formation of a bond between calcium from the NP and phosphate from the drug molecule. Similarly, free gemcitabine (dFdC) was poorly encapsulated into CPSNPs, 1.9 (±0.6)x10^-9 M with an EE of <0.01 mol%, while gemcitabine monophosphate (dFdCMP) was encapsulated with significantly higher efficiency, 2.3 (±0.4)x10^-5 M and an EE of 22 (±6) mol% (Table 4-1). Thus, while the prodrugs 5-FU and dFdC were poorly encapsulated into CPSNPS, we have demonstrated more efficient encapsulation of the bioactive monophosphate forms FdUMP and dFdCMP into CPSNPs.

4.3.3 CPSNP-encapsulated phopho-drugs block PDAC cell proliferation in vitro.

While others have reported that polymeric FdUMP has efficacy against other cancers, the efficacy of phosphorylated drug metabolites on the growth of pancreatic cancer cells has not been shown. In these studies, the anti-proliferative effects of 5-FU, FdUMP, dFdC and dFdCMP were compared to CPSNP-encapsulated FdUMP and CPSNP-encapsulated dFdCMP. The cultured
human pancreatic cancer cell lines BxPC-3 and PANC-1 were chosen, since each demonstrates some resistance to these drugs. Against both cell lines, 5-FU and FdUMP had similar efficacy, with a low μM EC₅₀ for both compounds and both cell lines (Figure 4-4). Similarly, dFdC and dFdCMP were both effective in blocking proliferation of BxPC-3 and PANC-1. The EC₅₀ for free dFdCMP against both cell lines was in the nM range and was lower than for 5-FU or FdUMP.

**Figure 4-4.** *In vitro* growth of human PDAC cell lines BxPC-3 and PANC-1 is effectively blocked by CPSNPs containing dFdCMP, with EC₅₀ values of 130 and 550 nM, respectively. BxPC-3 cells were more resistant to mPEG-FdUMP-CPSNPs than PANC-1 cells, which had an EC₅₀ of 1.3 μM. Empty mPEG-CPSNPs (light hatched bars), free drug (dark bars) or drug-containing CPSNPs (dark hatched bars) are expressed as relative proliferation (percent of vehicle controls, white bars). Values are the mean of 3-4 independent experiments with ***=p<0.001 and **=p<0.01.

Comparing FdUMP to CPSNP-encapsulated FdUMP, the response of BxPC-3 cells to encapsulated FdUMP plateaud between 2.5 μM and 50 nM, and higher doses failed to affect the proliferation of these cells (Figure 4-4). PANC-1 responded similarly to both free FdUMP and
encapsulated FdUMP, with an EC$_{50}$ for FdUMP-CPSNPs of 1.3 $\mu$M. Both BxPC-3 and PANC-1 were more sensitive to dFdCMP-CPSNPs than to FdUMP-CPSNPs. The EC$_{50}$ for dFdCMP-CPSNPs against PANC-1 was 550 nM, and proliferation of BxPC-3 was decreased to a much greater degree by dFdCMP-CPSNPs (EC$_{50}$ 130 nM) than by FdUMP-CPSNPs. There was no significant difference between the efficacy free dFdCMP and encapsulated dFdCMP for either cell line. Neither BxPC-3 nor PANC-1 proliferation was affected by empty mPEG-CPSNPs, consistent with previous work indicating that the particles themselves were non-toxic.$^{17-18}$ Thus, CPSNP encapsulation of FdUMP and dFdCMP did not affect the ability of these phospho-drugs to inhibit the growth of PDAC cell lines \textit{in vitro}.

### 4.3.4 Encapsulated FdUMP Inhibits Thymidylate Synthase (TS).

In the presence of folate, TS dimers and FdUMP create an irreversibly inactivated enzyme:drug complex,$^{186-187}$ and the inactive ternary complex (TS, FdUMP and THF) migrates more slowly than active TS on immunoblots.$^{188}$ Immunoblot analysis of TS in untreated cells, cells treated with vehicle or cells treated with mPEG-CPSNPs did not show formation of a TS ternary complex (only the lower molecular weight, active form of TS was present in these cells; Figure 4-5, Lanes 1, 2, and 4). Treatment with free FdUMP shifted most of the active TS into the inactive ternary complex, as expected, although FdUMP was slightly less effective for PANC-1 cells (80% inhibition) than for BxPC-3 cells (90% inhibition; Figure 4-5, Lane 3). However, nearly all TS was in the catalytically inactive TS:FdUMP:THF ternary complex when BxPC-3 or PANC-1 cells were treated with mPEG-FdUMP-CPSNPs (89%-91% inhibition; Figure 4-5, Lane 5). Thus, when delivered \textit{in vitro}, CPSNP-encapsulated FdUMP retained its capacity to bind to and inhibit its target enzyme, TS, in both BxPC-3 and PANC-1 cells.
Figure 4-5. Immunoblots of the FdUMP target enzyme thymidylate synthase (TS) from PANC-1 (upper panel) or BxPC-3 (lower panel) cells treated with 250 μM free FdUMP (Lane 3) or 2 μM mPEG-FdUMP-CPSNPs (Lane 5). Both cell lines showed significant (>80%) conversion of TS to an inactive ternary complex (TS:FdUMP) with free drug and with mPEG-FdUMP-CPSNP treatment. Controls that received no treatment (Lane 1), PBS vehicle (Lane 2) or mPEG-CPSNPs containing no FdUMP (Lane 4) exhibited only active TS with no evidence of TS:FdUMP ternary complex formation.

4.3.5 Cell Cycle Arrest in FdUMP-CPSNP Treated PANC-1 Cells.

Since TS provides the sole de novo source of cellular thymidylate, which is necessary for DNA replication and repair, inhibition of TS depletes nucleotide pools and leads to DNA strand breaks and arrest of the cell cycle since cells are unable to synthesize new DNA or repair damaged DNA. To demonstrate that this cellular activity of FdUMP was retained after CPSNP-encapsulation, PANC-1 cells treated with various CPSNP formulations were analyzed for cell cycle progression (Figure 4-6). Untreated cells, PBS vehicle-treated cells, and mPEG-CPSNP-treated cells all had equivalent percentages of cells in G0/G1, S, and G2/M-phase, indicating that the
CPSNPs themselves had no effect on cell cycle progression. Compared to these controls, cells treated with free FdUMP demonstrated a partial S-phase arrest and a reduced number of cells in the G0/G1 phase. The percentage of free FdUMP-treated cells in the G0/G1 phase decreased from 54% to 20%, and the percentage of cells in the S phase of the cell cycle increased from 25% to 56% compared to untreated cells. Similarly, cells treated with mPEG-FdUMP-CPSNPs showed virtually no G0/G1 phase cells, and the percentage of cells in G2/M and in S phase was nearly equal, consistent with the known mechanism of action of 5-FU and FdUMP. Despite the fact that a significantly lower concentration of FdUMP was used, the arrest of cell division in cells treated with mPEG-FdUMP-CPSNPs was more pronounced than in free FdUMP-treated cells.

<table>
<thead>
<tr>
<th>% of cells in</th>
<th>No treatment</th>
<th>Vehicle</th>
<th>mPEG-Ghost-CPSNP</th>
<th>Free FdUMP (250 µM)</th>
<th>mPEG-FdUMP-CPSNP (200 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1</td>
<td>54.1 ± 4.0</td>
<td>53.8 ± 4.0</td>
<td>52.0 ± 3.8</td>
<td>20.3 ± 3.0</td>
<td>0.1 ± 0.03</td>
</tr>
<tr>
<td>S</td>
<td>24.6 ± 1.5</td>
<td>23.6 ± 1.3</td>
<td>25.1 ± 1.6</td>
<td>56.3 ± 10.3</td>
<td>44.8 ± 7.0</td>
</tr>
<tr>
<td>G2/M</td>
<td>21.4 ± 1.2</td>
<td>22.7 ± 1.5</td>
<td>22.6 ± 1.0</td>
<td>23.5 ± 6.3</td>
<td>55.1 ± 5.3</td>
</tr>
</tbody>
</table>

**Figure 4-6.** The table indicates the percentage of cells in either G0/G1 or G2/M phase (red peaks in representative histograms) or in S-phase (hatch marked histogram peaks) after treatment. Control groups (No Treatment, PBS vehicle or empty mPEG-CPSNPs-without drug) have similar percentages of cells in all cell cycle phases. While cells treated with FdUMP demonstrated a reduced number of cells in G0/G1, only 0.1% of cells treated with mPEG-FdUMP-CPSNPs are in G0/G1. The percentage of cells in each cell cycle phase is expressed as the mean ±SEM of 3 independent experiments.
4.3.6 CCKBR-Targeted FdUMP-CPSNPS Deliver Active Drug to PDAC Tumor Cells in vivo.

Recently, we described a DNA aptamer (a small structured oligonucleotide) which can bind to the CCK-B receptor on the surface of PDAC tumor cells with high affinity yet does not activate receptor signaling.\textsuperscript{179} The CCK-B receptor is over-expressed on pancreatic cancer cells compared to normal pancreatic tissue.\textsuperscript{191} Here, either the selective CCKBR aptamer, or the endogenous CCKBR peptide ligand gastrin-16, were covalently attached to FdUMP-CPSNPS using established protocols.\textsuperscript{17} The ability of these targeted CPSNPs to deliver active FdUMP to PDAC tumor cells in vivo was assessed by TS immunoblotting of tumor tissues. Active TS protein levels in tumors from mice treated with empty mPEG-CPSNPs and untargeted mPEG-FdUMP-CPSNPS were not significantly different from each other (Figure 4-7), indicating that without tumor-specific targeting, CPSNPs are poorly taken up by PDAC tumors in vivo. Previous studies showed that gastrin-targeted fluorescent CPSNPS had enhanced PDAC tumor uptake in vivo.\textsuperscript{17} In this study, although the tumors from mice treated with gastrin-16 peptide targeted FdUMP-CPSNPs appeared to have reduced TS protein levels compared to untargeted mPEG-FdUMP-CPSNPs, the differences between these two treatment groups did not reach statistical significance. Tumors from mice treated with aptamer targeted-FdUMP-CPSNPs had a significant reduction in active TS levels: a 60\% reduction versus either empty CPSNPS or untargeted mPEG-FdUMP-CPSNP treatments (*p<0.05; Figure 4-7).
Figure 4-7. Levels of active thymidylate synthase (unbound TS) was determined by immunoblotting, and reflects that amount of the TS inhibitor FdUMP taken up by PANC-1 tumors in mice treated with various CPSNP formulations (n= 5 mice/treatment group). Tumors from mice treated with empty (non-drug containing) mPEG-CPSNPs (#1, black bar) or untargeted mPEG-FdUMP-CPSNPs (#2, grey bar) had equivalent amounts of unbound, active TS, suggesting that untargeted particles were not efficiently taken up by tumor cells in vivo. Although the mean TS levels in tumors from gastrin-16 peptide targeted-FdUMP-CPSNPs treated mice was decreased (#4, light hatched), only tumors in mice treated with CCKBR aptamer targeted-FdUMP-CPSNPs (#3, dark hatched) had significantly reduced TS levels (*p<0.05) compared to empty CPSNP or untargeted mPEG-FdUMP-CPSNP controls. Bars represent ±SEM of 2 independent experiments.

This demonstrates that in mice treated with aptamer-FdUMP-CPSNPs, the FdUMP cargo was internalized and released into PDAC tumor cells and that the biological activity of the FdUMP cargo against TS was retained. This result is again consistent with other recent data showing that this CCKBR aptamer increased fluorescent CPSNP accumulation in PDAC tumors in vivo compared to both untargeted or gastrin 16-targeted particles. However, the studies herein extend previous studies with aptamer-targeted fluorescent-CPSNPs, which did not specifically address cellular internalization of CPSNP cargo (as the fluorescent CPSNPS could have been associated
with CCKBR at the plasma membrane rather than inside of the tumor cells). These studies clearly demonstrate the improved efficacy of aptamer targeted-FdUMP-CPSNPs to be internalized by PDAC tumor cells and to release biologically active FdUMP into the cytoplasm.

### 4.4 Discussion and Conclusions

Our results demonstrate that CPSNP encapsulation of bioactive chemotherapeutic drug metabolites, such as the 5-FU metabolite FdUMP or the gemcitabine metabolite dFdCMP, can be an effective means to deliver these drugs to pancreatic tumors. Due to metal-ligand complexes between phosphate group on the phospho-drug molecule and calcium in the CPSNPs, phosphorylated, bioactive drugs, which cannot be effectively administered systemically, can be encapsulated within CPSNPs without compromising their cellular activity. This suggests a general strategy whereby other phosphorylated compounds can be effectively encapsulated into CPSNPs and suggests that CPSNPs can potentially be used as a vehicle to encapsulate and deliver other drugs that have previously been challenging to incorporate into NPs.

These unique CPSNP-drug formulations could have a straightforward application to PDAC patient treatment. A recent meta-analysis suggested that PDAC patients may benefit from 5-FU/dFdC combination therapy, although significant toxicities, including neutropenia, thrombocytopenia and diarrhea, can occur. The toxicities are due mainly to the high systemic drug concentrations required to achieve a therapeutic response. A treatment strategy which combines delivery of NP encapsulated and targeted FdUMP-CPSNPs with dFdCMP-CPSNPs should increase the anti-tumor efficacy of this drug combination with less toxicity and reduced side effects. Other opportunities for improving treatment could include administering drug encapsulated-CPSNPs with a combination of agents that normalize tumor vasculature, such as low-dose Cilengitide and Verapamil – which have been shown to enhance gemcitabine efficacy.
Resistance to dFdC often occurs by down-regulating proteins that facilitate drug transport, such as hENT1\textsuperscript{185} or hCNT1,\textsuperscript{195} or reducing the expression of the dFdC activating enzyme deoxycytidine kinase.\textsuperscript{196} Patients with inherently low deoxycytidine kinase activity do not effectively convert dFdC to its metabolically active monophosphate form and thus do not respond well to dFdC treatment. A strategy to encapsulate dFdCMP into CCKBR-targeted CPSNPs should bypass these inherent or acquired PDAC resistance mechanisms (i.e. reduced dFdC uptake due to transporter dysregulation or lack of metabolic activation) and make this drug far more effective.

NP encapsulation of FdUMP or dFdCMP could also make these drugs safer. For those patients with the genetic DPD deficiency (i.e. those with the DPYD*2A SNP), encapsulation and delivery of FdUMP could give this chemotherapy treatment better efficacy without increasing the risk of toxicities. Similarly, patients with low activity of cytidine deaminase, the enzyme that catabolizes dFdC to difluorodeoxyuridine, can develop severe adverse effects from dFdC treatment.\textsuperscript{197} Thus, replacing systemic dFdC with encapsulated dFdCMP should permit these PDAC patients to be more safely and effectively treated.

Beyond encapsulating effective concentrations of phospho-drugs within nanocarriers, this study demonstrated that CPSNPs deliver drugs such as FdUMP to tumor cells in a biologically active form both \textit{in vitro} and \textit{in vivo}. Evidence that CPSNP-encapsulated FdUMP retained activity included the formation of inactive thymidylate synthase ternary complexes and complete cell cycle arrest in mPEG-FdUMP-CPSNP-treated pancreatic cancer cells \textit{in vitro}. When surface bioconjugated with a PDAC tumor-targeting agent, specifically the CCKBR-targeting DNA aptamer,\textsuperscript{179} and administered via systemic circulation, the aptamer-FdUMP-CPSNPs were taken up by pancreatic tumors and delivered biologically active drug to tumor cells \textit{in vivo}. Significant aspects of this work include 1) the inferred stability of these particles in systemic circulation, 2) the ability of targeted-CPSNPs to be internalized by tumor cells and 3) the delivery of active drug to inhibit the intracellular drug target, the enzyme TS. Having demonstrated efficacy in delivering
active drug into PDAC tumor cells *in vivo*, future studies will examine dose-response curves with increased FdUMP (and dFdCMP) concentrations to further decrease active tumor cell TS and achieve therapeutic efficacy.

This investigation also opens the possibility of encapsulating synthetically phosphorylated drug compounds to improve their delivery and efficacy. If CPSNPs can effectively encapsulate phosphorylated compounds that have systemic toxicities and/or poor cellular internalization, a similar approach can be applied to the encapsulation of many other phospho-drugs. During systemic administration, the plasma concentration of chemotherapeutic drugs can increase rapidly and then drop quickly as the drug is metabolized. By encapsulating chemotherapeutics into CPSNPs, which are stable in circulation, a more uniform systemic drug concentration can be achieved, thus reducing the chances of both under-dosing and over-dosing. Surface modification of drug-containing CPSNPs with targeting aptamers that direct these particles to PDAC tumors adds specificity and should reduce off-target toxicities. This study demonstrates a promising new methodology for encapsulating and delivering bioactive, phosphorylated chemotherapeutic agents to pancreatic cancer cells.

4.5 Acknowledgements

This study was funded by the National Institutes of Health (NIH) grants R01CA167535 and R21CA170121 from the National Cancer Institute (NCI). The project was also supported in part under a grant with the Pennsylvania Department of Health using Tobacco CURE funds (SAP#4100072562 to GLM and JHA). The department specifically disclaims responsibility for any analyses, interpretations or conclusions. WSL was supported, in part, by the grants UL1 TR000127 and TL1 TR000125 from the National Center for Advancing Translational Sciences (NCATS). The authors also wish to acknowledge the assistance of Dr. Jason Liao, Director of the
Biostatistics Core of the Penn State Cancer Institute, for his help in designing these experiments and Dr. Bernd Kabius at the Penn State Materials Characterization Lab for his assistance in obtaining the HAADF images on the osmium-stained CPSNPs. Penn State Research Foundation has licensed CPSNP technology to Keystone Nano, Inc. (PA, USA). JHA and MK are co-founders of Keystone Nano and are CSO and CMO, respectively. All other authors declare that there are no conflicts of interest.
Chapter 5

Chloroauric acid reduction by sodium thiophosphate and development of Au-CPS core-shell nanoparticles by “encapsulation”

5.1 Introduction

Gold nanoparticles remain as a standard for platforms designed for biomedicine and come in interesting shapes for theranostic applications (e.g., nanoshells, cages, stars and rods). Optical effects from surface plasmon resonance (SPR), like surface enhance Raman scattering (SERS), offer benefits to tailor the particles to a wavelength for noninvasive imaging and photodynamic therapy. Changes in size, shape, morphology, and interparticle distance or orientation, of colloidal gold and other noble metals allow for the fine-tuning of this optical phenomenon. Further, the high-Z profile of gold that is responsible for efficient radiative properties, absorption and scattering, provides high contrast imaging and monitoring in live specimens. The biocompatibility of colloidal gold has not raised concern for some time until accumulation was studied for toxicity mechanisms.

Gold accumulation is a size-dependent event with longer clearance times for larger particles (100 nm range) from the liver, spleen, and mesenteric lymph nodes. One of the mechanisms by Ma et al. revealed that endocytosed particles (10, 25, and 50 nm) accumulate in lysosomes and disrupt the autophagic pathway. Before, autophagy was identified as a direct response to oxidative stress from gold nanoparticles due to processing of the autophagosome LC3

marker protein. The underlying reason was found to be related to blockages in the autophagy flux, autophagosome and lysosome fusion, by the enlargement of lysosomes. Enlargement reduces the lysosomal degradation capacity and leaves autophagosomes to engulf cell contents.

The effects of nanoparticles on living organisms is a topic with growing evidence that runs parallel to advances on electron and light microscopy techniques. New methods to improve nanoparticle platforms and biocompatible outcome depend on the knowledge of these effects. As a contrast agent, colloidal gold is a valuable material with optical properties within the visible light to near-infra red spectrum. This opens possibilities for improvements and downsizing of current detection methods into handheld multi-modal devices with enhanced sensitivity, reduced signal acquisition time, and on-site treatment capabilities.

Issues with biocompatibility and accumulation can be mitigated by encasing colloidal gold within a non-toxic matrix such as calcium phosphates, which allows options for different functionalities through surface decoration and encapsulation of therapeutics within the shell matrix. Silica-coated nanoparticles have been thoroughly documented for gold since the discovery by Liz Marzán et al., but studies on gold-calcium phosphate core-shell nanoparticles are limited. Cha et al. presented an exceptional example of the hybrid nanoparticle laced with doxorubicin (drug agent) in the calcium phosphate shell. To encapsulate the gold core, they modified citrate-gold with a custom polyethylene glycol-cysteine tether with repeated anionic aspartic acid residues to immobilize both doxorubicin and calcium ions. The negative charge is paramount for driving calcium ion-binding at the surface to seed the growth of calcium phosphate. Thus, the same challenge is present with colloidal gold as with encapsulating 5-fluorouracil or gemcitabine in Chapter 4, where encapsulation is unobtainable unless the gold core is surface-modified. The downside of using a custom PEG is the cost associated, so the interest here is to seek a direct method to reduce gold salt with an agent that can also create the ideal environment for calcium phosphate formation.
There are several robust ways to chemically synthesize colloidal gold with the strong SPR signature between 500 nm to 600 nm, corresponding to sub-100 nm size particles, such as photoreduction or reduction by sodium citrate, sodium borohydride, hydrazine, and alcohols, with a combination of stabilizing agents. Stabilizing agents usually consist of long chains or electron-withdrawing groups to maintain surface repulsion between particles. In preliminary findings, gold cores precipitated in reverse micelles from chloroauric acid by hydrazine were unencapsulated and only adhered externally to the CPS nanoparticle (Figure 5-1).

Figure 5-1. TEM image of a ~190 nm calcium phosphosilicate nanoparticle with gold nanoparticles agglomerated at the surface. Chloroauric acid was first reduced by hydrazine hydrate in reverse micelles and CPS precursors were subsequently added. Colloidal gold without further surface modification cannot seed CPS growth to produce dispersed core-shell particles and physically remained independent of CPS. This example provides the motivation for reducing gold salt with sodium thiophosphate to obtain a surface that can nucleate CPS.

It is expected that the reduction of gold salt from Au(III) → Au(I) → Au(0) yields inert gold that does not possess adequate surface charge for encapsulation in the CPS system. Thus, a phosphate-rich surface will assist encapsulation via calcium binding for the same reason phosphorylated chemotherapeutics can be encapsulated. The gold cores in this report were
precipitated with thiophosphate (Na$_3$PO$_3$S) at room temperature. This method also offers another safe and efficient reducing agent in gold synthesis.

Thiophosphates have not been previously reported for the direct reduction of tetrachloroauric acid to produce nanoparticles, but have been used as a phosphate source to thiophosphorylate substrates by protein kinase A (PKA). Xu et al. applied this chemistry to thiophosphorylate peptide substrates on an electrode with thiol-ATP as the phosphate donor. This allows for the assembly of gold nanoparticles at the peptide for signal transduction due to the high affinity between the nanoparticles and thiophosphate groups. It is worth noting that disputes around the true nature of thiol interactions at the gold nanoparticle interface for nanoclusters is gradually being resolved on the atomic scale. The Au-S bond had always been a widely accepted concept because sulfhydryl groups (SH) are good mediators for self-assembly or as capping ligands on flat and curved gold surfaces. The explanation is that deprotonation of R-SH yields a radical that can create R-S-Au covalent bonds similar in strength to Au-Au bonds. Au(III) salt first undergoes conversion to polymeric Au(I)-thiolate. Reductive decomposition then transforms Au(I)-thiolate into nanoparticles. Au-S models have been well-established to describe the two chemical states, oxidized and metallic, that divide Au atoms outside and inside of a nanocluster, respectively. Inner gold atoms are protected by the outer arrangement of R-S(Au-S-R)$_n$ units, so the mixture of Au(I) and Au(0) oxidation states in a cluster owes to this type of Au(I)-thiolate assembly.

Thoughts on the Au-S bond ultimately brings to the question on the oxidation-reduction mechanism of thiophosphate and chloroaauric acid to produce nanoparticles. While this reaction mechanism is unknown, we suspect this to proceed in similar ways to reduction by thiolates. It is important to probe the kinetics of the thiophosphate reaction at selected HAuCl$_4$ to Na$_3$PO$_3$S ratios in order to gain insight into the overall reaction and growth mechanism.

With the simple UV-visible absorbance information in this study, the Johnson-Mehl-Avrami-Kolmogorov (JMAK) equation was utilized to elucidate the relationship of the reactants in
this one mechanism. The JMAK equation works under several assumptions: (1) homogenous nucleation distributed randomly in space, (2) uniform nuclei growth rate in all directions into spheres, and (3) the extent of phase transformation does not influence the growth rate.\textsuperscript{218} This model consists of one rate constant \( k \), the Avrami exponent \( n \), and time \( t \). The JMAK model is summarized by Equation 5.1,

\[
A = 1 - \exp(kt^n)
\]

Experimental data from UV-visible spectroscopy will be used to determine the \( k \) and \( n \) parameters for each HAuCl\(_4\):Na\(_3\)PO\(_3\):S reaction by linearizing the maximum SPR absorption peaks \( A \) given the relationship in Equation 5.2,

\[
\ln(-\ln(1-A)) = \ln k + n \ln t
\]

The Avrami exponent is the slope and the rate constant is the intercept. The modeled kinetic curves will then be generated by fitting these parameters into the JMAK equation. The Avrami exponent traditionally refers to the dimensions of the transformation in solid-state reactions with \( n = 1 \) being a one-dimensional and \( n = 3 \) being a three-dimensional growth. However, \( n \) can be less than 1 or greater than 3 depending on the stage of the reaction. The physical meaning of the exponent can also differ depending on the reaction type. From Hancock and Sharp, Table 5-1 lists the reaction rate equations and implied mechanisms for solid-state reactions based on the \( n \) value.\textsuperscript{219}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Function & Mechanism & Equation & \( n \) \\
\hline
D\(_1\)(A) & Diffusion controlled & \( A^2 = kt \) & 0.62 \\
D\(_2\)(A) & Diffusion controlled & \((1 - A) \ln(1 - A) + A = kt\) & 0.57 \\
D\(_3\)(A) & Diffusion controlled & \([1 - (1 - A)^{1/3}]^2 = kt\) & 0.54 \\
D\(_4\)(A) & Diffusion controlled & \(1 - 2A/3 - (1 - A)^{2/3} = kt\) & 0.57 \\
F\(_1\)(A) & First order & \(\ln(1 - A) = kt\) & 1.00 \\
R\(_2\)(A) & Phase boundary & \(1 - (1 - A)^{1/2} = kt\) & 1.11 \\
R\(_3\)(A) & Phase boundary & \(1 - (1 - A)^{1/3} = kt\) & 1.07 \\
Z\(_1\)(A) & Zero order & \(A = kt\) & 1.24 \\
A\(_2\)(A) & Nucleation and growth & \([- \ln(1 - A)]^{1/2} = kt\) & 2.00 \\
A\(_3\)(A) & Nucleation and growth & \([- \ln(1 - A)]^{1/3} = kt\) & 3.00 \\
\hline
\end{tabular}
\caption{Reaction rate equations and mechanisms based on the Avrami exponent, \( n \) (Adapted from Ref. \textsuperscript{219})}
\end{table}
It should be interesting to see how the thiosphosphate reduction of tetrachloroauric acid fits with the JMAK model because there is limited information on the reaction and factors that control the rate. Studying the kinetics will also allow better size control for the core in Au-CPS nanoparticles. In addition to the characterization of the gold cores, the synthesis of Au-CPS core-shell is also discussed in later sections for the development of nanoparticles for multi-modal theranostic applications using the CPS material system. Due to differences between homogenous and heterogeneous nucleation, the starting calcium, phosphate, and silicate concentrations were reduced from our standard formulations to enable nucleation and growth of CPS on gold nanoparticle seeds.

5.2 Materials and Methods

5.2.1 Materials

Tetrachloroauric acid (HAuCl₄·xH₂O, 99.999%, Sigma-Aldrich), sodium thiophosphate (Na₃PO₃·xH₂O, ≥90%, Sigma-Aldrich), calcium chloride (CaCl₂, ≥99%, Sigma-Aldrich), sodium metasilicate (Na₂SiO₃, Sigma-Aldrich), sodium citrate (≥99%, Sigma-Aldrich), sodium hydrogen phosphate (Na₂HPO₄, ≥99%, Sigma-Aldrich), Igepal CO-520 (Rhodia), cyclohexane (Alfa Aesar), and ethanol (Koptec), were used as received. Water from High-Q 200PT-SYS (RO/IX2) purification system was degassed with argon and filtered with a 0.2 μm cellulose acetate membrane. The pH was measured with a HACH Isfet probe calibrated against aqueous standards. Conductivity was obtained from the YSI 3200 meter with a single-point calibration against a KCl standard. Stir bars were cleansed with aqua regia to prevent premature nanoparticle aggregation during gold synthesis.
5.2.2 Synthesis of gold cores and Au-CPS core-shell particles at room temperature

On the side, the micelle suspensions to synthesize CPSNPs were prepared with 1/10 the concentration of the starting calcium, phosphate, and silicate precursors. This consisted of two microemulsions, 650 μl of 10^{-3} M CaCl_2\cdot H_2O(aq) in 14.06 ml of 29 vol% Igepal CO-520/cyclohexane and 65 μl of 6.0x10^{-3} M Na_2HPO_4(aq) / 65 μl of 8.2x10^{-4} M Na_2SiO_3(aq) in 14.06 ml of 29 vol% Igepal CO-520/cyclohexane stirring at 200 rpm.

To prepare the gold core, aqueous solutions of 2 mM tetrachloroauric acid and 1 mM thiophosphate were aged for three days and then mixed with a 1:1.6 mol ratio, respectively, to a final volume of 5 mL. The reaction was capped airtight and stirred at 200 rpm at room temperature. A gradual change of color from golden yellow to orange brown, and to rusty red was observed. At 50 min, a 520 μl aliquot of the gold cores was transferred to the phosphate-containing microemulsion. The two microemulsions were equilibrated for 15 min and then combined for 2 min to grow the CPS shell. The reaction was quenched with 225 μl of 10^{-3} M citrate for 15 min. Finally, 50 ml neat ethanol was added to dissolve the micelles. The Au-CPS core-shell particles were laundered by centrifugation using the steps in Table 5-2, adapted from Tabakovic.^{34}

Table 5-2. Centrifugation laundering of nanoparticle suspension (Adapted from Ref. 34)

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Divide dissolved micelle suspension into two centrifuge tubes</td>
<td></td>
</tr>
<tr>
<td>Centrifuge at 6000 rpm for 20 min</td>
<td></td>
</tr>
<tr>
<td>Remove supernatant down to 10 mL and combine both aliquots</td>
<td></td>
</tr>
<tr>
<td>Add 20 mL 50/50 ethanol-cyclohexane (v/v)</td>
<td></td>
</tr>
<tr>
<td>Centrifuge at 6000 rpm for 20 min</td>
<td></td>
</tr>
<tr>
<td>Remove supernatant down to 10 mL and add 30 mL 50/50 ethanol-cyclohexane (v/v)</td>
<td></td>
</tr>
<tr>
<td>Centrifuge at 6000 rpm for 20 min</td>
<td></td>
</tr>
<tr>
<td>Remove supernatant down to 10 mL mark and add 30 mL neat ethanol</td>
<td></td>
</tr>
<tr>
<td>Centrifuge at 6000 rpm for 20 min</td>
<td></td>
</tr>
<tr>
<td>Remove supernatant down to 10 mL and add 30 mL 70/30 ethanol-water (v/v)</td>
<td></td>
</tr>
<tr>
<td>Centrifuge at 6000 rpm for 20 min</td>
<td></td>
</tr>
<tr>
<td>Remove supernatant down to 10 mL and add 30 mL 70/30 ethanol-water (v/v)</td>
<td></td>
</tr>
<tr>
<td>Centrifuge at 6000 rpm for 20 min</td>
<td></td>
</tr>
<tr>
<td>Resuspend and store particles in minimum 70/30 ethanol-water (v/v)</td>
<td></td>
</tr>
</tbody>
</table>
5.2.3 UV-Vis measurements for JMAK modeling

A final reaction volume of 5 mL for each 2 mM chloroauric acid to 1 mM sodium thiophosphate mole ratio (1:0.5, 1:0.7, 1:1, 1:1.3, 1:1.6, 1:2) was prepared at room temperature. The concentrations and reaction volumes are shown in Figure 5-2. Time measurements were taken in 10 min intervals, withdrawing 100 uL of the suspension at a time, for 60 min on the Thermo Scientific Multiskan spectrophotometer (with a working range up to 4.0 absorbance units) promptly after adding thiophosphate. The full scan range was 200-800 nm with absorbance taken at every 10 nm. After 60 min, turbid and purple suspensions formed visible aggregates that fall out of suspension.
Figure 5-2. Flow chart for gold nanoparticle synthesis and extraction times for optical density and particle size concentration measurements.
5.2.4 Nanoparticle characterization

A drop of the nanoparticle suspension was transferred onto a copper grid for TEM at 120 kV (FEI Tecnai G² Spirit BioTWIN, Materials Characterization Lab, Pennsylvania State University). at 120kV. Images were processed with Image J and Origin was used to fit the lognormal size distributions for about 300 particles. Zeta potential and dynamic light scattering measurements were conducted with aqueous parameters on the Brookhaven ZetaPlus v. 3.23 and DLS software (Holtsville, NY), respectively. Suspensions prepared for DLS were diluted to reduce multiple scattering.

To obtain images of the isolated gold cores, the colloidal suspension was laundered with a column. A third of a 5 ml syringe was slurry packed with solid glass microspheres with a 20 μm frit. Raw gold cores in water were eluted from the column once with minimum 70/30 ethanol-water.

The Microtrac equipped with the FLEX 11.1.02 software (Montgomeryville, PA) was programmed to acquire one measurement in 2 min. Assuming spherical particles, the transparency setting for the material was set with absorbing parameters in water at room temperature. The reactions were initiated on the side and 300 uL aliquots were extracted every 8 min (auto-zero background) for the particle size and particle number analysis.

5.3 Results and discussion

5.3.1 Gold core particle number characterization and laundering

At 60 min, the suspension color for each HAuCl₄:Na₃PO₃S ratio ranged from bright opaque yellow-orange, semi-transparent orange-red, and transparent purple-red mixtures (Figure #). The 1:0.5 and 1:0.7 ratios produced an opaque yellow-orange suspension. The 1:0.7 had identical color
transition as 1:0.5, but precipitated after 20 min. Visible aggregates appeared after 60 min when the 1:1, 1:1.3, 1:1.6, and 1:2, suspensions developed past the semi-transparent orange-red and transparent purple-red colors. In reference to the appearance, a lower ratio of thiophosphate than chloroaauric acid produced more opaque suspensions and is an indicator multiple scattering of larger gold nanoparticles.

The total particle number concentration, \( N \), in the reactions over time is shown in Figure 3-B. There are three specific regions in the curves: an initial increase in particle number (nucleation), particle number decrease (end of nucleation and beginnings of aggregation/coalescence), and a relatively constant region (growth). The regions described agree with the LaMer nucleation model where reaching a critical saturation point of gold nanoparticle monomers leads to a burst nucleation. Upon consumption of the monomers during nucleation, the free monomer concentration falls below the critical point and steady growth is controlled by diffusion of remaining monomers in solution.
Figure 5-3. Color descriptions of raw (not washed) reaction mixtures at various chloroauric acid to sodium thiophosphate ratios at 60 min: (A-1) 1:0.5 was opaque yellow to orange with blue hue under white light, (A-2) 1:0.7 contained agglomerated particles that precipitated after an opaque yellow to orange color, (A-3) 1:1 was semi-transparent orange-red with blue hue under white light, (A-4) 1:1.3 was transparent purple red, (A-5) 1:1.7 was transparent dark purple red, and (A-6) 1:2 was transparent purple blue. The total particle concentration vs. time plot in (B) shows a general increase in particle number during the initial nucleation period and decrease during growth and agglomeration. In the inset, nucleation for the 1:0.05 and 1:0.07 ratios occurred before 2 min and only partial growth and agglomeration kinetics were captured. Nucleation for the 1:1, 1:1.3, 1:1.6, and 1:2 ratios spanned over a period 10 min before the decrease in particle number was observed. An excess of thiophosphate seemed to have caused an increase in the particle concentration and transparency of the suspensions.
Nucleation peaked before 2 min for the 1:0.5 and 1:0.7 ratios and at 10 min for the 1:1, 1:1.3, 1:1.6, and 1:2 ratios. The particles then underwent further growth until the particles irreversibly precipitated. When chloroauric acid was in excess, the nucleation period occurred within a shorter period, but also created relatively less particles per volume. The addition of excess thiophosphate increased the particle number concentration during nucleation, with 1:2 ratio at the highest at $1.23 \times 10^{16}$ particles/mL in 10 min. The values for average particle size and particle concentration at each time point are listed in Table 5-3.
Table 5.3. Summary of raw gold nanoparticle average size distribution total particle number concentration (N) within 60 min, final suspension pH, and final SPR λ max at different HAuCl₄:Na₃PO₄S ratios. (The sizes are the majority that constitute >95% by particle number concentration. The standard deviation listed beside the diameter is a single measure of the distribution width and not the measurement variability on the Microtrac.)

<table>
<thead>
<tr>
<th>t (min)</th>
<th>HAuCl₄ particle size (nm)</th>
<th>N (particles/mL)</th>
<th>HAuCl₄ particle size (nm)</th>
<th>N (particles/mL)</th>
<th>HAuCl₄ particle size (nm)</th>
<th>N (particles/mL)</th>
<th>HAuCl₄ particle size (nm)</th>
<th>N (particles/mL)</th>
<th>HAuCl₄ particle size (nm)</th>
<th>N (particles/mL)</th>
<th>HAuCl₄ particle size (nm)</th>
<th>N (particles/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>9.2 (2.7) E-12</td>
<td>14.8 (3.1) E+12</td>
<td>15.2 (4.7) E+11</td>
<td>5.7 (0.4) E+14</td>
<td>9.1 (0.9) E-14</td>
<td>3.22 E+14</td>
<td>3.7 (0.5) E+14</td>
<td>8.41 E+14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>16.0 (1.1) E-12</td>
<td>49.4 (13.2) E+10</td>
<td>8.7 (1.6) E+14</td>
<td>6.1 (3.0) E+14</td>
<td>10.0 (0.8) E+15</td>
<td>1.84 E+15</td>
<td>5.8 (1.3) E+16</td>
<td>1.23 E+16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>66.7 (34.1) E-10</td>
<td>293.5 (161.7) E+9</td>
<td>17.0 (1.4) E+13</td>
<td>12.7 (4.0) E+14</td>
<td>12.5 (1.3) E+13</td>
<td>6.67 E+14</td>
<td>7.1 (1.5) E+15</td>
<td>6.3 E+15</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>30</td>
<td>25.4 (2.6) E-11</td>
<td>573* (5.14) E+9</td>
<td>31.6 (2.5) E+12</td>
<td>47.5 (3.6) E+14</td>
<td>23.1 (1.5) E+13</td>
<td>4.4 E+14</td>
<td>12.8 (1.3) E+13</td>
<td>2.5 E+14</td>
<td></td>
<td></td>
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<tr>
<td>40</td>
<td>35.6 (3.9) E-11</td>
<td>339.0* (66.7) E+10</td>
<td>20.0 (1.5) E+13</td>
<td>23.4 (2.3) E+13</td>
<td>18.7 (8.0) E+13</td>
<td>2.84 E+13</td>
<td>10.3 (6.2) E+13</td>
<td>9.72 E+13</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>50</td>
<td>69.3 (7.8) E-11</td>
<td>1.0* (0.1) E+8</td>
<td>26.4 (2.3) E+13</td>
<td>49.5 (5.1) E+10</td>
<td>32.4 (2.1) E+13</td>
<td>1.51 E+13</td>
<td>12.0 (1.5) E+13</td>
<td>1.59 E+14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>69.9 (78.7) E-10</td>
<td>1.61* (0.1) E+8</td>
<td>39.4 (2.87) E+12</td>
<td>198.3 (270) E+9</td>
<td>25.6 (2.6) E+13</td>
<td>1.51 E+13</td>
<td>17.5 (1.8) E+13</td>
<td>2.71 E+13</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

End pH

End λ max

<~600 nm (broad) | ~560 nm (broad) | ~550 nm | ~540-550 | ~540 nm | ~530 nm and agglomerates at ~700 nm (broad)

Larger  Smaller

Overall particle size distribution

* measurement unstable - particles precipitating
The semi-transparent and transparent suspensions, 1:1, 1:1.3, 1:1.6, and 1:2, contained finer nanoparticles than the 1:0.05 and 1:0.07 ratios. The 1:2 produced the smallest particles that averaged from 3.7 nm to 17.5 nm within 60 min and this can be attributed to its highest nucleation rate (~10^{15} \text{ particles-min/mL}, see Table 5-4) compared to others. The likely explanation is that excess thiophosphate stimulates the reduction of the chloroauroic acid species and rapid supersaturation. With more nuclei formed, the monomers are distributed amongst more material, so the particle size is smaller than in reactions with less reducing agent. Thus, narrow size distributions can be obtained in two ways, either by extracting particles at the peak of nucleation or by injecting excess thiophosphate to maximize the particle number concentration.

**Table 5-4.** Estimated nucleation and growth kinetics of chloroauroic acid and thiophosphate reactions at various ratios.

<table>
<thead>
<tr>
<th>HAuCl₄:Na₃PO₃S ratio</th>
<th>Nucleation rate* \text{(particles-min/mL)}</th>
<th>Growth rate* \text{(particles-min/mL)}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0.05</td>
<td>n/a**</td>
<td>10^{10}</td>
</tr>
<tr>
<td>1:0.07</td>
<td>n/a</td>
<td>7 \times 10^8</td>
</tr>
<tr>
<td>1:1</td>
<td>7 \times 10^{13}</td>
<td>10^{12}</td>
</tr>
<tr>
<td>1:1.3</td>
<td>6 \times 10^{13}</td>
<td>5 \times 10^9</td>
</tr>
<tr>
<td>1:1.6</td>
<td>2 \times 10^{14}</td>
<td>10^{12}</td>
</tr>
<tr>
<td>1:2</td>
<td>10^{15}</td>
<td>6 \times 10^{12}</td>
</tr>
</tbody>
</table>

* Extrapolated by linear regression from Figure 5-3
** Early kinetics not captured in Figure 5-3

The eventual formation of precipitates in all reactions, however, suggests that stabilizing agents are necessary for thiophosphate-reduced gold nanoparticles if colloidal stability is to be desired as-synthesized. Thiophosphate-reduced gold nanoparticles grow and flocculate continuously until the particles precipitate irreversibly. TEM characterization of the as-synthesized nanoparticles in Figure 5-4A showed 5-16 nm gold nanoparticles extracted from a 35 min 1:1.6 ratio reaction were embedded in solid byproducts that may have formed during solvent evaporation.
Figure 5-4. TEM image of the (A) as-synthesized gold cores from a 35 min 1:1.6 mole ratio embedded in artifacts from starting materials and image of the column-laundered cores (B) that are well-dispersed and appear spherical in shape. The lognormal size distribution of nanoparticles synthesized with the 1:1 mole ratio for 35 min is 47 ± 3 nm.

A column laundering method was employed to remove excess starting materials to perform proper particle size analysis by TEM. An example of the laundered gold suspension is shown in 5-4B for nanoparticles extracted at 35 min from a 1:1 ratio without the unknown solids. These nanoparticles were larger than those seen in the 1:1.6 ratio and span over a range of 10-90 nm in diameter with an average of 47 ± 3 nm by a lognormal distribution fit. Particle size analysis by TEM falls within a similar range for the 1:1 and 1:6 ratios at 30-40 min analyzed by the Microtrac. The washed particles were colloidally stable and can be re-suspended by agitation. The conductivities for the raw and washed gold nanoparticles were 885 μS/cm and 24 μS/cm, respectively. The presence of dissolved salts can neutralize the surface charge of the gold nanoparticles and cause the irreversible agglomeration over time. Therefore, both laundering and stabilizing agent (with net negative charge) are solutions to achieve long term colloidal stability.
Alternative to column purification, colloidal stability can be temporarily achieved by diluting the raw nanoparticles suspensions in water (i.e., 9 parts water and 1 part nanoparticles by volume). This method kept particles suspended for a period about 5-7 d, so reactions can be quenched to obtain particles with a specific size profile for immediate analysis.

**Figure 5-5A** is a summary of the nanoparticles before and after column purification. This process shifted the zeta potential from $-42 \pm 5$ mV to $-21 \pm 2$ mV in diluted nanoparticle suspensions. The SPR absorption band at 560 nm in **5-5B** was reduced post-launder. However, residual sodium thiosphosphate (230 nm) and chloroauric acid (310 nm) were not detectable in the washed particles. Majority of the sample was lost in the stationary phase and the laundered suspension was transparent, pink-red compared to the original dark red suspension.
**Figure 5-5.** The zeta potential shift of gold cores (A) before (in black) and after column laundering (in red) with values $-42 \pm 5$ mV and $-21 \pm 2$ mV in aqueous conditions, respectively. Column laundering is effective in the removal of starting materials as shown in (B) the UV-visible spectrum at 230 nm and 310 nm, but this method also reduces the absorption intensity of the gold nanoparticle peak at 560 nm due to the significant loss of particle number. The absorption intensity for the laundered cores did not change after 14 h. In (C), DLS was employed to obtain the effective diameter of the raw and laundered gold cores. The broad peaks in the 100-200 nm range suggest some agglomeration after laundering, but majority of the particles seem to flocculate over time within the 10-30 nm range.

A portion of the agglomerated particles were detected by DLS in 5-5C as a broad peak between 100 nm and 200 nm (red line) appeared after launder. There was a shift in size between the recorded time points, which indicate some reversible flocculation. While the suspensions lacked stabilizing agents, the thiophosphate groups (−SPO$_3^2$−) provided electronegative charge to maintain stability in diluted salt conditions. The majority of the particles analyzed by DLS fall within the 10-30 nm for the hydrodynamic size distribution. Because there was no shift in the SPR absorption wavelength or increased broadness in 5-5B, this rules out the possibility that column laundering causes significant agglomeration although present.
Spherical gold nanoparticles were successfully synthesized with sodium thiophosphate at room temperature. The suspension color was a reliable indicator for agglomeration and particle size and can be adjusted by the reactant ratios, final volume, and time. After 60 min, the nanoparticles typically agglomerate, either from unused thiophosphate or by salt effects, can be removed by column chromatography. However, the column laudering process significantly reduced the particle concentration and SPR absorption intensity and needs further refinement to minimize the loss. A different type of surface-treated stationary phase besides the solid glass spheres can perhaps be sought to improve particle elution.

**5.3.2 A kinetic assessment of chloroauric acid reduction by thiophosphate**

A final reaction volume of 5 mL was chosen so that small aliquots of 100 uL can be removed during the reduction to conduct time studies. The series of plots in Figure 5-6 shows the complete scans by UV-visible spectroscopy at every 10 min time point for all HAuCl₄:Na₃PO₃S ratios. At \( t = 0 \text{ min} \) across all spectra, no SPR peaks were detectable except for the absorbance corresponding to thiophosphate and \([\text{AuCl}_4]^-\) species at 230 nm and 310 nm, respectively. As time progressed, the starting materials were consumed and a new peak between 500 nm and 700 nm increased in intensity (red arrows). This absorbance range correlates with the reported SPR range for colloidal gold. When the particles grow in size or agglomerate, a red shift towards higher wavelengths in this region typically occur, vice-versa. A blue shift was identified from the 1:0.05 to the 1:2 ratios, with increasing amount of thiophosphate that resulted in a change of the wavelength maxima from 600 nm (broad) to approximately 530 nm. This corresponds well with the findings from the particle size distributions from the Microtrac instrument since a blueshift is to be expected with smaller particle sizes in principle (Table 5-3).
Figure 5-6. The full UV-visible spectrum of the HAuCl₄:Na₃PO₃S reactions occurring at 1:0.5, 1:0.07, 1:1, 1:1.3, 1:1.6, and 1:2 mole ratios recorded every 10 min for 60 min. The consumption of [PO₃S]⁺ (230 nm) and [AuClₓ(OH)₄₋ₓ] (x ≥ 2) (310 nm) forms gold nanoparticles that absorb between 500 nm and 600 nm that increased in intensity with time. A blueshift from 560 nm to ~530 nm is also identified from the increasing amount of Na₃PO₃S relative to [AuCl₄]. Agglomeration is suggested by peak broadening above 600 nm in the 1:2 ratio spectra. The relatively strongest SPR absorption bands corresponds to the 1:1 ratio.
In reference to the relative peak intensities, majority of the chloroauric acid seemed to deplete before thiophosphate. Consumption of thiophosphate was not observed until the 1:1 ratio, where the relatively strongest SPR band was produced. The reactions are most likely to be diffusion limited by the reagent with the highest molecular weight, \([\text{AuCl}_4]^-\), because given the excess chloroauric acid in 1:0.05 and 1:0.7 ratios, only broad SPR bands were produced around 560-600 nm. With excess thiophosphate, the kinetics for nucleation and growth are increased. The Avrami parameters, \(n\) and \(k\), obtained by replotting the UV-visible spectra using Equation 5.2 further explains this observation.

The SPR peak maxima for each ratio at all time points were first combined into the left graphs of Figure 5-7. Ideally, the curves are sigmoidal, but due to the large time gaps, early nucleation events are missing. The experimental curves, however, contain later nucleation and growth information that can be used to estimate the beginnings of the transformation with the JMAK equation. The data after \(t = 0\) min were linearized and plotted against \(\ln (t)\) as shown on the right.
Figure 5-7. The maximum absorbance at the SPR peak for each HAuCl₄:Na₃PO₄:S mole ratio was plotted against time \( t \) to obtain experimental kinetic curves (left). This data is then linearized by the relationship presented in Equation 5.2 and plotted against \( \ln t \). Linear regression gives the constants \( n \) as the slope and \( \ln k \) as the intercept (right). Two data points for the 1:1 ratio were excluded in the linear regression because the absorbance units were greater than 1.
The Avrami parameters are summarized in Table 5-5. The Avrami exponents, \( n \), are \(<1\) with the exception of the 1:1 ratio. The exponent describes the slope of the kinetic curve and as the reaction reaches limited nucleation and growth, \( n \) approaches zero. The exponent values obtained here describe a diffusion limited reaction mechanism that correlate to slow nucleation and growth. However, because an \( n \) of 2 or 3 is normally expected for nucleation and growth mechanism, the possibility for the data to only represent kinetics near the end of the reaction (diffusion-facilitated growth) is considered. Referring to the particle number concentration curves in Figure 5-3B, we know that at least partial nucleation events within the first 10 min were captured in these absorbance vs. time plots for the 1:1, 1:1.3, 1:1.6, and 1:2, ratios.

<table>
<thead>
<tr>
<th>H\text{AuCl}_3:\text{Na}_3\text{PO}_4\text{S} mole ratio</th>
<th>( k ) (s(^{-1}))</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0.5</td>
<td>(9.96 \times 10^{-3})</td>
<td>0.52</td>
</tr>
<tr>
<td>1:0.7</td>
<td>(4.82 \times 10^{-4})</td>
<td>0.99</td>
</tr>
<tr>
<td>1:1</td>
<td>(1.09 \times 10^{-4})</td>
<td>1.34</td>
</tr>
<tr>
<td>1:1.3</td>
<td>(2.92 \times 10^{-3})</td>
<td>0.83</td>
</tr>
<tr>
<td>1:1.6</td>
<td>(2.56 \times 10^{-2})</td>
<td>0.50</td>
</tr>
<tr>
<td>1:2</td>
<td>(8.78 \times 10^{-2})</td>
<td>0.38</td>
</tr>
</tbody>
</table>

From the values on the table, \( k \) and \( n \) are indirectly related and the 1:2 ratio has the highest \( k \), \(8.78 \times 10^{-2}\), and smallest \( n \), 0.38. The rate constant increases from the 1:1 ratio, with more thiophosphate and [AuCl\(_3\)]\(^-\) species to produce gold nuclei, which agrees with the measured particle number concentration. Knowingly, when the two starting reagents deplete, new materials cannot form and the nucleation rate decreases as the existing phase continues to grow. The Avrami rate constants in the table are comparable to reported values for various gold nanoparticle syntheses.\(^{220}\)

When the parameters are fitted into Equation 5.1, the complete kinetic curves are generated and shown in Figure 5-8. A closer examination of the modeled curves in 0.01-second increments reveal that the first fraction, where early growth occurs, takes place within 10 seconds. This time frame
appears shorter for larger rate constants because nucleation is accelerated by an increase of thiophosphate concentration. Conducting this reaction at lower temperatures may help seize early growth kinetics and having this model will help better understand the reaction over time.

![Model kinetic curves for the HAuCl₄:Na₃PO₃:S reaction at 1:0.5, 1:0.07, 1:1, 1:1.3, 1:1.6, and 1:2 mole ratios based on $k$ and $n$ parameters experimentally determined for the JMAK equation.](image)

**Figure 5-8.** Modeled kinetic curves for the HAuCl₄:Na₃PO₃:S reaction at 1:0.5, 1:0.07, 1:1, 1:1.3, 1:1.6, and 1:2 mole ratios based on $k$ and $n$ parameters experimentally determined for the JMAK equation.

Of course, one of the major drawbacks of using the JMAK model for LaMer-type nucleation and growth is that there is no physical meaning to the parameters, $k$ and $n$. The rate constant is also different from conventional chemical rate constants.²²¹ In spite of this limitation, the JMAK is a simple model to obtain relative growth kinetics with a sigmoidal profile. For the purpose of this analysis, the model is sufficient for understanding a single reduction reaction mechanism and comparing the results of different reactant ratios.

Based on existing literature on the reduction of chloroauroic acid to solid gold nanoparticles, it can be proposed that this reaction occurs similarly in two steps via covalent bonding with the sulfur anion of [SPO₃]³⁻: first thiophosphate reduction of Au(III) to Au(I) followed by the complete reduction of Au(I) to Au(0). The simplified reaction should be roughly the following,
(5.3) \[
\text{Au(III)} + \text{SPO}_3^{3-} \rightarrow [\text{−Au(I)}−\text{SPO}_3^{3-}]_n + \text{SPO}_3^{3-} \rightarrow \text{Au}_x(\text{SPO}_3^{2-})_y
\]

The product, \(\text{Au}_x(\text{SPO}_3^{2-})_y\), is a combination of Au at the interface and in the core of the nanocluster. Considering that the speciation of chloroauric acid is pH dependent, hydroxide and chlorine species will play a role in the reaction, a factor that is not often gauged enough in the gold-thiol reduction. This complicates the mechanism as the initial chloroauric acid species will influence chemical reactivity, and thus the final size and shape of the nanoparticles.\(^{222}\) Chloroauric acid at 2 mM in water is about pH 2.8-2.9 (no change when aged for 3 d), which can exist as several hydroxychloro gold (III) complexes, \([\text{AuCl}_4]^{-}\), \([\text{AuCl}_3(\text{OH})]^{-}\), and \([\text{AuCl}_2(\text{OH})_2]^{-}\).\(^{222}\) Reactions that participate in gold nucleation and growth rely on the coordination of the chlorine and hydroxy groups around \(\text{Au}^{3+}\). At basic pH where \([\text{AuCl(OH)}_3]^{-}\) and \([\text{Au(OH)}_4]^{-}\) species predominate, larger gold assemblies are produced because \([\text{AuCl(OH)}_3]^{-}\) and \([\text{Au(OH)}_4]^{-}\) are more difficult to reduce than \([\text{AuCl}_4]^{-}\), \([\text{AuCl}_3(\text{OH})]^{-}\), and \([\text{AuCl}_2(\text{OH})_2]^{-}\). This is according to cyclic voltammetry measurements by Wang et al. and based on the principle that \(\text{OH}^{-}\) produces a stronger ligand field than \(\text{Cl}^{-}\). The dissociation constants for each type are adapted into Table 5-6.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>(\text{Log } K)</th>
<th>(\text{Ref.})</th>
</tr>
</thead>
<tbody>
<tr>
<td>([\text{AuCl}_4]^{-} \rightleftharpoons \text{Au}^{3+} + 4\text{Cl}^{-})</td>
<td>−25.2</td>
<td>(224)</td>
</tr>
<tr>
<td>([\text{AuCl}_3(\text{OH})]^{-} \rightleftharpoons \text{Au}^{3+} + 3\text{Cl}^{-} + \text{OH}^{-})</td>
<td>−33.17</td>
<td>(225)</td>
</tr>
<tr>
<td>([\text{AuCl}_2(\text{OH})_2]^{-} \rightleftharpoons \text{Au}^{3+} + 2\text{Cl}^{-} + 2\text{OH}^{-})</td>
<td>−36.12</td>
<td>(226)</td>
</tr>
<tr>
<td>([\text{AuCl}(\text{OH})_3]^{-} \rightleftharpoons \text{Au}^{3+} + \text{Cl}^{-} + 3\text{OH}^{-})</td>
<td>−41.05</td>
<td>(226)</td>
</tr>
<tr>
<td>([\text{Au}(\text{OH})_4]^{-} \rightleftharpoons \text{Au}^{3+} + 4\text{OH}^{-})</td>
<td>−51.16</td>
<td>(227)</td>
</tr>
</tbody>
</table>

The absorption band at 310 nm is a ligand (\(\pi\))-to-metal (\(\sigma^*\)) charge transfer band (LMCT) of \([\text{AuCl}_4]^{-}\) at pH <3.\(^{222}\) The pH dependency of chloroauric acid speciation can be confirmed by the blue-shift of the LMCT band with decreased intensity at increasing pH. In the acidic conditions here, \([\text{AuCl}_4]^{-}\) is expected to be the major species. However, the likelihood of gold (III) hydroxide complexes to coexist is not to be excluded and \([\text{AuCl}_x(\text{OH})_{4-x}]^{-}(x≥2)\) should participate in the
reaction through coordination chemistry in **Equation 5.3.** There is insufficient evidence at this point to support the mechanisms that explain how thiophosphate reacts with the complex to form polymeric Au(I)-thiophosphate and solid nanoparticles. Even so, we can rationalize what the structures are, given these conditions: (1) thiophosphate can serve as a monodentate electron donor ligand for Au\(^{3+}\), (2) the final pH of all colloidal suspensions at 60 min remains acidic about pH 2.6-2.9, (3) other potential free ions in solution include Cl\(^-\) from chloroauric acid, OH\(^-\) from water, H\(^+\) from thiophosphate and chloroauric acid, and Na\(^+\) from thiophosphate, and (4) the dominant forms of gold are typically Au(I) with a 2-coordination linear geometry and coordination number of 2, or Au(III) with square planar geometry and coordination number of 4.

The prediction is that the first addition of a thiophosphate ligand to [AuCl\(_4\)]\(^-\) or [AuCl\(_3\)(OH)\(_4\)]\(^-\) will yield a higher energy transition state that forces a chlorine ligand to eject from the trigonal bipyramidal complex. The byproduct of this continuous exchange is HCl, which would support condition (2). Because charged species are more favored in polar solvents, H\(^+\) and Cl\(^-\) can also remain as ions. Studies using density functional theory calculations (DFT) gold-thiol reduction mechanisms and stable complexes propose similar predictions in **Equations 5.4-5.9.** The calculations were carried out in organic solvents that favor the proton transfer from the thiol to the ejected chlorine to form HCl, a step deemed necessary for the formation of polymeric gold-thiol. For the pathway that reduces Au(III) to linear Au(I), the second addition of the thiol group reacts with the gold complex to form a disulfide and an HCl in (5.6) to (5.7). Au(I) is formed from adding the third thiol in (5.8) and polymeric Au(I)-thiol is finally produced from the fourth thiol addition (5.9).

\[
\begin{align*}
(5.4) \quad [\text{AuCl}_4^-] + \text{HSR} & \rightarrow \text{AuCl}_3^-\text{HSR} + \text{Cl}^- \\
(5.5) \quad \text{AuCl}_3^-\text{HSR} + \text{Cl}^- & \rightarrow [\text{AuCl}_3^-\text{SR}]^- + \text{HCl} \\
(5.6) \quad [\text{AuCl}_3^-\text{SR}]^- + \text{HSR} & \rightarrow [\text{AuCl}_2^- + [\text{RSH}^-\text{SR}] + \text{Cl}^- \\
(5.7) \quad [\text{RSH}^-\text{SR}]^+ + \text{Cl}^- & \rightarrow \text{RSSR} + \text{HCl}
\end{align*}
\]
(5.8) \[ [\text{AuCl}_2^-] + \text{HSR} \rightarrow \text{AuCl}^-\text{HSR} + \text{Cl}^- \]

(5.9) \[ \text{AuCl}^-\text{HSR} + \text{HSR} \rightarrow [\text{Au}^+(\text{HSR})_2]^- + \text{Cl}^- \]

Whether the proton transfers will occur depends on the R residue on the thiol especially at (5.8).\textsuperscript{228-229} Electron-withdrawing ligands will create a more acidic proton that will dissociate from the sulfur. In another case, an electron-withdrawing ligand will also drive the reaction towards a different pathway that can reversibly produce 4-coordinated Au(III)-thiol crystals and the common Au(I)-thiol through oxidative addition and reductive elimination.\textsuperscript{230} In complex systems such as water, deprotonation of the thiol group can occur before the detachment of the chlorine ligand. This facile mechanism is difficult to predict in aqueous systems without further spectroscopic techniques and theoretical modeling.

In summary, this section presents the kinetic information on the thiophosphate reduction of chloroaurable acid using the JMAK model. The Avrami model reveal larger \( k \) constants from the increase of the chloroaurable acid to sodium thiophosphate ratio to 1:1 and the rate decreases when there are less reactants available to form new material over time. There is an indirect relationship between \( k \) and \( n \), with implications that the mechanism is a diffusion controlled process because \( n \) is mostly <1. Earlier time points for the model should be taken for \( n \) values to be more meaningful in terms of growth dimensionality. Based on established models from literature on gold-sulfur bonds, a mechanism was conjectured that is similar to thiolate reduction of Au(III) ions to form gold nanoclusters with a metallic Au(0) core and an oxidized surface for the oligomeric assembly of Au(I)-thiol. Contrary to the popular presentation of the mechanism as a two-step process, this pathway can occur with multiple intermediates from the exchange of the chlorine for thiophosphate ligands. Future work is needed to focus on calculations to determine the lowest energy conformation of gold-thiophosphate complexes in aqueous solvents and experimental work that can support this theory.
5.3.3 TEM morphology of Au-CPS core-shell particles

Au-CPS core-shell nanoparticles were synthesized with raw gold core suspensions that were extracted from a 1:1.6 reaction. Encapsulation of gold nanoparticles in a CPS matrix was observed for the first time. The procedure can be further improved to reduce random mixtures of core-shell and whole calcium phosphosilicate or gold nanoparticles. Because the gold core synthesis is not a controlled process solely by mixing aqueous thiosphosphate and chloroauric acid together, the reaction yields particles of other shapes such as cubes. There was an insufficient number of core-shell nanoparticles for statistical analysis in the sample of Figure 5-9A because the particles were not uniform. From the ~70 particles that were analyzed on Image J, the core-shell particles were 100 nm to 200 nm in diameter overall. The CPS shell coverages shown in 5-9B was uneven around the cores, but was consistently 15-20 nm in thickness. The core-shell suspensions after a centrifugation launder were dilute compared to the initial dark color of the gold core suspension, but the SPR absorbance peak was still detectable by spectroscopy.
Figure 5-9. TEM of dispersed Au-CPS core-shell nanoparticles in (A) and selected particles in the population shown in (B). The cores were subjected to 1/10 concentration of CPS precursors to seed shell growth on the thiophosphorylated gold surface. The hybrid particles were found to be around 100-200 nm in total diameter with shell thickness of about 15-20 nm (insufficient particle count for lognormal fitting). In (C), standard concentrations of CPS added to the gold cores only produced whole CPS nanoparticles. The 1/100 concentration of CPS precursors also did not yield core-shell nanoparticles in (D) and a mixture of gold morphologies were identified in the suspension.

From these image interpretations, there appears to be three features that should be strictly controlled in this synthesis: the core size, core shape, and the shell thickness. The gold core synthesis can be better controlled in micelles because the diameter is predetermined by the ratio of water to surfactant and particles formed within generally take the shape of the micelle. The micelles will also keep the particles suspended in the solvent phase to prevent aggregation before growing the shell.

The standard CPS precursor concentrations (5-9C) and 1/100 of the concentration (5-9D) did not produce the core-shell structures that were found in the 1/10 synthesis. (5-9A and B). The
calcium, phosphate, and silicate concentrations were lowered to reduce the rate of nucleation, thus a lower point of supersaturation, so that homogenous CPS nanoparticle nucleation and growth do not dominate. This allows thiophosphate-modified gold nanoparticles to serve as seeds to mediate heterogeneous CPS nucleation at the surface. The standard and 1/100 concentrations were high and too diluted, respectively, for the gold to seed the growth. Further experimentation should focus on the core to CPS precursor concentration ratio, starting with 1/10, to understand the influence of this relationship on shell thickness.

Core-shell Au-CPS nanoparticles were successfully synthesized with a 1/10 concentration of CPS precursors. The synthetic procedure, however, can use further refining to control the shape and size of the cores. A different laundering method, such as HPLC, to concentrate the particles would improve the recovery of the material and absorption signal. Although centrifugation laundering has been a straightforward procedure to remove surfactants in micelle synthesis, finer nanoparticles were inadvertently discarded in the supernatant.

5.3 Conclusions

In summary, colloidal gold synthesis by reduction with sodium thiophosphate at room temperature was presented in this study. The kinetics of the reaction using the JMAK equation suggest that excess thiophosphate plays a role in accelerating Au(III) reduction to Au(0) through a ligand exchange mechanism to eject chlorine ligands. A 1:1 mole ratio of HAuCl₄:Na₃PO₃S produced the strongest SPR band at ~550 nm in a 5 mL reaction. Experimentally determined Avrami exponents, $n$, ranged from 0.38 to 1.34 for the selected reactant ratios and point to a diffusion controlled reaction. Smaller time intervals are to be obtained to capture nucleation and early growth kinetics for the exponents to be fully interpretable within the definitions of the Avrami model. Owing to current knowledge on Au(I)-S bonds from thiolates for nanoclusters, we suspect
that the reduction progresses in a similar fashion with the reduction leading to metallic gold clusters protected by a oligomeric assembly of Au(I)-thiophosphate at the surface. And with exposed negatively charged functional groups such as phosphates, the gold clusters can seed the nucleation and growth of a CPS shell to create Au-CPS core-shell structures.

The very first synthesis of Au-CPS core-shell structures using a thiophosphorylated gold surface was presented in this report and verified by TEM. The reaction yielded large gold cores (110-140 nm in diameter) relative to the CPS shell (15-20 nm in thickness). CPS starting concentrations were reduced to enable nucleation on the surface of gold rather than nucleation into whole CPS nanoparticles. The 1/10 concentration of starting CPS yielded the desired architecture for the core-shell particles, but needs further experimentation to quantitatively understand the relationship between the gold clusters and CPS ratio to fine-tune the overall particle size distribution and morphology. Findings in this report will be useful for the development of nanoparticles for multimodal imaging and to reduce gold accumulation in biological systems by the modification of gold surfaces via phosphate-mediated encapsulation in CPS.

Acknowledgments

This work was made possible with assistance from Research Experiences for Undergraduates (REU) student, Jo Ann Martin from the University of Florida, Zachary Wileczynski, and Austin Ross for interesting discussions on the JMAK model.
Chapter 6
Considerations for CPSNPs and nanoparticles designed for translational science

6.1 Introduction

Presently, the price to develop one new drug compound is estimated to be over 2.6 billion dollars.\textsuperscript{231} While this is a rather controversial figure from an analysis led by Tufts (Center for the Study of Drug Development) for reasons stressed by Avorn,\textsuperscript{232,233} no one can disagree with the statement that drug development is a costly investment. Within the 10-year timeline to drug discovery, the process collectively involves funds from all three, academic, private, and government, sectors. And within a selection of promising treatments in development, only a few enter the market to impact patient lives. Most research on new compounds and biotech are abandoned when ideas transition from basic research and into industry, or known as crossing the valley of death.\textsuperscript{234} Discoveries can also be denied for the lack of incentives needed to intrigue industry partners, so the motive to achieve human benefits can easily fall out of the paradigm.

Since 2011, the National Institutes of Health (NIH) established a national initiative to bridge the gap between fundamental and applied sciences to improve global healthcare.\textsuperscript{235} This gave rise to what is now known as the National Center for Advancing Translational Sciences (NCATS) in the United States and the satellite programs, Clinical and Translational Science Institutes and Awards (CTSI/CTSA), to train translational scientists. Translational science is a field that is recognized within the biomedical community, but is unfamiliar to most professionals outside of the network. Through the bench-to-bedside practice, the goal is to take discoveries out of the laboratory and implement patient involvement and needs early in the process. What drives translational science is the desire to improve patient outcome through truly multidisciplinary collaborations to tackle scientific, ethical, industrial, and policy, problems.
Often, the work led by research scientists lack application even when the discovery amounts to publications. Without putting the full cycle of drug development into perspective, producing evidence within the scope of basic science limits contribution to the specialized field and not to real-world benefits. This chapter is an assessment of calcium phosphosilicate nanoparticle (CPSNP) system as a candidate for translational science from a STEM point-of-view. A discussion on where this overall development fits in the translational scheme is also engaged.

6.2 The conventional stages of translational research

6.2.1 Stages T0 and early T1

Defined by the NCATS and re-illustrated into Figure 6-1, there are five stages to translational science: discovery and basic research (T0), pre-clinical research (T1), clinical research (T2), clinical implementation (T3), and population-level outcomes research (T4), which all revolves around patient involvement to accomplish the ultimate goal of improved healthcare. This means the discovery will become part of clinical practice and in health decision-making. With complex layers, the entire process to drug discovery is a long-term investment on a product with clear expectations of the endpoint and benefits for patients.
Figure 6-1. The conventional approach to drug discovery, from basic science to human trials and then from data science to clinical practice. The four translational (T) stages (grey) overlap with the four phases of clinical trials (blue). Figure redrawn from Ref. 236.

The T0 level pertains to discoveries at the benchtop such as academic laboratories. A product is then tested *in vitro* and *in vivo* during part of the pre-clinical T1 stage for dose response, toxicities, selectivity, and efficacy. Species of cells and animals are chosen so that treatment conditions and results are in closest correlation to the human response. A safe dose is determined with quantitative studies on body-drug interactions (pharmacodynamics and pharmacokinetics). Biodistribution and sustainability (LD$_{50}$) in a physiological setting are also assessed. The evidence is all gathered to file an investigational new drug (IND) application in order to begin clinical trials.

At T0 to T1, new materials are constantly produced with potential medical applications. While innovative, the lack of a marketable profile will prevent most research at these stages from expanding past the non-profit regime. 234 For instance, consider genetics research: with the current boom in big data mining and genetic testing, just the discovery of a link to a disease will no longer
suffice because this is now a common ground. Merely a decade ago, genetic sequencing costed millions of dollars. \(^{237}\) Fast forward to 2016, the service is available to the public for $200 largely due to advances in modern computational power. The raw data that these services provide contains thousands of single nucleotide polymorphisms (SNPs) without definitive correlations. Although not very useful to the common audience, individuals with proper training and access to peer-reviewed literature or EST databases can conduct research with SNP data to draw statistical relationships. \(^{238}\) What is more compelling is that data mining research, with a testable hypothesis, can be performed at no cost and this has been done through the Penn State CTSI’s growing i2b2 informatics database (de-identified clinical records) by Schieffer et al.\(^{239}\)

The point is that getting past the early translational stages requires more than exceptional science and that the work must hold additional intrinsic value to pharma and biotech companies (e.g., intellectual property rights via PTO, the U.S. Patent and Trademark Office). Obtaining a patent on a gene is difficult now since popular research also suggests existing demands for solutions. This raises ethical concerns when an organization decides to own the rights to a discovery that is responsible for a number of yearly deaths. The long battle of gene patenting with Myriad Genetics on hereditary BRCA (the breast/ovarian cancer genetic marker) testing technology and therapeutics development, is a notorious example of this concern.\(^{240}\) In 2013, the supreme court finally reached a verdict that it is unlawful to patent genomic DNA (gDNA), or DNA that had been simply isolated from the body because the sequences are products of nature.\(^{241}\) This, however, does not bar the patenting of synthetic DNA (cDNA) and Myriad remains a symbol of what the future can hold when intellectual property is monopolized by private agencies.

Patented technology can be a major barrier to patient care and research - not only because the rights to generate new science will be prohibitively expensive, but it can also limit patient access to affordable care. However, one may rationally argue that patents drive innovation because these rights allow innovations to be profitable, which in turn, makes up for the sunk costs of research and
development. Thus, at the T0 to T1 levels, obtaining intellectual property rights may be more of a necessity than an option in this current situation for advancing ideas into large scale production for clinical trials, despite later ethical affairs. This is certainly a problem that most biomedical research will encounter when seeking FDA approval and private industry partners for funding outside of the NIH.

6.2.1 Continuation of T1 to T4

A clinical trial is divided into four phases: side effects and safe dosing range determined in a small healthy population (Phase 1, or later T1), safety and effectiveness are evaluated on a population that meets the condition/disease (Phase 2, or beginning of T2), continuation of a large population study and comparison to the effects of standard treatment (Phase 3, or later T2), and lastly, the drug is marketed and long-term use is monitored on various populations (Phase 4, or T3 to T4). From discovery to the clinical trial phases, the number of drug candidates decreases until there is less than 1% remaining of the initial candidate pool (Figure 6-2). When Phase III is complete, a new drug application (NDA) is filed to allow distribution and marketing. It is imperative that the therapeutic effect of the proposed drug is equal to or better than the standard treatment. A cost-benefit analysis will also be conducted to evaluate whether the new drug can replace current treatment.
Figure 6-2. A funnel diagram labeling the stages leading to FDA approved compounds. After rigorous clinical trials and reviews under stringent healthcare laws and guidelines, the process yielded 45 novel compounds in 2015. The development timeline was at least 10 years. Numbers for IND and NDA applications were extracted from the Food and Drug Administration (FDA) source in Ref. 242. Initial number of candidates and timeline were estimated from Ref. 243.

Phase 4 of clinical trials also exemplifies the T3 stage, clinical implementation and outcomes research. At this stage, the government sector becomes more involved in regulations and only 1 candidate typically survives this process. Investigators and public health services such as the Food and Drug Administration (FDA) collaborate to create guidelines and policies for translating the knowledge into clinical practice. Patient feedback can also be used to validate the positive impact of the new treatment or device. The merge of scientific and population-research knowledge promotes the understanding of human health and the nature of diseases. At T4, the
discovery proceeds to make a positive change to global healthcare through combined efforts of the scientific community and public.

6.2 Where CPSNPs fit in the translational scheme

CPSNPs were first introduced by the Adair Lab in 2008 and have been granted two patents since through Penn State University. The pH-sensitive property of CPNSPs allows for controlled dissolution of the carrier matrix for delivering agents in an environment that transitions from neutral to acidic pH. Therefore, this system is sustainable for transport in the bloodstream. There are many aspects of CPSNPs that can be probed analytically (the surface chemistry, composition and phase, size control, shape control, encapsulation efficiency, diffusion of encapsulated agents, laundering, and etc.), to understand the optimization required for delivering specific chemical compounds. Not only can this delivery method be tailored to experimental drugs, but it can also reformulate compounds that have been approved by the FDA.

In 2012, the NCATS established an initiative to repurpose and find novel uses for abandoned compounds. Approved drugs are incredibly valuable because there is a substantial amount of information from pre-clinical to clinical phases to be expanded upon. To revisit the bottlenecks and develop a completely new drug will consume a lot of time and funds. Studies such as CPSNPs modify the delivery mechanism of well-studied compounds without altering the original function. Thus, the review process for nanoparticles will primarily involve evaluating the the efficacy and safety of the new delivery method and less about approving the compound because the groundwork already exists (Figure 6-3).
Figure 6-3. Development process for nanoparticle delivery platforms (CPSNPs, in particular) using existing agents in the pharmaceutical market.
The target profile of CPSNPs that aims to improve the therapeutic effect of 5-fluorouracil and similar drug analogs in patients with pancreatic cancer is one of many. The design is versatile that it can be applied to other types of cancers or early detection applications. More importantly, there is demand for improving the outcome of treatments for aggressive cancers. It is important to note that the end goal does not have to be a cure, but a way to make the disease and treatment more manageable than current means.

6.3 Methods and thoughts on nanoparticle troubleshooting

Often there is uncertainty in the feasibility and safety of new nanomaterials that are introduced to biomedical research. While the nanomaterial system can carry out the intended function in cells on a plate, the same success cannot be guaranteed in a complex live animal. Nanoparticles on their own can be a challenge to characterize for a number of reasons (e.g., small size, sensitive elements, low contrast, or working in aggressive organic solvents/water), but incorporating them in living things demands another insight to elucidate the cause and effect of these properties. The sections that follow describe the types of characterization, failed cases and results that are helpful for maintaining nanoparticle quality, CPSNPs in particular, and for maintaining the consistency of biological outcomes during live cell tests and animal trials. It is especially important to understand common mishaps at the pre-clinical phase and have methods readily available to solve problems that can discredit the nanomaterial in question.

6.3.1 Non-specific toxicity

Inorganic nanoparticles synthesized by wet-chemical approaches typically need stabilizing ligands or reaction vesicles in suspension like micelles to control the reaction. The micelles for
CPSNPs are composed of surfactant molecules in bulk liquid. These single building blocks are Igepal CO-520, an amphiphilic molecule with a nonionic polar head structure and a hydrophobic hydrocarbon tail. Similar surfactants for nanoparticle synthesis such as sodium dodecyl sulfate (SDS), Brij, Triton, and Tween, are also routinely used as solutions for cell lysis in DNA/RNA and protein extraction.\textsuperscript{247} The cell membrane proteins solubilize when surfactant monomers partition into the lipid bilayer. At sufficient surfactant concentration, cells will undergo cell death from membrane disruption, so surfactants can skew cell proliferation assays by contributing to false-positives.

The washing of nanoparticles to remove residual surfactant is challenging when the process can also reduce the number of nanoparticles or disrupt colloidal stability, but the adequate removal of surfactants should be quantified. For CPSNPs, two cycles of vdW-HPLC laundering is sufficient to reduce Igepal CO-520 to negligible concentrations. Single wash cycles have previously led to non-specific toxicity observed \textit{in vitro} in formulations with dilution factors as high as 1/1600 on PANC-1 pancreatic cancer cells (\textbf{Figure 6-4}). Toxicity was independent of the encapsulated chemotherapeutic agent and was observed in the unloaded particles. The assays initially concluded that bare CPSNPs were inherently toxic to cells, which disagreed with the years of previously established \textit{in vitro} work.
Figure 6-4. *In vitro* cell proliferation of PANC-1 with non-specific toxicity from unloaded Cit-ghost-CPSNPs (labels, WSL and XT, correspond to formulation IDs with 1:100, 1:200, 1:400, 1:800, and 1:1600 dilution factors by volume). The first two treatment columns correspond to no treatment (NT) and phosphate buffered saline (PBS). There was a dose-dependent effect for toxicity which could have been caused by nanoparticle number fouling or an unknown contamination. The root cause was found to be residual Igepal CO-520 and the solution was to simply doubly-launder the CPSNPs by vdW-HPLC. Assay courtesy of G.L. Matters group.

Both alamarBlue® and MTS tetrazolium assays can be utilized to gain insight on cell viability. The MTS is similar to the commonly used MTT assay since both involve the conversion of a tetrazolium dye (yellow) to formazan (purple), but with additional steps for the MTT to dissolve crystalline formazan before analysis. Cells subjected to alamarBlue® convert resazurin (blue) to resorufin (red). In a typical assay for the purpose of this research, about 5,000 cancer cells were seeded into 96-well plate wells and incubated for 24 h in a CO₂ environment for cell adhesion to the surface. The cells are then treated with nanoparticles and controls and incubated for an additional 72 h. The assay solution is transferred to the wells and incubated until ready for absorbance readings.

PANC-1 and BxPC-3 reacted to Igepal CO-520 in similar ways as cell lysis detergent solutions. Live cell imaging on PANC-1 was conducted to observe the physical effects of the
surfactant on the cells (Figure 6-5). The cancer cells were naturally adherent to the culture flask. Minutes after Igepal CO-520 was introduced, the cells made conformational changes to detach from the surface. Disruption of cell adhesion suggested the loss of cell membrane structural integrity, which eventually led to cell death.
Figure 6-5. Live cell images of PANC-1 in culture media that were treated with Igepal CO-520. Cells were first plated overnight and then stained with orange CMRA. About 3 mM Igepal CO-520 was added to the well and images were extracted from a recorded video for 0, 30, and 180-second time points. Within 30 seconds, the adherent cells underwent membrane conformational changes and physically detached from the plate by 180 seconds. The cells gradually shrunk in size after 5 min (not shown), which was an indicator of cell death. Imaging was conducted on the Keyence BZ9000 Generation II microscope at 20x magnification (Huck Institutes of Life Sciences). Images were brightness and contrast-adjusted.
A simple dose response test of Igepal CO-520 on the cells was conducted to verify the surfactant concentration at which cyto-toxicity was significant. PANC-1 and BxPC-3 cells were treated with a range of Igepal CO-520 concentrations from 0.05 μM to 100 μM (Figure 6-6). From 10 μM and 50 μM, cell proliferation was significantly reduced. Concentrations ≤10 μM were tolerable for both the PANC-1 and BxPC-3.

**Figure 6-6.** The *in vitro* dose response of PANC-1 (left) and BxPC-3 (right) human pancreatic cancer cells treated with Igepal CO-520 solutions. The cells did not tolerate surfactant concentrations greater than 10 μM. Cell proliferation was reduced by half or more, relatively, with 50 μM. NT corresponds to no treatment and the vehicle is phosphate buffered saline. Assay courtesy of G.L. Matters group (*p<0.01)

A simple method to quantify surfactants is by measuring the surface tension when other routes such as mass spectrometry is too costly or when UV-VIS spectroscopy is inconclusive below 300 nm. The surface tension is expected to decrease from ~73 mJ/m² at 20°C with surfactants in water. The procedure to prepare samples for the dynamic tensiometry is outlined in **Appendix C**. The nanoparticle suspension was titrated into clean solvent and final surface tension was then compared to a standard curve to extrapolate the concentration. Changes in the surface tension of phosphate buffered saline with increasing additions of singly and doubly-launched nanoparticle suspension is shown in **Figure 6-7**. Singly-launched CPSNP suspensions contained at least 0.1 mM residual Igepal CO-520 while doubly-launched suspensions contained about 10 μM from
analysis by surface tension. LC-MS/MS verified that randomly selected doubly-laundered samples contained varied concentrations from 10 to 72 μM, but had negligible effects on cell proliferation when the formulations were further diluted into clean culture media for treatment dosing.

![Graph showing surface tension vs volume of CPSNPs](image)

**Figure 6-7.** Surface tension of phosphate buffered saline (PBS) titrated with singly-laundered CPSNPs (■) and doubly-laundered CPSNPs (▲). Igepal CO-520 concentration was significant in singly-laundered CPSNPs. This was qualitatively verified by the decline of PBS surface tension with increasing CPSNP additions. Doubly-laundered CPSNPs imposed negligible changes to the surface tension of PBS (~73.5 mJ/m²). Extrapolated from an Igepal CO-520 surface tension standard curve (see Appendix C), the surfactant concentrations were about 0.1 mM and 10 μM in singly and doubly-laundered CPSNP formulations, respectively.

In summary, the effects of residual starting materials, especially in inorganic synthesis, is unpredictable when incorporated in living organisms. Most biological assays are straightforward in procedure and analysis, but without proper characterization to investigate sources for false outcomes, nanomaterials can be mistaken as non-biocompatible.
6.3.2 Endotoxin contamination

To fulfill the United State Pharmacopoeia (USP) guidelines for injectable formulations in pre-clinical evaluations, endotoxins in deliverables should not exceed 5 EU/kg for a human dose.\textsuperscript{250} Endotoxins elicit an inflammatory response in animals because they are found in the outer membrane of gram-negative bacteria. Also known as lipopolysaccharides, endotoxins are composed of three parts: lipid A, ketodeoxyoctulosonic acids (KDO), and an O-antigen (Figure 6-8). Glucosamines join the lipid A to the core region. The compound is negatively charged in nature due to the phosphate groups linked to the glucosamines and other charged residues on the sugar backbone. The impact of endotoxin is a concern when specific surface chemistry on nanoparticles is desired. For CPSNPS, endotoxins can bind to calcium sites at the surface.

\textbf{Figure 6-8.} Three components to an endotoxin: O-antigen, ketodeoxyoctulosonic acid (KDO), and lipid A. Structure redrawn and generalized from Ref. \textsuperscript{252}.\textsuperscript{251}
The zeta potential is the metric for elucidating the net charge on CPSNPs before and after surface modification. This measurement also verifies the success of bioconjugation based on changes in surface polarity. Similar to the effects of high ionic strength in saline solutions (Figure 6-9), endotoxin fouling can cause double layer compression by the shielding of electrical charge. Regardless of pH, the zeta potential becomes less negative. Applications of this knowledge is commonly used to design filter membranes to absorb endotoxins from solution. Surface coverage of endotoxins will also prevent additional crosslinking reactions.

Figure 6-9. Example of electrical layer compression reflected on the zeta potential due to an increase of ionic strength. This is a scenario starting with a negatively charged surface and the zeta potential magnitude is gradually reduced by a high localization of counter ions at the diffuse layer. As a result, the diffuse layer compresses and the net force becomes attractive via van der Waals forces. This is also the driving force for colloid destabilization (agglomeration).

The method to quantify endotoxins is explained in Appendix E for a chromogenic limulus amebocyte lysate (LAL) assay. LAL is an extract from the blood cells of horseshoe crabs that coagulates on contact with endotoxins in water and remains as a reliable option for endotoxin detection. Sources for false positives of endotoxins for the chromogenic assay include high nanoparticle number in suspension and measurements at extreme pH values. The assay should be
conducted in absence of particulates and at relatively neutral pH in water. CPSNPs are to be digested with EDTA before adding the LAL reagent to prevent these errors.

In Appendix E.3, endotoxin data for multiple buildings at Penn State, University Park was obtained and significant contamination in the water source was revealed across campus (in red). While endotoxins can be reduced by distillation and reverse osmosis systems, endotoxin fouling reduces the expected lifetime of the filtration unit and may gradually leak into the purified water source and contaminate glass surfaces if not sanitized properly.

There is, unfortunately, limited reports on how endotoxins affect nanoparticles aside from toxicity. Endotoxins are a risk for active surfaces and on studies that involve the study of inflammatory responses in animals. Thus, it is worthwhile to investigate and test for endotoxins on a regular basis when the presence is widespread in the water source.

### 6.3.3 Size and particle number concentration

Due to the subjective nature of TEM imaging for nanoparticle size analysis and for general assessments, to acquire an image that represents a sample as a whole can be, at times, more of an art than science. Artifacts such as drying fronts, organic contamination, dust, beam damage, aberrations, and agglomeration, are details that can misguide the actual interpretation. Elemental analysis and diffraction on TEM offer additional information on composition and crystallinity for high Z-number nanomaterials, which are more quantitative than describing the size and shape. However, soft materials such as CPSNPs cannot take full advantage of high intensity beam analyses because the particles will ablate into hollow shells.253 This calls for more advanced imaging with a cryo-stage in order to map out CPSNP features and to understand its formation mechanism.

One of the challenges to obtaining a proper CPSNP image is agglomeration. A bright field image of CPSNPs presented in Figure 6-10 shows multiple clusters of smaller nanoparticles (red
arrows). There are two possible explanations to this scenario: (1) agglomeration is an inherent problem in the formulation or (2) the method used in TEM sample preparation is not suitable for the formulation. The first explanation completely discredits the quality of the particles, but the second is a simple fix. Being able to differentiate these two scenarios with supporting evidence can be the sole determinate factor for passing the pre-clinical phase. When a sensitive nanoparticle formulation is handed off to large scale production by scientists without the intimate technical knowledge, it is likely that agglomeration will be one of the encountered situations.

**Figure 6-10.** Nanoparticle clusters >100 nm composed of agglomerated CPSNPs (inset) from a sample that was diluted into neat ethanol before a droplet was transferred to the copper grid. Particles were sampled from WSL3-24-20 Cit-ghost-CPSNPs.

Agglomeration raises a red flag in drug delivery, with implications that the particles are not colloidally stable before entering the bloodstream. This is hazardous when the agglomerated mass circulates and clogs heart arteries. The final diameter is also a property that determines whether CPSNPs will be able penetrate dense fibrotic tissue or cell membrane to release active
agents. When results from imaging are ambiguous, colloidal stability can be suggested by the zeta potential. Other particle sizing methods such as dynamic light scattering (DLS) can be used as a secondary evaluation only if multiple scattering is minimized by reducing the particle number. For the case presented in Figure 6-10, temporary agglomeration was caused by diluting the nanoparticles into ethanol during sample preparation. Due to a change from 70/30 ethanol-water to a low dielectric constant solvent such as ethanol, the nanoparticles lacked adequate surface charge to remain dispersed and collectively deposited onto the copper TEM grid into a mass.

Obtaining the particle number concentration (particles per unit volume) relative to the diameter of nanoparticles in suspension is another way to monitor colloidal stability over time. This is also an important metric for tracking the number of particles per drug dose and for quantifying the number of drug molecules encapsulated per particle. Nanoparticle fouling by numbers can overwhelm cells and create a toxic environment for proliferation. Initial in vitro results with indocyanine green-encapsulated CPSNPs suggest that the LD$_{50}$ for colorectal cancers (HT29 and HTC116), breast cancer (MDA-MB-231), and pancreatic cancer (BxPC-3), is about 2x10$^8$ CPSNP/cell. Theoretically, 2.5x10$^{14}$ particles/ml is the expected yield per CPSNP formulation. The methods available to measure the particle concentration of CPSNPs is the Izon qNano (size is determined by electrical current blockages by nanoparticles on a single hole; detection limit ~50 nm) and the Microtrac (Mie theory; detection limit ~0.5 nm). Other common problems encountered with CPSNPs, aside from agglomeration, are low particle concentration and by similarities in the refractive index of CPSNPs to the parent solvent. This continues to be a present challenge that requires fine tuning of the instrument parameters to reliably detect and analyze the particles.

When dealing with small size range of nanoparticles <100nm that are as difficult to characterize like CPSNPs, what is observed under an electron microscope may not be representative of the actual state of the nanoparticles in suspension. This lesson applies to any nanoparticle platform that is delivered in liquid form, and it is best to understand the physical and
chemical properties of nanoparticles in the as-synthesized and in the intended environments. The particle number concentration is often not reported in \textit{in vitro} or \textit{in vivo} studies for nanomaterials and adds uncertainties in the reproducibility of treatment doses. This also complicates the analysis when non-specific toxicity is observed. Ultimately, employing the appropriate methods to characterize biomedical nanomaterials helps determine the proper dosing regimen for specific cell types.

\textbf{6.3.5 Long term storage and pH stability}

Ph-sensitive nanomaterials such as CPSNPs demand special care and monitoring for long-term storage. CPSNPs gradually dissolve over time when pH is not strictly maintained in aqueous suspensions. Degradation of the protective nanoparticle layer can lead to degradation of the encapsulated agent. For instance, CPSNPs loaded with indocyanine green enhance the fluorescence lifetime and intensity of the dye. Differences in fluorescence before and after encapsulation can be readily detected by a spectrophotometer. However, gradual degradation is not always obvious until the particles circulate in animals and the fluorescence intensity or drug potency is severely compromised.

Nanoparticles delivered intravenously are normally suspended in phosphate buffers, so the buffer capacity should account for additional H\(^{+}\) contributed by all reagents. The capacity of commercial PBS from Cellgro that is routinely used for CPSNPs is shown in Figure 6-11. Although the acid buffer capacity is about 7 mM before the pH drastically decreases from pH 6.0, the capacity between pH 7.4 and 6.0 is surprisingly narrow for CPSNPs. Between pH 7.0 and 6.0, CPSNPs will dissolve. It was discovered that the pH was significantly influenced by residual chemicals from bioconjugation - sulfo-NHS, in particular.
Figure 6-11. Titration of Dulbecco’s PBS solution (pH 7.4, Cellgro 21-031-CV) with 0.1 M HCl. The formulation contained about 2.7 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, 0.14 M NaCl. This gives a 3 mM acid buffering capacity from pH 7.4 to 6, which is a narrow buffering window for CPSNPs when the particles partially dissolve a pH <7. Titration conducted on dynamic mode on the Mettler Toledo.

Sulfo-NHS is a relatively strong acid as shown by the titration curve in Figure 6-12, so caution is to be taken when using the compound in excess for carbodiimide crosslinking reactions.

Figure 6-12. Titration of Sulfo-NHS (Thermo Scientific Pierce) with 0.01 M KOH.
Buffers should be selected with caution to maximize the storage life, but depending on the nanomaterial, pH will not be the only main contributor to degradation. With multiple factors that can contribute to an unsuccessful animal trial, basic material properties can be frequently overlooked when nanoparticles are expected to function in unfamiliar environments.

6.4 Conclusions

The fact that it is a challenge for nanoparticles to retain the desired characteristics in a biological setting emphasizes a need for more studies to analyze their response in uncommon situations. CPSNPs continue to reveal unexpected behaviors and these events serve as practical information to prevent future oversight in the development and pre-clinical phases. Here, four different considerations are summarized based on our experience with CPSNPs, which can be applied to similar nanoparticle systems. Failed experiments are equally valuable in creating nanomaterials for translational science when targeted medicine is still a growing area. Uncertainties in the safety, efficacy, and overall feasibility, of nanoparticles in medicine exist and it is up to experts in their respective disciplines to collectively provide evidence to refute these doubts.

Lastly, to pursue translational science as a STEM researcher is a decision that takes additional training to envision benchtop science through the perspective of healthcare providers. It is often an endeavor that is guided by passion and persistence to seek solutions to a disease. Given the traditional cycle of drug discovery, it is likely that most proposed science will not impact lives for a long time, deeming the purpose small and insignificant at the moment. New research is not always readily accepted, so grasping the significance can also be one of the hardest undertakings when translational research does not follow a conventional route. Nonetheless, efforts in translational research are rewarding as they offer creative outlets for better health management.
Without interdependent cooperation to improve healthcare, treatments will remain only as effective as they were first discovered like most chemotherapeutics of today.
Chapter 7

Conclusions and future work

7.1 Conclusions

This dissertation focused on the optimization of encapsulation efficiency for CPSNPs. The therapeutic capacity of CPSNPs on pancreatic cancer was evaluated for phosphorylated chemotherapeutic agents. The synthesis of thiophosphorylated gold nanoparticles and manipulation of secondary CPSNP growth on solid gold were also explored.

1. A method to condition and probe the adsorption of dissolved species in solution on active surfaces was developed specifically for CPSNPs. A model surface for CPSNPs was synthesized and verified by XRD to be brushite. ATP, citrate, 5-FU, FdUMP, and hydroxyl and proton ions, were introduced into the solution to observe trends in the zeta potential relative to pH. Hydroxyls, protons, and phosphates were potential-determining ions that influenced the hydrolysis products at the solid-liquid interface. Hydroxyl ions increased the (−) zeta potential magnitude at pH >7 (around the IEP of calcium phosphate) and protons increased the (+) zeta potential magnitude at pH <7. Increasing additions of ATP or citrate significantly increased the zeta potential magnitude towards more (−) values. Higher concentrations of FdUMP shifted the IEP and effects by 5-FU on brushite were negligible. A change in surface polarity was an indicator for adsorption activity and can be used as a metric to measure relative success in encapsulation for the CPSNP platform. The adsorption of drug agents and their phosphorylated counterparts are compared by this method to identify key functional groups for improving uptake in nanoparticles.
2. The encapsulation efficiencies (EE) for phosphorylated drug agents in CPSNPs, FdUMP and GemMP, were found to be several orders of magnitude higher than the non-phosphorylated 5-FU, FUdR, and Gem (<1% EE). A complete log of these samples with EE’s is archived in Appendix A. The analysis confirmed the significance of phosphorylated agents in nanoparticle uptake. Pyrimidine-based antimetabolites are a group of drugs that require intracellular phosphorylation pathways to become pharmacologically active agents. With encapsulation and direct delivery of the active agents, conversion pathways of 5-FU and Gem can be bypassed to minimize drug inactivation. The encapsulated FdUMP retained biological activity to inhibit thymidylate synthase, which is the mechanism of action for inducing cell death via damage of the DNA synthesis and repair pathways. Majority of the cells exhibited cell cycle arrest at the S-phase when treated with FdUMP-doped CPSNPs. Further, at a dose diluted 1000x to ~200 nM, encapsulated FdUMP had similar toxic effects to 250 μM unencapsulated FdUMP in vitro on human pancreatic cell lines. Normal pancreatic duct epithelial cells were relatively spared at 200 nM encapsulated FdUMP, indicating the delivery of an efficacious dose. A more potent effect can be achieved with nanoparticles at lower doses whereas cancer drugs are often administered at higher doses by conventional treatment in order to observe any therapeutic benefits. An in vivo trial on athymic mice with orthotopic PANC-1 tumors verified nanoparticle internalization in cancer cells for FdUMP to inhibit TS. Targeting with aptamer1153 FdUMP-CPSNPs showed significant TS inhibition than the untargeted particles.

3. Gold nanoparticles were produced by thiophosphate reduction in water at room temperature. The reaction mechanism was elucidated by the JMAK model and by the particle number concentration over time. The JMAK model was a simple solution for comparing the reaction kinetics at increasing molar additions of the reducing agent. Although the reaction was speculated to proceed in similar ways to the common thiosulphate reduction, there is still more to learn about the underlying mechanisms. By monitoring the particle number concentration, it
was found that the reaction underwent three specific stages within 60 min that were in line with LaMer nucleation and growth model: nucleation (creation of new material and phase - rapid increase in particle number), beginnings of coalescence (decline in particle number), and growth (relatively steady particle number). Within the Avrami definitions of the $n$ parameter, the reaction was mainly diffusion-limited and with increasing thiophosphate molar ratio, the reaction rate, $k$, increased. Interpretations with the JMAK model was limited in this scenario because early nucleation events were not captured, but there was more information on later growth kinetics. Additionally, core-shell nanoparticles that consisted of a gold core and thin calcium phosphosilicate (CPS) shell was synthesized at 1/10 concentration of the normal CPSNP precursor concentrations (standard formulation sheet in Appendix F). At standard concentrations, homogenous growth yielded whole CPSNPs and no core-shell structures were detected by TEM. At 1/100 concentration, the presence of gold nanoparticles was the majority. As expected, from our understanding of homogeneous and heterogeneous nucleation, it was found that the synthesis of Au-CPS core-shell nanoparticles was CPS-concentration dependent. While it was possible to nucleate CPS on thiophosphate-functionalized gold, refinement of the synthetic procedure will be needed so that CPSNP nucleation and growth does not dominate and that the gold cores are able grow a uniform coating of CPS.

In summary, the development of CPSNPs for drug delivery is an alternative route to conventional drug discovery because the main purpose here is to reformulate existing cancer drugs to improve drug efficacy and reduce side effects. These goals are achieved by reducing the administered dose, reducing premature metabolic breakdown of the prodrugs by enzymes, and directing the cargo with bioconjugated targets to accumulate at the tumor site.
7.2 Future work

The use of phosphorylated agents has opened a completely new avenue in encapsulation, which is not just limited to the antimetabolites discussed in this dissertation. This brings up suggestions for future work:

1. **Pathways for phosphorylation of various agents for encapsulation.** Phosphorylated cancer agents such as FdUMP and GemMP are expensive and not every candidate for encapsulation comes conveniently phosphorylated. However, there are two methods that can be further explored to achieve phosphorylation: enzymatically using protein kinase A (PKA) and ATP as the phosphate source, or encase the compound in a phosphate-rich “molecular cage” such as in cyclodextrin-phosphate. Challenges to the first method is modifying the compound without compromising fluorescence or biological function since PKA transfers γ-phosphoryl groups from ATP to any available hydroxyl groups on the receptor compound. A way to possibly employ this is by immobilizing the compound to be phosphorylated on a substrate (e.g. in a column stationary phase) and condition the substrate in a buffered solution with ATP, PKA, and cyclic adenosine monophosphate (cAMP), which activates the PKA. In the second method, the compound of interest needs to physically fit in the cage cavity, typically no more than 10 Å in diameter. A hydrophobic cavity gives the option to encapsulate hydrophobic compounds because the outer ring of cyclodextrin is hydrophilic to enhance solubility.

2. **In vivo targeting with non-athymic mice models.** The reason for using athymic mice models is the ability to implant human cancer cells without fear of tumor rejection. This creates a model that is closely related to a human organ possessing the cancer tumor. Hypothetically, effects from treatment in mice should reflect similar responses to treatment in humans. However, a downside to using an immunodeficient model is that one of the prominent indicators of reduced
drug side effects by nanoparticles, the lack of hair loss, cannot be observed because the mice are hairless. Mice with inherent genetic mutations leading to PDAC progression will be ideal for this purpose. Furthermore, genetic in vivo models are better representations of patients overall because cancer heterogeneity is a natural challenge in disease treatment. In vivo trials are currently ongoing for delivering FdUMP-doped CPSNPs to pancreatic cancer tumors with aptamer targets. Successful targeted delivery of ICG-doped CPSNPs with aptamers has already been verified in Tang’s findings with orthotopic PANC-1 athymic mice models.39

3. Encapsulation mechanism of CPSNPs via TEM tomography and spectroscopy. The work on osmium-stained CPSNPs to study particle formation and encapsulation is significant because imaging over the years for CPSNPs have been largely limited by beam damage and low contrast. This is the first time that 1-3 nm sub-particles on CPSNPs were observed by electron microscopy through the use of a high-Z element precursor, osmium (III) chloride, to substitute part of the calcium chloride. The next step is to perform tomography to map the distribution of osmium sub-particles in order to verify true encapsulation and not surface decoration. Preferably, cryo-EM should be employed because coarsening of the osmium particles was significant during EDS implementation (see Appendix G). This project, as well as drug encapsulation, will also greatly benefit from IR and Raman spectroscopy analyses. The process of encapsulation within a solid matrix should reduce the vibrational modes of the encapsulated compound. A method can be developed to measure encapsulation in real-time if interference from micelle formation and collision activity is mitigated.

4. Formulation scale-up. The scale-up of CPSNP production has been of interest because majority of our efforts and time are consumed by the (non-automated) vdW-HPLC washing process. The launder cycle for a single CPSNP formulation (yields 5-8 ml) takes about 4-6 h. In cases when larger volumes are needed for in vivo trials and characterization, a 10x-concentrated suspension is synthesized and needs at least 2 d to launder. The clogging of the mechanical
pump lines by nanoparticles requires the undivided attention of the user to ensure proper solvent flow to minimize sample loss. Thus, a scale-up procedure to create large batch formulations will shift efforts to focus more on characterization and development. Currently, the use of buoyant polymeric microspheres for laundering in a large vessel (VAML, or the van der Waals-assisted microsphere laundering) is in the refinement stages for rhodamine-WT and ICG-doped CPSNPs. With similar deposition and release mechanisms as with glass beads in vdW-HPLC, liter amounts can be yielded from one procedure. Scale-up of drug-doped CPSNPs is costly (especially for phosphorylated agents) and due to the toxicities of chemotherapeutics, stringent guidelines for safety, shelf life, and handling will be necessary. Lastly, another advantage of scale-up is enabling analysis of particle number concentration, which is a variable of CPSNPs that has yet to be characterized. Characterization by the Microtrac instrument (detection limit ~0.5 nm) is currently limited by insufficient particle count per formulation and is at least two orders of magnitude lower in particle loading index than the required amount.
Appendix A

Sample list with descriptions of CPSNPs doped with 5-FU, FdUMP, Gem, and GemMP

Table A-1. A complete list of drug-doped formulations that were assessed in vitro and/or in vivo with corresponding sample ID label, zeta potential and drug concentration as verified by LC-MS/MS.

<table>
<thead>
<tr>
<th>Sample ID*</th>
<th>Description</th>
<th>Batch size†</th>
<th>ζ- potential (mV)</th>
<th>Encapsulated drug conc. (M)</th>
<th>Mol% EE*</th>
<th>Mol% recovery*#</th>
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</thead>
<tbody>
<tr>
<td>WSL1-52-1A</td>
<td>Cit-5FU-CPSNP</td>
<td>1x</td>
<td>n/m</td>
<td>1.7E-07 (n=1)†</td>
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<td>n/m</td>
<td>2.1E-07 (n=1)†</td>
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<td>n/m</td>
<td>3.9E-07 (n=1)†</td>
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<td>3.6E-09 (n=1)†</td>
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<tr>
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<td>1.4E-06 (n=1)†</td>
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<td>−9±1 (n=5)</td>
<td>1.9E-07 (n=1)†</td>
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<td>Cit-FdUMP-CPSNP (1x washed)</td>
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<td>n/m</td>
<td>2.5E-04 (n=1)†</td>
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<td>Cit-FdUMP-CPSNP (2x washed from 5-54-2A)</td>
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<td>-33±5 (n=5)</td>
<td>5.5E-05± 1.8E-06 (n=3)†</td>
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<td>-3±5 (n=5)</td>
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<td>5.0±3.0E-08 (n=3)†</td>
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<td>-26±4 (n=10)</td>
<td>3.0±1.5E-08 (n=3)†</td>
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<td>WSL5-18-5B</td>
<td>Cit-FUdR-CPSNP</td>
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<td>1.1±0.7E-08 (n=3)†</td>
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<td>p-value</td>
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<td>8±13 (n=5)</td>
<td>Below detection limit†</td>
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<td>44±11 (n=5)</td>
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<td>16±3 (n=5)</td>
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<td>33±19 (n=5)</td>
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<td>15±9 (n=5)</td>
<td>2.00±0.06E-09 (n=3)*&lt;0.01</td>
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<td>mPEG-FdUMP-CPSNP</td>
<td>mPEG-Ghost-CPSNP</td>
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<td>1x</td>
<td>1.3±0.02E-05 (n=3)†</td>
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<td>2.20±0.04E-05 (n=3)†</td>
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<td>1.41±0.09E-05 (n=3)†</td>
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<tr>
<td>WSL5-90-45B</td>
<td>Cit-FdUMP-CPSNP</td>
<td>1x, 10x conc.*</td>
<td>3.99 ± 0.63E-04(n=3)†</td>
<td></td>
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</tr>
<tr>
<td>WSL5-92-5</td>
<td>mPEG-Ghost-CPSNP</td>
<td>1x, 10x conc.*</td>
<td>Below detection limit†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSL5-92-6</td>
<td>mPEG-FdUMP-CPSNP</td>
<td>1x, 10x conc.*</td>
<td>2.99 ± 0.61E-04 (n=3)†</td>
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<td></td>
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<tr>
<td>WSL5-92-8</td>
<td>AP1153-cPEG-FdUMP-CPSNP</td>
<td>1x, 10x conc.*</td>
<td>2.50 ± 0.27E-04 (n=3)†</td>
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<tr>
<td>WSL6-11</td>
<td>Cit-FdUMP-CPSNP</td>
<td>3x</td>
<td>3.69 ± 0.06E-04 (n=3)†</td>
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<tr>
<td>WSL6-12-4</td>
<td>mPEG-Ghost-CPSNP</td>
<td>3x</td>
<td>Below detection limit†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSL6-12-5</td>
<td>mPEG-FdUMP-CPSNP</td>
<td>3x</td>
<td>2.45 ± 0.01E-04 (n=3)†</td>
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<tr>
<td>WSL6-12-30</td>
<td>AP1153-cPEG-FdUMP-CPSNP</td>
<td>3x</td>
<td>1.92 ± 0.01E-04 (n=3)†</td>
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<tr>
<td>WSL6-12-31</td>
<td>AP1153-cPEG-FdUMP-CPSNP</td>
<td>3x</td>
<td>1.82 ± 0.03E-04 (n=3)†</td>
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<tr>
<td>WSL6-12-32</td>
<td>aCD71-cPEG-FdUMP-CPSNP</td>
<td>3x</td>
<td>1.94 ± 0.02E-04 (n=3)†</td>
<td>---</td>
<td>49</td>
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</table>

n/m, not measured
n/a, not available

* WSL(book no.)-(page no.)-(line or sample no.)
† Synthesis volume (e.g., 3x = three times the standard formulation precursor volumes)
‡ LC-MS analysis by Jenny Dai, formerly at the Penn State Hershey Medical Center
†† LC-MS analysis by Todd Fox at the University of Virginia
* 10x of precursor concentrations
► mol% Encapsulation Efficiency (EE) = moles of drug in formulation/initial moles of drug added to synthesis*100. Values for ghost particles were also not calculated.
*#mol% recovery of drug in PEGylated/bioconjugated formulations from parent citrate-functionalized particles
## Appendix B

### Index of Chemicals and supplies

**Table B-2.** Origin and details of chemicals used in CPSNP synthesis

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Abbrev.</th>
<th>Product Vendor</th>
<th>CAS #</th>
<th>CAT #</th>
<th>Lot #</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Fluorouracil, ≥99%</td>
<td>5-FU</td>
<td>Sigma-Aldrich</td>
<td>51-21-8</td>
<td>F6627</td>
<td>128K1409</td>
</tr>
<tr>
<td>5-fluoro-2'-deoxyuridine 5'-monophosphate, ≥85%</td>
<td>FdUMP</td>
<td>Sigma-Aldrich</td>
<td>103226-10-4</td>
<td>F3503</td>
<td>075K7015V</td>
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<tr>
<td>5-fluoro-2'-deoxyuridine</td>
<td>F UdR</td>
<td>ToCris</td>
<td>50-91-9</td>
<td>4659</td>
<td>Batch no: 1</td>
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<tr>
<td>Gemcitabine hydrochloride, ≥98%</td>
<td>GEM</td>
<td>Sigma-Aldrich</td>
<td>122111-03-9</td>
<td>G6423-10MG</td>
<td>014M4719V</td>
</tr>
<tr>
<td>Gemcitabine monophosphate formate salt, 95%</td>
<td>GemMP</td>
<td>TRC (Canada)</td>
<td>116371-67-6</td>
<td>G205010</td>
<td>11-JHY-174-1</td>
</tr>
<tr>
<td>Adenosine 5'-triphosphate disodium salt hydrate, 99%</td>
<td>ATP</td>
<td>Sigma-Aldrich</td>
<td>34369-07-8</td>
<td>A26209</td>
<td>MKBL7424V</td>
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<tr>
<td>Igepal CO-520</td>
<td>n/a</td>
<td>Rhodia</td>
<td>68412-54-4</td>
<td>N000715</td>
<td>MH0G11X02</td>
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<tr>
<td>Cyclohexane - ACS Grade</td>
<td>Chex</td>
<td>Alfa Aesar</td>
<td>110-82-7</td>
<td>BDH1111-191</td>
<td>L09Z032</td>
</tr>
<tr>
<td>Calcium chloride dihydrate, ≥99%</td>
<td>CaCl₂</td>
<td>Sigma-Aldrich</td>
<td>10035-04-8</td>
<td>223506-25g</td>
<td>MKBP4041V</td>
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<tr>
<td>Sodium hydrogen phosphate, ≥99%</td>
<td>HPO₄</td>
<td>Sigma-Aldrich</td>
<td>7558-79-4</td>
<td>S7907-500g</td>
<td>BCBH3675V</td>
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<tr>
<td>Sodium metasilicate</td>
<td>SiO₃</td>
<td>Sigma-Aldrich</td>
<td>6834-92-0</td>
<td>307815-25g</td>
<td>MKBG0743V</td>
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<tr>
<td>Sodium citrate</td>
<td>Cit</td>
<td>Sigma-Aldrich</td>
<td>6132-04-3</td>
<td>S4641-500g</td>
<td>SLBB9859V</td>
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<tr>
<td>Potassium hydroxide pellets, ≥98%</td>
<td>KOH</td>
<td>J.T. Baker</td>
<td>1310-58-3</td>
<td>3140-01</td>
<td>X44911</td>
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<tr>
<td>Hydrochloric acid, 37%</td>
<td>HCl</td>
<td>Sigma-Aldrich</td>
<td>7647-01-0</td>
<td>320331-500ml</td>
<td>SHBD8728V</td>
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<tr>
<td>Ethyl Alcohol 200 proof</td>
<td>EtOH</td>
<td>Koptec</td>
<td>64-17-5</td>
<td>V1005</td>
<td>083411</td>
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<tr>
<td>Acetonitrile, 99.8%</td>
<td>ACN</td>
<td>Sigma-Aldrich</td>
<td>75-05-8</td>
<td>271004-100ml</td>
<td>SHBD7106V</td>
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<tr>
<td>Formic acid, ≥95%</td>
<td>FA</td>
<td>Sigma-Aldrich</td>
<td>64-18-6</td>
<td>F0507-100ml</td>
<td>SHBD9036V</td>
</tr>
<tr>
<td>N-(3-Dimethylaminopropyl)-N'-ethyldiaimidemide hydrochloride</td>
<td>EDAC</td>
<td>Sigma-Aldrich</td>
<td>25952-53-8</td>
<td>E7750-1g</td>
<td>SLBH9924V</td>
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<tr>
<td>Amine-PEG-Carboxyl</td>
<td>cPEG</td>
<td>JenKem Technology</td>
<td>n/a</td>
<td>NH2-PEG2000-COOH</td>
<td>ZZ149P018</td>
</tr>
<tr>
<td>Methoxy-PEG-Amine</td>
<td>mPEG</td>
<td>JenKem Technology</td>
<td>n/a</td>
<td>M-NH2-2000</td>
<td>ZZ098P114</td>
</tr>
<tr>
<td>Maleimide-PEG-Amine</td>
<td>malPEG</td>
<td>JenKem Technology</td>
<td>n/a</td>
<td>MAL-PEG2000-NH2</td>
<td>ZZ128P173</td>
</tr>
<tr>
<td>N-Hydroxysulfo succimide</td>
<td>Sulfo-NHS</td>
<td>Thermo Scientific Pierce</td>
<td>106627-54-7</td>
<td>24510</td>
<td>OE185968</td>
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<tr>
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<td></td>
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<tr>
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<td>-----</td>
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<tr>
<td>G10-CYS</td>
<td>G10</td>
<td>Genscript</td>
<td>n/a</td>
<td>Custom sequence</td>
<td>P12921308</td>
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<tr>
<td>G16-CYS</td>
<td>G16</td>
<td>Genscript</td>
<td>n/a</td>
<td>Custom sequence</td>
<td>P15851310</td>
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<td>Aptamer-3449</td>
<td>AP1153</td>
<td>TriLink Biotechnologies</td>
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<td>Custom sequence</td>
<td>P6-AA01A P6-AI01A P6-AJ01A</td>
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<td>Aptamer-3450</td>
<td>AP3450</td>
<td>TriLink Biotechnologies</td>
<td>n/a</td>
<td>Custom sequence</td>
<td>P6-AA02A</td>
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<tr>
<td>Dulbecco's Phosphate Buffer Saline, 1x</td>
<td>PBS, DPBS</td>
<td>Corning Cellgro</td>
<td>n/a</td>
<td>21-031-CV 21031417</td>
<td></td>
</tr>
<tr>
<td>Pre-purified argon gas cylinder</td>
<td>Ar</td>
<td>Penn State General Store</td>
<td>n/a</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>deionized-distilled water</td>
<td>H2O</td>
<td>High-Q</td>
<td>n/a</td>
<td>103S Still 103C containers 200PT-SYS(RO/I X2) option #2</td>
<td>N/A</td>
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</tbody>
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Table B-3. Supplies and equipment specifications

<table>
<thead>
<tr>
<th>Supplies</th>
<th>Vendor</th>
<th>Catalogue no.</th>
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<tbody>
<tr>
<td>Sartorius analytical balance</td>
<td>Sartorius</td>
<td>MC210S</td>
</tr>
<tr>
<td>Finnpipette F2 variable volume pipettes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Pink: 2-20ul</td>
<td>Accumax A</td>
<td>AV700</td>
</tr>
<tr>
<td>• Purple: 20-200ul</td>
<td>Accumax A</td>
<td>AV800</td>
</tr>
<tr>
<td>• Blue: 100-1000ul</td>
<td>Sigma Aldrich</td>
<td>Z678341</td>
</tr>
<tr>
<td>• Green: 0.5-5ml</td>
<td>Sigma Aldrich</td>
<td>Z678368</td>
</tr>
<tr>
<td>VWR Gas Dispersion Tube</td>
<td>VWR</td>
<td>89083-472</td>
</tr>
<tr>
<td>VWR 60 psig Ar/He/N2 gas cylinder regulator</td>
<td>VWR</td>
<td>55850-474</td>
</tr>
<tr>
<td>VWR Safety Wash Bottles, low-density polyethylene</td>
<td>VWR</td>
<td>16649</td>
</tr>
<tr>
<td>VWR Four-Channel Alarm Timer (hr:min:sec)</td>
<td>VWR</td>
<td>N/A</td>
</tr>
<tr>
<td>Volumetric flasks with glass stoppers</td>
<td>VWR</td>
<td>89001-998</td>
</tr>
<tr>
<td>• 50ml</td>
<td>VWR</td>
<td>89001-986</td>
</tr>
<tr>
<td>• 100ml</td>
<td>VWR</td>
<td></td>
</tr>
<tr>
<td>Sterile polypropylene Falcon cups</td>
<td>Fisher Scientific</td>
<td></td>
</tr>
<tr>
<td>• Lids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• 110ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• 210ml</td>
<td></td>
<td></td>
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<tr>
<td>VWR Powder-free nitrile examination gloves (Sizes S, M, L)</td>
<td>VWR</td>
<td>82026-424, 426, 428</td>
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<tr>
<td>Disposable pipette tips for Finnpipette F2</td>
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<td></td>
</tr>
<tr>
<td>• Clear: 0.1-10ul</td>
<td>VWR</td>
<td>46620-312</td>
</tr>
<tr>
<td>• Yellow: 1-200ul</td>
<td>VWR</td>
<td>53508-810</td>
</tr>
<tr>
<td>• Blue: 100-1000ul</td>
<td>VWR</td>
<td>83007-376</td>
</tr>
<tr>
<td>• Clear: 1000-5000ul</td>
<td>Thermo Scientific</td>
<td>P2924</td>
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<tr>
<td>KIMWIPES Delicate Task Wipers</td>
<td>VWR</td>
<td>21905-026</td>
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<tr>
<td>VWR digital hotplate/stirrer</td>
<td>VWR</td>
<td>11301-074</td>
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<tr>
<td>GeneMate Magnetic Stirrers</td>
<td>Bioexpress</td>
<td>H-3000-S</td>
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<td>GeneMate Orbital Shaker MP4</td>
<td>Bioexpress</td>
<td>S-3500-1</td>
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<td>Disposable transfer pipettes - 3ml</td>
<td>Samco Scientific</td>
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<tr>
<td>VWR SuperClear Ultra-High Performance Centrifuge Tubes (sterile)</td>
<td>VWR</td>
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</tr>
<tr>
<td>• 15ml</td>
<td></td>
<td>21008-216</td>
</tr>
<tr>
<td>• 50ml</td>
<td></td>
<td>21008-242</td>
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<tr>
<td>20 mL Disposable Borosilicate Scintillation Vials with screw caps</td>
<td>VWR</td>
<td>66022-128</td>
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<tr>
<td>Amicon Ultra Centrifugal Filters Ultrace1 - 30K</td>
<td>Millipore</td>
<td>UFC803024</td>
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<tr>
<td>VWR Microcentrifuge Tubes - 1.7ml</td>
<td>VWR</td>
<td>87003-294</td>
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<tr>
<td>Carbon Film on 300 Mesh Copper Grids</td>
<td>Electron Microscopy Sciences</td>
<td>CF300-Cu</td>
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<tr>
<td>0.2um regenerated cellulose membrane syringe filters - 15mm</td>
<td>Corning</td>
<td>431215</td>
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<tr>
<td>0.2um cellulose acetate membrane syringe filters - 25mm</td>
<td>VWR</td>
<td>28145-477</td>
</tr>
<tr>
<td>Waters HPLC 600e with Shimadzu SPD-6A(V) UV-Vis detector performance plus sapphire check valves</td>
<td>Waters + Shimadzu</td>
<td>600e and SPD-6A(V)</td>
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<tr>
<td></td>
<td>Waters</td>
<td>700000254</td>
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<tr>
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<td>Brand</td>
<td>Code</td>
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<td>----------------------------------------------------------------------</td>
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<tr>
<td>Metal threaded ferrules (1/16&quot;)</td>
<td>VWR</td>
<td>10057-906</td>
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<tr>
<td>Syringe with Luer-Lok Tip - 5mL</td>
<td>BD</td>
<td>BD309646</td>
</tr>
<tr>
<td>EP Scientific Depyrogenated Vials, Sterile (pre-crimped)</td>
<td>Thermo Scientific</td>
<td>ST1-11</td>
</tr>
<tr>
<td>• 1ml</td>
<td></td>
<td>ST20-20</td>
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<tr>
<td>• 20ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spheriglass - solid glass spheres (HPLC stationary phase)</td>
<td>Potter's Industries</td>
<td>A-Glass 1922</td>
</tr>
</tbody>
</table>
Appendix C

Method developed for Igepal CO-520 concentration determination in CPSNP formulations by dynamic tensiometry

Procedure v. 7.29 designed by Gigliotti, Loc, and Adair for the Sensadyne PC9000.

A. Pre-testing operations
   1. Connect tubing from Argon tank to rear of tensiometer
   2. Turn on Argon gas tank to 10 psi—NEVER PUT TENSIOMETER PROBES INTO LIQUID WITHOUT THE GAS ON
   3. Open Sensadyne tensiometer program
   4. Heat a beaker of water to 70°C (enough to cover the tensiometer probes)
   5. Chill another cup of water to 2°C (enough to cover the tensiometer probes)
   6. Insert glass tensiometer probes a couple centimeters into hot water by loosening large black knobs on the tensiometer—be cautious
   7. In the software window select "calibration" and then "temperature"
   8. Enter the actual water temperature using a thermometer and select "high calibration"
   9. Insert glass probes into the cold water
   10. Enter actual water temperature and select "low calibration"
   11. Select "accept"
   12. Insert glass probes into room temperature water
   13. Select "analyze," "measure," and then "show signals"
   14. Using the small black knobs on the front of the tensiometer adjust the bubble frequency to between 1-2 bubbles per second (the larger glass probe should output a bubble every second)
   15. Select "calibration," "autocal," and then "autocal high"
   16. Dry probes completely
   17. Insert glass probes into neat ethanol
   18. Select "calibration," "autocal," and then "autocal low"
   19. Click "show signals" and then “measure” to stop analyzing data

B. Standard Curve
   1. Plug in and set the water jacket to 20°C (may have to adjust if temperature reading on tensiometer is not 20°C)
   2. Fill 110 mL -Falcon cup with 50 mL of PBS
   3. Insert Falcon cup with precut lid into the water jacket with the tensiometer probes in the smallest three holes
   4. Go to "File" "Data File" "new" and create "example.sdd"
   5. Select "analyze" "measure" "show signals" "record"
   6. Immediately after the data point is collected (every three minutes), transfer an aliquot of 1 mM Igepal stock and clean PBS into the Falcon cup to the desired concentration.
7. Continue this process until you have completed the calibration curve
8. Go to "File" Data File" "convert to ASCII" and open "example.sdd" Then create and save "example.txt"
9. Click "recording" "show signals" and then "measure" to stop analyzing data
10. Clean the glass probes using ethanol and discard the sample
11. Repeat the calibration curve steps four more times (do not turn off gas or program)
12. Open the text files for your five runs and record this data under the "calibration curve" tab. An example of the standard curve is presented below.

![Graph of standard curve](image)

**Table C-1.** Aliquots of a 1 mM stock solution of Igepal CO-520 in PBS or purified water (~22 mg/50 mL) is diluted into the corresponding bulk liquid to generate a standard curve. Each data point represents an average of five readings with 95% confidence intervals.

C. Sample Testing

1. Dry CPSNP sample down completely with Ar or heat and resuspend with equal volume of PBS
2. Fill Falcon cup with 50 mL of DPBS
3. Insert Falcon cup with precut lid into the water jacket with the tensiometer probes in the smallest three holes
4. Go to "File" "Data File" "new" and create "sample.sdd"
5. Select "analyze" "measure" "show signals" "record"
6. Immediately after the data point is collected (every three minutes), insert 100 uL of the sample into the Falcon cup
7. Continue this process until you have used up your sample
8. Go to "File" Data File" "convert to ASCII" and create "sample.txt"
9. Click "recording" "show signals" and then "measure" to stop analyzing data
D. Determination of Concentration in Samples

1. Insert the dilution factor and corresponding surface tension values from your sample into the “analysis” tab
2. Find a similar surface tension on your calibration curve and insert this value and its corresponding Igepal concentration into the “analysis” tab
3. This comparison will determine the concentration of Igepal present in your sample by adjusting the dilution factor of your sample to equal the dilution factor of the calibration curve that corresponds to the same surface tension measurement
4. Minitab Analysis:
   i. Paste the raw data on Minitab and select “assistant” on the tool bar and “hypothesis tests”
   ii. Select on the “One Way ANOVA” option
   iii. Select all your data and click OK (You will have to do this twice if using more than 12 points in your calibration curve)
Appendix D

Procedure for particle size analysis using Image J and Origin

D.1 Image processing on Image J

1. Open TEM Image on Image J
2. Image > Adjust > Threshold
3. Apply threshold
4. Use line tool to set the scale bar for known distance
   Analyze > set scale

5. Analyze > Analyze Particles
   Show the outlines. The size (nm²) can be adjusted to omit noise that are not particles from the analysis.
   The results will be in nm², area of a circle. Paste directly to Excel to calculate the diameter. The number corresponding to each area is used to locate a particle in the processed image.

   *Collect at least 300 particles for the distribution.*
D.2 Single peak fit on Origin

6. Paste the diameter data onto Origin into the Y column on the worksheet, ≥300 particles (X column is not needed)

Highlight the entire Y column to create a histogram (Plot > Statistics > Histogram)

To adjust the bin size, right-click on the histogram > Plot details…> Data

Notes: Not all particle distributions are lognormal. This can be checked with a hypothesis testing software, e.g. Minitab, for the goodness of fit (p-value) between the raw data and the distribution function. However, nanoparticle size distributions are normally presented as lognormal in papers within this field.

7. After applying the bin size, right-click on the histogram again > Go to Bin Worksheet

Highlight the first two columns and Plot > Columns/Bars > Column
8. While on the column graph window, select Analysis > Fitting > Fit Single Peak… Select the LogNormal Function. Click on “fit till converged”. The software will run multiple iterations for the best fitting. Sometimes the function will not converge. If that happens, go back to the histogram to change the bin size and repeat Steps 6-8.

Click OK and this summary table of the lognormal fitting will appear.

D.3 Multi-peak fit on Origin

A. Follow the same procedure up to Step 8, ending with a column graph. Select Analysis > Fitting > Fit Multi-peaks… Adjust the number of peaks
B. After clicking OK, a cursor will appear. Double-click where you think the two peak maximums are located. Select OK to validate the width of peaks.

C. A new side bar will appear in addition to the graph which displays all the fitting statistics.
Appendix E

Determination of endotoxin levels in building water using the Charles River LAL assay and depyrogenation procedure for contaminated lab equipment

E.1 Procedure for the LAL assay

Running water is collected and stored in a sterile centrifuge tube at room temperature. Endotoxin-free LAL reagent water is included in the Charles River Limulus Amebocyte Lysate Endochrome-K kit (R1710K) and Endosafe Control Std Endotoxin (E120). The Control Std Endotoxin is reconstituted with a specified volume of reagent water to 50 EU/ml. This endotoxin solution is then serially diluted to four standards, 1 EU/ml, 0.50 EU/ml, 0.25 EU/ml, and 0.10 EU/ml. For a lower concentration range, 0.25 EU/ml, 0.10 EU/ml, 0.07 EU/ml, and 0.05 EU/ml can be used to generate the standard curve. The 100 ul standards are transferred to a sterile 96-well plate, three wells each (see example of spreadsheet in Fig. F-2). LAL reagent water is used as the background solvent. A full plate with up to six water samples and four standards are placed in a cell culture incubator set to 37°C for 5 min. The Limulus Amebocyte Lysate (LAL) is reconstituted in 3.4 mL of reagent water during this incubation time and transferred to a sterile multichannel pipettor well. Three wells at a time, 100 ul aliquots of the LAL are pipetted to each background, standard, and sample. The assay plate is incubated for 15 min and immediately scanned at 386 nm every minute in a Multiskan UV-Vis plate reader until the highest concentration standard was ~1 absorbance unit.
Figure E-2. Example of excel spreadsheet for an LAL assay. Water samples are assayed on the same day or a day after collection. Endotoxin levels (EU/ml) in the samples are extrapolated from a linear standard curve. Levels ≥0.1 EU/ml are not acceptable for the purpose of this research. In such cases, water from alternative sources should be sought.
E.2 Endotoxin levels in selected building water on University Park campus

**Figure E-3.** A map of Pennsylvania State University highlighting buildings (circled in red) that were assayed for endotoxin in tap, distilled, nanopure, and/or deionized distilled water during the course of dissertation research. Water was sampled from Steidle Building (SDL, before renovation), Hallowell Building (HWL), Walker Building (WKR), Noll Building (NLL), Materials Science Complex (MSC), Modular Research Lab trailers next to the Forest Research Lab (FRL), and Pine Hall Labs (not shown on map). Image adapted from the Penn State Department of Geography webpage.

**Table E-1.** Endotoxin levels in water from selected buildings on campus

<table>
<thead>
<tr>
<th>Building</th>
<th>Date assayed</th>
<th>Water source</th>
<th>Endotoxins (EU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Old Steidle (SDL)</td>
<td>8/6/13</td>
<td>Adair Lab Millipore Nanopure</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Building hallway distilled</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td>8/15/13</td>
<td>Adair Lab Millipore Nanopure</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adair Lab distilled</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Building hallway distilled</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>8/23/13</td>
<td>Adair Lab Millipore Nanopure</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adair Lab distilled</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Building hallway distilled</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>9/30/13</td>
<td>Adair Lab Millipore Nanopure</td>
<td>0.052</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adair Lab distilled</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Building hallway distilled</td>
<td>0.107</td>
</tr>
<tr>
<td></td>
<td>10/9/13</td>
<td>Adair Lab Millipore Nanopure</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adair Lab distilled</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td>10/25/13</td>
<td>Adair Lab Millipore Nanopure</td>
<td>0.040</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adair Lab distilled</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Building hallway distilled</td>
<td>0.058</td>
</tr>
<tr>
<td></td>
<td>12/6/13</td>
<td>Adair Lab Millipore Nanopure</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adair Lab distilled</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>6/2/14</td>
<td>Adair Lab Millipore Nanopure</td>
<td>0.087</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adair Lab distilled</td>
<td>0.106</td>
</tr>
<tr>
<td>New Steidle</td>
<td>3/3/16</td>
<td><strong>Women’s bathroom west 1st floor</strong></td>
<td>&gt;1.000</td>
</tr>
<tr>
<td>Location</td>
<td>Date</td>
<td>Source</td>
<td>Conductivity (µS/cm)</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------</td>
<td>---------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Women’s bathroom west 3rd floor</td>
<td></td>
<td></td>
<td>&gt;1.000</td>
</tr>
<tr>
<td>Hallowell (HWL)</td>
<td>8/6/13</td>
<td>Hancock Lab deionized distilled</td>
<td>0.130</td>
</tr>
<tr>
<td></td>
<td>8/23/13</td>
<td>Hancock Lab deionized distilled</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>6/2/14</td>
<td>Hancock Lab deionized distilled</td>
<td>0.128</td>
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<tr>
<td>Building tap</td>
<td></td>
<td></td>
<td>3.516</td>
</tr>
<tr>
<td></td>
<td>8/28/14</td>
<td>Hancock Lab deionized distilled</td>
<td>0.070</td>
</tr>
<tr>
<td>Hancock Lab tap</td>
<td></td>
<td></td>
<td>2.766</td>
</tr>
<tr>
<td>Building tap</td>
<td></td>
<td></td>
<td>3.311</td>
</tr>
<tr>
<td></td>
<td>8/29/14</td>
<td>Hancock Lab deionized distilled</td>
<td>–0.027</td>
</tr>
<tr>
<td></td>
<td>10/17/14</td>
<td>Hancock Lab deionized distilled</td>
<td>0.058</td>
</tr>
<tr>
<td>Building tap</td>
<td></td>
<td></td>
<td>2.722</td>
</tr>
<tr>
<td></td>
<td>11/14/14</td>
<td>Hancock Lab deionized distilled</td>
<td>0.105</td>
</tr>
<tr>
<td>7/31/15 after distillation</td>
<td></td>
<td>Hancock Lab deionized distilled</td>
<td>0.199</td>
</tr>
<tr>
<td>column repair</td>
<td></td>
<td>(directly from column)</td>
<td></td>
</tr>
<tr>
<td>Hancock Lab deionized distilled</td>
<td></td>
<td>(left carboy)</td>
<td>0.151</td>
</tr>
<tr>
<td>Hancock Lab deionized distilled</td>
<td></td>
<td>(right carboy)</td>
<td>0.095</td>
</tr>
<tr>
<td>3/3/16</td>
<td></td>
<td>Hancock Lab (right carboy)</td>
<td>0.042</td>
</tr>
<tr>
<td>Modular Labs</td>
<td>5/14/15</td>
<td>Hickner Lab deionized distilled</td>
<td>0.050</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hickner Lab Millipore Nanopure</td>
<td>0.094</td>
</tr>
<tr>
<td>Walker (WKR)</td>
<td>5/14/15</td>
<td>Building hallway distilled</td>
<td>0.187</td>
</tr>
<tr>
<td>Building tap (bathroom)</td>
<td></td>
<td></td>
<td>1.201</td>
</tr>
<tr>
<td>Noll (NLL)</td>
<td>10/17/14</td>
<td>Building tap</td>
<td>1.556</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Korzick Lab distilled holding tank</td>
<td>0.277</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Korzick Lab Barnstead Nanopure Nalgene</td>
<td>0.046</td>
</tr>
<tr>
<td>5/14/15</td>
<td></td>
<td>Korzick Lab Barnstead Nanopure Nalgene</td>
<td>0.041</td>
</tr>
<tr>
<td>Materials Science Complex (MSC)</td>
<td>7/31/15</td>
<td>Zheng Lab distilled (N-031)</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Huang Lab distilled (N-023)</td>
<td>0.029</td>
</tr>
<tr>
<td>Huang Lab tap (N-023)</td>
<td></td>
<td></td>
<td>&gt;0.250</td>
</tr>
<tr>
<td>Pine Hall Lab</td>
<td>8/28/14</td>
<td>Building water fountain</td>
<td>0.091</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(city water)</td>
<td></td>
</tr>
</tbody>
</table>

**E.3 Depyrogenation**

Lab glassware are cleansed by a triple acid/base bath in 0.1M HCl and 0.1M NaOH with purified water in between rinses. The container openings were then covered with aluminium foil and baked in a 250°C oven for at least 1 hr. Plasticware can be rinsed with bleach. Water stored in
containers should be further sterilized with a 0.2μm cellulose acetate or regenerated cellulose filter membrane before use.
Appendix F

Drug encapsulation formulation and vdW-HPLC settings

F.1 Formulation sheet

Table F-1. Standard 1x tracking sheet of precursors and reaction times for CPSNP synthesis.

<table>
<thead>
<tr>
<th>R=water/surfactant</th>
<th>Starting conc. (M)</th>
<th>Microemulsion A</th>
<th>[ ] mL</th>
<th>[ ] grams</th>
<th>[ ] moles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final A or B volume</td>
<td>15</td>
<td>Igepal CO-520</td>
<td>4.063</td>
<td>4.051</td>
<td>9.19E-03</td>
</tr>
<tr>
<td>moles H2O total</td>
<td>0.07202</td>
<td>Cyclohexane</td>
<td>10.000</td>
<td>7.790</td>
<td>9.26E-02</td>
</tr>
<tr>
<td>moles Igepal (for both A &amp; B)</td>
<td>0.01800</td>
<td>CaCl2*2H2O with</td>
<td>0.6500</td>
<td>0.001</td>
<td>6.50E-06</td>
</tr>
<tr>
<td>Hydroxyapatite 10 [Ca] : 6 [PO4]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca:P Ratio</td>
<td>1.667</td>
<td>Microemulsion B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca:SiO2 Ratio</td>
<td>12.195</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>moles of ACP [Ca12(PO4)3(OH)2·(H2O)3]</td>
<td>4.33E-06</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>moles of HA [Ca9(PO4)3OH]</td>
<td>1.30E-06</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theoretical mass HA synthesized, grams:</td>
<td>6.53E-04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theoretical mass solid matrix, grams:</td>
<td>1.31E-03</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theoretical total mass, grams:</td>
<td>0.001%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theoretical volume percent:</td>
<td>0.01%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micelle equilibration time:</td>
<td>15 min</td>
<td>50 w/w</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micellar exchange equilibration time:</td>
<td>2 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface treatment time:</td>
<td>15 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthesis</td>
<td>Disodium Citrate (0.01 M aq. stock)</td>
<td>0.225</td>
<td>0.001</td>
<td>2.25E-06</td>
<td></td>
</tr>
<tr>
<td>Ca:Cit (by mol)</td>
<td>2.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug agent</th>
<th>Starting conc. X (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU</td>
<td>6.3</td>
</tr>
<tr>
<td>FdUMP</td>
<td>9.2</td>
</tr>
<tr>
<td>FUdR</td>
<td>9.2</td>
</tr>
<tr>
<td>Gem</td>
<td>2.5</td>
</tr>
<tr>
<td>GemMP</td>
<td>2.5</td>
</tr>
<tr>
<td>ATP:5-FU</td>
<td>100</td>
</tr>
</tbody>
</table>
F.2 Purification

The HPLC stationary phase consists of solid glass microspheres ~200 μM in diameter (Spheriglass A-Glass 1922, Potters Industries) that are soaked in purified water for 48 h and thoroughly rinsed with 10^{-3}M HCl and 10^{-3}M NaOH solutions before use. The microspheres are wet-packed in a 5 cm long x 3/8” OD, 1/4” ID polycarbonate tube (McMaster-Carr) with a 20 μm frit. The HPLC has three mobile phases on independent solvent lines: 70/30 ethanol-water with 0.3mM KOH (B-line), ethanol with 0.3mM KOH (C-line), and neat ethanol (D-line). The column is pre-rinsed with neat ethanol (3ml/min). Once the UV-Vis output is stable, the baseline is set to zero and the D-line is inserted into the ~80 mL micelle suspension. The UV-Vis reader is configured to detect Igepal CO-520 (see Table F-2). The sample is loaded onto the column for 10 min, followed by the wash cycle with the C-line until baseline is reached. Particles are eluted in 70/30 ethanol-water. The load and wash cycles are repeated until the entire suspension is purified.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>$\lambda_{\text{max}}$ in water (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Igepal CO-520</td>
<td>276</td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td>266</td>
</tr>
<tr>
<td>FdUMP</td>
<td>269</td>
</tr>
<tr>
<td>FUdR</td>
<td>270</td>
</tr>
<tr>
<td>Gem</td>
<td>262</td>
</tr>
<tr>
<td>GemMP</td>
<td>270</td>
</tr>
<tr>
<td>ATP</td>
<td>260</td>
</tr>
</tbody>
</table>

The eluted particles are then adjusted to 95/5 ethanol-water with neat ethanol for a second purification. The UV-Vis can be set to detect the $\lambda_{\text{max}}$ of the encapsulated drug molecule when Igepal is below the detection limit. The same load and wash steps are repeated for the final clean sample.
Appendix G

Synthesis and TEM characterization of osmium-stained CPSNPs

The following presents the synthesis particulars to obtain osmium (Os)-CPSNPs via a triple-microemulsion exchange and characterization on the FEI Titan™ TEM (Materials Characterization Lab, Penn State University). The “osmium-staining” method (Figure G-1) here refers to the partial substitution of the calcium chloride precursor with osmium (III) chloride hydrate (OsCl₃, 99.9%, Sigma-Aldrich). Differential centrifugation washing by Tabakovic³⁴ was adapted to Table G-1. A drop of the laundered suspension was directly transferred to a copper grid (Electron Microscopy Sciences) for TEM that operated at an accelerating voltage of 200 kV and given 5 min exposure in STEM (HAADF/EDS) mode.

![Flow schematic outlining the triple reverse micelle microemulsion synthesis of Os-CPSNPs.](image)

Figure G-1. A flow schematic outlining the triple reverse micelle microemulsion synthesis of Os-CPSNPs. The reaction was conducted at room temperature with constant stirring set at 200 rpm.
Table G-1. Differential centrifugation procedure for Os-CPSNPs

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Divide dissolved micelle suspension into two centrifuge tubes</td>
<td></td>
</tr>
<tr>
<td>Centrifuge at 6000 rpm for 20 min</td>
<td></td>
</tr>
<tr>
<td>Remove supernatant down to 10 mL and combine both aliquots</td>
<td></td>
</tr>
<tr>
<td>Add 20 mL 50/50 ethanol-cyclohexane (v/v)</td>
<td></td>
</tr>
<tr>
<td>Centrifuge at 6000 rpm for 20 min</td>
<td></td>
</tr>
<tr>
<td>Remove supernatant down to 10 mL and add 30 mL 50/50 ethanol-cyclohexane (v/v)</td>
<td></td>
</tr>
<tr>
<td>Centrifuge at 6000 rpm for 20 min</td>
<td></td>
</tr>
<tr>
<td>Remove supernatant down to 10 mL mark and add 30 mL neat ethanol</td>
<td></td>
</tr>
<tr>
<td>Centrifuge at 6000 rpm for 20 min</td>
<td></td>
</tr>
<tr>
<td>Remove supernatant down to 10 mL and add 30 mL 70/30 ethanol-water (v/v)</td>
<td></td>
</tr>
<tr>
<td>Centrifuge at 6000 rpm for 20 min</td>
<td></td>
</tr>
<tr>
<td>Remove supernatant down to 10 mL and add 30 mL 70/30 ethanol-water (v/v)</td>
<td></td>
</tr>
<tr>
<td>Centrifuge at 6000 rpm for 20 min</td>
<td></td>
</tr>
<tr>
<td>Resuspend and store particles in minimum 70/30 ethanol-water (v/v)</td>
<td></td>
</tr>
</tbody>
</table>

Figure G-2. A micrograph taken in STEM (HAADF/EDS) mode of an Os-CPSNP taken before (left) and after (right) elemental mapping. The unwanted coarsening of the osmium sub-particles at the surface was evident after 5 min of high intensity beam exposure. Organic contamination from residual surfactants was significant that higher magnification to examine the sub-particles was unobtainable.
Figure G-3. A bright-field micrograph (A) and HAADF micrograph (B) of selected Os-CPSNPs. These 40-70 nm nanoparticles were composed of 1-3 nm high contrast sub-particles that contain osmium. EDS mapping (C) of the nanoparticles from the HAADF image reveal a mixture of elemental calcium, oxygen, osmium, and phosphorus, which were expected for CPSNPs. The plots in (D) show the relative counts per element of interest for the left (i) and right (ii) particles. There were inconsistencies in the relative intensities due to insufficient counts of osmium and the low-Z elements P, Ca, and Si. However, the presence of these elements verified the composition of the nanoparticles, qualitatively.
Table G-2. EDS quantification of Os-CPSNPs (from Figure G-3, particles (i) and (ii))

<table>
<thead>
<tr>
<th>Element</th>
<th>Series</th>
<th>(i)</th>
<th>(ii)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Net</td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>K</td>
<td>144</td>
<td>46.1</td>
</tr>
<tr>
<td>P</td>
<td>K</td>
<td>28</td>
<td>8.91</td>
</tr>
<tr>
<td>Si</td>
<td>K</td>
<td>14</td>
<td>7.89</td>
</tr>
<tr>
<td>Os</td>
<td>L</td>
<td>22</td>
<td>23.99</td>
</tr>
<tr>
<td>Ca</td>
<td>K</td>
<td>86</td>
<td>13.11</td>
</tr>
</tbody>
</table>

Mass C. (wt%)  | Norm C. (wt%)  | Atom C. (at%) | Error, 3σ (wt%) | Mass C. (wt%)  | Norm C. (wt%)  | Atom C. (at%) | Error, 3σ (wt%) |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>46.1</td>
<td>46.1</td>
<td>69.98</td>
<td>9.77</td>
<td>46.1</td>
<td>46.1</td>
<td>69.98</td>
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<tr>
<td>8.91</td>
<td>8.91</td>
<td>6.99</td>
<td>3.98</td>
<td>8.91</td>
<td>8.91</td>
<td>6.99</td>
<td>3.98</td>
</tr>
<tr>
<td>7.89</td>
<td>7.89</td>
<td>6.82</td>
<td>3.56</td>
<td>7.89</td>
<td>7.89</td>
<td>6.82</td>
<td>3.56</td>
</tr>
<tr>
<td>13.11</td>
<td>13.11</td>
<td>1.67</td>
<td>10.96</td>
<td>23.99</td>
<td>23.99</td>
<td>14.54</td>
<td>7.10</td>
</tr>
</tbody>
</table>
Appendix H

Aptamer-1153 (AP1153) target for human pancreatic cancer

**Figure H-1.** The overlap (in red) of human and mouse CCKB receptor peptide sequence (A) at the N-terminus for amino acid at positions 5-21 and 40-57. Aptamer-1153 was selected specifically for these regions. Target sequence does not overlap with the CCKA receptor sequence, a receptor that is also expressed in the pancreas and gastrointestinal tract. A SELEX approach was conducted to down-select from a group of aptamers (red boxes) shown in the dendrogram (B). The predicted secondary structure (Mfold) of the single-stranded aptamer-1153 with 66 nucleotides consists of hairpin loops and short helical arms. In (D), the dissociation constant, $K_d$, for the aptamer-1153 was 15.5 pM, which corresponded to a higher binding affinity than a shorter variant of 1153 with 49 nucleotides ($K_d = 206.2$ pM, not shown).

**Figure H-2.** A pair (left and right) of athymic nude mice models with orthotopic PANC-1 tumors (highlighted with red arrows) that were imaged post-15 h tail vein injection of $10^{-5}$ M (100 μL) unencapsulated ICG and ICG-doped CPSNPs. The five test groups consisted of mice treated with unencapsulated ICG (control group 1), empty ghost-CPSNPs (control group 2), non-targeted methoxy-PEG terminated ICG-doped CPSNPs, and targeted gastrin-16 or aptamer-1153 ICG-doped CPSNPs. Specific accumulation of aptamer-targeted CPSNPs at the tumor site produced the highest transillumination signal compared to the non-targeted and gastrin-16 CPSNPs. Autofluorescence was negligible in the controls and the lack of targeting with free dye was evident compared dye-encapsulated nanoparticles.
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VITA

Welley Siu Loc was born in Brooklyn, NY as the fourth child to Dan Sin and Canh Loc. She received her bachelor’s degree in Chemistry from Binghamton University in 2012. That same summer, she began her doctorate studies at Penn State under the guidance of Dr. James H. Adair (Materials Science and Engineering) while being co-advised by Dr. Tom Mallouk (Chemistry). In 2014, she was one of the four recipients of the NCATS/CTSI TL1 Award between the University Park Campus and Hershey Medical Center to undergo didactic training in Translational Science. Her research mainly involves optimizing the therapeutic capacity of calcium phosphosilicate nanoparticles by encapsulating phosphorylated cancer drug metabolites. During her free time, Welley likes to paint and publish comics.

Listed below are technology disclosures and publications during her Ph.D. study:


