Title
Synthesis and Characterization of Janus Particle Nanocarriers and DNA Devices Toward Multifunctional Drug Delivery Systems

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Synthesis and Characterization of Janus Particle Nanocarriers and DNA Devices Toward Multifunctional Drug Delivery Systems

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy
in
Materials Science and Engineering
by
Alexander Han-Chung Mo

Committee in charge:
Professor Ratnesh Lal, Chair
Professor Renkun Chen
Professor Michael Sailor
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Professor Liangfang Zhang

2015
The dissertation of Alexander Han-Chung Mo is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2015
DEDICATION

To my wife and parents for all their love and support over the years.
EPIGRAPH

“Now faith is the substance of things hoped for,
the evidence of things not seen.
For by it the elders obtained a good report.
Through faith we understand that the worlds were framed by the word of God,
so that things which are seen were not made of things which do appear.”

— Hebrews 11:1-3, KJV
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Chapter 3, in full, is a reprint of the material as it appears in PB Landon, AH Mo, C Zhang, CD Emerson, AD Printz, AF Gomez, CJ DeLaTorre, DA Colburn, P Anzenberg, M Eliceiri, C O’Connell, R Lal. “Designing Hollow Nano Gold Golf Balls” ACS Appl. Mater. Interfaces, 2014, 6, 9937-9941. The dissertation/thesis author was the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

Synthesis and Characterization of Janus Particle Nanocarriers and DNA Devices Toward Multifunctional Drug Delivery Systems

by

Alexander Han-Chung Mo

Doctor of Philosophy in Materials Science and Engineering

University of California, San Diego, 2015

Professor Ratnesh Lal, Chair

Integrating a diagnostic moiety with a therapeutic payload promises improved prognoses in diseases such as cancer by simultaneously illuminating and treating cancerous tumors. However potential theranostic materials and chemicals must be biocompatible and evade rapid clearance from the body. Such strict constraints require an interdisciplinary approach to research. Working toward to address those constraints requires drawing on such disparate fields as DNA nanotechnology, Janus nanoparticle synthesis, and chemical conjugation in order to one day create a viable theranostic carrier.

Using an inosine-based partial strand displacement scheme is reported a nanoscale
positioning capabilities are used to provide on-demand activation and deactivation of a pair of split E6 DNAzymes on the device. The device also demonstrates a combined catalytic rate significantly higher than the original E6 DNAzyme under similar operational conditions. This approach can provide structural organization and spatially control the opening and closing of a theranostic particle.

Janus nanoparticles (JNP) synthesis methods were pursued to create potential theranostic carriers. Two particular morphologies are explored: (i) a gold-ball like core-shell carrier and (ii) a eccentrically encapsulated JNP. For the golf-ball structure, a hierarchical template synthesis scheme was used to create a carrier consisting of (i) solid silica core with a pitted gold surface and (ii) a hollow/porous gold shell without silica. Along with the golf-ball carrier, a carrier derived eccentric encapsulation of a one material around another. Using a sol-gel synthesis method, eccentric Janus nanoparticles composed of a silica shell partially encapsulating a carboxylate-modified polystyrene core (cPS). Nano-bowl-like structures were derived after the removal of the polystyrene core by organic solvent. The role of the polystyrene core in determining the eccentric JNP morphology and size was also elucidated.

These eccentric JPs and nanobowls can be further developed to create a magnetic gold nanobowl (mGNB). Such mGNBs consisted of a silica bowl whose outer surface is coated with iron oxide/gold/PEG in that order and a exposed silica bowl interior. The nanobowls demonstrated a surface enhanced Raman spectroscopy capability, showed cellular uptake in vitro and dose-dependent toxicity. Finally, we demonstrate preliminary work toward loading a payload (fluorophore, anti-cancer prodrug) in the silica bowl.
Chapter 1

Background

Drug delivery in the broadest sense is the distribution of a therapeutic agent to relieve, treat, or resolve a particular symptom, disease, or condition. How to best introduce a therapeutic agent to the patient’s body has been the subject of much debate, experimentation, and application for as long as humans have realized there are ways to aid and accelerate the body’s natural healing and disease fighting abilities. Ancient Egyptian, Chinese, and Incan cultural records demonstrate knowledge of treating disease and injury. Wound dressings in surgery were known to Egyptian physicians as early as the 16th century BCE[1]. Herbal knowledge drawn from traditional Chinese medicine texts resulted a whole family of anti-malarial therapeutics based on artemisinin[2]. Finally archeological excavations have shown how pre-Columbian Incan surgeons were skilled in repairing cranial wounds using quinine to disinfect and precious metals to perform cranioplasties[3].

Modes of drug delivery currently include topical, intranasal, eye drops, injection, or oral, depending on the therapeutic. While the first three options provide for local application of therapeutic, they are limited to ailments that afflict those particular regions. For access deep inside the body the latter two are often used for systemic delivery of a
pharmaceutical agent. However this requires a much higher dose of therapeutic agent in order to maintain a concentration a therapeutic concentration. This high concentration results in unwanted side effects with varying degrees of severity. In the most extreme circumstances, side effects such as severe weight and hair loss can seriously lower the patient’s quality of life[4]. It would be ideal for systemically delivered therapeutics to be selectively concentrated only to the site of greatest need. To that end much effort has been focus on localized and targeted delivery of therapeutics on difficult to treat diseases[5].

1.1 Theranostic

The term “theranostic” is a portmanteau of “therapeutic” and “diagnostic”. Advances in nanosynthesis methods have allowed for research groups to experiment with nanoscale entities that contain components capable of providing environmental information (imaging, pH, temperature) and releasing an active pharmaceutical ingredient[6]. The rationale for this approach is to provide a more granular approach to treatment of very heterogeneous diseases like cancer. Often such treatments are only effective for very specific patient sub-populations [7]. Integrating diagnostic information with therapeutic

Figure 1.1: Components of a generalized theranostic formulation. Reprinted with permission from Lin et al., Chemical Reviews, 2015, 115 (1), 327-394. Copyright 2015 American Chemical Society.
payload offers the hope of improved prognoses.

Along with hosting a diagnostic element and therapeutic agent, an idealized theranostic carriers would contain elements that allow the entity to maximize biocompatibility including remain stable in the biological serum, retain long circulation lifetime, and evade immune detection[7]. Components to direct and control the release of the therapeutic in a sustained or on-demand fashion are also essential. (Figure 1) Targeting molecules for specific delivery of particles into cells is essential for delivering therapeutics to the correct group of cells. A surface passivation molecule like PEG is necessary to evade immune detection and minimize surface fouling with serum proteins. Finally the size needs to be 10-200 nm. If the entity is too small ( <10 nm) than the entity is likely cleared by the kidneys[7]. However, if the entity is too large( » 200 nm) the particles liable to immune clearance[7].

Such strict requirements need an interdisciplinary approach to integrate facets of seemingly disparate lines of research. Areas such as Janus particles, DNA nanotechnology, and chemical conjugation can be combined together to form an smart, controllable, monitored, drug delivery carrier.

1.2 Use of inorganic materials in drug delivery

Nanoscale therapeutic carriers are attractive for drug delivery because they package drugs into confined volumes and can specifically release them to the desired locations if targeting moieties are attached. Many types of materials have been investigated as therapeutic carriers. Major classes include solid polymer nanoparticles, polymer micelles, liposomes, nano hydrogels, dendrimers and silica[6]. While each class has their compositional differences, all of these carriers can be broadly described as biocompatible, versatile in chemistry, store therapeutic molecules, and stable in bodily fluid. However,
these carriers become difficult to track and manipulate once inside the body. Inorganic materials like magnetic iron oxide or gold have properties that can augment therapeutic carriers with such abilities.

### 1.2.1 Iron Oxide

Iron oxide nanoparticles have been investigated for their use as an MRI contrast agent[8] and guidance mechanism[9]. Iron oxide particles less than 20 nm in diameter become superparamagnetic because thermal fluctuations of the ambient environment at room temperature are strong enough to randomly switch the particles’ magnetic moment. As such they display little hysteresis and are magnetically responsive in the presence of an externally applied magnetic field[10]. These particles have become useful as MRI contrast agents because they exhibit strong relaxation signals when pulsed by an MRI. However because of their size, these particles cannot carry too many therapeutic molecules on its surface, but may be coupled with a carrier to carry more therapeutics. In addition to MRI contrast agents, the particles have also been investigated as a guidance mechanism for local concentration of therapeutic carriers to a site of interest. Our laboratory and collaborator (Dr. Sungho Jin) have been able to demonstrate this guidance principle by localizing particles to murine breast cancer tumors [9] as well as across the blood-brain-barrier [11]. This delivery system can collect therapeutic carriers to a defined diseased area. However, it has a slow passive drug leakage.

### 1.2.2 Gold

Gold has also been extensively investigated for use both as a drug carrier and unique photophysical properties at the nanoscale [12, 13]. It is hydrophilic and biocompatible due to its chemical inertness in the body[6]. It also has a well understood and
developed chemistry for controlling its growth and properties. Depending on its size, gold will exhibit different plasmon resonances that can be tuned into the near infrared[13] and which in turn allow heating by a NIR laser causing hyperthermia in the local region[6].

Gold can also be integrated with other chemistries in order to form a core-shell structure. In a method pioneered by the Halas and co-workers, a continuous gold nanoshell can be formed on a particle surface in a two-step process12. First small gold seeds (2-3 nm) can be electrostatically or covalently attached to the surface of the core particle. Following the seeding, a complete gold shell can formed by reducing gold seeded cores in chloroauric acid solution buffered with potassium carbonate. The shell thickness can be controlled by reaction precursor conditions and reaction time[14].

1.2.3 Silica

As a therapeutic carrier, silica is a good material because i) it can chemically interface with iron oxide and gold and ii) store a lot of therapeutic agent. It is usually biocompatible, has a well-developed chemistry, and multiple structures have been developed 6. However it also has some short comings such as, varying issues with long term accumulation and concentration[6].

Using a sol-gel chemistry first described by Stober and coworkers, an alkoxyisilane in this case, tetraethyorthsilicate can be hydroylzed in a basic solution to form silica nanoparticle. The reaction as follows:

$$\text{Si}(\text{OC}_2\text{H}_5)_4 + 2 \text{H}_2\text{O} \rightarrow \text{SiO}_2 + 4 \text{C}_2\text{H}_5\text{OH}$$

results in silica and ethanol. The resulting silica forms particles whose sizes can adjusted based on reactant concentrations, pH, and temperature.

Many different structures have been made using this process including mesoporous silica, nanowires, shells, and Janus particles. This process is also highly amenable
to integration with other synthesis methods to form a multifunctional composite nanosstructure. Recently, magnetically responsive silica/(iron oxide-gold) core/shell particles have been reported [15, 16].

1.3 Janus Particle Development

Colloidal particles with two or more unique surface chemistries, commonly known as Janus particles (JP) named after the two-faced god of Roman mythology [17], are of interest because the combination of multiple surface chemistries can create a material with its own unique properties (Figure 1.2). These particles have a wide number of applications including catalysis, biomedical imaging[18], and drug delivery[19]. Strategies to make such particles are useful for making theranostic carriers because they naturally provide differential surface functionalization, an important trait that can make theranostic carriers easier to synthesize. While there are a plethora of strategies to make such particles there are several common synthesis mechanisms including: desymmetrization by masking template, solution phase separation, molecular self-assembly, and surface ligand manipulation.

![Figure 1.2: Different Janus particle morphologies. Spherical (a), two types of cylindrical (b,c), and disc-shaped (d,e) JPs. (f-k) Various kinds of dumbbell-shaped JPs with asymmetric or snowman character (f), symmetric appearance (g,k), attached nodes (h), and eccentric encapsulation (i). (l) Janus vesicles or capsules. Reprinted with permission from Walther and Müller, Chemical Reviews, 2013, 113 (7), 5194-5261. Copyright 2015 American Chemical Society.](image-url)
1.3.1 Desymmetrization

In order to form asymmetrical surfaces, a popular strategy is temporarily mask parts of the surface using a variety of different methods including, hierarchical templates, pickering emulsions [20, 21], or modification on planar surfaces. [22, 23]

JP can be also be formed with particles are embedded at the interface of a pickering emulsion. A pickering emulsion is a phase separated colloidal system stabilized by solid particles instead surfactants. Since the stabilizing particles have one side in hydrophobic and hydrophillic environments, each side is effectively masked from the other side. This allows for additional chemical modification on each surface that spatially limited. Many silica/polymer JP have been created in this fashion a high degree of control over how much of the silica covered by polymer.[20, 21]

Hierarchical templates are three dimensional equivalents of masking by planar surfaces. Instead of using a flat surface to hide approximately half of a particles surface, templates with core-satellite geometry can be used to create more dimpled or porous JP. Template particles consist of a single large core and many smaller particles attached to it. Such structures are often synthesized using electrostatic attraction or covalent attachment. Once a core-satellite template has been created, the surface of core can be further modified with oppositely charged polymers [24], silica [25], or metals [12]. Removal of the masking satellite exposes the previously covered surface of the core also forming circular dimples or pits on the newly synthesized JP.

The deposition of spherical particles in monolayers on planar surfaces allows for surface modification of the exposed surfaces the particles. This strategy is particularly simple and flexible to different materials and methods of modification. The one commonality among being that the method of deposition are directionally sensitive processes. Various deposition processes include microcontact printing, electron beam metal evaporation [22, 23] UV photopolymerization[26], and plasma treatments[27, 28, 26] ,
electroless deposition of metals [29, 30], binding of metal nanoparticles [31, 32, 33], and growth of metal oxide nanowires [34] are commonly used. Monolayers of particles are often loosely secured to the planar surface via van der Walls interactions. However more secure attachment can be made possible by deposition of a monolayer in a thin polymer film or gel [35].

1.3.2 Solution Phase Separation

Janus particles can also be synthesized in a confined phase separated environment. This is often used to blend two incompatible homopolymers into a single particle. Well defined polymer nanoparticles can be obtained by using phase separated mini emulsions or phase inversion.

The miniemulsion method relies on dissolving two dissimilar polymers chains in an organic solvent and stabilizing the emulsion with surfactant in an aqueous solution. Other factors that influence phase separation include molecular weight, interfacial tension of the polymer/water interface, and type of surfactant. By slowly evaporating the toluene, phase and particle geometry could be adjusted by surfactant concentration [36]. This method could also be used to create "mushroom-like" JP by using two different types of polystyrene, plain and initiator modified PS. By mixing the two different types of PS together, a phase separated particle could be created by whereby one side had a polymer initiator and the other side was plain. A secondary growth of PMMA was performed on the initiator side to create the mushroom-like cap [37, 38].

Phase inversion is uses polar organic solvent with water. The basic process involves dissolution of polymers in polar solvent with addition of water and no need for surfactant. The non-aqueous polar solvent is evaporated polymer particles can grow via nucleation, growth or precipitation. Particle size and morphology depends greatly on initial concentration and water/solvent ratio. If block copolymers are used complicated
microphase separated or compartmentalized particles can be synthesized depending on molecular weight of each component in the block copolymer [39, 40, 41].

1.3.3 Self-Assembly

Self-assembly processes refer to the self-organization of molecules into larger structures. A major class of molecules known to easily self-assemble into Janus particle structures are terpolymers. Terpolymers are block copolymers consisting of three different constituent monomer building blocks. The structure of such polymers is defined by the miscibility of each block with each other and volume fraction of each block. The polystyrene-block-polybutadiene-block poly(methyl methacrylate) system is well studied system of self-assembling Janus particle systems. Researchers found that by holding the polystyrene and poly(methyl methacrylate) blocks at the same weight faction, morphological changes could be induced solely by changing the weight fraction of the butadiene [35]. The terpolymer would take on lamellar [42], spherical[43], or cylindrical[44] shapes depending on the mass of the polybutadiene block. A wide of variety of cross linking groups could also be introduced to stabilize the terpolymer particle morphology for future use [35].

1.3.4 Ligand Manipulation

Surface coating the particle with a pair of immiscible ligands can allow for symmetry breaking properties. Detailed modelling has shown that different surface patterns can be obtained as a function of surfactant type, mixing ratios and size of the modified particle[44]. The surface coating can switch from a fully demixed to a stripe-like configuration as the particle size decreases. Janus particle character was also promoted when immiscible ligands were of short length, small bulkiness, and similar differences in
The symmetry breaking properties of a ligand shell with two immiscible ligands can be used to form asymmetric Janus particles. Chen and co-workers used an amphiphillic block copolymer (PS-b-PAA) to desymmetrize citrate-stabilized gold nanoparticles in a mixture of DMF and water at 150°C for 2 hours[45]. Further investigation showed that two low molecular weight ligands in combination in block copolymer can give rise to eccentric encapsulation of gold nanoparticles by the block copolymer [46, 45, 47]. Similar strategies have used gold and ruthenium core particles partially encapsulated by PEO-b-PPO[48] and PS-b-PEO [49] respectively. Another asymmetric particle using this strategy involved using with low molecular weight PAA and 4-mercaptophenylacetic acid on gold particles to create a gold particle eccentrically encapsulated by silica[46].

1.4 DNA nanotechnology

Strand displacement reactions have been used to actuate a variety of DNA based nanoscale devices including logic gates[50], switches[51], and walkers [52]. These devices have used Watson-Crick interactions and relied on long single stranded sections â€œh-holdsâ€œ in order operate. Recently a strand displacement reaction has been developed that utilizes inosine-cytidine bonding in place of traditional guanosine-cytidine pairing in the active area known as a "zipper"[53]. I-G bonds have only 2 hydrogen bonds as opposed to the 3 hydrogen bonds found in G-C bonds and give the zipper a lower binding energy when compared to a normal double stranded DNA of the same length and composition.

The zipper consists of two strands, the first on consists of A and G and the second consisting of I and Ts (Figure 1.3). This state is known as the "contracted" state The
Figure 1.3: Partial DNA Strand Displacement via Inosine. A) The inosine base forms 2 hydrogen bonds with cytosine (top) while guanine forms 3 hydrogen bonds with cytosine. The loss of one hydrogen bond weakens the over bond energy. B) Strategically replacing guanines with cytosines in a duplex (DNA zipper) allows a structure to remain in a stable state of partial DNA strand displacement (DNA tweezer). Reprinted with permission from Landon et al., Langmuir, 2012, 28 (1), 534-540. Copyright 2015 American Chemical Society.

natural complement (A and G) of the first strand have a stronger binding affinity it then the second strand. The second strand is thus displaced allowing the structure to which it is connected to unravel or "extend". This is a onetime reaction and not reversible at room temperature.

However, when the first and second strands on connected to each other by normal complementary base pairs, the system is able to cycle between this contracted and extended states. Specially designed extension strands are used to displace the zipper strands and a contraction strand is designed to remove the extension strand so that the zipper can hybridize.

1.5 Outlook- Trans Blood-Brain Barrier Drug Delivery

In particular, areas such as the brain, eyes, testes are particularly difficult to reach for the immune system.[54] Should a tumor or other ailment arise in these immune privileged regions, the immune system cannot easily resolve the issue. For the eyes and testes, therapeutics can usually be delivered through eye drops or injection respectively.
However the brain remains a difficult organ to access without major surgery. Internally the vasculature around the brain is especially tightly guarded by a system known as the blood-brain-barrier (BBB). The BBB is layer of tightly bound endothelial cells around the brain vasculature that restricts the passage of solutes into the brain.[55]

Several broad strategies have been proposed to deliver therapeutics into the brain. Chemical delivery systems involve modification of the active pharmaceutical that is more conducive to BBB- transcytosis. This can include making the API more lipid-like, conversion to prodrug form that promotes transcytosis, or a combination of the two to further enhance transcytosis. Biological based delivery systems rely on certain biophysical states, receptors. These can involve modification of API with cationic charges, modification with moiety capable of binding to a variety of transcytosis receptors, or modification with peptide sequences capable of directly penetrating cell membranes. Finally, alternate methods to bypass the BBB completely have been developed including the use of intranasal delivery.[57] While these approaches enhance the therapeutic uptake, they provide no diagnostic information about their presence simultaneously.

In order to accomplish this theranostic approach, a nanocarrier for the therapeutics must be developed. In order to deliver the nanocarrier past the BBB modification of the nanocarrier will need to utilize the abovementioned chemical and biological methods as well physical methods. Physical methods have been proposed including magnetically responsive carriers[11], and magnetic carriers with ultrasound [58]. The strategies that have been outlined in this thesis provide a path forward to developing more effective nanocarriers for future theranostic purposes. The gold golf ball or magnetic gold nanobowl can be used to for a four- fold purpose: i) magnetically guide particles across the BBB, ii) provide MRI localization diagnostics on its location, iii) heat surrounding tissue with a RF pulse, and iv) carry a payload of highly potent anti-cancer therapeutics. In addition the DNA switch currently adapted for enzymatic control can be readily
adapted in the future toward on controlled release of therapeutics.

1.6 Summary of Chapters

The chapters of this dissertation can be divided into three broad areas. The first is the development of an active DNA-based switch to possibly add on demand therapeutic release to the therapeutic. (Ch. 2) The second is the synthesis and study of nanocarriers with differential surface functionalization. (Ch 3-5) Finally, the second is the integration of diagnostic and therapeutic components into a theranostic carrier. (Ch 6-7)

Bibliography


Chapter 2

An on-demand four-way junction DNAzyme nanoswitch driven by inosine-based partial strand displacement

2.1 Abstract

A DNA four-way junction device capable of junction expansion and contraction cycles using an inosine-based partial strand displacement scheme is reported. These nanoscale positioning capabilities are used to provide on-demand activation and deactivation of a pair of split E6 DNAzymes on the device. The device also demonstrates a combined catalytic rate significantly higher than the original E6 DNAzyme under similar operational conditions. This approach can provide structural organization and spatially control other multicomponent molecular complexes


2.2 Introduction

DNA four-way junctions (4WJ) are found naturally as mobile junctions during DNA replication[1], and are also synthesized as immobile structural components of 2D DNA lattices. The 4WJ motif presents a way to precisely place different molecules in close proximity with a dynamic control over their spacing. Mobile 4WJ can spontaneously switch between different conformations[2, 3, 4, 5]. Immobile 4WJ, however, are usually static and have limited switchability, if any [6, 7, 8, 9]. With controllable shifts in conformation, a DNA 4WJ can be useful for the precise positioning of chemical moieties or a nanoscale switch to regulate other processes such as activity of E6 DNAzymes[10]. One way to design such a 4WJ is to make parts of the DNA junction stimuli responsive. DNA-based nanostructures with active elements responsive to light[11], metal ions [12], pH[13, 14], temperature [15, 16], and toehold strand displacement[17, 18] have been designed. The toehold strand displacement that has been used in actuation of various DNA nanomachines rely on the invasion of a single-stranded DNA into a double-stranded oligonucleotide until the toehold-less strand is completely displaced[19]. However, if this is applied to a 4WJ, full displacement of any portion of a 4WJ will disassociate the structure.

Here we report a 4WJ capable of repeatable junction point expansion and contraction via an inosine-based partial strand displacement scheme. The cycles of the 4WJ are monitored using a fluorophore and quencher pair. Furthermore, the nanoscale positioning capability of this structure was used for on-demand switching of a pair of opposing split E6 DNAzymes. A combined catalytic rate obtained with this device is significantly higher than a previously reported E6 DNAzyme[10] under similar conditions. The switching system of this device turns a chemical cue into a physical displacement, which in turn facilitates defined structural organization and enables controlling the behaviour
of enzyme complexes. In addition it may also be applied as a reusable platform for DNA-based biosensing because its conformational switching abilities give it the requisite functionality to operate like those reported in the literature[20, 21, 22, 23, 24].

2.3 Materials and methods

2.3.1 4WJ design

The device is driven by two independent inosine rich sequences indicated as "1" and "2". Strands containing only inosines and thymines in the sequence were indicated as such with "ẀI" Strands containing cytosines and adenines were denoted as such with āĂIJNāĂl. Four structural strands (N1, N2, W1, and W2) plus two cross over strands (N2/W1, N1/W2) assemble together to form the 4WJ (Figure 2.1). The crossover strands and all other regions do not contain any inosine. To monitor device functionality, two carboxyfluorescein (FAM) fluorophores and two Iowa Black FQ quenchers were attached to N2/W1 and N1/W2 respectively on both the 5' and 3' ends (Figure 2.1). Device opening was triggered by introduction of a single stranded DNA complementary to the "N" strands. Opening strands (Open N1, Open N2) also contain a 6 nucleotide (nt) sticky end enabling removal by a closing strand (Close N1, Close N2).

2.3.2 DNA strands

DNA strands were designed and ordered from Integrated DNA Technologies (Coralville, IA). The strands were resuspended in a buffer consisting of 30 mM of Tris (Fischer Scientific, Pittsburgh, PA) and 160 mM of NaCl (Sigma, St. Louis, MO) in MilliQ deionized water (Millipore, Billerica, MA). DNA strands were assembled via thermal annealing using a PCR thermocycler (Mastercycler Personal, Eppendorf,
Westbury, NY) programmed to raise the solution temperature to 94 °C and then cooled at rate of 1 °C every 2 minutes until the final temperature is 4 °C. A complete list of sequences is given in Table 2.1.

### 2.3.3 Gel electrophoresis

10 μL of 0.1 μM DNA solution was mixed with 2 μL of 10% v/v glycerin solution and placed in a 4% agarose gel (Lonza, Rockland, ME) preloaded with ethidium bromide (EtBr). DNA gel electrophoresis was performed with the gel placed in a 1ÅÊ TBE buffer (Fischer Scientific, Pittsburgh, PA) at temperatures between 20 and 25 °C with an electric field of 7.3 V cm\(^{-1}\) for 45 minutes. Gels are imaged with a Bio-Rad FX-Imager Pro Plus (Bio-Rad, Hercules, CA) and analyzed with the Quantity One software package (Bio-Rad). EtBr imaging is performed with the internal 532 nm laser and a 555 nm band pass filter, while Cy5 650/670 nm (Ex/Em) imaging used an external 635 nm laser and a 690 nm long pass filter. FAM imaging relied on an external 488 nm laser with a 530 nm band pass filter.

### 2.3.4 4WJ characterization

Measurements were performed at 480/530 nm (Ex/Em) in 96 well black plates (Nalgen Nunc, Rochester, NY) using an Infinite M200 Pro (Tecan, San Jose, CA) spectrophotometer at 37 °C. 50 μL of DNA 4WJs (0.1 μM) are placed in a well and 1 μL of increasingly concentrated solutions alternating between opening and closing strands (50, 100, 150, 200, 250 μM) were added to the solution as soon as the fluorescence signal started to level out. The molar excess in the cycling progression were equivalent to opening/closing strands 10, 20, 30, 40, and 50 times excess.
<table>
<thead>
<tr>
<th>Strand</th>
<th>Sequence</th>
<th>NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>CGC AAT CCT ACC ACC ATC CAA ACT CTC AGA ATC GCA CAC AAC ACC CAA CCA CAA CAA ACC AAA CCA ACT ATA CC GCA AAC TCT AACT ATA CC</td>
<td>92</td>
</tr>
<tr>
<td>W1</td>
<td>GGT ATA GTT AGA GTT TGC GGT ATA GT TII TTT IIT TTI TTI TII TII IIT TII IIT CCA TCA TAA ATT CCC ATC CTT CTT CGT CCA TCC CTA CCC TTA</td>
<td>92</td>
</tr>
<tr>
<td>N2</td>
<td>TAA GGG TAG GGA TGG ACG AA A TTA GTA GAG AGA GAA TAA TGA CAC AAC ACA ACC AAA CAA CAC ACC CGT CGA CTT CCT AAA TCC AAA ATC AG</td>
<td>92</td>
</tr>
<tr>
<td>W2</td>
<td>CTG ATT TTT GAT TTA GGA AGT CGA CG IIT ITI TTI TTT IIT TIT ITI AGA GTT TAT GAG CGA GGT AGA TTG GAT GGT GGT AGG ATT GCG</td>
<td>92</td>
</tr>
<tr>
<td>N1/W2</td>
<td>/FAM/ - TT GTG TGC GAT TCT GAG AAA ATC TAC CTC GCT CAT AAA CTC T-/FAM/</td>
<td>42</td>
</tr>
<tr>
<td>N2/W1</td>
<td>/IAbFQSp/TCA TTA TTC TCT CTC TAC TAA TTG GAA GGA TGG GAA TTT ATG ATG G/IAbFQSp/</td>
<td>46</td>
</tr>
<tr>
<td>E6 Target Strand</td>
<td>/TEX615/ GAC GAG TrAGG AGC AGT /IAbRQSp/</td>
<td>16</td>
</tr>
<tr>
<td>E6 Target Scrambled</td>
<td>/5TET/ AGA GTA TrAGG GAT ATC/3IABkFQ/</td>
<td>16</td>
</tr>
<tr>
<td>N1 E6</td>
<td>ACT GCT C AGC GAT GTT GTG TGC GAT TCT GAG AGT ATC TAC CTC GCT CAT AAA CTC T CAC CCA TGT CTC GTC</td>
<td>35</td>
</tr>
<tr>
<td>W2 E6</td>
<td>ACT GCT C AGC GAT GTT GTG TGC GAT TCT GAG AGT ATC TAC CTC GCT CAT AAA CTC T CAC CCA TGT CTC GTC</td>
<td>37</td>
</tr>
<tr>
<td>N2 E6</td>
<td>ACT GCT C AGC GAT TCA TTA TTC TCT CTC TAC TAA T GCAGG ATGGG AATTT ATGAT GG CAC CCA TGT CTC GTC</td>
<td>35</td>
</tr>
<tr>
<td>W1 E6</td>
<td>ACT GCT C AGC GAT TCA TTA TTC TCT CTC TAC TAA T GCAGG ATGGG AATTT ATGAT GG CAC CCA TGT CTC GTC</td>
<td>37</td>
</tr>
<tr>
<td>Open N1</td>
<td>TGG TTT GGT TTG TTG TGG TTG GGT TCA TAG</td>
<td>30</td>
</tr>
<tr>
<td>Close N1</td>
<td>CTA TGA ACC CAA CCA CAA CAA CAA ACC AAA CCA</td>
<td>30</td>
</tr>
<tr>
<td>Open N2</td>
<td>GGT GTG TTG TTT GTG GTG GTG GTG GTG GTC ATG</td>
<td>30</td>
</tr>
<tr>
<td>Close N2</td>
<td>CAT GAC CAC AAC ACA ACC AAA CAA CAC ACC</td>
<td>30</td>
</tr>
</tbody>
</table>


2.3.5 DNAzyme control

Using the sequences for a split E6-type DNAzyme adapted from Elbaz and Wilner, two identical DNAzymes were annealed into the device, one on each side of the device (Figure 2.6A, W1 E6, N1 E6, W2 E6 and N2 E6). The E6-type containing 4WJ were annealed at a concentration of 0.5 µM. A reporter nucleic acid strand consisting mostly of DNA with a single RNA adenine base (rA) in the middle is used to monitor DNAzyme activity. A fluorophore-quencher pair on the 5’ (Texas Red) and 3’ (Iowa Black RQ) of the reporter strand normally keeps the fluorescence of the molecule low. However, in the presence of the 4WJ DNAzyme, the reporter strand is hydrolyzed just after the rA. This separates the fluorophore from the quencher, allowing the fluorescent signal to increase.

Measurements were performed at 590/620 nm (Ex/Em) in black 96 well plates using the Infinite M200 Pro spectrophotometer at 25 ℃. 50 µL of the substrate strand of known concentrations was added in at least triplicate with 1 µL of a buffered magnesium chloride solution (600 mM MgCl₂, 30 mM Tris, 16 mM NaCl). This raised the Mg²⁺ concentration of the well to 12 mM. Incubation of the well plate took place at 25 ℃ for at least 15 minutes followed by at least 30 minutes of background readings. 1 µL of 4WJ DNAzymes was added to each measurement well after background readings were completed. This raised the concentration of 4WJ DNAzyme to 0.01 µM.

2.3.6 DNA modeling assumptions

To characterize the device different reaction schemes were used to describe the opening and closing of the device. The following reaction described the opening:

\[ S_1 + O_1 \xrightleftharpoons[k_{10}]{k_{-10}} F_1 \]
\[ S_2 + O_2 \xrightleftharpoons[k_{20}]{k_{-20}} F_2 \]
Where $S_1$ and $S_2$ represent the two closed sides of the 4WJ, $O_1$ and $O_2$ represent opening strands for the respective sides, and $F$ is the normalized fluorescent signal representing an open side 1 or side 2 of the 4WJ. The fluorescence signal for the full device can be represented as an average of the normalized signal for side 1 and side 2.

$$[F] = \frac{[F_1] + [F_2]}{2}$$

And the rate at which the fluorescent signal changes is thus given by:

$$\frac{d[F_1]}{dt} = -\frac{d[S_1]}{dt} = k_{1o}[S_1][O_1]$$

$$\frac{d[F_2]}{dt} = -\frac{d[S_2]}{dt} = k_{2o}[S_2][O_2]$$

The follow assumptions were made for both reactions:

$$[S_1]_i = [S_2]_i = 1$$

$$[S_1] = 1 - [F_1], [S_2] = 1 - [F_2]$$

$$[O_1] = [O_1]_i - [C_1]_i - [F_1], [O_2] = [O_2]_i - [C_2]_i - [F_2]$$

The first assumption states that the maximum normalized fluorescent level during an experiment represents a completion of the reaction and thus represents the concentration of devices in solution. The second assumption is based on the one-to-one conversion of closed side 1 or 2 to fluorescent side 1 or 2. Likewise the concentration of opening strands at any particular time is the number of excess opening strands subtracted from the fluorescent open side 1 or side 2 strands. Nonlinear curve fitting of equations 5 and 6 can be used to find $k_{1o}$ and $k_{2o}$.
\[
\frac{d[F_1]}{dt} = k_1 O (1 - [F_1]) ([O_1]_i - [C_1]_i - [F_1]_i)
\]
\[
\frac{d[F_2]}{dt} = k_2 O (1 - [F_2]) ([O_2]_i - [C_2]_i - [F_2]_i)
\]

Solving equations 5 and 6 respectively yields:

\[
\frac{[S_1]}{[O_1]} = \frac{[S_1]_i}{[O_1]_i} \exp(([S_1]_i - [O_1]_i)k_1 t)
\]
\[
\frac{[S_2]}{[O_2]} = \frac{[S_2]_i}{[O_2]_i} \exp(([S_2]_i - [O_2]_i)k_2 t)
\]

Substituting in the appropriate assumptions, analytical equations can be derived for \( F_1 \) and \( F_2 \):

\[
[F_1] = \frac{[O_1]_i - ([O_1]_i - [C_1]_i) \exp((1 - [O_1]_i)k_1 t)}{[O_1]_i - \exp((1 - [O_1]_i)k_1 t)}
\]
\[
[F_2] = \frac{[O_2]_i - ([O_2]_i - [C_2]_i) \exp((1 - [O_2]_i)k_2 t)}{[O_2]_i - \exp((1 - [O_2]_i)k_2 t)}
\]

The average of these two signals represents the behavior of the full device opening.

\[
[F] = \frac{1}{2} \left( \frac{[O_1]_i - ([O_1]_i - [C_1]_i) \exp((1 - [O_1]_i)k_1 t)}{[O_1]_i - \exp((1 - [O_1]_i)k_1 t)} + \frac{[O_2]_i - ([O_2]_i - [C_2]_i) \exp((1 - [O_2]_i)k_2 t)}{[O_2]_i - \exp((1 - [O_2]_i)k_2 t)} \right)
\]

The closing reaction is modeled as a reversible process following the below reaction equations:

\[
F_1 + C_1 \xrightarrow{k_{1C}} S_1
\]
\[
F_2 + C_2 \xrightarrow{k_{2C}} S_2
\]
where C1 and C2 represent the closing strands for their respective sides. The opening closing strands form a stable duplex O1C1 and O2C2 respectively that do not participate in the reaction. The full process can be also be modeled as the average of the normalized fluorescent closing of side 1 and side 2 like in equation 3. The kinetic equation for the closing reaction is given by

$$\frac{d[F_1]}{dt} = -k_{+1c}[F_1][C_1]$$

$$\frac{d[F_2]}{dt} = -k_{+2c}[F_2][C_2]$$

And the following assumptions were made for the closing part of the cycle:

$$[S_1] = 1 - [F_1], [S_2] = 1 - [F_2]$$

$$[C_1] = [C_1]_i - [O_1]_i - [F_1], [C_2] = [C_2]_i - [O_2]_i - [F_2]$$

The first assumption is as before; each 4WJ is composed of two sides which contribute to half the fluorescence. The second assumption is that the duplex waste product was created at the same rate and quickly and can be assumed to be concentration of opening strand in solution previously added subtracted by the amount of open 4WJ at a particular time. From these assumptions the following equation was used for fitting the closing reaction to find the reaction constants $k_{1c}, k_{2c}$.

$$\frac{d[F_1]}{dt} = -k_{+1c}[F_1]([C_1]_i - [O_1]_i - [F_1])$$

$$\frac{d[F_2]}{dt} = -k_{+2c}[F_2]([C_2]_i - [O_2]_i - [F_1])$$

The integrated forms of these rate equations are the following with the initial condition $F(t=0) = 1$: 
\[ [F_1] = \frac{([O_1]_i - [C_1]_i)exp(([O_1]_i - [C_1]_i)k_{1c}t)}{[O_1]_i - [C_1]_i + 1 - exp(([O_1]_i - [C_1]_i)k_{1c}t)} \]

\[ [F_2] = \frac{([O_2]_i - [C_2]_i)exp(([O_2]_i - [C_2]_i)k_{2c}t)}{[O_2]_i - [C_2]_i + 1 - exp(([O_2]_i - [C_2]_i)k_{2c}t)} \]

And thus the full device can be represented as:

\[ [F] = \frac{1}{2} \left( \frac{([O_1]_i - [C_1]_i)exp(([O_1]_i - [C_1]_i)k_{1c}t)}{[O_1]_i - [C_1]_i + 1 - exp(([O_1]_i - [C_1]_i)k_{1c}t)} + \frac{([O_2]_i - [C_2]_i)exp(([O_2]_i - [C_2]_i)k_{2c}t)}{[O_2]_i - [C_2]_i + 1 - exp(([O_2]_i - [C_2]_i)k_{2c}t)} \right) \]

2.4 Results and Discussion

In order to develop the controllable 4WJ nanodevices, we have designed an inosine-based strand displacement system (SDS). This SDS is capable of partial strand displacement by introducing energetic differences between the original and the displaced duplex[25]. In this system, one strand is limited to inosines and thymines, while its complementary strand is limited to adenines and cytosines. The duplex is held together by two hydrogen bonds across its entire length. A third strand called a displacement strand (the opening strand) consists of thymines, and guanines in place of inosines (similar to the above mentioned complementary strand but with guanine instead of inosine)[25]. Because the guanine-cytosine bond is more energetically favorable than the inosine-cytosine bond, the displacement strand is able to dislodge the inosine containing strand without use of a toehold. By using this inosine-rich active region, a contractile system capable of maintaining a stable, partially displaced state with multiple opening and closing cycles is accomplished (Figure 2.1).

The structure of the 4WJ shape was imaged by a transmission electron microscope.
Figure 2.1: 4WJ layout. The closed DNA 4WJ (A) and the open DNA 4WJ (B). Two inosine containing strands (red/blue duplex) were on opposite sides of the 4WJ. The device can be cycled between the closed state and the open state by the introduction of the appropriate strands. The conformation change is monitored by a fluorophore (red dot) and quencher (black dot) pair placed near the junction point of the 4WJ. The insets show how a difference in bond strength between I-C and G-C allows the 4WJ to open. The opening strand is removed by a toehold (arrows coming off opening strands) that binds with the closing strand, thus resetting the device. (C) A representative TEM image shown reveals that the structure has assembled as designed. Scale bar is 20 nm.

Figure 2.1 showed a four arm X-shape about 50 nm long and 30 nm wide. This confirms that the structure has assembled as designed. Additional TEM images can be found in Figure 2.2.

To demonstrate the thermodynamic stability of the 4WJ in their different conformations, a full opening and closing cycle was annealed in separate batches and compared using gel electrophoresis (Figure 2.3). A comparison of the 4WJ annealed by itself to 4WJs annealed with excess opening strands showed an increase in the molecular weight and increased FAM fluorescence of the latter due to incorporation of opening strands into the device. Comparison of the first two samples with 4WJs annealed with excess of opening and closing strands revealed that 4WJ switched back to its closed state. This was indicated by a drop in the molecular weight and the disappearance of the FAM fluorescence signal.

The kinetic behavior of the 4WJ was observed using time lapsed fluorescence measurements at 37 °C. In order to determine the range of mole excess appropriate for kinetic cycling experiments, various concentrations of opening strands were attempted
Figure 2.2: TEM of DNA switch. Uranyl acetate staining of inosine containing DNA four-way junctions without fluorophores in the A) closed and B) open conformation examined under TEM. A model of each conformation is shown next to zoomed in images of each state. A wide field image of each conformation is provided on the far right. Scale bar: 20 nm.

(Figure 2.4). At mole excesses tested (10, 50, 75 times) the fluorescence intensity levels out to the same place indicating that a population of 4WJs is fully opened.

The device was then cycled multiple times by alternating addition of opening and closing strands at successively higher concentrations (Figure 2.5 A). Opening of the device was driven purely by the collision of the opening strand with the inosine section as no toehold was present to further speed up the reaction. Closing of the device was mediated by a 6 nucleotide (nt) toehold. It took about 8-10 minutes to reach half fluorescence when the device was opening but only less than 1 minute to drop to half
when closing. Comparing rate constants between opening and closing legs of the cycle, the closing rate is 2 orders of magnitude faster than the opening strand. The 4WJ structure was faster when closing than when opening, however, not every device closed after each cycle. The fluorescence levels upon closing remain at 10% of the normalized fluorescence value (Figure 2.5A and B). This would indicate that approximately 10% of 4WJ did not fully close indicating that the devices cannot be cycled with perfect efficiency. This behavior was consistent with other strand displacement devices where device cycling efficiency decreases with the cycle number[18].

We also examined the ability of each side of the 4WJ for independent actuation. This was tested by sequential addition of opening strands for side 1 followed by side 2 at 10 times excess followed by sequential addition of closing strands of side 1 and side 2 at 20 times excess. In Figure 2.5B, two sharp increases in the fluorescence intensity followed by two sharp drops in the fluorescence intensity demonstrated that each side contributed to roughly half the function of the device, as well as that each side’s opening and closing strands retained their strand specificity. Rate constants extracted by curve

![Figure 2.3: Fluorescence Gel Electrophoresis. Lane 1: 25 bp Ladder, Lanes 2 & 3: Closed DNA 4WJ, Lane 4 & 5: Open DNA 4WJ, Lanes 6 & 7: Closed DNA 4WJ with excess closing strands. Fluorescent gel electrophoresis demonstrate thermodynamic stability of DNA 4WJ by itself Lanes (2 & 3), with opening strands (Lanes 4 & 5), with opening and excess closing strands (Lanes 6 & 7). The bands below the 50 bp markers were the duplexed waste product from the closing strands binding with the opening strands.](Image)
Figure 2.4: Opening strands. Different amounts of opening strands were mixed with the same amount of dual spring actuator and monitored simultaneously. It was discovered that full opening within the experiment’s observation time was achieved with an amount of opening strands 10x concentration. T= 37 °C.

fitting of Figure 2.5B, using assumptions described in the methods, are listed in Table 1. The closing reaction was significantly faster (~100 times faster) than the opening reaction for both sides. This can be explained by the different mechanisms by which the opening and closing are achieved. The opening strand operates based on a currently unknown mechanism by which the opening strand presumably infiltrates the device’s helix and displaces the weak side. The closing strand operates on the better understood toehold strand displacement. This infiltration method takes a greater time to complete than the toehold mechanism.

The 4WJ’s utility as a switch was then demonstrated by annealing two identical split E6-type DNAzymes into the 4WJ in place of fluorophore/quencher attached strands of DNA. When the 4WJ was opened the DNAzymes were inactive because the two halves of the split DNAzyme on both sides of the device were separated (Figure 2.6A). When the
4WJ was closed, the DNAzyme became active because the two halves of the DNAzyme were close enough to cut its target: the reporter strand (Figure 2.6B). This reporter strand consisted of complementary DNA bases at the ends that bind to the DNAzyme, a single RNA adenine (rA) at the center of the strand, and a fluorophore and a quencher on the 5’ and 3’ ends respectively. Thus, when DNAzymes cut the reporter after rA, the fluorophore separated from the quencher and allowed the reaction to be monitored.

Initially active in the closed state, the 4WJ DNAzyme was allowed to cut reporter strands at 25 °C (Figure 2.7A, I). The device was inactivated by injection of 10 times excess opening strands at 120 minutes (Figure 2.7A, II). The device gradually became

![Figure 2.5](image)

**Figure 2.5**: Cycling 4WJ Open and Closed. A) Real time monitoring of the four-way junction is performed by measuring the fluorescence intensity upon introduction of an opening or closing strand. The device can be cycled between closed and open states by introduction of successively higher amounts of opening and closing strands at 37 °C. In this figure the opening strands were introduced at 10, 30, and 50 times the amount of dual-spring actuators. And closing strands were introduced at 20 and 40 times the amount of dual-spring actuators. Additionally approximately 10% of opened strands never fully close again. This behavior was attributed to competition between the closing strand and the device with the opening strand. B) Independent functionality of the two inosine-rich sequences was confirmed by sequentially adding opening strands for each side and then sequentially closing each side at 37 °C. Upon addition of side 1’s opening strands the fluorescence increased approximately 50% and leveled off. Upon addition of side 2’s opening strands, the fluorescence rises sharply again and levels off. This behavior is confirmed again when sequential addition of closing strands yields the same behavior. The appearance of these fluorescence levels indicates that inosine-rich sequences were sufficiently different in the sequence to be independently operated. It also demonstrates that both inosine-rich sequences contribute to the opening of the 4WJ. Error bars represent one standard deviation.
Table 2.2: Kinetic Rate Constants for 4WJ switch

<table>
<thead>
<tr>
<th>Opening cycle</th>
<th>$K_o$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Side 1</td>
<td>190 ± 10</td>
</tr>
<tr>
<td>Side 2</td>
<td>380 ± 40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Closing cycle</th>
<th>$K_c$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Side 1</td>
<td>14,000 ± 5000</td>
</tr>
<tr>
<td>Side 2</td>
<td>20,000 ± 7000</td>
</tr>
</tbody>
</table>

inactive and remained so until 20 times excess closing strands were added at 390 minutes (Figure 2.7A, III) to reanimate the 4WJ DNAzyme. This behavior was consistent with opening and closing cycles found in Figure 2.5A. The DNAzyme was also able to cut with specificity when tested against a reporter with a scrambled sequence (Figure 2.8A). Analysis of 4WJ annealed DNAzymes on one or both sides revealed that side 1’s DNAzyme cutting velocity was about three times as fast as side 2’s (Figure 2.8B).

By design, both sides should have the same cutting speed because they were binding the same reporter strand and had the same catalytic core sequences. 4WJ DNAzymes with 0, 4, and 8 bp complementary stabilizer sections between the two halves of the DNAzyme showed direct gains in cutting velocities with stabilizer sections length (Figure 2.8C). Based on these data, the longer the stabilizer sections, the higher the melting point, the more stable the stabilizer section and the accompanying DNAzymes were. This strongly suggested that the conformational stability of the DNAzyme catalytic core was related to its cutting velocity. Upon inspection of the sequences in side 1 and side 2, it can be seen that 3 bases of the catalytic core for side 2 adjacent to the inosine-rich section on W2 was complementary to 3 bases in the inosine-rich section.

The DNAzyme strand on W2 was partially competing with part of the normal side. This competition would lead to lower catalytic core stability and reduce the overall cutting velocity of side 2’s DNAzyme. No such complementary bases were present on
side 1, and thus the side 1’s catalytic core was more stable and able to cut reporter strands more often. This competition for bases on side 2 was the most likely reason for the cutting speed discrepancy between the two sides. Thus taking advantage of inosine’s wobble base nature and with careful placement in the inosine SDS sequence, the sequences can be used to modify the cutting velocity of other split DNAzymes.

In addition to monitoring 4WJ DNAzyme switching ability, the kinetics of the

![Figure 2.6: 4WJ DNAzymes. (A) The DNAzymes attached to the 4WJ can be inactivated by introduction of opening strands that widen the structure and separate the two halves of the DNAzyme. Side 1’s DNAzyme strands (W1 E6, N2 E6) and side 2’s DNAzyme strands (W2 E6, N2 E6) were now so far apart, any target strand that diffuses into the area may bind with one side of the DNAzyme but will never be cut. The target strand consists of a fluorophore and a quencher at opposite ends of the nucleic acid strand. The strand is mostly DNA with the exception of one RNA adenine (rA) in the middle. (B) The DNAzyme is active when the both sides of the 4WJ were in the closed position, bringing the two split parts of the DNAzyme close together. This close proximity at all times allows a target strand that diffuses into it to be cut by the waiting DNAzymes. It is at the rA where the DNAzyme breaks the target strand into two pieces, resulting in an increase of the fluorescent signal.](image-url)
Figure 2.7: 4WJ DNAzyme operation. (A) Demonstration of a 4WJ E6-type DNAzyme function at 25 °C. (I) Addition of 1 µL active DNAzyme (0.5 µM) into 50 µL of the target strand (0.1 µM) with Mg$^{2+}$ (10 mM). (II) Shut off DNAzyme activity with addition of 1 µL solutions of Open N1 and Open N2 (both 5 µM). (III) Restoration of DNAzyme activity with 1 µL solutions of Close N1 and Close N2 (both 10 µM). Error bars represent one standard deviation. (B) Michaelis-Menten fitting of an active 4WJ DNAzyme with both sides closed at 25 °F. Error bars represent two standard deviations.

device’s cutting ability was characterized with Michaelis-Menten kinetics (Figure 2.7B). Using substrate concentrations ranging from 0.1-1 µM and an enzyme concentration of 0.01 µM, initial velocities were found by a linear fit to the first 10% of data points in the reaction. Michaelis-Menten constants were determined by plotting substrate concentration and initial velocities using the Hanes-Woolf plot. When both sides were activated, their combined catalytic rate ($k_{cat}$) was 0.052 min$^{-1}$, the effective binding constant ($K_m$) was 110 nM, and their ratio ($k_{cat}/K_m$) was 480,000 M$^{-1}$ min$^{-1}$. Under similar buffer (magnesium ion levels (10 mM vs. 12 mM), sodium ion levels (160 mM vs. 160 mM), and temperature (23 vs. 25 °C), the collective catalytic activity of the 4WJ DNAzymes was an order of magnitude higher than the originally developed E6 DNAzyme with a catalytic rate of ~0.005 min$^{-1}$[10].
(a) E6 Cutting Target vs. Scrambled Sequences

(b) E6 Side 1 vs. Side 2

(c) Comparison of 4WJ DNAzyme Stabilizer Length

**Figure 2.8**: E6 D NAzyme behavior. A) A test to check for strand specific cutting. A scrambled target was prepared and mixed in with the target strand to check what would be cut. This figure shows that no scrambled target was cut by the DNAzyme and only the target strand was cut by the DNAzyme. B) Four-way junctions with E6-type DNAzymes annealed into both sides (black), side 1 (red), or side 2 (green). 4WJ DNAzymes were prepared at a concentration of 0.5 µM and 1 µL of it was added into 50 uL of target strands (0.6 µM) in triplicate at 25 °C. Addition of side 1 and side 2 data points (△) reveal that two sidesâ€”cutting rates were linearly superimposable. C) Comparison between 4WJ DNAzymes with different length stabilizer section lengths between the 2 parts of the DNAzyme when the 4WJ is closed. With a reporter strand concentration of 0.8 µM, the length of the stabilizer strand positively correlates with the rise in cutting velocity.

### 2.5 Conclusion

We have successfully constructed a stable 4WJ with conformational switching abilities. The non-complementary sequences that make up the 4WJ prevent the device from spontaneously switching between conformations. Addition of the inosine rich sequences in two of the arms introduces switchable elements in the junction without
disassociating the whole junction. Using gel electrophoresis and time lapsed fluorescence, the 4WJ is able to open and close multiple times and each inosine-rich section is able to function independently of the other. The 4WJ is demonstrated to be a competent nanoswitch, able to turn on and off the activity of split DNAzymes attached to it. The 4WJ DNAzyme structure also had an order of magnitude increase in its catalytic rate over its single stranded form with all else being the same. The inclusion of the inosine inside a four-way junction opens up the center to allow for a variety of processes to happen. These features can be readily expanded into other applications in quantitative biology, nanomedicine, and nanomanipulation.

DNA strands dissociation and re-association processes are essential components of DNA double helix dynamics in vivo during replication, transcription, and reciprocal dynamic transitions between right-handed B-DNA and high-energy left-handed Z-DNA conformations. Successful development of nano-devices demonstrating on-demand dissociation and re-association of DNA strands in response to induced 4WJ expansion and contraction cycles reported here should facilitate a single molecule level mechanistic analyses of DNA strand displacement reactions during these fundamental biological processes.

2.6 Acknowledgements

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This chapter, in full, is a reprint of the material as it appears in AH Mo, PB
Landon, B Meckes, MM Yang, GV Glinsky, R Lal. “An on-demand four-way junction DNAzyme nanoswitch driven by inosine-based partial strand displacement” *Nanoscale*, 2014, 6, 1462-1466. The dissertation/thesis author was the primary investigator and author of this paper.

**Bibliography**


Chapter 3

Designing Hollow Nano Gold Golf Balls

3.1 Abstract

Hollow/porous nanoparticles, including nanocarriers, nanoshells, and mesoporous materials have applications in catalysis, photonics, biosensing, and delivery of theranostic agents. Using a hierarchical template synthesis scheme, we have synthesized a nanocarrier mimicking a golf ball, consisting of (i) solid silica core with a pitted gold surface and (ii) a hollow/porous gold shell without silica. The template consisted of 100 nm polystyrene beads attached to a larger silica core. Selective gold plating of the core followed by removal of the polystyrene beads produced a golf ball-like nanostructure with 100 nm pits. Dissolution of the silica core produced a hollow/porous golf ball-like nanostructure.

3.2 Introduction

Porous and hollow/porous nanostructures of various materials with sizes ranging from 100 nm to 100 µm are of current interest in catalysis [1], photonics [2], biosensing [3], nanodelivery [4, 5], cell culture [6, 7], and toxin scavenging [8]. The unique
properties of nanostructured gold are of current interest. Hollow/porous particles made of gold are desirable because of gold’s surface plasmon resonance [9] and use in surface enhanced Raman spectroscopy [10]. Gold is relatively biocompatible and can be chemically modified using a variety of moieties. Gold has been the material of choice in the fabrication of many nanostructures including spheres [11], rods [12], core/shell structures [13, 14, 15], assembled vesicles [16], and spiky gold shells [17]. However, particle diameter and pore size determine many potential applications of the particle and control of these two features has been difficult.

Over a decade ago, a variety of different approaches were employed to fabricate large, porous gold nanostructures. One previous approach filled the voids of a colloidal crystal template with colloidal gold and sintered the gold together prior to removal of the template [18, 19]. A different approach used colloidal gold and emulsion-templated polymers to fabricate macroporous gold beads with diameters ranging from 0.5-1 mm [20]. Another approach filled the matrix in porous polystyrene spheres with colloidal gold. Subsequent removal of the polystyrene by calcination resulted in macroporous porous gold spheres with diameters $\sim 9 \, \mu$m [19]. The latter two synthesis processes could perhaps be scaled down to produce particles with smaller diameters; however, it remains to be demonstrated.

In the past decade, several routes leading to the realization of hollow/porous gold particles have emerged. These routes have offered better control over particle diameter and pore size. One notable route employed electroless plating to grow a silver shell on top of a previously grown gold shell on a spherical polystyrene core [21]. The particles then underwent a calcination process which alloyed the gold to the silver and decomposed the polystyrene. This was followed by acid etching of the silver from the shells. The removed silver resulted in a restructuring of the gold atoms in the shell creating nanoporous rifts throughout most of gold the shell [21].
Recently, a technique for creating colloidal hierarchical templates has emerged. These templates consist of a core particle with smaller satellite particles attached to it. A shell is then selectively grown on the exposed surface of the core particle. Removal of the template results in shells containing pores with a morphology defined by the shape of the attached satellite particles. Colloidal hierarchical templates have been used (by Zhao and Collinson) to fabricate porous gold shells [22]. Also, Wan et al. used the process to create nanosized colloidal hollow/porous silica shells [23].

Previous synthesis processes employing colloidal hierarchical templates used the same material for the satellite and core particles [22, 23]. In this work, we extend the hierarchical template strategy by using different core (silica) and satellite (polystyrene) materials to create two different types of particles (core-shell golf balls and hollow gold golf balls). We demonstrate the process is also size scalable by using cores with diameters of 200 and 1000 nm. We also present the effect of different gold plating conditions on the morphology of the gold shells formed.

### 3.3 Materials and methods

#### 3.3.1 Materials

Colloidal polystyrene spheres with carboxylate-modified surfaces (2.73 wt % in water) with 100 nm diameters, spherical colloidal silica with 200 and 1000 nm diameters (prepared as 2 wt % in water); poly(diallyldimethylammonium chloride) (PDDA, Mw ∼ 8,500) were purchased from PolySciences. Tetrakis(hydroxymethyl)phosphonium chloride (THPC, 80% solution in water), and sodium hydroxide (NaOH, 10 M) were purchased from Aldrich. Potassium carbonate (K$_2$CO$_3$), formaldehyde (37%), ammonium hydroxide (NH$_4$OH, 29%), and hydrofluoric acid (HF, 48%) were purchased from Fisher Scientific. Dimethylformamide (DMF) was purchased from Macron Chemicals. Anhy-
drous ethyl alcohol (EtOH) was purchased from JT Baker. Chloroauric acid (HAuCl₄) was purchased from Sigma and prepared as a 1 wt % solution in water. The water used in all experiments was produced using a Millipore Advantage A10 system with a resistance of 18.2 MΩ.

3.3.2 Core-Satellite Template Synthesis

Colloidal silica has a net negative surface charge at pH values above 2.2. The net charge on the surface of colloidal silica can be reversed using a cationic polyelectrolyte such as PDDA [24, 25]. This was performed by adding 320 µL of ammonium hydroxide to 5 mL of 2 wt % dispersions of colloidal silica (pH ≥ 11). Subsequently, the solution was placed in an ultrasonic ice bath at 4 °C, and then 5 mL of 1 wt % aqueous PDDA solution was added into the mixture. The resulting solution was left in the ultrasonic bath for 20 min. The tube containing the solution was then centrifuged at 3200 g for 10 min to remove unadsorbed polymer. Centrifugation and redispersion was repeated four times and transferred to a clean container. On the final rinse the silica was redispersed in 5 mL of water.

Carboxylate-modified polystyrene spheres with 100 nm diameters were electrostatically attracted to the 1000 nm PDDA-functionalized silica as follows. In a 2 mL centrifuge tube, 1 mL of the PDDA-functionalized silica was centrifuged at 3200 g and redispersed in 1 mL of EtOH. The tube containing PDDA-functionalized silica solution was placed in an ultrasonic ice bath at 4 °C for 10 min. Then 25 µL of aqueous carboxylate-modified colloidal polystyrene was added to the PDDA-functionalized silica and left in the ultrasonic ice bath for an additional 5 min. The mixture was then centrifuged at 1000 g for 5 min, decanted and the pellet was redispersed in 1 mL of water.
3.3.3 Gold seed solution

Colloidal gold nanoparticles with \(\sim 3\) nm diameters were used as seeds for the gold plating process [26, 27]. The utility of the colloidal gold seeding solution was dependent on the order and method by which the reactants were mixed during their synthesis [26]. They were prepared by mixing 54 mL of water and 50 µL of 10 M sodium hydroxide together. In a separate container 12 µL of 80% THPC was diluted in 1 mL of water and aged for 5 min before being added to the aqueous sodium hydroxide solution. The mixture was stirring for an additional 5 min prior to the addition of 2 mL of 1 wt % HAuCl4. The solution quickly turned a brown-red and was stirred for 30 min prior to storage at 4 °C (for at least 24 h before use).

3.3.4 Gold plating

Gold seeds were attached to the hierarchical templates by adding 100 µL of the template containing solution and 5 mL of gold seed solution together while vigorously stirring. The solution was stirred at 45 °C for at least 30 min. To remove excess gold seeds from the mixture, the solution was centrifuged at 1000 g for 10 min. This centrifugation step was repeated at least 2 times. Finally, a reddish white pellet formed and was redispersed in 1 mL of water.

The gold seeds were grown into an interconnected gold shell through an electroless plating process as follows. A gold hydroxide (\(\text{Au(OH)}_3\)) stock solution (183 µM) was prepared by stirring 70 mL of 5.37 mM \(\text{K}_2\text{CO}_3\) aqueous solution for 10 min prior to the addition of 3.15 mL of 1 wt % HAuCl4. The gold hydroxide solution was initially light yellow and became clear after 1 h of stirring prior to refrigeration. The solution was aged in dark at 4 °C for 24 h prior to use in plating. Then 500 µL of gold seeded template particles were added to 15 mL of gold hydroxide stock solution and vigorously
stirred for 5 min prior to the addition of 50 µL of formaldehyde (37%). This step was quickly followed by the addition of 5 µL of ammonium hydroxide (29%). The resulting solution was then transferred to a rotisserie and tumbled for approximately 3 h. The plated particles were centrifuged at 1000 g for 5 min, decanted to remove waste products, and redispersed in water. This step was repeated at least 4 times and finally redispersed in 2 mL of water.

### 3.3.5 Gold Golf Balls

Gold golf balls were created when the 100 nm polystyrene satellites were removed. This was done by solvent dissolution of the polystyrene by redispersing them in 5 mL of DMF after centrifugation. The tube containing the solution was placed in an ultrasonic water bath for 20 min at 60 °C followed by centrifugation at 3200 g for 10 min and redispersing in DMF. Centrifugation and rinsing with DMF was repeated twice more before redispersing in water.

### 3.3.6 Hollow Gold Golf Balls

Hollow gold golf balls are produced by the subsequent removal of the silica core. After removal of the polystyrene, the gold golf balls were dispersed in a 10% HF solution in 2 mL centrifuge tubes for 24 h. The golf balls were centrifuged and etched with HF once more. The hollow gold golf balls were then rinsed 6 times by centrifugation with water. Scanning electron microscopy (SEM) images for all samples were obtained using a FEI XL30 SFEG UHR SEM.
3.4 Results and Discussion

The preparation schemes of the gold golf ball and hollow gold golf ball particles are presented (Fig. 7.1). The process consists of three major parts: (i) template synthesis, (ii) gold plating, and (iii) template dissolution. The first template was synthesized by attaching 100 nm carboxylate-modified polystyrene to a 1000 nm PDDA-functionalized silica core. By varying the concentration of the polystyrene spheres during the synthesis process, the amount of polystyrene attached to the silica core was controllable up to a limit of saturation (Fig. 7.2a). A saturation limit was previously observed [23, 28] and has been attributed to electrostatic repulsion limiting the number of polystyrene satellites that can attach to the surface.

**Figure 3.1:** Hierarchical template scheme used in the synthesis of gold golf balls and hollow gold golf ball. (a) Silica core (gray) functionalized with a cationic polyelectrolyte (PDDA) with smaller polystyrene (PS) satellite spheres electrostatically attached. (b) Nanosized colloidal gold (red) selectively attached onto the PDDA-functionalized silica core (gold seeding). (c) Electroless plating process grows the nanosized gold seeds into an interconnected gold shell. (d) Dissolution of the polystyrene satellites completes the synthesis of the gold golf ball particles. (e) Subsequent dissolution of the silica core completes the synthesis of the hollow gold golf ball particles.

Electroless gold shell growth is chemically driven by the reduction of gold ions to gold atoms. The gold atoms preferentially accumulate on preexisting gold surfaces, i.e., the gold seeds on the template cores (Fig. 7.2b). Eventually the growing gold seeds come into contact with each other forming an interconnected gold shell over the core and
Figure 3.2: SEM images from stages of the gold golf ball synthesis process using 1000 nm core. (a) Pollen mimicking structure formed by the hierarchical self-assembly of PDDA-functionalized 1000 nm silica and 100 nm carboxylate-modified polystyrene. (b) Nanosized colloidal gold (gold seeds) preferentially attaches to the template core. (c) After the plating process, the gold seeds can be seen as an interconnected gold shell formed around the 100 nm polystyrene satellite spheres (indicated with arrows). (d) Removal of the polystyrene leaves behind pits (arrows), resulting in the gold golf ball particle. (e) Subsequent removal of the silica core results in hollow gold golf ball particles (a broken hollow gold golf ball). (f) Wide field view of 1000 nm gold-plated template particles around the satellites particles (Fig. 7.2c). After the gold shell is formed, removal of the polystyrene satellites created a gold golf ball particle (Fig. 7.2d). Subsequent removal of the silica core produced a hollow gold golf ball particle (Fig. 7.2e). A wide-angle image of the gold golf balls is presented (Fig. 7.2f).

The thickness and completeness of the gold shells are determined by two main factors, the surface coverage of seeds on the template and the gold ion/template ratio. Dense gold seeding of the template cores allows gold seeds to grow into contact with neighboring seeds with minimum growth. The saturation limit for gold seeds on silica has been observed to be about 30% surface coverage in water and without salt [13, 29].
Figure 3.3: Gold plating of core-satellite templates. SEM images of gold shells plated on gold seeded template particles using various concentrations of gold hydroxide or formaldehyde. Gold plated template particles using 15 mL of gold hydroxide stock solution (183 Î¼M) as prepared with the following amount of formaldehyde (37%): (a) 7, (b) 50, and (c) 100 µL. Gold plating of template particles by adding 50 µL of formaldehyde (37%) with the following amounts of gold hydroxide stock solution: (d) 2.5, (e) 5, and (f) 15 mL.

The density of gold seeding on the template cores is expected to be near the saturation limit.

Gold shell growth on the template particles was controlled by varying two factors in our samples, formaldehyde concentration (Fig. 7.3a-c) and gold ion concentration (Fig. 7.3d-f). If there are insufficient amounts of either formaldehyde or gold ions in solution the seeds will not grow to form a single interconnected shell. Insufficient shell growth results in a patchwork of large gold islands on the surface. Likewise, significant over growth of the shell will envelope the polystyrene satellites.

The limits of the size range over which colloidal hierarchical template particles can be produced has not been determined; however, the gold golf ball synthesis pro-
cess was also performed using 200 nm PDDA-functionalized silica cores with 100 nm carboxylate-modified polystyrene satellites (Fig. 7.4a). Subsequent gold seeding and plating of the smaller template particles was also successful (Fig. 7.4b).

![Figure 3.4: 200 nm gold plated core-satellite templates. SEM images from stages of the gold golf ball synthesis process using 200 nm cores. (a) Colloidal hierarchical template particles containing PDDA-functionalized 200 nm silica cores and 100 nm carboxylate-modified polystyrene satellites. (b) After completion of the electroless gold plating process and before the removal of the polystyrene satellites.](image)

Using a hierarchical template constructed of two different materials, two variations of the same particle were produced with potentially very different applications. The pores of the gold golf ball have a silica bottom and may allow them to be selectively functionalized. The vacant center of the hollow gold golf ball offers storage capacity unavailable to the gold golf ball and may be used to store and release theranostic agents in the future.

### 3.5 Conclusion

Two different variants of a golf ball mimicking nanostructure were fabricated using a colloidal hierarchical template. The hierarchical template particles were fabricated by attaching smaller negatively charged colloidal polystyrene spheres (satellites) onto larger, positively charged colloidal silica spheres (cores). The PDDA-functionalized silica cores of the templates preferentially absorbed ~3 nm colloidal gold creating a dense
layer of gold scattered on the surface. An electroless plating process grew the individual surface bound gold colloids into a single interconnected gold shell. Dissolution of the polystyrene satellites resulted in a gold golf ball with a silica core. Subsequent removal of the core created the hollow/porous gold golf ball. In addition, two different-sized cores were used to create templates and demonstrate the size scalability of the process. We anticipate this nanostructure may be used as a platform for site-specific functionalization or theranostic delivery.

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Bibliography


Chapter 4

Synthesis of Nano-Bowls with a Janus Template

4.1 Abstract

Colloidal particles with two or more different surface properties (Janus particles) are of interest in catalysis, biological imaging, and drug delivery. Eccentric nanoparticles are a type of Janus particle consisting of a shell that envelops the majority of a core particle, leaving a portion of the core surface exposed. Previous work to synthesize eccentric nanoparticles from silica and polystyrene have only used microemulsion techniques. In contrast we report the sol-gel synthesis of eccentric Janus nanoparticles composed of a silica shell around a carboxylate-modified polystyrene core (Janus templates). In addition, we have synthesized nano-bowl-like structures after the removal of the polystyrene core by organic solvent. These Janus templates and nanobowls can be used as a versatile platform for site-specific functionalization or controlled theranostic delivery.
4.2 Introduction

Colloidal particles with two or more unique surface chemistries, commonly known as Janus particles named after the two-faced god of Roman mythology[1], are of interest because the combination of multiple surface chemistries can create a material with its own unique properties. These particles have a wide number of applications including catalysis, biomedical imaging[2], and drug delivery[3].

A wide variety of physical[4, 5] and chemical methods[6, 7, 8, 9] have been used to synthesize Janus particles. Early Janus particle morphologies typically consisted of large spheres with different surface chemistries on each hemisphere. The past few decades have seen a large exploration of different Janus particle morphologies and synthesis techniques[10, 11, 5, 12, 13, 14]. There is current interest in developing eccentrically encapsulated Janus nanoparticles[15, 16]. Such efforts have included the synthesis of eccentric nanoparticles where a silica shell is formed around a partially exposed polystyrene core [17, 18] or a polystyrene shell around a silica core [19, 20, 21]. Dissolving the core from this structure would create a nanobowl and a potential starting point for building a controlled drug delivery system.

There are several methods to synthesize eccentric particles with a polystyrene shell around a silica core. However, silica shells around a polystyrene core have required the precise tuning of reactants in a microemulsion. Wang and coworkers balanced concentrations of styrene, tetraethylorthosilicate (TEOS), and iron oxide nanoparticles in nano-micelles to create the necessary phase separation needed for synthesis of a magnetically responsive silica shell eccentrically encapsulating a polystyrene core [18]. Microemulsions however require the addition of a surfactant and require multiple wash steps to remove post-synthesis. A non-emulsion process would be more simple to scale up for future industrial use and have a simpler reaction chemistry.
Chen and coworkers recently demonstrated the eccentric encapsulation of silica around a carboxylate modified gold core using Stöber’s method. By varying the ratio of two carboxylate containing surface modification agents on the gold, they were able to control the degree to which silica would encapsulates the gold. [16]. Feyen and coworkers also demonstrated eccentric encapsulation of silica around carboxylate modified iron oxide [22]. This literature thus suggests partial encapsulation of cores by silica can be accomplished more widely on different materials.

Here, we report a sol-gel, non-microemulsion method for controlled synthesis of a silica shell eccentrically encapsulating a carboxylate-modified polystyrene core (Janus template). The effect of polystyrene core size, surface functionalization, and tetraethylorthosilicate (TEOS) concentration on the Janus template-like particle morphology was examined. In addition, we synthesized nano-bowl-like structures after the removal of the polystyrene core by organic solvent. The nanobowls have a cavity that can be used for storage of therapeutics and capped with a biocompatible materials and can be used for efficient and controlled delivery and release of theranostics (imaging contrast molecules and therapeutics).

4.3 Materials and methods

4.3.1 Materials

Various polystyrene cores of different sizes and functional groups were purchased from Polysciences. The following nominal and actual diameters listed are those reported by the manufacturer. Spherical colloidal polystyrene with carboxylate (PS-COOH) modified surfaces of 50 nm (actual 45 ± 6.2 nm) diameters, 2.6% in water; 100 nm (actual: 85 ± 6.7 nm) diameters, 2.62% in water; and 200 nm (actual: 190 ± 6.5 nm) diameters, 2.65% in water were obtained. Polystyrene with amine (PS-NH\textsubscript{2}) modified
surfaces of 200 nm (actual: 230 nm ±16.1) diameter, 2.5% in water; sulfate (PS-SO₄) modified surfaces of 200 nm (actual: 194 ± 9 nm) diameter, 2.65% in water; and hydroxyl (PS-OH) modified surfaces of 200 nm diameter (actual: 190 ± 16.1 nm), 2.6% in water were also purchased. Ammonium hydroxide (NH₄OH, 29.79%) was purchased from Fisher Scientific. Dimethylformamide (DMF) was purchased from Macron Chemicals. Tetraethyloorthosilicate (TEOS, 98%) and anhydrous isopropanol (IPA) was purchased from Sigma. Deionized water used in all experiments was produced using a Millipore Advantage A10 system with a resistance of 18.2 MΩ.

### 4.3.2 Particle formation

All silica Janus templates were synthesized in 20 mL glass scintillation vials with 700 µL of H₂O, 4 mL of IPA and 1.3 mL of NH₄OH. To this mixture 100 µL of polystyrene spheres and 83 µL of TEOS (60 mM) were simultaneously added while stirring (unless stated otherwise). All solutions were allowed to react for 2 hours prior to reaction termination by centrifugation (unless stated otherwise). Reaction mixtures were centrifuged for 5 min at 500 g; the supernatant was transferred (the pellet discarded) to a fresh 15 mL centrifuge tube and centrifuged at 3221 g for 5 min. The resulting pellet was dispersed in 15 mL of IPA. The particles were washed by centrifugation twice in IPA (at 3221g) prior to being redispersed and washed twice in H₂O (by centrifugation at 3221g).

### 4.3.3 Particle diameter determination

Particle diameters were measured using either dynamic light scattering (DLS) or by averaging 100 measurements from SEM images. A Brookhaven ZetaPlus DLS instrument was used to measure particle dimensions in solution. Each sample was measured five times to obtain an average signal. The largest majority peak is reported.
Histograms were obtained from DLS or SEM measurements and plotted using OriginPro 7.0.

### 4.3.4 Imaging

Samples were sputter coated with palladium to improve imaging using an Emitech K575X Sputter Coater. SEM images were obtained using an FEI XL30 fitted with an FEI Sirion column to enable ultra-high resolution.

### 4.4 Results and Discussion

Janus templates were made by modifying Stöber’s method with addition of carboxylated polystyrene nanoparticles at the start of the reaction. In a solution of ammonium hydroxide, IPA, DI water, TEOS, and PS-COOH, silica condensed around the PS-COOH in an eccentric fashion (Fig. 7.1).

**Figure 4.1**: Janus template synthesis scheme. A one pot aqueous synthesis scheme produces eccentric silica/polystyrene particles (Janus templates). A representative SEM image is shown (middle). Dissolving the polystyrene in DMF leaves an exposed cavity in the silica creating silica nanobowls (bottom right).

An excess of PS-COOH yielded Janus templates with one or more cores embedded in the silica. The cores were removed using an organic solvent like DMF to create a silica nanobowl (an Janus template without the polystyrene core) (Figure 7.1). The actual formation of the Janus templates occurred rather quickly; within the first 15-20
minutes, the reaction transitioned from a translucent solution to an opaque white one. Additional processing time did not affect the morphology of the Janus templates; it only allowed time to solidify the particle and did not cover up the exposed polystyrene. Janus templates were reacted for as long as 18 hr and SEM images revealed no major changes in morphology between any of the reaction times tested (1, 2, 3, 18 hr). (Figure 7.2). However the reaction was usually terminated and washed after 2 hr.

During the washing process, the reaction solution is first spun relatively slowly (500g) to separate out larger Janus template aggregates and enrich the supernatant with smaller, well-formed Janus templates. The supernatant was subsequently centrifuged at higher speeds (3221g) and washed as reported. Comparison of the Janus template aggregates with the Janus template supernatant by DLS showed enrichment of the supernatant with well-formed Janus templates consistent with sizes as observed with

![Figure 4.2](image)

**Figure 4.2:** Janus template formation as function of time. Janus templates produced with 60 mM TEOS after (a) 1 hr, (b) 2 hr, (c) 3 hr, and (d) 18 hr in reaction solution. The polystyrene cores remains partially exposed at all times.
SEM (Figure 4.3).

**Figure 4.3**: Janus template size distribution before and after centrifugation. The particle size distribution of Janus templates found in the supernatant and the pellet of an Janus template reaction after slow centrifugation (500g, 5 minutes) are compared against each other. 60 mM TEOS Janus template sample reacted for 2 hr was used. Smaller well-formed Janus templates were concentrated in the supernatant while larger aggregate Janus templates were left behind in the pellet.

Adjusting the TEOS concentration while holding everything else the same changed how much the silica enveloped the PS-COOH. As the TEOS concentration and silica coverage of the polystyrene increased, so did the particle size as measured by DLS (Figure 7.3a). At low TEOS concentrations (20, 40 mM), half to three quarters of the polystyrene surface was enveloped by the silica (Figure 7.3 b-c). At higher concentrations (80, 100 mM), the silica almost engulfed the polystyrene (Figure 7.3 d-f).

Janus templates may have one or more PS-COOH cores incorporated with the silica. A rough survey of two wide-field SEM images revealed 70-95% of all particles counted were Janus templates (Fig. 4.5). A closer examination of the wide field images in SEM (Fig. 4.5) and TEM (Figure 4.5) also shows a heterogeneous population in regard to the number of cores incorporated into an Janus template. Counting the number of cores embedded in Janus templates also revealed similar results regardless of imaging modality (Figure 4.7). Janus templates embedded with a single PS core represented only 30% of the surveyed population. Janus templates with two cores represented
Figure 4.4: Janus template as a function of TEOS concentration. Janus templates were formed using increasing amounts of TEOS (all other reactants constant) and two trends emerged: i) increasing Janus template diameter with more TEOS and ii) increasing encapsulation of the polystyrene by the silica. The following TEOS concentrations were used to form Janus templates. Each condition’s average Janus template diameter and coefficient of variation were measured with DLS and reported in parentheses. (a) 20 mM (193 nm, 0.22), (b) 40 mM (236 nm, 0.12), (c) 60 mM (244 nm, 0.15), (d) 80 mM (298 nm, 0.18), and (e) 100 mM (539 nm, 0.34) of TEOS. Multiple polystyrene cores may incorporate into an Janus template particle at different times during the reaction. If they incorporate near the beginning of the process (3c and 3e) they can be well encapsulated. However if the polystyrene core incorporates much later in Janus template formation, the cores are poorly encapsulated as evidenced by the darker grey spheres in 3b and 3d.

the largest plurality (40%), and Janus templates with three or more cores made up the remaining 30%. Attempts to improve homogeneity by varying ammonium hydroxide, water, and core concentration individually resulted in similar or worse distributions
Figure 4.5: Wide-field SEM images to determine reaction yield. Wide field SEM images of (a) 20 mM, (b) 60 mM, (c) 80 mM TEOS Janus template samples were used to estimate Janus template reaction yield. A blue "1" identify Janus templates; a green "2" identify non-Janus templates. The 20 mM sample resulted in 71% of 117 particles being Janus templates. The 80 mM sample showed 95% of 95 particles counted to be Janus templates. Uncounted particles are unincorporated polystyrene templates.

(data not shown). Therefore improving the yield of the single core Janus templates will require the changing of two or more reactants simultaneously. It seems reasonable that the formation process is driven by particle surface energies. An ideal recipe leading to the formation of predominately single core Janus templates of this size is no straight forward and requires more investigation. Otherwise, more advanced particle separations techniques are required to isolate single core Janus templates from the current mixture.

Three different sizes of PS-COOH were used to investigate the effect of core size
Figure 4.6: TEM images to evaluate PS inclusion in Janus template distribution. Using wide-field TEM images of 40 mM TEOS Janus templates, the number of polystyrene templates in one Janus template was tallied manually and a population breakdown was found. '1', '2', '3', '4', and '5' indicate the number of templates observable in the counted Janus templates. A total of 529 Janus templates were included in the count.

on Janus template formation. Using 60 mM TEOS with the standard reaction conditions, particles were synthesized with no PS present, 50 nm, 100 nm and 200 nm diameter PS-COOH cores (Figure 7.4). 50 nm and 100 nm PS-COOH cores successfully made Janus template nanoparticles. These Janus templates were smaller on average than a pure
Figure 4.7: Janus template incorporated polystyrene distribution. A pie chart shows the distribution of polystyrene cores embedded within the Janus templates as measured by a) SEM in figure 4.5 and b) TEM in figure 4.6. Both distributions regardless of imaging technique are similar. Single core Janus templates represented ~30% of the population, double core Janus templates were ~40% of the sample, and Janus templates with three or more cores were the remaining ~30%.

silica particle (Figure 7.4e). Attempting to make Janus templates with 200 nm PS-COOH resulted in poor silica encapsulation. From the SEM images, a large percentage of silica attempted to wrap around the PS-COOH unsuccessfully, leaving round indents and heavily agglomerated particles.

It may suggest that the interaction between the silica and the PS-COOH interact during formation with multiple weak molecular bonds. Comparing the diameter of the pure silica particle (350 nm) with the size of the 200 nm PS-COOH, suggests that core size in addition to surface functionality plays an important role in Janus template formation. Successful formation of an Janus template would thus hinge on growing enough silica to physically entrap the core. If the silica particle formed from Stöber’s method alone is not larger than the core by a certain amount, Janus templates will not form properly. Surface functionality of the core particle appeared to play a central role in
Janus template morphology. The addition of 200 nm PS-NH$_2$, PS-OH, or PS-SO$_4$ into a 60 mM TEOS reaction solution yielded particles that were completely encapsulated by silica (Fig. 6.5). This was observed by an increase in the diameter of the resultant particles over the size of the polystyrene cores and by SEM images showing spherical particles with smooth morphology. Therefore, some undetermined interaction between TEOS and PS-COOH enabled the formation of Janus templates which did not occur with polystyrene cores containing other surface functional groups.

In a previous study by Lu et al.[23], silica was grown around polystyrene of different surface functionalities using Stöber’s method. Uniform shells grew around amine-terminated polystyrene while silica islands grew around sulfate terminated polystyrene. The discrepancy between Lu et al. and our study is likely due to the relative amounts of water and ammonium hydroxide used. The previous study used 3.5 mL DI water and 0.5 mL ammonium hydroxide; 6 times more volume of water than ammonium hydroxide. In our study the volume of water used was about half the volume of ammonium hydroxide used. The diameter of silica nanoparticles formed with the Stöber method is highly sensitive to even small changes in reaction conditions[24] and the same is likely true for shell formation.

More insight into Janus template formation was acquired from Chen et al. who were able to create Janus template like structures using carboxylated gold cores and a silica shell[16]. In their study, they functionalized the gold core with mercaptophenylacetic acid (MPAA, a small carboxyl containing compound) and a polystyrene-polyacrylic acid block copolymer (PS-PAA, a large, carboxyl rich polymer). By changing the MPAA:PS-PAA ratio and thus the carboxylic acid surface density, they were able to achieve different types of silica encapsulation. The silica coverage ranged from concentric, to oblong, and finally Janus template like. In our study we believe the manufacturer’s carboxylate polystyrene spheres have a carboxylate surface density such that our process can form
Figure 4.8: Janus template as function of PS core size. Using 60 mM TEOS concentration, Janus template synthesis was attempted using PS-COOH cores of different diameters: (a) 50 nm, (b) 100 nm, (c) 200 nm in diameter, and (d) no core present in reaction. (e) Janus templates made with 50 nm and 100 nm cores show a smaller average diameter when compared to a silica sample made with no cores. No size distribution is reported for Janus template synthesis using 200 nm cores because the silica wrapped poorly around many of the 200 nm cores. They instead agglomerated leaving imprints where the polystyrene spheres once were. Size distributions were obtained by measuring the diameter of 100 distinct particles in SEM wide-field images.

Janus templates. However since the manufacturer does not titrate their polystyrene spheres, this surface density is currently unknown. For a better understanding of the formation process, experiments to monitor the silica formation as a function of carboxyl surface density will be required and are beyond the scope of the work presented here.

Because the Janus templates contain both silica and carboxyl groups on the
same particle, chemical moieties like a targeting agent and a therapeutic can be attached independently in a spatially defined manner. The Janus templates may be of use as a multifunctional delivery platform because it can spatially organize the different chemical moieties on the nanoscale, a feature not commonly found in previous drug delivery systems like nanoliposomes[25] or silica[26, 27]. Alternatively by removing polystyrene the Janus templates become a nanobowl with more surface area and internal carrier space for therapeutic or diagnostic agents. Nanobowls can then be capped with a sphere made of biocompatible materials, including PLGA[28], liposomes[29], and chitosan[30]. For conditional and controlled release of the therapeutic from the bowl, such a cap could be released by specific interaction with DNA[31, 32], enzymatic processes, or environmental triggers like temperature and pH. Existing delivery systems usually have pores or open surfaces that allow passive and/or continuous release of their loads (imaging contrast molecules or therapeutic agents). Our nanobowls can be used for a controlled release of imaging contrast molecules and therapeutic (theranostics) agents.
4.5 Conclusion

The addition of a carboxylate modified polystyrene core added at the beginning of a modified Stöber’s method resulted in polystyrene partially encapsulated in silica. Both the size and carboxylate surface groups of the polystyrene were found to be important in formation of a Janus template. The polystyrene core was dissolved from an organic solvent leaving a nanobowl behind. These Janus templates and nanobowl can be used as a versatile platform for site-specific functionalization or theranostic delivery.

4.6 Acknowledgements

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Chapter 4, in full, is a reprint of the material as it appears in AH Mo, PB Landon, CD Emerson, C Zhang, P Anzenberg, S Akkiraju, and R Lal, “Synthesis of nano-bowls with a Janus template” Nanoscale, 2015,7, 771-775. The dissertation/thesis author was the primary investigator and author of this paper.

Bibliography


Chapter 5

Asymmetric Colloidal Janus Particle Formation is Core Size Dependent

5.1 Abstract

Colloidal particles with asymmetric surface chemistry (Janus particles) have unique bifunctional properties. The size of these particles is an important determinant for their applications in diverse fields from drug delivery to chemical catalysis. The size of Janus particles, with a core surface coated with carboxylate and a partially encapsulating silica shell, depends upon several factors, including the core size and the concentration of carboxylate coating. The role of the carboxylate coating on the Janus particle size is well-understood; however, the role of the core size is not well defined. The role of the carboxylated polystyrene (cPS) core size on the cPS-silica Janus particle morphology (its size and shape) was examined by testing two different silica sizes and five different cPS core sizes. Results from electron microscopy (EM) and dynamic light scattering (DLS) analysis indicate that the composite cPS-silica particle acquires two distinct shapes: (i) when the size of the cPS core is much smaller than the non-cPS silica (b-SiO$_2$) sphere,
partially encapsulated Janus particles are formed, and (ii) when the cPS core is larger than or equal to the b-SiO$_2$ sphere, a raspberry-like structure rather than a Janus particle is formed. The cPS-silica Janus particles of $\sim$100-500 nm size were obtained when the size of the cPS core was much smaller than the non-cPS silica (b-SiO$_2$) sphere. These scalable nanoscale Janus particles will have wide application in a multifunctional delivery platform and catalysis.

5.2 Introduction

Asymmetric colloidal particles with different chemical compositions (Janus particles) have drawn much attention because they form a structure with bifunctional physico-chemical properties [1, 2]. A wide variety of physical [3, 4, 5] and chemical [6, 7, 8, 9, 10] methods have been used to synthesize asymmetric particles. Silica-polymer asymmetric particles have been of great interest [7, 11, 12, 13, 14] because they are relatively inert materials and are suitable templates to build more complicated structures for use in photonics [15], therapeutic delivery [16], and functional devices [17].

A common route of synthesis for Janus particles is using electrostatic assembly[18, 19, 20, 21, 22, 23] and spontaneous formations [6, 7, 8, 9, 10, 24]. This method is frequently used to make hierarchical templates that can then be selectively coated to make asymmetric colloidal structures [18, 19, 20, 21, 22, 23]. Such templates are usually synthesized by mixing in the same solution a core particle of one charge and satellites of the opposite charge. These opposite charged particles electrostatically self-assemble into structures consisting of a central core particle with multiple satellite particles attached to it. Colloidal hierarchical templates have been made of polystyrene [18, 19] and polystyrene with silica [20]. Templates showing controlled assembly with varying core and satellite sizes [21, 22] have been used to create silica particles with anisotropic
Janus particles have also been synthesized through spontaneous chemical interactions via microemulsion, with polystyrene-silica colloids being one of the most commonly made particles using this technique [8, 11, 12, 25, 26, 27]. Recently, we used a sol-gel method to form a composite particle consisting of a silica shell partially encapsulating a carboxylated polystyrene (cPS) core [28]. This morphology has also been demonstrated in Janus particles with gold [6] and iron oxide [7] cores. The common factor in all of these Janus particles is the presence of the carboxylate surface. Because of these commonalities, a greater understanding into the formation of the cPS-silica Janus particle should provide insight into forming other Janus particles with a carboxylated core and partial silica shell more generally.

Current research has focused mainly on the carboxyl surface density of the cores [6], but any encapsulation process will also be affected by the size of the core being encapsulated. The size of non-cPS silica (b-SiO$_2$) particles is known to be self-limited by reactant concentrations [29]. Therefore, any cPS-silica Janus particles made with the same reaction conditions as the b-SiO$_2$ particles cannot have a cPS core larger than that of b-SiO$_2$. Therefore, we hypothesize that cPS-silica Janus particles only form when the cPS core size < b-SiO$_2$ particle. To better understand the silica encapsulation process, we performed a detailed examination of the role of the core size on the morphology (e.g., size and shape) of the cPS-silica Janus particles.

The cPS core size dependence upon the cPS-silica morphology was explored by synthesizing cPS-silica particles with cPS cores of varying sizes (50-1000 nm). To better understand the core size dependence upon cPS-silica particle formation, reaction conditions were selected from the published work [30] to make “small” (180 nm) and “large” (380 nm) b-SiO$_2$ nanoparticles. The reaction conditions required to make a small b-SiO$_2$ and a large b-SiO$_2$ were referred to as the “small recipe” and “large recipe”. The
core size dependence upon the morphology of the cPS-silica particle was evaluated using the size of b-SiO$_2$ nanoparticles as a reference. The particle morphology was evaluated using transmission electron microscopy (TEM), scanning electron microscopy (SEM), and dynamic light scattering (DLS). Without any cPS cores, the previously mentioned recipes created small ($\sim$180 nm) and large ($\sim$380 nm) diameter b-SiO$_2$ nanoparticles. These scalable nanoscale Janus particles potentially have applications in theranostics and catalysis.

5.3 Materials and methods

5.3.1 Materials

Spherical polystyrene with carboxylate (cPS) modified surfaces of 50 nm [coefficient of variation (CV) of 15%] diameter, 2.6% in water; 100 nm (CV of 15%) diameters, 2.62% in water; 200 nm (CV of 8%) diameter, 2.65% in water; and 1000 nm (CV of 3%), 2.5% in water were obtained from Polysciences. The cPS modified surfaces of 400 nm (CV of 2%) diameter, 10% in water were acquired from Seradyn. Ammonium hydroxide (NH$_4$OH, 29.79%) was purchased from Fisher Scientific. Tetraethylorthosilicate (TEOS, 98%) and N,N-dimethylformamide (DMF) was purchased from Sigma. Anhydrous ethanol was purchased from Koptec. Deionized water used in all experiments was produced using a Millipore Advantage A10 system with a resistance of 18.2 M$\Omega$.

5.3.2 Particle formation

Two different size silica particles were created that could be grouped as “small” and “large”. The small particles used 300 $\mu$L of TEOS, 207 $\mu$L of NH$_4$OH, 324 $\mu$L of water, and 5815 $\mu$L of ethanol. The large particles used 227 $\mu$L of TEOS, 1286 $\mu$L of
NH₄OH, 540 µL of water, and 3946 µL of ethanol. Typically, a certain amount of water, ammonium hydroxide, and isopropyl alcohol (IPA) were mixed together and stirred using a magnetic stir bar for 2 min. The reaction volume was kept constant, and the TEOS/PS ratio was varied.

In holding the reaction volume constant, the amount of TEOS was 300 or 227 µL (for the small or large particles) and 100 µL of 50-1000 nm size cPS was added to the solution at the same time. The reaction was allowed to proceed for 2 h before being washed in water 3 times and then resuspended in 2 mL of water.

The TEOS/PS ratio was held constant across all core sizes in both the small and large recipes. Two different ratios (10⁹ and 10¹⁰) and three cPS cores of 200, 400, and 1000 nm were selected for the sake of convenience and a proper reaction yield. To hold TEOS/PS ratios the same, different volumes of cPS had to be used because of differences in cPS stock concentrations. Therefore, all other reactant volumes were scaled as necessary to preserve the proper reactant concentrations.

5.3.3 Particle diameter determination

Particle diameters were measured using DLS as described previously [28]. Briefly, a Brookhaven ZetaPlus DLS instrument was used to measure particle dimensions in solution. Particles were suspended in 1 mL of water and measured in a standard 1 cm disposable cuvette. Using the accompanying acquisition software, each sample was measured 5 times to obtain an average signal. Histograms were obtained from DLS data and plotted using OriginPro 7.0.
5.3.4 Imaging

Samples were sputter-coated with iridium to improve imaging using an Emitech K575X sputter coater as described previously [28]. Briefly, the particle SEM post was placed in the machine and sputtered for 5 s with a plasma voltage of 80 mV. SEM images were obtained using a FEI XL30 fitted with a FEI Sirion column to enable resolution up to 1 nm. TEM samples were prepared on copper grids coated with Formavar and carbon and dried with blotting paper. The samples were imaged in JEOL 1200 EX II TEM at 80 kV.

5.4 Results and Discussion

The formation of cPS-silica Janus particles was first observed after the addition of cPS at the start of a sol-gel reaction [28] (Fig. 7.1). As shown in Fig. 7.1, these particles can be identified by a smooth silica shell with distinct depressions at the surface where the silica meets the cPS. These depressions are easily recognizable in SEM images by the circular mark in the silica.

![Figure 5.1](image.png)

**Figure 5.1:** Janus template synthesis scheme. cPS-silica Janus particles are formed when carboxylated polystyrene particles are added to a silica sol-gel reaction.

Reaction conditions were selected from the published work [30] to make “small” (180 nm) and “large” (380 nm) b-SiO₂ nanoparticles (Fig. 6.7). Different cPS-silica particles were synthesized by adding cPS cores of varying size into small and large recipes.

Size dependence was first investigated by keeping the reaction volume constant.
Four cPS cores of different diameters (50-400 nm) were examined for the small recipe, while five different cPS cores (50-1000 nm) were examined for the large recipe. For the smaller ethanol recipe, cores smaller (50 nm) than the small b-SiO$_2$ formed well-defined Janus particles (Fig. 7.2a). However, when the PS core diameter became similarly sized or much larger than b-SiO$_2$ particles, the silica started to coat the cPS in clumps (Fig. 7.2b). The clumps became greater, and a raspberry-like shell formed as the core sizes increased (panels c and d of Fig. 7.2). The silica completely coated the cPS, leaving the excess TEOS to form pure silica particles surrounding it. The formation of the raspberry shell and Janus particles were also relatively uniform, as seen in the wide-field images in Fig. 7.2. The corresponding DLS distribution also supports this observation for each of the core sizes (Fig. 7.2e).

A similar trend emerged when the cPS core size was varied using the large silica recipe. When 50 nm cores were used, multiple cores appeared to be almost fully encapsulated in a single Janus particle (Fig. 7.3a). When 100 nm cores were used,

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**Figure 5.2**: "Large" and "small" recipe silica. SEM images of bare silica made with Stöoer’s Method for the a) large and b) small recipe. The accompanying DLS measured size distributions for the c) large and d) small recipe.
encapsulation of the cPS by silica is noticeably reduced (Fig. 7.3b). Additionally, cPS-silica Janus particles formed with smaller cPS cores (50-100 nm) but with a larger diameter than corresponding particles in the smaller recipe. Using mid-sized cores (200 and 400 nm) that approached the particle size of large b-SiO₂, a complete silica shell was formed with varying levels of bumps. The 200 nm core showed a complete silica shell with a few defined bumps around the shell (Fig. 7.3c). The 400 nm core showed a greater progression of this trend, as more bumps can be seen forming on the outside of the cPS cores (Fig. 7.3d). When the core is much larger (1000 nm) than b-SiO₂, the bumps appeared to be very pronounced on the silica surface (Fig. 7.3e). Wide-field images (Fig. 5.5) also demonstrate uniformity in the particle formation for each of the core sizes using the large recipe.

**Figure 5.3**: Small silica recipe with different diameter cPS cores. (a) 50 nm, (b) 100 nm, (c) 200 nm, and (d) 400 nm. (Inset) Wide-field images are shown with a more detailed image of cPS-silica particles. The small silica process with the addition of 50 nm core sizes created uniformly sized Janus particles. The addition of 100 nm cores created particles with multiple silica bumps attempting to cover the core. As the cores become larger (200 and 400 nm), silica formed around the larger cores with an even more bumpy shell and excess silica coalesced into smaller particles. (e) Creation of silica shells and excess pure silica particles for larger cPS diameters (c and d) is confirmed with DLS histograms. Smaller core sizes (a and b) demonstrated no such behavior.
Figure 5.4: Large silica recipe with cPS cores of increasing diameter. (a) 50 nm, (b) 100 nm, (c) 200 nm, (d) 400 nm, and (e) 1000 nm. The 50 and 100 nm core sizes correspond with partial encapsulation around the core. The medium-sized core (200 nm) demonstrates formation of raspberry-like shells. The much larger cores (400 and 1000 nm) show the cPS cores covered with raspberry-like shells with larger clumps of silica than the shell formed with the small silica processes. Corresponding DLS histograms reveal the formation of larger structures and confirmed smaller silica particles as well.

Figure 5.5: Wide field EM images of large recipe silica-cPS particles under constant volume. Wide field images of volume constant samples using the large recipe with a) 50 nm, b) 100 nm, c) 200 nm, d) 400 nm, e) 1000 nm carboxylate polystyrene cores.

For the conditions that formed Janus particles, yields for such particles were found by tallying Janus and non-Janus particles in wide-field SEM images. The 50 and 100 nm cPS with the large recipe (panels a and b of Fig. 5.6) and the 50 nm cPS with the small recipe (Fig. 5.6c) demonstrated Janus particle formation. The 50 and 100 nm cPS with the large recipe both had yields of 42%, while the 50 nm cPS with the small recipe had a yield of 70% (Fig. 5.6d). The lower incorporation rate of cPS into silica particles formed with the large recipe may be due to the higher concentration of ammonium hydroxide than the small recipe. Ammonium hydroxide acts as a catalyst during the sol-gel process and speeds up the growth of the silica particles. (31) This accelerated rate of growth may account for fewer cPS becoming embedded in the silica.
The internal structure of formed Janus particles is revealed in TEM (Fig. 5.9). To obtain better contrast on the structure, cPS particles were dissolved from Janus particles using DMF. The DMF-treated particles were then washed in water and placed on TEM grids for imaging. All conditions that formed Janus particles show lighter circular impressions in the silica particle body where the cPS used to be. Janus particles formed with 100 and 50 nm cPS cores in the large recipe (panels a and b of Fig. 5.9, respectively) showed multiple circular impressions where multiple cPS cores incorporated into a single Janus particle. The small recipe with 50 nm cPS cores (Fig. 5.9c) on the other hand only showed one circular impression, indicating the incorporation of only one cPS core in a Janus particle.

The formation of raspberry-like shells (cPS core > b-SiO₂ particle) in panels b-d of Fig. 7.2 and panels c-e of Fig. 7.3 could be a result of different TEOS/cPS ratios instead of the core size. To account for this possibility, the ratio of TEOS/cPS was held constant for different core sizes made with the same recipe. For the small recipe (i.e., the condition to make the small b-SiO₂ particle), the TEOS/PS ratio was held at 10¹⁰.
(panels a-c of Fig. 7.4) and $10^9$ (panels d-f of Fig. 7.4) using cPS cores with diameters of 200 nm (panels a and d of Fig. 7.4), 400 nm (panels b and e of Fig. 7.4), and 1000 nm (panels c and f of Fig. 7.4). From these SEM images, raspberry-like silica shells coated all cPS cores and any excess TEOS in solution formed silica nanoparticles. Changing the TEOS/PS ratio did not affect the overall morphology of the shells. Furthermore, at the same TEOS/PS ratio, the shell developed more noticeable bumps as well as a greater number of bumps as the core size increases.

Using the large recipe (i.e., the condition to make the large b-SiO$_2$ particle), the TEOS/PS ratio was held at $10^{10}$ (panels a-c of Fig. 6.5) and $10^9$ (panels d-f of Fig. 6.5), using cPS cores with diameters of 200 nm (panels a and d of Fig. 6.5), 400 nm (panels b and e of Fig. 6.5), and 1000 nm (panels c and f of Fig. 6.5). Similar to Fig. 7.4, raspberry-like shells coated all cPS cores and excess TEOS formed silica nanoparticles as well. These results suggest that, in general, changing the TEOS/PS ratio does not greatly affect the overall morphology of the raspberry shells. Also, the shell developed more numerous bumps as the core size increased. However, in comparison of samples

![Figure 5.7](image_url): Silica-cPS particle morphology as function of PS core size with small recipe. Small silica process PS/silica composites created by varying the TEOS/PS ratio at (a-c) $10^{10}$ and (d-f) $10^9$. As the core size increases from (a and d) 200 nm, (b and e) 400 nm, to (c and f) 1000 nm, the bumps are more noticeable and numerous than the larger silica process. It appears that, as the core size increases, the silica shell begins to smoothen out (c) or coalesce (f) depending upon the TEOS/PS ratio (a-e). The excess silica coalesces into smaller silica particles and is noticeable as smaller spheres.
Figure 5.8: Silica-cPS particle morphology as function of PS core size with large recipe. Large silica process cPS-silica composites created by varying the TEOS/PS ratio at (a-c) $10^{10}$ and (d-f) $10^9$. Silica-encapsulated cPS is distinguishable from solid silica particles by their larger diameter and multiple bumps on the surface. As the core size increases from (a and d) 200 nm, (b and e) 400 nm, to (c and f) 1000 nm, the bumps become more noticeable and numerous to the point where they may not completely cover the surface of the particles.

with the same TEOS/PS ratio and cPS core size between the small (i.e., the condition to make the small b-SiO$_2$ particle) and large (i.e., the condition to make the large b-SiO$_2$ particle) recipes (e.g., Fig. 7.4a versus Fig. 6.5a), the individual bumps on the silica shell were reduced in size using the small recipe compared to the large recipe.

To confirm the coating of silica on top of cPS, the cPS was dissolved by placing cPS-silica samples (made with 200, 400, and 1000 nm cores using the large recipe) in DMF. The subsequent particles were then imaged by TEM (Fig. 5.6). These dissolved samples showed a contrast difference between the edge and the center of the particle, indicating the successful removal of cPS. Smaller particles in the images show a single contrast and, thus, are most likely pure silica particles. The TEM data (Fig. 5.6) corroborates the overall morphology as observed with SEM (Fig. 6.5).

On the basis of the above observations, we believe that the following model can be used to explain the effect of the core in Janus particle formation (Figure 6a). When the core size is much smaller than the b-SiO$_2$ particle size, Janus particles will form. As the core and b-SiO$_2$ diameter approach the size of each other, a raspberry-like shell
Figure 5.9: cPS dissolved from Janus templates. Internal structure of Janus particles after removal of polystyrene by DMF of a) 100 nm polystyrene with the large recipe, b) 50 nm polystyrene with the large recipe, and c) 50 nm polystyrene with the small recipe.

will form around the cPS with only a few bumps. When the core is much larger than b-SiO$_2$, the TEOS will form a raspberry-like shell with numerous rounded protrusions around the PS core. The larger the size difference between the core and b-SiO$_2$, the more pronounced and numerous the bumps. Additionally, any excess TEOS that did not incorporate into a raspberry shell then goes into forming silica nanoparticles similar to the size of b-SiO$_2$. If the cPS core diameter is kept the same and the TEOS/PS ratio is increased, an increasingly bumpy silica shell is formed that continues to coalesce together to form a smoother shell (Figure 6b).

The partial encapsulation of a carboxylate-modified gold nanoparticle with silica

Figure 5.10: cPS dissolved from silica/PS particles. TEM images of silica shells post THF removal of the polystyrene core with a diameter a) 200 nm, b) 400 nm, and c) 1000 nm.
Figure 5.11: cPS-silica morphology model. (a) cPS-silica Janus particles form when the diameter of the cPS is much smaller than the silica. When the diameter of the cPS is about the same as the silica, multiple silica bumps attempt to form on a single core and a shell of these silica bumps begin to dominate. (b) When the PS core size is much larger than the silica, bumps of silica form on the surface of the core until the shell become so numerous the surface is smooth.

using the sol-gel method was also demonstrated by Chen et al.[6]. The carboxylate surface density on the gold was varied to control the degree of silica encapsulation around the gold [6].(6) A similar partial encapsulation by silica around a core particle was demonstrated by Feyen et al [7]. The silica partially wrapped around an asymmetric iron oxide/cPS core, wherein the iron oxide surface was carboxylate-modified, while the cPS was not. While the authors did not report the carboxylate surface density, it was enough to form a partial silica shell [6]. The work by Chen et al.[6] and Feyen et al. [7] did not investigate the role of core size and b-SiO₂ size in the partial encapsulation process.

The control over encapsulation demonstrated by Chen et al[6]. and core size dependence described in this paper suggest that partial encapsulation is driven by a wetting process. Data from these studies lead us to hypothesize that, as TEOS condenses in the presence of carboxylated particles, new silica particles would much prefer to nucleate on another surface as opposed to on their own. However, condensing TEOS (precursor for forming silica) is not able to readily wet the cPS, possibly because of the charge repulsion. This results in a range of carboxyl densities and core sizes where a
carboxylated core becomes partially wetted by a first layer of silica and the silica shell begins to grow around it. If the core is much larger, one silica droplet is not able to encapsulate the particle. Therefore, multiple silica droplets attach to the surface and grow into a raspberry-like shell.

Understanding this process opens the door to creating Janus particles of different sizes. The Janus particles may be of use as a multifunctional delivery platform because one can selectively attach different moieties to the silica and cPS. These are features not commonly found in previous drug delivery systems, like nanoliposomes [31] or silica [32, 33]. Additionally, removal of the cPS could turn Janus particles into nanobowls with greater carrier volume and surface area. The bowls could then potentially carry therapeutic or diagnostic agents and be capped with biocompatible materials, including chitosan, poly(lactic-co-glycolic acid) (PLGA), and liposomes [34, 35, 36].

5.5 Conclusion

The role of the cPS core size on the cPS-silica Janus particle morphology was examined, including its size and shape. We tested two different silica sizes and five different cPS core sizes. Results from electron microscopy (EM) and DLS analysis indicate that the cPS-silica particle acquires two distinct shapes: (i) when the cPS core size was much smaller than that of b-SiO$_2$, the cPS-silica particles formed Janus particles, and (ii) cPS cores with a diameter greater than or equal to b-SiO$_2$ formed raspberry-like silica shells. The size and coverage of the bumps in the silica shells were determined by the core size, recipe reactant concentrations, and TEOS/PS ratio. The change in morphology from Janus particles to raspberry-like shells as the core size increased was believed to be due to TEOS wetting of the cPS surface. Understanding the factors that determine the growth of a silica shell around a carboxylated core revealed that they can
vary the morphology of a cPS-silica particle. Such knowledge is of use in developing nanoscale chemistries for applications, including multifunctional theranostic delivery.

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Bibliography


Chapter 6

Magnetically responsive Silica-gold Nanobowls for targeted delivery and SERS-based sensing

6.1 Abstract

Composite colloidal structures with multi-functional properties have wide applications in targeted delivery of therapeutics and imaging contrast molecules and high-throughput molecular bio-sensing. We have constructed a multifunctional composite magnetic nanobowl using bottom-up approach on an asymmetric silica/polystyrene Janus template consisting of a silica shell around a partially exposed polystyrene core. The nanobowl consists of a silica bowl and a gold exterior shell with iron oxide magnetic nanoparticles sandwiched between the silica and gold shells. Nanobowls were characterized by electron microscopy, magnetometry, UV-vis and IR spectroscopy. Magnetically vectored transport of these nanobowls was ascertained by time-lapsed imaging of their flow in fluid through a porous hydrogel under a defined magnetic field. These
magnetically-responsive nanobowls show distinct surface enhanced Raman spectroscopy (SERS) imaging capability. PEGylated magnetically-responsive nanobowls show size-dependent cellular uptake in-vitro.

6.2 Introduction

The ability to create colloidal structures with control over their size, geometrical architecture, material composition, and surface chemical functionalities are of immense interest. Such structures have potential applications in photonic[1], therapeutic drug delivery[2] and functional devices[3]. Composite colloids are of interest because two or more different materials are co-localized onto the same particle and their properties can be used in a complementary or synergistic fashion [4, 5, 6]. Composite colloidal structures have been synthesized using various physical[7, 8, 9, 10, 11, 12, 13] and chemical [14, 15, 16, 17, 18, 19] methods. Composite colloids consisting of silica and different polymers are currently of great interest, because they can be readily synthesized [15, 20, 21, 22, 23], consist of relatively inert materials, and are well suited as templates for the bottom-up fabrication of more intricate architecture such as core-shell nanostructures.

The bottom-up approach has been used to create a three layer silica-iron oxide-gold magnetic bowls by attaching magnetite iron oxide and gold nanoparticles to a silica core, followed by growth of gold shell over the silica [24, 25]. Also, hierarchical colloidal templates with a pollen-like shape have been used to create silica whiffle balls[26] and gold-silica golf balls [27]. Colloidal templates were created by electrostatic absorbance of smaller spheres (satellites) to a larger spherical core [26, 27, 28, 29, 30, 31] and then the desired materials were selectively grown on the core of these templates. Also, asymmetric silica/polystyrene colloids have been synthesized that contains a polystyrene shell around a partially exposed silica core [16, 20, 32] or a silica shell around a partially
exposed polystyrene core [21, 33, 34].

In the present study, we have used an emulsion-free method[35] to create a multifunctional nanobowl. Starting with a template of silica with partially exposed polystyrene core, we added magnetic iron oxide nanoparticles and gold on top of the template to create a composite nanobowl with a gold exterior, a silica bowl interior, and iron oxide particles sandwiched in the middle. Nanobowl morphology and functional properties were evaluated by electron microscopy, AFM, magnetometry, and spectroscopy (UV-vis, IR, Raman). Using a defined magnetic field, the transport of nanobowls through a porous hydrogel was monitored at different time points. The biosensing and drug delivery potential of the nanobowls was evaluated through surface-enhanced Raman scattering (SERS) detection of rhodamine B and size-dependent cellular uptake of nanobowls in vitro.

6.3 Materials and methods

6.3.1 Materials

Carboxylate modified polystyrene (PS-COOH) spheres (100 nm 2.7% in water) were purchased from PolySciences. Tetrakis(hydroxymethyl) phosphonium chloride(THPC, 80% in water) sodium hydroxide(NaOH,10M), and chloroauric acid trihydrate (HAuCl₄ • 3H₂O 99.9%) were purchased from Aldrich. Potassium carbonate (K₂CO₃), formaldehyde (37%) and ammonium hydroxide (NH₃OH, 29.79%) were purchased from Fisher Scientific. Anhydrous Ethyl alcohol (EtOH) was purchased from JT Baker. (3-Aminoethylamino)propyl-trimethoxysilane (AEAPTMS), anhydrous isopropanol, tetrahydrofuran (THF), O-(2-Mercaptoethyl)-O-methylpolyethylene glycol (10 kDa, mPEG-SH) was purchased from Sigma. Deionized (DI) water used in samples was produced using a Millipore Advantage A10 system with a resistance of 18.2 MΩ.
Phosphate buffered saline (PBS, 1x) was purchased from Corning.

### 6.3.2 Janus template formation

In a 250 mL glass vial with a magnetic stir bar, we added, in order, 7 mL DI water, 40 mL isopropanol alcohol, and 13 mL of ammonium hydroxide. We then added 40 mM TEOS (550µL) and 1 mL PS-COOH spheres at the same time while the solution stirring and allowed to stir for 2 hrs. The actual formation of the Janus template happened rather quickly; within the first 15-20 minutes of the reaction. The solution was then transferred to a centrifuge tube and centrifuged for 5 min @ 500 g to separate out agglomerated particles from lighter particles. The pellet was disposed of while the supernatant was transferred to a fresh centrifuge tube and centrifuged again at 3221 g for 15 minutes to settle the single particles. The supernatant was washed twice in 30 mL DI water and resuspended in 20 mL of anhydrous ethanol.

### 6.3.3 Preparation of gold seeds

In 54 ml of water 50µL of 10M sodium hydroxide was added and stirred well. In a separate container, 1 ml of water and 12µL of 80% THPC were added to the original solution and the reaction was stirred for 5 min before 1.5 ml of chloroauric acid (10 mg/mL) was added. The solution was stirred for another 30 min at room temperature and incubated at 4°C for 24 hours before use.

### 6.3.4 Gold plating solution

50 mg of K₂CO₃ was dissolved in 47 mL of DI water and stirred for 5 minutes. 3 mL of chloroauric acid (10 mg/mL) was then added to the stirring solution. The gold solution was initially light yellow and became clear after 30 min of stirring. Afterwards,
the gold hydroxide solution was incubated for 24 hr at 4°C before use.

6.3.5 Janus template surface modification

Asymmetric Janus templates were functionalized with amines by placing Janus templates into a solution containing 20 mL of anhydrous ethanol and 40µL of AEAPTMS (final: 0.2 % v/v). The solution was stirred at 60°C for 2 hrs. The amine functionalized Janus templates were washed 3 times in ethanol and resuspended in 5 mL of anhydrous ethanol. The particles are the transferred to a glass vial and dried under vacuum under mild heating overnight.

6.3.6 Attachment of iron oxide and gold nanoparticles to Janus templates

5 mg of dried amine modified Janus templates were weighed out and dispersed 1 mL of water with sonication as needed. 200µL of 10 nm iron oxide solution was added to 1 mL of THPC-gold seed solution. The pH was adjusted to 8 by using sodium hydroxide or hydrochloric acid before adding the 1 mL of amine modified Janus templates into solution. The combined solution was allowed to tumble overnight, allowing proper time for attachment gold and iron oxide nanoparticles. The attached particles were then washed 3 times in water and a dark red pellet was redispersed in 2 mL of water.

6.3.7 Gold plating

Gold and iron oxide seeded Janus templates (1 mg) were added into well-stirred solution of gold plating solution (5 mL), deionized water (4 mL) and 10 mM sodium citrate (1 mL). Then 26µL of 37% formaldehyde was added to the solution and stirred for approximately 5 minutes. The solution proceeded from clear to pink to a greenish-blue
during this time. The final solution had a dark green tint. Gold plated templates were then washed once and centrifuged at 2000g for 10 minutes before being resuspended in 1 mL of water.

6.3.8 **PEGylation protocol**

50 mg of mPEG-SH (10 kDa) was dissolved in 1 mL of water and added to a 1 mL solution of gold shell Janus templates. The solution was allowed to tumble overnight before washing twice at 2000 g for 10 minutes.

6.3.9 **Nanobowl Formation**

Nanobowls were formed by dissolving the exposed polystyrene in gold plated templates. The 2 mL of gold was centrifuged and re-suspended in 2 mL of ethanol before being transferred into a glass centrifuge vial containing 10 mL of THF. The solution was allowed to stir overnight before washing and re-suspension in 2 mL of water. Synthesis of gelatin-methacrylate-co-N-acryloyl 6-aminocaproic acid (GelMA-co-A6ACA) porous hydrogels: Gelatin-methacrylate (GelMA) and N-acryloyl 6-aminocaproic acid (A6ACA) were synthesized as previously described 56, 57. GelMA-co-A6ACA porous hydrogels were prepared by using polymethylmethacrylate (PMMA) porogen leaching method. Cylindrical polypropylene mold was filled with 65 mg of PMMA microbeads (Bangs Laboratories, diameter: 165µm). 60µl of a precursor solution containing 15% (w/v) GelMA and 9.25% (w/v) A6ACA as well as a photoinitiator of 0.3% (w/v) Irgacure 2959 was cast into the PMMA-packed mold and photopolymerized under 365 nm UV light for 10 min. The porous hydrogels were obtained by dissolving PMMA microspheres in acetone for 3 days. The porous hydrogels were equilibrated in PBS and cut into a cylindrical shape with diameter and thickness of 5 mm and 2 mm, respectively, prior to
the introduction of magnetic nanobowls.

6.3.10 FTIR

Fourier transform infra-red measurements were carried out with an Alpha series (Bruker) FTIR spectrometer equipped with a single reflection attenuated total reflectance module. Colloidal suspensions were dried under vacuum and the resulting sample was placed in the FTIR. 32 signals were acquired in transmittance mode and averaged to produce a spectrum.

6.3.11 SEM

SEM samples were prepared by spreading out 20µL of sample into four to five spots on an aluminum SEM post and allowed to air-dry. Images were taken on a FEI XL30 with an FEI Sirion column to enable higher resolution with an accelerating voltage of 15 kV. SEM imaging was carried out to examine a porous structure of the freeze-dried hydrogels. The pore diameter of the hydrogels in the dried state was estimated from 10 pores of each of three SEM images (n=30) using ImageJ. The data are shown as mean ± standard errors.

6.3.12 TEM

Samples were prepared by placing a holey carbon coated TEM grid on a sheet of blotting paper. 20µL of sample was added to the grid and allowed to sit for 15 min to allow the sample to adsorb. The sample was quickly dried using another sheet of blotting paper. The TEM grid was allowed to dry before imaging. The TEM grids were loaded into the sample holder of a JEOL 1200 EX II TEM operating at 60 kV and images were acquired using a Gatan Orius 600 digital camera. Vibrating Sample Magnetometer:
The sample was dried in glass vial under vacuum and placed in a vibrating sample magnetometer (Quantum Design) to measure the magnetic behavior at 300 K.

6.3.13 SERS measurement

Raman spectra were obtained using a Jobin Yvon/HORIBA LabRam ARAMIS Raman spectrometer integrated with a confocal microscope. Raman Spectra were collected in the range from 600 to 1700 cm⁻¹ with an accumulation time of 30 seconds using a 785 diode laser. AFM Nanoparticles characterization: A multimode Nanoscope (Vecco Instruments) was used in tapping mode to characterize the magnetic nanobowls under ambient conditions, using a cantilever with a spring constant of 48 N/m. A clean mica substrate was coated with a drop of Poly-l-Lysine (0.01%) for 2 minutes and rinsed with ultrapure distilled water, and dried. 50 mg of nanobowls was deposited in the mica substrate during 15 minutes at room temperature. Samples were washed with ultrapure water and dried. AFM images were recorded in 50, 20 and 3μm scan areas for the particles size distribution.

6.3.14 Visualization of the in vitro uptake of magnetically-responsive nanobowls after incubation with human WPMY-1 cells

Normal prostate human cells (WPMY-1) were obtained from the American Type Culture Collection (ATCC CRL-285, Manassas, VA, USA). WPMY-1 cells were cultured using Dulbecco’s Modified Eagle’s Medium (DMEM) containing 5% of fetal bovine serum (FBS) at 37°C in humidified atmosphere of 5% of CO₂. Cells were grown in 60 mm petri dishes to reach a confluence of 70-80%. WPMY-1 was treated with GNBs coated with Polyethylene glycol (PEG) in a Phosphate-buffered saline (PBS) solution (0.001 mg/ml). After 2 hours of incubation, cells were washed with PBS, further fixed
using 0.4% glutaraldehyde for 5 minutes, and washed with PBST (PBS with 5% of Tween-20) solution three times. 3 ml of PBS was added. Atomic force microscopy (AFM) images were obtained using a Bioscope AFM integrated with a Zeiss Axiovert 135TV inverted light microscope, using the Nanoscope3 software. Imaging of WPMY-1 treated and untreated cells was performed in PBS using the contact mode AFM. Cantilevers with nominal spring constant (k) of 0.02 N/m were utilized for imaging height and deflection modes. Images in scan area of 50 and 15 mm were recorded in 512 pixels format.

6.4 Results and Discussion

Gold shell magnetic nanobowls were created by using a multistep process summarized in Fig. 1. The process started with the synthesis of a silica/polystyrene Janus template using Stöber’s method for silica particle formation modified with the addition of carboxylate polystyrene nanoparticles. The silica in the templates was then functionalized with an amino alkoxy silane to render the silica surface positively charged. An outer shell was grown around the template in a two-step process. The template was first seeded with negatively charged iron oxide (IONP) and gold (AuNP) nanoparticles. Then a gold shell was grown by reducing seeded templates in a gold chloride solution. The gold surface was modified with polyethylene glycol to keep the particles well-dispersed. Finally, the gold-covered templates were turned into magnetically-responsive bowls by dissolving the exposed polystyrene in organic solvent.

In order to confirm the effectiveness of every step in the bowl synthesis process, changes in morphology at each step was visualized by electron microscopy imaging. The first step consisted of an one-pot reaction to form the silica/polystyrene Janus templates (Fig. 2A). Modification of the Janus templates by an (3-aminoethylamino)propyltrimethoxysilane (AEAPTMS) resulted in a positive surface charge, typically +20-30
Figure 6.1: Schematic of magnetic gold nanobowl formation. A silica/polystyrene template is amine-modified and seeded with small gold and iron oxide nanoparticles. Later, a gold shell is grown in a plating solution, filling in the spaces between the gold nanoparticles. The magnetic gold bowl is finished by dissolving away the polystyrene in organic solvent.

mV as measured by zeta potential (data not shown). This allowed for the attachment of negatively charged 15 nm-sized IONP and 3-5 nm-sized AuNP. IONPs were modified by the manufacturer (Ocean Nano, see Experimental for more details) with a strong negative surface charge. AuNPs were synthesized from the reduction of chloroaauric acid (HAuCl4) with tetrakis(hydroxymethyl)phosphonium chloride (THPC) in aqueous sodium hydroxide solution (pH >10). TEM images of well-washed IONP-AuNP coated templates show dense surface coverage of both the larger IONPs and the smaller AuNPs (Fig. 2B).

A combination of nanoparticle attachment order and pH played a critical role in attaining dispersed and densely attached templates. In order for the seeded templates to remain dispersed in solution, a rapid reversal of charge is required when using electrostatic assembly methods. Amine modified templates would often aggregate when solely in the presence of IONPs. Manufacturer supplied IONPs were at a sub-saturation concentration and thus neutralized the surface change (zeta potential typically, -10 to +10 mV) rather than completely reversing it. We reasoned that it was necessary to perform seeding with both IONP and gold at the same time, because the gold seeding concentration was sufficiently high to reverse the surface charge and stabilize the seeded templates. By adjusting the ratio of IONPs and Janus templates, the amount of IONPs on the
Figure 6.2: Electron microscopy images of the synthesis process. A) Janus template formation, B) gold and iron oxide nanoparticle attachment on silica, C) gold shell formation, and D) removal of polystyrene core. E) Gold plated particles in solution. F) Magnetic gold/silica particles in solution attracted to magnet placed outside the container.

Janus template can be further increased if needed. Another important factor in our nanobowl synthesis was pH, because the gold seeding solution was synthesized in basic pH condition and remained at highly basic (pH>9). At such high pH values, aminated templates were generally neutral due to deprotonation of the majority of amines on the silica surface. Addition of IONPs into the seeding solution did not lower the pH significantly and attempts to seed in these conditions resulted in heavy agglomeration. Adjustment of the seeding solution to pH 7-8 with the addition of small amounts of 10 mM HCl to the IONP/gold solution resulted in non-agglomerated templates and successful seeding of both IONP and gold as evident in TEM image (Fig. 2B).
A complete shell was formed by suspending the IONP-AuNP coated templates in a HAuCl4 plating solution and reducing the gold onto the templates (Fig. 2C). After formation of the gold shell, the gold-plated Janus template was suspended in thiolated polyethylene glycol (PEG) solution for 24 hours and then transferred to a solution of THF for another 24 hr to dissolve polystyrene (Fig. 2D). The composite magnetically-responsive nanobowls are a deep teal color when suspended in solution (Fig. 2E) and were magnetically attracted to the side wall of the container (Fig. 2F).

Figure 6.3: FTIR of amine-modified Janus template. FTIR spectra shows the presence of peaks corresponding to amine (1500 and 3600 cm\(^{-1}\)), siloxane bonds (1100 cm\(^{-1}\)), and silyl hydride bonds (2100 cm\(^{-1}\)).

Various characterization modalities were used to confirm the completion of different steps of the synthesis process. After the Janus templates were formed, the surface of the Janus templates was modified with AEAPTMS and confirmed by FTIR analysis (Fig. 3). The FTIR spectrum showed peaks that correspond to primary amines at 1500 and 3600 cm\(^{-1}\). In addition, the siloxane and silylhydride bonds were seen more prominently at 1100 and 2100 cm\(^{-1}\), respectively. The absorbance measurements of the gold nanobowls showed the formation of absorbance peak at 840 nm, indicative of a gold shell around a silica core [36] (Fig. 4A). In addition, the magnetic hysteresis of the particles was measured with a vibrating sample magnetometer and the particles
were found to be slightly ferromagnetic (Fig. 4B). The saturating magnetization for the magnetic gold shell nanobowls was between 0.4-0.6 emu/g. The 15 nm IONPs were usually superparamagnetic at the 15 nm size, but in this particular case, the composite particles appeared to have retained some ferromagnetic character with a noticeable hysteresis.

![Magnetic Hysteresis](image1)

**Figure 6.4**: Magnetic hysteresis and UV-vis of shell. A) The magnetic hysteresis of the particles indicates that the composite particle retains some ferromagnetic character despite using 15 nm-sized ferromagnetic particles. B) UV-Vis spectrum of the gold plated nanobowls. The peak at the wavelength of 840 nm is indicative of successful gold shell formation.

Wide-field SEM images and DLS of the gold-plated Janus templates and nanobowls showed the monodispersity of the nanobowls (Fig. 5). The PEGylated gold plated templates showed individual particles formed a complete gold shell over the external silica
surface (Fig. 5A). After removal of polystyrene by THF, dark holes in the gold shells appeared indicating removal of the polystyrene and no noticeable changes in dispersity were noted (Fig. 5B). DLS particle sizing before THF revealed two peaks at 266 nm and 669 nm of the sample (Fig. 5C, green). Particle sizing after THF (Fig. 5C, red) showed a slight increase in diameter of the two peaks (279 nm, 765 nm) when compared to pre-THF measurement (Fig. 5C, green). The 266 and 279 nm peaks in both curves of Fig. 5C represented approximately 80% of the population and matched well with the diameter of individual nanobowls in SEM images (Fig. 5A-B). The larger diameter peaks corresponded to some aggregates that were also visible in SEM images of pre-THF gold Janus templates (Fig. 5A) and post-THF nanobowls (Fig. 5B). A paired two-sample t-test on the two DLS curves showed that the two populations are not statistically different (p<0.05) from each other.

Figure 6.5: Wide field images of PEGylated magnetic gold-silica A) Before and B) after removal of the polystyrene with accompanying C) DLS data for before (solid red, 266 nm, and 669 nm) and after removal of polystyrene (dotted green, 279 nm, 765 nm) in THF.

We then verified the ability of the nanobowls for directed transport under a magnetic field by acquiring time course images of their passage through porous hydrogels in PBS under a permanent magnetic field over a span of 52 hr (Fig. 6). A cylindrically shaped gelatin-methacrylate-co-N-acryloyl 6-aminocaproic acid hydrogel with approximately 50 micron-sized pores (Fig S1) was laid on its side in a glass vial containing PEGylated nanobowls in PBS and a strong rare earth magnet at one end of the glass vial. PEGylated magnetically-responsive nanobowls over the time course were attracted
toward the magnet and the infiltration of the particles in the hydrogel was visualized as teal tint within a previously clear gel. The gel became progressively more teal colored over the time (Fig. 6A-E). On closer examination, a front of particles was seen infiltrating the porous hydrogel from the right to the left side (toward the magnet) and becoming more diffuse in the gel as time progressed (Fig. 6F-H). This suggests a potential use of the nanobowls for magnetically controlled delivery through soft tissues. An arrow in Fig. 6 F-H indicates the direction and movement of nanobowls towards the left side of each image.

We examined the application of gold/silica nanobowls as SERS platform for the detection of specific molecules. The SERS activity of our magnetically-responsive nanobowls was tested using Rhodamine B (Rho B) as a probe molecule. The Raman
Figure 6.7: GelMA-co-A6ACA microstructure. SEM images revealed that GelMA-co-A6ACA porous hydrogels contain interconnected pores. The pore diameter of the hydrogels was determined to be $46.7 \pm 2.8$ µm in their dried state.

Enhancement effect can be maximized when the frequency of the excitation laser approach the resonance frequency of the localized surface plasmon in metallic nanoparticles[37]. In Fig. 4, our nanobowls demonstrated a maximum absorption peak at 840 nm. Based on this, we used a 785 nm laser for Raman excitation. Fig. 7 showed the Raman spectra of Rho B with and without gold/silica nanobowls. The Raman signal of the Rho B in the presence of the nanobowls (Fig. 7C) was found to be superior by several orders of magnitude. We identified each peak in the measured Raman spectrum and found they matched well with known Raman spectra of Rho B (Table S1).

In vitro cellular uptake of nanobowls was evaluated by atomic force microscopy (AFM)[38, 39]. For the in vitro uptake analysis, WPMY-1 cells were incubated with PEGylated-gold nanobowls for 2 hours. PEG is usually used to reduce the macrophage uptake and prolong nanoparticles circulation half-life [40]. Earlier studies suggested that PEGylation increased nanoparticle stability and improved their cellular biocompatibility [41, 42]. As seen in AFM images (Fig. 8), after 2 hours of incubation, the magnetic nanobowls were distributed heterogeneously into the cytoplasm in a size-dependent manner. Larger nanobowls were located on the cell periphery (Fig. 8C, blue circles), whereas smaller nanobowls appeared to be endocytosed into the cytoplasm (Fig. 8C,
black circles). These results are consistent with previous observations that endocytosis of colloids is primarily related to size, shape and surface properties of nanostructure [43, 44]. AFM images of WPMY-1 cells treated with magnetic gold nanobowls show the presence of spots in the treated cells (Fig. 8D). The diameter of the nanobowls (denoted by spots in Fig. 8D) located inside the cells is $274 \pm 3$ nm, similar to the size obtained from AFM of the magnetic gold nanobowls by themselves (Fig. 9) and the size distribution of magnetic gold nanobowls in DLS (Fig. 5). Typically, a total of 43 spherical nanoparticles per cell were observed. No significant changes in the normal morphology of the WPMY-1 cells or visible nanoparticles aggregation were observed after 2 hours of incubation of magnetic nanobowls in a 0.001 $\mu$g/ml dilution. The presence of larger (275 nm diameter) nanobowls in the cytoplasm suggests that even large nanobowls can eventually be endocytosed. In addition we have demonstrated previously that the Janus template diameter can be controlled[45] and correspondingly the nanobowls can also be controlled as well. The mechanism of such cellular uptake, though important is beyond the scope of this manuscript.

Although not studied in this work, nanobowls can be used as a drug delivery

**Figure 6.8**: Raman spectra of nanobowls. A) Raman Spectra of magnetically-responsive nanobowls. B) Raman spectra of 0.1 M Rhodamine B in aqueous solution. C) SERS spectra of 1 $\mu$M Rhodamine on gold/silica nanobowls, 150 mW of 785 nm excitation, 30 s acquisition.
Figure 6.9: Magnetic Gold Nanobowls uptake by WPMY-1. WPMY-1 cell morphology imaged by AFM contact mode in PBS for controls in scan areas of 50µm A) and 15µm B), as well as C-D) after the uptake of nanobowls. Magnetic gold nanobowls in different sizes are non-uniformly distributed throughout the cytoplasm. Smallest size nanoparticles (274 ± 3 nm) are located inside the cell (black circles), whereas largest nanoparticles did not enter the cells or remain in the periphery (blue circles). All images were recorded in AFM contact mode in PBS solution.

vehicle by putting a biocompatible cap on the bowl, including liposomes [46], chitosan [47], and PLGA [48]. For conditional and controlled release of the therapeutic agent from the bowl, such a cap could be released by specific interaction with nucleic acid molecules, enzymatic processes, or environmental triggers like temperature and pH. Existing delivery systems usually have pores or open surfaces that allow passive and/or continuous release of their loads (imaging contrast molecules or therapeutic agents) [38, 49]. Our nanobowls can be used for a magnetically-guided delivery and controlled on-demand release of imaging contrast molecules and therapeutic (theranostic) agents.
Figure 6.10: AFM images of Magnetically-responsive gold nanobowls. A) AFM image demonstrating magnetic nanobowls circular shape and size distributed from 250 to 700 nm. B) Smallest size of a nanobowl is 250 nm. All images were recorded in AFM air tapping mode.

Table 6.1: Rhodamine B typical peaks and their assignments.

<table>
<thead>
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<th>Reference 1 (cm(^{-1}))</th>
<th>SERS (cm(^{-1}))</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1647</td>
<td>1645</td>
<td>arom C-C str</td>
</tr>
<tr>
<td>1596</td>
<td>1595</td>
<td></td>
</tr>
<tr>
<td>1570</td>
<td>1580</td>
<td>arom C-C str</td>
</tr>
<tr>
<td>1532</td>
<td>1531</td>
<td>C-H str</td>
</tr>
<tr>
<td>1503</td>
<td>1507</td>
<td>arom C-C str</td>
</tr>
<tr>
<td>1359</td>
<td>1358</td>
<td>arom C-C str</td>
</tr>
<tr>
<td>1308</td>
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</tr>
<tr>
<td>1269</td>
<td>1279</td>
<td>C-O-C str</td>
</tr>
<tr>
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<td>1182</td>
<td>C-H ip bend</td>
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<tr>
<td>1120</td>
<td>1128</td>
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<tr>
<td>1084</td>
<td>1076</td>
<td>-(CH)</td>
</tr>
<tr>
<td>919</td>
<td></td>
<td>C-H str</td>
</tr>
<tr>
<td></td>
<td>932</td>
<td>C-H str</td>
</tr>
<tr>
<td></td>
<td>734</td>
<td>arom ring ip bed</td>
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<tr>
<td>766</td>
<td>766</td>
<td>C-H op bend</td>
</tr>
<tr>
<td>608</td>
<td>622</td>
<td>C-C-C ip bend</td>
</tr>
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</table>

6.5 Conclusion

In conclusion, we have synthesized magnetic gold nanobowls using an asymmetric silica/polystyrene Janus template. The template was covered by small iron oxide nanoparticles coated with a gold shell. However, since gold only covered the silica and not the polystyrene, the symmetry of the shell is broken providing access to the interior
of the bowl once the polystyrene is dissolved. Such a bowl has many advantages such as its interior volume for storage, differential functionalization with gold (exterior) and silica (interior), and magnetic guidance due to its embedded iron oxide nanoparticles. Present experimental evidence revealed a distinct SERS capability and size-dependent in vitro cellular uptake of our magnetically-guided nanobowls. These advantages make them an attractive candidate as a theranostic (therapeutics and diagnostics) vehicle.

6.6 Acknowledgements

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Chapter 7

Functionalization of Magnetic Gold Nanobowls

7.1 Introduction

In the previous chapter, we demonstrated the synthesis of a magnetic gold-iron oxide-silica nanobowl (mGNB). The formation of the magnetic gold nanobowl is adapted from the work of Mo et al. [1], Stoeva et al. [2], and Kim et al. [3]. In this chapter we demonstrate that bowls can be further modified by using silica-alkoxysilane chemistry to attach desired molecules onto the exposed silica in the magnetic gold nanobowl. Along with attaching fluorophore, the prodrug valine-citruline-monomethylauristatin E is used as a demonstration of application toward cancer chemotherapy for the mGNB. This prodrog contains a dipeptide cleaving agent that is sensitive to enzymatic degradation by the lysosomal enzyme cathepsin B. [4] Additionally MMAE itself is more potent by a factor of 100 to 1000 than previous generation of drugs such as doxorubicin and paclitaxel. [4] Thus using this next generation prodrug would allow maximum efficacy with minimum payload.
7.2 Materials and methods

7.2.1 Janus template formation

Adapted from the protocol in Mo et al [1], in a 250 mL glass vial with a magnetic stir bar, we added, in order, 7 mL DI water (Millipore MilliQ) + 40 mL isopropanol alcohol (Sigma) + 13 mL of ammonium hydroxide (Fisher). We then added 40 mM TEOS (550 µL, Sigma)) and 1 mL PS-COOH spheres (Polysciences) at the same time while the solution stirring and allowed to stir for 2 hrs. The actual formation of the Janus template happened rather quickly; within the first 15-20 minutes of the reaction. The solution was then transferred to a centrifuge tube and centrifuged for 5 min @ 500 g to separate out agglomerated particles from lighter particles. The pellet was disposed of while the supernatant was transferred to a fresh centrifuge tube and centrifuged again at 3221 g for 15 minutes to settle the single particles. The supernatant was washed twice in 30 mL DI water and resuspended in 20 mL of anhydrous ethanol (JT Baker). Preparation of gold seeds: In 54 ml of DI water 50 µL of 10M sodium hydroxide (Fisher) was added and stirred well. In a separate container, 1 ml of water and 12 µL of 80% THPC (Sigma) were added to the original solution and the reaction was stirred for 5 min before 1.5 ml of chloroauric acid (10 mg/mL, Sigma) was added. The solution was stirred for another 30 min at room temperature and incubated at 4°C for 24 hours before use.

7.2.2 Gold plating solution

50 mg of K$_2$CO$_3$ (Fisher) was dissolved in 47 mL of DI water and stirred for 5 minutes. 3 mL of chloroauric acid (10 mg/mL) was then added to the stirring solution. The gold solution was initially light yellow and became clear after 30 min of stirring. Afterwards, the gold hydroxide solution was incubated for 24 hr at 4°C before use. Janus template surface modification. Asymmetric Janus templates were functionalized with
amines by placing Janus templates into a solution containing 20 mL of anhydrous ethanol and 40 µL of AEAPTMS (final: 0.2 %v/v, Sigma). The solution was stirred at 60°C for 2 hrs. The amine functionalized Janus templates were washed 3 times in ethanol and resuspended in 5 mL of anhydrous ethanol. The particles are the transferred to a glass vial and dried under vacuum under mild heating overnight.

### 7.2.3 Attachment of iron oxide and gold nanoparticles to Janus templates

5 mg of dried amine modified Janus templates were weighed out and dispersed 1 mL of water with sonication as needed. 200 µL of 10 nm iron oxide solution was added to 1 mL of THPC-gold seed solution. The pH was adjusted to 8 by using sodium hydroxide or hydrochloric acid before adding the 1 mL of amine modified Janus templates into solution. The combined solution was allowed to tumble overnight, allowing proper time for attachment gold and iron oxide nanoparticles. The attached particles were then washed 3 times in water and a dark red pellet was redispersed in 2 mL of water. Gold plating: Gold and iron oxide seeded Janus templates (1 mg) were added into well-stirred solution of gold plating solution (5 mL), deionized water (4 mL) and 10 mM sodium citrate dihydrate (1 mL, Sigma). Then 26 µL of 37% formaldehyde was added to the solution and stirred for approximately 5 minutes. The solution proceeded from clear to pink to a greenish-blue during this time. The final solution had a dark green tint. Gold plated templates were then washed once and centrifuged at 2000g for 10 minutes before being resuspended in 1 mL of water.
7.2.4 PEGylation protocol

50 mg of mPEG-SH (10 kDa, Sigma) was dissolved in 1 mL of water and added to a 1 mL solution of gold shell Janus templates. The solution was allowed to tumble overnight before washing twice at 2000 g for 10 minutes.

7.2.5 Nanobowl Formation

Nanobowls were formed by dissolving the exposed polystyrene in gold plated templates. The 2 mL of gold was centrifuged and re-suspended in 2 mL of ethanol before being transferred into a glass centrifuge vial containing 10 mL of THF. The solution was allowed to stir overnight before washing and re-suspension in 2 mL of water.

7.2.6 Nanobowl formation / Coumarin-silane attachment

O-4-methylcoumarinyl-n-[3-(triethoxysilyl)propyl]carbamate (mCTES, Gelest) was dissolved in THF (Sigma) at a concentration of 100 mg/mL. 4 mL of THF and 1 mL of 20 mg/mL mCTES solution were added to freshly PEGylated gold-iron oxide-silica-polystyrene particles suspended in 1 mL of water. The solution is stirred in the dark overnight in order to simultaneously create magnetic gold nanobowls and functionalize the particles with mCTES. The particles were then washed in water 3 times with 10 mL water and centrifuged at 2000 g for 5 minutes each time. The particles were then resuspended in 2 mL of water.

7.2.7 Nanobowl formation/ FITC-silane attachment

Freshly PEGylated particles were synthesized in a manner as described above. Fluorescein isothiocyanate (FITC, Sigma) and aminopropyltrimethoxysilane (APTES, Sigma) were suspended together in 1 mL of ethanol and tumbled together over night in
the dark. Afterwards the FITC-APTES conjugate solution was added to the 4 mL of THF and 1 mL of PEGylated gold-iron oxide-silica-polystyrene particle suspended in water. Again the magnetic gold nanobowls are formed while the FITC-APTES is attached to the bowl of the particle. The particles were then washed in water 3 times in 10 mL water and centrifuged at 2000 g for 5 minutes each time. The particles were then resuspended in 2 mL of water.

### 7.2.8 Nanobowl formation/ APTES-vc-MMAE attachment

Succinimide -valine-citruline-monomethylauristatin E (OSu-vc-MMAE, Concortis) is an amine reactive prodrug of the anti-mitotic monomethylauristatin E. OSu-vc-MMAE dissolved in DMSO (10mg/ml) was suspended in 450 µL of ethanol with addition of 9 µL APTES and 4 µL of triethylamine. The solution was tumbled in the dark overnight and the dialyzed another 24 hours. The conjugation of APTES-vc-MMAE is monitored using electrospray ionization/ atmospheric-pressure chemical ionization mass spectroscopy (ESI/APCI-MS).

### 7.2.9 Fluorescent measurement

Fluorescence was confirmed using fluorescent spectrophotometry and fluorescence microscopy. 50 µL of magnetic gold nanobowls were added in triplicate to a 96 well plate. Courmarin (ex/ex 350/450) and FITC signals were measured.

### 7.2.10 Cell work

WPMY-1, a human prostate cell line, and LNCaP, a human carcinoma line, were used to test cell viability upon exposure to FITC-PEG-mGNB for increasing amounts of time. WPMY-1 and LNCaP were obtained from the American Type Culture Collection
WPMY-1 and LNCaP cells were cultured using Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) containing 5% of fetal bovine serum (FBS, Gibco) at 37°C in humidified atmosphere of 5% of CO₂. Cells were grown in 60 mm petri dishes to reach a confluence of 70-80%. For imaging WPMY-1 and LNCaP were treated with FITC conjugated, pegylated, mGNB in a Phosphate-buffered saline (PBS, Corning) solution at different concentrations (0, 0.001, 0.01, 0.1 mg/mL). After different time intervals (0, 2, 4, 24 hr), cells were washed with PBS, further fixed using 0.4% glutaraldehyde (Sigma) for 5 minutes, and washed with PBST (PBS with 5% of Tween-20) solution three times. 3 ml of PBS was added afterwards.

Additionally WPMY-1 and LNCaP cells were exposed to same concentrations of FITC conjugated, pegylated mGNB (0, 0.001, 0.01, 0.1 mg/mL) and times (0, 2, 4, 24 hr) were assayed for cell viability with the MTT assay (ThermoFisher). MTT absorbance measurements were made in triplicate to arrive at an average cell viability.

7.3 Results and Discussion

7.3.1 Conjugation of Molecules to Magnetic Gold Nanobowls

mCTES and FITC-silane were first conjugated to the magnetic gold nanobowl surfaces to test the viability of the attachment chemical moieties via silane chemistry. This is in preparation for attachment of OSu-vc-MMAE to an amine containing silane. mCTES was selected first for attachment to the mGNBs because mCTES is a commercially available dye conjugated silane stimulated in the UV and fluorescent in the blue. Repeated conjugated trials showed mCTES attached mGNB surfaces with a signal greater than background against controls (Fig 7.1A). In order test two part conjugation and attachment process similar OSu-vc-MMAE conjugation, FITC was reacted with APTES in anhydrous ethanol in order to create FITC-silane conjugate. Then FITC-silane was added to a THF
solution containing mGNB in order to react. Fluorescence spectroscopy and microscopy were used to determine FITC-silane to particle surface. Figure 7.1B shows emission counts when compared to relevant controls. There is a 100-fold signal difference over DI water and small error bars showing high confidence in the results (n=3). Figure 7.2 shows FITC-PEG-mGNB particles on a glass slide.

Succinimide-valine-citruline-monomethylauristatin E (OSu-vc-MMAE) is a amine reactive prodrug of the anti-mitotic monomethylauristatin E. The prodrug is designed to be released upon cleavage by the enzyme cathepsin B which is found in abundance in the lysosome of a cell after uptake. [5] In anhydrous organic solvent conditions, APTES can be attached to the OSu-vc-MMAE in order to form a conjugate capable of attachment to the magnetic gold nanobowls. Using mass spectrometry preliminary results show a possible match with APTES-vc-MMAE (Fig. 7.3). Major peaks were identified at 1259,

Figure 7.1: Fluorescence emissions demonstrating attachemnt of fluorophore to mGNB.A) Fluorescence emission measurements showing successful attachment of mCTES on to the PEG-mGNB. B) Fluorescence emission measurements show successful attachment of FITC-silane onto PEG-mGNB.
**Figure 7.2:** FITC-PEG-mGNB on glass slides. Fluorescence and optical images of FITC-PEG-mGNB particles on glass slides at different dilutions. The false color green see in the images indicates successful modification of the mGNB

**Figure 7.3:** Mass Spectrometry of APTES-vc-MMAE. Peaks A, B, C correlate to the structures on the right. They are all within 1 amu of the recorded masses with known commonly found salts (Na and K) in solution.

1378 and 1493 m/z. Assuming all major peaks had a single positive charge, these masses can be correlated with the products listed in the figure. Peak A is likely vc-MMAE (1235 amu) after the succinimide group has been knocked off with a sodium atom in transit (1235 +23 (Na⁺) = 1258). Peak B likely corresponds to Osu-vc-MMAE (1333 amu) with two sodium atoms in transit (1333 + 46 (2 Na⁺) = 1379). Peak C likely corresponds to the conjugated product APTES-vc-MMAE with a potassium atom in transit (1455 + 39 (K⁺) = 1494). While none of the measured masses lines up exactly up with predicted masses, they are within 1 amu or the mass of one hydrogen atom, and with known contaminant salts.
Figure 7.4: Cell viability after treatment with PEG-GNBs. Relative cell viability for WPMY-1 (A) and LNCaP (B) cells after 2, 4 and 24 hours of incubation with GNBs using the MTT assay. C) WPMY-1 and D) LNCaP (fluorescence microscopy GNBs uptake by after 4 hours of incubation. C and D are bright-field and fluorescence image merge. The fluorescence was obtained using the FITC filter $\lambda_{\text{ex}} = 482 \pm 17\text{nm}$ and $\lambda_{\text{em}} = 536 \pm 20\text{nm}$.

### 7.3.2 Cell Testing

FITC-PEG-mGNB were tested with WPMY-1 normal human prostate and LNCaP cancerous human prostate cell lines. The mGNBs were introduced at different initial concentrations and incubated with the cells for varying lengths of time up to 24 hours. Overall the cells exhibited a dose dependent response to mGNB particles. At the lower concentration measured (0.001 mg/mL) no cellular toxicity is observed in either cell line even after 24 hr. (Fig. 7.4 A and B) At the highest concentration measured (0.1 mg/mL) approximately 60% of cells were still alive after 2 hr in both cell lines. After 24 hours, only 50% of cells were alive in the WPMY-1 cell line and about 30% were still alive in LNCaP cells. Merged phase contrast-fluorescent images show cell uptake in WPMY-1 (Fig. 7.4C) and LNCaP (Fig 7.4D) cell as well.
7.4 Conclusion

Initial modification of the particles with fluorophore and its combination with cell testing shows uptake by cancerous and non-cancerous cells. However there is dose dependent toxicity as the mGNB concentration increases. The prodrug attachment is still on going. Conjugation was successfully confirmed by mass spectrometry and awaits conjugation.

7.5 Acknowledgements

The authors thank Professor Jan Talbot for access to the DLS instrument. The authors also thank NANO3 for access to SEM. Finally, the authors thank Timothy Merloo and Ying Jones for support in using TEM. Thanks to Professor Seth Cohen for access to the FTIR instrument. This work was funded by University of California, San Diego (UCSD) startup funds and National Institutes of Health (NIH) Grants R01DA024871, R01DA025296, and R01AG028709 to Ratnesh Lal. Post-doctoral Fellowship (237085) provided by Consejo Nacional de Ciencia y Tecnología (CONACyT) to K.S-G.

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