DNA and Peptide Functionalized Gold Nanoparticles for Biological Imaging and Transfection

Ziyan Zhang
Washington University in St. Louis

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WASHINGTON UNIVERSITY IN ST. LOUIS

Department of Chemistry

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DNA and Peptide Functionalized Gold Nanoparticles for Biological Imaging and Transfection

By

Ziyan Zhang

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

August 2012

St. Louis, Missouri
ABSTRACT

DNA and Peptide Functionalized Gold Nanoparticles
for Biological Imaging and Transfection

By
Ziyan Zhang

Doctor of Philosophy in Chemistry
Washington University in St. Louis, 2011
Professor John-Stephen A. Taylor, Chair

This dissertation focuses on the development of DNA and peptide functionalized gold nanoparticles. Three nanoparticle systems have been developed: (1) nucleic acid-directed self-assembly of a gold nanoparticle PET imaging agent; (2) cationic peptide functionalized gold nanoparticles; (3) gold nanoparticle templated peptide nanoshells.

The first functionalized gold nanoparticles were constructed by assembling ODN (oligodeoxynucleotide)-derivatized gold nanoparticles with functionalized complementary PNAs (peptide nucleic acids). The PNAs were conjugated with DOTA for chelating chelating $^{64}$Cu for PET imaging, PEG for conferring stealth properties, and Cy5 for fluorescent imaging. The resulting functionalized nanoparticles showed good stability both in vitro and in vivo and had biodistribution properties expected for a PEGylated gold nanoparticle rather than that for the functionalized PNAs used in the construction.
The second nanoparticle system was arginine-rich peptide and lysine-rich peptide functionalized cationic gold nanoparticles as transfection agents. A series of cysteine containing oligoarginine was synthesized by automated solid phase Fmoc synthesis. Chloroauric acid was reduced in the presence of the peptides. The resulting gold nanoparticles were around 13 nm in diameter with zeta potential around 29 mV. Compare to other reported cationic gold nanoparticles, the arginine-rich gold nanoparticles were highly stable in up to 0.6 M NaCl solutions and up to pH 8.5 in non-phosphate containing buffers. In phosphate buffers, however, the nanoparticles started to aggregate at above pH 6, though aggregation was reversible by lowering the pH. The strong affinity between arginine and phosphate groups also resulted in the nanoparticle’s strong ODN binding ability. Despite of the reported cytotoxicity of oligoarginines, the arginine-rich gold nanoparticles showed no cytotoxicity even at a very high concentration. An improved assembly approach was also investigated. Cationic peptides were exchanged with cetyltrimethylammonium bromide (CTAB) on gold nanoparticles fabricated by seeded growth method. This approach allowed quick assembly of a library of different peptide on the gold surface. The approach was validated by synthesizing three cationic gold nanoparticles, R13C-AuNP, K10Y2C-AuNP and R13C/K10Y2C-AuNP. All of the nanoparticles retained the monodispersity of the original CTAB•AuNP colloid. Their stability and ODN binding ability showed distinct difference due to their different surface composition. All of the colloids were proved to be non-toxic at high concentration but further optimization is needed in the transfection efficiency.

The third nanoparticle system was developed based on the ligand exchange approach. A cysteine containing lysine-rich peptide was deposited on CTAB•AuNP.
Then the lysines were crosslinked by a biodegradable crosslinker, Sulfo-EGS (Ethylene glycol bis[sulfosuccinimidylsuccinate]), at various peptide/crosslinker ratio. The gold core was then removed by potassium cyanide. The resulting peptide nanoshells were about 40 nm in diameter, compare to the 15 nm diameter before the gold core was removed. Crosslinking also resulted in dramatic decrease of the ODN binding ability of the nanoparticle and the nanoshells. This approach showed great potential of making a combinatorial library of biodegradable nanostructures quickly for a broad range of applications.
ACKNOWLEDGEMENTS

I am incredibly grateful to my advisor, Prof. John-Stephen A. Taylor, for his guidance and support, especially during the first three years of my graduate study, when the projects weren’t going smoothly. Thank you for your patience and belief in me and finally leading me to the area of gold nanoparticles. I am also benefited tremendously from you passion and persistence in science, which raised me up when I encounter problems and calmed me down when I got overly excited and not very rigorous. I believe that’s the most important gifts a young scientist can receive from her mentor. I would like to thank my research committee members, Prof. Kevin D. Moeller and Prof. Joshua A Maurer, for their constructive advices and encouragement over the past 6 years. I also want to thank the rest of my defense committee members, Prof. Liviu Mirica, Prof. Steven Brody and Prof. Mikhail Berezin.

I also would like to extend my appreciation to the past and present members of the Taylor Group. In particular, I would like to express my gratitude to Dr. Gang Shen and Dr. Huafeng Fang, who were both knowledgeable and nurturing postdocs. They helped me with many of the basic techniques and ideas. Further, I had the pleasure of working with Dr. Rongsheng Wang, Dr. Zhenghui Wang, Dr. Vincent Cannistraro, Yuefei Shen, Qian Song, Jillian Smith, Zifan Li and Alexandra Loftis who have been great friends and source of constant inspiration and support.

I would also like to acknowledge my collaborators in the PEN project, especially Prof. Michael Welch and Prof. Karen Wooley, who have been widely respected scientists and role models to me. I would also like to thank Dr. Yongjian Liu and Ritu Shrestha.
whom I have worked with closely on different projects. Thank you for your brilliance, dedication and hard work.

I would also like to thank my peer friends in the Chemistry Department, Matthew Hynes, Dr. Hannah Malcolm, Dr. Dawn Johnson, Dr. Matthew Strulson, Natalie LaFranzo, Dr. Dian Su, Dr. Xinliu Gao, Hai Yue, Dr. Hao Zhang, Dr. Libo Hu, Xing Yang, Bo Bi, Dr. Haichao Xu, Dr. Guojian Lu, Guoxi Xu, Allison Reddon, Dr. Ke Zhang, and Dr. Zhou Li, who shared this experience with me from the beginning through the good times and the bad times, who have provided constant support both professionally and personally.

I would like to express my special appreciation to McDonnell International Scholars Academy, which provided me with not only 5 years of generous financial support, but also exposure to countless events, leaders around the global in different areas, and outstanding students. Interacting with so many brilliant young scholars from all over the world have been tremendously beneficial to me and will continue to affect me in the years to come. I would like to extend my greatest gratitude to Prof. James V. Wertsch, Director of the McDonnell Academy. I can’t say enough about how much I have learned from you, from all aspects of life. I would also like to thank Mary E. Wertsch, who has been an important mentor and role model in my life and has always been available and supportive for me through happiness and difficulties. I would also like to thank Mr. John McDonnell, who is a generous benefactor and more importantly, a great mentor and role model to me. I am honored to have befriended with you and exchanged ideas with you. Last but not least, I would also like to thank Chancellor Mark Wrighton, who influenced me greatly both as a talented chemist and as a great leader.
Finally, I would like to thank my family, especially my mom and dad, Xiaoli Ma and Ning Zhang, my grandparents, Xide Ma, Zhiying Wang, Yun Zhang and Yulan Liu, for their unconditional love and support. I would also like to thank my parents-in-law, Gang Sun and Xiulan Chang, who have been very loving and helpful in the busiest time of writing this dissertation. In the end, I would like to thank my dear husband, Hongtao Sun.
This thesis is dedicated to my husband Hongtao Sun and our unborn son.
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<th>Full Form</th>
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<tbody>
<tr>
<td>AuNP</td>
<td>gold nanoparticle</td>
</tr>
<tr>
<td>C, Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>cAuNP</td>
<td>cationic gold nanoparticle</td>
</tr>
<tr>
<td>CIA</td>
<td>collagen-induced arthritis</td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-Diisopropylethylamine</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DMF</td>
<td>N, N’-dimethyl formamide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid</td>
</tr>
<tr>
<td>EPR</td>
<td>enhanced permeation and retention</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>inductively-coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>K, Lys</td>
<td>lysine</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>mPEG</td>
<td>methyl polyethylene glycol</td>
</tr>
<tr>
<td>MPS</td>
<td>mononuclear phagocytic system</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>NIR</td>
<td>near infrared</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>NP</td>
<td>nanoparticle</td>
</tr>
<tr>
<td>ODN</td>
<td>oligodeoxynucleotide</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAMAM</td>
<td>poly(amido amine)</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PNA</td>
<td>peptide nucleic acid</td>
</tr>
<tr>
<td>R, Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>shRNA</td>
<td>small hairpin ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SPB</td>
<td>surface plasmon band</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
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<tr>
<td>Sulfo-EGS</td>
<td>ethylene glycol bis[sulfosuccinimidylsuccinate]</td>
</tr>
<tr>
<td>T, Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TOF</td>
<td>time of flight</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>V, Val</td>
<td>valine</td>
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Chapter 1

An Introduction to Biomolecule Modified Gold Nanoparticles

1.1 Nanoparticles

Nanotechnology involves structures in the sub-micrometer, or more typically, 1-100nm dimensions. This scale is much smaller than what is studied in conventional material science, which is several microns and above. And yet it is quite large compared to that investigated by traditional physicists and chemists, who study atoms and bonds at the angstrom level (Figure 1.1). Modern physics has found that materials in this range exhibit unique properties, different from both the bulk material and the molecules and atoms that they are composed of. These properties include decreased melting temperature [1], color (Figure 1.2), quantum confinement in semiconductor particles (Figure 1.3), surface plasmon resonance in some metal particles and superparamagnetism in magnetic materials [2], etc. These properties provide unique opportunities in material science, making nanoparticles highly desirable candidates as novel catalysts, paints, lubricants, optical devices, etc.

![Figure 1.1. The scale of some common structures](image)

Human hair
Viruses
Nanoparticles
Glucose molecule
Atoms
Figure 1.2 The diameter of gold nanoparticles determines the wavelengths of light absorbed. The colors in this diagram illustrate this effect.

(Copyright © Nanotechnology eLearning Center)

Figure 1.3 Fluorescent properties of nanosized semiconductor due to quantum confinement effect (quantum dots). The top picture was shot in ultraviolet light and the bottom was shot in ambient light. The length of the synthesis reaction determines particle size for CdSe, increasing from left to right. (Copyright © The University of Wisconsin-Madison Materials Research Science and Engineering Center)
The special size range and large surface to volume of nanoparticles not only results in interesting physical behaviors that benefit material industry, but also enables biomedical scientists to develop novel diagnostic and therapeutic agents. First of all, the intercellular space between renal blood vessel epithelial cells is on the scale of 1-10 nm. For this reason, small molecule drugs usually have very limited blood retention and are cleared through the kidneys within the first hour or even a few minutes after administration. To compensate, large doses or repetitive administration of small molecule drugs are usually used, which can create serious side effects and high expense. In contrast, nanoparticles (usually above 10 nm) can easily bypass renal clearance and are usually cleared through mononuclear phagocytic system (MPS). If the surface of the nanoparticles are appropriately modified, blood retention can be greatly extended, from several hours to days [3]. For targeted nanoparticles, this property provides longer time for the targeting ligands to interact with the target site and hence increase specificity. For oncology applications, this property allows the nanoparticles with a therapeutic payload to take full advantage of the enhanced permeation and retention (EPR) effect. Secondly, the large surface area allows multiple drugs as well as different functional groups to be attached to one single nanoparticle. One nanoparticle can carry hundreds to hundreds of thousands of functional groups on the surface. This enables the particle to carry contrast agents for different imaging techniques, like MRI/optical imaging [4], PET/MRI imaging [5], etc. These multimodal approaches combine the advantages of multiple imaging techniques such as high resolution, quick data acquisition, low dosage and easy application, and achieve results that are difficult if not impossible to achieve through any single technique. One single particle may also carry multiple copies of targeting moieties
to enhance specificity and facilitate delivery. Ligands that bind specifically with surface receptor on the targeted cells were incorporated in the nanoparticles, such as RGD peptide [6], tumor necrosis factor-alpha (TNF-alpha) [7, 8], aptamers [9], etc. Similar to the multimodal nanoparticle imaging agents, some nanoparticles also carry several kinds of targeting ligands that bind to different receptors on same type of cells[10]. The simultaneous binding greatly enhanced the binding affinity and specificity to a level that can hardly be achieved by any single ligand.

1.2 Gold Nanoparticles

Among all the nanomaterials, pseudo-spherical gold nanoparticles (AuNP) are one of the most widely studied. For many centuries, people have recognized the distinct properties of gold nanoparticles apart from the bulk material and have used the “soluble gold” as components in medicinal remedies and, due to their astonishing red color, as dyes and pigments in architecture and art (Figure 1.4).
Figure 1.4 Lycurgus Cup, a Roman glass cage cup crafted in 4th to 5th century B. C. The cup showed ruby red color in transmitted light (left panel) and green color in reflected light (right panel) due to the colloid silver-gold alloy (about 70nm) dispersed in the glass. [11] (Copyright © Johnbod from Wikipedia)

Modern research rediscovered gold nanoparticles’ unique physical properties, which have become the basis of advanced applications in many fields. The deep-red color of gold nanoparticle colloids in water and glasses reflects the surface plasmon band (SPB), a broad absorption band in the visible region around 520 nm. The SPB is due to the coherent oscillation of an electron gas in the conduction band at the surface of the nanoparticles (the 6s shell of the conduction band for gold nanoparticles) in response to the oscillating electromagnetic field of the incoming light [12]. Because the surface plasmon band is very sensitive to the distribution of electrons on the particle surface, the study of the SPB has remained an area of very active research from both a scientific and technological standpoint. The SPB of gold nanoparticle is affected by surface ligands, the distance between the nanoparticles, the particle sizes, etc. Thus, the SPB has become a sensitive and reliable reporter of surface modification, interactions, aggregation, and
particle growth. As a result, gold nanoparticles have been used to probe antibody-antigen [13, 14] and nucleic acid interactions [15].

1.2.1 The Photothermal Effect

The photothermal effect is also derived from surface plasmon resonance. When the surface electrons on the nanoparticles oscillate in resonance with the incoming light, part of the energy from the photons is transferred to heat and generates a fast and highly localized thermal effect. Modern lithography uses noble metal nanoparticles to form high-resolution patterns that cannot be achieved by conventional methods. When a laser beam shines on the substrate, the exposed noble metal nanoparticles will generate enough heat to melt or degrade the surrounding polymers. Because the photothermal effect is very fast and highly localized, the pattern can be precisely controlled by the laser beam. The most important application of the photothermal effect in the biomedical field is to use noble nanoparticles, especially gold nanoparticles, to thermally destroy tumor tissue. The excitation wavelength of the SPB is largely determined by the size and geometry of the nanoparticle. Unfortunately, the SPB of spherical gold nanoparticles fall between 510-570 nm which is outside the optimal window for tissue penetration of 700-900 nm. It wasn’t until Cathy Murphy of University of Illinois discovered how to make gold nanorods that useful biological applications of the photothermal effect were possible. While the axial SPR peaks of a nanorod remain at 500 nm, the longitudinal SPR peak can range from 500 nm to 1000 nm by adjusting the aspect ratio [16].

El-Sayed and co-workers found that they were able to achieve destruction of cancer cells by the photothermal effect with gold nanorods which are preferably
internalized with a laser power that is low enough to be safe for normal cells [17]. In their recent studies using a mouse model [18], the nanorods were conjugated to mPEG-SH 5000 and injected into mice both intravenously and subcutaneously. Using the transmission imaging of the NIR (near infrared) laser with a customized camera, the tumor could be well visualized after nanorod accumulation following either intravenous or subcutaneous injections. After exposure to a CW red laser at 808 nm with an energy of 1 W cm$^{-2}$ for 10 min, tumor volumes in both delivery methods did not grow while the untreated tumor kept growing at a rapid rate. The intravenous treated tumors show lower photothermal therapy efficiency due to lower amount of gold nanorods inside the tumor.

1.2.2 Fluorescence Modulation

Fluorescence modulation is another important physical property of gold nanoparticles. When a fluorophore is placed at a relatively short distance, e.g., within 10 nm, from a metal particle possessing a strong plasmon field, the electrons of the fluorophore participating in the excitation/emission interacts with the plasmon field. The interaction results in either quenching or enhancement of the fluorescence emission intensity level. Establishing the relationship between the plasmon field and the resulting fluorescence intensity level can be beneficial in developing highly efficacious optical contrast agents for bio-sensing and imaging. Schneider and co workers studied the distance dependence of fluorescence quenching effect on the surface of gold nanoparticles with layer-by-layer assembled polyelectrolytes [19] and found that the quenching efficiency decreases rapidly with the increasing distance. At 1 nm from the surface, the quenching efficiency could reach over 90% whereas when the distance
increased to 3 nm, only 30% of the efficiency remained. This property provides the basis of many gold nanoparticle based fluorescent probes [20-22]. Interestingly, in the cases of certain ions, e.g. Eu\(^{3+}\) [23], or beyond a certain distance, the fluorescence of the fluorophores is greatly enhanced. Like the quenching effect, this enhancement is also very sensitive to changes in distance and thus provides valuable information on substance interactions around the gold nanoparticle. The enhancement effect, though, is less widely used in biological applications than the quenching effect.

**1.2.3 Gold NP Toxicity**

For biomedical applications, gold nanoparticles have another desirable property compared to other nanomaterials—low toxicity. Because of its inertness, gold has shown great compatibility inside the biological systems. Paciotti and coworkers showed that although gold nanoparticles accumulated in the mouse liver in high concentration, the mouse still survived without apparent harm [8]. Ligands such as CTAB and oligoarginines, that are known to have high cytotoxicity in their free form appeared harmless in high concentration once they were tightly bound to gold nanoparticle surfaces [24].

**1.2.4 Preparation of Gold NP**

Because of the desirable properties discussed above, gold nanoparticles have been used for medical purposes since ancient times. Historically, alchemists would make potions out of gold nanoparticles (“Aurum Potabile” or “potable gold”) to treat many diseases [25]. Rheumatoid arthritis was among the first modern conditions where gold
was part of the therapy [25]. The gold nanoparticles were implanted near the arthritic side to help relieve pain. Studies have shown that intraarticular administration of nanogold ameliorates the clinical course of collagen-induced arthritis (CIA) in rats [26]. Nanogold exerted antiangiogenic activities (inhibition of the growth of new blood vessels) and subsequently reduced macrophage infiltration and inflammation, which resulted in attenuation of arthritis [27]. In the recent years, gold nanoparticles and their properties have led to new and exciting developments with great potential in biology and medicine. Gold nanoparticles have been used as carriers for drugs like Paclitaxel [28]. Contrast agents have also been complexed with gold nanoparticles for cellular detect and in vivo tumor detection. Colloidal gold itself can be used as therapeutics in the photothermal therapy to destruct tumors.

The first scientific report describing the preparation of colloidal gold nanoparticles appeared in 1857 when Michael Faraday found that the “fine particles” formed from the aqueous reduction of gold chloride by phosphorus could be further stabilized by the addition of carbon disulfide, giving a “beautiful ruby fluid” [29]. Nowadays, most colloid synthetic methods for production of gold nanoparticles follow a similar strategy, where solvated gold salt is reduced in the presence of surface capping molecules, which prevent particle aggregation by electrostatic and/or physical repulsion. The size of the particles is usually controlled by temperature, pH, the capping ligands, and most importantly, the ratio of the gold ion/reducing agent or gold ion/stabilizer, which smaller (and usually more monodispersed) sizes obtained from smaller ratios.

Citrate reduction is one of the most widely used methods for obtaining gold nanoparticles ranging from 9 nm to 120 nm. This method was first reported by J.
Turkevich[30] and then further improved by G. Frens [31] and J. Kimling [32]. In this method, sodium citrate serves both as reducing agent and capping agent. The size of the gold nanoparticles is determined by the ratio between chloroauric acid and sodium citrate. The reduction only happens at elevated temperature (boiling point of the chloroauric acid solution) and results in monodispersed aqueous gold colloid with negative zeta potential.

Sodium borohydride is another widely used reducer in making gold nanoparticles. Compared to sodium citrate, sodium borohydride is much stronger and can reduce the gold ion to gold nanoparticle at room temperature. However, sodium borohydride alone is a very weak capping agent. Gold nanoparticles synthesized by sodium borohydride alone are relatively small and polydispersed. Stronger capping agents are usually used to achieve a narrower size distribution. In the seeded growth method reported by Jana et al. [33], sodium citrate was used as capping agent while the gold ion was reduced by sodium borohydride at room temperature. More stable nanoparticles can be obtained by using thiolated ligands as capping agents during reduction. The Brust-Shiffrin method [34, 35], first published in 1994, utilized a two-phase system. AuCl₄⁻ is transferred to toluene using tetraoctylammonium bromide as the phase-transfer reagent and is reduced by sodium borohydride in the presence of dodecanethiol. The organic phase changes color from orange to deep brown within a few seconds upon addition of NaBH₄. The resulting gold nanoparticles are capped by dodecanethiol and can be repeatedly isolated and redissolved in common organic solvents without irreversible aggregation or decomposition. They can be easily handled and functionalized just as stable organic and molecular compounds.

Another category of gold nanoparticle synthesis is seeded growth strategy. Murphy’s group first reported the seeded growth approach for gold nanorod preparation
and gold nanoparticle preparation [33]. Small roughly spherical gold seeds with a mean diameter between 3 and 4 nm were prepared first by NaBH$_4$ reduction in the presence of citrate. Then the seeds were mixed with a growth solution containing a gold salt and cetyl-tri-methyl ammonium bromide (CTAB) as the capping agent and ascorbic acid as the reducing agent. In the preparation of gold nanorods, silver ions were also added for aspect ratio control. Ascorbic acid is a very weak reducing agent that can’t reduce gold$^{III}$ to gold$^0$ without the presence of the seeds. Therefore, nucleation is controlled and the growth only happens on the surface of the seeds. The size of the particles is mainly dependent on the gold seed to gold salt ratio.

Various ligands have been used to stabilize gold nanoparticles in different synthetic strategies, such as PPh$_3$ in the Brust-Schiffrin method [37], citrate ion in citrate reduction [31], tannic acetate in a modified citrate reduction method [38], centrimonium bromide [33] and polyethyleneimine [39]. Among all the explored ligands, sulfur-containing ligands have proven to form the strongest bonds to the surface, due to the soft character of both sulfur and gold. All other ligands that are used to protect gold nanoparticles in the synthetic process can be replaced by sulfur-containing ligands to increase stability, such as in the two phase synthesis [37] and seeded growth synthesis [33] methods.

1.2.5 Functionalization of Gold NP

Functionalization of gold nanoparticles is usually achieved through deposition of thiolated ligands on the gold surface. One of the deposition strategies involves displacement of non-sulfur containing capping ligands with sulfur containing ligands on
the gold surface. This process is usually complete within 24 hours. In the preparation of oligodeoxynucleotide (ODN) functionalized gold nanoparticles reported by Mirkin and coworkers in 1996, citrate on the gold surface was displaced by thiolated ODNs overnight [15, 40-44]. Since thiol groups are ubiquitous among proteins, this approach is the most widely used method for preparing protein functionalized gold nanoparticles. Paciotti et al reported a tumor necrosis factor (TNF) functionalized gold nanoparticle for tumor targeting. The TNF was also deposited on the gold surface by displacing the citrate [45].

Another strategy is to exchange the thiolated ligands used in synthetic step with thiolated ligands of a desired function. This method is particularly useful when the desired functional ligands are hydrophobic, or smaller particles are needed. This strategy usually takes much longer time, usually several days. If the two ligands are similar in solubility and sulfur containing group, a large excess of the incoming ligands is needed to ensure the complete exchange [46].

Once the gold nanoparticles are stabilized by thiolated ligands, further functionalization can be done through the reactive groups on the ligands, such as amino groups, carboxyl groups, nucleotides, and alkynes. Astruc et al. reported a “clickable” gold nanoparticle [47]. Dodecanethiolate stabilized gold nanoparticles were first synthesized by the two-phase method. Then the surface dodecanethiolate was exchanged with bromoundecanethiol. The terminal bromide was further substituted by azido group using sodium azide. The resulting gold nanoparticles can be conveniently functionalized by acetylene bearing molecules through the classic copper catalyzed click reactions. Brust et. al reported the synthesis of lipase functionalized gold nanoparticle [48]. In their
approach, a bifunctional linker with thiol and azide on either end was directly deposited on the citrate•AuNP. Then, acetylene modified lipase was “clicked” on the gold surface through a copper catalyzed click reaction. Agarose gel electrophoresis assay proved the success of the conjugation and a fluorometric lipase activity assay proved that the lipase on the gold particle still retained its enzymatic activity.

Sulfur containing molecules are ubiquitous in biological systems. Most of the natural proteins contain cysteine. It is also very convenient to conjugate cysteine to a synthetic peptide or add a terminal thiol to synthetic oligonucleotide. People have fabricated gold nanoparticles with thiolated oligodeoxynucleotides (ODNs) [15, 40-44], peptides [43, 49, 50], antibodies [51-54], and other proteins [55, 56]. Biomolecule modified gold nanoparticles show excellent stability compared to individual biomolecules, good biocompatibility compared to other synthetic nanomaterials and versatile functionality compared to conventional formulation. Biomolecule modified gold nanoparticles are now heavily utilized in chemistry, biology, engineering, and medicine.

1.3 Focus of Dissertation

This dissertation is focused on the design, synthesis, characterization and application of biomolecule functionalized gold nanoparticles and its derivatives. Although great progress has been made in this field, the full potential of the biomolecule modified gold nanoparticle is far from thoroughly investigated. Rather than functionalizing gold nanoparticles with protein, this dissertation focuses on functionalization with synthetic ODNs and peptides whose primary and secondary
structures are easier to manipulate and thus the properties are more predictable and versatile.

**Chapter 2** explores a self-assembling strategy to form multifunctional gold nanoparticles based on Watson-Crick base paring. Since the introduction of ODN protected gold nanoparticles by Chad Mirkin from Northwestern University [15], much work has been done using this type of gold nanoparticle for *in vitro* gene detection and delivery. However, the multivalency of the ODN-AuNP hasn’t been fully utilized on functionalities in a broader range. In addition, no *in vivo* study has been done with ODN-AuNP, therefore, its potential as delivery agent has never been explored. The work described in **Chapter 2** demonstrates that oligodeoxynucleotide protected gold nanoparticles assemble readily with functionalized complementary peptide nucleic acids in a sequence specific manner. The hybridization yields multifunctional nanoparticles that are stable under physiological conditions. We also show that the non-specific cell uptake of gold-ODN nanoparticles can be greatly reduced by PEGylation. The resulting nanoparticle showed typical biodistribution pattern of gold nanoparticles. The majority of the particles were cleared from the blood stream by MPS system and retained in the liver and spleen. With improvements in circulation and the addition of targeting moieties, this self-assembling system could be used in development of imaging and therapeutic agents in the future.

ODN protected gold nanoparticles are also used for *in vitro* ODN delivery [57]. However, this approach requires that the gold particle be designed individually for each payload. This approach is limited to smaller ODNs like siRNA, shRNA or antisense DNA but will be less efficient for larger types of genetic material like plasmid DNA due
to the spatial constraints of the DNA duplex. **Chapter 3** reports a library of arginine-rich peptide functionalized gold nanoparticles for nucleic acid delivery. Oligoarginines have been studied and utilized intensively for cell permeation and gene delivery due to the unusually high affinity between guanidinium groups and phosphate groups [58]. Many types of nanoparticles have been made with oligoarginines for transfection purposes [59-63]. However, the effect of oligoarginine on gold nanoparticles has never been explored. It was expected that gold nanoparticles functionalized with arginine rich peptide should be highly stable due to the high basicity of the guanidinium group. It was also expected to enter the cells with high efficiency and bind to DNA with strong affinity. A library of cysteine containing oligoarginines were synthesized by automated solid phase Fmoc synthesis. The number of arginines on each strand was altered to investigate the effect of arginine density. Meanwhile, threonine or valine was also incorporated into some of the strands to optimize nanoparticle stability. A library of Arg-AuNP were synthesized accordingly by NaBH₄ reduction. The stability, DNA binding ability, transfection efficiency and cytotoxicity of the cationic AuNPs (cAuNPs) were evaluated and compared. It was found that all the cAuNPs tested were non-toxic to the cells. AuNP-CR13 showed the highest DNA binding affinity and stability under high salt concentration and high pH in non-phosphate containing buffer. All the arginine-rich nanoparticles were found to aggregate reversibly in high pH phosphate buffer, presumably due to the hydrogen bonding between guanidinium groups and phosphate groups. Besides direct reduction, another synthetic approach was investigated. Three peptide-AuNPs, R13C-AuNP, K10Y2C-AuNP and R13C/K10Y2C-AuNP, were synthesized through ligand-peptide exchange on gold nanoparticles fabricated by citrate
reduction and seeded growth method. Nanoparticles fabricated this way retained the morphology and monodispersity of the original gold colloid and yet showed distinct properties consistency with their surface composition. All the cationic gold nanoparticles studied in this dissertation were non-toxic to cells, however, no transfection was observed using any of the gold nanoparticles, which requires further investigation.

Although numerous studies including the studies in this dissertation have shown that gold nanoparticles are highly tolerated in biological systems, their clearance is still a major concern when used systemically. This was the intrinsic drawback of gold nanoparticles that can’t be overcome. To be useful for nanomedicine nanoparticles must be biodegradable. Chapter 4 describes the construction of degradable nanoparticles based on a combinatorial approach that uses gold nanoparticles as templates for assembling a shell of crosslinked peptides after which the gold was removed. The size, surface properties and DNA binding ability of the nanoshells were studied. It was shown that crosslinked peptide nanoshells remained intact after the gold cores were removed although the size expands a little presumably due to inter-strand repulsion. Crosslinking of the peptides, however, greatly decreased the DNA binding ability of the nanoparticles, indicating that extra DNA binding moiety such as arginine should be incorporated into the peptide shell. The results in Chapter 4 demonstrate that this combinatorial approach can yield stable peptide-shell nanoparticles. Many parameters in this approach could be optimized for different applications in the future, such as particle size, peptide composition, peptide modification, crosslinking strength, etc.
This dissertation concludes with a short summary of the biomolecule functionalized gold nanoparticles prepared and suggestions for how this work can be moved forward in the future in both the fundamental and applied arenas (Chapter 5).

1.4 References


Chapter 2

Nucleic Acid-directed Self-assembly of a Gold Nanoparticle PET Imaging Agent

2.1 Introduction

Nanotechnology offers great potential for the development of highly sensitive diagnostic and targeted therapeutic agents. The particular size range of 10-100 nm gives nanoparticles extraordinary loading capacity for therapeutics and/or contrast agents as well as other functions such as targeting. Mutifunctional and multimodal nanoparticles have been developed as MRI, PET, photoacoustic and optical imaging agents, as well as photodynamic, photothermal, and chemotherapeutic agents among others [1-6]. Functionalization of nanoparticles is largely carried out by covalent bond formation using classical bioconjugation methods such as amide bond formation, addition or substitution reactions of thiols, hydrazone formation, and metal-thiol reactions, and more recently via various click reactions [7-10]. These strategies generally suffer from difficulty in controlling the extent of the reactions and in characterizing the products making it difficult to precisely tune the composition and properties of the nanoparticles. In addition, the covalent nature of the conjugations can inhibit their biodegradation. Functionality has also been incorporated through weaker and less specific non-covalent self-assembly mechanisms such as electrostatic interactions, micelle or liposome formation, and host guest interactions [11-14]. Nanoparticles produced in this way, however, have variable stability and are less well defined.
A potentially more promising approach to assembling nanoparticles with programmable composition and properties would be to use the higher specificity and tunable stability afforded by nucleic acid hybridization. Though nucleic acid hybridization has been extensively used for assembling materials for structural and analytical purposes [15-17], it has seen very little use for constructing highly functionalized nanoparticles for in vivo use. Nucleic acid hybridization has been shown to assemble targeting agents with imaging agents in vivo for pretargeting strategies aimed at improving specificity [18, 19]. More recently nucleic acid hybridization was used to pre-assemble PAMAM nanoparticles with antibodies for pretargeting cells in culture which were then recognized by a complementary radiolabeled nucleic acid [20]. We envision that nucleic acid hybridization could be used as a reversible click reaction to rapidly and combinatorially assemble more complicated NP containing any desired combination of targeting, imaging, therapeutic, endosome disrupting, and stealth functionalities (Figure 2.1).
Figure 2.1 Self-assembly of functionalized gold nanoparticles. Gold nanoparticles conjugated to thiolated ODNs are hybridized with any combination of functionalized PNAs to rapidly assemble a library of functionalized gold NP.

Gold nanoparticles are an ideal platform for nucleic acid directed self-assembly because they can be readily produced in a variety of sizes and shapes and easily derivatized with thiolated oligodeoxynucleotides (ODNs) [21-24]. Instead of using a functionalized complementary ODN to hybridize to the ODN on the gold NP, we decided to make use of functionalized PNAs (peptide nucleic acid) because their unnatural polyamide backbone confers a number of advantages over ODNs [25, 26]. PNAs are highly resistant to degradation by biological systems and also protect the DNA or RNA to which they are bound from degradation. They also hybridize to DNA with higher affinity than does DNA making it possible to use shorter nucleic acid sequences for assembly. Most importantly, they can be conveniently synthesized by solid phase automated peptide synthesis, which allows the easy preparation of PNA-peptide hybrids and the incorporation of a wide variety of amino acid analogues and auxiliary agents. Herein, we will describe the assembly of gold nanoparticles functionalized with PEG,
$^{64}$Cu-DOTA, and fluorescent molecules, and demonstrate that they behave like nanoparticles in vivo.

<table>
<thead>
<tr>
<th>ODN</th>
<th>TGGTGCTTTTGTGGATG</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODN-SH</td>
<td>TGGTGCTTTTGTGGATG-SH</td>
</tr>
<tr>
<td>ODNnc-SH</td>
<td>ACCACACGAAACACCTAC-SH</td>
</tr>
</tbody>
</table>

**Cy5-PNA**

**PNA-DOTA**

**PNA-Cy5**

**PNA-PEG$_3$**

**Figure 2.2** Functionalized ODNs and PNAs used in this study. All ODNs are written in a 5’→3’ direction and all PNAs in an amino → carboxy direction.
2.2 Results and Discussion

2.2.1 ODN-Gold Nanoparticle Synthesis

Citrate gold nanoparticles, citrate-AuNP, were synthesized by standard methods [27] and were 10.2 ± 0.8 nm in diameter as measured by TEM (Figure 2.3a). Dynamic light scattering gave a very similar hydrodynamic radius of 10.3 ± 2.6 nm (Figure 2.3a). The particles had an expected highly negative zeta potential of -22.5 ± 2.8 mV. Conjugation of the gold nanoparticles to a 3’-thiolated ODN (Figure 2.2) by standard methods [21], gave ODN-AuNP. TEM of a negatively stained sample showed particles with a diameter of about 16.1 ± 0.9 nm consisting of a dark core and a bright outer circle corresponding to the ODN layer (Figure 2.3b). Dynamic light scattering gave a similar hydrodynamic diameter of 16.2 ± 4.1 nm (Figure 2.3b). The thickness of the ODN layer was about 2.5-2.8 nm deduced from the difference in size of the ODN-AuNP and the citrate-AuNP from TEM and DLS experiments. The thickness of the ODN layer is less than half the length of a helical 18-mer ODN (6.3 nm) indicating that the ODN is not fully extended on the gold surface and may be partially contacting the surface. The zeta potential of the ODN-AuNP increased to -31.7 ± 0.1 mV due to the large number of negatively charged phosphate groups contributed by the ODN.
Figure 2.3 TEM pictures of gold NPs at various stages of construction. TEM of A) Citrate-AuNP, B) ODN18-AuNP, and C) PNA15-PEG₃/PNA15-DOTA-ODN18-AuNP with a PNA-PEG/PNA-DOTA ratio of 3:1. All three samples were stained with uranyl acetate. Panel C) was assembled from four representative pictures.
Figure 2.4 Dynamic light scattering analysis of gold NP at various stages of construction.
2.2.2 Sequence Specific Self-Assembly with PNA

Gold has been known as an effective fluorescent quencher and the fluorescent quenching efficiency decreases drastically as the distance between the fluorophore and the gold surface increases [28]. We utilized this unique property to demonstrate the self-assembly of the ODN-AuNP with PNA. We synthesized a 15-mer PNA that was labeled with Cy5 at its amino end, Cy5-PNA (Figure 2.2), which is complementary (antisense) to the ODN on the AuNP. Upon PNA-ODN hybridization, the fluorophore is positioned three nucleotides away from the gold surface, or about one nm if the PNA-ODN duplex were perpendicular to the surface [29]. This distance is close enough to cause significant fluorescent quenching [28]. As shown in Figure 2.5, when the Cy5-PNA was mixed with ODN-AuNP, the fluorescence of the Cy5 was almost completely quenched and remained so until the concentration of the Cy5-PNA-mer was about 90 times the concentration of the gold nanoparticle. Beyond this point, the fluorescence started to increase linearly indicating that all the complementary binding sites on the gold NP had been saturated. The gold NP without the ODN shell, however, also quenched the fluorescence of the PNA. To demonstrate that the quenching was due to sequence specific binding of the PNA to the ODN shell, and not simply to the gold surface, a gold NP bearing the non-complementary ODN, ODNnc (Figure 2.2) was synthesized. When titrated with Cy5-PNA very little quenching was observed (Figure 2.5).
**Figure 2.5** Fluorescence titration of ODN-AuNPs with Cy5-PNAs.  
A) Fluorescence of increasing concentration of Cy5-PNA in presence (■) or absence (□) of 5 nM ODN-AuNP. B) Fluorescence of increasing concentration of Cy5-PNA in (♦) buffer alone, or the presence of: (Δ) 10 nM ODN-AuNP; (▲) 10 nM ODNnc-AuNP; (■) 10 nM citrate-AuNP.
To determine the stability of the PNA·ODN hybrid on the gold NP, temperature dependent fluorescence measurements were made between room temperature and 85°C. Following an initial heating to 85°C to degas the sample, the fluorescent heating and cooling curves for ODN-AuNP·Cy5-PNA were quite reproducible and indicated that the complexes had a melting temperature of 67°C (Figure 2.6). This can be compared to a melting temperature of 71.5 °C for ODN·Cy5-PNA determined by temperature dependent absorbance measurements.

### 2.2.3 PEGylation of the ODN-Gold NP

For imaging specific targets *in vivo* it is important that the NPs are not recognized by the MPS (mononuclear phagocyte) system, often referred to in the literature as the RES (reticulo-endothelial) system, or taken up by non-target cells [30]. It has been found that PEGylation can greatly reduce binding of NP to proteins and confer stealth character [31]. To study the interaction of the PNA·ODN-AuNP with cells and the ability of PEGylation to reduce non-specific binding and uptake we synthesized a fluorescently labeled PNA for tracking the NP and a PEGylated PNA for conferring stealth character. To minimize quenching of the fluorescence, Cy5 was conjugated to the carboxy terminus of PNA to give PNA-Cy5 (Figure 2.2) via a carboxy-terminal lysine group so that the fluorophore would be directed away from the surface of the gold NP. To create a PEGylated PNA with sufficient PEG to cover the surface, the PNA was derivatized with three PEG2K molecules via acylation of three lysines at the carboxy terminus of PNA and spaced apart by two beta-alanines to give PNA-PEG₃ (Figure 2.2). The ODN-AuNP was hybridized with a stoichiometric amount of PNA with differing
ratios of PNA-PEG₃:PNA-Cy5. TEM analysis under negative staining of 75/25 ratio of PNA-DOTA/PNA-PEG₃:ODN-AuNP showed a dark center of about 12 nm, representing the gold core, surrounded by a narrower bright band than was seen with the ODN-AuNP, and a large and diffuse dark area of average diameter 41.7 ± 1.1 nm that must correspond to the ODN-PNA duplex linked to the PEG-2k layer. Dynamic light scattering gave a hydrodynamic radius of and 30.2 ± 9.1 nm which when subtracted from the original diameter of 10.1 nm for the citrate-AuNP gives an increased diameter of 17.1, or an increased radius of 8.5 nm. The increased radius corresponds very well to 6 nm for the PNA-ODN helix (18 bp × 0.33 nm/bp) [29] plus 3.8 nm corresponding to the Flory radius of PEG-2k [32]. Simple calculations suggest that there is ample surface area to accommodate the PNA·ODN duplex and the three PEG2K chains. A PNA·ODN duplex has a diameter of 2.2 nm corresponding to a cross-sectional area of 0.95 nm² which when multiplied by 100 ODNs/AuNP gives and area of 95 nm² which is 3 times less than the calculated surface area of 314 nm² for a 10 nm diameter gold particle. If fully extended, the PNA·ODN duplex would extend the radius of the gold NP by 6 nm, resulting in an outer surface area of 1520 nm², or 152 nm² per PNA·ODN duplex. Given that the Flory radius of PEG2K is 3.8 nm and that the center of radius for each PEG2K would be an additional 1 nm further out, then the available surface area is calculated to be 3157 nm². Since three PEG2K accommodate a cross-sectional surface area of 114 nm², the available surface would become saturated upon addition of about 28 PNA-PEG₃ units. Above this amount, one would expect the PEG chains to adopt a more extended, brush-like structure. In accord with this analysis, the zeta potential of the particle decreases to -4.1±0.4mV as the PEG layer significantly shields the charges [33].
Figure 2.6 Melting curves for Cy5-PNA•ODN-AuNP and Cy5-PNA-ODN. Fraction single strand form for Cy5-PNA•ODN-AuNP (●) from analysis of temperature dependent Cy5 fluorescence measurements, and for Cy5-PNA-ODN (○) from analysis of temperature dependent absorbance measurements. Data from two heating/cooling cycles were used to generate each melting temperature curve.
Figure 2.7 Effect of PEGylation on uptake of PNA-Cy5•ODN-AuNP by MCF-10A cells.  A) fluorescence imaging of MCF10A cells incubated with PNA-Cy5•ODN-AuNP and FAM-dextran (MW 25,000) . B) quantification of gold NP uptake by cells by ICP-MS.
2.2.4 Cellular Uptake of the PEGylated ODN-Gold NP

The interaction of the AuNPs with a normal human breast cell line (MCF10A) was examined by confocal microscopy and quantified by ICP-MS. As expected from previous studies with ODN-AuNP [34], Cy5-PNA-ODN-AuNP was internalized by MCF-10A cells efficiently in complete growth media without any cell permeation enhancing ligand (Figure 2.7A). The particle (red) colocalized with FAM-dextran (green) which is considered to enter the cells through an endosomal pathway. Upon complexation with PNA-PEG$_3$, the uptake decreased with increasing density of the PNA-PEG$_3$ on the gold NP (Figure 2.7B). MCF-10A uptake was reduced by 60% at 60% of PEGylation and further reduced by 80% with almost complete PEGylation. The required percentage of ODN could possibly be further driven down by utilizing PEG of higher molecular weight or higher density per PNA.

2.2.5 Bio-Distribution Studies of the PEGylated Gold NP.

To determine whether or not the non-covalently assembled PNA-PEG$_3$-ODN-AuNP would remain intact in vivo, we carried out biodistribution studies with a radiolabeled nanoparticle. To radiolabel the particle, we synthesized a PNA functionalized with DOTA at the carboxy end of PNA via coupling of tri-$t$-butyl-DOTA ester to a carboxy-terminal lysine to give PNA-DOTA (Figure 2.2) following deprotection. PNA-DOTA was first radiolabeled with $^{64}$Cu, purified, and then hybridized with ODN and ODN-AuNP. Biodistribution of the labeled species at 1 h, 4 h and 24 h post injection (p.i) showed very similar profiles. Both PNA-DOTA-$^{64}$Cu and PNA-DOTA-$^{64}$Cu-ODN hybrid were cleared very fast from the system through the renal
system. There was some minor accumulation in the liver, lung and gastrointestinal tract (stomach and intestine), and negligible uptake in other organs (Figure 2.8). At 1 h p.i., the blood retention of PNA-DOTA\textbullet^{64}\text{Cu} and PNA-DOTA\textbullet^{64}\text{Cu}\cdot\text{ODN} hybrid were both less than 0.5 \% ID/g while the kidney uptakes were about 5\%. The liver uptakes were constant over 24 h, at less than 2\% ID/g. PNA-DOTA\textbullet^{64}\text{Cu}/PNA-PEG_{3}\cdot\text{ODN-AuNP} also showed rapid clearance from the systemic circulation (0.31 ± 0.09 \%ID/g at 1 h p.i.) but much higher retention in liver. The liver uptake was dominant but gradually decreased during the 24 h with 32 ± 3\% ID/g, 28 ± 7\% ID/g, and 21 ± 2\% ID/g at 1 h, 4 h and 24 h p.i., respectively. The spleen accumulations, however, were stable with about 10\% ID/g through the study. The sharp contrast between the biodistribution profile of PNA-DOTA\textbullet^{64}\text{Cu} by itself or hybridized to ODN and when it was hybridized to PNA-PEG_{3}\cdot\text{ODN-AuNP} proves that both the ODN-Au conjugate and PNA/ODN hybridization were stable under physiological conditions. The biodistribution at 24 h (21\% liver, 11\% spleen, 4\% kidney) was very similar to that reported in mice at 48 h for a 20 nm gold nanoparticle coated with thiotic acid-anchored 5K PEG with a hydrodynamic radius of 45 nm (30\% liver, 15\% spleen and 5\% kidney)[35]. It is less than optimal, however, for potential \textit{in vivo} delivery application due to its rapid depletion from the blood. Further surface passivation will be needed before it can be used for cell specific targeting, and is the focus of our further work.
Figure 2.8 Biodistribution studies of AuNP and components.

A) PNA-DOTA-\(^{64}\)Cu, B) PNA-DOTA-\(^{64}\)Cu•ODN and

C) PNA-DOTA/PEG\(_3\)•ODN-AuNP for 1h (a), 4h (b) and 24h (c) p.i.

A) PNA-DOTA

B) PNA-DOTA•ODN

C) PNA-DOTA/PEG\(_3\)•ODN-AuNP
2.3 Conclusions

In summary, we have developed a DNA hybridization-based self-assembling nanoparticle system. We have shown that the oligodeoxynucleotide protected gold nanoparticle assembles readily with functionalized complementary peptide nucleic acids in a sequence specific manner. We also showed that hybridization yields a stable multifunctional nanoparticle under physiological conditions and that non-specific cell uptake can be greatly reduced by PEGylation. The resulting non-covalently PEGylated gold nanoparticle showed a similar biodistribution pattern in mice to that of a dithiol bound PEGylated gold nanoparticle of a similar size. These results suggest that DNA-directed self-assembly could be used for the combinatorial development of and optimization of nanoparticle-based imaging and therapeutic agents.

2.4 Experimental

Materials  All solvents and chemicals were purchased from Sigma-Aldrich and used without further purification unless otherwise indicated. Fmoc-Lys(mtt)-OH was purchased from EMD Chemicals, Inc. PEG2K-NHS ester was purchased from JenKem Technology Co., LTD (M-PEG-SCM, MW2000). Cy5-NHS ester was synthesized by Jillian Smith according to a published procedure [36]. The MCF-10A cell lines were purchased from ATCC and cultured under the guidelines provided by ATCC. All cell culture media were purchased from Invitrogen, Inc.

Size and zeta potential measurements Samples for transmission electron microscopy (TEM) measurements were dropped onto PELCO formvar/carbon coated copper grids (Ted Pella, Inc.). After 15 min, the excess colloid was removed by filter paper and 2%
uranyl acetate was then dropped on the same grid and incubated for another 2 min. Then the excess solution was again removed by filter paper. The resulting grids were imaged by a FEI Spirit Lab6 TEM. Hydrodynamic diameters and zeta potential were determined by Malvern Zetasizer Nano. Surface plasma resonance bands of gold nanoparticles were measured by Varian Cary 100.

_Synthesis of ODN capped gold nanoparticles ODN-AuNP and ODNnc-AuNP_. The ODN capped gold nanoparticles were synthesized according to a reported procedure [21]. Briefly, sodium citrate was added to boiling chloroauric acid solution to yield gold nanoparticles about 11 nm in size [27]. TGGTGTGCTTTGTGGATG-O-C₃H₆-SS-C₃H₆-OH and ACCACACGAAACACCTAC-O-C₃H₆-SS-C₃H₆-OH were purchased from IDT and reduced with tris(2-carboxyethyl)phosphine at room temperature for one hour to produce ODN-SH and ODNnc-SH respectively. The ODNs were then isolated by ethanol precipitation and redissolved in water. These freshly prepared thiolated ODN or ODNnc solutions were added to the citrate gold nanoparticles (3 nmol per 1 mL of 7 nM citrate•AuNP). After 20 min, 10% SDS solution was added to bring the mixture to 0.1% SDS. Phosphate buffer (0.1 M, pH 7.4) was added to bring the mixture to 0.01 M phosphate and sodium chloride (2.0 M) was added in 30 min intervals to bring the final mixture to 0.3 M NaCl. The final mixture was gently shaken for 24 h in the dark under room temperature. The ODN-AuNP or ODNnc-AuNP was centrifuged (13500 rcf, 30 min; 3X) and resuspended in PBS.
Determination of the extinction coefficient of citrate•AuNP and ODN-AuNP: 10uL of citrate•AuNP or ODN-AuNP was dissolved in 90uL of freshly made aqua regia under room temperature. The mixture was diluted to 5mL with 1% nitric acid. The standard solutions was prepared with HAuCl₄ standard (Perkin Elmer) at 0.1, 0.5, 1, 5, 10 and 100 μg/L in 1% nitric acid. The gold concentration was measured on Perkin Elmer Elan DRC II ICP-MS using Bi as external reference. The concentration of gold nanoparticles was calculated by the following equation.

\[
\text{Particle Mass} = \rho \times V = \rho \times \frac{4}{3} \pi r^3
\]

\[
\text{Particle Concentration} = \frac{\text{Gold concentration (uL)}}{\text{particle mass (ug) \times N}_A}
\]

\(\rho\) is the density of gold, 19.3g/cm³; \(r\) is the radius of the AuNP measured by TEM, 5.1nm; \(N_A\) is Avogadro Constant.

Another 100uL of citrate•AuNP or ODN-AuNP was diluted with 0, 100, 300, 700 or 1500uL 1X PBS the absorbance of each colloid at 520nm for citrate•AuNP or 524nm for ODN-AuNP was measured and plotted against the corresponding particle concentration calculated from the equation. The extinction coefficient was calculated from linear regression. (Figure A 2.1)

Determination of the ODN loading of the ODN gold nanoparticles ODN-AuNP and ODNnc-AuNP. ODN-AuNP or ODNnc-AuNP (0.5 mL of 10 nM, determined by the absorbance at 520nm) was dissolved in 2 mM KCN solution and 1 μL of this solution was mixed with 1 μL of \(^{32}\)P-ATP, 2 μL of T4 polynucleotide kinase buffer A, 1 μL of T4 polynucleotide solution and 15 μL of water, incubated at 37 °C for an hour and then boiled for 10 min. ODN and ODNnc of known concentrations were also labeled following
the same procedure. $^{32}$P-labeled ODN or ODNS 50 nM and 1 µL of the radiolabeled ODN-AuNP solution were then separately incubated with known amounts of antisense PNA in Tris buffer (10 mM, pH 7.5) and NaCl (50 mM), boiled for 5 min and slowly cooled down to room temperature. The mixtures was electrophoresed on a 10% native PAGE and the radiolabeled bands were imaged on a Biorad Personal Molecular Imager and quantified by Biorad Quantity One software. The titration curve between ODN or ODNnc and PNA was used to determine the concentration of ODN in the dissolved ODN-AuNP solution. The concentration he ODN-AuNP colloid was then determined by the absorbance at 520 nm (Figure A 2.2).

*General procedure for PNA synthesis* All PNAs and conjugates were synthesized on an Expedite 8900 PNA synthesizer on 2 µmol of Fmoc-PAL-PEG-PS according to the standard automated Fmoc PNA synthesis procedure utilizing commercial PNA and peptide monomers (Panagene Inc., Korea). Following the final step of synthesis, the resin was washed with dry CH$_2$Cl$_2$ (2 × 3 mL), followed by drying under a stream of N$_2$. The resin was then shaken in a vial with trifluoroacetic acid (160 µL) and m-cresol (40 µL) at room temperature for 1.5 h twice to release and deprotect the PNA. The solution from each deprotection was separated from the resin, combined and added to ice-cold Et$_2$O (1.5 mL). The resulting precipitate was collected by centrifugation and purified by reverse-phase HPLC on a Waters XBridge Prep C18 column (130 Å) with buffer A (0.1% TFA in H$_2$O) and buffer B ( 0.1% TFA in CH$_3$CN) on a Beckman System Gold instrument equipped with a UV-vis array detector. The flow rate was 2.5 mL/min and the gradient was 1% buffer B/min starting from 5% buffer B. The fractions were collected
and concentrated to dryness in a SpeedVac (Savant) and characterized by UV-vis and MALDI-TOF which was carried out on a PerSpective Voyager mass spectrometer with α-cyano-4-hydroxycinnamic acid as the matrix and insulin as the internal reference.

*Synthesis of Cy5-PNA.* PNA CAT CCA CAA AGC ACA CCA was synthesized with removal of the amino terminal Fmoc group. Cy5-NHS ester (20 µmol) were dissolved in anhydrous DMF (100 µL) and mixed with DIPEA (2 µmol). The mixture was incubated with the solid support under room temperature for 2 h. Then the solid support was separated, washed with DMF. The Cy5-PNA was cleaved and purified by the general procedure described above. MALDI: exact [M + H]^+ expected 4413.0, found 4415.2 (Figure A 2.3).

*Synthesis of PNA-DOTA and PNA-Cy5.* CAT CCA CAA AGC ACA CCA-Lys(Mtt) was synthesized with deprotection and capping of the the N terminal amine with 5% acetic anhydride and 6% lutidine in DMF. The Mtt protecting group was removed by washing the solid support with 1 mL of 1% TFA in DCM several times, 5 min each time, until no yellow color was detected. The solid support was then washed with DMF until the eluant was no longer acidic, as measured by wet pH paper. For PNA-Cy5 synthesis, Cy5-NHS ester (20 µmol) and DIPEA (2 µmol) were dissolved in 100 µL DMF and incubated with the solid support at room temperature for 2 h. For PNA-DOTA synthesis, tri-tert-butyl 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate (20 µmol), EDC (20 µmol) and DIPEA (2 µmol) in 100 µL DMF was incubated with the solid support under room temperature overnight. After both syntheses, the reaction mixtures were removed and the
solid supports were washed with 1 mL of DMF four times. PNA-DOTA or PNA-Cy5 was cleaved and purified by HPLC as described above. MALDI of PNA-DOTA: [M + Na]$^+$ expected 5365.3, found 5364.8 (Figure A 2.5). PNA-Cy5 did not give rise to an identifiable MALDI peak but PAGE analysis showed hybridization of PNA-Cy5 with ODN (Figure A 2.4).

**Synthesis of PNA-(PEG2K)$_3$.**

CATCCACAAAGCACACCA-Lys(Mtt)-bAla-bAla-Lys(Mtt)-bAla-bAla-Lys(Mtt) was synthesized followed by removal of the amino terminal Fmoc group, the amino terminus was acetylated with 5% acetic anhydride and 6% lutidine in DMF. The PNA was cleaved from the solid support and purified by HPLC. PEG2K-NHS ester (10 μmol) was dissolved in 100 μL DMF and mixed with 1 μL of DIPEA. 0.0963 μmol of Ac-PNA-(Lys-bAla)$_2$Lys was dissolved in 10 μL DMF and added to the PEG solution, 1 μL at a time. Following each addition, the pH of the reaction of the mixture was checked with wet pH paper and additional DIPEA was added to maintain the basicity. The reaction mixture was incubated under 55°C for 20 min before the next aliquot was added. After all the PNA was added, the reaction mixture was left under room temperature overnight and then precipitated with ether. The crude product was separated with HPLC. Each fraction was collected, hybridized with $^{32}$P labeled ODN and characterized by native PAGE (Figure A 2.6).

**Fluorescence quenching studies** Various amounts of 36.6 μM Cy5-PNA were added to 60 μL of 5.23 nM ODN-AuNP or ODNnc-AuNP or citrate•AuNP) in 1X PBS. The mixture
was incubated under room temperature for 5 min and then the fluorescent was measured at 663 nm with excitation at 633 nm. The fluorescence of Cy5-PNA in 1X PBS was measured at each concentration as control.

**Fluorescence melting temperature studies** The Cy5-PNA·ODN-AuNP complex (0.357 μM Cy5-PNA, 4.7 nM ODN-AuNP in 1X PBS buffer) was heated from 25° to 80 °C at 1 °C per min and held at 80 °C for 5 min and then cooled down to 25 °C. After 5 min, the heating-cooling cycle was repeated. Fluorescence was excited at 633 nm and detected at 663 nm. The temperature-dependence of fluorescence of Cy5-PNA was measured under the same condition in the absence of ODN-AuNP (Figure A 2.7).

**Absorbance melting temperature of Cy5-PNA·ODN:** The complex from Cy5-PNA (26.4 μM) and ODN (40 μM) in 1X PBS buffer was heated from 25° to 85 °C at 1 °C per min and held at 85 °C for 5 min and then cooled down to 25 °C. After 5 min, the heating-cooling cycle was repeated. Absorbance was measured at 260 nm.

**Cellular uptake of ODN-AuNP** MCF10A cells were seeded in 35 mm glass bottom dish and allowed to grow to 70% confluence. PNA50S-Cy5 (1 μL of 1.18 μM) was mixed with 15 μL 10 nM ODN50-AuNP and incubated at 50 °C for 10 min. After cooling down to room temperature, the mixture was mixed with 135 μL of fresh complete media containing 0.5ug/mL FAM-dextran (MW 25,000, Invitrogen). Then the cell media was removed and replaced with this mixture and incubated at 37 °C with 5% CO₂ for 6 h. After 5 h, 2 μL of 10 μg/mL Hoechst 3342 was added to the mixture. After 6 h, the
mixture was removed and the cells were washed with 1X PBS buffer 5 times and then incubated with 2 mL of fresh media. The cellular uptake was visualized by confocal microscopy.

Reduction of nonspecific cell binding by PEGylation: MCF10A cells were seeded in a 12 well plate and grown to 90% confluence. PNA-PEG₃ was mixed with ODN-AuNP in various ratios. The mixtures were incubated at room temperature for 30 min and added to the cells with complete cell media. The final concentration of AuNP was 0.9 nM. After 6 h of incubation at 37°C, cell media was removed and the cells were washed with PBS twice. The cells were trypsinized, counted with hemacytometer and centrifuged. Each cell pellet was digested with 100 µL nitric acid and hydrogen peroxide mixture (9:1, v/v) overnight followed by addition of 200 µL aqua regia. The resulting solution was then diluted to 7 mL with 1% nitric acid and analyzed by Perkin Elmer Elan DRC II ICP-MS using Bi as external reference.

Radiolabeling: Radiolabeling and experiments were done by Yongjian Liu and Chad Jarreau from Department of Radiology, Washington University School of Medicine. Copper-64 (t₁/₂=12.7h, β⁺=17%, β⁻=40%) was produced on the Washington University Medical School CS-15 cyclotron by the ⁶⁴Ni(p,n) ⁶⁴Cu nuclear reaction at a specific activity of 1.85-7.40 GB1/µg at the end of bombardment[37]. PNA-DOTA (320 pmol) was incubated with ⁶⁴Cu (185 MBq) in 100 µL of 0.1 M ammonium acetate buffer (pH 5.5) at 65 °C for 1 h, with a yield of 52.5 ± 2.4%. The PNA-DOTA⁶⁴Cu was purified by solid-phase extraction after ethylenediaminetetraacetic acid (EDTA, 10 mM in 50 mM pH
7.4 phosphate buffer) and the specific activity was 301 ± 14 MBq/nmol [38]. The PNA-DOTA used for annealing with ODN and ODN-AuNP was radiolabeled and purified following the same procedure with the specific activity of 285 ± 11 MBq/nmol. Then 37 MBq of PNA-DOTA\(^{64}\)Cu was mixed with ODN. Another 37 MBq (9.1 nmol) of PNA-DOTA\(^{64}\)Cu was mixed with ODN-AuNP (0.52 nmol) followed by addition of PNA-(PEG2K)_3 (27.3 nmol). The mixture was purified with Zebu size exclusion column (GE Healthcare).

_Bio-Distribution._ Bio-distribution study was done by Yongjian Liu and Chad Jarreau from Department of Radiology, Washington University School of Medicine. All animal studies were performed in compliance with guidelines set forth by the NIH Office of Laboratory Animal Welfare and approved by the Washington University Animal Studies Committee. Biodistribution studies were performed in male C57BL/6 mice weighing 20-25 g (n=4/group) and about 370 kBq of PNA-DOTA\(^{64}\)Cu, PNA-DOTA\(^{64}\)Cu·ODN and PNA-DOTA\(^{64}\)Cu, PEG-ODN50-AuNP in 100 µL saline (APP Pharmaceuticals) was injected via the tail vein. The mice were anesthetized with inhaled isoflurane and were re-anesthetized before being euthanized by cervical dislocation at each time point (1 h, 4 h, and 24 h) post injection (p.i.). Organs of interest were collected, weighed and counted in a well gamma counter (Beckman 8000). Standards were prepared and measured along with the samples to calculate the percentage of the injected dose per gram of tissue (%ID/gram) [39].
Acknowledgments. This work was supported by Kay Yow V Foundation Grant to JST and MJW, and in part by a PEN grant (HHSN268201000046C) and by the Washington University NIH Mass Spectrometry Resource (Grant No. P41 RR000954). We thank Jillian Smith for synthesizing Cy5-NHS ester. We also thank Nano Research Facility of Washington University for providing the DLS, TEM and ICP-MS facility.

2.5 Appendix

Figure A 2.1 Determination of extinction coefficient of citrate•AuNP (●) and ODN-AuNP (×)
Figure A 2.2 Determination of the average ODN loading of the ODN·AuNP. A) Titration of ODN of known concentration and ODN from ODN·AuNP with PNA-DOTA analyzed by gel electrophoresis. B) Calibration curve generated from Lane 1-6 of A)
Figure A 2.3 Preparative HPLC and MALDI-TOF mass spectrum of Cy5-PNA
Figure A 2.4 Preparative HPLC trace and gel binding assay of PNA-Cy5.

The first lane on the left is ODN alone. Upper bands are ODN•PNA-Cy5 hybrid. Lower bands are free ODN. Only the fraction eluted at 39mins hybridized with the ODN and thus was used to label ODN-AuNP.
Figure A 2.5 Preparative HPLC and MALDI-TOF mass spectrum of PNA-DOTA
Figure A 2.6 Preparative HPLC trace and gel binding assay of PNA-PEG₃. Lane 1: ODN; Lane 2: PNA-PEG; Lane 3-5: fraction 1-3. Fraction 1-3 all hybridized with ODN. Fraction1•ODN hybrid showed the same mobility as PNA-PEG•ODN hybrid; Fraction2•ODN was a mixture of PNA-PEG and a species with higher molecular weight. Fraction3•ODN showed the lowest mobility, indicating that three PEG chains have been attached on the PNA.
Figure A 2.7 A) Original melting curves for Cy5-PNA-ODN-AuNP B) Temperature-dependent fluorescence intensity of Cy5-PNA.
2.6 References


Chapter 3

Cationic Peptide Functionalized Gold Nanoparticles

3.1 Introduction

Gene transfection is the process of introducing foreign DNA into cells and has emerged as a powerful technique for studying gene functions, treating and controlling diseases, and cell engineering among other applications [1]. A virus is nature’s solution for high gene transfection efficiency and viral methods have been successfully utilized in many applications. However, viral methods have an intrinsic problem of immunogenicity and induction of mutation responses [2]. Non-viral methods that utilize physical or chemical methods have attracted more and more interest as gene transfection agents [3, 4]. Physical methods like particle bombardment, electroporation, and fine-needle injection utilize physical forces to deliver a gene into a cell. Chemical methods use cationic carriers such as cationic peptides, cationic lipids, and cationic synthetic polymers to form complexes with DNA and penetrate cellular membranes. However, many of the existing cationic non-viral transfection agents also show high cytotoxicity. Development of new non-viral methods to facilitate high DNA binding and transfection efficiency with low cytotoxicity is strongly desirable.

In the past two decades, a number of approaches to gene transfection have been developed based on gold and other inorganic nanoparticles [5-12]. Most of the cationic gold nanoparticles rely on thiolated primary or tertiary amines as capping agents to provide charges. Sandhu et al. reported gene transfection using gold nanoparticles
modified with N,N,N-trimethyl(11-mercaptoundecyl) ammonium chloride and alkylthiol with several chain lengths [10]. Niidome et al. reported a gold nanoparticle based transfection agent with 2-aminoethanthiol as a capping agent. These nanoparticles suffer from poor DNA binding affinity due to the inherent low charge density and the low binding affinity between amino group and phosphate group. The guanidinium group is a much better binder for ODNs and various oligoarginine derivatives have been developed for gene transfection due to their high ODN binding affinity and cell permeation ability [13]. In this chapter, a series of oligoarginine peptide modified gold nanoparticles were prepared and characterized. The effect of the length of the oligoarginine chain and the function of other amino acids in the sequence were investigated.

3.2 Results and Discussion

3.2.1 Arginine-rich Peptide Modified Gold Nanoparticle Synthesis

Chloroaauric acid (HAuCl₄) was reduced by sodium borohydrate (NaBH₄) in the presence of arginine-rich peptides. The resulting nanoparticles are similar in size and surface plasmon band. Figure 3.1 is the TEM image of all the cationic gold nanoparticles (cAuNP) synthesized. Table 3.1 is the summary of the diameters and surface plasmon resonance band of all the cAuNP studied. AuNP-CTTR5 measured 11.6 ± 1.8 nm; AuNP-CTTR9 measured 17.0 ± 2.6 nm; AuNP-CTTR13 measured 13.5 ± 2.4 nm; AuNP-CVVR9 measured 12.1 ± 1.9 nm; AuNP-CR13 measured 11.8 ± 3.1 nm; AuNP-CTTR17 measured 10.9 ± 2.1 nm. The cAuNPs are smaller compared to gold nanoparticles synthesized by similar methods [14, 15], presumably due to more surface charges provided by the arginines in the sequences. The zeta potential of AuNP-CTTR9 was 29.4 ± 2.4 mV; AuNP-CVVR9 was 41.2 ± 4.9 mV; AuNP-CR13 was 26.8 ± 2.9 mV.
Figure 3.1 TEM images of cAuNP studied in this chapter. A) AuNP-CTTR5; B) AuNP-CTTR9; C) AuNP-CTTR13; D) AuNP-CR13; E) AuNP-CVVR9; F) AuNP-CTTR17.
**Figure 3.2** Absorption of the cAuNPs. ( ) AuNP-CTTR5; (×) AuNP-CTTR9; (★) AuNP-CTTR13; (+) AuNP-CR13; (■) AuNP-CVVR9; (○) AuNP-CTTR17. All the absorption curves are normalized to 1 at SPB.

**Table 3.1** Diameter and surface plasmon band of each cAuNP.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Diameter (nm)</th>
<th>SPB (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AuNP-CTTR5</td>
<td>11.6 ± 1.8</td>
<td>528</td>
</tr>
<tr>
<td>AuNP-CTTR9</td>
<td>17.0 ± 2.6</td>
<td>532</td>
</tr>
<tr>
<td>AuNP-CTTR13</td>
<td>13.5 ± 2.4</td>
<td>529</td>
</tr>
<tr>
<td>AuNP-CR13</td>
<td>11.8 ± 3.1</td>
<td>523</td>
</tr>
<tr>
<td>AuNP-CVVR9</td>
<td>12.1 ± 1.9</td>
<td>528</td>
</tr>
<tr>
<td>AuNP-CTTR17</td>
<td>10.9 ± 2.1</td>
<td>528</td>
</tr>
</tbody>
</table>
3.2.2 Stability of the cAuNPs in Different Ionic Strengths

High ionic strength is known to induce aggregation of electrostatically stabilized colloids as counter ions form ion pairs with the surface ions and thus neutralize the charges. For transfection purposes, the colloid needs to be stable at or above the inter- and intra-cellular salt concentration, which could be over 0.2M [16]. Therefore, the stability against increasing salt concentration is an important measurement of colloid stability. We used dynamic light scattering (DLS) to measure the increase in diameter of the cAuNPs as an indication of aggregation. Figure 3.3 shows that all the cAuNPs stayed well dispersed at 0.5 M of NaCl. At 0.6 M NaCl, AuNP-CTTR5 started to aggregate. The stability of cAuNPs increased as the number of arginine per sequence increased. AuNP-CR13 showed the best stability and it remained non-aggregated at 1 M NaCl. When threonine was replaced with valine, the stability appeared to be lower. When threonine was removed from CTTR13, the stability increased. This result is not very consistent with previous reported studies [17], maybe due to the influence of arginines at the C terminus of the peptides.
3.2.3 Stability of the cAuNPs as a Function of pH

To evaluate the suitability of the cAuNPs as potential in vitro transfection agents, we measured the stability of the cAuNPs under different pHs in 10 mM phosphate buffer with DLS. As shown in Figure 3.4, all of the cAuNPs remained dispersed below pH 5. However, as the basicity increased to pH 6, all the cAuNPs except for AuNP-CR13 started to aggregate. At physiological pH, all the cAuNPs showed various degrees of aggregation and the colloid visibly turned purple. In general, the stability increases as number of arginines per sequence increases. The observed aggregation at pH 7 was unexpected since the arginine is the most basic amino acid among all natural amino acids.
acids. The pKa of the guanidinium group of the side chain of arginine is 12.8, so nearly 100% of the guanidinium groups should be protonated at pH 7. To further probe the influence of pH, 10mM HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) buffer (pH 7.0 and 7.5) and 10mM borate buffer (pH 8.0 and 8.5) were also tested (Table 3.2). Unlike phosphate buffer, HEPES buffer did not induce aggregation at pH 7 and pH 7.5. The colloid remained well dispersed even at pH 8 and pH 8.5 in borate buffer. This observation indicates that the pH-induced aggregation is largely due to the strong affinity between guanidinium group and phosphate group (Figure 3.5). As the pH increases, more phosphate ions are deprotonated and form hydrogen bonds with the guanidinium groups and hence cause inter-particle bridging. HEPES and borate, on the other hand, do not form strong hydrogen bonds with arginines.

**Figure 3.4** Diameters of cAuNPs in 10 mM phosphate buffer of different pHs. (○) AuNP-CTTR5; (×) AuNP-CTTR9; (●) AuNP-CVVR9; (■) AuNP-CTTR13; (◆) AuNP-CR13.
**Figure 3.5** Proposed interaction between guanidinium groups and phosphate groups.

**Table 3.2** Summary of the hydrodynamic diameters of AuNP-CR13 in different buffers and pH.

<table>
<thead>
<tr>
<th>Buffer (10mM)</th>
<th>Phosphate</th>
<th>HEPES</th>
<th>Borate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>8.0</td>
</tr>
<tr>
<td>pH 7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>8.5</td>
</tr>
<tr>
<td>d (nm)</td>
<td>707.9</td>
<td>676.9</td>
<td>16.5</td>
</tr>
<tr>
<td>d (nm)</td>
<td></td>
<td>15.7</td>
<td>17.3</td>
</tr>
<tr>
<td>STDEV</td>
<td>206.3</td>
<td>4.4</td>
<td>5.2</td>
</tr>
<tr>
<td>STDEV</td>
<td>209.4</td>
<td>4.2</td>
<td>5.0</td>
</tr>
</tbody>
</table>
3.2.4 Reversibility of the Phosphate-Induced Aggregation

To better understand the nature of the phosphate-induced aggregation, acid was added to the aggregated colloid. When the pH dropped below 5, the colloid immediately returned to the original red color, indicating that the particles were dispersed again. TEM was performed on the colloid at pH 7.5 and pH 3 (Figure 3.6). The images clearly showed that the aggregation was completely reversible. This enabled the colloid to be purified conveniently by inducing aggregation at higher pH. When the supernatant was removed and the aggregate was washed, the colloid could then be re-dispersed with the addition of acid.

![TEM images](image)

**Figure 3.6** TEM images of AuNP-CTTR9 before and after acid addition
A) at pH7.5; B) acidified to pH 3 by addition of HCl.

3.2.5 ODN Binding Ability of the cAuNPs

ODN binding ability of the cAuNPs was evaluated by a gel retardation essay. cAuNPs were mixed with $^{32}$P labeled ODN in different weight ratios and separated by
native PAGE (**Figure 3.7**). Peptide CTTR13 was also tested as a control to determine the retardation effect of cationic peptide alone. All the cAuNPs showed different gel shift patterns than the peptide alone, indicating that the retardation effect was because of assembly of the peptides on the cAuNP. Except for the case of AuNP-CTTR17, the ODN binding ability increased as the arginine content of the capping peptide increased. Among all the cAuNP tested, AuNP-CR13 showed the highest ODN binding affinity, which bound the majority of the ODN at 1:1 w/w ratio. The presence of threonine or valine in the sequences did not seem to affect the binding ability.

![Figure 3.7](image_url)

**Figure 3.7** Gel retardation assay of cAuNP/ODN complexes at different AuNP/ODN weight ratios. Radioactive $5'\text{-}^{32}\text{P}$-labeled d(TGTGACACACAGCGCTACAA) (25 nM) was incubated with cAuNPs in 10 mM Tris buffer (pH 7.5) and 50 mM NaCl for 30 min and then loaded to 10% native polyacrylamide gel. The band at the top of the gel is the ODN bound to the cAuNP, and the band at the bottom is free ODN.

### 3.2.6 cAuNP Cytotoxicity

To evaluate the cytotoxicity of the two best ODN binding cAuNPs, AuNP-CVVR9 and AuNP-CR13 on pLuc705 HeLa cells, the MTT assay was employed (**Figure**)
This assay is performed by adding a premixed, optimized Dye Solution to cell culture in a 96-well plate. During a 4-hour incubation, living cells convert the MTT tetrazolium component of the Dye Solution into a formazan product. The Solubilization/Stop Solution is then added to the culture wells to solubilize the formazan product, and the absorbance at 570 nm is recorded using a 96-well plate reader. For comparison, peptides CVVR9 and CR13 were also evaluated simultaneously (Figure 3.9). The cytotoxicity of arginine-rich cell permeating peptides is well documented [18, 19], which also agrees with our observation. The cytotoxicity increased dramatically for CR13 as the concentration increased, while CVVR9 showed much less but significant toxicity. Interestingly, AuNP-CVVR9 and AuNP-CR13 did not show any toxicity at all even at a very high concentration. We estimated that the peptide strands were packed on the gold surface at a density of about 500 peptides/AuNP. Both AuNP-CVVR9 and AuNP-CR13 showed much higher cell viability than the peptide alone at an equivalent concentration. This is likely because that the membrane rupture caused by oligoarginine on the cAuNP is localized compare to the global damage caused by free peptide and a localized rupture may be easier to recover from. This observation indicates that cAuNP could potentially provide much higher DNA binding strength and cell penetrating ability with much lower toxicity.
Figure 3.8 Relative cell viability of pLuc705 HeLa cells in the presence of various concentrations of AuNP-CVVR9 and AuNP-CR13. “Cell” is the negative control with no additives.

Figure 3.9 Relative cell viability of pLuc705 HeLa cells in the presence of various concentrations of (☒) CVVR9 and (☐) CR13.
3.2.7 Preparation of Peptide Gold NP by a Ligand Exchange Method

3.2.7.1 Synthetic Approach

Instead of forming the peptide nanoparticles by incubating the gold salt with the peptide during reduction with NaBH₄, we decided to see if ligand-peptide exchange of gold nanoparticles fabricated by other methods would also be feasible. Gold nanoparticles made from citrate reduction (Figure 3.10A) and CTAB seeding-growth method (Figure 3.10C) were tested as templates for peptide assembly. After ligand-peptide exchange, both colloids retained the low polydispersity of the original nanoparticle. The CTAB•AuNP appeared to be a better candidate for cAuNP synthesis than citrate•AuNP because a much larger amount of the peptide is required to be added to negatively charged citrate•AuNP to prevent aggregation. In comparison, the positively charged surfactant CTAB is a stronger stabilizer and more compatible with positively charged peptides. The displaced CTAB in the colloid could be removed by centrifugation. We find this approach to be much more convenient and versatile than the direct reduction method. The size of the colloid is more controllable and tunable in the seed-growth process and a combinatorial library of peptide-AuNP could be prepared quickly.
Figure 3.10 TEM images of citrate gold nanoparticles A) before and B) after CR13 exchange. CTAB gold nanoparticles C) before and D) after CR13 exchange.

3.2.7.2 Choice of Peptide

To further tune the surface properties of the cAuNP, a lysine rich peptide, K10Y2C was synthesized. Three colloids were prepared by exchanging with R13C only, R13C-AuNP (Figure 3.11A), K10Y2C only, K10Y2C-AuNP (Figure 3.11C), and equal amount of K10Y2C and R13C together, R13C/K10Y2C-AuNP (Figure 3.11B), with
CTAB•AuNP following the same procedure. All three nanoparticle colloids retained the polydispersity of the original CTAB•AuNP.

![Figure 3.11](image1.png)

**Figure 3.11** TEM images of A) R13C-AuNP; B) R13C/K10Y2C-AuNP; C) K10Y2C-AuNP made from CTAB•AuNP.

### 3.2.7.3 Properties of the New cAuNP

A series of experiments were conducted to evaluate the properties of R13C-AuNP, K10Y2C-AuNP and R13C/K10Y2C-AuNP synthesized with the new approach. **Figure 3.12** shows that R13C-AuNP (CTAB) behaves in the same way as R13C-AuNP (direct reduction) in phosphate buffers. However, K10Y2C-AuNP did not aggregate at all even in pH 8 phosphate buffer, which further supports the idea that the aggregation of arginine-rich gold nanoparticle in phosphate buffer was due to the affinity between phosphate groups and guanidinium groups. The hybrid R13C/K10Y2C-AuNP exhibited intermediate stability, starting to aggregate moderately at pH7.

R13C-AuNP from the CTAB method showed similarly strong ODN binding ability shown by R13C-AuNP from the direct reduction method (**Figure 3.13**). When all the arginine was replaced with lysine, the binding ability decreased dramatically. Again, the hybrid R13C/K10Y2C-AuNP showed intermediate affinity, though not as high as what would be expected for 50% R13C loading, which indicates that the actual peptide
composition on the gold surface might not be the same as that used in the synthesis and K10Y2C might be more preferable.

To rapidly and quantitatively evaluate the cell transfection efficiencies of all three AuNPs, we adopted the luciferase splice correction assay developed by Kole and coworkers. This assay relies on a luciferase gene (pLuc705) that results in a longer, mis-spliced mRNA that encodes a defective luciferase. In the presence of a phosphorothioate 2′-O-methyl- oligodeoxyribonucleotide (ps-MeON) CCUCUUACCUCAGUUACA, complementary to the aberrant splice site, correct splicing is restored in a dose-dependent and sequence-specific manner, resulting in an active luciferase [20]. We employed MTT assay for cytotoxicity assessment. Their cytotoxicities were also assessed by the MTT assay (Figure 3.14). Although all the particles were non-toxic to the cells even at a very high concentration, none of them appeared to be able to transfec the cells effectively at an AuNP/ODN w/w ratio of 10 or 20. Further investigation is needed to understand and optimize the transfection process.
Figure 3.12 Hydrodynamic diameter of (•) R13C-AuNP, (■) R13C/K10Y2C-AuNP, and (◆) K10Y2C-AuNP in phosphate buffer of different pH.
Figure 3.13 A) Gel retardation essay of cAuNP/ODN complexes at different AuNP/ODN weight ratios. Radioactively $^{32}$P-labeled d(TGTGACACACAGCGCTACAA) (25 nM) was incubated with cAuNPs in 10 mM Tris buffer (pH 7.5) and 50 mM NaCl for 30 min and then loaded to 10% native polyacrylamide gel. The band at the top is the ODN bound to the cAuNP, and the band at the bottom is free ODN. B) Quantification of the ODN binding, analyzed by BioRad Quantity One. (●) R13C-AuNP, (■) R13C/K10Y2C-AuNP and (◆) K10Y2C-AuNP.
**Figure 3.14** A) Luciferase splice correction activity and B) cytotoxicity of cAuNP-ps-MeON at different Au/ODN w/w ratios, compared to the commercially available transfection agents Lipofectamine, after 24 h incubation with 0.2 μM ps-MeON.
3.3 Conclusions

In summary, we have synthesized a library of arginine-rich peptide functionalized gold nanoparticles through direct sodium borohydride reduction. We have shown that these gold nanoparticles are stable in high salt concentration and physiological pH in non-phosphate containing buffers. The strong affinity between the arginine and the phosphate group resulted in reversible aggregation under high pH phosphate buffers as well as high ODN binding affinity. Unlike the free oligoarginine peptide, the gold nanoparticles showed no cytotoxicity in pLuc705 HeLa cells even at a high concentration. An improved post-synthetic ligand exchange strategy proved to be more convenient and tunable than the direct reduction method. A library of three cationic gold nanoparticles was synthesized using the new strategy with different surface peptide combinations. The new cAuNP showed improved stability, decreased ODN binding affinity, and comparable transfection capability and cytotoxicity compare to the arginine-rich AuNP. With further optimization of the surface properties by the combinatorial strategy described, this cationic peptide gold nanoparticle system may eventually find use as a stable, safe and effective transfection agent. Moreover, this combinatorial strategy can be a powerful tool to prepare a large library of gold nanoparticles quickly and conveniently for other applications.

3.4 Experimental

Materials All solvents and chemicals were purchased from Sigma-Aldrich and used without purification, unless otherwise indicated. Fmoc amino acids were purchased from AAPTEC. Fmoc-PAL-PEG-PS resin was purchased from Applied Biosystems. A luciferase splice-correcting phosphorothioate 2’-O-methyl-oligoribonucleotide (ps-
MeON, CCUCCUACCUCAGUUACA) and the oligodeoxynucleotide d(TGTGACACACAGCGCTACAA) used in gel retardation assay were purchased from Integrated DNA Technologies, Inc and used directly without further purification. The pLuc705 HeLa cell line was a generous gift from Dr. R. Kole (University of North Carolina, Chapel Hill, NC). Lipofectamine2000 was obtained from Invitrogen Laboratories, Inc. Steady-Glo® Luciferase Assay reagent and CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) kit were purchased from Promega Co. All cell culture media was purchased from Invitrogen, Inc.

**Measurements** UV-Vis spectra were recorded on a Varian 100 UV-Vis spectrometer. Samples for transmission electron microscopy (TEM) measurements were prepared on Formvar/carbon coated copper grids. Micrographs were collected at 15,000 × magnifications on a FEI Spirit Lab6 TEM. Dynamic light scattering (DLS) was measured on Malvern Nano Sizer. Measurements were made at 25 ± 1 °C. Scattered light was collected at a fixed angle of 173°.

**Synthesis of CTTR5, CTTR9, CTTR13, CTTR17, CVVR9, CR13** Arginine-rich cationic peptide were synthesized using standard solid phase automated Fmoc chemistry on a 6 µmol scale. After the synthesis, the Fmoc group on the amino terminus was removed by mixing 30 mg of resin with 1 mL 20% piperidine in DMF for 30 min. The resin was then washed with 1 mL DMF four times and 1 mL of DCM once, then dried under nitrogen. A cleavage cocktail (440 µL of trifluoroacetic acid, 25 µL of water, 25 µL of phenol and 10 µL of triisopropylsilane was added into the resin and the mixture was incubated under
room temperature for 3 h. After the cleavage cocktail was separated from the resin, 1.2 mL of cold anhydrous diethyl ether was added to the cleavage cocktail to precipitate the crude peptide. The resulting precipitate was collected by centrifugation and purified by reverse-phase HPLC on a Waters XBridge Prep C18 column (130 Å) with buffer A (0.1% TFA in H$_2$O) and buffer B (0.1% TFA in CH$_3$CN) on a Beckman System Gold instrument equipped with a UV-vis array detector. The flow rate was 2.5 mL/min and the gradient was 1%/min starting from 5% buffer B. The fractions were collected and concentrated to dryness in a Savant SpeedVac and characterized by UV-vis and MALDI-TOF which was carried out on a PerSpective Voyager mass spectrometer with α-cyano-4-hydroxycinnamic acid as the matrix and insulin as the internal reference. MALDI: CTTR5 exact (M+H)$^+$ expected: 1103.6 found: 1103.5 (Figure A 3.1); CTTR9 exact (M+H)$^+$ expected: 1728.0 found: 1734.0 (Figure A 3.2); CTTR13 exact (M+H)$^+$ expected: 2351.4 found: 2352.2 (Figure A 3.3); CTTR17 exact (M+H)$^+$ expected: 2976.8 found: 2977.1; CR13 exact (M+H)$^+$ expected: 2150.3 found: 2150.0 (Figure A 3.4) CVVR9 exact (M+H)$^+$ expected: 1723.9 found: 1724.0 (Figure A 3.5)

*Synthesis of AuNP-CTTR5, AuNP-CTTR9, AuNP-CTTR13, AuNP-CTTR17, AuNP-CVVR9, and AuNP-CR13* 1mM HAuCl$_4$ solution (1 mL) was mixed with 5 eq of the corresponding peptide solution at room temperature for 15 min, followed by addition of 1 µL of 10 mM NaBH$_4$ solution under vigorous stirring. The reaction mixture was left stirring under room temperature for 4 h in the dark. The resulting gold colloid was stored at 4 °C in the dark.
Determination of colloid stability under different salt concentration Serial amounts of 2 M NaCl solution was added to 100 µL of 2 nM cAuNP colloid to achieve 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1 M of final NaCl concentration. The mixtures were then incubated at room temperature for 30 min and the hydrodynamic diameters of the resulting gold nanoparticles were measured by dynamic light scattering.

Determination of colloid stability under different buffer condition Phosphate buffer (10 µL of 1 M pH 5, 6, 6.5, 7, 7.5, 8) or 1 M HEPES buffer (pH 7, 7.5) or 20 µL of 0.5 M borate buffer (pH 8, 8.5) was added to 100 µL of 2 nM cAuNP colloid. The mixture was then incubated at room temperature for 30 min and the hydrodynamic diameters of the resulting gold nanoparticles were measured by dynamic light scattering.

Gel retardation assay The oligodeoxynucleotide d(TGTGACACACAGCGCTACAA) was 5’-labeled by [³²p-γ]-ATP with T4 polynucleotide kinase. Serial amounts of cAuNPs were mixed with 50 nM radiolabeled ODN at the w/w ratio indicated in Figure 3.6 in 10 mM Tris pH 7.5 buffer with 50 mM NaCl for 30 min. The mixture (20 µL) was then mixed with 5 µL of 50% glycerol and 5µL of the final mixture was loaded onto a10% native polyamide gel.

Cytotoxicity Cytotoxicity of cAuNPs was evaluated on pLuc705 HeLa cells (human cervical cancer cell line). Cells were maintained in DMEM containing 10% FBS, streptomycin (100 µg/mL), and penicillin (100 units/mL) with additional G418 (100 µg/mL) and hygromycin B (100 µg/mL) at 37 °C in a humidified atmosphere with 5%
CO₂. The cells were seeded in a 96-well microtiter plate at a density of 2×10⁴ cells/well and cultured for 24 h in 100 µL complete growth media. At the time of the cytotoxicity experiment, the media was replaced with 100 µL of DMEM containing various concentrations of AuNP-CVVR9 or AuNP-CR13, CVVR9, CR13, or no additive (negative control) as indicated in Figure 3.7. After 24 h incubation at 37°C, 15 µL CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) dye solution was added. The contents were mixed and the plate was allowed to incubate at 37 °C temperature for 4 h followed by addition of 100 µL of the stabilizing solution and 1 h additional incubation at 37 °C. Absorbance at 600 nm was recorded on a SpectraMax Plus 380 (Molecular Devices). The relative cell viability was calculated by the following equation:

\[
\text{Cell viability (\%)} = \left( \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{negative control}}} \right) \times 100
\]

Where \( \text{Abs}_{\text{negative control}} \) was obtained in the absence of particles or peptides and \( \text{Abs}_{\text{sample}} \) was obtained in the presence of cAuNP or peptide.

**Synthesis of citrate gold nanoparticles and CTAB gold nanoparticles.** The citrate gold nanoparticles were synthesized according to a reported procedure [21]. Briefly, sodium citrate was added to boiling chloroauric acid solution to yield gold nanoparticles about 11 nm in size. The CTAB gold was also synthesized following a published procedure [22]. Briefly, a 3.5 nm seed was prepared by adding sodium borohydride into a mixture of chloroauric acid and sodium citrate at room temperature. Growth stock solution was made by mixing chloroauric acid and cetyltrimethylammonium bromide (CTAB). Ascorbic solution was added to the growth solution followed by the seed solution. The
colloid turned red immediately and was stirred for another 10 min. The excess CTAB was removed by centrifugation.

**Determination of the extinction coefficient of CTAB•AuNP** 10 µL of CTAB•AuNP colloid was dissolved in 90 µL of concentrated nitric acid and then diluted 100, 1000 and 10000 times with 1% nitric acid. The standard solutions was prepared with HAuCl₄ standard (Perkin Elmer) at 0.1, 0.5, 1, 5, 10, 100 µg/L in 1% nitric acid. The gold concentration was measured on Perkin Elmer Elan DRC II ICP-MS using Bi as external reference. Another series of CTAB•AuNP colloid was diluted by 2, 2.5, 3.3, 5, and 10 times in double distilled water. The absorbance of the diluted colloid at 517 nm was measured and plotted against corresponding concentration. The extinction coefficient was calculated from linear regression (Figure A 3.6).

**Synthesis of R13C-AuNP, K10Y2C-AuNP and R13C/K10Y2C-AuNP** 0.66 µL of R13C (202 mM) or 3 µL of K10Y2C (44 mM) or a mixture of 0.33 µL of R13C and 1.5 µL of K10Y2C was first mixed with 5 µL of 50 mM TCEP followed by addition of 1 mL of 29.3 nM CTAB•AuNP and then incubated at room temperature. NaCl (50 µL of a 2M solution) was added to the mixture every 20 min until the final concentration of NaCl reached 0.3 M. The mixture was then allowed to incubate under room temperature in the dark overnight. To remove the unreacted peptide, the colloid was centrifuged at 126,000 rcf for 30 min. The supernatant was removed. The pellet was re-dispersed in double distilled water. This process was repeated twice. The final concentration was determined
by absorbance at 520 nm ($\lambda_{\text{max}}$) using the extinction coefficient of CTAB-AuNP. 
$\varepsilon_{517} = 0.0746 \text{ nM}^{-1} \text{cm}^{-1}$.

*Cell culture* cAuNP mediated transfection and cytotoxicity was evaluated on pLuc705 HeLa cells by luciferase expression. Cells were maintained in DMEM supplemented with 10% FBS, G418 (100 µg/mL) hygromycin B (100 µg/mL) streptomycin (100 µg/mL), and penicillin (100 units/mL) at 37 °C in a humidified atmosphere with 5% CO$_2$.

*Luciferase antisense splicing correction assay* pLuc705 HeLa cells were seeded in a 96-well microtiter plate at a density of $2 \times 10^4$ cells/well and cultured for 24 h in 100 µL complete media. ps-MeON (phosphorothioate 2′- O-methyl-oligoribonucleotide: CCUCUUACCUCAGUUACA) was complexed with cAuNPs at predetermined AuNP/ODN w/w ratios and incubated for 30 min before use. At the time of the transfection experiment, the medium was replaced with 80 µL of DMEM, to which the cSCK/ps-MeON complexes were added. Following 24 h incubation periods, 100 µL Steady-Glo® Luciferase assay reagent was added. The contents were allowed to incubate at room temperature for 10 min to stabilize the luminescence signal. Luminescence intensities were recorded on a Luminoskan Ascent® luminometer (Thermo Scientific) with an integration time of 1 second per well.
3.5 Appendix

Figure A 3.1 Preparative HPLC trace and MALDI-TOF mass spectrum of CTTR5.
Figure A 3.2 Preparative HPLC trace and MALDI-TOF mass spectrum of CTTR9.
Figure A 3.3 Preparative HPLC trace and MALDI-TOF mass spectrum of CTTR13.
Figure A 3.4 Preparative HPLC trace of CR13.
Figure A 3.5 Preparative HPLC trace and MALDI-TOF mass spectrum of CVVR9
Figure A 3.6 Calculation of the extinction coefficient of CTAB\textbullet{}AuNP.

The calibration equation and R squared value are shown on the chart. $\varepsilon_{517} = 0.0746 \text{ nM}^{-1} \text{ cm}^{-1}$. 
Optimizing the synthetic conditions for AuNP-CTTR5, AuNP-CTTR9, AuNP-CTTR13, AuNP-CTTR17, AuNP-CVVR9, and AuNP-CR13: The optimization of the reaction conditions was done in a clear 96 well plate. The color of the resulting colloid and TEM images were used to determine the outcome of each reaction. A bright red colloid and narrow size distribution from TEM were considered as successful outcomes. Each reaction was done in 50 µL scale and the mixing was done on a shaker at room temperature. The following parameters were investigated:

1. Concentration of HAuCl₄: 50 µL of 1 mM HAuCl₄ solution (A) or 0.6 mM HAuCl₄ solution (B) were mixed with 3.75 µL of CTTR5 solution (20 mM), incubated at room temperature for 15 min followed by addition of 0.5 mM NaBH₄ solution (1 µL or 0.6 µL respectively). Within 20 min, colloid A developed red color while colloid B developed purple color (Figure A 3.7).

**Figure A 3.7** TEM images of AuNP-CTTR5 synthesized from different HAuCl₄ concentration. A) 1 mM B) 0.6 mM
2. Peptide to HAuCl₄ molar ratio: 50 µL of 1 mM HAuCl₄ solution was mixed with A) 1.6 µL (1.5 eq), B) 3.2 µL (3 eq), C) 4.3 µL (4 eq), D) 5.3 µL (5 eq) and E) 7.5 µL (7 eq) of 46.8 mM CTTR5 solution, incubated at room temperature for 15 min, followed by addition of 1 µL of 0.5 mM NaBH₄ solution. Within 20 min, Colloid A and B developed purple color while Colloid C, D and E developed red color. (Figure A 3.8)

![Image](image1.png)

**Figure A 3.8** TEM images of AuNP-CTTR5 synthesized from different equivalence of CTTR5 solution. A) 5 eq B) 6 eq C) 7 eq
3. Reducing agent: two reducing agents were tested in the synthesis: sodium borohydride and tryptophan. When HAuCl₄ solution (0.2 mM, 6.5 mL) was mixed with tryptophan (14 mM, 0.35 mL) and shaken at room temperature, 28 nm monodispersed gold nanoparticles formed within 20 min (Figure A 3.9A). However, when the peptide was added, no nanoparticles with defined size was formed (Figure A 3.9B). When sodium borohydride was used as reducing agent, 0.5%, 1% or 2% equivalent of NaBH₄ was added (0.5, 1, or 2 µL of 0.5 mM NaBH₄ solution into a mixture of 50 µL of 1 mM HAuCl₄ and 5.4 µL of 46.8 mM CTTR5 solution), 1% equivalence yielded best dispersed colloid while 0.5% equivalence yielded slow reduction and ununiformed particle size and 2% equivalence resulted in ununiformed particle size (Figure A 3.9 C-E). The optimized synthetic conditions of AuNP-CTTR5 were applied to the rest of the cAuNP library.
Figure A 3.9 TEM images of AuNP-CTTR5 synthesized with different reducing agent. A) tryptophan (without CTTR5) B) tryptophan (with CTTR5) C) 0.5% NaBH$_4$ D) 1% NaBH$_4$ E) 2% NaBH$_4$
3.6 References


Chapter 4

Biodegradable Peptide Nanoshell

4.1 Introduction

Nanoshells have gained significant interest in recent years due to their unique chemical, physical, and biological properties and their versatility to carry payload. They have been used in a wide spectrum of applications in imaging [1, 2], drug delivery [3, 4] and catalysis [5]. Many different methods have been employed to prepare these structures based on layer-by-layer process [6], cross-linking of micelles [7-9], nanoparticle self-assembly [10, 11] and templating techniques [12-15]. Among all existing strategies, the templating method is especially attractive because it takes advantage of both the convenience to synthesize and control the morphology of the template and the readiness to assemble the nanoshell material on the template. The resulting nanoshells are usually homogeneous and well-defined.

Gold nanoparticles (AuNPs) are one of the most popular nanoparticle systems for surface modification, due to the unique affinity between gold and sulfur. Thiolated ligands such as polymers [16], oligodeoxynucleotides (ODNs) [17], peptides [18-20] and proteins [21] have been deposited on the gold surface for different functionalities. In some studies, the gold nanoparticles were used as templates and were removed after the crosslinking of the surface ligands. Zhang and his coworkers developed a polymeric nanopod through gold nanoparticle templated and catalyzed crosslinking of polymers [22]. They demonstrated that after removal of the AuNP template, the crosslinked...
nanopod still remained its spherical geometry and by using AuNPs of different diameters, they were able to obtain hollow nanopod of similar morphology. Cross-linking took place between the propargyl groups on the surface of the particle (Figure 4.1). Inspired by that work, Culter and his coworkers crosslinked ODNs on the AuNP template [23]. Each ODN had 10 alkyne-modified thymines next to the thiol terminus. The resulting polyvalent nucleic acid nanostructures were well-defined in shape, size and ODN loading. The cellular permeation and transfection ability of the nanostructure were also comparable to the ODN modified gold nanoparticles.

Figure 4.1 Tentative cross-linking mechanism of propargyl groups on the surface of the gold nanoparticle. Adapted with permission from [22] Zhang, K., et al., Nanopod Formation through Gold Nanoparticle Templated and Catalyzed Cross-linking of Polymers Bearing Pendant Propargyl Ethers. Journal of the American Chemical Society, 2010. 132(43): p. 15151-15153. Copyright 2010 American Chemical Society.
Compared with ODNs and polymers, peptides could provide even more tunability in surface functions and properties. Wide selection of the side chains of natural and unnatural amino acids could satisfy a variety of chemical and biological purposes. Moreover, different combinations of peptides could be deposited simultaneously in gold colloids to yield a library of multifunctional gold nanoparticles for further screening for specific functions [20, 24].

This chapter reports the proof-of-principle study on a combinatorial approach to prepare a library of biodegradable nanoshells (Figure 4.2). The nanoshells were prepared by crosslinking the peptide-modified gold nanoparticles and then removing the gold template. The resulting nanoshells were characterized and the future direction of this approach is discussed. With the promising preliminary result obtained in this chapter, we envision that this synthetic strategy can be a powerful tool for preparing biodegradable nanostructures for in vitro and in vivo delivery.

Figure 4.2 Synthetic scheme for peptide nanoshells. Cationic peptides are first deposited on the gold nanoparticle template. The lysines in the peptide sequences are then crosslinked by a biodegradable crosslinker. The gold nanoparticle template is then dissolved by potassium cyanide. The resulting cationic peptide nanoshell can then be complexed with siRNA, DNA or DNA/PNA complex for transfection purposes.
4.2 Results and Discussion

4.3.1 Synthesis of 8 nm Gold Nanoparticle Templates

We synthesized the 8 nm gold nanoparticle template following a published procedure [25]. Briefly, a 3.5 nm seed was made by reducing chloroauric acid solution with sodium borohydride at room temperature in the presence of sodium tricitrate as capping agent. This seed was then added to a growth media containing cetyl trimethylammonium bromide (CTAB) as capping agent, ascorbic acid as reducing agent and chloroauric acid. The resulting nanoparticle was 8.1 ± 0.7 nm measured by transmission electron microscopy (TEM) (Figure 4.3) and 13.3 ± 3.7 nm measured by dynamic light scattering (DLS). The difference between the DLS measurement and the TEM measurement accounts for the bulky surfactant layer on the gold surface that increased the hydrodynamic diameter of the gold nanoparticle but was invisible under TEM. The 8 nm CTAB-AuNP was chosen as the template because 1) smaller nanoparticles particles (d < 100 nm) showed the best cell uptake properties with diameter around 50 nm being the optimal [26-30]. Peptide nanoshells formed on smaller templates are most likely to remain small enough once the gold cores are removed; 2) AuNPs smaller than 8 nm cannot be purified by centrifugation so the synthetic process will be less convenient.
4.3.2 Ligand Exchange with the Lysine-Rich Peptide on the Gold Template

KKKKKKKKKKKYYC was exchanged for citrate on the surface of the gold template to give K10Y2C-AuNP. Atomic force microscopy (AFM) showed a particle with a height of about 8 nm (Figure 4.4a) and the modulus channel (Figure 4.4b) showed that each particle consisted of a hard core measuring about 14.2 ± 1.2 nm in diameter corresponding to the gold core and a donut-shaped outer layer around the hard core measuring 29.2 ± 1.2 nm in diameter corresponding to the peptide layer around the gold nanoparticle. The hydrodynamic diameter measured by dynamic light scattering is 10.9 ± 3.3 nm, indicating that the peptide layer was more tightly associated with the gold template than the CTAB layer. The difference between the measurement by
AFM and DLS could result from the different states that the K10Y2C-AuNP was in (dried on the mica vs in water) and the resolution limit of the AFM probe. The TEM image (Figure 4.5) showed that after peptide deposition, the colloid is more evenly dispersed than CTAB AuNP, indicating that the lysine rich peptides provide more effective repulsion among particles than CTAB. The average peptide loading of the K10Y2C-AuNP was 511peptide/AuNP.

**Figure 4.4** AFM images of K10Y2C-AuNP. a) Height image. b) Modulus image
4.3.3 Crosslinking of the K10Y2C-AuNP

K10Y2C-AuNP was crosslinked with sulfo-EGS (ethylene glycol bis[sulfosuccinimidylsuccinate]) (Figure 4.6) at peptide/crosslinker ratio of 1:1, 1:5, 1:10, 1:30, 1:50 and 1:100. The surface plasmon resonances of the crosslinked AuNPs remained around 517 nm except for particles prepared with a 1:100 ratio, indicating that the colloid remained well dispersed without aggregation. At 1:100 peptide/crosslinker ratio, inter-particle crosslinking may have started to occur which would have changed the color of the colloid. DLS measurement confirmed that the crosslinked colloids were still well dispersed, with hydrodynamic diameters around 13 nm, except for the 1:100 ratio sample, whose hydrodynamic diameter increased to 40 ± 12 nm. However, both the AFM images (Figure 4.7) and the TEM images (Figure 4.8) showed that all the crosslinked
particles were less evenly distributed once they were dried on the imaging substrate than the non-crosslinked nanoparticles. This could be due to the fact that the amino groups that provide the inter-particle repulsion were transformed to much less basic amide groups to various degrees during the crosslinking process.

Figure 4.6 a) Structure of the crosslinker sulfo-EGS (ethylene glycol bis[sulfosuccinimidylsuccinate]); b) The crosslinking reaction.
Figure 4.7 AFM modulus images of a) K10Y2C-AuNP crosslinked at peptide/crosslinker ratio of 1:1; b) K10Y2C-AuNP crosslinked at peptide/crosslinker ratio of 1:10.
4.3.4 Removal of the Gold Nanoparticle Template

The gold nanoparticle core templates of the K10Y2C-AuNP crosslinked at 1:10, 1:50 and 1:100 peptide/crosslinker ratios were dissolved by KCN and the DLS measurements of the resulting peptide nanoshells are shown in Figure 4.9. After the removal of the template, the hydrodynamic diameter of the crosslinked nanoshells increased from around 13 nm to around 40 nm (Table 4.1). No difference was observed in the nanoshells crosslinked at different peptide/crosslinker ratios. The non-crosslinked peptide-AuNP was treated with the same procedure and the product showed a very large diameter by DLS, indicating that no nanostructures were left after the gold template was removed. The difference in the diameters before and after gold removal is probably the
result of the repulsion between the positively charged peptide strands of the same nanoshell. The length of the crosslinker (16 Å) also allowed some flexibility in the nanoshell.

**Figure 4.9** Hydrodynamic diameter of CTAB-AuNP, K10Y2C-AuNP, crosslinked K10Y2C-AuNP before and after removal of the gold template.
Table 4.1 Hydrodynamic diameter of CTAB-AuNP, K10Y2C-AuNP, crosslinked K10Y2C-AuNP before and after removal of the gold template. Error is the standard deviation of the size distribution.

<table>
<thead>
<tr>
<th>Particle</th>
<th>Number mean diameter with gold (nm)</th>
<th>Number mean diameter without gold (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K10Y2C-AuNP</td>
<td>10.9±3.3</td>
<td>736.4±196.7</td>
</tr>
<tr>
<td>K10Y2C-X10-AuNP</td>
<td>12.5±3.5</td>
<td>34.6±19.6</td>
</tr>
<tr>
<td>K10Y2C-X50-AuNP</td>
<td>14.3±4.3</td>
<td>41.1±13.0</td>
</tr>
<tr>
<td>K10Y2C-X100-AuNP</td>
<td>40.2±12.1</td>
<td>37.2±10.1</td>
</tr>
</tbody>
</table>

4.3.5 DNA Binding Affinity of the Crosslinked Peptide-AuNP and the Nanoshells

ODN binding abilities of the peptide-AuNP crosslinked at 1:1, 1:10 and 1:30 ratios before and after the removal of the gold template were evaluated by a gel retardation assay. Peptide-AuNPs and peptide nanoshells were mixed with $^{32}$P-labeled ODN in N/P ratios of 0, 2, 4, 16 and 32 and separated by native PAGE (Figure 4.10). Peptide K10Y2C alone was also tested as negative control. K10Y2C-AuNP showed relatively strong binding affinity. However, after only 20% of the lysines were crosslinked (K10Y2C-X1-Au), the binding affinity decreased dramatically. When all the lysines were crosslinked (K10Y2C-X10-Au and K10Y2C-X30-Au), the ODN binding affinity was completely lost. Interestingly, after the gold template was removed, all nanoshells including K10Y2C-X1 failed to bind ODN even at an N/P ratio of 32. The
peptide alone showed a completely different ODN binding pattern from that of peptide gold nanoparticles or nanoshells.

**Figure 4.10** Gel shift of ODN/gold nanoparticle complex and peptide nanoshell/gold complex at different N/P ratios. Lane 1 is ODN alone. Lane 29 is peptide/ODN complex.

**4.3 Conclusions**

In summary, we have demonstrated a novel method for synthesizing well-defined biodegradable nanoshells from crosslinking lysine-rich peptide, using AuNP as the template. Based on the design, the nanoshells are tunable in size by using AuNPs of different sizes; tunable in surface properties by depositing different peptides on the surface as discussed in Chapter 2; tunable in charge by altering the surface peptide and peptide-to-crosslinker ratio; and tunable in degradability through the use of different crosslinkers. This approach is also suitable for automated combinatorial applications. We envision that this general strategy can be applied to create a variety of complex and functional systems that are applicable in a broad range of disciplines.
4.4 Experimental

Material All solvents and chemicals were purchased from Sigma-Aldrich and used without purification, unless otherwise indicated. Fmoc amino acids were purchased from AAPTEC. Fmoc-PAL-PEG-PS resin was purchased from Applied Biosystems. Sulfo-EGS (Ethylene glycol bis[sulfosuccinimidylsuccinate]) was purchased from Thermo Scientific. The oligodeoxynucleotide d(TGTGACACACAGCGCTACAA) used in gel retardation assay were purchased from idtDNA and used directly without further purification.

Measurements UV-Vis spectra were recorded on a Varian 100 UV-Vis spectrometer. Samples for transmission electron microscopy (TEM) measurements were prepared on Formvar/carbon coated copper grids. Micrographs were collected at 42,000× magnifications on a FEI Spirit Lab6 TEM. Dynamic light scattering (DLS) was measured on Malvern Nano Sizer. Measurements were taken at 25 ± 1 °C. Scattered light was collected at a fixed angle of 173°. The gold concentration of CTAB-AuNP colloid was determined on Perkin Elmer Elan DRC II ICP-MS using Bi as external reference. Atomic force microscopy (AFM) was done using quantitative nanomechanical mapping (QNM) on a MultiMode 8 scanning probe microscope (Bruker, Santa Barbara, CA) with NP-10 gold probe (Bruker, Santa Barbara, CA) with the assistance of Matthew Hynes and Professor Joshua Maurer. Molecular structures were generated by MarvinSketch (ChemAxon).
Synthesis of K10Y2C: The peptide were synthesized using standard solid phase automated Fmoc chemistry on a 6 µmol scale. After the synthesis, the Fmoc group on the amino terminus was removed by mixing 30 mg of resin with 1 mL 20% piperidine in DMF for 30 min. The resin was then washed with 1 mL DMF four times and 1 mL of DCM once, then dried under nitrogen. A cleavage cocktail (440 µL of trifluoroacetic acid, 25 µL of water, 25 µL of phenol and 10 µL of triisopropylsilane) was added into the resin and the mixture was incubated under room temperature for 3 h. After the cleavage cocktail was separated from the resin, 1.2 mL of cold anhydrous diethyl ether was added to the cleavage cocktail to precipitate the crude peptide. The resulting precipitate was collected by centrifugation and purified by reverse-phase HPLC on a Waters XBridge Prep C18 column (130 Å) with buffer A (0.1% TFA in H₂O) and buffer B (0.1% TFA in CH₃CN) on a Beckman System Gold instrument equipped with a UV-vis array detector. The flow rate was 2.5 mL/min and the gradient was 1%/min starting from 5% buffer B. The fractions were collected and concentrated to dryness in a Savant SpeedVac and characterized by UV-vis and MALDI-TOF which was carried out on a PerSpective Voyager mass spectrometer with α-cyano-4-hydroxycinnamic acid as the matrix and insulin as the internal reference. MALDI: exact (M+H)⁺ expected: 2085.4, found 2085.2.

Synthesis of CTAB-AuNP: A 20 mL aqueous solution containing 2.5 × 10⁻⁴ M HAuCl₄ and 2.5 × 10⁻⁴ M trisodium citrate was prepared in a conical flask. Next, 0.6 mL of ice-cold, freshly prepared 0.1 M NaBH₄ solution was added to the solution while stirring. The solution turned orange immediately after adding NaBH₄, indicating particle formation. The particles in this solution were used as seeds within 1-2 h after preparation. A 200 mL
aqueous solution of $2.5 \times 10^{-4}$ M HAuCl$_4$ was prepared in a conical flask. Next, 6 g of solid cetyltrimethylammonium bromide (0.08 M final concentration) was added to the solution, and the mixture was slightly heated until the solution turned into a clear yellow color. The solution was cooled to room temperature and used as a stock growth solution. 90 mL of growth solution and 0.5 mL of 0.1 M ascorbic acid solution were mixed, and 10 mL of seed solution was added while vigorously stirring. After stirring continued for an additional 10 min the solution developed a deep red color. The resulting particles were centrifuged at 40 °C, 126,000 rcf for 30 min. The pellet was separated and re-dispersed in double distilled water and centrifuged again. This procedure was repeated twice. Finally, the colloid was re-dispersed in double distilled water and stored under 4°C in the dark.

**Determination of the extinction coefficient of CTAB-AuNP** 10 µL of CTAB-AuNP colloid was dissolved in 90 µL of concentrated nitric acid and then dilute 100, 1000 and 10000 times with 1% nitric acid. The standard solutions was prepared with HAuCl$_4$ standard (Perkin Elmer) at 0.1, 0.5, 1, 5, 10 and 100 µg/L in 1% nitric acid. The gold concentration was measured on Perkin Elmer Elan DRC II ICP-MS using Bi as external reference. Another series of CTAB-AuNP colloid was diluted by 2, 2.5, 3.3, 5, and 10 times in double distilled water. The absorbance of the diluted colloid at 517nm was measured and plotted against corresponding concentration. The extinction coefficient was calculated from linear regression.

**Synthesis of K10Y2C-AuNP** K10Y2C (5 µL of 44.2 mM solution) was mixed with 5 µL of 50 mM TCEP followed by addition of 1 mL of 29.3 nM CTAB-AuNP and then
incubated at room temperature. NaCl (50 µL of 2M solution) was added to the mixture every 20 min until the final concentration of NaCl reached 0.3 M. The mixture was then allowed to incubate under room temperature in the dark overnight. To remove the unreacted peptide, the colloid was centrifuged at 126,000 rcf for 30 min. The supernatant was removed. The pellet was re-dispersed in double distilled water. This process was repeated twice. The final concentration was determined by absorbance at 520 nm ($\lambda_{\text{max}}$) using the extinction coefficient of CTAB-AuNP. To determine the peptide loading, 30 µL of K10Y2C-AuNP was dried in a Savant Speedvac and then dissolved in 3 µL of 100 mM KCN (CAUTION: HIGHLY TOXIC). The absorbance of the resulting solution was measured at 277 nm on NanoPhotometer (Implen) and the concentration was calculated according to the extinction coefficient of tyrosine.

Crosslinking of K10Y2C-AuNP Sulfo-EGS (Ethylene glycol bis[sulfosuccinimidylsuccinate]) 3.75, 18.75, and 37.5 µL of 2 mM solution was added to K10Y2C-AuNP (final concentration: 10 nM, final volume: 100 µL) for K10Y2C-X10-AuNP, K10Y2C-X50-AuNP and K10Y2C-X100-AuNP. The mixture was shaken at room temperature overnight. The resulting colloids were dialyzed against 1 L of double distilled water in mini Slide-A-Lyer (MWCO 10,000) for 2 h.

Removal of the gold template KCN (10 µL of 100 mM solution, CAUTION: HIGHLY TOXIC) was added to 100 µL of 10 nM crosslinked peptide gold nanoparticle. The mixture was incubated at room temperature for 1 h (when the red color disappeared). The hydrodynamic diameter of the peptide nanoshells was quickly measured by DLS. The
nanoshells were then dialyzed in 100 µL Slide-A-Lyzer dialysis cup (MWCO: 10,000) against double distilled water for 1 h. The volume of the final colloid was measured and the concentration of the nanoshell was calculated according to the concentration of the crosslinked gold nanoparticle.

*Gel retardation assay* The oligodeoxynucleotide d(TGTGACACACAGCGCTACAA) was 5’-labeled with [Υ-32p-ATP] with T4 polynucleotide kinase. Serially diluted amounts of peptide gold nanoparticles and peptide nanoshells were mixed with 50 nM radiolabeled OND at N/P ratio indicated in Figure 4.9 in 10 mM Tris pH 7.5 buffer with 50 mM NaCl for 30 min. The mixtures (20 µL) were then mixed with 5 µL of 50% glycerol and 5 µL of the final mixtures were loaded to 10% native polyamide gel.

### 4.5 References


Chapter 5

Concluding Remarks and Future Work

Despite of the increasing interest in biomolecule-functionalized nanoparticles, many challenges still remain unsolved. There has always been a compromise between structural definedness and tunability, transfection efficacy and cytotoxicity, stability and biodegradability, etc.

This dissertation has addressed some of the challenges with novel approaches for nanoparticle design and preparation. In Chapter 2 a DNA hybridization-based self-assembling nanoparticle system was developed, which is both highly well defined and tunable. The tunability was demonstrated through the ready assembly between oligodeoxynucleotide-protected gold nanoparticles (ODN•AuNP) and functionalized peptide nucleic acids (PNA). The resulting nanoparticles were highly stable in vivo, as shown by bio-distribution in mice. The surface PEGylation also successfully reduced non-specific cell uptake. With PEGylation, most of the nanoparticles were still cleared within one hour from the blood circulation and remained in the liver. These initial results paved the road for further development as nanoparticle-based imaging and therapeutic agents. In future studies, more functions could be assembled in the same manner. First, to extend the number of accessible sites for functionalization, one or more layers of branched ODN or PNA can be assembled on the ODN•AuNP. By adjusting the length of each branch, the ligand geometry on the gold surface can be controlled. For example, with a longer ODN-binding stem, the ligands hybridized on the branches could be farther
apart from one another and hence allow more space for bulky ligands such as PEG or protein. With two shorter branches, the ligands attached to the same branched PNA or ODN could be closer than they are from other PNA or ODN. If the sequences on the two branches are different, two different ligands could be precisely hybridized on each branch. The short distance between these two ligands could facilitate cooperative functionalities. (Figure 5.1).

Figure 5.1 Illustration of branched PNA or ODN assembled on the ODN-AuNP. The black lines represent the ODN attached on the gold surface. The stems (blue lines) and branches (red lines and green lines) could be different in length and sequence.

Secondly, the bio-distribution pattern shown in Chapter 2 is very promising for liver targeting. However, the blood retention of the nanoparticles needs to be significantly improved for targeting other organs via systematic administration. For nanoparticles with a given size, there are two ways to improve circulation: increase the surface density of the passivation ligand, i.e. increase of the loading or the size of the passivation ligand, or use a different type of passivation ligand. In our case, longer and more branched PEG can be
used in the place of PNA-(PEG2K)$_3$. More PEG can also be loaded if the ODN-AuNP is derivatized by the branched ODN/PNA mentioned above. Besides PEG, a variety of natural polymers such as heparin, dextran, and chitosan, along with many synthetic polymers have also been used in a wide range of drug-delivery systems [1, 2]. These alternatives might provide us opportunities to improve the circulation of gold nanoparticles.

Once the blood retention is increased, targeted delivery would become possible. Targeting moieties such as antibodies, aptamers, peptides, or other small molecules can be conjugated to peptide nucleic acids and assembled on the ODN•AuNP. The linker between the targeting ligand and PNA can be adjusted to avoid loss of activity caused by PNA interference. If two factors are needed for a particular binding process, they can be hybridized onto one branched PNA or ODN so that the adjacency is ensured.

The nature of DNA hybridization provides another advantage of this approach. The ODN sequence could be designed to be partially complementary to the PNA sequence and completely complementary to the target mRNA or DNA. Once the nanoparticle enters the desired cell, the PNA-payload conjugate could be displaced by the target mRNA and function independently of the nanoparticle. Mirkin et al. reported a similar approach, where EGFP antisense ODN was hybridized on an ODN•AuNP. Once inside the cell, EGFP antisense ODN was displaced and hybridized with EGFP mRNA to knockdown the expression of EGFP [3]. When a fluorescent dye is used as payload, this system can be used for in vivo mRNA imaging. Mirkin’s group reported a study on in vitro mRNA imaging using this strategy. Fluorescently labeled ODN was hybridized with ODN•AuNP. The dye was quenched by the gold nanoparticle. When the nanoparticle
comes in contact with the target mRNA, the fluorescent ODN was displaced and become fluorescent. The complementary-probe-treated cell line with target mRNA showed much higher fluorescence than the control cell line or non-complementary-probe-treated target cell line [4]. This type of system could be applied in vivo using the design described in Chapter 2.

The accuracy of DNA hybridization could be better taken advantage of by attaching several ODN sequences simultaneously on one gold nanoparticle. Different functional ligands could be conjugated to the different antisense PNAs and hybridized simultaneously on the ODN-AuNP. In this way, the composition of the ligands on the gold surface could be more precisely controlled.

In Chapter 3, a series of arginine-rich peptide functionalized gold nanoparticles were synthesized by reducing goldIII with sodium borohydride. The resulting cationic gold nanoparticles (cAuNPs) exhibited excellent stability in high salt solution and broad pH range, which makes them suitable for biological applications. Due to the high affinity between guanidinium group and phosphate group, the arginine-rich gold nanoparticles showed very high ODN binding constant on the gel shift assay. Unlike the individual oligoarginine peptides, the arginine-rich gold nanoparticles didn’t exhibit any cytotoxicity even at high concentration. With the knowledge gained in the cAuNP study, a better and more universal synthetic strategy was proposed. Cationic peptides synthesized by combinatorial peptide synthesizer were deposited directly on gold nanoparticles synthesized by citrate reduction or seeded growth method. Nanoparticles fabricated this way retained the morphology and monodispersity of the original gold colloid and yet showed distinct properties consistent with their surface composition. For proof-of-
principle study, three peptide-AuNPs were synthesized based on CTAB protected gold nanoparticles, R13C-AuNP, K10Y2C-AuNP and R13C/K10Y2C-AuNP. The R13C-AuNP behaved the same as R13C-AuNP synthesized by NaBH₄ reduction, which bound to ODN effectively but aggregated in phosphate buffer at higher pH reversibly. The K10Y2C-AuNP, however, was stable in a broader range of buffers and pH’s but a much weaker ODN binder. The combination of K10Y2C and R13C on R13C/K10Y2C-AuNP resulted in intermediate ODN binding ability and buffer stability. This approach indicated a possibility of quickly assembling a library of peptide-AuNPs whose functions could be easily tuned by altering the peptide sequences by use of a combinatorial automated peptide synthesizer. Although none of the peptide-AuNP tested in this study effectively transfected pLuc705 HeLa cells in the splice correction assay, I believe that the transfection ability could be optimized by changing the peptide composition and sequence.

Based on the combinatorial synthesis described in Chapter 3, a biodegradable peptide nanoshell was synthesized by crosslinking the lysine-rich peptide on the gold nanoparticle and then removing the gold template. As a proof-of-principle study, only one peptide-AuNP, K10Y2C-AuNP was synthesized with different extents of crosslinking. DLS showed that after the gold template was removed, the nanoshells remained assembled, although the hydrodynamic diameter of the nanoshells increased by around 20 nm, probably due to the electron static repulsion between the oligolysine strands and the length of the crosslinker (~16 Å). The crosslinking, however, caused a dramatic decrease in the ODN binding ability, even at low crosslinking ratios, indicating that the number of amines on the surface is much more important than the number of total
amines. Based on this preliminary data, several optimizations could be envisioned for the future. To improve the ODN binding ability, arginines could be added to the peptide strand between the lysines and the cysteine. When the gold template is removed, the arginines would be exposed and available for ODN binding and cell entry. To better regulate the size of the nanoshell, other crosslinking strategies could also be employed. Dash et al. reported a hollow sphere crosslinked by microbial transglutaminase (mTGase). In their study, Elastin-like polypeptide was assembled electrostatically on polystyrene (PS) beads of different sizes. Then mTGase was used to crosslink between lysines and glutamines. The resulting nanostructure was robust and remained spherical even after the PS bead was dissolved by THF [5]. Their approach, however, allows less tunability in peptide functions due to the amorphic assembly on the template surface.

Once the nanoshell is formed, there are several ways to encapsulate the payload, besides the electrostatic association mentioned in Chapter 4. During the dissolution of the gold templates, the thiol group on the cysteine will be exposed. Payloads functionalized with thiol reacting groups such as maleimide and bromo acetyl could be covalently conjugate to the nanoshell. If the payloads are functionalized with thiol groups, they can form disulfide bonds with the nanoshell and be released inside the endosomes. Payloads functionalized with multiple thiol groups could further stabilize the nanoshell structure as well. pH-responsive peptides could be incorporated into the nanoshell. For example, at lower pH, the peptides are protonated and repel from one another. The nanoshell will be “open” and the payload can enter the hollow center. Then when the pH is higher, the peptides would be neutral and more hydrophobic and the hydrophilic payload would be trapped inside the nanoshell. Once the complex enters the cell via
endocytosis, as the pH in the endosome decreases, the nanoshell would be “open” again and release the payload. In future cell studies, targeting peptide or endosomal escape peptides could also be incorporated in the nanoshell to facilitate diagnostic or therapeutic purposes. To take a step forward, the stealth design that was used in the ODN-AuNP study could be applied to the nanoshell in vivo.

Biomolecule modified nanoparticles have great potential in many biomedical applications. Although a lot of interesting work has been conducted, further research must be continued in this area to realize the full potential of these nanoscale materials.

5.1 References


