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Plasmonic Nanostructures for Controlled Drug Delivery

by

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ABSTRACT

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Drug delivery and photothermal ablation based on resonant illumination of near-infrared-absorbing noble metal nanoparticles that have accumulated in tumors are highly promising cancer therapies. Crucial aspects of these therapies include the nanoparticle size and biocompatibility, and the ability to remotely trigger the release of therapeutic cargo, once the particles have relocated to the tumor site. Yet, maximizing tumor uptake, reducing non-specific toxicity, and achieving flexible drug loading and release strategies remain challenges in developing a nanocarrier system.

Here, a class of nanoparticles known as hollow Au nanoshells (HGNS) is investigated because near-IR resonances are achievable in this system with diameters less than 100 nm. However, we report a surprising finding that in vivo HGNS are unstable, fragmenting with the Au and remnants of the sacrificial Ag core accumulating differently in various organs. Stability studies across a wide range of pH environments and in serum confirmed HGNS fragmentation. These results demonstrate the importance of tracking both materials of a galvanic replacement in biodistribution studies and of performing thorough nanoparticle stability studies prior to any intended in vivo applications.

Using biocompatible nanoshells, near-IR light-induced DNA release was studied as a platform for controlled drug delivery wherein therapeutic drugs can be released from a DNA host. Yet, tailorability of this system is limited. Understanding the mechanism of
DNA release will allow easier control of release for various molecular cargos. Our studies have shown that under irradiation by a continuous wave (CW) laser, nanoparticle heating, as opposed to hot electrons, is responsible for DNA dehybridization and is highly dependent on nanoparticle concentration, requiring the bulk solution temperature to rise above the DNA dehybridization temperature to induce release at particle concentrations feasible in tumors. Alternatively, DNA release due to femtosecond irradiation can be achieved by breaking the Au-S bond via a hot-electron transfer process without any considerable bulk temperature increase. This is critically important for cancer treatment, as the cellular environment is very sensitive to temperature fluctuations and nanoparticle uptake in tumors is highly variable. Remotely triggering release of host DNA with no bulk temperature increase can enable selective drug release, drastically reducing the nonspecific side effects of typical chemotherapy treatments.

Intracellular light-triggered release of chemotherapy drugs was investigated using low levels of CW and pulsed NIR light from both DNA and protein scaffolds. The results showed a higher percent cytotoxicity for CW laser-induced release from a DNA scaffold. Using the protein scaffold, increased cytotoxicity was observed for release using pulsed light in cancerous cells, while non-cancerous cells were unaffected. These results show that light-triggered drug release is an effective non-toxic drug delivery vehicle to selectively kill cancers cells. Furthermore, by simply exchanging the chemotherapy drug, this system can be extended to treatments for many cancer types. Achieving the flexible loading and release strategies shown here will allow release to be more easily controlled and tailored for various chemotherapeutic drugs and cancer types.
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# Contents

Acknowledgments ................................................................................................................iv

Contents ............................................................................................................................... vii

List of Figures ......................................................................................................................... ix

List of Tables ........................................................................................................................... xv

Nomenclature ........................................................................................................................ xvi

Introduction ............................................................................................................................. 17

Background ............................................................................................................................. 21

2.1. Plasmonic Nanoparticles ............................................................................................... 21

2.1.1. Nanoshells and Plasmon Resonance Tunability ......................................................... 22

2.1.2. Plasmon Decay ........................................................................................................... 27

2.2. Ideal Nanoparticle System for Biomedical Application .................................................. 28

2.2.1. Biocompatibility .......................................................................................................... 28

2.2.2. NIR Absorption and Light-to-Heat Conversion ......................................................... 30

2.2.3. Nanoparticle Uptake in Tumors ................................................................................ 31

2.2.3.1. Size ......................................................................................................................... 31

2.2.3.1.1. Challenges in Designing Small Nanoparticles ................................................... 32

2.2.3.2. Charge and Surface Coating .................................................................................. 34

2.2.3.3. Targeting ............................................................................................................... 35

2.2.4. Enhanced Imaging Capabilities .................................................................................. 36

2.2.5. Active Drug Release ................................................................................................. 37

2.3. DNA as a Drug Release Scaffold ................................................................................... 38

2.3.1. DNA Sequence Design .............................................................................................. 39

2.3.2. DNA Preparation and Nanoparticle Functionalization ............................................ 41

2.3.3. DNA Characterization .............................................................................................. 41

2.4. Continuous Wave vs. Pulsed Wave Light Sources ......................................................... 42

The Surprising in Vivo Instability of Near-IR-Absorbing Hollow Au-Ag Nanoshells ................. 45

3.1. Introduction .................................................................................................................... 45

3.2. Experimental Methods ................................................................................................. 48

3.3. Results and Discussion ................................................................................................. 51
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4. Conclusions</td>
<td>68</td>
</tr>
<tr>
<td>Understanding Near-IR Light-Triggered DNA Release with Continuous Wave and Pulsed Lasers</td>
<td>69</td>
</tr>
<tr>
<td>4.1. Introduction</td>
<td>69</td>
</tr>
<tr>
<td>4.2. Experimental Methods</td>
<td>72</td>
</tr>
<tr>
<td>4.3. Results and Discussion</td>
<td>75</td>
</tr>
<tr>
<td>4.4. Conclusions</td>
<td>91</td>
</tr>
<tr>
<td>Intracellular Light-Triggered Drug Release for Metastatic Breast Cancer Treatment</td>
<td>92</td>
</tr>
<tr>
<td>5.1. Introduction</td>
<td>92</td>
</tr>
<tr>
<td>5.2. Experimental Methods</td>
<td>92</td>
</tr>
<tr>
<td>5.3. Results and Discussion</td>
<td>99</td>
</tr>
<tr>
<td>5.4. Conclusions</td>
<td>115</td>
</tr>
<tr>
<td>Conclusions</td>
<td>116</td>
</tr>
<tr>
<td>References</td>
<td>119</td>
</tr>
</tbody>
</table>
List of Figures

**Figure 2.1** Schematic of the plasmon resonance showing excitation of the collective oscillation of the conduction band electrons by incident light. .......................................................... 22

**Figure 2.2** Tuning of the plasmon resonance frequency of gold nanoshells by controlling the shell thickness and schematic of a gold nanoshell with variable core ($\varepsilon_1$), shell ($\varepsilon_2$), and medium ($\varepsilon_3$) dielectric constants and an inner and outer radius of $[r_1,r_2]$. .......................... 23

**Figure 2.3** Schematic and corresponding TEM images of nanoshell fabrication showing (A,E) 120 nm silica cores, (B) amination of the silica core with APTES, (C,F) formation of seeded precursor by attachment of 2-3 nm Au colloid, and (D,G) growth of a terminal 22 nm thick gold shell using hydrogen tetrachloroaurate, potassium carbonate, and formaldehyde. .......................................................... 24

**Figure 2.4** Plasmon hybridization model of a gold nanoshell. (A) Detailed model showing the interaction of the sphere ($\omega_{sp}$) and cavity ($\omega_c$) modes to form the observable bonding ($\omega_b$) and antibonding ($\omega_a$) modes. (B) Illustration of the resulting bonding mode plasmon energy based on the strength of the interaction between the sphere and cavity plasmon modes determined by the thickness of the gold shell. .................................................. 26

**Figure 2.5** Characteristics of an ideal nanoparticle system. The nanoparticle system should be (1) biocompatible, (2) absorb light in the near-infrared (NIR) and exhibit efficient light-to-heat conversion, (3) have high tumor uptake, which depends on nanoparticle size, charge and surface coating, and tumor targeting, (4) encompass enhanced imaging capabilities, and (5) be able to actively release drugs. ......................... 29

**Figure 2.6** Plasmon resonance frequency dependence on nanoparticle size, geometry, and dielectric constant. Schematics and corresponding extinction spectra of a 50 nm Au sphere (black), 150 nm nanoshell (red), 90 nm nanoshell (blue), 55 x 14 nm nanorod (green), 82 nm HGNS (magenta), 90 nm nanomatryoshka (cyan), and 100 nm nanoshell with an iron oxide core (orange). The highlighted sections in the extinction spectra represent the desired NIR wavelength range. .......................................................... 33

**Figure 2.7** Comparison of continuous and pulsed wave lasers. (A) Output beam of a continuous wave laser showing constant power as a function of time. (B) Output beam of a pulsed wave laser showing pulses of duration, $\tau$, a period of $1/f_{rep}$, repetition rate of $f_{rep}$, with a peak power, $P_{peak}$, and an average power, $P_{avg}$. .......................................................... 43

**Scheme 3.1** Schematics showing the synthesis of HGNS by galvanic replacement. (A) Starting with Ag colloid, AuCl$_3$ is added, causing (B) Ag to oxidize and Au$^{3+}$ ions to
simultaneously reduce. (C) As the replacement continues, mass flows from the central region of the nanoparticle, forming a complete HGNS. (D) Then, dealloying occurs, resulting in fragmentation of the nanoparticle shell. (E) After complete Ag oxidation and diffusion, only pure Au fragments remain.

Figure 3.1 Extinction spectra and TEM images of Ag nanoparticle precursors and HGNS. (A) Extinction spectra of Ag colloid with 80, 60, and 40 nm diameters (top panel) and resulting HGNS of 82, 62, and 43 nm diameters (bottom panel). Representative TEM images of (B) 80, 60, and 40 nm Ag colloids and (C) 82, 62, and 43 nm HGNS are shown. All scale bars are 50 nm.

Figure 3.2 Plasmon resonance position as a function of time. (A) Extinction spectra versus time of 82 nm diameter HGNS showing a continuous blue-shift of the plasmon due to silver oxidation. (B) Extinction maximum of 82 nm HGNS as a function of reaction time.

Figure 3.3 (A) XPS atomic percentages and (B) TEM images of the formation of an 82 nm diameter HGNS illustrating the atomic percent of silver decreases and gold increases as a function of reaction time.

Figure 3.4 Finite element method (FEM) modeling and Mie theory of HGNS showing the influence of material porosity on the plasmon properties. Extinction cross sections calculated with the FEM of 82 nm diameter HGNS comparing the presence of holes in the particle surface with (A) nonporous (80% Ag, 20% Au) and (B) porous materials (43.2% Ag, 10.8% Au, and 46% H₂O). Spectra are plotted for the average E field polarization. (C) Extinction cross section spectra of the three sizes of HGNS with a H₂O core, alloy shell, and zero holes calculated with Mie theory show similar plasmon resonances in the visible region. (D) The three-hole porous model is used to calculate with FEM the extinction cross section spectra of 82, 62, and 43 nm diameter HGNS with extinction maxima of 800, 815, and 815 nm, respectively.

Figure 3.5 Comparison of the dielectric functions of non-porous (solid) and porous materials (dashed). The real (black) and imaginary (red) parts of the dielectric are shown for the Ag core (A) and the Ag/Au alloy shell (B). The material percentages used in the alloy are 80% Ag and 20% Au for the non-porous case, and 43.2% Ag, 10.8% Au, and 46% H₂O in the porous case.

Figure 3.6 Extinction cross section spectra of the porous three-hole 82 nm HGNS shows a plasmon red-shift as the percentage of water in the porous material is increased.

Figure 3.7 Effect of laser irradiation on HGNS with an 800 nm CW laser at 2.0 W/cm² for 25 min for three diameters: 82 nm (black), 62 nm (red), and 43 nm (blue). Extinction
spectra of HGNS (top panel) and HGNS@PEG (bottom panel) (A) before and (B) after laser irradiation. TEM images of 82, 62, and 43 nm diameter (C) HGNS and (D) HGNS@PEG after laser irradiation. All scale bars are 50 nm. 62

**Figure 3.8** Biodistribution of 82, 62, and 43 nm diameter HGNS@PEG determined by ICP-MS of three mice for each size of particle with xenografts transplanted from U87 cell lines. Biodistribution is shown independently for the gold content in each organ in µg/g of organ (A) and silver content in each organ in µg/g of organ (B). 64

**Figure 3.9** Comparison of the Ag:Au ratio in each organ determined by ICP-MS for 82 (A), 62 (B), and 43 nm diameter HGNS@PEG (C). Ag and Au content were calculated in µg/g of organ. The green line indicates the expected Ag:Au ratio of 4:1 as determined by XPS analysis of the particles prior to injection. 64

**Figure 3.10** Effect of a range of pH and human serum on HGNS with and without PEG. Extinction spectra of 82 nm diameter HGNS (A) and HGNS@PEG (B) after 24 h incubation in water adjusted to various pH. (C) TEM images of HGNS@PEG after 24 h incubation at pH 2 – 7. (D) HRTEM image of HGNS@PEG after 24 h incubation in human serum at physiological pH. All scale bars are 50 nm. 66

**Scheme 4.1** Formation of NS@dsDNA by hybridization of a thiolated ssDNA with a complementary fluorescein tagged ssDNA, followed by attachment to the NS surface through Au-thiol bonds, and subsequent DNA release when irradiated with NIR light. CW irradiation results in dehybridization and release of the fluorescein tagged ssDNA, while pulsed irradiation results in Au-S bond breakage and release of dsDNA. 76

**Figure 4.1** Nanocomplex characterization. (A) Extinction spectrum of bare NS (green) and NS@dsDNA (blue), TEM images of (B) bare NS and (C) NS@dsDNA, and (D) zeta potential of bare NS (green) and NS@dsDNA (blue). Scale bars are 50 nm. 77

**Figure 4.2** Determination of dsDNA Surface Coverage. dsDNA calibration curve where error bars represent triplicate fluorescence measurements of fluorescein tagged dsDNA (ex. = 485 ± 10 nm, em. = 535 ± 12.5 nm). Linear regression y = 409.7x + 312.0, R² = 0.998. 77

**Figure 4.3** DNA characterization. (A) Circular dichroism spectra of dsDNA as a function of temperature. (B) Change in ellipticity at 246 nm from dsDNA melting with a Boltzmann fit calculating a melting temperature of 55.2 ± 0.7 °C. 78

**Figure 4.4** Investigation of dsDNA melting. ssDNA release profile from solutions heated via a (A) temperature controlled cuvette stage and (B) CW laser at various laser powers.
A Boltzmann fit estimates a melting temperature of 61.1 ± 2.3 and 59.1 ± 4.5 °C, respectively. (C) Temperature controlled CW laser induced DNA release under 1.6 (red) and 3.4 W (black) laser irradiation with the stage set to 4 °C showing no DNA release. Inset: corresponding heating profile showing minimal increase in solution temperature.

**Figure 4.5** Non-temperature controlled CW laser induced ssDNA release. (A) Heating profile of DNA release at various laser powers, (B) thermal image of DNA release experiment at 1.5 W, (C) DNA released as a function of time at various laser powers, and (D) DNA release as function of cumulative incident energy.

**Figure 4.6** Femtosecond pulsed laser induced ssDNA release. (A) DNA release profile showing a maximum release at 25 mW. At powers above 25 mW, particle degradation occurs. (B) Heating profile of ultrafast pulsed laser DNA release at laser powers from 2-50 mW showing no bulk solution temperature increase. Inset: FEM simulation of the maximum temperature in a 6.6 nm layer around the NS.

**Figure 4.7** FEM simulation results. Spatially averaged temperature as a function of time in (A) a 6.6 nm layer surrounding the Au NS, which we identify as the domain of the dsDNA and (B) the Au shell.

**Figure 4.8** DNA release profile resulting from femtosecond pulsed laser irradiation using DNA that is thiolated on the 5’-end and tagged with fluorescein on the 3’-end and is hybridized with a non-tagged complementary strand. Release is linear from 2-35 mW with degradation occurring at powers above 35 mW.

**Figure 4.9** Nanoparticle characterization after pulsed laser induced DNA release. (A) Extinction spectroscopy showing a red-shift in the plasmon resonance at powers above 25 mW. (B) FEM simulation of the maximum average temperature of the Au shell after a single 150 fs laser pulse. Inset: temperature map for 25 mW incident power 100 ps after laser pulse (scale in °C). TEM images after laser irradiation at (C) 2, (D) 25, (E) 30, (F) 35, and (G) 50 mW showing particle degradation above 25 mW. Scale bars are 200 nm.

**Figure 4.10** Approximate energy density in the Au shell as a function of laser power. The dependent axis is calculated assuming all of the energy absorbed by a NS in a single cycle of the laser is deposited in the Au before any thermal relaxation occurs. The bulk melting energy density for Au is 190 J/g, while nanoscale reshaping values have been reported as much lower.

**Figure 4.11** Time and concentration dependence of pulsed laser induced DNA release. Variation of release as a function of (A) irradiation time at 25 mW with 1x10⁹ particles/mL fit with an exponential that reveals a time constant of 2.2 min, and (B)
concentration of NS@dsDNA irradiated at 25 mW and 10 min irradiation showing no appreciable change in release at different concentrations.

**Scheme 5.1** Nanocomplex formation. (A) Formation of NS@DNA@Drug nanocomplex by functionalization of NS with thiolated dsDNA through Au-thiol bonds, followed by attachment of drug molecules. Release triggered by CW irradiation results in DNA dehybridization and release of the drug, while pulsed irradiation results in Au-S bond breakage and release of the DNA-drug complex. (B) Formation of NS@HSA@Drug by coating NS with HSA, and attachment of drug molecules. Drug release from the protein is triggered by CW or pulsed irradiation.

**Figure 5.1** Nanocomplex characterization: (A) TEM images of bare NS (orange), NS@scaffold (blue), and NS@scaffold@drug (green) for DNA1 scaffold – DTX drug, DNA2 scaffold – LAP drug, and HSA scaffold – LAP drug coatings. Scale bars are 50 nm. (B) Table of corresponding extinction maxima, zeta potentials, and hydrodynamic diameters for scaffold and drug coatings.

**Figure 5.2** Nanocomplex surface characterization. TEM images (A) and extinction spectra (B) of bare NS (orange), NS@DNA2 (blue), and NS@DNA2@LAP (green) showing a minor red-shifts in the plasmon resonance and the formation of a thin DNA layer around the NS. Scale bars are 20 nm.

**Figure 5.3** SERS characterization to confirm drug loading. Normal Raman spectra of DTX (black) and LAP (black), and SERS spectra of NS@scaffold (blue) and NS@scaffold@drug (green) for (A) DNA1-DTX, (B) DNA2-LAP, and (C) HSA-LAP.

**Figure 5.4** XPS characterization to confirm LAP loading. (A) Survey scan and (B) S 2p element scan of NS@DNA2 (blue) and NS@DNA2@LAP (green).

**Figure 5.5** DNA characterization. (A) Circular dichroism spectra of DNA1 and DNA1-DTX. DNA melting curve for (B) DNA1 at 279 nm with a $T_m$ of 71.0 ± 0.5 °C and (C) DNA1-DTX at 277 nm with a $T_m$ of 69.0 ± 0.5 °C. (D) Circular dichroism spectra of DNA2 and DNA2-LAP. DNA melting curve for (E) DNA2 at 279 nm with a $T_m$ of 55.0 ± 1.2 °C and (F) DNA2-LAP at 278 nm with a $T_m$ of 57.3 ± 2.2 °C.

**Figure 5.6** Cellular uptake of nanocomplexes. Dark field spectroscopy of NS@HSA incubated with RAW 264.7 cells for (A) 0 h, (B) 1 h, and (C) 4 h. Brightfield images (20x objective, 4 averages) measured in transmission (D) without and (E) with nanocomplexes. (F) Orthogonal view of the 3D maximum intensity projection image of
cells incubated overnight with nanocomplex. Cells were stained for nucleus (Hoechst 33342, blue). ........................................................................................................ 109

Figure 5.7 Comparison of cell viability after DTX release from a DNA scaffold without (blue) and with (orange) CW and pulsed lasers in (A) MDA-MB-231 and (B) RAW 264.7 cells. .................................................................................................................. 111

Figure 5.8 Comparison of cell viability after LAP release in SKBR3 cells without (blue) and with (orange) CW and pulsed lasers from a (A) DNA scaffold and (B) protein scaffold. ........................................................................................................ 113

Figure 5.9 Comparison of cell viability after LAP release in RAW 264.7 cells without (blue) and with (orange) CW and pulsed lasers from a (A) DNA scaffold and (B) protein scaffold. ........................................................................................................ 114
List of Tables

Table 2.1 Melting temperatures of double-stranded and hairpin forms of various DNA sequences with 1 µmole oligo. ................................................................. 40

Table 2.2 Parameters used to characterize pulsed laser output ............................................. 43

Table 3.1 Particle dimensions and hole sizes used in the theoretical simulations. .......... 58

Table 3.2 Extinction Maximum and Zeta Potential of 80, 60, and 40 nm Diameter Ag Colloids and the Corresponding HGNS and HGNS@PEG........................................ 61

Table 5.1 SERS peak assignments for nanocomplex formation by the attachment of DNA, HSA, DTX, and LAP to NS surfaces ........................................ 104
**Nomenclature**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>APTES</td>
<td>(3-amino)propyltriethoxysilane</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced Permeability and Retention Effect</td>
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<tr>
<td>FDTD</td>
<td>Finite Difference Time Domain</td>
</tr>
<tr>
<td>FEM</td>
<td>Finite Element Method</td>
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<tr>
<td>HGNS</td>
<td>Hollow Gold Nanoshells</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively Coupled Plasma-Mass Spectrometry</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NIR</td>
<td>Near Infrared (~700 – 1500 nm)</td>
</tr>
<tr>
<td>NS</td>
<td>Nanoshells</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PTT</td>
<td>Photothermal Therapy</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<tr>
<td>SERS</td>
<td>Surface Enhanced Raman Spectroscopy</td>
</tr>
<tr>
<td>TEOS</td>
<td>Tetraethyl Orthosilicate</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>THPC</td>
<td>Tetrakis(hydroxymethyl)phosphonium Chloride</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet (~200 – 400 nm)</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray Photoelectron Spectroscopy</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

Nanoparticles are an area of intense research due to the unique optical, electronic, magnetic, and mechanical properties that arise from their small size. For instance optically, gold appears yellow and shiny in its bulk form, while nano-scale gold changes color depending on its size. The reason nanoparticles’ optical properties can be influenced by their physical properties is due to the plasmon resonance. The plasmon resonance is the collective oscillation of the conduction band electrons in a metal nanostructure. The plasmon energy decays either through scattering or absorption.

Nanoparticles can be engineered to exhibit the different properties of scattering and absorption for various applications including enhanced spectroscopy\(^1,^2\), energy harvesting\(^3\), photocatalysis\(^4,^5\), solar steam generation\(^6\), nano-circuits\(^7\), and biomedical applications\(^8,^9\). One major aspect of biomedical research is cancer treatment.

Cancer is one of the most devastating diseases facing the human population. Typical therapies for cancer include a combination of surgery, chemotherapy, and
radiation therapy. However, surgery typically requires removal of portions of healthy tissue, muscles, nerves, or blood vessels to resect all of the tumors cells. Chemotherapy is nonspecific and highly toxic. Radiation often damages the surrounding skin and soft tissue.¹⁰

Today as an alternative to traditional cancer therapies, nanoparticles are being engineered to selectively target, diagnose, and treat diseased cells.⁸,¹¹,¹² Nanoparticles therapies are non-invasive and cause minimal damage to the surrounding environment. They can treat tumors that are inoperable due to their size or location. Or can be used when surgery may also not be an option due to metastasis of the cancer to multiple organs or due to poor health conditions of the patient. Nanoparticles can also treat cancers, such as triple negative breast cancer, which cannot be treated with targeted chemotherapy drugs due to their lack of receptors.

Various types of nanoparticles have been utilized for cancer therapy including liposomes,¹³,¹⁴ hydrogels,¹⁵,¹⁶ and polymeric,¹⁷–¹⁹ magnetic²⁰,²¹ and metallic nanoparticles.²² Each nanoparticle type has their advantages and disadvantages. However, metallic nanoparticles, and in particular nanoshells, are of interest for cancer therapy because their optical properties, arising from the plasmon, can be exploited for a wide range of applications from tumor ablation to drug delivery. This thesis explores how to overcome the limitations of conventional cancer treatments by using metallic nanoparticles, specifically focusing on the synthesis of biocompatible nanoparticles and their applications in non-toxic nanoscale therapeutics, including drug delivery.
Chapter 2 contains background information on the fundamental concepts of plasmonic nanoparticles including plasmon resonance tunability and plasmon decay, and key characteristics to consider when designing a nanoparticle system for cancer therapy. Ideally, the nanocomplex should be biocompatible, absorb light in the near-infrared (NIR), exhibit efficient light-to-heat conversion, have high tumor uptake, encompass enhanced imaging capabilities such as MRI and fluorescence, and be able to actively release drugs. Factors that influence tumor uptake like nanoparticle size, charge, surface coating, and targeting ligands are also discussed. The use of DNA as a drug release scaffold is examined with specifics on DNA sequence design, hybridization, and characterization. Lastly, the differences in nanoparticle heating resulting from continuous wave and pulsed wave light sources are explored.

Chapter 3 investigates the surprising in vivo instability of a class of nanoparticles known as hollow gold nanoshells (HGNS). These particles are appealing for biomedical applications because near-infrared (NIR) resonances are achievable with diameters less than 100 nm. However, this study shows in vivo fragmentation with the Au and the remnants of the sacrificial Ag core accumulating differently in various organs. The origin of the NIR resonance is confirmed with theoretical modeling to be residual porous Ag in the nanoparticle core. The HGNS stability is investigated under continuous wave (CW) laser irradiation and in a wide range of pH environments and in serum, which confirmed HGNS fragmentation and leaching of Ag. These results demonstrate the importance of performing thorough nanoparticle stability studies prior to any intended in vivo trial application.
Chapter 4 explores the mechanism of NIR light-triggered DNA release from plasmonic nanoparticles under CW and pulsed wave laser irradiation as a platform for controlled drug delivery. Under CW irradiation, nanoparticle heating, as opposed to hot electrons, is shown to be responsible for the dehybridization. Additionally, release is shown to be highly dependent on nanoparticle concentration, requiring the bulk solution temperature to rise above the DNA dehybridization temperature to induce release at particle concentrations feasible in tumors. Alternatively, a femtosecond pulsed laser induces release through a hot electron mediated process without increasing the bulk temperature and is relatively independent of particle concentration. This is critically important for cancer treatment, as the cellular environment is very sensitive to temperature fluctuations and nanoparticle uptake in tumors is highly variable.

Chapter 5 builds upon the previous chapter by investigating intracellular NIR light-triggered release of metastatic breast cancer drugs. To attain therapeutic efficacy, a flexible drug loading and release strategy are essential. DNA and protein scaffolds are examined as hosts for the chemotherapy drugs and CW and pulsed wave lasers are investigated as release triggers. The results showed a higher percent cytotoxicity for CW laser-induced release from a DNA scaffold. Using the protein scaffold, increased cytotoxicity was observed for release using pulsed light in cancerous cells, while non-cancerous cells were unaffected. These results show that light-triggered drug release is an effective non-toxic drug delivery vehicle to selectively kill cancers cells.

Finally, Chapter 6 presents general conclusions of the thesis.
2.1. Plasmonic Nanoparticles

Metallic nanoparticles exhibit unique optical properties arising from localized surface plasmon resonances. When incident light strikes a metal nanostructure it can induce a collective oscillation of the conduction band electrons, known as the plasmon resonance (Figure 2.1). The electrons oscillate against the restoring force of the positive nuclei. The frequency at which the oscillation occurs is known as the plasmon resonance frequency and must match the frequency of the incident light for resonance to occur. Properties of both the nanoparticle and its local environment influence the plasmon frequency. For instance, large nanoparticles have lower plasmon resonance frequencies than smaller nanoparticles. Many of the factors influencing the plasmon resonance frequency are discussed in section 2.1.1. Once the plasmon is excited, its energy
dissipates through both radiative (scattering) and non-radiative (absorption) processes, which are discussed in section 2.1.2.

**Figure 2.1** Schematic of the plasmon resonance showing excitation of the collective oscillation of the conduction band electrons by incident light.

### 2.1.1. Nanoshells and Plasmon Resonance Tunability

Nanoshells (NS) are spherical particles, composed of a dielectric silica (SiO$_2$) core surrounded by a continuous metallic gold shell, which support a localized surface plasmon.$^{23}$ The nanoshell plasmon resonance frequency can be tuned from visible to near-infrared (NIR) wavelengths by changing the core-to-shell dimensions, i.e. the ratio of the sphere radius to the nanoshell radius ($r_1/r_2$ ratio) (Figure 2.2). The plasmon resonance is shifted to shorter wavelengths by decreasing the core size or by increasing the shell thickness. Conversely, the plasmon resonance is shifted to NIR wavelengths by increasing the core size or by thinning the gold shell.
Figure 2.2 Tuning of the plasmon resonance frequency of gold nanoshells by controlling the shell thickness and schematic of a gold nanoshell with variable core ($\varepsilon_1$), shell ($\varepsilon_2$), and medium ($\varepsilon_3$) dielectric constants and an inner and outer radius of $[r_1, r_2]$.

To fabricate nanoshells, first, silica cores are synthesized by the Stöber method. In a water/ethanol mixture, upon addition of ammonium hydroxide, tetraethyl orthosilicate (TEOS) undergoes hydrolysis followed by condensation to form SiO$_2$ nanoparticles (Figure 2.3A,E). The surface of the SiO$_2$ nanoparticles is aminated by silanization with 3-aminopropyltriethoxysilane (APTES) (Figure 2.3B). Small 2-3 nm gold colloid are attached to the SiO$_2$ surface through the amine groups to form the seeded precursor (Figure 2.3C,F). The small gold colloid are previously synthesized through reduction of hydrogen tetrachloroaurate(III) with tetrakis(hydroxymethyl)phosphonium chloride (THPC). Next, the seeded precursor is added to a solution of Au$^{3+}$ and
potassium carbonate (plating solution). Addition of formaldehyde reduces Au\(^{3+}\) to Au\(^0\) causing the gold seeds on the SiO\(_2\) surface to coalesce into a shell (Figure 2.3D,G). The thickness of the shell is determined by the ratio of seeded precursor to plating solution. The addition of more seeds with a constant volume of plating solution and formaldehyde results in thinner shells and red-shifted plasmon resonances.

**Figure 2.3** Schematic and corresponding TEM images of nanoshell fabrication showing (A,E) 120 nm silica cores, (B) amination of the silica core with APTES, (C,F) formation of seeded precursor by attachment of 2-3 nm Au colloid, and (D,G) growth of a terminal 22 nm thick gold shell using hydrogen tetrachloroaurate, potassium carbonate, and formaldehyde.

The tunability of the nanoshell plasmon resonance is described by plasmon hybridization.\(^{26,27}\) Plasmon hybridization is the electromagnetic analog of molecular orbital theory, which describes the interaction of atomic orbitals to form molecular orbitals. Similarly, plasmon hybridization describes the interaction of two individual plasmon modes to form two new hybrid plasmon modes (Figure 2.4A). In the case of nanoshells, both the outer surface (sphere mode) and the inner surface (cavity mode) of
the nanoshell can support plasmons. The sphere and cavity modes interact to form new lower energy, symmetric bonding and higher energy, anti-symmetric antibonding nanoshell plasmon modes. The anti-symmetric plasmon mode has a net zero dipole moment and is therefore a dark mode, meaning it weakly couples to light and is typically not observable. The bonding mode, on the other hand, has a large dipole moment and strongly couples to light. The strength of the coupling between the sphere and cavity modes is determined by the gold shell thickness (Figure 2.4B). A thinner shell results in stronger coupling and a larger energy splitting of the hybrid modes, lowering the energy of the bonding mode and increasing the energy of the antibonding mode.

The energy of the plasmon resonance depends on the nanoparticle’s material, local environment, size, geometry, and on its proximity to other nanoparticles. For instance, silver nanoparticles have higher energy plasmon resonances than gold nanoparticles and nanoshells with higher dielectric cores (e.g. iron oxide vs. silica) have lower energy plasmons.

Nanoparticles in water as compared to air have lower energy plasmon resonances, while smaller nanoparticles have higher energy plasmons. Breaking the symmetry (e.g. a nanorod) or increasing the number of layers in a nanoparticle (e.g. a nanomatryushka) creates new higher and lower energy modes. Bringing nanoparticles into close proximity to each other, couples their plasmons, shifting the resonance to lower energies. For example, the appearance of a low energy plasmon peak indicates nanoparticle aggregation. All of these criteria are important to consider when designing a nanoparticle system.
Figure 2.4 Plasmon hybridization model of a gold nanoshell. (A) Detailed model showing the interaction of the sphere ($\omega_{sp}$) and cavity ($\omega_c$) modes to form the observable bonding ($\omega_-$) and antibonding ($\omega_+$) modes. (B) Illustration of the resulting bonding mode plasmon energy based on the strength of the interaction between the sphere and cavity plasmon modes determined by the thickness of the gold shell.
2.1.2. Plasmon Decay

The plasmon energy decays through either scattering or absorption. Scattering is a radiative process that produces photon emission, which can be used for characterizations such as UV-Vis spectroscopy. Absorption is a non-radiative process that produces “hot” electron-hole pairs, which can go on to produce heat or the electrons can be transferred on to do work. Upon non-radiative decay of a plasmon, an electron is elevated to an excited energy state above the Fermi energy. The hot electron then relaxes to equilibrium through electron-electron scattering, causing a temperature increase of the electron gas of the nanoparticle. This process occurs on the femtosecond timescale. Within a few picoseconds, the electrons couple to phonons (a nanoparticle lattice vibration) causing the electron gas to cool and the nanoparticle lattice to heat up. The phonons then undergo phonon-phonon coupling in several hundred picoseconds, which transfers heat from the nanoparticle lattice to the surrounding medium. It is important to note that the nanoparticle can melt under laser irradiation if the rate at which energy is absorbed exceeds the rate at which the particle can cool from energy transfer through phonon-phonon coupling to the environment.

The rate of heat dissipation from the nanoparticle to the surrounding medium is highly dependent on the medium characteristics. For instance, if a nanoparticle is suspended on a substrate where most of its contact is with air, heat dissipation from the nanoparticle to air has low efficiency due to air’s low thermal conductivity (0.024 W/(m•K)). This can allow for a large amount of heat to build up in the nanoparticle, causing it to melt. Conversely, a nanoparticle suspended in water and irradiated under the same condition as in air can remain stable since water has a much higher thermal
conductivity (0.6 W/(m•K)). The more efficient heat transfer causes a high temperature build up in the water as opposed to the nanoparticle. This process of heat production on the nanoparticle surface and subsequently the surrounding medium is used for cancer therapies to both induce photothermal cell death and to induce dehybridization of DNA on the nanoparticle surface.

2.2. Ideal Nanoparticle System for Biomedical Application

Many types of nanoparticle complexes have been investigated for biomedical applications like tumor ablation and drug delivery. For these applications, there are a number of characteristics that are favorable to incorporate in a multifunctional nanoparticle system; these include biocompatibility, absorption of NIR light, high uptake in tumors, imaging capability, and an active drug release scaffold. These characteristics are illustrated in Figure 2.5 and discussed in detail below.

2.2.1. Biocompatibility

The most important characteristic of the nanoparticle system is biocompatibility. The particles must consist of stable, non-toxic materials. Gold nanoparticles have been studied extensively and many studies have not reported cytotoxic effects.\textsuperscript{32–34} Nanoshells, composed of silica core and gold shell, have undergone long-term toxicity studies in dogs and found to have no bioincompatibilities for over 10 months.\textsuperscript{35} Silver nanoparticles, on the other hand, can aggregate or dissolve into Ag ions \textit{in vivo}, which has known cytotoxic effects.\textsuperscript{36–40}
Figure 2.5 Characteristics of an ideal nanoparticle system. The nanoparticle system should be (1) biocompatible, (2) absorb light in the near-infrared (NIR) and exhibit efficient light-to-heat conversion, (3) have high tumor uptake, which depends on nanoparticle size, charge and surface coating, and tumor targeting, (4) encompass enhanced imaging capabilities, and (5) be able to actively release drugs.

If toxic materials are included in the nanocomplex, then they need to be protected within the complex to prevent leaching. Gadolinium(III), for instance, is a useful magnetic resonance imaging (MRI) contrast agent. However, gadolinium ions are similar in size to Ca$^{2+}$ ions and as a result can bind competitively with higher affinity to many biological systems such as enzymes.$^{41,42}$ Furthermore, gadolinium is suggested to cause nephrogenic systemic fibrosis (NSF).$^{43-45}$ To use gadolinium in vivo, efforts have been
made to encapsulate it within a gold shell. Methods like this are necessary to limit systemic toxicity.

2.2.2. NIR Absorption and Light-to-Heat Conversion

Photothermal therapy (PTT) is a heavily studied nanoparticle-based method of tumor ablation. In PTT, nanoparticles are irradiated with light at their plasmon resonance, the absorbed light is transduced into heat, which causes local heating of the tumor. The increased heat triggers cell hyperthermia, resulting in cell death. To maximize PTT efficiency, the nanoparticle should have a large absorption cross section in order to efficiently convert light into heat. In other words, the nanoparticle should have a high ratio of absorption to scattering, which is dependent on the nanoparticle size. Large nanoparticles scatter more light than they absorb but as the particle size shrinks the scattering efficiency decreases and eventually absorption dominates. For instance, 150 nm nanoshells have only 15% absorption efficiency, while 90 nm nanomatryoshkas have 77% absorption efficiency.

In addition to large absorption cross sections, the nanoparticles should absorb light in the NIR. At visible wavelengths, skin and tissue strongly absorb light. However, in the NIR, there is a region from ~700-900 nm where hemoglobin and water are maximally transparent and penetration of NIR light into tissue is maximal. This region is known as the NIR water window. Because of the low absorption of NIR light in tissue, the light itself does not damage the tissue; instead, the heat from the nanoparticle causes the desired cellular damage. By using NIR light, deeper tumors can be accessed and PTT efficiency increased.
2.2.3. Nanoparticle Uptake in Tumors

Maximizing nanoparticle uptake in tumors is an important clinical interest. Nanocomplex designs are becoming more sophisticated incorporating imaging and treatment agents. However, their efficacy is limited by their deliverability. Facilitating greater uptake can improve diagnostic and therapeutic applications. The magnitude of nanoparticle accumulation in tumors depends on a number of factors including nanoparticle size, charge, and surface coating, as well as targeting agents.

2.2.3.1. Size

For biological applications, it is widely believed that permeation and accumulation of nanoparticles in tumors is dependent upon nanoparticle size, and should be enhanced for nanoparticles with sizes below 100 nm. Specifically, nanoparticles 10-100 nm in diameter will accumulate in tumors because of the enhanced permeability and retention (EPR) effect, which explains that nanoparticles will preferentially naturally accumulate in tumor cells over normal tissue due to the leaky vasculature and poor lymphatic drainage present in tumors.\(^{52,53}\) Particles smaller than 5 nm have short circulation times because they easily diffuse out of the blood vessels because the average pore size in endothelium is 5 nm.\(^{17}\) Furthermore, particles smaller than 10 nm may be excreted from the body by renal filtering.\(^{54}\)

Yet, the optimal size reported varies across the literature, depending on nanoparticle type and cancer cell line or tumor type. For instance, \textit{in vitro} it has been demonstrated that 50 nm gold nanoparticles show optimal uptake in HeLa (lung cancer) cells.\(^{55}\) \textit{In vivo}, 40 nm iron oxide nanoparticles had higher uptake in skin cancer tumors.
(SCCVII cells) than 20 nm particles, which is contrary to a different in vivo study of skin cancer tumors (A431 cells) showing 20 nm gold nanoparticles to have higher uptake than 40 and 80 nm nanoparticles. Furthermore, a study of polymeric micelles revealed that 30, 50, 70, and 100 nm micelles accumulated similarly in highly permeable colon cancer tumors (C26 cells) but only 30 and 50 nm micelles entered poorly permeable pancreatic cancer tumors (BxPC3 cells). These studies underscore the variability in nanoparticle uptake depending on tumor biology.

2.2.3.1.1. Challenges in Designing Small Nanoparticles

It is not trivial to have nanoparticles that are smaller than 100 nm and exhibit a NIR resonance (Figure 2.6). For instance, a 50 nm gold colloid has a resonance in the visible region around 520 nm. Alternatively, nanoshells composed of a 120 nm silica core and 30 nm Au shell have a resonance at 800 nm but the particles are ~150-180 nm in size. If the overall size of the nanoshell is shrunk to 90 nm, the plasmon resonance shifts to the visible region around 650 nm. Blue-shifting plasmon resonances with decreasing nanoparticle sizes pose a challenge in nanoparticle design.

One type of particle that can both have a physical size below 100 nm and exhibit a NIR plasmon resonance is nanorods. Nanorods exhibit a high-energy transverse plasmon resonance from excitation of the short axis of the nanorod and a lower energy longitudinal plasmon resonance from excitation of the long axis of the nanorod. The longitudinal resonance can easily be tuned across the near-IR by changing the aspect ratio, while maintaining sizes below 100 nm. However, nanorods are synthesized using the surfactant, cetyltrimethylammonium bromide (CTAB), which is highly toxic to
Furthermore, nanorods can reshape under both pulsed and continuous wave laser illumination, causing them to lose their NIR resonance, making them undesirable for photothermal therapy.\textsuperscript{61,62}

Figure 2.6 Plasmon resonance frequency dependence on nanoparticle size, geometry, and dielectric constant. Schematics and corresponding extinction spectra of a 50 nm Au sphere (black), 150 nm nanoshell (red), 90 nm nanoshell (blue), 55 x 14 nm nanorod (green), 82 nm HGNS (magenta), 90 nm nanomatryoshka (cyan), and 100 nm nanoshell with an iron oxide core (orange). The highlighted sections in the extinction spectra represent the desired NIR wavelength range.

Hollow gold nanoshells (HGNS) are another type of particles that can achieve these criteria. HGNS are made by a galvanic replacement between two metals with varying reduction potentials. Nanoparticles of the metal with the lower reduction potential are used as a template that oxidize as ions of the higher reduction potential metal are reduced onto the template, resulting in a hollow nanoparticle with a thin shell of the higher reduction potential metal. The templates are typically silver or cobalt, with gold being used for the shell.\textsuperscript{63–70} However, it has been shown that the template metal
does not always completely dissolve, bringing up toxicity concerns. These particles’ \textit{in vivo} applicability is investigated in detail in Chapter 3.

There are two alternate ways to address the issue of small sizes and visible plasmon resonances. The first is to use a >2 layer particle geometry. Nanomatriyoshkas are a three-layer particle composed of a gold core and outer gold shell, with an intermediate silica layer. By using three layers instead of two layers, the plasmon resonance splits into two new modes, one of higher and one of lower energy. Nanomatriyoshkas can be made 88 nm in diameter with plasmon peaks at 560 and 780 nm.\cite{9} The second method is by using a high dielectric material. For instance in the case of nanoshells, the silica core has a dielectric constant ($\varepsilon_c$) of 2.08. By using copper oxide ($\varepsilon_c = 7$) or mixed valency iron oxide ($\varepsilon_c = 12$), the nanoshell resonances can be red-shifted to the NIR.\cite{72}

\subsection*{2.2.3.2. Charge and Surface Coating}

Nanoparticle charge is another important factor that dictates tumor uptake. Highly charged particles are readily recognized and sequestered by the immune system exhibiting increased accumulation in the reticuloendothelial system (RES) (liver and spleen).\cite{52,73} Furthermore, positively charged nanoparticle typically have adverse effects on cell viability.\cite{74,75} However, particles with close to neutral charge readily aggregate as their electrostatic repulsion is minimized. Particles with slightly negative zeta potentials of -8 to -15 mV have been shown to maintain colloid stability while maximize tumor uptake and avoiding uptake by macrophages.\cite{75,76}
Surface coatings can also help to maximize tumor uptake. Specifically, coating nanoparticles with polyethylene glycol (PEG) has been shown to be advantageous \textit{in vivo}. PEG improves circulation time of the particles by reducing nonspecific binding of proteins onto the nanoparticles and by allowing for precise control over the surface charge.\textsuperscript{77,78} In addition, PEG inhibits opsonization, the process by which the particles are branded for ingestion by phagocytes.\textsuperscript{52,77,78} The molecular weight of PEG also plays a role with higher molecular weights (5000 vs. 2000 Da) resulting in increased blood circulation half-life.\textsuperscript{57}

\subsection*{2.2.3.3. Targeting}

Targeting allows therapeutics to be delivered selectively to the diseased region leaving healthy regions unaffected and accelerates particle accumulation in tumors. The surface ligands used are chosen to target receptors that are overexpressed in the tumor. One such type of strategy is to functionalize nanoparticles with antibodies. Antibodies can bind specifically with high affinity to a particular antigen. Many tumors express certain antigens, allowing for effective antibody targeting through a specific lock and key mechanism. For instance, human epidermal growth factor receptor 2 (HER2) is a protein over-expressed in breast cancer cells. Anti-HER2 antibody has been conjugated to the surface to selectively target HER2, increasing nanoparticle accumulation in tumors.\textsuperscript{8}

Another approach is the use of aptamers. Aptamers are short single-stranded oligonucleotides that are specifically designed to bind to a particular target, such as a protein or small molecule, with high affinity and specificity. Large libraries of oligonucleotides with random sequences are screened to determine the exact aptamer
sequence for a chosen target. Farokhzad et al. demonstrated a 62% reduction in tumor size by coating polymeric nanoparticles with an aptamer that targets prostate-specific membrane antigen (PSMA) on the surface of prostate cancer cells (LNCaP cells).

Incorporation of too many targeting agents though can have adverse effects. Dense coverage of targeting agents increases surface charge and as a result reduces tumor accumulation. Gu et al. increased polymeric nanoparticles uptake in pancreatic tumors (LNCaP cells) 47% by functionalizing the particles with a 1% surface coverage of aptamers. By increasing the surface coverage to 5%, the uptake increased 68%. However, when the surface coverage was increased to 10%, uptake dropped by 59%.

It is important to note that the targeting agent must be in close proximity to the receptor site in order for the interaction to occur. Therefore, even targeted nanoparticles still rely on the EPR effect to reach the tumors. Though nanoparticle clearance can be minimized once the binding occurs, in effect anchoring the nanoparticle to the tumor. To increase the opportunities for the particles to interact with the intended receptor, nanoparticle blood circulation half-lives should be maximized. Therefore, the effective use of targeting agents requires not only the appropriate choice of targeting ligand and ligand density, but also surface charge optimization to extend circulation times.

2.2.4. Enhanced Imaging Capabilities

Ideally, imaging agents are incorporated in the particles to track them in vivo. Tracking enables a non-invasive means to determine the optimal time for maximum nanoparticle uptake prior to treatment. Particles can be tracked by fluorescence or magnetic resonance imaging (MRI). Fluorescence imaging involves the detection of
photons emitted from a fluorescent molecule when excited by light. In nanoparticle systems, the fluorescence signal of fluorophores on the surface of metallic nanoparticles is quenched; however, when placed a few nanometers from the nanoparticle surface, the fluorescence can be strongly enhanced.\textsuperscript{81,82} Fluorescence imaging is an inexpensive and rapid technique but is low resolution due to the diffraction limit of light and the penetration depth of light into tissue is low.

MRI, on the other hand, has high spatial resolution, enabling deep tissue imaging. It has relatively low sensitivity, but the sensitivity can be increased by the use of contrast agents. Typically, either superparamagnetic iron oxide is utilized as a negative ($T_2$) contrast agent, darkening the MR image, or paramagnetic gadolinium(III) is used as a positive ($T_1$) contrast agent, brightening the MR image. Either contrast agent can be incorporated into the nanovehicle to enable tracking.

\textbf{2.2.5. Active Drug Release}

A nanoparticle delivery vehicle should be able to deliver high amounts of chemotherapeutic drugs to tumor sites without releasing any of its cargo to the environment during transport. Once the particles reach the diseased region, drug delivery can be achieved either through passive or controlled release. Passive release exploits variations in the intracellular environment between healthy and diseased tissues, including pH\textsuperscript{19,83–85} and temperature,\textsuperscript{16,18,83–85} to prompt release. Yet, pH and temperature can vary with cell type and location causing premature or erratic release, increasing the possibility of non-specific cytotoxicity. Instead, controlled release systems allow for precise spatial and temporal release by application of an external trigger such as
ultrasound,\textsuperscript{86} magnetic\textsuperscript{87,88} and electric\textsuperscript{89,90} fields, and UV to NIR light.\textsuperscript{91–95} Active drug release using NIR light is discussed in chapter 5.

\subsection*{2.3. DNA as a Drug Release Scaffold}

Light-triggered release from plasmonic nanoparticles has been shown to be an effective way to deliver oligonucleotides and molecules complexed to DNA.\textsuperscript{93,96–98} The surface of nanoparticles can be functionalized with double-stranded DNA (dsDNA), where one strand is thiol terminated and bound to the gold surface and the complementary strand is hybridized to the thiolated strand through non-covalent interactions. When the particles are irradiated with NIR light, the dsDNA is dehybridized releasing the nonthiolated single-stranded DNA (ssDNA). Controllable DNA release is useful for gene therapies including plasmid therapy (synthesizes new healthy proteins), aptamer therapy (inhibits malfunctioning proteins), antigene therapy (blocks transcription), and antisense therapy (blocks translation).\textsuperscript{97}

In addition, dsDNA functionalized nanoshells have been shown as an effective delivery vector for molecules that reversibly bind to the DNA helix. For instance, upon irradiation of the nanoparticle, the dsDNA dehybridizes releasing the dye molecule DAPI (4',6- diamidino-2-phenylindole) and the ssDNA.\textsuperscript{93} Light-triggered release from dsDNA can be employed for the numerous molecules that associate with DNA, including many chemotherapeutic molecules.

Two mechanisms have been proposed as possible driving forces of DNA dehybridization: (1) non-radiative decay of the plasmon, which causes high temperatures
in the direct, nanoscale vicinity of the particle surface, resulting in thermal dehybridization or (2) “hot” electrons generated from radiative plasmon decay, which are injected into the DNA strands, increasing electrostatic repulsion and facilitating dehybridization.\textsuperscript{98} The mechanism of DNA dehybridization is investigated in chapter 4.

2.3.1. DNA Sequence Design

In order to effectively control DNA dehybridization, the DNA sequence itself must be specifically designed to have an optimal melting temperature for \textit{in vivo} use. The melting temperature ($T_m$) at which dsDNA dehybridizes depends on a number of factors including nucleic acid base sequence, oligonucleotide strand length, and salt concentration. For instance, sequences with higher guanine-cytosine (GC) content have higher melting temperatures than adenine-thymine (AT) rich sequences due to increased stacking interactions and a third hydrogen bond. The $T_m$ needs to be higher than the body temperature of 37 °C but not too hot to result in widespread cell damage. An online calculator, Integrated DNA Technologies’ OligoAnalyzer, can predict the $T_m$ of a particular DNA sequence and salt concentration.\textsuperscript{99} However, it is important to note when designing the sequence for a specific temperature that attachment of DNA to a nanoparticle surface will alter the $T_m$. The dsDNA $T_m$ has been observed to increase when the dsDNA is tethered to the NS surface likely as a result of increased steric hindrance and a difference in salt concentration \textit{vs.} free dsDNA in solution.\textsuperscript{100}

In addition to careful choice of the dsDNA melting temperature, the melting temperature of any hairpin structures must be considered. Also called stem-loop base pairing, hairpin structures form when ssDNA has two regions that are complementary
when read in opposite directions. The ssDNA self-binds to form a double helix region and a loop region composed of unpaired bases. The DNA sequence must be chosen such that all of the hairpin structures have a lower melting temperature than the dsDNA. In order to hybridize two DNA strands, the strands are heated to a high temperature to unfold any secondary structures. If the melting temperatures have appropriately been chosen, as the DNA cools, it will preferentially hybridize over forming hairpins.

Various DNA sequences were simulated to attain a $T_m$ around 55 °C with the double-stranded form having a higher $T_m$ than the hairpin form. Table 2.1 shows the variability in DNA melting temperatures based on changes in sequence and salt concentration. In most cases, multiple hairpin structures are possible. The table shows only the highest melting temperature of the hairpin structures. Highlighted in red are the bases that have been altered from the original DNA sequence. These sequences illustrate the large shifts in melting temperature that can occur by changing as little as one base.

**Table 2.1** Melting temperatures of double-stranded and hairpin forms of various DNA sequences with 1 µmole oligo.

<table>
<thead>
<tr>
<th>DNA Sequence</th>
<th>$[Na^+]$ (mM)</th>
<th>Double-Strand $T_m$ (°C)</th>
<th>Hairpin $T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAT GAT CTG TCA CAG CTT GA</td>
<td>70</td>
<td>55.4</td>
<td>28.9</td>
</tr>
<tr>
<td>TAT GAT CTG TCA CAG CTT GA</td>
<td>50</td>
<td>52.7</td>
<td>27.6</td>
</tr>
<tr>
<td>TAT GAT CTG TCA CAG CTT GA</td>
<td>30</td>
<td>48.2</td>
<td>25.6</td>
</tr>
<tr>
<td>TAT GAT CTG TCA TAG CTT GA</td>
<td>50</td>
<td>49.7</td>
<td>40.9</td>
</tr>
<tr>
<td>TAT GAT CTA TCA TAG CTT GA</td>
<td>50</td>
<td>46.6</td>
<td>43.3</td>
</tr>
<tr>
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<td>50</td>
<td>60.4</td>
<td>33.5</td>
</tr>
<tr>
<td>TCT GAT CTG GCA CAG CCT GA</td>
<td>50</td>
<td>60.4</td>
<td>51.7</td>
</tr>
</tbody>
</table>
2.3.2. DNA Preparation and Nanoparticle Functionalization

DNA is prepared and functionalized to nanoparticles according to previously published methods. Briefly, DNA strands are purchased from Integrated DNA Technologies in 1 µmole quantities of oligo and are purified by high-performance liquid-chromatography (HPLC). DNA strands thiolated on their 5'-end are reduced with 1,4-dithio-DL-threitol (DTT) and purified with NAP25 purification columns. Fluorescent strands are redispersed in TE buffer. The DNA strands are hybridized by mixing the two complementary stands in a 1:1 M ratio in buffer (TE/33 mM NaCl, pH = 7.5). The solution is boiled in a water bath for 4 min and slowly cooled to room temperature overnight. The dsDNA is incubated with the nanoparticles in a ratio of 20,000 DNA strands/particle overnight with gentle rocking. The DNA is attached to the nanoparticle surface through a gold-thiol bond. The mixture is centrifuged and resuspended in TE buffer to remove excess free DNA in solution. The nanoparticle concentration is determined from the extinction spectrum and the extinction cross section calculated from Mie theory.

2.3.3. DNA Characterization

Once the DNA has been hybridized the actual melting temperature in solution can be characterized by circular dichroism (CD). A dsDNA solution with the desired buffer and salt concentration is heated from 25 °C to 90 °C. CD spectra are collected typically in 5 °C temperature increments after 180-300 s equilibration at each temperature. The maximum or minimum amplitude of ellipticity (θ) is extracted from each spectrum and plot as a function of temperature to obtain the melting curve. The first derivative of a
Boltzmann fit of the melting curve reveals the melting temperature.

2.4. Continuous Wave vs. Pulsed Wave Light Sources

For applications such as light-triggered drug release and photothermal therapy, the heating dynamics of a nanoparticle and its surrounding environment under laser irradiation is an important factor in determining the response achieved. The temperature increase of a nanoparticle is a function of the energy absorbed by that nanoparticle. The amount of energy the nanoparticle absorbs depends on the absorption cross section of the nanoparticle and the laser fluence (energy per unit area). Both continuous wave (CW) and pulsed wave lasers have been used to increase the temperature of nanoparticles, but in differing manners.

Continuous wave lasers emit light with constant output power over time (Figure 2.7A). Conversely, pulsed lasers emit light through a series of pulses over time. The pulses have a defined duration, $\tau$, repetition rate, $f_{\text{rep}}$, and peak power, $P_{\text{peak}}$, with a resulting average power over time of $P_{\text{avg}}$ (Figure 2.7B). A few important parameters used to characterize pulsed lasers and their mathematical relationships are listed below in Table 2.2.\textsuperscript{101,102}

At long length (distance from the nanoparticle surface) and time (time from a single pulse) scales, heating of the nanoparticle from CW and pulsed lasers is the same. However, on short length and time scales, the nanoparticle experiences very different heating with pulsed irradiation as compared to CW. For instance, in the some of the light-triggered release experiments discussed in chapter 4, an ultrafast laser is used with a
Figure 2.7 Comparison of continuous and pulsed wave lasers. (A) Output beam of a continuous wave laser showing constant power as a function of time. (B) Output beam of a pulsed wave laser showing pulses of duration, $\tau$, a period of $1/f_{rep}$, repetition rate of $f_{rep}$, with a peak power, $P_{peak}$, and an average power, $P_{avg}$.

Table 2.2 Parameters used to characterize pulsed laser output.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
<th>Units</th>
<th>Key Relationships</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\tau$</td>
<td>Pulse Duration</td>
<td>sec (s)</td>
<td>$\tau = D / f_{rep}$</td>
</tr>
<tr>
<td>$f_{rep}$</td>
<td>Repetition Rate</td>
<td>Hz = s$^{-1}$</td>
<td>$f_{rep} = D / \tau$</td>
</tr>
<tr>
<td>$D$</td>
<td>Duty cycle</td>
<td>dimensionless</td>
<td>$D = \tau \times f_{rep}$</td>
</tr>
<tr>
<td>$P_{peak}$</td>
<td>Peak Power</td>
<td>Watts (W) = Joules (J)/s</td>
<td>$P_{peak} = E / \tau$</td>
</tr>
<tr>
<td>$P_{avg}$</td>
<td>Average Power</td>
<td>W = J/s</td>
<td>$P_{avg} = E \times f_{rep}$</td>
</tr>
<tr>
<td>$E$</td>
<td>Pulse Energy</td>
<td>J</td>
<td>$E = P_{peak} \times \tau$ ; $E = P_{avg} / f_{rep}$</td>
</tr>
<tr>
<td>$A$</td>
<td>Area of Laser Spot</td>
<td>cm$^2$</td>
<td>$A = \pi r^2$</td>
</tr>
<tr>
<td>$I$</td>
<td>Intensity</td>
<td>W/cm$^2$</td>
<td>$I_{peak} = P_{peak} / A$</td>
</tr>
<tr>
<td>$F$</td>
<td>Fluence</td>
<td>J/cm$^2$</td>
<td>$F = E / A$ ; $F = I_{peak} \times \tau$</td>
</tr>
</tbody>
</table>

pulse duration of 160 femtoseconds and a repetition rate of 250 kHz (or 4 µs). Because of the short pulse duration, very high peak powers can be achieved, while maintaining a low average power. As a result, the nanoparticle surface can reach extremely high temperatures without heating the bulk solution. This is important to induce DNA dehybridization without heating the surrounding environment, which in vivo could damage healthy tissue. Though the peak power must be carefully regulated as to not melt the nanoparticle. The other notable difference is that with a CW laser, the nanoparticle
and environment reach equilibrium quicker because it does not push the system as far out of equilibrium as a pulsed laser.
Chapter 3

The Surprising \textit{in Vivo} Instability of Near-IR-Absorbing Hollow Au-Ag Nanoshells


3.1. Introduction

Cancer is one of the most devastating diseases facing the human population. There is currently intense interest in engineering nanoparticles for diagnosis and/or treatment of this disease.\textsuperscript{8,11,12} In particular, photothermal therapy (PTT) has emerged as an effective way to selectively ablate tumors. When nanoparticles are taken up into tumors, the tumor is irradiated with near-infrared (NIR) light at the nanoparticle plasmon
resonance. The absorbed light is converted into heat, resulting in cell hyperthermia, subsequent cell death, and tumor remission.\textsuperscript{8,11,12} Because of the low inherent absorption of NIR light in tissue, the light itself does not significantly damage the tissue in the absence of nanoparticles, rendering this approach minimally invasive.\textsuperscript{51} Photothermal therapy has been successfully demonstrated with many NIR-absorbing gold nanostructures including silica-gold nanoshells,\textsuperscript{11,48,103} nanorods,\textsuperscript{50,104,105} branched nanoparticles,\textsuperscript{49} nanohexapods,\textsuperscript{106} nanocages,\textsuperscript{73,107} and hollow gold nanoshells.\textsuperscript{108,109}

It is widely believed that passive (non-antibody-targeted) uptake of nanoparticles in cells and in tumors is dependent upon nanoparticle size, in addition to other factors such as zeta potential, and should be enhanced for nanoparticles with sizes below 100 nm. For example, 45 nm gold nanoparticles have been shown with optical sectioning microscopy to have higher uptake in lung cancer and HeLa cells than 70 and 100 nm gold nanoparticles.\textsuperscript{110} Passive uptake in tumors is known as the enhanced permeability and retention (EPR) effect and occurs due to the leaky vasculature and poor lymphatic drainage characteristic of tumors.\textsuperscript{52,53} Hollow core-shell nanoparticles, whose internal geometry can be adjusted to achieve strong NIR absorption across a broad particle size range, can provide this property in the sub-100 nm nanoparticle size range.

Multiple groups have reported that hollow Au nanoshells (HGNS) can be made successfully in the sub-100 nm size range, with NIR plasmon resonances, using a galvanic replacement reaction between Au and Ag, or Au and Co.\textsuperscript{63–70} In a galvanic replacement synthesis, a salt solution of a given metal with a high reduction potential is added to a colloidal solution of a metal with a lower reduction potential. The difference in reduction potentials causes the metal in the salt solution to be reduced, while the metal in
the nanoparticle template simultaneously oxidizes. For the case of Au and Ag, the galvanic replacement occurs according to the following reaction.\textsuperscript{111}

\[ 3\text{Ag}(s) + \text{AuCl}_4^-(aq) \rightarrow \text{Au}(s) + 3\text{Ag}^+(aq) + 4\text{Cl}^-(aq) \]

The standard reduction potential of the \( \text{AuCl}_4^-/\text{Au} \) pair is 0.99 V \textit{vs} standard hydrogen electrode (SHE) compared to 0.80 V \textit{vs} SHE for \( \text{Ag}^+/\text{Ag} \).\textsuperscript{111} Xia and co-workers have shown that, by starting with Ag cubes, NIR sub-100 nm Au nanocages can be formed by galvanic replacement and subsequently show applicability for biomedical applications.\textsuperscript{112} Following this work, HGNS were synthesized using sacrificial Co cores\textsuperscript{66,113–115} and have also shown potential for biomedical applications.\textsuperscript{108,109,116–119} However, the \textit{in vivo} stability of HGNS has not been well studied, and to our knowledge, published biodistribution studies have focused on only the shell material. The fate of the sacrificial core material has yet to be investigated in this context.

Here we show that HGNS with sizes varying from 43 to 82 nm in diameter can be synthesized using sacrificial Ag cores, with virtually identical NIR resonances across this size range. We examined the NIR resonance properties of this nanoparticle in detail including the use of theoretical simulations, where we observed that alloying of the two constituent metals, porosity of the remnant sacrificial layer, and the presence of surface defects all play an important role in determining the plasmon resonance frequency and line width. We investigated the stability of the nanoparticles before and after functionalization with polyethylene glycol (PEG) under laser irradiation, preliminarily to \textit{in vivo} studies. We then performed biodistribution studies of the HGNS nanoparticles in mice. Here we made the unexpected observation that the constituent metals of the
nanoparticle have dramatically different biodistribution profiles. To better understand the origin of this variation in biodistribution, nanoparticle stability was investigated across a range of pH environments and in human serum at physiological pH. In serum and in strong pH environments, the structural integrity of the nanoparticles is strongly compromised. Our results indicate that disintegration of HGNS nanoparticles in vivo may quite possibly be a common occurrence. Therefore, careful characterization of multicomponent particles needs to be thoroughly pursued prior to any planned in vivo application.

3.2. Experimental Methods

Materials. Potassium carbonate and hydrogen tetrachloroaurate(III) trihydrate (HAuCl₄ • 3H₂O) were purchased from Sigma Aldrich. Citrate-stabilized silver colloids (0.02 mg/mL) of 40, 60, and 80 nm diameters were purchased from nanoComposix. Polyethylene glycol was purchased from Laysan Bio, Inc. Water was deionized and filtered by a Milli-Q water system (Millipore).

Synthesis and Functionalization of Hollow Gold Nanoshells. A 1% HAuCl₄ solution was made in H₂O (500 mL). A KCarb/Au solution was prepared by adding K₂CO₃ (0.025 g, 0.181 mmol) and 2 mL of 1% HAuCl₄ to 100 mL of H₂O, stirred for 30 min at room temperature, and left at 4 °C for an additional 30 min. For synthesis using 80 nm Ag cores, 24 mL of KCarb/Au was added to 72 mL of silver colloid under vigorous stirring. The solution was stirred for ~2 min, then sonicated for 30 min. For synthesis using 40 and 60 nm silver cores, 24 mL of KCarb/Au was added to 72 mL of silver colloid under vigorous stirring. The solutions were stirred 30 min at 4 °C. All solutions
were left at room temperature for a further 30 min to obtain the desired plasmon resonance. Solutions were centrifuged for 30 min at 800 g. The 40 and 60 nm supernatant was centrifuged an additional 30 min at 800 g. Particles were redispersed in H2O. HGNS were functionalized first with 10,000 MW thiol-mPEG and subsequently with 10,000 MW thiol-PEG-amine. A water bath was heated to 50 °C. A solution of HGNS was combined with thiol-PEG (15 µM final concentration) in a small vial and vortexed. The vial was floated in the water bath, covered with foil, and agitated in a Styrofoam box overnight. The solutions were centrifuged to remove excess PEG and redispersed in H2O. The procedure was repeated with the HGNS functionalized with thiol-mPEG and a thiol-PEG-amine solution (70 µM final concentration) to achieve a mixed monolayer.

**pH and Serum Studies.** Aliquots of water were adjusted to various pH values from 2 to 12 using 0.1 M NaOH or 0.1 M HCl. The 82 nm HGNS and HGNS@PEG were centrifuged and redispersed into separate vials with varying pH. HGNS@PEG of 82 nm were centrifuged and redispersed into human serum AB obtained from Lonza. UV-vis measurements were obtained for each solution, to track the response of the plasmon resonance to the pH environments, and TEM images were taken to observe the integrity of the HGNS.

**Irradiation with a NIR Laser.** Solutions (2.5 mL) of each size of HGNS, bare and PEGylated, were diluted with H2O to the same optical density. Extinction spectra and TEM images were obtained before resonant illumination. A solution was placed in a vial with a stir bar and thermocouple, and the vial was sealed with Parafilm. While stirring, the solution was irradiated with an 800 nm CW laser at 2.0W/cm² for 25 min.
Extinction spectra and TEM images were obtained from aliquots removed from the solution after irradiation.

**In Vivo Studies.** The protocol for xenograft tumor creation and nude mice imaging was approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine. Athymic nude female mice (4-5 weeks old, Nude-Foxn1nu, Harlan Sprague-Dawley) were injected sc on the right flank with $1 \times 10^6$ U87_luc cells per mouse, in a total volume of 200 µL of serum-free medium. After 21-28 days, tumors grew to 8-12 mm in diameter and mice were randomized into 3 groups ($n = 3$). Nanoparticles were administered via 200 µL tail vein injection with a concentration of $2 \times 10^{11}$ particles/mL. Three different nanoparticles were injected, one particle type per group: HGNS@PEG 82 nm, HGNS@PEG 62 nm, and HGNS@PEG 43 nm. To compare the biodistribution and the passive accumulation in the tumor by enhanced permeability and retention, all mice were euthanized with CO$_2$ asphyxiation 24 h after nanoparticle injection. The tumor, brain, heart, lung, liver, spleen, gut, and kidneys were collected, washed in phosphate-buffered saline, and then stored at -80 °C until analysis.

**Inductively Coupled Plasma Mass Spectrometry.** The organs were weighed into clean vials and digested in aqua regia for two days. After digestion, the samples were purified and all samples diluted in 10 mL of 1% aqua regia. The liver was diluted 100 times more, and the spleen was diluted 10 times more. The gold content was analyzed three times in the organs for statistical analysis. Subsequently, the silver content was analyzed three times in the organs. The ICP-MS analysis was performed using a Perkin-Elmer inductively coupled plasma mass spectrometer.
Instrumentation. Extinction spectra were measured on a Cary 5000 UV/vis/NIR spectrometer. Zeta potential measurements were taken with a Malvern Zetasizer Nano ZS. TEM images were obtained on a JEOL 2010 transmission electron microscope. HRTEM was performed on a JEOL 2100 field emission gun transmission electron microscope. SEM images were taken on a FEI Quanta 650 at an accelerating voltage of 25 kV. XPS measurements were taken on a PHI Quantera X-ray photoelectron spectrometer.

3.3. Results and Discussion

Synthesis of HGNS. HGNS were synthesized with sacrificial Ag cores through a galvanic replacement reaction (Scheme 3.1). The reaction starts with the oxidation of Ag colloid (Scheme 3.1A) through the formation of pinholes. Since an Au/Ag alloy is more thermodynamically stable than either of the pure metals, as Au reduces on the surface of the Ag nanoparticle, a thin, incomplete alloy shell forms (Scheme 3.1B). The oxidation of the Ag continues through the pinholes and empties the central region of the nanoparticle via the Kirkendall effect. Net mass flow toward the shell occurs because Ag diffuses faster into Au than vice versa. The shell then reshapes, with the pinholes reducing in size through Ostwald ripening (Scheme 3.1C). At this stage, Ag is still present in the nanoparticle. Next, dealloying occurs (Scheme 3.1D). The dealloying process produces many holes, since each Au atom replaces three Ag atoms. The Ag is fully oxidized once the dealloying process has transformed the nanoparticle into smaller components composed of pure Au (Scheme 3.1E). In fact, pinhole-free particles were
found to commonly retain ~30% of their initial Ag content. The pinhole-free HGNS exhibit a plasmon resonance located at 634 nm for a diameter of 50 nm.

**Scheme 3.1** Schematics showing the synthesis of HGNS by galvanic replacement. (A) Starting with Ag colloid, AuCl$_3$ is added, causing (B) Ag to oxidize and Au$^{3+}$ ions to simultaneously reduce. (C) As the replacement continues, mass flows from the central region of the nanoparticle, forming a complete HGNS. (D) Then, dealloying occurs, resulting in fragmentation of the nanoparticle shell. (E) After complete Ag oxidation and diffusion, only pure Au fragments remain.

Three different diameters (40, 60, and 80 nm) of Ag cores were used. By adding a solution of gold chloride and potassium carbonate to a colloidal suspension of the Ag nanoparticles, HGNS of 82.6 ± 4.2, 61.8 ± 3.3, and 43.1 ± 3.9 nm were formed. Figure 3.1A shows the extinction spectra of the Ag colloid (top panel) and the HGNS (bottom panel), and corresponding transmission electron microscopy (TEM) images are shown in Figure 3.1B and C.

The optical signature of HGNS formation was studied and then correlated with its structural and compositional evolution during growth. After adding the AuCl$_3$ solution, HGNS with a resonance at ~820 nm were formed. Over time, Ag continued to be oxidized from the core, as Au$^{3+}$ continued to be reduced. As the ratio of Ag:Au decreased, there was an accompanying blue-shift of the plasmon resonance (Figure 3.2). Using X-ray photoelectron spectroscopy (XPS), the atomic percent of Ag and Au at various reaction times was determined (Figure 3.3). At early reaction times of an 82 nm...
HGNS, the ratio of Ag to Au was high, 4:1. However, if the reaction was allowed to proceed for 24 h, that ratio was reduced to ~1:1 and the plasmon resonance shifted to the visible region. Given the desire to produce NIR-active HGNS, for all further experiments, HGNS were allowed to react for only 1 h, which resulted in NIR resonances and a corresponding Ag:Au ratio of 4:1.

![Figure 3.1](image)

**Figure 3.1** Extinction spectra and TEM images of Ag nanoparticle precursors and HGNS. (A) Extinction spectra of Ag colloid with 80, 60, and 40 nm diameters (top panel) and resulting HGNS of 82, 62, and 43 nm diameters (bottom panel). Representative TEM images of (B) 80, 60, and 40 nm Ag colloids and (C) 82, 62, and 43 nm HGNS are shown. All scale bars are 50 nm.
Figure 3.2 Plasmon resonance position as a function of time. (A) Extinction spectra versus time of 82 nm diameter HGNS showing a continuous blue-shift of the plasmon due to silver oxidation. (B) Extinction maximum of 82 nm HGNS as a function of reaction time.

Figure 3.3 (A) XPS atomic percentages and (B) TEM images of the formation of an 82 nm diameter HGNS illustrating the atomic percent of silver decreases and gold increases as a function of reaction time.
**Theoretical Modeling.** Calculations based on the finite element method (FEM, COMSOL) were performed to better understand the origin of the NIR resonance in this family of nanoparticles and to provide further insight into their structural composition prior to in vivo studies. Experimentally determined dielectric functions for both Au and Ag were used (Figure 3.5). Based on our theoretical modeling, for the nanoparticle to support a plasmon resonance in the NIR, it must have a structure similar to that shown in Scheme 3.1B, where the nanoparticle shell is composed of Au/Ag alloy with multiple large defects on its surface (Figure 3.4A, inset SEM image). Because three Ag atoms are removed for every Au atom added, the metallic alloy and core must be porous. To account for the corresponding modification to the metallic dielectric functions that this compositional change would induce, simulations were performed where the HGNS was modeled as a spherical, porous silver core coated with a similarly porous Au/Ag alloy shell. The porous materials were modeled using the Bruggeman effective medium theory.

The Bruggeman effective medium theory has been widely used to obtain effective material parameters for two dissimilar, porous materials. The Bruggeman effective approximation formula can be written as

\[ f_1 \frac{\varepsilon_1 - \varepsilon_{\text{eff}}}{\varepsilon_{\text{eff}} + g(\varepsilon_1 - \varepsilon_{\text{eff}})} + (1 - f_1) \frac{\varepsilon_2 - \varepsilon_{\text{eff}}}{\varepsilon_{\text{eff}} + g(\varepsilon_2 - \varepsilon_{\text{eff}})} = 0 \]

where \( \varepsilon_1 \) and \( \varepsilon_2 \) are the complex dielectric functions of the constituent materials. The coefficient \( f_1 \) represents the volume fraction of one material in the composite, with the remaining material comprising a fill fraction of \( (1 - f_1) \). The constant \( g \) is a geometric factor; for spherical inclusions \( g = 1/3 \). In the case of porous Ag, the effective dielectric is...
calculated as a compositional mixture of Ag with H2O. To obtain an effective dielectric for the porous alloy, a more general form of the Bruggeman effective model is used, to include more than two different composite materials (i.e., Ag, Au, and H2O):

$$\Sigma_i f_i \frac{\varepsilon_i - \varepsilon_{\text{eff}}}{\varepsilon_{\text{eff}} + g(\varepsilon_i - \varepsilon_{\text{eff}})} = 0$$

Now $i = 3$, and $\varepsilon_i$ and $f_i$ are the complex dielectric function and the volume fraction of the $i$th constituent material, respectively.\textsuperscript{125} The above equation yields a unique physical solution for $\varepsilon_{\text{eff}}$ in the absence of gain.

To investigate the role of the nanoporous alloy in determining the plasmon resonance properties, the nanoparticle geometries, with their constituent materials, were simulated independently as being composed of either a nonporous or porous medium and compared to nanoparticles simulated with H2O cores. The nanoparticles were also simulated with zero, one, and three holes to isolate the influence of shell defects on peak position. For an asymmetric nanoparticle, the extinction cross section can depend upon the orientation of the particle relative to the polarization of incident light. To account for this, the nanoparticles were simulated with the E field polarized in the x, y, and z directions independently, then averaged; for the spectra shown in Figure 3.4, the average of all three polarizations is shown. All nanoparticles were simulated with a background medium of H2O. The inset in Figure 3.4A illustrates the geometry of the model used, where $d$ is the diameter of the core and $D$ is the outer diameter including the shell. To emulate the large holes in the surface of the HGNS, the modeled geometry included three spherical holes: one large hole with a diameter of $h_1$ and two smaller holes each with diameter $h_2$. The parameters used for each particle size are listed in Table 3.1. The
simulations showed that the presence of holes of either large or small diameter has a minimal effect on the plasmon resonance.

The case of a nonporous HGNS with an 82 nm diameter, a solid Ag core, and a solid Ag/Au alloy shell is shown in Figure 3.4A. For this structure, the plasmon resonance peak wavelength occurs at $\lambda = 480$ nm, far from our experimentally observed resonance. The introduction of defects into the shell layer red-shifts the plasmon resonance slightly, but only by $\sim 50$ nm (Figure 3.4A). However, when porosity is introduced, it induces a much larger red-shift of the plasmon resonance, by 275 nm (Figure 3.4B). The addition of large surface defects also results in an additional red-shift of the plasmon resonance, to a wavelength region corresponding to our experimental observations, nominally 800 nm (Figure 3.4B). The dielectric functions used to calculate Figure 3.4A and Figure 3.4B for the nonporous and porous materials, respectively, are shown in Figure 3.5.

The role of porosity is further emphasized by comparing the porous nanoparticle simulations to simulations of the three sizes of HGNS with pure H$_2$O cores, which is the expected composition for HGNS (Figure 3.4C). In this case, Mie theory was used to simulate the shell as a continuous alloy composed of 80% Ag and 20% Au with the dimensions listed in Table 3.1. The inside core and background media are H$_2$O. The core to shell ratio for each particle size is $\sim 0.8$, and as a result, their plasmon resonances are expected to be similar. Interestingly, when the nonporous nanoparticles are hollow, their plasmon resonances are only at $\sim 600$ nm. This set of simulations demonstrates that it is highly likely that all three factors of (1) alloying, (2) porosity, and (3) surface defects contribute, in concert, to the NIR resonances observed in the solution phase absorption
Table 3.1 Particle dimensions and hole sizes used in the theoretical simulations.

<table>
<thead>
<tr>
<th>Ag Core Size</th>
<th>HGNs outer diameter (D, nm)</th>
<th>HGNs inner diameter (d, nm)</th>
<th>Large hole diameter (h₁, nm)</th>
<th>Small holes diameter (h₂ = h₃, nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 nm</td>
<td>82.8</td>
<td>66.6</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>60 nm</td>
<td>61.8</td>
<td>49.8</td>
<td>45.4</td>
<td>22.8</td>
</tr>
<tr>
<td>40 nm</td>
<td>43.1</td>
<td>35.1</td>
<td>31.2</td>
<td>15.6</td>
</tr>
</tbody>
</table>

Figure 3.4 Finite element method (FEM) modeling and Mie theory of HGNS showing the influence of material porosity on the plasmon properties. Extinction cross sections calculated with the FEM of 82 nm diameter HGNS comparing the presence of holes in the particle surface with (A) nonporous (80% Ag, 20% Au) and (B) porous materials (43.2% Ag, 10.8% Au, and 46% H₂O). Spectra are plotted for the average E field polarization. (C) Extinction cross section spectra of the three sizes of HGNS with a H₂O core, alloy shell, and zero holes calculated with Mie theory show similar plasmon resonances in the visible region. (D) The three-hole porous model is used to calculate with FEM the extinction cross section spectra of 82, 62, and 43 nm diameter HGNS with extinction maxima of 800, 815, and 815 nm, respectively.
Figure 3.5 Comparison of the dielectric functions of non-porous (solid) and porous materials (dashed). The real (black) and imaginary (red) parts of the dielectric are shown for the Ag core (A) and the Ag/Au alloy shell (B). The material percentages used in the alloy are 80% Ag and 20% Au for the non-porous case, and 43.2% Ag, 10.8% Au, and 46% H$_2$O in the porous case.

spectrum of the HGNS, but that the presence of a porous Ag core is required to shift the plasmon to the NIR.

The degree of porosity of the constituent materials of the nanoparticle also strongly affects the plasmon peak position. Using the three-defect porous HGNS model geometry, the percentage of H$_2$O (i.e., the porosity) in the nanoparticle was gradually increased. This increase introduces a significant red-shift of the plasmon resonance: by varying the porosity from 40% to 46%, an additional shift of the plasmon resonance from 660 nm to 800 nm is obtained (Figure 3.6).

Figure 3.4D shows the average polarization spectrum for each of the three sizes of HGNS. The extinction maxima for the averaged spectra are 800, 815, and 815 nm, and the percentages of water are 46%, 46.3%, and 46.4% for 82, 62, and 43 nm HGNS,
respectively. Because the percentage of H$_2$O in the nanoparticle cannot be determined experimentally, the H$_2$O composition was determined theoretically by the percent required to achieve the desired plasmon resonance.

![Graph](image)

**Figure 3.6** Extinction cross section spectra of the porous three-hole 82 nm HGNS shows a plasmon red-shift as the percentage of water in the porous material is increased.

**Surface Functionalization.** For biological applications, the HGNS need to be functionalized with polyethylene glycol (PEG). Coating the particles in PEG is important for several reasons.$^{52,73,77,78}$ First, PEGylation improves circulation time of the nanoparticles *in vivo*.$^{77,78}$ Second, it inhibits opsonization, the process by which foreign structures such as nanoparticles are identified for endocytosis by phagocytes.$^{52,77,78}$ Third, PEGylation allows the surface charge of the particles to be reduced to near neutrality, since highly charged nanoparticles have shown increased accumulation in the reticuloendothelial system (liver and spleen).$^{52,53}$

The HGNS were functionalized with PEG in two steps to achieve a zeta potential of approximately 10 mV, appropriate for tumor uptake studies *in vivo*. The HGNS were
first coated with 10,000 MW thiol-mPEG (thiol-PEG-methyl), then subsequently with 10,000 MW thiol-PEG-amine using a warm water bath to increase the packing density without furthering Ag oxidation. The two-step coating allowed the zeta potential to be reduced to about 10 mV, while avoiding a highly positively charged surface that would have resulted from thiol-PEG-amine alone. In contrast, one-step coating with thiol-mPEG alone only reduced the highly negative zeta potential of the bare HGNS to about 23 mV. The unusual high negative zeta potential observed for HGNS@mPEG supports the idea that the surface is indeed composed of an alloy of Au and Ag, since mPEG thiolation of Au nanoparticles typically results in a much lower zeta potential than we observe for the uncoated nanoparticles. Table 3.2 shows the extinction maximum and zeta potentials of the Ag colloid precursor, HGNS, and HGNS@PEG for the three particle core sizes. After PEGylation, the HGNS retain their similar plasmon resonances.

### Table 3.2 Extinction Maximum and Zeta Potential of 80, 60, and 40 nm Diameter Ag Colloids and the Corresponding HGNS and HGNS@PEG

<table>
<thead>
<tr>
<th>Particle Type</th>
<th>80 nm</th>
<th>60 nm</th>
<th>40 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\lambda_{\text{max}}) (nm)</td>
<td>(\zeta_{\text{pot}}) (mV)</td>
<td>(\lambda_{\text{max}}) (nm)</td>
</tr>
<tr>
<td>Ag colloid</td>
<td>458</td>
<td>-49.7 ± 2.2</td>
<td>430</td>
</tr>
<tr>
<td>HGNS</td>
<td>811</td>
<td>-30.6 ± 0.2</td>
<td>826</td>
</tr>
<tr>
<td>HGNS@PEG</td>
<td>804</td>
<td>-12.3 ± 0.1</td>
<td>806</td>
</tr>
</tbody>
</table>

**NIR Laser Irradiation.** A primary application for chemically synthesized NIR-active particles is photothermal therapy. HGNS are desirable for photothermal therapy because their thinner shells have a low thermal mass, which should enable efficient heat dissipation to the surrounding tissues.66 The effect of laser irradiation was tested on solutions of bare and PEGylated HGNS (Figure 3.7). The nanoparticles were irradiated with a continuous wave 800 nm diode laser at 2.0 W/cm² for 25 min. Figure 3.7A shows
the extinction spectra before irradiation, and Figure 3.7B shows the extinction after irradiation. After irradiation, there is a drastic size-dependent blue-shift of the plasmon resonance of the bare HGNS, with the smallest nanoparticles being affected most dramatically. The TEM images reveal that the bare nanoparticles have changed in morphology and have also likely leached Ag into the solution (Figure 3.7C). Alternatively, the HGNS@PEG extinction spectra are stable after irradiation and the TEM images confirm that the HGNS@PEG remain intact (Figure 3.7D). These results suggested that HGNS@PEG would be viable nanoparticles for photothermal therapy.

![Figure 3.7](image)

**Figure 3.7** Effect of laser irradiation on HGNS with an 800 nm CW laser at 2.0 W/cm² for 25 min for three diameters: 82 nm (black), 62 nm (red), and 43 nm (blue). Extinction spectra of HGNS (top panel) and HGNS@PEG (bottom panel) (A) before and (B) after laser irradiation. TEM images of 82, 62, and 43 nm diameter (C) HGNS and (D) HGNS@PEG after laser irradiation. All scale bars are 50 nm.

**Biodistribution.** Here, the three sizes of HGNS were PEGylated for in vivo studies in nine mice, with three mice for each nanoparticle size. Nanoparticles were injected into the tail vein of nude mice, each with a glioma xenograft transplanted from a U87 cell line. The mice were sacrificed 24 h post-injection. The biodistribution of the
HGNS was examined by inductively coupled plasma mass spectrometry (ICP-MS) to quantify the Ag and Au content in each organ (Figure 3.8). The error bars in Figure 3.8A represent the standard deviation of Au content in each organ for the three mice injected with HGNS of 82, 62, or 43 nm. Error bars in Figure 3.8B were calculated in the same manner for Ag content. For all nanoparticle sizes, there is a high uptake in the liver and spleen, which is common and has been observed by other groups.\textsuperscript{54,77,126} Notably, however, there is a variable ratio of Ag:Au in each organ. The average Ag:Au ratio was determined for each nanoparticle size and is shown in Figure 3.9. Assuming that the particles have a ratio of 4:1 Ag:Au (as determined by XPS), it would be expected in each organ to find three times more Ag than Au. However, the ratio varies considerably between organs. In fact, in the liver and spleen the Au content is actually higher than the Ag content. The variable ratio of Ag:Au in each organ indicates that the particles are fragmenting at some point after injection. Furthermore, data from Figure 3.7 suggest that the fragmentation of the particles is size dependent. The 43 nm HGNS shows a very large amount of Ag surrounding the particle after laser irradiation. As the overall particle size decreases, the shell becomes thinner. This thinner shell allows the 43 nm particle to fragment quicker, leading to the more drastic variation in Au and Ag distribution seen in mice injected with the 43 nm HGNS. For biological applications it is imperative that the Ag be unaffected by the environment and stay confined within the particles, due to potential cytotoxic effects.\textsuperscript{39,40,127}
Figure 3.8 Biodistribution of 82, 62, and 43 nm diameter HGNS@PEG determined by ICP-MS of three mice for each size of particle with xenografts transplanted from U87 cell lines. Biodistribution is shown independently for the gold content in each organ in µg/g of organ (A) and silver content in each organ in µg/g of organ (B).

Figure 3.9 Comparison of the Ag:Au ratio in each organ determined by ICP-MS for 82 (A), 62 (B), and 43 nm diameter HGNS@PEG (C). Ag and Au content were calculated in µg/g of organ. The green line indicates the expected Ag:Au ratio of 4:1 as determined by XPS analysis of the particles prior to injection.

**Stability Studies.** To explain the fragmentation of the HGNS *in vivo*, stability studies were performed on the 82 nm HGNS in a range of pH and in serum. In vivo, the nanoparticles are subjected to a range of pH values as they traffic throughout the organism; therefore, we first investigated their pH stability. The 82 nm HGNS were dispersed in aqueous solutions with pH ranging from 2 to 10 for 24 h, and the extinction
spectrum of the nanoparticles was monitored for changes resulting from each environment. When bare 82 nm diameter HGNS were incubated in various pH solutions, a large shift of the plasmon resonance from 805 to 712 nm was observed (Figure 3.10A). With HGNS@PEG, we observe a large shift of the plasmon resonance from 811 to 769 nm from pH 2 – 4, but only a small shift from 769 to 756 nm for pH 4 – 10 (Figure 3.10B). This suggests that PEG cannot protect the particles in acidic pH. In both cases, we see a decrease in the extinction at low pH, suggesting aggregation or breakdown of nanoparticles. TEM images of HGNS@PEG incubated in pH 2 – 7 for 24 h reveal that the particles are in fact destroyed at low pH (Figure 3.10C). Within physiological environments, the nanoparticles are exposed to many other factors in addition to various pH environments. In order to simulate a complex biological environment, the HGNS were dispersed in serum at physiological pH. Serum is the portion of the blood that does not contain the blood cells, platelets, or fibrinogens. However, it does contain dissolved gases such as oxygen and carbon dioxide, proteins including albumin and antibodies, as well as antigens, salts such as sodium, chloride, and potassium ions, hormones, oligonucleotides, etc.\textsuperscript{129,130} The HGNS@PEG were incubated in human serum for 24 h, and high-resolution transmission electron microscope (HRTEM) images were taken to investigate the structural integrity of HGNS in the \textit{in vivo}-like environment. The HRTEM image reveals that the particles break apart in serum at physiological pH, similarly to the nanoparticle fragmentation observed at low pH (Figure 3.10D). The particles were also incubated in fetal bovine serum, which showed fragmentation of the particles as well (TEM not shown).
Figure 3.10 Effect of a range of pH and human serum on HGNS with and without PEG. Extinction spectra of 82 nm diameter HGNS (A) and HGNS@PEG (B) after 24 h incubation in water adjusted to various pH. (C) TEM images of HGNS@PEG after 24 h incubation at pH 2 – 7. (D) HRTEM image of HGNS@PEG after 24 h incubation in human serum at physiological pH. All scale bars are 50 nm.

It is probable that the cause of the destruction of the particles originates with the presence of Ag on the surface of the nanoparticles. Many studies have shown the instability of Ag nanoparticles.\textsuperscript{131} Lok et al. demonstrated Ag nanoparticle aggregation in media with high ionic strength.\textsuperscript{36} Bouwmeester et al. showed that Ag nanoparticles incubated in cell culture media dissociated into Ag ions,\textsuperscript{37} and Stebounova et al. showed that Ag nanoparticles aggregate in artificial interstitial and lysosomal fluid with only 1% of particles remaining suspended after 24 h.\textsuperscript{38} Moreover, Hurt and co-workers have shown pH-dependent dissolution of Ag nanoparticles, with higher degrees of Ag dissolution occurring at lower pH.\textsuperscript{128,132} Most notably, Hurt and co-workers concluded that dissolved O$_2$ in solution is crucial for Ag dissolution.\textsuperscript{132} In deoxygenated acetate buffer at pH 4, Ag dissolution is negligible, but it is drastically increased in air-saturated
acetate buffer at pH 4. The protons and dissolved O\textsubscript{2} cooperatively oxidize the Ag. In the case of HGNS, dissolved O\textsubscript{2} is present in all solutions, resulting in some degree of oxidation; but as the pH is decreased, more protons are available to cooperatively oxidize the Ag in the shell layer, and subsequently at very low pH Ag from the core is oxidized, destroying the integrity of the HGNS.

Another factor potentially influencing stability is the protein corona that immediately forms on the surface of nanoparticles when introduced \textit{in vivo}.\textsuperscript{133} Any one of the more than 3700 proteins in plasma\textsuperscript{134} could have a degradative effect on the nanoparticles. However, because biological media are so complex, it is difficult to deduce one specific cause of HGNS fragmentation; instead, it is likely that a variety of factors in this complex environment contribute to the fragmentation, including high ionic strengths, proteins, low pH environments, and dissolved O\textsubscript{2}.\textsuperscript{36,38,128,132,133}

This study shows that it is critically important that the remnant sacrificial core material and the shell material of HGNS both be monitored independently when performing biodistribution studies. The results shown here indicate that, although this nanoparticle family has a highly promising set of characteristics for \textit{in vivo} use, it may be substantially degraded \textit{in vivo} even when PEGylated. Consequently, further investigation into the fragmentation and toxicity of HGNS is necessary to determine their ultimate suitability for nanomedical applications.
3.4. Conclusions

HGNS of three sizes below 100 nm in diameter were synthesized with plasmon resonances in the NIR region as potential photothermal transducers for nanomedical applications. Using the Bruggeman effective medium theory, resonant behavior of these nanoparticles is well described by including porosity of the core and shell, as well as surface defects, in the theoretical model. The theoretical model confirmed that there is a large amount of residual Ag in the HGNS, in order for them to be NIR resonant. In this study, the HGNS showed potential for photothermal therapy due to their stability when PEGylated under laser illumination on their plasmon resonance frequency, which corresponded well to the great potential previously shown in the literature. However, tracking the biodistribution of both component species, Ag and Au, from our HGNS following injection in mice strongly suggests that the nanoparticles are fragmenting after injection. Testing the HGNS@PEG stability over a large pH range and in serum at pH 7 showed that even when PEGylated, the nanoparticles fragment in low pH environments and in human serum at physiological pH. These early studies indicate that due to the remaining sacrificial Ag core, the loss of structural integrity, and the known toxicity of Ag, the stability and toxicity of HGNS made by galvanic replacements should be more carefully studied prior to their use in vivo. These results should strongly impact the assessment of ongoing efforts to design biocompatible plasmonic nanoparticles with compositions and structural properties appropriate for nanomedicine.
4.1. Introduction

Targeted drug delivery is an area of intense interest for cancer treatments to reduce the systemic toxicity of chemotherapy drugs. Nanoparticle-based systems are ideal carriers for the toxic chemicals, allowing for controllable release once the particles have relocated to the tumor site. A number of nanoparticle platforms have been investigated including liposomes,\textsuperscript{13,14} hydrogels,\textsuperscript{15,16} and polymeric,\textsuperscript{17–19} magnetic\textsuperscript{20,21} and metallic nanoparticles.\textsuperscript{22} Many targeted delivery strategies utilize changes in the intracellular environment, including pH\textsuperscript{19,83–85} and temperature,\textsuperscript{16,18,83–85} to induce release; however, these stimuli vary with cell type and location resulting in unreliable release.
Alternatively, externally applied triggers such as ultrasound, magnetic and electric fields, and light allow for precise spatial and temporal release.

Metallc nanoparticles (MNPs) have been used for light-triggered release, where in response to continuous wave near-infrared (NIR) illumination, double-stranded DNA (dsDNA) is dehybridized resulting in release of single-stranded DNA (ssDNA) into the solution, while the complementary strand remains tethered to the NP surface. Light-induced DNA dehybridization has shown to be promising for gene therapy and drug delivery. In gene therapy, the released ssDNA prevents transcription or translation, or inhibits protein function, in order to treat and prevent diseases. In addition, molecules can be associated with the DNA duplex and released upon DNA dehybridization using low levels of NIR irradiation. Specifically, the nuclear stain, DAPI, was delivered to cells upon irradiation with low levels of NIR light. This system can be utilized for drug delivery as chemotherapeutic molecules can be loaded on the DNA scaffold in a similar manner as DAPI.

The experimental conditions employed for such schemes vary widely from study to study, as do the proposed mechanisms. Many studies use ultraviolet (UV) light to trigger release, while others utilize visible or near-infrared radiation. Both continuous-wave (CW) and pulsed (fs, ns, and ps) lasers have been utilized. Two main mechanisms have been proposed to explain the release under such conditions. Many studies have proposed simple melting of the DNA from the increased temperature of the nanoparticles. Others suggest that hot-electron transfer from plasmon decay causes either dehybridization or Au-S bond breaking, which results in release of the entire dsDNA.
Here, we use nanoshells (NS) functionalized with dsDNA to investigate the physics of plasmon-driven DNA release (Scheme 4.1). We do not consider release induced by UV light because of its inapplicability \textit{in vivo}. High energy UV photons are strongly absorbed by bulk tissue, which can result in protein and DNA damage.\textsuperscript{139} On the other hand, NIR light enables deep tissue penetration due to the low absorption of biological tissue in this region.\textsuperscript{51} We also do not consider nanoparticle concentrations above $10^9$ particles/mL as a recent study has shown that the uptake of nanoparticles into a tumor is on the order of $10^8 - 10^9$ particles/mL.\textsuperscript{9,140} We find that release can be achieved in two distinct regimes: high power CW illumination and low average power, high intensity pulsed laser excitation. Each regime features distinctive mechanisms that drive release. DNA release upon CW illumination is due to global temperature increase arising from the collective heating effects of the nanoparticles, causing dehybridization and release of ssDNA. This limits the use of CW illumination \textit{in vivo} because the high global temperatures are likely to result in non-specific cell death and also because it is highly concentration dependent. Alternatively, we investigate low average-power femtosecond pulsed laser-induced release. DNA release is found to occur via a hot-electron transfer process, which breaks the Au-S bond, releasing dsDNA. No bulk temperature increase is observed and the process is approximately independent of nanoparticle concentration. This makes pulsed laser-induced release more favorable for \textit{in vivo} applications where non-specific cell death needs to be minimized and nanoparticle uptake is uncontrollable. While Au-S bond breakage has been demonstrated with high energy UV pulsed lasers, to our knowledge no previous work has demonstrated the pulsed release mechanism with NIR light.
4.2. Experimental Methods

Materials. Potassium carbonate anhydrous was purchased from Fisher Scientific. Hydrogen tetrachloroaurate(III) trihydrate (HAuCl$_4$·3H$_2$O), DL-dithiothreitol (DTT), and sodium chloride were purchased from Sigma Aldrich. 120 nm diameter aminated silica cores in ethanol (40 mg/mL) were purchased from nanoComposix, Inc., San Diego, CA. HPLC-purified DNA strands and 10 mM Tris, pH 7.5, 0.1 mM EDTA buffer (1X TE buffer) were purchased from Integrated DNA Technologies (IA, USA). Water was deionized and filtered by a Milli-Q water system (18.2 MΩ·cm at 25 °C, Millipore).

DNA purification. Prior to nanoshell (NS) functionalization, thiolated ssDNA [HS-C$_6$H$_{12}$-TATGATCTGTCACAGCTTGA] was incubated with 10 mM DTT reducing agent in 10 mM TE buffer for 1 h at room temperature. The thiolated DNA was purified with a Sephadex G-25 column (NAP 25, GE Healthcare), eluting with TE buffer. The complimentary fluorescein tagged ssDNA [ATA CTA GAC AGT GTC GAA CT-Fluor] was dispersed in 600 µL TE buffer. DNA concentration of both strands was determined by measuring their absorbance at 260 nm. The two DNA strands were mixed in a 1:1 molar ratio with 33 mM NaCl and boiled for 4 min. The mixture was cooled slowly overnight to hybridize the strands.

Nanoshell synthesis and functionalization. Nanoshells (NS) were synthesized by a previously published procedure resulting in dimensions of [r1, r2] = [60, 81] nm.$^{23,141}$ The nanoshell surface was functionalized by mixing dsDNA with bare NS in a ratio of 20,000 dsDNA strands/NS and stirred overnight. The particles were washed three times by centrifugation and redispersed in TE buffer, pH 7.5. Extinction spectra were
obtained using a Cary 5000 UV/Vis/NIR Varian spectrophotometer. Zeta potential measurements were taken with a Malvern Zetasizer Nano ZS. TEM images were obtained on a JEOL 1230 high contrast transmission electron microscope.

**DNA surface coverage determination.** dsDNA coverage was determined by displacement with 2-mercaptoethanol in TE buffer$^{142}$ and quantified using fluorescence. Displaced dsDNA strands were separated from the NS by centrifugation and quantified using a fluorescence filter-based microplate reader (Infinite F200 PRO TECAN; excitation = 485 ± 10 nm, emission = 535 ± 12.5 nm). dsDNA concentration was determined by comparison to a standard curve performed in triplicate. Surface coverage was calculated after subtraction of the dsDNA signal in the background sample and using a NS concentration of 1x10$^9$ particles/mL.

**Circular dichroism measurements.** CD spectra of 5 μM dsDNA in 33 μM NaCl TE buffer (pH 7.5) was acquired in a 1 cm path length quartz cell. Measurements were made using a JASCO J-810 Spectropolarimeter equipped with a Peltier-type temperature control system at wavelengths from 200 to 320 nm. Thermal dsDNA dehybridization curves were obtained by monitoring the decrease in ellipticity with temperature in the desired wavelength range. The temperature was varied between 25 °C and 90 °C in 5 °C increments with an equilibration time of 180 s. The melting CD spectra of dsDNA, which show structural transitions from the double-strand state to the single-strand state, are shown in Figure 4.3A. The melting curve was extracted from the change in ellipticity at 246 nm ($\theta_{246}$) and the first derivative of a Boltzmann fit determined the T$_m$ to be 55.2 ± 0.7 °C (Figure 4.3B).
**Thermal studies.** While stirring, 1 mL of $1 \times 10^9$ particles/mL NS@dsDNA was heated to the desired temperature in a 1 x 1 x 4 cm methacrylate cuvette using a Peltier temperature controlled cuvette stage. After 20 min of heating, a 300 µL aliquot was removed and immediately centrifuged at 5000 g for 2 min at 6 °C to separate the released ssDNA from the particles. The process was repeated in 5 °C increments up to 65 °C. A fresh sample was used for each temperature point. Released ssDNA in the supernatant was quantified by fluorescence.

**Temperature controlled CW laser release studies.** 2.5 mL of $1 \times 10^9$ particles/mL NS@dsDNA was cooled to 7 °C using a Peltier temperature controlled cuvette stage. While cooling and stirring, the NS@dsDNA solution was irradiated with an 808 nm CW diode laser (Diomed, 15Plus, Angio Dynamics) at 1.6 and 3.4 W with a full-width half-max of 3.5 mm. The sample was irradiated from the side to minimize vaporization. Temperature was monitored with an infrared thermocouple (Omega OS-801-MT-K) with a 0.5 cm spot size. After the desired time, a 300 µL aliquot was removed and immediately centrifuged at 5000 g for 2 min at 6 °C. A fresh sample was used for each time point. Fluorescence measurements were taken on the supernatant to quantify released ssDNA.

**Non-temperature controlled CW laser release studies.** 1 mL of $1 \times 10^9$ particles/mL NS@dsDNA was stirred on a custom built plastic cuvette stage. NS@dsDNA solutions were irradiated from the side with the CW laser at 0.4, 0.7, 0.8, 1.0 and 1.5 W/cm². For each laser power, the saturation temperature was determined and samples were then irradiated for 0, 2.5, 5, 7.5, 10, and 15 min after the saturation temperature was achieved. Samples were also taken 2.5 and 5 min after the laser was
turned on, before the saturation temperature was reached. Temperature was monitored with a thermal camera (FLIR A5). After irradiation, a 300 µL aliquot was removed and immediately centrifuged at 5000 g for 2 min at 6 °C. Spatial and temporal average temperatures were used to as the temperature value in the analysis. The supernatant of all samples was measured by fluorescence.

**Femtosecond pulsed laser release studies.** 1 mL of 1x10^9 particles/mL NS@dsDNA was stirred on cuvette stage and irradiated from the side with a Ti:Sa Laser Amplifier (Coherent, RegA) operating at 250 kHz with an average power of 1 W and a pulse length of ~150-180 fs. The laser spot had a full-width-half-max of 44 µm. Temperature was monitored with a thermal camera (FLIR A5). Samples were irradiated for 10 min at 2-50 mW. After irradiation, a 300 µL aliquot was removed and immediately centrifuged at 3000 g for 3 min. Fluorescence measurements were taken of the supernatants. SEM images were taken of the particles before and after release (FEI Quanta 650, accelerating voltage of 25 kV).

### 4.3. Results and Discussion

**Nanocomplex Synthesis and Characterization.** Nanoshells, composed of a silica core surrounded by a thin gold shell, were employed in this study as they are strong absorbers of NIR light and are biocompatible and easily functionalized. The nanocomplexes (NS@dsDNA) were formed by first hybridizing a thiolated ssDNA with a non-thiolated fluorescein tagged ssDNA (Scheme 4.1). NS, with a 120 nm SiO_2 core and a 21 nm Au shell were functionalized with the dsDNA *via* a gold-thiol bond.
Scheme 4.1 Formation of NS@dsDNA by hybridization of a thiolated ssDNA with a complementary fluorescein tagged ssDNA, followed by attachment to the NS surface through Au-thiol bonds, and subsequent DNA release when irradiated with NIR light. CW irradiation results in dehybridization and release of the fluorescein tagged ssDNA, while pulsed irradiation results in Au-S bond breakage and release of dsDNA.

Functionalization of the NS surface was verified with extinction spectroscopy, zeta potential and transmission electron microscopy (TEM). The bare NS extinction spectrum red-shifted from 775 to 781 nm after dsDNA functionalization resulting from the change in the dielectric environment around the NS (Figure 4.1A). TEM images show the formation of a thin layer of DNA around the NS (Figure 4.1B,C). The zeta potential decreased from -44.1 ± 0.7 to -59.4 ± 1.7 mV due to the increased negative charges associated with the phosphate backbone of the DNA (Figure 4.1D). The number of dsDNA on the NS surface was quantified with a mercaptoethanol displacement method. Displaced dsDNA concentration was calculated using a linear standard curve of dsDNA fluorescence (Figure 4.2), showing the surface coverage to be ~4100 dsDNA strands per NS.
Figure 4.1 Nanocomplex characterization. (A) Extinction spectrum of bare NS (green) and NS@dsDNA (blue), TEM images of (B) bare NS and (C) NS@dsDNA, and (D) zeta potential of bare NS (green) and NS@dsDNA (blue). Scale bars are 50 nm.

Figure 4.2 Determination of dsDNA Surface Coverage. dsDNA calibration curve where error bars represent triplicate fluorescence measurements of fluorescein tagged dsDNA (ex. = 485 ± 10 nm, em. = 535 ± 12.5 nm). Linear regression y = 409.7x + 312.0, R² = 0.998.
Thermal and CW Laser-Induced DNA Melting. The melting temperature of the dsDNA in solution was characterized by circular dichroism (CD). Spectra were collected for 5 µM oligonucleotide in 33 mM NaCl TE buffer from 25 °C to 90 °C in 5 °C increments and 3 min equilibration time (Figure 4.3A). The amplitude of ellipticity (θ) at 246 nm from each spectrum was analyzed as a function of temperature to obtain the dsDNA melting curve (Figure 4.3B). The first derivative of a Boltzmann fit of the CD curve showed a melting temperature (T_m) of 55.2 ± 0.7 °C, which is close to the theoretically calculated T_m of 51.3 °C in 33 mM NaCl TE buffer.99

Figure 4.3 DNA characterization. (A) Circular dichroism spectra of dsDNA as a function of temperature. (B) Change in ellipticity at 246 nm from dsDNA melting with a Boltzmann fit calculating a melting temperature of 55.2 ± 0.7 °C.

To investigate the dehybridization temperature when dsDNA is tethered to the NS surface, a thermal release profile at 5 °C increments was acquired. The nanoparticle concentration used was 1 x 10^9 NS/mL to mimic achievable tumor concentrations. The NS@dsDNA solution was heated in a temperature-controlled stage; after 20 min of
equilibration at the chosen temperature, an aliquot was removed and centrifuged. A fresh sample was used for each temperature point. A fluorescein tag on the non-thiolated strand is used for quantification of released ssDNA. The fluorescence of the supernatant was measured and converted to DNA/NS released using a linear standard curve. The ssDNA/NS released vs. temperature was fit with a Boltzmann equation showing the T_m achieved through heating the samples was 61.1 ± 2.3 °C (Figure 4.4A). The slightly higher T_m from thermal release compared to the T_m observed in CD can be attributed to the dsDNA being anchored to the NS surface vs. free dsDNA in CD, resulting in increased steric hindrance and a difference in salt concentration.

Here we compare the thermal release to release induced by an 808 nm CW laser. The NS@dsDNA solution was irradiated at 0.4, 0.7, 0.8, 1.0, and 1.5 W for times ranging from 1.5 – 25 min. During irradiation the sample temperature is monitored with a thermal camera (Figure 4.5A,B). When plot as a simple function of time, the release follows linear trends at each laser power (Figure 4.5C). However, we also observe that when plot as a function of temperature, the DNA release profile shows a remarkable trend that matches the thermal release profile with a T_m of 59.1 ± 4.5 °C (Figure 4.4B). These results suggest that thermal effects play a dominant role, but they do not unambiguously separate thermal and hot electron effects.

We separate the two mechanisms by keeping the sample below the DNA melting temperature via a Peltier stage. The NS@dsDNA solution was cooled to 7 °C and illuminated from the side with an 808 nm CW laser at 1.6 and 3.4 W for durations of 3 - 24 min at 3 min intervals. A fresh sample was used for each time point. The sample temperature stayed below 20 °C for all experiments (Figure 4.4C, inset). No release was
observed for either laser power indicating that hot electrons do not play a measurable role in release (Figure 4.4C). A significant fraction of hot electrons from plasmon decay should have energies on the same order to one order of magnitude smaller than the photon energy (1.53 eV), which is 2-3 orders of magnitude higher than the average thermal energy increase required for dehybridization, $k_b (T_m - 25 \degree C) \sim 3$ meV. Even with cooling the stage to 7 °C, the energy requirement for dehybridization is only 4.6 meV, which is still an insignificant fraction of the energy of the hot electrons. Therefore the absence of release when the sample is maintained below room temperature supports that thermal effects drive the DNA dehybridization.

In this study, no increased release is observed below the DNA dehybridization temperature under CW irradiation. We believe this is because of the low nanoparticle concentration used. In previous studies, the NS concentration used was $10^{10}$ NS/mL. At high particle concentrations, multiple scattering events have been shown to drastically increase the absorption efficiency of NS solutions. Also, at high concentrations the collective heating of the nanoparticles can result in large temperature increases with temperature gradients. These temperature gradients can explain the previously observed release below the dehybridization temperature where the temperature was probed at the bottom of the temperature gradient. In order to accurately describe release, one must consider the full temperature profile of the absorbing medium, which is very likely to be non-uniform.

**Pulsed Laser-Induced Release.** The results seen in the CW laser experiments provide strong evidence that the release mechanism is thermal in nature and requires
significant bulk heating to dehybridize DNA. Still, preventing global temperature increases is imperative to avoid non-specific damage to surrounding tissues during DNA
Figure 4.4 Investigation of dsDNA melting. ssDNA release profile from solutions heated via a (A) temperature controlled cuvette stage and (B) CW laser at various laser powers. A Boltzmann fit estimates a melting temperature of 61.1 ± 2.3 and 59.1 ± 4.5 °C, respectively. (C) Temperature controlled CW laser induced DNA release under 1.6 (red) and 3.4 W (black) laser irradiation with the stage set to 4 °C showing no DNA release. Inset: corresponding heating profile showing minimal increase in solution temperature.

Figure 4.5 Non-temperature controlled CW laser induced ssDNA release. (A) Heating profile of DNA release at various laser powers, (B) thermal image of DNA release experiment at 1.5 W, (C) DNA released as a function of time at various laser powers, and (D) DNA release as function of cumulative incident energy.
release in vivo. Many previous studies of pulsed laser-induced DNA release have used high energy UV lasers to break the Au-S bond or large powers in the NIR to melt the particles in order to release DNA.

Here, NS@dsDNA solutions were irradiated for 10 min with a ~160 fs 800 nm wavelength pulsed laser at powers from 2 – 50 mW (Figure 4.6A). By using a small spot size, 44 µm FWHM, the average power could be decreased from W in the CW case to mW in the ultrafast case to remove collective heating effects, resulting in no global temperature rise (Figure 4.6B). DNA release increased linearly from 2-25 mW, but decreased above 25 mW. The linear increase in release as a function of laser power suggests a hot-electron mediated process, which differs distinctively from the thermal release profiles in the CW case. However, we investigate the possibility of both mechanisms explicitly.
Figure 4.6 Femtosecond pulsed laser induced ssDNA release. (A) DNA release profile showing a maximum release at 25 mW. At powers above 25 mW, particle degradation occurs. (B) Heating profile of ultrafast pulsed laser DNA release at laser powers from 2-50 mW showing no bulk solution temperature increase. Inset: FEM simulation of the maximum temperature in a 6.6 nm layer around the NS.

The expected transient temperature rise of the DNA due to the pulsed irradiation is in principle enough to dehybridize the DNA. To understand the temperature experienced by the DNA during illumination, we employed the finite-element method (FEM, Comsol) to calculate the spatial and temporal dependence of the temperature field of a single nanoparticle in aqueous solution. Because of the dependences of the temperature field, we employ both spatial and temporal averages to obtain average
temperatures (Figure 4.7). Averaging the temperature in a shell around the nanoparticle with dimensions corresponding to the length of the dsDNA (6.6 nm) gives average temperatures ranging from essentially room temperature at 2 mW to around 140 °C at 50 mW (Figure 4.6B, inset). For laser powers where considerable release is observed (10-25 mW), the calculated temperatures fall between 45-80 °C, which is consistent with the $T_m$ results from the thermal and CW laser experiments. However, thermal processes are much slower than hot electron processes by at least an order of magnitude. Therefore, if we definitively observe Au-S bond breakage then dehybridization never has the chance to occur.

We investigate the breaking of the Au-S bond by employing dsDNA that is fluorescently tagged on the thiolated strand. A similar release profile to that shown in Figure 4.6A is observed with a linear increase in release up to 25 mW (see Figure 4.8). Because the binding energy of the Au-S bond has been shown to be ~ 0.1 eV in such systems, it is highly unlikely that the increased temperature of the nanoparticle surface could induce the breakage. Temperatures in excess of 890 °C ($k_b T = 0.1$ eV) would be required to thermally break the bond, a temperature above which nanoparticle damage and reshaping is expected to occur. These results indicate that hot carriers from plasmon decay cause breakage of the Au-S bond, resulting in release of the entire dsDNA.
Figure 4.7 FEM simulation results. Spatially averaged temperature as a function of time in (A) a 6.6 nm layer surrounding the Au NS, which we identify as the domain of the dsDNA and (B) the Au shell.

Nanoparticle damage is the reason for the decrease in DNA release observed above 25 mW of irradiation, as evidenced by extinction spectroscopy and scanning electron microscopy (SEM). The extinction spectra of samples irradiated at 0-25 mW display a dipole peak centered at ~785 nm and a well-defined higher-energy quadrupole peak. From 30-50 mW, the extinction maximum red-shifts to 688 nm, accompanied by the disappearance of the quadrupole and the appearance of a second peak at ~1015 nm (Figure 4.9A). These changes indicate loss of shell integrity and particle aggregation. SEM images show no change in the Au shells from 2 to 25 mW, but reveal an increase in holes and cracks in the shells at 30 and 35 mW, with widespread holes in the shell at 50 mW (Figure 4.9C-G). These results agree well with previously reported reshaping of nanoshells under femtosecond pulsed irradiation.\textsuperscript{149}
Figure 4.8 DNA release profile resulting from femtosecond pulsed laser irradiation using DNA that is thiolated on the 5’-end and tagged with fluorescein on the 3’-end and is hybridized with a non-tagged complementary strand. Release is linear from 2-35 mW with degradation occurring at powers above 35 mW.

FEM calculations are also useful in explaining why beginning at 30 mW we see a degradation of the DNA release and a breakdown of the gold particles. Figure 4.9B displays the average temperature increase of the NS as a function of incident laser power. At the highest power of 50 mW, the gold shell reaches calculated temperatures around 1000 °C, close to the 1064 °C melting point of bulk gold. It is well known that at the nanoscale the melting and reshaping energy densities are a fraction of their bulk values. Our calculations show that at 30 mW, the gold shell reaches a maximum temperature of ~550 °C, which seems to be the onset of nanoscale reshaping for this structure. Over the range of laser powers used the gold is reaching between 3-70% of the bulk melting energy density of 195 J/g (Figure 4.10), with the onset of reshaping occurring at ~40%. These values will vary depending on nanoparticle morphology and composition as well as temporal pulse width.
Figure 4.9 Nanoparticle characterization after pulsed laser induced DNA release. (A) Extinction spectroscopy showing a red-shift in the plasmon resonance at powers above 25 mW. (B) FEM simulation of the maximum average temperature of the Au shell after a single 150 fs laser pulse. Inset: temperature map for 25 mW incident power 100 ps after laser pulse (scale in °C). TEM images after laser irradiation at (C) 2, (D) 25, (E) 30, (F) 35, and (G) 50 mW showing particle degradation above 25 mW. Scale bars are 200 nm.

**Time and Concentration Dependence of DNA Release.** Using 25 mW of irradiation, the time to release the maximum DNA was investigated (Figure 4.11A). ~40% of release occurs after 2 min of irradiation with saturation achieved after only 5 min. Achieving substantial DNA release with low powers for short irradiation times can minimize non-specific side effects *in vivo*.

Nanoparticle uptake in tumors is known to vary widely, making it critical to have a release method that is relatively insensitive to nanoparticle concentration. Under 10 min of pulsed laser illumination at 25 mW, as nanoparticle concentration was
increased, no appreciable change was seen in the amount of DNA released per NS (Figure 4.11B). Ultrafast irradiation produces high, localized temperatures in the nanoscale vicinity of the particle that relax quickly to equilibrium. The thermal coupling between adjacent nanoparticles is minimized because of low averaged powers: the environment can dissipate absorbed energy fast enough such that the global temperature does not appreciably increase. Thus, DNA release induced by ultrafast irradiation is unlike CW induced release, which depends completely on collective nanoparticle heating. In CW induced release the thermal coupling between many particles is required to raise the solution above the DNA dehybridization temperature, making CW induced release is highly dependent on nanoparticle concentration. Solutions with high nanoparticle concentrations heat faster, allowing more release at shorter times but with the side effect of large volumes of tissue being exposed to high temperatures which are non-ideal for therapy. Conversely, ultrafast induced release is a single particle effect arising from carrier dynamics and the magnitude of release is approximately independent of nanoparticle concentration. Some concentration dependence is expected when high nanoparticle concentrations attenuate the incident beam at the focus. However, as our experiments illustrate, this effect is not relevant over the range of applicable concentrations for therapy.
Figure 4.10 Approximate energy density in the Au shell as a function of laser power. The dependent axis is calculated assuming all of the energy absorbed by a NS in a single cycle of the laser is deposited in the Au before any thermal relaxation occurs. The bulk melting energy density for Au is 190 J/g, while nanoscale reshaping values have been reported as much lower.

Figure 4.11 Time and concentration dependence of pulsed laser induced DNA release. Variation of release as a function of (A) irradiation time at 25 mW with 1x10⁹ particles/mL fit with an exponential that reveals a time constant of 2.2 min, and (B) concentration of NS@dsDNA irradiated at 25 mW and 10 min irradiation showing no appreciable change in release at different concentrations.
4.4. Conclusions

In this study, we showed that NIR CW light-triggered DNA dehybridization is thermally induced as opposed to hot-electron induced. Significantly, we found that collective heating effects of the nanoparticles make the DNA release highly dependent on nanoparticle concentration. At the low particle concentrations achievable in tumors, we demonstrated that the global temperature must rise above the DNA dehybridization temperature to cause DNA release. Alternatively, we illustrated using a NIR pulsed laser that DNA release can be induced with no global temperature increase at lower nanoparticle concentrations through hot-electron induced Au-S bond breakage. Furthermore, with a pulsed laser, DNA release is a single particle phenomenon and is independent of particle concentration. These results are very promising for release of therapeutic molecules without inducing local temperature increase, preserving the integrity of healthy tissues.
Chapter 5

Intracellular Light-Triggered Drug Release for Metastatic Breast Cancer Treatment

5.1. Introduction

Cancer is one of the most devastating diseases facing the human population. What is more, when cancer metastasizes, originally encouraging survival rates dramatically drop. Breast cancer is the second most frequently diagnosed type of cancer and is the second leading cause of cancer deaths in women in the United States.\textsuperscript{151,152} However, deaths from breast cancer rarely result from the primary tumor, but rather as a result of metastases to other organs.\textsuperscript{153} For breast cancer, the 5-year survival rate catastrophically drops from above 75\% to below 10\% when bone metastases are present.\textsuperscript{154} The most common sites of breast cancer metastases are the bone, lungs, brain, and liver.\textsuperscript{155} Typical therapies for cancer include a combination of surgery, chemotherapy, and radiation...
therapy. However, surgery may require removal of portions of healthy tissue, muscles, nerves, or blood vessels, while chemotherapy is nonspecific, and radiation often damages the surrounding skin and soft tissue.\textsuperscript{10}

Today, theranostic nanoparticles are being engineered to selectively target and treat diseased cells.\textsuperscript{8,11,12} In particular, nanoshells (NS), with a thin gold shell around a silica core, are proving promising for theranostics.\textsuperscript{23} A major advantage of NS systems is the tunability of their optical plasmon resonance. By varying the core-shell dimensions, the plasmon resonance can be tuned to the near-infrared (NIR) window where hemoglobin and water are maximally transparent and penetration of NIR light into tissue is maximal.\textsuperscript{51} Recently, light-triggered release from NS has been shown to be an effective way to deliver oligonucleotides and molecules complexed to DNA.\textsuperscript{93,96-98} The surface of NS is functionalized with double-stranded DNA (dsDNA), where one strand is thiol terminated and bound to the Au surface and the complementary strand is hybridized to the thiolated strand through non-covalent interactions. When the particles are irradiated with continuous wave (CW) NIR light, the dsDNA is dehybridized releasing the nonthiolated single-stranded DNA (ssDNA). In addition, dsDNA functionalized NS have been shown as an effective delivery vector for molecules that reversibly bind to the DNA helix. For instance, upon irradiation of the NS, the dsDNA dehybridizes releasing the dye molecule DAPI (4',6-diamidino-2-phenylindole) and the ssDNA.\textsuperscript{93} Light-triggered release can allow for many types of chemotherapeutic molecules to be delivered selectively only to diseased regions.

Previous studies in chapter 4 have shown that continuous wave (CW) induced light-triggered release requires the bulk temperature to rise above the DNA
dehybridization temperature in order to release DNA, while pulsed light induced release breaks the Au-S bond with no bulk temperature increase. Here, we investigate the in vitro effects of these two release mechanisms on drug delivery. Two FDA approved metastatic breast cancer drugs, docetaxel (DTX) and lapatinib (LAP), were delivered to two breast cancer cell lines and a non-cancerous macrophage cell line. DTX is an anti-mitotic chemotherapy drug that binds to the inside of microtubules preventing their disassembly, which disrupts cell division.\textsuperscript{156} LAP is a tyrosine kinase inhibitor that works by binding to the intracellular ATP binding site of the tyrosine kinase domain, blocking phosphorylation.\textsuperscript{157} Tyrosine kinases are proteins on the cell surface that signal it to grow and divide. Some forms of breast cancer over-express the HER2 tyrosine kinase. LAP acts to block the HER2 protein from signaling cell growth.\textsuperscript{158} Release is performed in MDA-MB-231 and SKBR3 (over expresses HER2) breast cancer cells and is compared to release in non-cancerous RAW 264.7 macrophage cells.

Cellular cytotoxicity is compared when drugs are released from a DNA scaffold and a protein scaffold. Currently only hydrophilic molecules have been released from a DNA scaffold, but many chemotherapy drugs are highly hydrophobic. Here we investigate the release of hydrophobic molecules from a DNA scaffold. We also employ a protein scaffold since many proteins can easily bind insoluble drugs in their hydrophobic pockets. We anticipate that highly insoluble, hydrophobic drug can be loaded onto the particles and be released with NIR light.
5.2. Experimental Methods

**Materials.** Potassium carbonate, hydrogen tetrachloroaurate(III) trihydrate (HAuCl$_4$·3H$_2$O), DL-dithiothreitol (DTT), essentially fatty acid free albumin from human serum (HSA), and sodium chloride were purchased from Sigma Aldrich. Docetaxel (DTX) and lapatinib di-p-toluenesulfonate salt (LAP) were purchased from LC Laboratories, Woburn, MA and dissolved in 100% DMSO. 120 nm diameter aminated silica cores in ethanol (10 mg/mL) were purchased from nanoComposix, Inc., San Diego, CA. HPLC-purified ssDNA strands and 10 mM Tris, pH 7.5, 0.1 mM EDTA buffer (1X TE buffer) were purchased from Integrated DNA Technologies (IA, USA). Pierce LDH Cytotoxicity Assay Kit was purchased from ThermoFisher Scientific. Phosphate buffer saline (PBS, 1X) was obtained from Gibco. Water was deionized and filtered by a Milli-Q water system (18.2 MΩ·cm at 25 °C, Millipore).

**DNA purification and hybridization.** The oligonucleotide sequences used in this study for DTX loading were 22-bp ssDNA 5’-HS-C$_6$H$_{12}$-GGA ATA CAC GCG CGA AAT CAC G-3’ and the complementary sequence 5’-CGT GAA TTC GCG CGT GTA TTC C-3’, and for LAP loading were 27-bp ssDNA 5’-HS-C$_6$H$_{12}$-AAA AAA ATA TAT AAT TAA AAG TTG AAA-3’ and the complementary sequence 5’-TTT TTT TAT TTA ATT TTC AAC TTT-3’. The thiolated ssDNA strands were incubated with 10 mM DTT reducing agent in 10 mM TE buffer. The thiolated ssDNA were allowed to react with DTT for 1 h at room temperature, to allow the reduction of the disulfide bonds, followed by purification with a Sephadex G-25 column (NAP 25, GE Healthcare), eluted with TE buffer. The complimentary ssDNA strands were dispersed in 600 µL TE buffer without further purification. The oligonucleotide concentrations were determined by
measuring their absorbances at 260 nm, using a UV-vis spectrophotometer. To form dsDNA, two complementary ssDNA sequences were mixed in a 1:1 molar ratio in a TE buffer solution with 33 mM NaCl, heated at 100 °C in a large water bath for 4 min, and then cooled slowly overnight to room temperature. The dsDNA for DTX loading is referred to as DNA1 and the dsDNA for LAP loading is referred to as DNA2.

**DNA nanocomplex formation.** Nanoshells with core-shell dimensions of \([r1, r2] = [60, 86] \text{ nm}\) were synthesized according to a previously published procedure, and correspond to a nanoshell plasmon resonance at \(\sim 770 \text{ nm}\) in aqueous solution.\(^{23,141}\) The dsDNA was attached to the nanoshell surface via thiol-gold interaction by overnight incubation of dsDNA with aqueous NS in an excess of 20,000 dsDNA strands/NS and stirred. The complex was centrifuged twice at 300 g for 20 min to remove unbound dsDNA strands and was redispersed in TE buffer at pH 7.5. The NS@DNA complex was then incubated with three drug molecules, docetaxel (DTX) or lapatinib (LAP), per dsDNA strand, assuming 20,000 dsDNA strands/NS, on an orbital shaker at 175 rpm overnight. After incubation, the NS@DNA@drug solutions were centrifuged three times at 300 g for 20 min and resuspended in a minimal volume of TE buffer, pH 7.5. The particles were diluted to a final concentration of \(2 \times 10^8 \text{ NS/mL}\) with DMEM media.

**Protein nanocomplex formation.** A 200 \(\mu\text{g/mL}\) solution of HSA in 10 mM PBS (pH 7) was rocked for 30 min to dissolve HSA. The HSA solution was then filtered with a 0.8/0.2 \(\mu\text{m}\) PES (hydrophilic polyethersulfone) filter. Nanoshells were synthesized as described above at a concentration of \(10^{10} \text{ NS/mL}\). A solution of 800 \(\mu\text{L}\) filtered HSA and 800 \(\mu\text{L}\) PBS was pre-heated to 37 °C. 500 \(\mu\text{L}\) NS were added to the solution and stirred for 1 h at 37 °C. The particles were centrifuged twice at 200 g for 15 min,
resuspended in PBS, and filtered through a 0.8/0.2 µm PES filter. The NS@HSA complex was then incubated with 20,000 LAP/NS and rocked overnight. After incubation, the NS@HSA@LAP solution was centrifuged three times at 300 g for 10 min, resuspended in PBS and filtered. The particles were diluted to a final concentration of $2 \times 10^8$ NS/mL with DMEM media.

**Circular dichroism measurements.** CD spectra of 5 µM dsDNA (DNA1 and DNA2) in 33 µM NaCl TE buffer (pH 7.5) and 5 µM dsDNA with 30 µM drug (DTX for DNA1 and LAP for DNA2) in 33 µM NaCl TE buffer (pH 7.5) were acquired in a 1 cm path length quartz cell. The melting CD curves were measured by varying the temperature between 20 °C and 95 °C after an equilibration time of 300 s at a heating rate of in 5 °C per minute. Measurements were made for wavelengths ranging from 200 to 350 nm. The dsDNA melting temperature was extracted from the first derivative of a Boltzmann fit of the melting curve and determined to be 71.0 ± 0.5 °C DNA1, 69.0 ± 0.5 °C DNA1-DTX, 55.0 ± 1.2 °C DNA2, and 57.3 ± 2.2 °C DNA2-LAP.

**Cell Culture.** SKBR3, MDA-MB-231-eGFP breast cancer cells, and RAW 264.7 macrophage cells (Sigma-Aldrich) were cultured in Dulbecco’s Modified Essential Media (DMEM, Gibco) with 25 mM HEPES, 4.5 g/L glucose, and L-glutamine. Media was supplemented with 10% fetal bovine serum (FBS, Gibco), 1% sodium pyruvate (100 mM, Gibco), and 1% antibiotic-antimycotic solution (100X, Gibco). Cells were incubated at 37 °C in a 5% CO₂ environment.

**Nanocomplex cellular uptake verification.** Cells were grown on 8 well µ-slides (Ibidi) for 24 h followed by incubation with the NS@HSA@LAP complex for 4 h (37
°C, 5% CO₂). The cells were washed three times with 1X PBS and a 4% paraformaldehyde fixing solution was added to the chamber slide and incubated for 15 min at room temperature. The fixing solution was then aspirated and the cells were washed two times in 1X PBS, followed by 5 min of incubation with a Hoechst 33342 solution to stain the nuclei of the cells. After incubation with the dye, the cells were washed with 1X PBS and imaged using an epifluorescence microscope. The plasmonic nanoparticles were measured by reflectance (excitation and emission 640 nm).

**Intracellular release of chemotherapy drugs.** Cells were seeded in 96-well plates at a concentration of 5.5 x 10⁴ cells/well 24 h prior to incubation with nanoparticles. Wells for spontaneous and maximum LDH activity controls, and media alone negative controls were added. All samples were performed in triplicate. After 24 h, the media was removed and cells were incubated with 100 µL of media containing 2 x 10⁸ NS/mL of either NS@scaffold or NS@scaffold@drug for 4 h. Media was then removed and cells were washed once with 1X PBS and 100 µL of fresh media was added to each well. 10 µL of sterile water was added to each spontaneous LDH activity well.

**Laser treatment.** Cells were then irradiated with either an 808 nm CW laser at 1.5 W for 2 min or and 800 nm 160 fs pulsed laser at 25 mW for 2 min. After laser treatment, the samples were incubated for 24 h (37°C, 5% CO₂) to allow released drug (DTX or LAP) molecules to induce cell death.

**Lactate dehydrogenase (LDH) cytotoxicity assay.** 23.25 h after laser treatment, 10 µL of lysis buffer (10X) was added to each maximum LDH activity well. After 45 min of incubation (37°C, 5% CO₂), 50 µL of media from each well was transferred to new 96
well plates. 50 µL of reaction mixture was added to each well and pipette mixed. The plate was incubated for 30 min at room temperature in the dark, followed by addition of 50 µL of stop solution to each well. Absorbance measurements were taken at 490 ± 9 and 680 ± 9 nm. The background absorbance at 680 nm was then subtracted from the absorbance at 490 nm. Percent cytotoxicity was calculated using the following equation:

\[
\% \text{ Cytotoxicity} = \frac{\text{Sample LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \times 100
\]

**Instrumentation.** Extinction spectra were taken on a Cary 5000 UV/Vis/NIR Varian spectrophotometer. Zeta potential measurements were obtained using a Malvern Zetasizer Nano ZS. TEM images were taken with a JEOL 1230 high contrast transmission electron microscope. Circular dichroism was measured on a JASCO J-810 spectropolarimeter equipped with a Peltier-type temperature control system. LDH assay absorbance readings were taken on an Infinite M200 PRO TECAN plate reader. An Angio Dynamics Diomed 15Plus 808 nm continuous wave diode laser with a full-width half-max of 3.5 mm and an 800 nm Ti:Sa Laser Amplifier (Coherent, RegA) operating at 250 kHz with an average power of 1 W, a pulse length of ~150-180 fs, and a full-width-half-max of 44 µm were used for release studies. Imaging for cellular studies were taken with a Nikon A1-Rsi Confocal microscope with a 60X 1.6 numerical aperture oil immersion objective.

### 5.3. Results and Discussion

A schematic representation of the nanocomplex fabrication and the light-triggered drug release process is shown in Scheme 5.1. First, dsDNA is attached to nanoshells (NS)
through gold-thiol bonds, followed by DNA loading with drug (DTX or LAP) molecules. The NS@DNA@drug nanocomplexes are then incubated with cells and irradiated with a NIR laser. To maximize drug release efficiency, two illumination sources were investigated. CW laser irradiation, results in dehybridization and release of the drug, while pulsed irradiation results in Au-S bond breakage and release of the dsDNA-drug complex. The laser wavelength was selected to match the NS plasmon resonance and the NIR window.

**Scheme 5.1** Nanocomplex formation. (A) Formation of NS@DNA@Drug nanocomplex by functionalization of NS with thiolated dsDNA through Au-thiol bonds, followed by attachment of drug molecules. Release triggered by CW irradiation results in DNA dehybridization and release of the drug, while pulsed irradiation results in Au-S bond breakage and release of the DNA-drug complex. (B) Formation of NS@HSA@Drug by coating NS with HSA, and attachment of drug molecules. Drug release from the protein is triggered by CW or pulsed irradiation.
A protein layer is also investigated as a drug release scaffold. Human serum albumin (HSA) is the most abundant protein in the blood plasma and can bind highly insoluble drug molecules in its hydrophobic pockets. Additionally due to its large size and multiple binding regions, HSA has the potential to significantly increase the payload capacity of the nanocomplexes. Here, NS are coated with HSA, which forms a protein corona around the particles, which are subsequently incubated with drug molecules (Scheme 5.1B). The NS@HSA@drug nanocomplexes are then incubated with cells and irradiated with either a CW or pulsed NIR laser in the same manner as the NS@DNA@drug nanocomplexes.

Functionalization of the NS surfaces was verified with transmission electron microscopy (TEM), extinction spectroscopy, zeta potential, and dynamic light scattering (DLS) (Figure 5.1). The TEM images of NS (orange) showed spherical particles with smooth regular surfaces, while TEM images of NS@DNA1 and NS@DNA2 (blue) showed the formation of a thin DNA layers around the NS (Figure 5.1A). TEM images of NS@DNA1@DTX and NS@DNA2@LAP (green) showed no morphological changes. Zoomed in TEM images of the NS surface are seen in Figure 5.2A. In the case of NS@HSA and NS@HSA@LAP, a hard and a soft corona around the NS was observed (Figure 5.1A).

The extinction maxima red-shift when coated with DNA and further red-shift when the drug molecules are loaded due to the change in the dielectric environment around the NS (Figure 5.1B). Protein coating induces a larger red-shift as a result of the larger size of the protein layer. Representative extinction spectra of NS, NS@DNA2, and NS@DNA2@LAP is shown in Figure 5.2B. No changes in the peak shape or width of
the extinction spectra is observed, indicating that the nanocomplexes do not aggregate during the functionalization process. Zeta potential measurements further confirm modification of the NS surface, for example the zeta potential became more negative after DNA attachment due to the increased negative charges associated with the phosphate backbone of the DNA and even more negative with drug loading (Figure 5.1B). DLS measurements show the hydrodynamic diameter of bare NS decreases after DNA functionalization possibly due to a change in the solvent from water to TE buffer. The hydrodynamic diameter further decreases after drug loading likely as a result of compaction of the DNA. In the case of protein functionalization the hydrodynamic diameter slightly increases after protein coating and shows minimal change upon drug loading.

Figure 5.1 Nanocomplex characterization. (A) TEM images of bare NS (orange), NS@scaffold (blue), and NS@scaffold@drug (green) for DNA1 scaffold – DTX drug, DNA2 scaffold – LAP drug, and HSA scaffold – LAP drug coatings. Scale bars are 50 nm. (B) Table of corresponding extinction maxima, zeta potentials, and hydrodynamic diameters for scaffold and drug coatings.
Figure 5.2 Nanocomplex surface characterization. TEM images (A) and extinction spectra (B) of bare NS (orange), NS@DNA2 (blue), and NS@DNA2@LAP (green) showing a minor red-shifts in the plasmon resonance and the formation of a thin DNA layer around the NS. Scale bars are 20 nm.

Confirmation of drug loading. Surface enhanced Raman spectroscopy (SERS) was used to confirm drug loading on the NS@scaffold complexes (Figure 5.3). The SERS spectrum of the thiolated DNA1 monolayer on the NS surface shows several characteristic SERS bands at 1346 and 1480 cm\(^{-1}\). The SERS mode at 737 cm\(^{-1}\) is weak due to the low number of adenine bases in the DNA sequence (Figure 5.3A). Moreover, several strong SERS peaks at 1003, 1271, 1520, and 1543 cm\(^{-1}\) assigned to the benzene ring modes (\(\nu_C-C\), \(\nu_C-O\), \(\nu_C=C\), and ring stretching) are seen in the NS@DNA1@DTX SERS spectrum. This indicates the DNA1-DTX complex formation. The bands at 1340 and 1475 cm\(^{-1}\) are enhanced in the complex spectrum in comparison to DNA1. In the case of the NS@DNA2 monolayer on the NS surface the SERS spectrum is dominated by the adenine ring-breathing mode at 737 cm\(^{-1}\) (Figure 5.3B). LAP Raman peaks including C-O-C mode of a substituted furan at 1136 cm\(^{-1}\) are seen in the NS@DNA2@LAP SERS spectra. The protein-drug nanocomplex system
SERS data confirms that LAP has associated with the HSA protein corona on the particles surface from peaks including a quinazoline ring stretch at 1369 cm\(^{-1}\) (Figure 5.3C). The observed SERS frequencies and their assignments of DNA1, DNA2 and NS@DNA1@DTX, NS@DNA2@LAP complexes are listed in Table 5.1.

**Table 5.1** SERS peak assignments for nanocomplex formation by the attachment of DNA, HSA, DTX, and LAP to NS surfaces.

<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>737</td>
<td>A, ring breathing</td>
</tr>
<tr>
<td>758</td>
<td>C-H out-of-plane deformation</td>
</tr>
<tr>
<td>949</td>
<td>C-H out-of-plane deformation</td>
</tr>
<tr>
<td>1003</td>
<td>Benzene C-C-C in-plane ring breathing</td>
</tr>
<tr>
<td>1136</td>
<td>C-O-C of substituted furan</td>
</tr>
<tr>
<td>1271</td>
<td>Benzene ring</td>
</tr>
<tr>
<td>1346</td>
<td>A, G</td>
</tr>
<tr>
<td>1369</td>
<td>Quinazoline ring stretch</td>
</tr>
<tr>
<td>1480</td>
<td>A, G</td>
</tr>
<tr>
<td>1520</td>
<td>Benzene C-C ring mode</td>
</tr>
<tr>
<td>1543</td>
<td>Benzene ring mode</td>
</tr>
</tbody>
</table>

\(^{a}\)A, adenine; G, guanine.

Further confirmation of LAP loading onto NS@DNA2 was performed with X-ray photoelectron spectroscopy (XPS) (Figure 5.4). A survey scan shows Au 4p\(^3\), 4d\(^3\), 4d\(^5\), 4f\(^6\), and 4f\(^7\) from the Au shells of NS. Si 2s and 2p peaks, as well as O 1s peaks are from the SiO\(_2\) core of the NS. C 1s comes from the DNA. An elemental scan of the S 2p reveals a peak at a binding energy of 168 eV, which indicates S in a sulfone group as opposed to a thiol, which further confirms LAP loading onto the DNA scaffold.
Figure 5.3 SERS characterization to confirm drug loading. Normal Raman spectra of DTX (black) and LAP (black), and SERS spectra of NS@scaffold (blue) and NS@scaffold@drug (green) for (A) DNA1-DTX, (B) DNA2-LAP, and (C) HSA-LAP.
Figure 5.4 XPS characterization to confirm LAP loading. (A) Survey scan and (B) S 2p element scan of NS@DNA2 (blue) and NS@DNA2@LAP (green).

**DNA characterization.** Circular dichroism was performed to reveal the temperature at which the dsDNA is expected to dehybridize under CW laser illumination. CD spectra were acquired in TE buffer (pH = 7.5) with 33 mM NaCl using dsDNA concentrations of 5 µM. Thermal curves were obtained by monitoring the decrease in ellipticity with temperature at the peak maximum. The temperature was varied between 20 °C and 100 °C in 5 °C increments using an equilibration time of 300 s. The CD spectra of free DNA1 and DNA1-DTX complex are shown in Figure 5.5A. The CD spectrum of the free DNA1 has two positive peaks at 223 and 279 nm, consistent with a B conformation. Upon DTX complexation, a shift in the CD positive peak position from 279 to 277 nm and negative peak position from 247 to 249 nm was observed. This spectral shifting is due to a structural perturbation of the dsDNA helix as a result of drug interaction. Further, the differences in melting curves of DNA1 and DNA1-DTX complex (Figure 5.5B, C) indicate a structural perturbation from drug loading. The first derivative of a Boltzmann fit of the CD melting curve was used to determine melting
temperatures ($T_m$) of $71.0 \pm 0.5 \, ^\circ C$ and $69.0 \pm 0.5 \, ^\circ C$ for DNA1 and DNA1-DTX, respectively. In the case of the DNA2-LAP complex, no spectral shift was observed in the CD spectra and the melting temperature curves showed no differences (Figure 5.5D-F). This indicates no major structural changes occurred upon complexation of LAP with DNA2.

**Figure 5.5** DNA characterization. (A) Circular dichroism spectra of DNA1 and DNA1-DTX. DNA melting curve for (B) DNA1 at 279 nm with a $T_m$ of $71.0 \pm 0.5 \, ^\circ C$ and (C) DNA1-DTX at 277 nm with a $T_m$ of $69.0 \pm 0.5 \, ^\circ C$. (D) Circular dichroism spectra of DNA2 and DNA2-LAP. DNA melting curve for (E) DNA2 at 279 nm with a $T_m$ of $55.0 \pm 1.2 \, ^\circ C$ and (F) DNA2-LAP at 278 nm with a $T_m$ of $57.3 \pm 2.2 \, ^\circ C$.

**Verification of nanocomplex cellular uptake.** Cellular uptake of the NS@scaffold@drug complexes was investigated using dark field, confocal, and reflectance (Em. and Ext. at 640 nm) microscopy in RAW 264.7 macrophages cells, and
in MDA-MB-231-eGFP and SKBR3 breast cancer cells. For simplicity only the RAW 264.7 images are shown (Figure 5.6). In the darkfield images, time dependent internalization of the nanocomplexes is easily seen as diffraction-limited bright spots (Figure 5.6B, C). As a control, cells not incubated with nanoparticles showed no observable bright spots (Figure 5.6A). Because the darkfield images alone do not give clear evidence whether the NS@HSA@drug are adsorbed onto the outer membrane of the cells or internalized into the cells, brightfield images were taken. Varying depths of field within an individual cell allows clear 3D visualization of the nanocomplex distribution within the cell. Figure 5.6E is a slice from the middle of the cell showing clear diffraction-limited dark spots corresponding to the nanocomplexes, verifying that the NS are internalized within the cell. As a control, cells not incubated with the nanocomplexes showed no observable dark spots when imaged in the same mode (Figure 5.6D). To further investigate the nanocomplex cellular uptake and localization inside the cells, confocal images were measured in transmission and reflection mode (em. and ext. at 640 nm) at varying depths. The details in the orthogonal YZ and XZ planes illustrate the location of the nanoparticles within the cell boundary around but not inside the nucleus (the nucleus was stained with Hoechst 33342 dye; Figure 5.6F).
Figure 5.6 Cellular uptake of nanocomplexes. Dark field spectroscopy of NS@HSA incubated with RAW 264.7 cells for (A) 0 h, (B) 1 h, and (C) 4 h. Brightfield images (20x objective, 4 averages) measured in transmission (D) without and (E) with nanocomplexes. (F) Orthogonal view of the 3D maximum intensity projection image of cells incubated overnight with nanocomplex. Cells were stained for nucleus (Hoechst 33342, blue).

**Intracellular Light-Triggered Drug Release Studies.** Cellular cytotoxicity resulting from light-triggered release of DTX and LAP was evaluated using DNA and protein scaffolds. Cells were grown for 24 h in 96 well plates, incubated for 4 h with nanocomplexes, washed with PBS, then given fresh media. Cells were irradiated with 1.5 W of CW or 25 mW of femtosecond pulsed laser power for 2 min then incubated at
37°C, 5% CO₂ for 24 h to allow time for the released drug molecules to interact with the cells. Cellular death was evaluated with a lactate dehydrogenase (LDH) cytotoxicity assay. Percent cytotoxicity values were calculated considering the NS@scaffold with no laser treatment samples as 10%. DTX release from the DNA1 scaffold was investigated in MDA-MB-231 breast cancer cells (Figure 5.7A) and in RAW 264.7 macrophage cells (Figure 5.7B). The NS@DNA1@DTX nanocomplex is shown to be non-toxic as no increase in cytotoxicity was observed as compared to the NS@DNA1 nanoparticles. This indicates that DTX stays confined within the complex and no leaching occurs. An increase in cell death is seen when DTX is released with the CW laser, while no increased death is seen for pulsed laser induced release. This is due to the different DNA release mechanisms resulting from CW vs. pulsed lasers. In the case of a CW laser, the nanocomplex and surrounding environment heat up causing the dsDNA to dehybridize, releasing the drug and ssDNA. Contrarily, irradiation with a pulsed laser induces hot-electron mediated breakage of the Au-S anchoring bond of the dsDNA. As a result, the entire dsDNA-drug complex is released with the drug likely remaining sequestered within the DNA, preventing it from producing cell death. It is important to note that there is an increase in cytotoxicity for the cells with NS@DNA1 after CW laser treatment from the large global temperature increase caused by the high average powers needed to induce release with CW illumination. Similar results are seen in both the cancer and macrophage cells, which is to be expected as DTX is not a targeted cancer drug and does not discriminate between cancerous and healthy cells.
Figure 5.7 Comparison of cell viability after DTX release from a DNA scaffold without (blue) and with (orange) CW and pulsed lasers in (A) MDA-MB-231 and (B) RAW 264.7 cells.

Pulsed induced drug release is preferable over CW laser release as lower average powers can be used, while maintain high peak powers. As a result the global temperature can remain low, reducing non-specific cell death. Since pulsed laser induced drug release from the DNA scaffold releases the entire scaffold-drug complex, protein was
investigated as an alternative scaffold. LAP was released from both a DNA and protein (HSA) scaffold in SKBR3 breast cancer cells and RAW 264.7 macrophage cells. LAP was chosen to see if cell death could be preferentially induced in cancer cells over healthy cells, as LAP specifically targets the HER2 receptor that is overexpressed in the SKBR3 cells. Using the DNA scaffold, no increased cytotoxicity is observed for either CW or pulsed laser induced release in SKBR3 cells (Figure 5.8A). There is also no increase in cytotoxicity for CW laser induced release from the protein scaffold; however, there is a large increase in cytotoxicity for pulsed laser induced LAP release from the protein scaffold in SKBR3 cells (Figure 5.8B). The low average power (25 mW) does not increase global temperatures and therefore cells with NS@HSA do not show increased death after pulsed laser treatment. But, due to the high peak powers achieved by a pulsed laser, irradiation causes very high temperatures in the direct nanoscale vicinity of nanoparticle (~500 °C on the NS surface and ~80 °C in a 7 nm layer around the NS for 25 mW). It is probable that these high temperatures cause the protein to unfold, releasing LAP from the nanocomplex.

LAP release in the macrophage cells was compared to the cancer cells (Figure 5.9). No increased cytotoxicity was observed for CW or pulsed laser release from either the DNA or protein scaffolds. This shows that the released LAP can selectively induced cell death in HER2 expressing breast cancer cells, without affecting non-cancerous cells.
Figure 5.8 Comparison of cell viability after LAP release in SKBR3 cells without (blue) and with (orange) CW and pulsed lasers from a (A) DNA scaffold and (B) protein scaffold.
Figure 5.9 Comparison of cell viability after LAP release in RAW 264.7 cells without (blue) and with (orange) CW and pulsed lasers from a (A) DNA scaffold and (B) protein scaffold.
5.4. Conclusions

Low levels of both CW and pulsed near-IR light have been shown to release DNA from plasmonic nanoparticles as a strategy for controlled drug delivery. Here, we show that *in vitro* release can be achieved using both types of laser sources from DNA and protein scaffolds. Non-targeted, docetaxel (DTX), and targeted, lapatinib (LAP), breast cancer drugs were released in both cancerous and non-cancerous cells. The results show that there is a higher percent cytotoxicity for CW vs. pulsed laser-induced DTX release from a DNA scaffold in cancerous and non-cancerous cells, as DTX is a non-targeted drug; however, some non-specific cell death is induced by the CW laser itself. Using the protein scaffold, increased cytotoxicity was observed for LAP release using pulsed vs. CW light in cancerous cells, while non-cancerous cells were unaffected. Released lapatinib was specifically able to kill the HER2+ breast cancer cells, showing that light-triggered drug release is a very promising non-toxic and selective drug delivery vehicle for cancer treatment. By simply exchanging the chemotherapy drug, this system can be extended to treatments for many cancer types. Achieving the flexible loading and release strategies shown here will allow release to be more easily controlled and tailored for various chemotherapeutic drugs and cancer types.
Chapter 6

Conclusions

The use of plasmonic nanoparticles for controlled drug delivery necessitates the particles to be biocompatible, absorb light in the near-infrared (NIR), exhibit efficient light-to-heat conversion, have high tumor uptake, and be able to actively release drugs. These factors were taken into consideration in the projects discussed here. In Chapter 3, HGNS with sizes below 100 nm and NIR resonances were synthesized for biomedical applications; however, when there is a large amount of residual Ag in the HGNS to achieve the NIR resonance. When injected in mice, the biodistribution of both component species, Ag and Au, strongly suggests that the nanoparticles are fragmenting after injection. These early studies indicate that due to the remaining sacrificial Ag core, the loss of structural integrity, and the known toxicity of Ag, the stability and toxicity of HGNS made by galvanic replacements should be more carefully studied prior to their use in vivo. These results should strongly impact the assessment of ongoing efforts to design
biocompatible plasmonic nanoparticles with compositions and structural properties appropriate for nanomedicine.

Using biocompatible nanoshells, we investigated light-triggered release as a method of controlled drug delivery. It was critical to understand the release mechanism with CW and pulsed laser to enable control and tailorability of the system for various chemotherapy drugs. We showed that CW light-triggered DNA dehybridization is thermally induced as opposed to hot-electron induced. Significantly, we found that DNA release is highly dependent on nanoparticle concentration. At the low particle concentrations achievable in tumors, we demonstrated that the global temperature must rise above the DNA dehybridization temperature to cause DNA release. Alternatively, we illustrated that DNA release via Au-S bond breakage can be induced with no global temperature increase at lower particle concentrations using a pulsed laser. Furthermore, with a pulsed laser, DNA release is a single particle phenomenon and is independent of particle concentration. These results are very promising for release of therapeutic molecules without inducing local temperature increase, preserving the integrity of healthy tissues.

Finally, light-triggered drug release was tested in vitro from DNA and protein scaffolds using CW and pulsed lasers. Non-targeted, docetaxel (DTX), and targeted, lapatinib (LAP), metastatic breast cancer drugs were released in both cancerous and non-cancerous cells. The results show that there is a higher percent cytotoxicity for CW-induced DTX release from the DNA scaffold in both cancerous and non-cancerous cells, as DTX is a non-targeted drug. Using the protein scaffold, increased cytotoxicity was seen for LAP release using pulsed vs. CW light in cancerous cells, while non-cancerous
cells were unaffected, which illustrated that light-triggered drug release can selectively kill cancer cells. This system can be extended to many cancers by exchanging the chemotherapy drug for ones specific to each cancer type. Achieving a flexible loading and release strategy will allow release to more easily controlled and tailored for various chemotherapeutic drugs and cancer types.
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