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Nanostructures for Plasmon Enhanced Fluorescence Sensing: From Photophysics to Biomedicine

by

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Abstract

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Metallic nanostructures exhibit unique plasmonic properties when optically excited, which includes modification of the spontaneous emission and lifetime of fluorophores in their vicinity. Here we utilize silica (SiO$_2$) core encapsulated in gold (Au) shell nanoshells for emission enhancement of weak near-infrared (NIR) emitting fluorophores, including Indocyanine green (ICG) and IR800. The fluorescence enhancement of ICG molecules as a function of distance from the surface of nanoshells was studied. A maximum enhancement of 50X at a distance of 7 nm from the nanoshells surface, and minimum enhancement of 7X at 42 nm from nanoshells surface was achieved. Additionally, fluorescence enhancement of IR800 molecules induced by nanoshells was compared with that of Au nanorods. The quantum yield of IR800 was enhanced from 7% to 86% in the case of nanoshells and 74 % for nanorods. The native lifetime of IR800 decreased from 564 ps to 121 ps when conjugated to nanorods and 68 ps for nanoshells.

We then demonstrated a biomedical application of plasmon enhanced fluorescence sensing by utilizing nanoshell based complexes (nanocomplexes) for simultaneous fluorescence optical imaging as well as magnetic resonance imaging of cancer cells in vitro and in vivo. Nanocomplexes were fabricated by encapsulating nanoshells with a SiO$_2$ epilayer doped with iron oxide nanoparticles and ICG molecules,
which resulted in a high $T_2$ relaxivity (390 mM$^{-1}$sec$^{-1}$) and 45X fluorescence enhancement of ICG. The nanocomplexes were covalently conjugated with antibodies to enable active targeting \textit{in vitro} and \textit{in vivo}. In addition they were utilized for photothermal therapy of cancer cells \textit{in vitro}.

Furthermore, other plasmonic nanostructures relevant for biomedical applications were also synthesized in the sub-100 nm regime including Au/SiO$_2$/Au nanoshells and cuprous oxide core coated with Au shell nanoshells. Excellent agreement between their experimental and theoretical optical properties was achieved. Additionally, physical and chemical properties of mesostructures relevant for photonic devices including sub-micrometer zinc oxide structures and Mesostars composed of a mixture of iron oxides and Au were also studied.
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# Table of Contents

Abstract...........................................................................................................................................i

Acknowledgements.........................................................................................................................iii

Table of Contents..............................................................................................................................v

List of Figures.....................................................................................................................................x

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

Chapter 1: Introduction......................................................................................................................1  
  1.1 Plasmon Enhanced Fluorescence............................................................................................3
  1.2 Nanoshell Background.............................................................................................................5
    1.2.1 Fabrication and Characterization of Nanoshells............................................................6
    1.2.2 Optical Properties of Nanoshells and Plasmon Hybridization.................................8
    1.2.3 Near Field and Far Field Properties of Nanoshells.................................................13
    1.2.4 Photothermal Properties of Nanoshells...................................................................14
  1.3 Frequency Domain Fluorescence Lifetime........................................................................16
  1.4 Non-Invasive Biological Imaging Techniques....................................................................19
    1.4.1 Fluorescence Optical Imaging...................................................................................21
    1.4.2 Magnetic Resonance Imaging.....................................................................................23
  1.5 Cancer Biology and Therapy.................................................................................................26
    1.5.1 Cancer Biology.........................................................................................................28
    1.5.2 Cancer Therapy Strategies.........................................................................................30
  1.6 Thesis Outline..........................................................................................................................33

Chapter 2: Nanoscale Control of Near-Infrared Fluorescence Enhancement Using  
Au Nanoshell.....................................................................................................................................35
2.1 Introduction ...........................................................................................................35
2.2 Fabrication of Nanoshell-ICG Conjugates .........................................................39
2.3 Characterization of Nanoshell-ICG Conjugates ..............................................40
2.4 Experimental Fluorescence Enhancement .......................................................43
2.5 Calculated Fluorescence Enhancement ..........................................................45
2.6 Calculated Quantum Yield and Lifetime .......................................................49
2.7 Conclusions ..........................................................................................................50

Chapter 3: Fluorescence Enhancement by Au Nanostructures: Nanoshells and
Nanorods ..................................................................................................................52
3.1 Introduction ..........................................................................................................52
3.2 Fabrication of Nanoparticle-Fluorophore conjugates .....................................56
3.3 Characterization of Nanoparticle-Fluorophore conjugates ............................60
3.4 Experimental Fluorescence Enhancement .......................................................64
3.5 Calculated and Experimental Scattering Efficiency .........................................65
3.6 Frequency Domain Lifetime Decay ...................................................................68
3.7 Experimental Quantification of Quantum Yield ..............................................71
3.8 Conclusions ..........................................................................................................74

Chapter 4: Nanoshells for Simultaneous Magnetic and Optical Imaging and
Photothermal Therapeutic Response ......................................................................75
4.1 Introduction ..........................................................................................................75
4.2 Experimental Methods ......................................................................................79
4.3 Characterization of Nanocomplexes ...............................................................84
4.4 Magnetic Resonance Analysis and Relaxivity ...............................................90
4.5 Antibody Conjugation and Quantification of number of Antibodies ........................................... 94
4.6 In vitro Magnetic Resonance Imaging ......................................................................................... 95
4.7 In vitro Fluorescence Optical Imaging ....................................................................................... 98
4.8 In vitro Photothermal Therapy .................................................................................................. 99
4.9 Conclusions ............................................................................................................................ 101

Chapter 5: A Molecularly Targeted Theranostic Probe for Ovarian Cancer ................................. 102
5.1 Introduction ............................................................................................................................ 102
5.2 Experimental Methods .......................................................................................................... 106
5.3 Properties of Nanocomplexes ............................................................................................... 109
5.4 In vitro Fluorescence Optical Imaging ...................................................................................... 110
5.5 In vitro Magnetic Resonance Imaging ....................................................................................... 112
5.6 In vitro Photothermal Therapy and Cytotoxicity Studies ....................................................... 115
5.7 Discussion .............................................................................................................................. 118
5.8 Conclusions ............................................................................................................................ 120

Chapter 6: In vivo Multimodal Imaging of Breast Cancer with Magneto-Fluorescent Gold Nanoshells ................................................................. 121
6.1 Introduction ............................................................................................................................ 121
6.2 Experimental methods .......................................................................................................... 125
6.3 Properties of dual modal nanocomplexes ............................................................................... 128
6.4 Fluorescence optical imaging in vivo ...................................................................................... 130
6.5 Magnetic resonance imaging in vivo ....................................................................................... 133
6.6 Biodistribution after 72 hours .................................................................................................. 135
6.7 TEM analysis of nanocomplexes in tumor sections ............................................................... 139
Chapter 10: Facile Chemical Approach to ZnO Submicrometer Particles with Controllable Morphologies

10.1 Introduction

10.2 Fabrication of ZnO Particles

10.3 Characterization of ZnO Particles

10.4 Growth Mechanism of ZnO Particles

10.5 Emission Properties and Defect States

10.6 Conclusions

Chapter 11: Summary and Prospects

References

Appendix A – Publications related to the research described in this thesis

Appendix B – Presentations related to the research described in this thesis
List of Figures

Figure 1.1 Simple Jablonski diagram describing emission processes .............................................. 3

Figure 1.2 Jablonski diagram showing the fluorescence of a molecule in free space and in proximity to a metallic surface .......................................................... 5

Figure 1.3 Schematic diagram and corresponding TEM images showing nanoshell fabrication ................................................................. 7

Figure 1.4 Low resolution SEM image of nanoshells [r_1, r_2] = [60, 74] nm in size with high resolution SEM image provided as inset .............................................. 8

Figure 1.5 Experimental extinction spectra of Au shell growth on 60 nm radius silica cores by varying the ratio of the precursor particles to the plating solution .................................................. 8

Figure 1.6 Energy level diagram depicting plasmon hybridization in nanoshells resulting from interacting sphere and cavity plasmons .................................................. 9

Figure 1.7 Schematic diagram showing nanoshell geometry with various parameters.... 12

Figure 1.8 The near field of nanoshells with dimensions [r_1, r_2] = [60, 76] nm plotted as a function of distance from the surface and .................................................. 14

Figure 1.9 Schematic showing the two different fluorescence lifetime measurement techniques ........................................................................................................ 18

Figure 1.10 The NIR window showing minimal light absorption by hemoglobin and water .................................................................................................................. 22

Figure 1.11 Schematic diagram explaining the relaxation processes in MRI ........................................ 24

Figure 1.12 Schematic explaining TE in a spin echo sequence ................................................................................. 25

Figure 1.13 Schematic representation of iron oxide interaction with H_2O molecule...... 26

Figure 1.14 Hallmarks of cancer describing the genetic alterations which characterize the progression of malignant cells ........................................................................ 29

Figure 2.1 Absorption–emission spectra (λ_{max-Ab} = 780 nm and λ_{max-Em} = 820 nm) and chemical structure (inset) of ICG ........................................................................................................ 40

Figure 2.2 Schematic diagram of fabrication procedure, and TEM micrographs (B – F) of NS coated with varying thicknesses of silica epilayers ......................................................................... 40
Figure 2.3 Observed shift in the plasmon resonance peak with increasing thickness of silica layer surrounding the NS.................................................................41

Figure 2.4 Schematic diagram of ICG conjugation on NS@SiO₂ nanoparticles, excitation and observed emission........................................................................43

Figure 2.5 Fluorescence enhancement factor of ICG molecules adsorbed on NS without any silica layer is represented..........................................................46

Figure 2.6 Experimental and calculated values of fluorescence enhancement factor of ICG conjugated to NS@SiO₂ nanoparticles........................................47

Figure 2.7 Calculated values of distance dependent fluorescence of ICG as a function of silica spacer thickness surrounding the NS....................................49

Figure 3.1 Absorption - emission profile (λ_max-Ab ~ 782 nm and λ_max-Em ~ 804 nm) of IR800 conjugated with HSA.................................................................60

Figure 3.2 TEM micrographs of NSs and NSs coated with HSA-IR800.................................................................62

Figure 3.3 Surface plasmon resonance shift when nanoparticles are conjugated with HSA-IR800.................................................................62

Figure 3.4 Extinction spectra of NSs-HSA-IR800 and NRs-HSA-IR800.................................................................63

Figure 3.5 Emission spectra of control, HSA-IR800, nanoshells and nanorods coated with HSA but without IR800.................................................................64

Figure 3.6 Calculated scattering spectra of NSs and NRs.................................................................65

Figure 3.7 Schematic diagram representing angle-resolved fluorescence emission apparatus and experimental angle-resolved scattering spectra........66

Figure 3.8 Frequency domain fluorescence decay of IR800, HSA-IR800, NRs-HSA-IR800 and NSs-HSA-IR800.................................................................67

Figure 4.1 Schematic diagram illustrating fabrication of nanocomplexes for imaging and therapy, low and high resolution SEM images........................................85

Figure 4.2 Powder XRD patterns of nanocomplexes and corresponding XRD intensity profile of Au and Fe₃O₄ from powder diffraction database........87

Figure 4.3 TEM image of Fe₃O₄ nanoparticles, powder X-ray diffraction patterns and magnetization results of Fe₃O₄ nanoparticles.........................................89
Figure 4.4 T₂-weighted MR images of nanocomplexes in aqueous media at various concentrations, MR image intensity and Spin–Spin relaxation rate (T₂⁻¹).................................91

Figure 4.5 T₂-weighted MR images of Fe₃O₄ nanoparticles and Spin – Spin relaxation rate as a function of Fe concentration.................................................................93

Figure 4.6 Schematic diagram showing anti-HER2 conjugation to nanocomplexes via streptavidin-biotin binding and ELISA results.........................................................94

Figure 4.7 MR image of HER2 positive SKBR3 cells and HER2 negative MDAMB231 cells suspended in 0.5% agarose.................................................................96

Figure 4.8 Fluorescence images of SKBR3 and MDAMB231 with nanocomplexes-anti-HER2 conjugates and unconjugated nanocomplexes..............................................98

Figure 4.9 Photothermal ablation and live/dead stain of SKBR3 cells incubated with nanocomplexes-anti-HER2 conjugates and unconjugated nanocomplexes..................99

Figure 5.1 Schematic representation of anti-HER2 conjugated nanoshell contrast agents, extinction and emission spectra of nanocomplexes.................................109

Figure 5.2 Fluorescence optical images of OVCAR3 and MDAMB231 cells with nanocomplex-anti-HER2 conjugates, and unconjugated nanocomplexes.......................111

Figure 5.3 Schematic representation of sample preparation for in vitro MRI studies. MR image of OVCAR3 and MDAMB231 cells suspended in 0.5 % agarose.............113

Figure 5.4 Photothermal ablation and live/dead stain of OVCAR3 and MDAMB231 cells incubated with nanocomplex-anti-HER2 conjugates and control.......................116

Figure 5.5 Cytotoxicity studies of OVCAR3 cells incubated with nanocomplex-anti-HER2 conjugates and unconjugated nanocomplexes...........................................117

Figure 6.1 Characterization of the magneto-fluorescent nanocomplexes.............................................129

Figure 6.2 NIR fluorescence images of mice with MDAMB231 and BT474AZ xenografts.................................................................131

Figure 6.3 MR images of mice with BT474AZ and MDAMB231 xenografts.................................133

Figure 6.4 NIR fluorescence images and gold distribution in mice organs...................................138

Figure 6.5 TEM images of tumor sections retrieved from mice 72h post-injection.................139

Figure 7.1 Theoretical analysis of Au/SiO₂/Au nanoshells and energy level diagram describing plasmon modes of Au/SiO₂/Au nanoshells.................................149
Figure 7.2 Fabrication procedure of Au/SiO$_2$/Au nanoshells and SEM images........152

Figure 7.3 Experimental, theoretical extinction spectra and SEM images of sub-150 nm Au/SiO$_2$/Au nanoshells.................................................................155

Figure 7.4 Experimental, calculated extinction spectra and SEM images of sub 100 nm Au/SiO$_2$/Au nanoshells with different...............................................................157

Figure 7.5 Calculated absorption and scattering efficiencies of Au/SiO$_2$/Au nanoshells of different sizes..............................................................................................159

Figure 8.1 SEM images of Cu$_2$O nanoparticles of different sizes.................................167

Figure 8.2 XRD patterns of Cu$_2$O nanoparticles of different sizes...............................169

Figure 8.3 Optical image, experimental and calculated absorption spectra of Cu$_2$O nanoparticles of increasing sizes.................................................................171

Figure 8.4 Self-assembled arrays of Cu$_2$O nanoparticles of increasing diameter............172

Figure 8.5 SEM images of Cu$_2$O/Au-NS of different sizes........................................173

Figure 8.6 Experimental and calculated extinction spectra of Cu$_2$O/Au-NS................175

Figure 8.7 Experimental and calculated extinction spectra of Cu$_2$O/Au-NS for core radius $r_1 = 40 \pm 2$ nm with varying Au shell thicknesses.................................177

Figure 8.8 Calculated extinction, absorption, and scattering efficiency spectra of Cu$_2$O/Au compared with SiO$_2$/Au nanoshells.........................................................180

Figure 8.9 Dielectric function of Cu$_2$O obtained from ref. 236.....................................181

Figure 8.10 Calculated extinction absorption and scattering spectra of Cu$_2$O/Au nanoshells with a constant permittivity of 8 for the cores........................................181

Figure 8.11 Calculated extinction absorption and scattering spectra of various semiconductor cores/Au shell nanoshells..........................................................183

Scheme 9.1 Schematic representation of experimental set-up.......................................187

Figure 9.1 Electron microscopy and crystallographic studies of mesostar......................189

Figure 9.2 TEM micrographs of a star-shaped structure and mesostars, ED and SAED patterns showing evidence of $\alpha$-FeOOH, $\alpha$-Fe$_2$O$_3$ and Au.................................192
Figure 9.3 Schematic representation of mesostar formation mechanism. (B) SEM images supporting the mesostar formation mechanism

Figure 9.4 Additional SEM images supporting the mesostar formation mechanism

Figure 9.5 SEM image of long chain branched structures obtained both at 5 V and 10 V in addition to the mesostars

Figure 9.6 SEM image of one electrode utilized in the electrolytic cell is shown

Figure 9.7 SEM images of mesostructures obtained by varying the voltage while keeping electrolysis time constant at 24 hrs

Figure 9.8 Extinction spectra of α-Fe₂O₃ cores, nanorice particles and mesostars

Figure 9.9 SEM images of α-Fe₂O₃ cores and pyramidal mesostructures

Figure 9.10 XRD and extinction spectra of α-Fe₂O₃ cores and pyramidal mesostructures

Figure 10.1 Low- and high-magnification SEM micrographs and corresponding TEM images of ZnO particles

Figure 10.2 Irregular ZnO nanoparticles obtained at 0.001 M of Zn precursor and agglomerated ZnO particles obtained at 0.04M of Zn precursor

Figure 10.3 Crystallographic studies of the ZnO particles obtained at different concentrations of the Zn precursor

Figure 10.4 Distorted bowl-like shapes obtained at lower concentration of NH₄OH (1.12 wt %) and inhomogeneous matrix obtained at 5.6 wt % of NH₄OH

Figure 10.5 ZnO matrix obtained at different concentrations of Zn precursor and KOH (pH ~ 8.2)

Figure 10.6 Fluorescence image of ZnO hemispheres emitting white light upon UV excitation and PL spectra of the observed ZnO particles
List of Tables

Table 1.1 Overview of different non-invasive imaging modalities..............................20

Table 1.2 Overview of cancer cases and deaths in 2009.............................................27

Table 3.1 Multiexponential analysis of intensity decay of IR800 with nanoparticles showing molecular fraction, observed lifetime, amplitude weighted lifetime.......................69

Table 3.2 Quantum yield, radiative decay rate and nonradiative decay rate of IR800, HSA-IR800 and HSA-IR800 with nanoparticles...............................................................71

Table 4.1 Comparison of size and T₂ relaxivity of nanocomplexes with various USPIO/ SPIO agents at increasing magnetic field strengths.......................................................93

Table 8.1 Experimental parameters for synthesizing Cu₂O nanoparticles of various sizes............................................................................................................................169

Table 10.1 Synthesis parameters and morphology of different ZnO Structures...........211

Table 10.2 Concentration of Ammonia and Morphology of ZnO Structures..............214
Chapter 1: Introduction

Fluorescence sensing is a versatile technique widely applicable in many different disciplines from physiological imaging and clinical chemistry to environmental monitoring of gaseous molecules. In particular, fluorescence sensing and the subsequent use of clinically relevant fluorescent media have revolutionized the field of biomedical research for diagnosing disease markers \textit{in vitro} and \textit{in vivo}. In biological imaging the use of organic fluorophores, which emit in the near-infrared (NIR), has rapidly developed in the past few decades due to their low cost and biocompatibility when used in small doses. However, most organic fluorophores in the NIR have a relatively low quantum yield, they are susceptible to photobleaching and their excitation-emission profiles are often prone to changes in local chemical environment such as pH, H$_2$O, O$_2$, and ions.\textsuperscript{1,2} There are several approaches to improving the properties of NIR organic fluorophores: (1) complex organic synthesis techniques to synthesize physiologically safe fluorescent molecules or (2) use metallic nanostructures to enhance the quantum efficiency of existing fluorescent molecules which have already been approved by U.S. Food and Drug Administration (FDA) for clinical applications. In this thesis, we have engineered a metal nanoparticle substrate to enhance the fluorescence of weak NIR fluorophores and subsequently employed these fluorescent nanoparticles for diagnosing and treating cancer at the cellular (\textit{in vitro}) and molecular (\textit{in vivo}) level.

Since the pioneering work of Chance at al.\textsuperscript{3} and Drexhage\textsuperscript{4} it has been known that the emissive properties and lifetime of fluorophores are modified in the presence of metals. Metallic surfaces and nanostructures support surface plasmons, which are the
collective oscillations of the conduction electrons. Plasmon resonance when coupled to molecular fluorescence provides one of the most effective routes for enhancing the photostability and increasing the quantum yield of fluorophores. The metal/fluorophore interaction results from (i) the modification of the electromagnetic field and (ii) the photonic mode density in the vicinity of the fluorophores. For small metal/fluorophore distances (≤ 4 nm), the damping of dipole oscillation coupled to plasmon resonances leads to a strong quenching of fluorescence. This is also attributed to the increase in nonradiative decay rate for short fluorophore-metal distances because the nonradiative energy transfer rate depends on the inverse cube of the molecule-surface separation. However, for larger metal/fluorophore distances, an increase in the fluorescence intensity results primarily from a combination of (i) increased absorption by the molecule due to interaction with the enhanced near-field, (ii) increased radiative decay rate of the molecule without significant changes in the nonradiative decay rates, and (iii) increased coupling efficiency of the fluorescent emission to the far-field.

Beyond the quenching regime, the enhanced near-field persists for tens of nanometers which implies that an optimal distance d > 4 nm exists where the fluorescence enhancement is at a maximum. As the distance from the metal surface increases the near-field decays and consequently the fluorescence enhancement decreases. Due to the dipole-dipole interaction between the fluorophore and the metal, the radiative decay rate also reduces as the distance from the metal surface increases, contributing towards the diminishing fluorescence enhancement.
1.1 Plasmon Enhanced Fluorescence

Fluorescence is defined as the emission of light from a singlet-excited electronic state to the ground state of a material. In excited singlet states, the electron in the excited orbital is paired to the second electron in the ground state orbital. As a result, return to ground state is spin-allowed and occurs by emission of a photon (Fig. 1.1).

![Jablonski diagram](image)

**Figure 1.1.** Simple Jablonski diagram describing the emission processes of a material.  

In a homogenous solution, fluorophores emit light into free space and are observed in the far field. The observed emission of the fluorophore in the absence of any quenching interactions is described in terms of its quantum yield ($Q_0$) and lifetime ($\tau_0$). The quantum yield, given by

$$Q_0 = \frac{\Gamma}{\Gamma + k_{nr}}$$

is the fraction of the excited fluorophores which relaxes by radiative decay ($\Gamma$) relative to the total relaxation rate ($\Gamma + k_{nr}$). The observed lifetime is simply the inverse of the total decay rate of the excited state:

$$\tau_0 = \frac{1}{\Gamma + k_{nr}}.$$
When a sample of molecules with molecular absorptivity $\varepsilon$ is excited with light of intensity $I_{exc}$, the total amount of energy absorbed by the molecules is simply $I_{exc} \varepsilon$.\textsuperscript{9} The quantum yield determines what portion of this energy is re-emitted as fluorescence; therefore, the observed emission intensity is

$$I_o = I_{exc} \varepsilon \cdot Q_o.$$ \hspace{1cm} (3)

In the presence of a metallic surface or nanoparticles, the enhanced local field will increase the amount of light absorbed by the molecule. In the case of spherical particles, the surface average of the near field intensity enhancement $\langle |E|^2 \rangle$ can be calculated directly using Mie theory, leading to a fluorescence emission which is $(\langle |E|^2 \rangle \cdot I_{exc}) \varepsilon \cdot Q_o$.

In addition, electromagnetic coupling occurs between the fluorophore and the nanoparticle plasmon, causing an increase in the radiative decay rate of the molecule at the emission wavelength by a factor $\gamma_r$. As a result the effective radiative decay rate is equivalent to $\gamma_r \Gamma$.\textsuperscript{3,4,10} For spherical symmetrical systems, this can also be evaluated using Mie theory following the method in Gibson et al.\textsuperscript{11} The modified quantum yield $(Q_M)$ and lifetime ($\tau_M$) in the presence of the metallic surface are then given by:

$$Q_M = \frac{\gamma_r \Gamma}{\gamma_r \Gamma + k_{nr}}$$ \hspace{1cm} (4)

$$\tau_M = \frac{1}{\gamma_r \Gamma + k_{nr}}.$$ \hspace{1cm} (5)

The observed emission of the fluorophore-metal system is therefore given by,

$$I_M = (\langle |E|^2 \rangle \cdot I_{exc}) \varepsilon \cdot Q_M.$$ \hspace{1cm} (6)

The fluorescence enhancement can be evaluated by taking the ratio of the emission from the metal fluorophore system ($I_M$) to the emission intensity of the fluorophore alone ($I_o$):

$$\frac{I_M}{I_o} = \frac{\langle |E|^2 \rangle}{Q_o} = \frac{\gamma_r \Gamma + k_{nr}}{\gamma_r \Gamma + k_{nr}}.$$ \hspace{1cm} (7)
The Jablonski diagram for metal enhanced fluorescence is shown in Figure 1.2.

![Jablonski Diagram](image)

**Figure 1.2.** Jablonski diagram showing the fluorescence of a molecule in free space and in proximity to a metallic surface

### 1.2 Nanoshell Background

In this thesis, the metallic nanoparticle of choice for enhancing the fluorescence quantum yield of biologically relevant fluorophores is gold nanoshells. Nanoshells are symmetric spherical nanostructures consisting of dielectric silica (SiO₂) core uniformly coated with a thin gold (Au) shell. The plasmon resonance frequencies of nanoshells are dependent on the relative ratio of the core and shell dimensions, as well as the dielectric constant of the core, shell and medium. The size dependent properties of nanoshells have been explored for various technologies. Particularly, tunability of the nanoshell plasmon resonance in the NIR has enabled numerous diagnostic and therapeutic applications including NIR bioimaging and photothermal cancer therapy. Nanoshells are also interesting for biomedicine due to the inert, biocompatible Au layer which enables simple conjugation chemistry relevant for binding molecules as well as biopolymers such as antibodies, oligonucleotides, and peptides. The non-toxicity, stability *in vivo*, and
straightforward surface functionalization allows the use of nanoshells as a successful targeting, imaging, and therapeutic agent at the cellular and molecular level.

1.2.1. Fabrication and Characterization of Nanoshells

The SiO₂/Au nanoshell is fabricated following previously reported protocols.¹²,¹⁸ For the sake of this thesis, only the synthesis of nanoshells which are resonant in the NIR (~ 770 – 810 nm) are discussed. Briefly, monodisperse silica nanospheres of 120 ± 4 nm diameter are synthesized using the Stöber method of particle growth.¹⁹ The Stöber method involves the base catalyzed hydrolysis and condensation of tetraethylorthosilicate (TEOS, Sigma). Typically, ~ 4.5 mL of TEOS is mixed with ~11.5 mL of NH₄OH in the presence of 180 mL of 200 proof ethanol (Fisher) to yield silica nanospheres of the desired size regime. The silica nanospheres are then washed several times to remove excess TEOS and NH₄OH and finally redispersed in 10 mL ethanol. Silica nanospheres are then functionalized with aminated silanes using 3-aminopropyltriethoxysilane (APTES, Sigma) overnight under vigorous stirring. The silane groups on the APTES molecule binds to the silica surface while the amine moiety is now available to attach to Au nanoparticles. Small gold nanoparticles ~2 nm in diameter are synthesized using tetrakis(hydroxymethyl)phosphonium chloride (THPC) as the reducing agent from the method reported by Duff, et al.²⁰ The THPC reduced Au nanoparticles (THPC-Au) are attached to the surface of the silica nanospheres and used for metal shell growth. The amount of silica particles to THPC-Au is calculated from the total surface area of the silica particle solution, the concentration and physical cross-section of the THPC-Au, and assuming 25 – 30 % coverage of THPC-Au on the silica surface. The silica-Au precursor
particles are typically prepared by mixing 40 mL of THPC-Au aqueous solution with 250-350 µL amine terminated silica nanospheres and 1 mL, 1 M NaCl. The salt increases the isoelectric point of the solution mixture by increasing the ionic strength, thereby decreasing Coulombic repulsion and increasing surface coverage of THPC-Au nanoparticles on the silica surface.

**Figure 1.3.** Schematic diagram and corresponding TEM images showing nanoshell fabrication: (i) 2 nm Au particles attached to amine terminated silica nanospheres, (ii) gold reduction using electroless plating technique, and (iii) more gold reduction resulting in a complete Au shell

Next, a plating solution is prepared for Au shell growth. Typically, 3 mL of 1% HAuCl₄ solution (which was aged for 14 days) is mixed with 50 mg K₂CO₃ in 200 mL of deionized H₂O and aged for 24–72 hours. By varying the ratio of the silica-Au precursor particles’ volume to the plating solution volume nanoshells with different Au shell thicknesses can be fabricated. The smooth Au shell on the silica-Au precursor particles is obtained by either mixing formaldehyde as a reducing agent¹² or by bubbling with CO.¹⁸

A schematic diagram and corresponding transmission electron microscopy (TEM) images of nanoshell fabrication (Fig. 1.3) and scanning electron microscopy (SEM) image of several nanoshells are shown (Fig. 1.4).
Figure 1.4. Low resolution SEM image of nanoshells $[r_1, r_2] = [60, 74]$ nm in size with high resolution SEM image provided as inset.

1.2.2. Optical Properties of Nanoshells and Plasmon Hybridization

Figure 1.5. Experimental extinction spectra of Au shell growth on 60 nm radius silica cores by varying the ratio of the precursor particles to the plating solution.
Figure 1.6. (A) Energy level diagram depicting plasmon hybridization in nanoshells resulting from interacting sphere and cavity plasmons. The two hybridized plasmon modes are an anti-symmetric plasmon resonance ($\omega^+$) and a symmetric plasmon resonance ($\omega^-$). (B) Energy diagrams illustrating the dependence of nanoshell plasmon energies on the strength of the interaction between the sphere and cavity plasmons, determined by the thickness of the Au shell.

The nanoshell plasmon resonance shifts to longer wavelengths as the thickness of the Au shell decreases (Fig. 1.5). Experimentally this is achieved by simply decreasing the ratio of Au salt plating solution to the SiO$_2$-Au precursor particles. Theoretically, this highly
tunable optical property of nanoshells can be explained in terms of the plasmon hybridization model of nanoshells. Plasmon hybridization theory is an electromagnetic analog of how atomic orbitals interact to form molecular orbitals in electronic structure theory.\(^{21}\) Plasmon hybridization theory principally deconstructs a complex nanostructure into more elementary shapes, and analyzes the interaction of the primitive plasmons supported by these elementary geometries with each other, to form the hybridized plasmons of the complex nanostructure.

The geometry dependent plasmon resonance of nanoshells results from the interaction of two elementary geometries, a metallic sphere and a spherical cavity inside a bulk metal (Fig. 1.6A).\(^{22,23}\) From classical Mie theory the plasmon resonance of a metal sphere and a cavity are given by,

\[
\omega_{S,l} = \omega_B \left( \frac{l}{2l+1} \right)^{1/2},
\]

and

\[
\omega_{C,l} = \omega_B \left( \frac{l+1}{2l+1} \right)^{1/2},
\]

where \(\omega_B\) corresponds to the bulk plasma frequency of metal and \(l\) is the angular momentum. In a nanoshell, the deformation fields associated with the sphere and cavity plasmons introduce surface charges at both the inner and outer boundaries of the shell.

These surface charges couple the sphere and cavity modes, resulting in hybridized plasmons. The hybridization of the cavity and the sphere plasmons depends on their interaction, which is determined by the thickness of the Au shell. The nanoshell plasmon hybridization gives rise to two hybridized plasmon modes \(|\omega_+\rangle\) and \(|\omega_-\rangle\) for each \(l > 0\). The frequencies of these modes are given by,
\[ \omega_{ps}^2 = \frac{\omega_B^2}{2} \left\{ 1 \pm \frac{1}{2l+1} \left[ 1 + 4l(l+1) \left( \frac{r_1}{r_2} \right)^{2l+1} \right]^{1/2} \right\} \]  

(17)

where, \( |\omega_+\rangle \) mode corresponds to antisymmetric or antibonding coupling between the sphere and cavity modes and the \( |\omega_-\rangle \) mode corresponds to symmetric or bonding coupling between the two modes. The lower energy \( \omega_- \) plasmon interacts strongly with the incident optical field, while the \( \omega_+ \) mode interacts weakly since the antisymmetric plasmon possesses a very weak net dipole moment. The interaction of the two plasmons is governed by the ratio \( r_1/r_2 \) (equation 17), from which the nanoshell plasmon derives its inherent tunability. The plasmon hybridization model of nanoshells also provides a simple explanation as to why the energy of the optically active plasmon resonance shifts to lower energies with decreasing shell thickness. A thinner shell leads to a stronger coupling between the sphere and cavity plasmons, increasing the splitting between the bonding and antibonding hybridized plasmons (Fig. 1.6B).

### 1.2.3. Near Field and Far Field Properties of Nanoshells

The nanoshell near field and far field properties can be best understood in terms of Mie scattering theory.\(^{24-26}\) Mie theory provides analytical solutions to Maxwell's equations for concentric spherical geometries by expressing electromagnetic waves as expansions of the vector spherical harmonic basis functions \( L, M, \) and \( N \). The light scattering by nanoshells can be understood as a plane wave scattering off a spherical nanoshell. In the vector basis formalism the electric and magnetic fields associated with the incident plane wave, and the different layers of a nanoshell are written in terms of the spherical
harmonic functions. By satisfying Maxwell’s equations at the interfaces between the core and shell and between the shell and embedding medium the scattering coefficients for the electric and magnetic fields can be determined.

Figure 1.7. Schematic diagram showing nanoshell geometry with various parameters.

The incident plane wave is assumed to be propagating along the z-axis and is linearly polarized in the x direction. The electromagnetic field can hence be expressed as linear combinations of the spherical vector basis function given by,

\[
E_i = \sum_{n=1}^{\infty} \left\{ a_{n+1/2} M^{(1)/}_{n,1} + b_{n+1/2} N^{(1)/}_{n,1} \right\}
\]

\[
H_i = -i \sqrt{\mu_0 \varepsilon_0} \sum_{n=1}^{\infty} \left\{ a_{n+1/2} M^{(1)/}_{n,1} + b_{n+1/2} N^{(1)/}_{n,1} \right\}
\]

(18)

where the scattering coefficients \(a\) and \(b\) are given by,

\[
a_{n+1} = b_{n+1} = i^{n+1} \frac{2n+1}{2n(n+1)}
\]

\[
a_{n+1} = b_{n+1} = n(n+1)a_{n+1}
\]

(19)

For a nanoshell, the series expansion can be expressed in the 3 regions (Fig. 1.7): the dielectric core forms region 1, with radius \(R_1\) and dielectric constant \(\varepsilon_1\), the Au shell forms region 2 with radius \(R_2\) and dielectric constant \(\varepsilon_2\) and the embedding medium forms region 3 with dielectric constant \(\varepsilon_3\). The electric fields in the three regions are given by,
\[ E_1 = \sum_{n=1}^{\infty} \left\{ a_{n,1}^{(1)/j} M_{n,1}^{(1)/j} + b_{n,1}^{(1)/j} N_{n,1}^{(1)/j} \right\} \]

\[ E_2 = \sum_{n=1}^{\infty} \left\{ a_{n,1}^{(2)/j} M_{n,1}^{(2)/j} + b_{n,1}^{(2)/j} N_{n,1}^{(2)/j} + a_{n,1}^{(2)h} M_{n,1}^{(2)h} + b_{n,1}^{(2)h} N_{n,1}^{(2)h} \right\} \]

\[ E_3 = \sum_{n=1}^{\infty} \left\{ a_{n,1}^{(3)h} M_{n,1}^{(3)h} + b_{n,1}^{(3)h} N_{n,1}^{(3)h} \right\} \]

and the magnetic fields are given by,

\[ H_1 = -i \sqrt{\frac{\epsilon_1}{\mu_1}} \sum_{n=1}^{\infty} \left\{ a_{n,1}^{(1)/j} M_{n,1}^{(1)/j} + b_{n,1}^{(1)/j} N_{n,1}^{(1)/j} \right\} \]

\[ H_2 = -i \sqrt{\frac{\epsilon_2}{\mu_2}} \sum_{n=1}^{\infty} \left\{ a_{n,1}^{(2)/j} M_{n,1}^{(2)/j} + b_{n,1}^{(2)/j} N_{n,1}^{(2)/j} + a_{n,1}^{(2)h} M_{n,1}^{(2)h} + b_{n,1}^{(2)h} N_{n,1}^{(2)h} \right\} \]

\[ H_3 = -i \sqrt{\frac{\epsilon_3}{\mu_3}} \sum_{n=1}^{\infty} \left\{ a_{n,1}^{(3)h} M_{n,1}^{(3)h} + b_{n,1}^{(3)h} N_{n,1}^{(3)h} \right\} \]

where the subscripts represent the individual regions (Fig. 1.7). It is noteworthy that region1 only has radial contributions from the spherical Bessel functions (j), region3 contain only the Hankel functions of the first kind (h) and region2 has contributions from both types of functions. Assuming that the materials are nonmagnetic so that the relative permeability \( \mu = 1 \) simplifies the above equations. Then solving these equations by continuity equation at the boundaries, coefficients \( a \) and \( b \) can be determined.

The local near field at the nanoshell surface is calculated as \( E_3 \) from eq. 20. The nanoshell near field can be evaluated at appropriate distances from the surface by calculating the average of the field at 2500 different points on the nanoshell surface (50 different \( \theta \) and 50 different \( \phi \) angles). For example, for a nanoshell with dimensions \( [r_1, r_2] = [60, 76] \) nm, the surface average near field calculated at 2, 7, 13, 21, 30 and 42 nm from the nanoshell surface is shown in Figure 1.8A. The near field decays off with increasing distance from the nanoshell surface. A pictorial depiction of nanoshell near field (Fig. 1.8B), where the \( k \) vector is perpendicular to the E-field, clearly demonstrates highest field are generated near the nanoshell surface.
Figure 1.8. (A) The near field of nanoshells with dimensions \([r_1, r_2] = [60, 76]\) nm plotted as a function of distance from the surface and (B) Pictorial depiction of nanoshell near field.

The far field properties of nanoshells including its absorption, scattering and extinction cross-sections can be straightforwardly determined from \(a\) and \(b\) coefficients and the wave vector \(k\) evaluated in region 3. The cross-sections are given by,

\[
\begin{align*}
\sigma_{sca} &= \frac{2\pi}{k^2} \sum_{n=1}^{\infty} (2n+1)(|a_{ln}^{(3)h}|^2 + |b_{ln}^{(3)h}|^2) \\
\sigma_{ext} &= -\frac{2\pi}{k^2} \text{Re} \sum_{n=1}^{\infty} (2n+1)(a_{ln}^{(3)h} + b_{ln}^{(3)h}) \\
\sigma_{abs} &= \sigma_{ext} - \sigma_{sca}
\end{align*}
\]

(22)

1.2.4. Photothermal Properties of Nanoshells

Nanoshells have been extensively used in therapeutic applications including photothermal cancer therapy\(^{27}\) and light assisted gene therapy.\(^{28}\) In this thesis, nanoshells have been utilized for cancer therapy (chapter 4, 5). It is therefore important to understand the physical process attributing to the photothermal properties of nanoshells.

Upon illumination with resonant light, the plasmon resonance in metal nanostructures either decays radiatively resulting in light scattering, a phenomenon
highly useful in biological sensing and bioimaging, or the plasmon decays nonradiatively resulting in light absorption.\textsuperscript{26} As absorbers, metallic nanostructures efficiently convert light to heat. The absorption and scattering characteristics of a nanoparticle is a function of its size: small nanoparticles absorb strongly, while with increasing size the absorption to scattering ratio decreases, and ultimately larger particles are better scatters.

The fundamental principle underlying the photothermal response of nanoshells has been studied with ultrafast laser spectroscopic studies.\textsuperscript{24} The photoexcitation of the electrons by an ultrashort laser pulse leads to a perturbation of the electron distribution in the metal, which is given by its Fermi distribution.\textsuperscript{29} This is followed by relaxation with electron-electron scattering which results in rapid increase in surface temperature (subpicosecond timescale). This initial rapid heating is followed by cooling back to equilibrium by energy exchange between the electrons and the lattice via electron-phonon coupling (few picosecond timescale). At very fast rates of energy dissipation relative to lattice cooling, the photothermal heating can result in the melting or reshaping of the nanostructure changing its optical absorption characteristics irreversibly. However, at slower rates (on the order of several hundred picoseconds), the lattice cools via phonon-phonon coupling resulting in heating of the medium surrounding the nanostructure,\textsuperscript{30-32} which can be used in cancer therapy to induce photothermal cell death. If nanostructures are immersed in a medium, following illumination, a non-equilibrium condition will exist between the hot nanoparticle and the cooler surrounding medium resulting in large temperature increases in the surrounding medium. The characteristics of the surrounding medium strongly influence the cooling dynamics of the hot electrons in the nanostructures. The relaxation time (electron-phonon and phonon-phonon) decreases
when the thermal contact between the nanostructures and the surrounding medium reduces or when the thermal conductivity of the medium is decreased, implying that the cooling dynamics would be fastest in aqueous media. Nanoshells, due to their large absorption cross-section, are efficient light-to-heat convertors. Even at low illumination intensities, specifically of interest in biomedical applications, photothermal response of nanoshells results in heating of the surrounding tissue inducing hyperthermia and subsequent tumor cell death.

### 1.3 Frequency Domain Fluorescence Lifetime

A steady-state measurement of fluorescence only provides an averaged measurement of a fluorophores absorption and resulting emission of light. To effectively characterize the fluorophore-nanoshell complexes and determine the changes in the emissive properties of the fluorophore due to the presence of the metal, fluorescence lifetime measurements are employed. The behavior of a material’s fluorescence as a function of time, can systematically explain the fluorophore’s environment, intermolecular distances, and many other molecular parameters. When a fluorophore is excited with an infinitely sharp pulse of light, it results in an initial population \( n_0 \) of fluorophores in the excited state. The excited state population then decays with a rate \( \Gamma + k_m \) according to,

\[
\frac{dn(t)}{dt} = -(\Gamma + k_m) n(t)
\]  

where, \( n(t) \) is the number of excited molecules in time \( t \) following excitation. So the exponential decay of the excited state population can be understood by,
\[ n(t) = n_0 e^{-\frac{t}{\tau}} \]  

where \( \tau = (\Gamma + k_w)^{-1} \). However, when fluorescence emission is measured, the no. of excited molecules is not observed, rather a fluorescence intensity is measured given by,

\[ I(t) = I_0 e^{-\frac{t}{\tau}} . \]  

This time dependent intensity is the expression for a single exponential (SE) decay. Decay profiles for most exogenous fluorophores employed for biomedical applications can be explained with SE kinetics. A multi exponential (ME) model, however, describes the fractional contribution of decay time for each component present in a heterogeneous sample. The ME model for analyzing the intensity decay is given by

\[ I(t) = \sum \alpha_i e^{-\frac{t}{\tau_i}} \]  

where \( \tau_i \) are observed lifetimes with amplitude or molecular fraction \( \alpha_i \), and \( \sum \alpha_i = 1 \).

The amplitude-weighted lifetime is then

\[ \langle \tau \rangle = \sum \alpha_i \tau_i . \]  

Fluorescence lifetime can be measured in the time domain or in the frequency domain as shown in Figure 1.9. This thesis will focus on the frequency domain technique. In the frequency domain technique, lifetime measurements are obtained by exciting the fluorescent sample with intensity-modulated light at a high frequency comparable to \( \tau^{-1} \) of the sample.\(^9\) Subsequent to excitation, the fluorescence emission of the sample follows the same modulation frequency as the excitation, but it is delayed in time relative to the
excitation. The time delay is measured as a phase shift ($\Phi$) from which the lifetime ($\tau_\Phi$) is determined given by

$$\tau_\Phi = \frac{1}{\omega} \tan(\Phi),$$

where $\omega$ is angular frequency.

**Figure 1.9.** Schematic showing the two different fluorescence lifetime measurement techniques

The finite time response of the sample also results in demodulation of the emission by a factor defined as modulation ($m$). As the frequency increases the lifetime of the excited state results in a decrease in the amplitude of the modulated emission. The modulation decreases because a fraction of the fluorophores excited at the peak of the excitation continue to emit even when the excitation is at a minimum. The extent to which modulation decreases depends on the frequency and also the intrinsic lifetime of the fluorophore. The lifetime can also be calculated from the modulation given by,
The difference between time domain and frequency domain is simply that in the time domain technique, the sample is excited with a pulse of light (instead of modulated light) which is much shorter than the decay time $\tau$ of the sample (Fig. 1.9). The time dependent intensity is measured following the excitation pulse and the decay time $\tau$ is calculated from the slope of the plot of $\log I(t)$ versus $t$, so $\text{slope} = -1/\tau$.\textsuperscript{9,33}

\[ \tau_n = \frac{1}{\omega} \left[ \left( \frac{1}{m^2} \right) - 1 \right]^{\frac{1}{2}}. \]  

(14)

1.4 Non-Invasive Biological Imaging Techniques

The plasmon enhanced nanoshell-fluorophore complexes are utilized in an exciting biomedical application: diagnosing cancer using dual modal non-invasive imaging modalities including fluorescence optical imaging (FOI) and magnetic resonance imaging (MRI). The development of non-invasive imaging techniques that diagnose cancer at its earliest stages is vital to cancer therapy and patient survival rate. Non-invasive imaging techniques are benign and safe as they do not involve physical incision into the body. Powerful non-invasive imaging techniques are necessary to precisely identify tumor location, tumor size, whether tumor has spread to lymph nodes, tumor treatment stage, and to assess therapeutic strategies in real time.\textsuperscript{34} An overview of different non-invasive imaging modalities commonly utilized in clinic are shown in Table 1.1.\textsuperscript{34} The conventional anatomical imaging methods currently a mainstay in the clinic include computed x-ray tomography (CT), MRI, and ultrasound (US). These imaging technologies allow for non-invasive visualization of the body based on different forms of energy interacting with tissues including physical (absorption, scattering, relaxation.
rates), metabolic, and physiologic characteristics of tissue. While these traditional imaging methods have high spatial resolution, they are inadequate in acquiring complete physiological information due to lack of sensitivity, specificity, and inefficiency in identifying specific molecular events responsible for diseases.

**Table 1.1** Overview of different non-invasive imaging modalities adapted from Ref. 34

<table>
<thead>
<tr>
<th>Technique</th>
<th>Resolution</th>
<th>Depth</th>
<th>Time</th>
<th>Imaging agents</th>
<th>Cost</th>
<th>Primary use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetic resonance imaging (MRI)</td>
<td>10-100 μm</td>
<td>No limit</td>
<td>Min/hours</td>
<td>Gadolinium, dysprosium, iron oxide</td>
<td>$$$</td>
<td>Best all-round imaging system with high contrast; used in phenotyping,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>particles</td>
<td></td>
<td>physiological imaging and cell tracking</td>
</tr>
<tr>
<td>X-ray computed tomography (CT) imaging</td>
<td>50 μm</td>
<td>No limit</td>
<td>Min</td>
<td>Iodine</td>
<td>$$</td>
<td>Lung and bone-tumour imaging</td>
</tr>
<tr>
<td>Ultrasound imaging</td>
<td>50 μm</td>
<td>mm</td>
<td>Min</td>
<td>Microbubbles</td>
<td>$$</td>
<td>Vascular and interventional imaging</td>
</tr>
<tr>
<td>Positron emission tomography (PET) imaging</td>
<td>1-2 mm</td>
<td>No limit</td>
<td>Min</td>
<td>$^{18}F, ^{11}C, ^{15}O</td>
<td>$$</td>
<td>Imaging metabolism of molecules, such as glucose, thymidine and so on</td>
</tr>
<tr>
<td>Single photon emission tomography (SPECT) imaging</td>
<td>1-2 mm</td>
<td>No limit</td>
<td>Min</td>
<td>$^{99m}$Tc, $^{111}$In</td>
<td>$$</td>
<td>Imaging of probes such as antibodies, peptides and so on</td>
</tr>
<tr>
<td>Fluorescence reflectance imaging (FRI)</td>
<td>1-2 mm</td>
<td>&lt; 1 cm</td>
<td>Sec/min</td>
<td>Fluorescent proteins, NIR fluorochromes</td>
<td>$</td>
<td>Rapid screening of molecular events in surface-based tumours</td>
</tr>
<tr>
<td>Fluorescence-mediated tomography (FMT)</td>
<td>1-2 mm</td>
<td>&lt; 10 cm</td>
<td>Sec/min</td>
<td>NIR fluorochromes</td>
<td>$$</td>
<td>Quantitative imaging of targeted or 'smart' fluorochrome reporters in deep</td>
</tr>
<tr>
<td>Biotumour imaging (BLI)</td>
<td>Several mm</td>
<td>cm</td>
<td>Min</td>
<td>Luciferin</td>
<td>$$</td>
<td>Gene expression, cell tracking</td>
</tr>
<tr>
<td>Intravital microscopy (confocal, multiphoton)</td>
<td>1 μm</td>
<td>&lt; 400 μm</td>
<td>Sec/min</td>
<td>Fluorescent proteins, $$$ photoproteins, fluorochromes</td>
<td>All of the above at higher resolutions but at limited depths and coverage</td>
<td></td>
</tr>
</tbody>
</table>

Cost of system: $ < 100 K; $$ 100-300 K; $$$ > 300 K. C, carbon; F, fluorine; In, indium; NIR, near infrared; O, oxygen; $^{99m}$Tc, technetium metastable.

Molecular imaging, a rapidly emerging technique, effectively integrates biology, chemistry, medical physics, and pharmacology with novel technologies to overcome some of the challenges involved in the conventional imaging methods. It is primarily defined as the "visual representation, characterization, and quantification of biological
processes at the cellular and sub-cellular levels within intact living organisms". Molecular imaging techniques can be classified under two categories, (i) nuclear imaging which include positron emission tomography (PET) and single photon emission tomography (SPECT), and (ii) optical imaging which includes fluorescence optical imaging (FOI), and fluorescence mediated tomography. MRI and ultrasound can be used for molecular imaging as well using appropriate contrast agents such as gadolinium and iron oxide based agents for MRI and microbubbles for ultrasound.

Molecular imaging provides routes for obtaining rapid, reproducible, high resolution images with high sensitivity which are lacking in the conventional imaging methods. In combination with innovative probes, targeting ligands and novel tools, molecular imaging can thus provide earlier detection of disease and evaluation of treatment long before phenotypic changes occur. Additionally, developing imaging probes which incorporate the advantages of molecular imaging techniques with conventional imaging methodologies into a single platform can potentially modify the way in which cancer is clinically diagnosed and treated. This dissertation will focus on two non-invasive molecular imaging modalities: FOI and MRI, by employing receptor targeted fluorescence and MR contrast agents.

1.4.1 Fluorescence Optical Imaging (FOI)

Among the many known optical imaging techniques, FOI is the most inexpensive and rapid way of imaging at the cellular and molecular level. FOI in tissues is employed mainly via two strategies: (i) detection of endogenous fluorescence (fluorescent proteins, luciferase bioluminescence system), and (iii) detection of exogenous fluorescence
(organic dyes, lanthanide compounds, quantum dots, inorganic nanoparticles) that is either untargeted, or targeted via antibodies, peptides, or other biopolymers. Exogenous contrast agents are more popular today among scientists and clinicians for molecular imaging. FOI specifically *in vitro* and *in vivo* entails injecting a fluorescent agent into cells or the body, which emits photons upon activation with excitation light. The emitted photons are then detected externally either by image intensifying cameras or other sensitive detectors.

![Figure 1.10. The NIR water window showing minimal light absorption by hemoglobin and water, adapted from ref 38.](image)

The fundamental obstacles to FOI of tissue are high absorption, scattering and autofluorescence by hemoglobin in the visible region of the spectrum. The use of NIR light is particularly advantageous due to the large penetration depth of NIR light in soft tissue.\(^1\) NIR light has been reported to penetrate ~10 cm through breast tissue, and 4 cm of brain tissue using microwatt laser sources.\(^{38}\) Moreover, excitation and emission in this physiologically relevant “water window” (680–900 nm) is crucial to obtaining functional images since hemoglobin and water have their lowest absorption coefficient here (Fig.
Autofluorescence of tissues and Rayleigh scattering are also minimal in the NIR water window.

An effective fluorescent agent should have the following attributes: (i) biocompatibility and non-toxicity, (ii) stability in vitro and in vivo, (iii) high quantum yield, (iv) resistance to rapid metabolic disintegration, (iv) resistance to photobleaching, and (v) availability of bioconjugation and targeting sites. While quantum dots combine a number of the desired properties listed above, the difficulty in sample preparation and toxicity poses a major challenge for them. The excitation of quantum dots are also limited to the UV and visible region of the spectrum where tissue absorbs maximally and UV light causes irreversible damage to tissue. Moreover, the penetration of excitation light is limited at UV and visible wavelengths, making quantum dots unsuitable for imaging deeper tissue. This thesis thus focuses on the use of NIR organic fluorophores for FOI and a simple approach to achieving enhanced stability. By enhancing their quantum yield using plasmonic nanostructures, lower concentrations of the fluorophore can be used, which is clinically safe and non-toxic.

1.4.2 Magnetic Resonance Imaging (MRI)

MRI is based on the fundamental principles of nuclear magnetic resonance, commonly known as NMR. When an external magnetic field $B_0$ is applied to a system, each proton rotates around its axis with an angular frequency $\omega_0$, which is proportional to the applied magnetic field strength. This is given by the Larmor equation: $\omega_0 = \gamma B_0$ where $\gamma$ is the gyromagnetic ratio. The net magnetization vector, $M$, is described by its 3 components: $M_z$ is the component parallel to $B_0$ defined as longitudinal magnetization. At
equilibrium, $M_z$ is maximal and termed as equilibrium magnetization, $M_0$, where $M_0 = M_z$. $M_{xy}$ is the component perpendicular to $B_0$ known as transverse magnetization. At equilibrium, $M_{xy} = 0$ since the vector components of the spins are randomly oriented about 360° in the x-y plane and cancel each other.\(^{39}\)

In MRI, radiofrequency (rf) energy is applied synchronized (or in "resonance") to the $\omega_0$ of the protons causing displacement of the tissue magnetic moment from equilibrium conditions. Return to equilibrium results in emission of MR signals proportional to the number of excited protons in the sample as shown in Figure 1.11. The MR signal strength also depends on the characteristics of the tissues. $T_1$ relaxation, also known as spin-lattice relaxation, is the loss of energy from the excited state (spin) to its surrounding tissue (lattice), mostly exhibited by gadolinium based contrast agents. Since this thesis discusses iron oxide based agents, which are $T_2$ contrast agents, only $T_2$ relaxation will be elaborated in details.

**Figure 1.11.** Schematic diagram explaining the relaxation processes in MRI.

$T_2$ or transverse relaxation is an exponential decay which represents the spin-spin interactions of processing nuclei resulting in the loss of phase coherence. This arises from the intrinsic magnetic properties of the sample. After the rf sequence is applied, the 90° rf pulse produces phase coherence of the individual protons and generates the maximum
\(M_{xy}\). As \(M_{xy}\) rotates with \(\omega_0\), the receiver antenna coil is induced by magnetic induction to produce a damped sinusoidal electronic signal, known as free induction decay (FID). FID arises due to loss of phase coherence of the individual spins caused by magnetic field variations. The \(T_2\) decay constant is generally expressed as:

\[
M_{xy}(t) = M_0 e^{-t/T_2}.
\] (15)

There are three terms which essentially explain \(T_2\) relaxation process: echo, spin echo sequence, and time of echo (TE). An echo is the emission of energy in form of an electromagnetic resonance signal of nuclei after its excitation. Spin echo sequence describes a sequence of events: the excitation of the magnetized protons in a sample with an RF pulse, FID production, followed by a 2\(^{nd}\) RF pulse to produce an echo. The spin echo sequence starts with a 90\(^\circ\) pulse and produces a FID that decays according to \(T_2\) relaxation. A 180\(^\circ\) pulse inverts the spins that reestablishes the phase coherence and produces an echo at a time equivalent to TE (Fig. 1.12). In other words the time difference between the 90\(^\circ\) pulse and the maximum in the echo is TE.\(^{39}\)

![Figure 1.12. Schematic explaining TE in a spin echo sequence adapted from ref 39.](image)

25
The T$_2$ values are distinct for each tissue in the body which results in the contrast between tissues of various types. Due to the low sensitivity of MRI, tumors are difficult to distinguish from normal tissues in the body using an MR image. Therefore patients are often injected with contrast agents, such as iron oxide particles that selectively highlight the tumors. Effective MRI contrast agents significantly change the T$_1$ and/or T$_2$ values of the protons in the vicinity of the agent and generate image contrast (bright/dark) for diagnosis. In the case of T$_2$ contrast, the presence of iron oxide based agents leads to inhomogeneities in the external magnetic field resulting in a rapid dephasing of spins and T$_2$ shortening which generates strong contrast. Depending on the concentration of the contrast agent, either fast relaxation occurs producing strong contrast or slow relaxation occurs resulting in weak contrast (Fig. 1.13).

![Schematic representation of iron oxide interaction with H$_2$O molecule](image)

**Figure 1.13.** Schematic representation of iron oxide interaction with H$_2$O molecule

### 1.6 Cancer Biology and Therapy

Cancer is one of the most lethal diseases known to human kind and the second most common cause of death in the U.S., exceeded only by heart disease. In 2009, ~ 1.5 million new cases of cancer were reported in the U.S. with an estimated death of 38%. Since this thesis will discuss the diagnosis and therapy of breast and ovarian cancer, an understanding of the cause and implications of cancer is important. Among the many
known types of cancer in women, breast cancer is the most frequently diagnosed with a mortality rate of 21% in 2009.\footnote{\textsuperscript{40}} While fewer cases of ovarian cancer are reported compared to breast cancer, most are at advanced stages of the disease and has a 68% mortality rate. An estimate of cancer cases and death in 2009 are shown (Table 1.2).

While cancer not only represents significant threat to human health, it also incurs tremendous costs in diagnosis, treatment and loss to economy. The National institutes of health (NIH) estimates overall costs of cancer in 2008 at $228.1 billion: $93.2 billion for direct medical costs, $18.8 billion for indirect cost of lost productivity due to illness and $116.1 billion for indirect mortality costs attributed to lost productivity due to premature death. Due to this enormous human and economic cost, improved diagnostic and therapeutic strategies are imperative for early detection and treatment of cancer.

**Table 1.2.** Overview of cancer cases and deaths in 2009 adapted from Ref. 40

<table>
<thead>
<tr>
<th>Leading Sites of New Cancer Cases and Deaths – 2009 Estimates</th>
<th>Estimated New Cases*</th>
<th>Estimated Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td><strong>Female</strong></td>
<td><strong>Male</strong></td>
</tr>
<tr>
<td><strong>Prostate</strong></td>
<td>192,280 (25%)</td>
<td>192,370 (27%)</td>
</tr>
<tr>
<td><strong>Lung &amp; bronchus</strong></td>
<td>118,090 (15%)</td>
<td>Lung &amp; bronchus</td>
</tr>
<tr>
<td><strong>Colon &amp; rectum</strong></td>
<td>82,340 (10%)</td>
<td>Colon &amp; rectum</td>
</tr>
<tr>
<td><strong>Urinary bladder</strong></td>
<td>52,810 (7%)</td>
<td>Urethra corpus</td>
</tr>
<tr>
<td><strong>Male breast</strong></td>
<td>32,490 (4%)</td>
<td><strong>Female breast</strong></td>
</tr>
<tr>
<td><strong>Non-Hodgkin lymphoma</strong></td>
<td>33,600 (4%)</td>
<td><strong>Melanoma of the skin</strong></td>
</tr>
<tr>
<td><strong>Kidney &amp; renal pelvis</strong></td>
<td>33,430 (5%)</td>
<td>Thyroid</td>
</tr>
<tr>
<td><strong>Leukemia</strong></td>
<td>23,630 (3%)</td>
<td>Kidney &amp; renal pelvis</td>
</tr>
<tr>
<td><strong>Oral cavity &amp; pharynx</strong></td>
<td>23,240 (3%)</td>
<td>Ovary</td>
</tr>
<tr>
<td><strong>Pancreas</strong></td>
<td>21,830 (3%)</td>
<td>Pancreas</td>
</tr>
<tr>
<td><strong>All sites</strong></td>
<td>756,350 (100%)</td>
<td>All sites</td>
</tr>
</tbody>
</table>

*Includes basal and squamous cell skin cancers and in situ carcinoma except urinary bladder.
1.6.2 Cancer Biology

Cancer is the unremitting and uncontrolled cell proliferation in the body forming a tumor. The underlying cause for neoplastic progression (cancer) is either due to deregulated cell proliferation or suppressed apoptosis, both of which can lead to pathological disorders. Cancer genes are categorized into two groups: (1) oncogenes, mutations of which result in suppressed apoptosis, uncontrolled cell growth and incorrect differentiation, and (2) tumor suppressor genes or anti-oncogenes, which when mutated results in aberrant cell proliferation. In the case of breast and ovarian cancer, the human epidermal growth factor receptor 2 (HER2) proto-oncogene (normal gene), becomes oncogenic due to overexpression of the gene leading to tumorigenesis. The processes that drive the progression of normal human cells into malignant cells can be summarized into six genetic alterations, termed as the “hallmarks of cancer”: self-sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Fig. 1.14). Independent of how these genetic pathways are acquired by normal cells to become malignant, the biological endpoints that are ultimately reached is shared by almost all tumor types.

Tumors pose a less threat when they are localized and can either be surgically removed or successfully treated with radiotherapy. The major cause of mortality is not due to these localized tumors but due to metastasis, which is spread of tumor cells from primary site to form secondary tumors at other locations in the body. Metastasis occurs by a complex multi-step process: (1) cancer cells separation from the primary tumor site, (2) penetration through basement membrane of blood vessels, (3) migration of tumor
cells through stroma, (4) disruption of lymphatic vasculature and circulation, (5) extravasation through blood vessels into tissues of a different location and (6) secondary tumor formation and metastatic growth.\textsuperscript{44} While it is difficult to predict the location of metastatic spread for a specific tumor, carcinomas (cancers of the epithelial or endothelial cells) mainly spread to lymph nodes. Metastasis in lung, liver, and bone is usually common due to the presence of small blood vessels where tumor cells often get trapped.\textsuperscript{41,44}

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{figure1.14.png}
\caption{Hallmarks of cancer adapted from Ref. 41 describing the genetic alterations which characterize the progression of malignant cells.}
\end{figure}

There is a general consensus that blood vessels in tumors have structural irregularity, heterogeneity and leaky endothelium.\textsuperscript{45-47} The endothelial cells are disorganized, irregularly shaped, have loose interconnections and intercellular openings as large as 4.7 \textmu m.\textsuperscript{45} It is due to this leakiness of the tumor vessels that allow flow of tumor cells into the bloodstream and formation of metastasis. There are multiple factors that influence the leakiness of blood vessels: luminal surface area, permeability of the
vessel wall, concentration and driving forces (hydrostatic and osmotic) across the endothelium and blood flow. In addition to abnormal blood vessel structures, the rapid growth of tumors often does not allow lymphatic drainage to be well developed. However, the combination of leaky blood vessels and poor drainage has also led to the enhanced permeability and retention (EPR) effect which is vital for the accumulation of many diagnostic and therapeutic agents in tumor cells.

While nanoparticles and other therapeutic agents < 400 nm may be delivered by the EPR effect (passive targeting), targeting proteins that are preferentially expressed on specific tumor types (active targeting) is more effective. Among the many known potential targets on tumor vessels, antibody targeting against phosphatidylserine are effective and non-toxic. Phosphatidylserine are expressed on the cytoplasmic surface but flips to external surface in tumor vessels due to hypoxic conditions. Antibodies can be conjugated with therapeutic agents to target the vasculature of tumors as well as destroy the tumors. This thesis focuses on the use of anti-HER2 antibodies for targeting breast and ovary cancer cells in vitro and in vivo.

1.6.3 Cancer Therapy Strategies

The leading cancer therapy strategies being followed in clinic are chemotherapy, which is systemically toxic, and radiation therapy, which has serious side effects such as radiation induced necrosis of surrounding healthy tissue. Surgical oncology, a highly invasive approach, is usually the treatment of choice for primary tumors and metastasis in locations other than brain. However, several non-invasive and less-toxic strategies have also been developed for cancer treatment such as antibody based therapies, which are
directed at reducing systemic toxicity, the overexpression of a tumor receptor is down regulated by directly binding a monoclonal antibody to the receptor. For example, Herceptin was developed for down regulating overexpression of HER2, which is found in majority of human breast and ovarian cancer cases.\textsuperscript{49} Aptamer based therapies, which are short chain of nucleotides developed from DNA and RNA, have also proven effective since aptamers are resistant to degradation in tissue, allowing longer circulation time. Aptamers can selectively identify internalized surface markers, thus be imported into cells and used as a delivery vehicle for transporting therapeutic cargoes to cancer cells.\textsuperscript{50} These therapeutic strategies are, however, limited to only a single functionality. As cancer continues to aggressively take human lives, novel agents which can detect and treat the disease in one setting is impending.

In the past decade various nanoparticles have been explored for diagnosis and therapy of cancer including polymeric nanoparticles, liposomes, iron oxide agents and gold nanostructures. Polymeric nanoparticles are designed by incorporating biodegradable polymers such as polylactic acid (PLA), polyethylene glycol (PEG) or polylactic-co-glycolic acid and encapsulating chemotherapeutic drugs or photosensitizers within the polymeric capsules.\textsuperscript{51,52} These nanoparticles can be engineered to release drug based on pH, temperature, diffusion through polymer surface and diffusion due to polymer swelling. Liposomes are spherical micellar structures formed by one or several lipid bilayers with an aqueous phase inside and between the lipid bilayers. They can entrap hydrophilic agents within the internal compartment and hydrophobic agents into the membrane. These nanocarriers can be labeled with immunoglobulin and probes for targeting and imaging or incorporated with DNA, proteins, peptides and drugs for
therapeutics. Nonetheless, both liposomes and polymeric nanoparticles are restricted due to their larger size, and in some cases inability to perform multiple functionalities. Iron oxide nanoparticles have been traditionally used as MRI contrast agents. When exposed to alternating magnetic fields (AMFs), magnetic particles can generate heat by hysteresis loss resulting in hyperthermia and tumor ablation, a process referred as magnetodynamic therapy. Heat generation in the range of 100-1000 W/g has been reported using AMFs at physiologically safe frequencies and magnetic field strengths. Additionally, magnetic fields in the KHz to MHz frequencies can easily penetrate tissues up to several centimeters, making the magnetodynamic therapy a promising strategy for tumor treatment. However, a high dosage of iron oxide particles is necessary for inducing hyperthermia which may have severe toxic effects.

Gold nanostructures, both solid and in a core-shell geometry, of different shapes and sizes have been explored extensively as therapeutic agents due to their photothermal properties. When illuminated with resonant light, gold nanostructures efficiently convert light to heat causing high local temperatures on the surface which result in hyperthermia and tumor cell death. Unlike magnetic nanoparticles, these noble metal heat-generators only require pico-molar concentrations for inducing hyperthermia. Gold colloids resonant in the visible, and NIR resonant structures including nanorods nanocages, and nanoshells are the commonly used particles for cancer therapy. Gold nanostructures synergistically combine a biocompatible surface which can be straightforwardly conjugated with PEG for stealth character, or antibodies for active targeting. Nanostructures with large scattering cross-sections, for example nanoshells, can be used as optical contrast agents, or in combination with iron oxide particles they can be used
for MRI and photothermal therapy.\textsuperscript{60,61} Recently, a promising new paradigm termed as “Theranostic”, which entails the efficient integration of therapeutic and diagnostic moieties into a single agent, is rapidly emerging as an imminent alternative to traditional drugs and imaging agents.\textsuperscript{62} The ability to controllably manipulate the properties of gold nanostructures for targeting, imaging and therapy, hence provides a full theranostic spectrum of capabilities in a single nanostructure.

\textbf{1.7 Thesis Outline}

The research covered in this thesis may be categorized into four aspects: (1) nanoshells for plasmon enhanced fluorescence sensing in the NIR, (2) plasmon enhanced fluorescent nanoshell complexes for dual modal imaging, antibody targeting \textit{in vitro} and \textit{in vivo}, and for photothermal therapy \textit{in vitro}, (3) novel plasmonic nanoparticles for biomedicine and (4) photonic nanostructures developed via electrolytically induced assembly and wet chemical synthesis.

Chapter 2 discusses plasmon enhanced fluorescence of weak NIR fluorophore, Indocyanine green, as a function of distance from the nanoshell surface. The distance between the fluorophore and the nanoshell surface was controlled by growing silica epilayers of varying thicknesses. In Chapter 3, the fluorescence enhancement efficiency of nanoshells has been compared with gold nanorods for NIR fluorophore IR800, to understand the detailed photophysics and analyze the factors contributing towards fluorescence enhancement. In Chapter 4 and 5, the plasmon enhanced fluorescent nanoshells incorporated with iron oxide nanoparticles were demonstrated in an exciting biomedical application: \textit{in vitro} diagnostics and therapeutics of breast and ovarian cancer
cells respectively. The antibody conjugated nanoshells targeted the cancer cells, diagnosed via multimodal imaging using MRI and FOI and finally treated the cells using photothermal hyperthermia. Chapter 6 extends the *in vitro* studies to targeting, multimodal imaging and biodistribution of nanocomplexes *in vivo* in breast cancer xenograft mouse models. In Chapter 7, novel Au/SiO$_2$/Au plasmonic nanostructures were fabricated in the sub-150 and sub-100 nm size regime to expand the therapeutic possibilities of nanoshells to diseases where smaller nanoparticles are necessary. Plasmon hybridization was used to analyze and assign the optical modes observed for these complex layered nanostructures. Chapter 8 discusses the fabrication and plasmonic properties of a high refractive index semiconductor core, Cu$_2$O (n=7), coated with Au shell nanoshells. The Cu$_2$O/Au nanoshells were fabricated in the sub-100 nm size regime by a straightforward wet-chemistry approach and are highly promising for biomedical applications where smaller sizes are needed. Their optical properties were explored experimentally and theoretically. Chapter 9 describes the electrolytically induced formation, growth mechanism and optical properties of mesostars formed from Fe$_2$O$_3$-core/Au-shell nanorice structures. Chapter 10 illustrates the fabrication, growth mechanism and photoluminescence studies of ZnO submicrometer particles relevant for photonic applications.
Chapter 2: Nanoscale Control of Near-Infrared Fluorescence Enhancement Using Au Nanoshells

2.1 Introduction

Metal enhanced fluorescence is highly relevant for understanding the fundamental physical phenomena contributing towards emission enhancement as well as for developing technologies based on these nanoparticle-fluorophore assemblies. Metal-fluorophore interaction is a sensitive function of the distance between them. While at short distances (≤ 4 nm) fluorescence quenching is inevitable, at an optimum distance from the nanoparticle surface strong plasmon-enhanced fluorescence can be observed.\(^3\) The enhancement will decrease as the fluorophore-metal distance continues to increase until the metal’s optical field does not affect the fluorophore emission properties. In this chapter, we have qualitatively measured the fluorescence of NIR fluorophore, Indocyanine Green (ICG), as a function of distance from the Au nanoshell surface and determined the optimum distance where a maximum enhancement of 50X is achieved. Beyond the optimal distance from the nanoshell surface, the emission and hence the quantum yield decreases. All nanoparticle synthesis, characterization and experimental fluorescence spectra were obtained by Rizia Bardhan and Nathaniel K. Grady contributed towards theoretical analysis and discussion. Reproduced with permission from Rizia Bardhan, Nathaniel K. Grady, Naomi J. Halas, *Small*, 2008, 4, 1716–1722. Copyright 2008 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Water-soluble and biocompatible NIR emitting fluorescent molecules have provided an important new tool for biomedical imaging\(^6^3\) and for the detection of disease markers *in vivo*. The relatively large penetration depth of NIR light in most biological
media offers the potential for imaging deeply into the organs and soft tissues of living systems. This property is aided by the use of NIR-excitable fluorescent molecules as agents for contrast enhancement.\textsuperscript{64} The goal of designing and synthesizing NIR fluorescent molecules with large absorption coefficients and high quantum yields that are also safe for physiological environments is quite challenging, due at least in part to the complex synthetic routes required for these large, complex molecules.\textsuperscript{65, 66} A highly promising alternative approach is to enhance the emission of currently available NIR fluorescent molecules by combining them with appropriately designed metallic “nanoantennas”. For example, ICG, currently the only FDA-approved, commercially available NIR emitting dye, is used extensively as an exogenous fluorescent marker in clinical imaging applications\textsuperscript{67} such as the diagnosis of cardiac and hepatic function,\textsuperscript{68} measurement of plasma volume,\textsuperscript{69, 70} ophthalmic scanning laser angiography\textsuperscript{71, 72} and optical tomography.\textsuperscript{73} However, ICG is a relatively weak fluorophore with a quantum yield of only 1.3 \%,\textsuperscript{74} and toxicity limits the maximum concentration appropriate for clinical use. Enhancing the quantum efficiency of ICG significantly without compromising its biocompatibility will lead to significant improvements in the detection limits of NIR fluorescence-based imaging, enabling, for example, the detection of significantly smaller tumor volumes than is currently possible. Recently, we have shown that the fluorescence of ICG can be significantly enhanced when the fluorescent molecules are positioned near Au nanoshells, when the plasmon resonance of the nanoshell is tuned to the emission wavelength of the fluorophore.\textsuperscript{75} Fluorescence enhancements as large as 50 were achieved, for a nanoshell designed with a significant scattering cross section at that wavelength. In this system the ICG molecules act like
local sources that feed the optical “nanoantenna”, which then transmits their light efficiently into the nanoparticle far field.

Noble metal nanoparticles and nanostructures exhibit unique, remarkably vivid optical properties due to excitation of their surface plasmons by incident light. Plasmon excitation results in significantly enhanced local fields at the nanoparticle surfaces, which give rise to fundamentally interesting phenomena and technologically important applications. Noble metal nanoparticles are known to significantly enhance the emission rates of vicinal fluorophores by decreasing their radiative lifetime, thereby increasing their quantum yield. The photostability of fluorophores can also be increased in this manner. Enhancing the emission of molecular fluorophores is a highly useful strategy for improving detection sensitivity and selectivity in many emerging applications such as DNA screening and single molecule detection, in addition to image enhancement. Consequently, designing and developing nanoparticle-molecule complexes to enhance molecular fluorescence is of broad interest and general importance.

The influence of metallic surfaces (and subsequently metal nanoparticles) on fluorescence emission and molecular excited-state lifetimes has been a topic of interest since the pioneering work of Drexhage. The lifetime of a molecular excited state varies as a function of distance from the surface of a noble metal due to a modification of the enhanced absorption (due to the near field), radiative decay rate (due to the modified photon density of states near the metal surface) and nonradiative decay channels (due to energy dissipation). While fluorescence from a molecule directly adsorbed onto a metal surface or nanoparticle is quenched, for a molecule positioned a few nanometers from the metal, it can be strongly enhanced. As the fluorophore-metal distance is
increased, longer-range interactions between the molecule and the metal can lead to oscillatory enhancement and quenching behavior as a function of separation distance for bulk metals. For large separation distances from a bulk metal or metallic nanoparticle, the molecular fluorescence returns to its unperturbed value.\textsuperscript{85,86}

In this study, we have investigated the fluorescence enhancement of ICG molecules as a function of distance from the surface of Au nanoshells (NS). The distance between ICG molecules and the NS surface is controlled by varying the thickness of silica shell spacer layers grown on the nanoshell surface. We observe a fluorescence enhancement of 50 when ICG molecules are spaced 7 nm from the Au nanoshell surface, which decreases with increasing distance of the molecule from the nanoshell surface. Au nanoshells are excellent plasmonic nanostructures for these experiments for several reasons. First, their plasmon resonances can be controllably tuned across the visible and infrared region of the spectrum by modification of their core and shell dimensions.\textsuperscript{12,87} Their relative absorption and scattering cross sections can also be controlled by varying total nanoparticle size.\textsuperscript{88,89} The spherical symmetry provides a highly uniform geometry for studying phenomena requiring a systematic variation of molecule-metal nanoparticle distance. Growing a silica shell spacer layer around the Au nanoshell core, then binding molecules onto the silica layer, is a controllable and reproducible technique for positioning fluorophores at well controlled, nanometer-scale distances from the nanoparticle surface over the length scales appropriate for modifying molecular fluorescence. The chemistry also imparts a robust, chemically inert, and biocompatible surface for the nanoparticle.\textsuperscript{90} This approach allows us to measure the spatial extent of
the fluorophore – nanoparticle interaction quantitatively over a length scale relevant to fluorescence enhancement.

2.2 Fabrication of Nanoshell-ICG Conjugates

NS@SiO$_2$ fabrication: Au nanoshells $[r_1, r_2] = [60, 76]$ nm were fabricated as described in Chapter 1. The NS were then coated with a thin layer of silica to facilitate a biocompatible surface as well as to bind the fluorophore on the silica layer. NS@SiO$_2$ nanoparticles were fabricated by slight modification of a previously reported protocol. \cite{91} 4 mL of the NS (2 x 10$^9$ particles/mL) were added to 40 mL of 200 proof fresh ethanol, followed by adding 500 uL of 28% NH$_4$OH (Fisher). Subsequently, 6 - 70 µL of fresh TEOS was quickly added to the reaction mixture under vigorous stirring. The vessel was immediately sealed and vigorously stirred for 45 minutes. Note: A new bottle of ethanol should be opened every time this synthesis is done. The volume ratio between NS and ethanol should be between 1:7-1:10. The amount of NH$_4$OH depends on how it is, 400 µL is sufficient for fresh ammonia, more should be added if not fresh. The NS@SiO$_2$ nanoparticles were stored in the refrigerator at 4 °C overnight without agitation. After 24 hours the particles were centrifuged twice and re-dispersed in 20 mL ethanol.

ICG Binding to NS@SiO$_2$ Nanoparticles: ICG molecules were adsorbed onto the NS@SiO$_2$ by a two step procedure. (1) 1 mg ICG was mixed with 500 µL degassed 200 proof ethanol and 100 µL APTES was subsequently added and the mixture was stirred for 8 hrs at room temperature. (2) The APTES-ICG mixture was added to the NS@SiO$_2$ nanoparticles dispersed in ethanol and stirred for 24 hours at room temperature. The ICG bound nanoparticles were centrifuged in microcentrifuge tubes in small aliquots of 1 mL for 5 minutes at 280 rcf to remove unbound ICG molecules. Absorbance of the
supernatant was monitored to account for unbound dye and we determined that ~ 400 nM ICG molecules were bound to the NS@SiO₂ nanoparticles. ICG molecules were bound to silica nanospheres similarly and ~ 400 nM ICG were attached to the silica nanospheres as well. The final nanoparticle solutions were redispersed in water and stored at 4 °C. Fluorescence measurements of ICG molecules adsorbed on NS@SiO₂ nanoparticles and silica nanospheres were obtained in solution with 10⁷ particles/mL.

2.3 Characterization of Nanoshell-ICG Conjugates

![Figure 2.1](image)

**Figure 2.1.** Absorption–emission spectra (λ<sub>max-Ab</sub> = 780 nm and λ<sub>max-Em</sub> = 820 nm) and chemical structure (inset) of ICG.

The nanoparticles were characterized by obtaining transmission electron microscope (TEM) images using a JEOL JEM-2010 TEM, and absorption measurements using a Varian Cary 5000 UV-Vis-NIR spectrometer. Fluorescence emission spectra were obtained using Jobin Yvon Spex Fluorolog 3 and the samples were excited at 785 nm. The absorption-emission spectrum and chemical structure of ICG (Fig. 2.1), shows
that ICG, a negatively charged tricarbocyanine dye, strongly absorbs at 780 nm and emits at 820 nm. The presence of the charged sulfonate groups increases the solubility of ICG in aqueous media and minimizes significant ground-state aggregation, a phenomenon commonly observed in carbocyanine-type dyes.92

Figure 2.2. (A) Schematic diagram of fabrication procedure, and TEM micrographs (B – F) of NS coated with varying thicknesses of silica epilayers (B) 7 ± 2 nm (C) 13 ± 2 nm (D) 21 ± 3 nm (E) 30 ± 3 nm (F) 42 ± 3 nm. Scale bar is 50 nm for all TEM images.

The fabrication procedure of NS@SiO2 nanoparticles is illustrated in Figure 2.2A. TEM micrographs of NS coated with different thicknesses of SiO2 are shown in Figure 2.2B –
2F, where the thickness of the silica shell was varied from 7 – 42 nm. The NS@SiO₂ nanoparticles range from 166 ± 3 nm in diameter to 236 ± 4 nm where the silica thickness was controlled by concentration of tetraethylorthosilicate (TEOS) after alkaline initiation.

![Graph showing the observed shift in the plasmon resonance peak with increasing thickness of silica layer surrounding the NS.](image)

**Figure 2.3.** Observed shift in the plasmon resonance peak with increasing thickness of silica layer surrounding the NS. (■) Experimental, and (—) calculated values for NS \([r_1, r_2] = [60, 76] \text{ nm}\) and NS@SiO₂ nanoparticles, with \(7 ± 2 \text{ nm}, 13 ± 2 \text{ nm}, 21 ± 3 \text{ nm}, 30 ± 3 \text{ nm}, 42 ± 3 \text{ nm}, 61 ± 4 \text{ nm}, \text{ and } 90 ± 4 \text{ nm}\) thick silica epilayers are shown.

The plasmon resonance of the NS nanoparticles shifts to longer wavelengths as the silica layer thickness is increased. The experimentally obtained plasmon resonance frequencies as a function of silica layer thickness are compared directly to theoretical values calculated using Mie Theory for a concentric, three-layer, SiO₂-Au-SiO₂ spherical nanoparticle (Fig. 2.3). The plasmon redshift occurs due to the presence of the silica layer because of the higher refractive index of silica relative to water. The increasing thickness of the silica epilayer surrounding the NS screens the electron oscillation in the metal, decreasing the plasmon energy. A redshift in the plasmon resonance peak from 790 nm (without silica shell layer) to 834 nm (with 60 nm silica shell layer) is observed, with the plasmon shift remaining constant for the thicker silica shell layers. This behavior
indicates that changes to the local dielectric environment beyond a certain distance from the surface do not apparently affect the surface plasmons of the NS nanoparticles. However, the silica layer thicknesses used in this experiment do not extend to the longer length scale where oscillatory behavior of the SPR shift with increasing dielectric layer thickness has been reported. The experimentally obtained values of the plasmon resonance wavelength shown in Figure 2.3 correlate very well with the theoretically obtained values. The linewidth of the plasmon peak of these nanostructures allows good overlap with the ICG absorption-emission profile for all the NS@SiO₂ nanoparticles synthesized for this experiment.

2.4 Experimental Fluorescence Enhancement

A schematic of ICG adsorption on the NS@SiO₂ nanoparticles through APTES surface functionalization, and subsequent fluorescence emission, is illustrated in Figure 2.4A. The ICG molecules are adsorbed onto the silica surface of the nanoparticle electrostatically. To quantify the fluorescence enhancement due to the silica coated nanoshells, ICG bound in the same manner to silica nanospheres (radius 60 ± 2 nm) was used as a control sample. Comparison of the fluorescence of ICG adsorbed to silica-coated nanoshells vs. ICG on silica nanoparticles, rather than ICG in solution, allows us to examine the fluorescence enhancement process for molecules in equivalent chemical environments. The fluorescence spectra of ICG bound to silica (λ_{max} = 810 nm) at varying distances from NS surface, as determined by the silica spacer layer, is shown (Fig. 2.4B).
Figure 2.4. (A) Schematic diagram of ICG conjugation on NS@SiO₂ nanoparticles, excitation and observed emission. (B) Fluorescence spectra of ICG conjugated at various distances from the NS surface. (—) Emission spectrum of control, ICG deposited on silica nanospheres is also included. (C) Fluorescence enhancement factor of ICG conjugated on NS@SiO₂ nanoparticles relative to silica nanospheres is shown as a function of thickness of silica layer surrounding the NS (color coordinated with reference to figure 4B).

The fluorescence spectra were collected under identical excitation and detection conditions, allowing direct comparison of the various nanoparticle-ICG complexes. The concentration of ICG molecules was maintained at ~ 400 nM in each of the nanoshell and silica nanoparticle solutions used in the deposition of ICG onto the nanoparticle surfaces. Based on our deposition protocol, near-saturation coverage of ICG on the nanoparticle surfaces is likely, corresponding to nominally 10³ ICG molecules per nanoparticle, increasing as nanoparticle size (and therefore surface area) is increased. A maximum enhancement of 50X is achieved relative to ICG molecules adsorbed on silica nanospheres for a silica spacer layer of 7 ± 2 nm on the nanoshell surface. As the
thickness of the silica spacer layer (13 ± 2 nm, 21 ± 3 nm, 30 ± 3 nm, 42 ± 3 nm) was increased, the fluorescence enhancement decreased, with the lowest enhancement factor of 7 observed for the largest spacer layer. This distance-dependent decay of the fluorescence enhancement is shown in Figure 2.4C as a function of silica layer thickness.

2.5 Calculated Fluorescence Enhancement

The Au nanoshells influence ICG emission by a combination of two processes: absorption enhancement via the high intensity nanoparticle near field, and radiative rate enhancement, which increases its quantum yield. Since the nanoparticle complexes used in this study have spherical symmetry, their properties can be calculated quite straightforwardly using Mie theory. In particular, both the near field enhancement and the radiative rate enhancement of molecules near the nanoshell surface can be calculated for this spherical geometry. To obtain the near field enhancement, the electromagnetic fields around and within the particle are expressed in terms of a spherical harmonic basis set and the scattering is obtained in response to an incident plane wave. To evaluate the radiative rate enhancement, a similar procedure is followed, except that the plane wave excitation is replaced with a point dipole adjacent to the nanoparticle. The far-field Poynting vector is then integrated over all angles and compared directly to the amount of energy radiated by the same dipole in free space. This theoretical model agrees very well with the distance dependence of the fluorescence enhancement we have observed (Fig. 2.5A). We will now examine the relative contribution of these processes in more detail.

Emission from a fluorophore is experimentally quantified in terms of its quantum yield ($Q_o$) and lifetime ($\tau$). Intrinsically, these processes are best described in terms of the
radiative emission rate \( (\Gamma) \) and non-radiative decay rate \( (k_{nr}) \) as discussed in chapter 1. For most dye molecules, including ICG, these parameters are highly dependent on local environment.\(^{96}\) For ICG adsorbed onto silica-coated NS and ICG on solid silica nanospheres, we can assume that the intrinsic radiative and non-radiative decay rates of ICG are the same for these equivalent environments. The emission intensity of reference, ICG adsorbed on silica, is \( I_0 = I_{exc} \cdot e^* \cdot Q_o \) where \( e \) is molecular adsorptivity and \( I_{exc} \) is intensity of excitation source. In the presence of the metal, the quantum yield increases and lifetime decreases. Because the chemical environment is unchanged from the reference sample and quenching is not observed, we assume that \( k_{nr} \) is unaffected by the presence of the metal shell inside the silica coating. The observed emission of the ICG-NS system is therefore \( I_{NS} = (\langle |E|^2 \rangle \cdot I_{exc}) \cdot e^* \cdot Q_{NS} \) where \( \langle |E|^2 \rangle \) is the surface average near field enhancement of nanoshells. The fluorescence enhancement is evaluated following the same procedure as for the experiment, by taking the ratio of the emission from the ICG-NS \( (I_{NS}) \) to the reference ICG on the silica nanosphere \( (I_0) \):

\[
\frac{I_{NS}}{I_0} = \frac{\langle |E|^2 \rangle \cdot Q_{NS}}{Q_o} = \langle |E|^2 \rangle \frac{\chi_r (\Gamma + k_{nr})}{\chi_r \Gamma + k_{nr}}, \tag{1}
\]

where both the dye concentration and excitation intensity are taken to be the same in both cases. \( \chi_r \) is the factor by which the radiative decay rate of the molecules increases due to electromagnetic coupling. From this expression, one can see that the observed enhancement can be separated into the effect of the field enhancement \( (\langle |E|^2 \rangle) \) and the quantum yield enhancement \( (Q_{NS}/Q_o) \). This expression indicates that fluorescence enhancement depends on the properties of the nanoparticle, and also the dye molecule, via \( \Gamma \) and \( k_{nr} \). A third effect should come into play when the molecule is very close to the metal surface, where the fluorescence is quenched by an increase in the nonradiative
decay rate. In the experimental regime studied here, no evidence of quenching is observed and is therefore not included in this analysis. We performed a control experiment with ICG molecules bound to nanoshells without any silica layer, and observed fluorophore quenching, shown in Figure 2.5. However, direct comparison of the fluorescence enhancement factor of ICG molecules bound to NS@SiO₂ nanoparticles relative to ICG adsorbed on NS surface would not be quantitative since the chemical environment of the fluorophore is different in each solution.

![Figure 2.5](image)

**Figure 2.5.** Fluorescence enhancement factor of ICG molecules adsorbed on NS without any silica layer (•) is represented showing strong quenching of ICG molecules. ICG molecules bound to NS@SiO₂ nanoparticles relative to silica nanospheres is also shown as a function of silica layer thickness surrounding the NS. The data points representing ICG-NS@SiO₂ nanoparticles are color coordinated with ref. to Figure 2.4B and 2.4C.

The theoretical model we have used yields excellent agreement with the observed fluorescence enhancement as a function of distance (Fig. 2.6A). To obtain this fit, we first calculated the surface-averaged near field intensity enhancement $\langle |E|^2 \rangle$ as a function of distance from the nanoshell surface using Mie theory (Fig. 2.6B). This term appears quite similar to the observed distance-dependent fluorescence enhancement. However, it was shown previously that near field enhancement alone does not explain the fluorescence enhancement as a function of plasmon energy for ICG at a fixed distance from the
Therefore we need to investigate to what extent the properties of the molecule affect the fluorescence enhancement. We also calculate the radiative rate enhancement factor $\chi_r$ (Fig. 2.6C).

To calculate the quantum yield enhancement $Q_{NS}/Q_o$, $\Gamma$ and $k_{nr}$ for ICG in a silica environment are required. To determine these parameters, the total fluorescence enhancement (Eqn. 1) was fit to the experimental data with $\Gamma$, $k_{nr}$, and a scale factor.
(interpreted as molecular orientation) as free parameters, using a generalized reduced gradient minimization algorithm. The initial values of $\Gamma$ and $k_{nr}$, were chosen based on the rates previously reported. From this approach, values of $\Gamma = 0.19 \text{ ns}^{-1}$, $k_{nr} = 2.1 \text{ ns}^{-1}$, and a scale factor of 0.53, yielding values of $\tau_o = 0.44 \text{ ns}$ and $Q_o = 8.6\%$ for the ICG-on-silica-nanoparticle control sample, were obtained. The increase in quantum yield for ICG from nominally 1.3% for the molecule in solution to 8.6% for ICG adsorbed on silica nanoparticles is quite reasonable and consistent with several aspects of the molecule’s new environment: stabilization of the polymethine chain due to adsorption on the silica surface, and the reduction in polarity and free oxygen exposure relative to an aqueous phase environment. The resulting fit, i.e. the product of Figures 2.6B, 2.6D, and a scale factor on the order of 0.5, is shown in Figure 2.6A. The scale factor is justifiable due to the random orientation of ICG on the nanoparticle surface. The agreement between our analysis and the experimental data shown in Figure 6A demonstrates that this modeling approach does an excellent job describing the system, and also confirms that the sample uniformity is quite good. Due to the tractability of this system, this approach is highly promising for further experimental studies and analysis of fluorescence modification on nanoshell-based structures.

2.6 Calculated Quantum Yield and Lifetime

Examining the contribution from the near field enhancement (Fig. 2.6B) and quantum yield enhancement (Fig. 2.6D), it can be seen that the radiative rate enhancement is actually more important than the near field enhancement at all distances studied here, and decreases quite slowly with distance. This is quite an important point to consider, since a
fluorophore with intrinsically high quantum yield can only be enhanced due to $\langle |E|^2 \rangle$, and would therefore experience much weaker enhancement. The effective quantum yield and lifetime of the ICG-NS system can be calculated using these obtained parameters and Equations 4 and 5 from chapter 1, and is shown in Figure 2.7. From Figure 2.7A it can be seen that the quantum yield is strongly enhanced, approaching unity, relative to the unenhanced 8.6% quantum yield without the presence of the nanoshell. In this analysis, the quantum yield enhancement (Fig. 2.6D) is relatively flat for thin spacer layers. The lifetime of the dye decreases significantly as the silica layer is decreased (Fig. 2.7B), resulting in an increased quantum yield. Further studies to test this model using direct lifetime measurements are currently underway.

![Figure 2.7.](image)

**Figure 2.7.** Calculated values of distance dependent fluorescence of ICG as a function of silica spacer thickness surrounding the Au-NS (a) quantum yield and (b) lifetime.

### 2.7 Conclusions

In conclusion, we have shown that fabrication of silica epilayers surrounding NS nanoparticles of controlled thicknesses allows us to examine the distance-dependent fluorescence enhancement of the low quantum yield molecular fluorophore ICG in a
quantifiable manner. A maximum fluorescence enhancement of 50 is achieved at a distance of \(~ 7 \text{ nm}\) from the NS surface, and even for the thickest silica layer fabricated on the NS (\(~ 42 \text{ nm}\)) a 7 fold enhancement is still observed. The quantum yield of ICG molecules decrease with increasing distance from the NS surface due to a decrease in the NS near field at the excitation wavelength and a decrease in coupling of the fluorophore to the NS plasmon at the emission wavelength. The strongly enhanced fluorescent emission of ICG in this nanoparticle configuration is potentially very valuable in biomedical imaging and clinical diagnostic applications. This strategy for enhancing the emission of low-quantum-yield fluorescent emitters can be straightforwardly generalized to other types of fluorescent emitters and media.
Chapter 3: Fluorescence Enhancement by Au nanostructures: Nanoshells and Nanorods

3.1 Introduction

Plasmonic fluorescence enhancement is not only critically dependent on the distance between the metal surface and the fluorophores, but also on the geometry, dimensions and corresponding near-field and far-field properties of the nanoparticles. In this chapter, we have studied in details the fundamental photophysics contributing towards plasmon enhanced fluorescence of molecular systems. We have examined the enhancement of NIR fluorophore, IR800-NHS ester (IR800), with Au nanoshells and compared that with Au nanorods and experimentally determined the quantum yield and lifetime of the fluorophore. All nanoparticle synthesis, characterization, fluorescence, lifetime and experimental angular scattering measurements were obtained by Rizia Bardhan. Nathaniel K. Grady contributed in building the angular scattering set-up, data interpretation and discussion. Reproduced with permission from Rizia Bardhan, Nathaniel K. Grady, Joseph R. Cole, Amit Joshi, Naomi J. Halas, ACS Nano, 2009, 3, 744-752. Copyright 2009 American Chemical Society.

Fluorescence imaging has seen widespread use in clinical diagnosis and monitoring processes in biological systems. The development of contrast agents, such as fluorescent probes with engineered biomarker functionalities, has become integral to the advancement of new bioimaging technologies. Fluorescent molecules emitting at wavelengths in the physiologically relevant “water window” (700 nm – 900 nm), are of particular interest due to the large penetration depth of near infrared (NIR) light in most biological media, and offer the potential for imaging at significant depths in living
tissues. However, achieving bright fluorescent emission with photostable and biocompatible NIR fluorophores has proven to be extremely difficult. It has long been known that in the proximity of a metallic surface, fluorescence emission of molecules can be enhanced; this is also the case for metallic nanostructures and nanoparticles adjacent to a fluorophore. The presence of a nearby metallic nanoparticle can not only enhance the quantum yield but also stabilize adjacent fluorophores against photobleaching, further enhancing their practical use in bioimaging applications. In new and emerging light-assisted therapeutic applications such as photothermal cancer therapy, the addition of bright NIR fluorescence to a therapeutic nanostructure complex could provide addition diagnostic imaging capabilities for this treatment strategy that could facilitate clinical use. Understanding precisely how metallic nanostructures enhance molecular fluorescence is of general fundamental interest, and may ultimately provide practical routes to enhancing light emission from a variety of materials systems and devices far beyond the specific application of bioimaging.

Metal nanostructures exhibit remarkable optical properties due to excitation of their surface plasmons by incident light, which results in a significant enhancement of the electromagnetic field at the nanoparticle surface. This enhanced near field can be used to design highly sensitive chemical and biosensors with specific plasmon resonances tailored by the nanoparticle geometry. Metallic nanoparticles have been shown to enhance the fluorescence emission and decrease the molecular excited-state lifetimes of vicinal fluorophores. The fluorescence enhancement is attributable to a combination of processes including enhanced absorption by the molecule, modification of the radiative
decay rate of the molecule, and enhanced coupling efficiency of the fluorescent emission to the far field. \(^3\) \(^{10}\)

The plasmon resonant properties of metallic nanoparticles can be controlled by optimizing the nanoparticle topology, dimensions, and composition. \(^{56}\) \(^{101-103}\) When plasmonic nanoparticles are much smaller than a wavelength of light they are absorbers, much like molecules. Nanoparticles larger than a few tens of nanometers both absorb and scatter light. While both the absorption and scattering cross sections of a plasmon resonant nanoparticle increase with increasing particle size, scattering begins to dominate in this larger-size regime. \(^{89}\) Au nanorods (NRs) and Au nanoshells (NSs) are both particularly useful for biological applications since their near field and far field optical properties can be tuned controllably throughout the NIR water window by varying their geometry. For NRs, the aspect ratio defines two distinct plasmon resonance frequencies associated with the longitudinal and transverse dimensions of the nanostructure. \(^{104}\) For NSs, symmetric spherical nanoparticles consisting of a dielectric core and a metal shell, the plasmon resonances are determined by the relative size of the core and the metal shell layer. \(^{87}\) NSs can be fabricated both in a small size regime (d~50 nm) using Au$_2$S/Au and sizes up to a micron in silica/Au core/shell structures, accessing NIR resonances over a large size range. \(^{89}\) NRs tuned to the same NIR plasmon resonance frequency as silica/Au NSs are significantly smaller in size than the corresponding nanoshell, resulting in a much smaller contribution of scattering to the overall extinction cross section. Studying fluorescence enhancement by these two types of structures allows us to examine how the properties of both nanostructures contribute to this effect.
We have recently shown that the fluorescence of NIR fluorophores can be significantly enhanced when the molecules are in close proximity to NSs. Fluorescence enhancements as large as 50 were obtained when the plasmon resonance of the nanoshell was tuned to the emission wavelength of the fluorophore, for a nanoshell designed with a significant scattering cross section at that wavelength. In the study we report here, we compare the fluorescence enhancement of IR800 positioned a small, controlled distance from NSs and NRs. We quantified the fluorescence enhancement of IR800 experimentally by measuring the angle resolved scattering intensities, frequency domain fluorescence decay, and quantum yield of IR800 before and after binding to the nanoparticles. We also calculated the scattering cross sections of NSs and NRs to determine the scattering intensities of the nanoparticles and relative contribution of nanoparticle scattering towards molecular fluorescence enhancement.

When a fluorophore is adsorbed directly onto a metal surface, its fluorescence is quenched. However, at a distance of a few nanometers from the nanoparticle surface, the fluorescence can be strongly enhanced. IR800 molecules were placed in close proximity of NS and NR surfaces by coating the nanoparticles with a 5-11 nm layer of human serum albumin (HSA) in order to prevent quenching of IR800. Serum albumin, a large multi-domain protein relevant to many physiological functions, has been conjugated to Au nanoparticles extensively for cell-targeting applications. It binds to Au by electrostatic attraction between the amine groups of the protein and the negative charge on the gold surface, or, alternatively, by covalent attachment between the Au surface and amino acid functional groups present in the protein. In this study HSA acts as both a spacer layer as well as a linker of the fluorophore to the nanoparticle. It contains an abundance of α-amines at N-terminals and ε-amines on lysine side chains, which
covalently bind to IR800 via the N-hydroxsuccinimide (NHS) ester group to form a stable protein-dye complex.\textsuperscript{111} The NHS ester group facilitates protein conjugation, and the presence of the negatively charged sulfonate groups makes IR800 soluble in aqueous media. While these properties make IR800 a suitable dye for bioimaging,\textsuperscript{64} it is limited by low quantum efficiency compared to commonly used visible dyes such as Boron Dipyrromethene (BODIPY) and Fluorescein isothiocyanate (FITC). Therefore, by binding IR800 to metal nanoparticles with a suitably determined spacer layer, the quantum yield can be significantly enhanced.

3.2 Fabrication of Nanoparticle-Fluorophore Conjugates

**Section-I. Nanoparticle Fabrication:** All chemicals were purchased from Sigma Aldrich and IR800 NHS ester was purchased from Licor Biosciences. Au nanoshells (NSs) \([r_1, r_2] = [63, 78]\) nm were fabricated as described in Chapter 1. The fabricated NSs were centrifuged several times and finally redispersed in phosphate buffer (Na\textsubscript{3}PO\textsubscript{4}, pH \(\sim 8.0\)).

Au nanorods (NRs) \([w, l] = [11, 46]\) nm were fabricated by the method reported by Murphy and co-workers.\textsuperscript{112} Briefly, the seed solution was prepared by gently mixing 7.5 mL of 0.1 M Cetyl trimethylammonium bromide (CTAB) with 0.25 mL of 0.01 M H\textsubscript{Au}Cl\textsubscript{4}. 0.6 mL of ice-cold 0.01 M NaBH\textsubscript{4} was injected into the solution and mixed rapidly. The solution turns from a golden yellow color to pale brown color. It is essential that the temperature of NaBH\textsubscript{4} is maintained at \(\sim 0\) °C prior to mixing with CTAB and H\textsubscript{Au}Cl\textsubscript{4} for proper growth of nanorods. The seed solution was then stored at 27 °C until further use. The growth solution was prepared by gently mixing 47.5 mL of 0.1 M CTAB, 2 mL of 0.01 M H\textsubscript{Au}Cl\textsubscript{4}, 0.3 mL of 0.01 M AgNO\textsubscript{3} and 0.32 mL of 0.1 M Ascorbic Acid in the order mentioned. Addition of ascorbic acid turns the solution from
golden yellow to colorless. 0.25 mL of the seed solution was added to the growth solution and mixed gently and then the solution was stored at 27 °C for 3 hours without agitation. Within 20 minutes the solution changes from colorless to pink and eventually changes to dark pinkish brown after 3 hours. The NRs were washed twice at 8000 rpm to remove excess CTAB and finally redispersed in 10 mL Na3PO4 buffer.

Section-II. Protein and Fluorophore Conjugation: HSA was conjugated to IR800 by mixing equal volumes of 5 µM HSA aqueous solutions in Na3PO4 buffer and 30 µM IR800 aqueous solution at room temperature for 5 hours, in the dark. The protein-fluorophore complex was then dialyzed in Na3PO4 buffer for 24 hours at room temperature protected from light, in a 3500 MW dialysis bag (MW of HSA is 67 kDA and MW of IR800 is 1166 g/mol) to remove excess dye. Absorbance peaks of the protein-dye complex (λmax = 782 nm) and protein (λmax = 280 nm) were monitored to account for free-dye removal. After sufficient removal of unbound dye, the ratio of fluorophore to protein concentration (NIR800:NHSA) of the complex is determined by utilizing the absorbance of fluorophore at 782 nm (AIR800), molar extinction coefficient of IR800 (εIR800), absorbance of HSA at 280 nm (AHSA), molar extinction coefficient of HSA (εHSA) given by:

\[
\frac{N_{IR800}}{N_{HSA}} = \frac{[A_{IR800}/\varepsilon_{IR800}]}{[A_{HSA}/\varepsilon_{HSA}]}
\]

where \( \varepsilon_{IR800} = 240,000 \text{ M}^{-1}\text{cm}^{-1} \) and \( \varepsilon_{HSA} = 42,864 \text{ M}^{-1}\text{cm}^{-1} \). The final protein concentration was calculated using the molecular weight (M.W.HSA) and dilution factor (d. f.) of original solution given by:

\[
N_{HSA} = [A_{HSA}/\varepsilon_{HSA}] \times \text{M.W.HSA} \times \text{d. f.}
\]

The final protein concentration was ~ 4 µM and fluorophore concentration was ~ 10 µM.
Section-III. Protein-Fluorophore Complex Binding to Nanoparticles: The protein-fluorophore complex obtained was conjugated to the nanoparticles by adding 1 mL of HSA-IR800 solution in Na₃PO₄ buffer to 10 ml nanoshells, and 10 ml nanorods dispersed in Na₃PO₄ buffer respectively under constant stirring. After 7 hours conjugation at room temperature, in the dark, the nanoparticles were centrifuged in small aliquots of 500 μL to remove excess dye and resuspended in Na₃PO₄ buffer and we closely monitored the absorbance of the supernatant to account for unbound fluorophore concentration in the supernatant (9.55 – 9.65 μM). The concentration of the supernatant was then subtracted from the initial concentration of HSA-IR800 which was added to the nanoparticles to calculate the amount of HSA-IR800 on the nanoparticles. The final NSs concentration after resuspension in buffer was at ~ 10⁸ particles/mL and final NRs concentration was at ~ 10⁹ particles/mL. The surface area available for the HSA-IR800 complex to bind to the nanoparticles was normalized (see below) and hence the concentration of fluorophore was equivalent for both the NSs-HSA-IR800 and NRs-HSA-IR800, ~ 400 ± 50 nM.

Section-IV. Calculation of surface area available for HSA-IR800 to bind to nanoparticles

The amount of protein and fluorophore bound to the nanoparticles were quantified by assuming that IR800 binds to HSA similarly for both nanoshells and nanorods. The available surface area for fluorophore binding is kept equivalent for both nanoparticle solutions. The concentration (C_NS) and extinction co-efficient (σ_NS) for NSs were calculated utilizing Mie theory:

\[ σ_{NS} = 9.6072 \times 10^{-10} \text{ cm}^2 \text{ for NSs } [r_1, r_2] = [63, 78] \text{ nm} \]

\[ C_{NS} = \ln(10) \times \text{Absorbance} / σ \times \text{cuvette path length} \]

\[ = 2.303 \times 0.052 / 9.6072 \times 10^{-10} \text{ cm}^2 \times 1 \text{ cm} \]
The Surface Area of one NS (SA_{NS}) = 4\pi [r^2 - r_1^2] = 2.855 \times 10^{-14} \text{ m}^2.

The Surface Area of NSs solution = (SA_{NS}) \times C_{NS} = 3.568 \times 10^{-6} \text{ m}^2/\text{mL}

The extinction coefficient (\sigma_{NR}) for NRs \([w, l] = [11, 46]\) nm resonant at \(\sim 800\) nm have been reported previously as \(\sigma_{NR} \sim 4 \times 10^{-11} \text{ cm}^2\). The concentration of NRs (C_{NR}) were calculated utilizing Gan’s theory,

\[
C_{NR} = 2 \times \text{Absorbance} / \sigma \times \text{cuvette path length}
\]

\[
= 2 \times 0.043 / 4 \times 10^{-11} \text{ cm}^2 \times 1 \text{ cm}
\]

\[
= 2.154 \times 10^9 \text{ particles/ mL}
\]

The Surface Area of one NR (SA_{NR}) = 2\pi r^2 + 2\pi rl = 1.727 \times 10^{-15} \text{ m}^2.

The Surface Area of NRs solution = (SA_{NR}) \times C_{NR} = 3.719 \times 10^{-6} \text{ m}^2/\text{mL}.

However, experimentally the available nanoparticle surface area for fluorophore binding would vary slightly due to non-uniform size distribution of nanoparticles in solution.

**Section V. Calculation of no. of HSA-IR800 molecules bound to the nanoparticles**

The diameter of a HSA molecule is approximately \(\sim 8 \pm 3\) nm and we assume the diameter of a IR800 molecule is roughly \(\sim 2\) nm, resulting in a hydrodynamic diameter of \(\sim 12\) nm for the protein-fluorophore complex. We have determined the number of fluorophore molecules per protein molecule by taking a ratio of their surface area:

The surface area of one HSA molecule (SA_{HSA}) = 4\pi r^2 = 4\pi (4 \times 10^{-9})^2 = 2.011 \times 10^{-16} \text{ m}^2

The surface area of one IR800 molecule (SA_{IR800}) = 4\pi (1 \times 10^{-9})^2 = 1.256 \times 10^{-17} \text{ m}^2

The no. of IR800 molecules per HSA molecule = \(SA_{HSA} / SA_{IR800}\)

\(\approx 16\) dye molecules
We have also calculated the number of molecules of protein-fluorophore complex per nanoparticle, for both NSs as well as NRs:

The surface area of one HSA-IR800 molecule \( (SA_{\text{HSA-IR800}}) \) = \( 4\pi (6 \times 10^{-9})^2 \)

\[ = 4.523 \times 10^{-16} \text{ m}^2 \]

The no. of HSA-IR800 molecule per NS = \( \frac{SA_{\text{NS}}}{SA_{\text{HSA-IR800}}} \)

\[ = 2.855 \times 10^{-14} \text{ m}^2 / 4.523 \times 10^{-16} \text{ m}^2 \]

\[ \approx 63 \text{ HSA-IR800 molecules} \]

The no. of HSA-IR800 molecule per NR = \( \frac{SA_{\text{NS}}}{SA_{\text{HSA-IR800}}} \)

\[ = 1.727 \times 10^{-15} \text{ m}^2 / 4.523 \times 10^{-16} \text{ m}^2 \]

\[ \approx 4 \text{ HSA-IR800 molecules} \]

However, as described above the surface area available for HSA-IR800 complex to bind to NSs and NRs have been normalized by adjusting the nanoparticle concentration. This provides almost equivalent amounts of protein-dye molecules to bind to each nanoparticle suspension.

### 3.3 Characterization of Nanoparticle-Fluorophore Conjugates

The nanoparticles were characterized by obtaining transmission electron microscope (TEM) images using a JEOL JEM-2010 TEM, and absorbance measurements using a Varian Cary 5000 UV-Vis-NIR spectrometer. Fluorescence emission spectra and frequency domain lifetime measurements were obtained using Jobin Yvon Fluorolog 3 and the samples were excited at 780 nm. The dynamic range of the Fluorolog 3 for lifetime measurements is 0 – 300 MHz. The absorption-emission profile of IR800 conjugated to HSA in sodium phosphate buffer (\( \text{Na}_3\text{PO}_4, \text{pH} \sim 8.0 \)) is shown in Figure
3.1a. The absorbance maximum of the protein-fluorophore complex is observed at 782 nm, and the fluorescence emission maximum at 804 nm. The IR800 molecules were conjugated to HSA to form the HSA-IR800 complex, which was then bound to NSs (NSs-HSA-IR800) and NRs (NRs-HSA-IR800) as demonstrated in Figure 3.1b.

Figure 3.1. (a) Absorption - emission profile ($\lambda_{\text{max-Ab}} \sim 782$ nm and $\lambda_{\text{max-Em}} \sim 804$ nm) of IR800 conjugated with HSA. Chemical structure of IR800 is provided as inset. (b) Schematic diagram illustrating the conjugation of HSA-IR800 protein-fluorophore complex to Nanoshells (NSs) and Nanorods (NRs) to form NSs-HSA-IR800 and NRs-HSA-IR800 respectively. The blue curved line represents HSA and red dots represent IR800 dye.
HSA-IR800 conjugation was performed at room temperature, and the protein-fluorophore complex was dialyzed for 24 hours to remove excess dye (see 3.2 section II). It was determined that approximately 16 molecules of IR800 were bound to each HSA molecule (see 3.2 section V). The bioconjugation of the HSA-IR800 complex to the nanoparticles was performed in Na₃PO₄ buffer at room temperature and protected from light, and excess protein-fluorophore mixture was removed by centrifuging the nanoparticle conjugates in small aliquots (see 3.2 section III). We determined that ~ 63 HSA-IR800 complexes were conjugated to each NS and ~ 4 HSA-IR800 complexes were bound to each NR (see 3.2 section V). However, the total number of HSA-IR800 complexes bound to NSs or NRs was kept constant by adjusting the nanoparticle concentration, such that the total surface area available for conjugation was equivalent for both NSs and NRs (see 3.2 section IV).

The geometry of the nanoparticles coated with HSA-IR800 was characterized with transmission electron microscopy (TEM). TEM micrographs of NSs with dimensions \([r_1, r_2] = [63, 78]\) nm, where \(r_1\) is the radius of the silica core and \(r_2\) is the radius of the Au shell, are shown in Figure 3.2a. NRs, \([w, l] = [11, 46]\) nm, where \(w\) is the width and \(l\) is the length, are shown in Figure 3.2c. The NSs and NRs coated with a uniform 8 ± 3 nm layer of HSA are shown in Figure 3.2b and 3.2d, respectively.
Figure 3.2. TEM micrographs of (a) NSs $[r_1, r_2] = [63, 78]$ nm, (b) NSs coated with $8 \pm 3$ nm HSA-IR800, (c) NRs $[w, l] = [11, 46]$ nm, and (d) NRs coated with $8 \pm 3$ nm HSA-IR800.

Figure 3.3. Surface plasmon resonance shift when nanoparticles are conjugated with HSA-IR800 (a) NSs (solid red), $\lambda_{\text{max}} \sim 800$ nm and NSs coated with HSA-IR800 (dash), $\lambda_{\text{max}} \sim 805$ nm, and (b) NRs (solid blue), $\lambda_{\text{max}} \sim 796$ nm and NRs coated with HSA-IR800 (dash), $\lambda_{\text{max}} \sim 804$ nm. The peak at 280 nm is protein absorbance peak.
3.4 Experimental Fluorescence Enhancement

Figure 3.4. (a) Extinction spectra of (i) NSs-HSA-IR800, plasmon maximum at ~ 805 nm, and (ii) NRs-HSA-IR800, plasmon maximum at ~ 804 nm are shown. Spectra are offset for clarity. (b) Fluorescence (FL) spectra, λmax ~ 804 nm of (i) NSs-HSA-IR800, (ii) NRs-HSA-IR800, and (iii) control sample HSA-IR800 are shown.

The plasmon resonance of NSs and NRs shifts to longer wavelengths by 5 – 8 nm when HSA-IR800 is adsorbed, due to the higher refractive index of HSA than H2O113,114 shown in Figure 3.3. The plasmon resonances of the nanoparticles were tuned to the emission wavelength of the fluorophore-protein complex to maximize the fluorescence enhancement.75 The extinction maximum of NSs-HSA-IR800 was observed at ~ 805 nm, and that of NRs-HSA-IR800 was observed at ~ 804 nm, as shown in Figure 3.4a. The corresponding emission spectra of HSA-IR800 conjugated to the nanoparticles are shown in Figure 3.4b. HSA-IR800 was used as the control sample rather than IR800 in aqueous solution to ensure that the fluorophore was in essentially the same chemical environment in all measurements. The emission spectra of nanoshells coated with HSA without IR800 and nanorods coated with HSA without IR800 are also provided in Figure 3.5. The
nanoparticles exhibited no fluorescence in the near-infrared without the fluorophore. The reference sample as well as the nanoparticle-conjugates was excited at 780 nm and the fluorescence spectra were collected in solution under identical excitation and detection conditions, allowing direct comparison of the various nanoparticle-fluorophore complexes. A maximum fluorescence enhancement of ~ 40 was measured for NSs-HSA-IR800 relative to the control sample, and an enhancement of ~ 9 was found for NRs-HSA-IR800.

![Emission spectra](image)

**Figure 3.5.** Emission spectra of control, HSA-IR800 (black), nanoshells coated with HSA without IR800 (red), and nanorods coated with HSA without IR800 (blue) are shown. Fluorescence is not observed from the nanoparticles without the fluorophore.

### 3.5 Calculated and Experimental Scattering Efficiency of Nanostructures

Since the nanoparticles used in this experiment vary in size, their relative scattering cross sections also differ significantly, which directly affects their fluorescence enhancement efficiency. We determined the scattering intensities of the NSs and NRs by calculating their scattering cross sections using experimentally obtained nanoparticle sizes. The calculated scattering intensities for NSs \([r_1, r_2] = [63, 78]\) nm in aqueous medium were obtained using Mie theory, and the maximum plasmon resonance peak was
observed at 800 nm. The theoretical scattering intensity of NRs was calculated by using the finite element method (FEM) (as implemented by COMSOL Multiphysics). The nanorods were modeled as cylindrical objects with hemispherical end-caps in H₂O, estimating size obtained from TEM \( [w, l] = [11, 46] \) nm. The dielectric function used for Au was obtained from literature values. The calculated longitudinal plasmon mode of NRs, showed a maximum at a wavelength of 824 nm in water. The experimentally observed longitudinal plasmon resonance of NRs (804 nm) differs from that calculated theoretically because the energy of the longitudinal mode depends strongly on the end cap geometry. Nonetheless, the calculated scattering spectra shown in Figure 3.6 indicate that NSs scatter more efficiently than NRs for the sizes of nanoparticles studied.

![Calculated scattering spectra of (i) NSs \([r_1, r_2] = [63, 78]\) nm, \(\lambda_{max} = 805\) nm and (ii) NRs \([w, l] = [11, 46]\) nm, \(\lambda_{max} = 824\) nm.](image)

The scattering measurements were also obtained experimentally by utilizing solutions with similar concentrations of NSs and NRs (Fig. 3.7A). The nanoparticle solutions were positioned in a 6.25 mm path length cylindrical cuvette and placed on a
goniometer which rotated the collection point around the sample while keeping the angle of the sample fixed. A 785 nm diode laser was used as the excitation source, along with appropriate combination of filters and linear polarizers for light propagation and detection. The scattered light from the nanoparticles was detected by a CCD array connected to a spectrograph. The scattering intensities of the nanoparticles were then determined by obtaining the scattered light intensity as a function of polar angle. The measurements range from 0° to 360°, however measurements between 130° - 180° were not obtained due to constraints in the experimental set-up. The spectra are not completely symmetric for rotation over 90° attributable to a small misalignment in the apparatus. We observe that NSs scatter by a factor of ~ 34 more than NRs. (Fig. 3.7B) The larger scattering efficiency of NSs relative to NRs studied here correlates with the larger fluorescence enhancement observed for IR800 by NSs.

Figure 3.7. (a) Schematic diagram representing angle-resolved fluorescence emission apparatus. M1: Mirror1, M2: Mirror 2, P1: Polarizer1, P2: Polarizer2, λ/2: Half-wavelength plate, F1: Laser selection filter, F2: Long pass filter, RM: Rotating mount with knob to control the angle of the sample relative to the excitation light (Red circle). Black solid line indicates laser light path. (b) Experimental angle-resolved scattering spectra of NSs (red circles) and NRs (blue circles). Spectra are normalized by concentration of nanoparticles. The radial axis ranges from 0 – 4.
3.6 Frequency Domain Lifetime Decay

Figure 3.8. Frequency domain fluorescence decay of (a) IR800 (τ ~ 564 ps), (b) HSA-IR800 (τ ~ 427 ps), (c) NRs-HSA-IR800 (τ ~ 121 ps), and (d) NSs-HSA-IR800 (τ ~ 68 ps). The curves referring to phase and modulation are shown with arrows.

In addition to the scattering characteristics of the nanoparticles, increasing the radiative decay rate of the fluorophore also contributes substantially to fluorescence enhancement. The change in radiative lifetime for IR800 conjugated to NSs and NRs via HSA was determined experimentally by monitoring the frequency domain fluorescence decay in aqueous solution. The lifetime spectra of IR800, HSA-IR800, NRs-HSA-IR800, and NSs-HSA-IR800 are shown in Figure 3.8. The intrinsic lifetime of the fluorophore was measured to be ~ 564 ps, which corresponds well to the previously reported value. The lifetime decreased to 427 ps when IR800 molecules were bound to HSA (Fig. 3.8b). This
is due to a small enhancement in quantum yield of the fluorophore when conjugated to the protein, attributable to an increased steric stabilization of IR800 bound to HSA. This reduces the mobility of the fluorophore in aqueous solution, resulting in higher stability and a decreased lifetime. The lifetime of IR800 bound to the nanoparticles via HSA was reduced significantly to 121 ps for NRs-HSA-IR800 and 68 ps for NSs-HSA-IR800. These measured lifetimes confirm the theoretical prediction of enhanced quantum yield and diminished lifetime of fluorophores near metallic surfaces.

The fluorescence lifetime data can be evaluated in terms of a single exponential (SE) model or a multi exponential (ME) model. A SE model is appropriate for samples consisting of a single fluorophore in a homogenous environment, while a ME model describes the fractional contribution of decay time for each component present in a sample mixture. The results of fitting to a ME decay analysis are represented in Table 3.1. The decay rate for IR800 in aqueous media, in the absence of any other component in the solution, fits a SE decay model. However, the HSA-IR800 complex required a ME decay model, demonstrating that two different environments are available to the fluorophore with distinct lifetimes in each individual environment. About 90% of the IR800 molecules were covalently attached to HSA with a fluorescence decay of 418 ps. The lifetime of the remaining fraction was similar to that observed for free IR800 in aqueous media, suggesting that a small fraction of unbound fluorophores remain in the HSA-IR800 solution. The ME analysis of the fluorescence decay of IR800 conjugated to nanoparticles indicates three distinct environments, which we interpret as IR800 molecules conjugated to the nanoparticle-HSA, IR800 molecules bound to HSA only, and the free fluorophore in solution. Most of the fluorophores in these samples exhibited
a significantly reduced lifetime, indicating that nearly all of the dye molecules were attached to the nanoparticles via the HSA layer. This analysis reveals that both nanoparticle samples had less than 2% of unbound HSA-IR800 complex and less than 1% of free fluorophore in the solution mixture. This is attributable to the high affinity of the protein for Au nanoparticle surface as well as the use of a fluorophore with a chemical linker which can covalently attach to the protein. Therefore, the nanoparticle-HSA-IR800 system provides a useful complex for determining individual lifetime components. The $\chi^2_R$ values shown in the last column of Table 3.1 are the goodness of fit parameter, obtained by fitting calculated values to experimentally obtained parameters by a nonlinear least-squares deconvolution method. The $\chi^2_R$ values represented here are within 5–10% of the random deviations in the data.\textsuperscript{118}

Table 3.1. Multiexponential analysis of intensity decay of IR800 with nanoparticles showing molecular fraction ($\alpha$), observed lifetime ($\tau_i$, ns), amplitude weighted lifetime ($\langle \tau \rangle$, ns), and goodness of fit parameter ($\chi^2_R$).

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\alpha_i$</th>
<th>$\tau_i$</th>
<th>$\langle \tau \rangle$</th>
<th>$\chi^2_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR800</td>
<td>1</td>
<td>0.564</td>
<td>0.564</td>
<td>1.57</td>
</tr>
<tr>
<td>HSA-IR800</td>
<td>0.076</td>
<td>0.547</td>
<td>0.427</td>
<td>1.54</td>
</tr>
<tr>
<td>NRs-HSA-IR800</td>
<td>0.008</td>
<td>0.550</td>
<td>0.121</td>
<td>1.42</td>
</tr>
<tr>
<td>NSs-HSA-IR800</td>
<td>0.005</td>
<td>0.568</td>
<td>0.068</td>
<td>1.39</td>
</tr>
</tbody>
</table>
3.7 Experimental Quantification of Quantum Yield

In order to determine the radiative rate enhancement from the experimentally measured changes in lifetime, the nonradiative decay rate must be known. By combining and rearranging quantum yield, $Q_0 = \frac{\Gamma}{\tau_0 + k_{nr}}$ and lifetime $\tau_o = \frac{1}{\Gamma + k_{nr}}$ equations (chapter 1), the radiative and nonradiative decay rate can be experimentally obtained:

$$\Gamma = \frac{Q_0}{\tau_0}$$  \hspace{1cm} (1)

$$k_{nr} = \frac{1}{\tau_0} - \Gamma.$$  \hspace{1cm} (2)

The quantum yield of IR800 ($Q$) was determined experimentally using Indocyanine green (ICG) as a reference sample with a known quantum yield ($Q_R$) of $\sim$ 1% in aqueous media.96 The NIR excitation-emission profile of ICG (excitation: 780 nm, emission: 820 nm) is similar to that of IR800 and is therefore ideal as a reference sample for quantum yield determination. The quantum yield was computed by measuring the optical density of solutions with equivalent concentrations of IR800 ($OD$) and ICG ($OD_R$) and by calculating the integrated fluorescence intensity of IR800 ($I$) and ICG ($I_R$):

$$Q = Q_R \cdot \frac{I}{I_R} \cdot \frac{OD}{OD_R} \cdot \frac{\eta}{\eta_R},$$  \hspace{1cm} (3)

where $\eta$ is the refractive index of sample medium and $\eta_R$ is the refractive index of the reference medium, which are equivalent in this experiment ($\eta = \eta_R = \eta_{H2O} = 1.33$). By using Eqn. (3), the quantum yield of IR800 was determined to be 7% in H$_2$O, and that of the HSA-IR800 complex was determined to be 11%. Using the quantum yield and lifetime measured independently, Eqns. 1 and 2 allow determination of the radiative and nonradiative decay rates of the fluorophore as shown in Table 3.2. The quantum yield

71
values obtained for IR800 in aqueous media as well as HSA-IR800 are comparable to those reported in the literature.\textsuperscript{119}

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|}
\hline
Sample & Q.Y. & $\Gamma$ & $k_{nr}$ \\
\hline
IR800 & 0.07 & $1.241 \times 10^8$ & $1.648 \times 10^9$ \\
HSA-IR800 & 0.11 & $2.576 \times 10^8$ & $2.084 \times 10^9$ \\
NRs-HSA-IR800 & 0.74 & $6.180 \times 10^9$ & $2.084 \times 10^9$ \\
NSs-HSA-IR800 & 0.86 & $1.262 \times 10^{10}$ & $2.084 \times 10^9$ \\
\hline
\end{tabular}
\caption{Quantum yield (Q.Y.), radiative decay rate ($\Gamma$) and nonradiative decay rate ($k_{nr}$) of IR800, HSA-IR800 and HSA-IR800 with nanoparticles.}
\end{table}

The radiative rate enhancement of IR800 induced by NSs and NRs and the improved quantum yields can now be calculated by rearranging the modified quantum yield $Q_M = \gamma_r \Gamma / (\gamma_r \Gamma + k_{nr})$ and lifetime $\tau_M = 1 / (\gamma_r \Gamma + k_{nr})$ near metallic surfaces (chapter 1):

$$\gamma_r \Gamma = \frac{1}{\tau_M} - k_{nr} \quad (4)$$

$$Q_M = \gamma_r \Gamma \tau_M \quad (5)$$

where $\tau_M$ is obtained from Table 3.1, and $k_{nr}$ is the non-radiative decay rate for HSA-IR800. We assume that $k_{nr}$ is unaltered from HSA-IR800 for both NS-HSA-IR800 and NR-HSA-IR800 complexes because no fluorescence quenching was observable and the chemical environment of the fluorophore was equivalent in all cases. The nonradiative decay rate does increase when the chemical environment of the fluorophore is changed\textsuperscript{6} from solution phase to being bound to HSA. Table 3.2 shows that the $k_{nr}$ increases when IR800 is bound to HSA compared to the free fluorophore in solution. On a metallic surface, the nonradiative decay rate also increases for short fluorophore-metal distances,
< 4 nm, since the nonradiative energy transfer rate depends on the inverse cube of the molecule-surface separation. However, in our nanostructure complexes, HSA provides a spacer layer of ~ 8 nm between the fluorophore and the metal nanoparticle surface. For this significantly larger metal-molecule distance, a significant increase in $k_{nr}$ due to the metal nanoparticle surface is not anticipated. Therefore we assume that $k_{nr}$ is essentially the same for HSA-IR800 in solution and for HSA-IR800 adsorbed sequentially onto a nanoshell or nanorod surface. The radiative decay rates of NRs-HSA-IR800 and NSs-HSA-IR800 and the quantum efficiencies are also shown in Table 3.2. The high quantum yield of 86% for NSs-HSA-IR800 demonstrates that plasmonic enhancement can be used to create NIR-fluorescent species with similar intensities as visible dyes. Although the NRs achieve a lower quantum yield of 74% due to the lower scattering efficiency of the particles, this is still a very high value for a NIR fluorophore, and these structures would certainly also be useful as markers in fluorescence-based bioimaging where the smaller physical size of the NR-based nanoparticle complex would be desired.

The precise relative contribution of each process responsible for IR800 fluorescence enhancement, including absorption enhancement, scattering enhancement, and radiative decay rate enhancement, is difficult to determine since these processes are interdependent. Nevertheless, for the experimental parameters and nanoparticle geometries discussed here, the scattering efficiency of a nanoparticle appears to provide the most important mechanism for improving the quantum yield of a fluorophore. NRs predominantly enhance the emission of the fluorophore by absorption enhancement, owing to the high-intensity near field resulting from the longitudinal plasmon resonance.
However, due to the significant difference in scattering cross sections of NSs and NRs it is apparent that NSs increase the coupling efficiency of the fluorescence emission to the far field more efficiently than NRs. This explains the 40-fold fluorescence enhancement observed for IR800 bound to NSs compared to the 9-fold enhancement for IR800 bound to NRs. The radiative decay rate enhancement of the fluorophore is dependent on both the scattering efficiency as well as the absorption efficiency of nanoparticles. This explains why NRs enhance the quantum yield of IR800 by 74% as well as decrease the fluorophore’s lifetime considerably.

3.8 Conclusions

In conclusion, we have examined the fluorescence enhancement of IR800 conjugated to NSs and NRs, and we have shown that both NS and NR lead to large increases in quantum yield relative to the isolated fluorophore. We have observed that nanoshells are more efficient in improving the emissive properties of a fluorophore due to their significant scattering cross section at the emission wavelength of the fluorophore. Additionally, the near-field response of NSs gives rise to a considerable enhancement in the absorption, and the radiative decay rate of IR800, resulting in 40 fold enhancement and 86% quantum yield. IR800 molecules bound to NRs demonstrate a 9-fold emission enhancement and a 74% quantum yield, attributable to the high local field enhancement at the longitudinal plasmon wavelength. Utilizing Au nanoparticles with appropriate geometry and dimensions for emission enhancement is a useful strategy for enhancing the detection sensitivity of low-quantum-yield fluorescent emitters. This approach is also potentially valuable in biomedical imaging, and moreover can be conveniently generalized to enhance other fluorescent media.
Chapter 4: Nanoshells for Simultaneous Magnetic and Optical Imaging and Photothermal Therapeutic Response

4.1 Introduction

Fluorescence spectroscopy is a widely used research tool in molecular and translational biology which has enabled significant progress in medical diagnostics. In this chapter, we show an exciting biomedical application of plasmon enhanced Au nanoshell-ICG molecular conjugates for NIR fluorescence optical imaging (FOI) at the cellular level. Iron oxide nanoparticles were also integrated on the Au nanoshell surface to simultaneously enable MRI in addition to FOI. These novel nanoshell complexes are not only effective diagnostic probes but also enable photothermal tumor ablation when illuminated with resonant light. The design and development of the nanoshell complexes, their characterization, MRI data analysis, FOI and photothermal cancer therapy in vitro was performed by Rizia Bardhan. Wenxue Chen performed the cell culture and contributed in FOI, MRI and cancer therapy in vitro. Carlos Perez-Torres obtained MR images of nanoshell complexes and MRI in vitro. Reproduced with permission from Rizia Bardhan*, Wenxue Chen*, Carlos Perez-Torres, Marc Bartels, Ryan M. Huschka, Liang L. Zhao, Emilia Morosan, Robia G. Pautler, Amit Joshi and Naomi J. Halas, Adv. Func. Mater. 2009, 19, 3901-3909 (*equal contribution). Copyright 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

The rapid development of noninvasive diagnostic imaging modalities such as MRI and FOI has revolutionized biomedical research and practice. Each imaging technique has its own merits and drawbacks in terms of sensitivity, resolution, data acquisition time, and complexity. While numerous contrast agents for biological image
enhancement have been developed in the past decade, they have most often been limited to the enhancement of a single modality.\textsuperscript{124, 125} Contrast agents that enhance more than one imaging method provide a very important advance, enabling the use of multiple modalities to probe the same system, yielding more information than any single imaging method alone.\textsuperscript{126, 127} For example, multimodal contrast agents that simultaneously enhance MRI and FOI would combine the high sensitivity of FOI with the high spatial resolution of MRI.\textsuperscript{128} In practice, such a dual-modality contrast agent could be used in a single clinical procedure, for pre- and post-operative MRI, then for intraoperative FOI, providing enhanced imaging before, during, and after the procedure.\textsuperscript{126, 129} The addition of antibody targeting, so that the nanocomplex can bind to the surface receptors of specific cell types in the case of cancer, along with a therapeutic function, such as photothermal heating to induce cell death, would provide a full theranostic spectrum of capabilities in a single, practical nanocomplex. The availability of multiple diagnostic and therapeutic modalities in a single agent will streamline the regulatory processed in the pharmaceutical drug development pipeline, and thus significantly reduce the cost and complexity involved in translating novel therapies from in vitro and in vivo settings to human applications.

Here we report the design and demonstration of Au nanoshell-based, targeted, multimodality contrast agents in the NIR for MRI and FOI which also possess an effective photothermal therapeutic response. Nanoshells, consisting of a spherical dielectric core coated with a thin metallic shell, are ideal nanostructures for fabrication of these multifunctional probes. Their plasmon-derived optical resonance can be easily tuned to the NIR region of the spectrum, where tissue is maximally transparent, by
modification of their core and shell dimensions. This enables a broad range of diagnostic and therapeutic applications including optical imaging and photothermal cancer therapy. The plasmonic properties of nanoshells can also provide a significant fluorescence enhancement of NIR fluorescent ICG molecules when they are positioned at an appropriate distance (~10 nm) from the nanoshell surface. This can be accomplished by growing a dielectric layer around the nanoparticle to serve as a spacer between the fluorophore and the metallic nanoparticle surface. ICG, although extensively used for contrast enhancement in molecular imaging, is limited by a low quantum yield of ~1% in aqueous media. Recently, we have shown that the emission of weak NIR fluorophores such as ICG can be dramatically enhanced to ~80% for a weak fluorophore in proximity to nanoshells designed with a significant scattering cross section at the emission wavelength of the fluorophore. The enhancement is attributable to a combination of several processes including enhanced absorption, enhanced coupling efficiency of the fluorescence emission to the far-field, and enhanced radiative decay rate of the fluorophores which increases the quantum yield.

In this geometry, the spacer layer that separates the fluorophores from the metallic nanoparticle surface can also incorporate Fe₃O₄ nanoparticles for MRI enhancement. This minor modification in the chemical structure of the nanoparticle complex enables it to provide simultaneous MR and fluorescence enhancement integrated naturally into the same nanostructure. It is relatively straightforward to incorporate Fe₃O₄ nanoparticles at a sufficient density to promote increased magnetic interaction between neighboring Fe₃O₄ nanoparticles, resulting in efficient MR contrast. Binding the Fe₃O₄ nanoparticles to the nanoshell surface, rather than encapsulating them within the Au shell, preserves their
large magnetic moment and superparamagnetic behavior.\textsuperscript{132} Au coatings on iron oxide nanoparticles have been known to compromise the magnetic properties of iron oxide due to the large diamagnetic mass of Au.\textsuperscript{133}

The tunability of these nanocomplexes in the NIR region (700–900 nm) is highly advantageous since NIR light has been reported to penetrate deep into soft tissue, nearly \(\sim 10\) cm through breast and 4 cm through brain tissue using microwatt laser sources.\textsuperscript{35, 38, 134} The therapeutic response of nanoshells results from their ability to absorb NIR light resonant with the nanoshell plasmon energy and convert the light to heat.\textsuperscript{15, 17} The heat generated by the nanoshells raises the local temperature of tissues resulting in thermal ablation of cancer cells. Unlike current cancer treatment strategies such as chemotherapy and radiation therapy, whose toxicity leads to deleterious side effects, these benign, nontoxic nanoshell-based complexes are far less likely to induce side effects in clinical applications.

Molecular targeting of the nanocomplexes to cancer cells via antibodies against cell surface markers will selectively accumulate these agents in tumors, retard their clearance, and enable extended periods of imaging as well as photoinduced thermal ablation therapy.\textsuperscript{16, 135} Among the many known cell surface markers for targeting breast tumor, human epidermal growth factor receptor (HER2) has been identified as a promising target for antibody-based therapy. HER2 is expressed on healthy cells at \(\sim 10^4\) receptors per cells; however, in tumors overexpressing this marker, roughly \(10^6\) HER2 receptors per cell may be present.\textsuperscript{136, 137} HER2 has been used to target breast cancer cells because of its stable overexpression on \(~30\%\) of breast cancers: it is currently considered to be the best prognostic marker for breast cancer.\textsuperscript{138} Here, the nanocomplexes are conjugated with
anti-HER2 to target HER2-expressing breast carcinoma cells in vitro, imaged via MRI and FOI, and used for photothermal therapy.

4.2 Experimental Methods

**Fe$_3$O$_4$ Nanoparticles Fabrication:** The 10 ± 3 nm magnetic Fe$_3$O$_4$ nanoparticles were fabricated by following a procedure previously reported. Briefly, 0.85 mL of 12.1 N HCl (Fisher Scientific) and 25 mL of deionized, deoxygenated H$_2$O (bubbled with N$_2$ for 30 mins) were mixed together. Subsequently, 5.2 g of anhydrous FeCl$_3$ (Sigma) and 2 g of FeCl$_2$ (Sigma) are added in the solution under vigorous stirring. The brownish yellow solution is then added drop wise into 250 mL of 1.5 M NaOH solution under vigorous stirring. This step generated an instant black precipitate which was isolated in the magnetic field and the supernatant was removed by decantation. The precipitate was purified with deionized, deoxygenated H$_2$O and centrifuged at 1000 rcf several times and the final precipitate was stored in a N$_2$ environment for further characterization.

**Nanocomplexes Fabrication:** Au nanoshells (NS) [$r_1$, $r_2$] = [60, 74] nm were fabricated as described in Chapter 1. The Fe$_3$O$_4$ nanoparticles were functionalized with APTES overnight under mild stirring. After 24 hours of stirring, the nanoparticles were centrifuged twice at 1000 rcf and re-dispersed in H$_2$O. The amine terminated Fe$_3$O$_4$ nanoparticles were then added to the NS (2x10$^9$ particles/mL) drop-wise under vigorous stirring and left overnight. The NS coated with the Fe$_3$O$_4$ nanoparticles (NS@Fe$_3$O$_4$) were washed to remove excess APTES and unbound Fe$_3$O$_4$ nanoparticles. NS@Fe$_3$O$_4$ nanoparticles were then coated with a thin layer of silica to facilitate a biocompatible surface as well as to trap the fluorophore within the silica layer. The dye doped silica
layer was grown around the NS@Fe₃O₄ nanoparticles by slight modification of a previously reported protocol. Briefly, NS@Fe₃O₄ (2x10⁹ particles/mL) were mixed with 200 proof ethanol and 28% NH₄OH (Fisher) was subsequently added. Fresh TEOS was immediately added to the solution mixture under vigorous stirring followed by 500 uL of an ethanolic solution of ICG-APTES mixture. The vessel was sealed and vigorously stirred for 45 minutes. The nanocomplexes were stored in the refrigerator at 4 °C overnight protected from light. After 24 hours the particles were centrifuged and redispersed in ethanol. The absorbance of the supernatant was monitored to account for unbound fluorophore concentration in the supernatant. The concentration of the supernatant was then subtracted from the initial concentration of ICG which was added to the nanoparticles to calculate the amount of ICG doped within the silica layer of the nanocomplexes. The final nanocomplexes concentration after resuspension in ethanol was at ~ 10⁹ particles/mL and the ICG concentration was ~ 500 ± 50 nM. The control sample, 180 nm SiO₂ nanospheres doped with ICG molecules was prepared similarly and contained almost equivalent amount of ICG.

**Anti-HER2 conjugation to nanocomplexes:** The antibody conjugation is a three-step process; (i) anti-HER2 is biotinylated, (ii) nanocomplexes are coated with streptavidin and (iii) biotinylated antibodies are added to the streptavidin conjugated nanocomplexes.

**A. Anti-HER2 Biotinylation:** A 1 mM solution of Sulfo-NHS-Biotin (Pierce) reagent was added to the antibody solution, anti-HER2 (c-erbB-2 / HER-2 / neu epitope specific rabbit antibody 200 µg/mL, Thermo Scientific). The biotin-antibody conjugate is incubated at 4 °C for 3 hours followed by dialysis in PBS (phosphate buffered saline, pH
7.4) to remove excess biotin. The ratio of biotin/antibody was determined with an HABA colorimetric assay and the measured concentration was 5–7 biotins per antibody.

B. Streptavidin binding to nanocomplexes: The nanocomplexes were conjugated to streptavidin by initially functionalizing the nanocomplexes with (3-mercaptopropyl) triethoxysilane (MPTES, Sigma) to generate thiol terminated nanoparticles. These thiol terminated nanoparticles were re-dispersed in phosphate buffer at pH 7.5. Subsequently, a solution of streptavidin maleimide (Sigma) was added to the nanocomplexes and mildly stirred for 4 hours at 4 °C. The nanoparticles were centrifuged and resuspended in 5 mL phosphate buffer.

C. Biotinylated anti-HER2 binding to Streptavidin conjugated nanocomplexes: The antibody-biotin conjugate prepared in part B is added to the streptavidin conjugated nanocomplexes and incubated overnight at 4 °C. The nanoparticles are centrifuged at 300g for 5 mins in to remove unbound antibody and redispersed in phosphate buffer.

D. Number of antibodies per nanocomplexes: The number of antibodies per nanocomplex was quantified by utilizing ELISA. The nanocomplexes-anti-HER2 conjugates were incubated with horseradish peroxidase-labeled Anti-Rabbit IgG (HRP-AR, Sigma A0545) for 1 hour after non specific reaction sites were blocked with 3 % solution of bovine serum albumin (BSA, Sigma). The HRP bound nanocomplexes were developed with 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) and compared with a HRP anti-rabbit IgG standard curve ranging from 0.02–0.0003125 μg/ml. Results were analyzed using a spectrophotometer.

Cell culture for FOI: SKBR3 Breast Adenocarcinoma Human (Homo sapiens) cells were grown in DMEM/F-12 50/50 1X (Dulbecco’s Mod. of Eagle’s Medium/Ham’s F-12
50/50 mix with L-glutamine), 1% antibiotics and 10% fetal bovine serum (FBS). Cells were incubated at 37 °C in a 5% CO₂ environment and were detached from culture with trypsin (0.05%) and EDTA (0.02%) and re-suspended in media for passing to wells. The control cell line, MDA-MB-231 Breast Adenocarcinoma Human (Homo sapiens) cells were grown similarly at 37 °C in a 5% CO₂ environment and were detached from culture with trypsin and EDTA and re-suspended in media for passing to wells. 3x10⁵ cells of SKBR3 and MDA-MB-231 were plated in each well of 4 well plates respectively, and allowed to incubate. Subsequently, cells were washed with 1X PBS twice and fixed with PFA (3.7% paraformaldehyde in PBS). Cells were then quenched with Lysine-periodate and permeabilized with 0.2 % triton, following which they were washed twice with PBS. Normal goat serum solution is PBS was added to each well plate and incubated for 15 mins, following which excess NGS was removed and the cells were incubated with nanocomplexes conjugated with anti-HER2 and control, unconjugated nanocomplexes at particle concentration 2x10⁹ particles/mL for 2 hrs at 4 °C. After 2 hours, the cells were washed with PBS to remove unbound nanocomplexes, and subsequently the secondary antibody, Goat Anti-rabbit IgG-Alexa Fluor 488 (Invitrogen) was added to the wells and incubated for 1 hr at 4 °C. The cells were again washed with PBS while protected from light and excess secondary antibody was removed. The cell plates were then mounted on slides with mounting media containing DAPI and prepared for fluorescence imaging.

**Cell Culture for MRI:** 1x10⁶ cells of SKBR3 and MDA-MB-231 were plated in each well of 6 well plates respectively, and allowed to incubate. A similar procedure was followed as described in section V. After removing excess normal goat serum, the cells were incubated with nanocomplexes conjugated with anti-HER2, and unconjugated
nanocomplexes at particle concentration $2 \times 10^9$ particles/mL with $\sim 0.18$ mM Fe for 2 hrs at 4°C. After 2 hours, the cells were washed with PBS to remove unbound nanocomplexes. Subsequently, the cells were scraped from the bottom of the petridish, dispersed in 500 uL PBS, and centrifuged at 1100 rpm for 5 mins. The supernatant was then removed leaving $\sim 100$ uL cells containing nanocomplexes conjugated with anti-HER2 and unconjugated nanocomplexes respectively in the eppendorf tubes. 400 uL of 0.5% agarose gel was added to each tube and the samples were left at 4°C for 10 mins to allow the agarose to solidify. The tubes containing the solidified agarose gel with the nanocomplexes bound to cells suspended within the gel were directly utilized for MRI.

**Cell Culture and Photothermal Therapy:** $1 \times 10^6$ cells of SKBR3 and MDA-MB-231 were plated in each well of 6 well plates respectively, and allowed to incubate. Nanocomplexes-anti-HER2 conjugates as well as unconjugated nanocomplexes were dispersed in media (without FBS) at a concentration of $2 \times 10^9$ particles/mL and allowed to incubate with the SKBR3 cells and MDA-MB-231 cells for 2 hours. Following incubation, the cells were gently rinsed with PBS 3 times to remove unbound nanocomplexes and resuspended in media. The cells were then treated with a 200 mW laser diode with a wavelength of 808 nm at a power density of 3.72 W/cm$^2$ and spot size of $\sim 1$ mm diameter for 10 minutes each. After laser irradiation the cells were incubated with media for 4 hrs. Subsequently, the cells were rinsed with PBS 3 times and then cell viability was assessed. Calcein-AM and propidium iodide (PI) was purchased from Sigma Aldrich. A 2 mM solution of calcein was prepared in anhydrous DMSO (Sigma) and stored at -20 °C. Calcein-AM in extremely sensitive to moisture and hydrolyses rapidly. Immediately before use, a solution mixture with 2 µM calcein and 3 µM PI in
PBS was prepared and the cells were incubated with the dye mixture for 30 minutes. The cell plates were then mounted on slides and directly used for imaging without rinsing.

**Instrumentation:** The nanocomplexes were characterized using scanning electron microscope (SEM). Images were obtained on a FEI Quanta 400 environmental SEM at an accelerating voltage of 25 kV. Extinction spectra were obtained using a Cary 5000 UV/Vis/NIR spectrophotometer. X-ray diffraction (XRD) patterns were obtained using a Rigaku Ultima II vertical 0-0 powder diffractometer (CuKα, λ = 1.5418 Å). Magnetization measurements as a function of applied field were performed in a Quantum Design Magnetic Properties Measurement System (MPMS) superconducting quantum interference device (SQUID) magnetometer (H_{max} = 70 kOe). The ICP-OES analysis were done using a Perkin Elmer inductively coupled plasma optical emission spectrometer the spectral range of 165-800 nm. Fluorescence emission spectra were obtained using Jobin Yvon Fluorolog 3 and the samples were excited at 780 nm. Fluorescence images were obtained on a Leica fluorescence microscope. The images were obtained using cutoff filters with appropriate excitation and emission wavelengths:

<table>
<thead>
<tr>
<th>Dye</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI</td>
<td>360</td>
<td>470</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>480</td>
<td>530</td>
</tr>
<tr>
<td>ICG</td>
<td>720</td>
<td>820</td>
</tr>
<tr>
<td>Calcein</td>
<td>480</td>
<td>530</td>
</tr>
<tr>
<td>PI</td>
<td>520</td>
<td>620</td>
</tr>
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</table>

### 4.3 Characterization of Nanocomplexes

A schematic representing the fabrication procedure of the nanocomplexes is shown in Figure 4.1A. Water soluble Fe₃O₄ nanoparticles were synthesized as previously reported^{139} and functionalized with (3-aminopropyl) triethoxysilane (APTES) molecules.
NS were then coated with amine terminated Fe₃O₄ nanoparticles (NS@Fe₃O₄) and subsequently surrounded with a thin silica epilayer with the fluorophore, ICG, doped within the silica layer, forming the nanocomplexes. The SiO₂ layer not only traps the fluorophore but also encapsulates the Fe₃O₄ nanoparticles, providing a chemically inert and biocompatible surface. ICG is stabilized within the protective silica shell which decreases photobleaching of the fluorophore due to interaction with aqueous media. The silica shell also allows straightforward conjugation of antibodies and other biomolecules for in vitro applications.

Figure 4.1. (A) Schematic diagram illustrating fabrication of nanocomplexes for imaging and therapy. Au Nanoshells (NS) were coated with Fe₃O₄ and SiO₂ with the fluorophore, ICG, doped within the SiO₂ layer. Low resolution SEM images of (B) NS [r₁, r₂] = [60, 74] nm, (C) NS@Fe₃O₄, where the Fe₃O₄ nanoparticles are 10 ± 3 nm in diameter, and (D) Nanocomplexes, where the SiO₂ layer is 10 ± 4 nm. Corresponding high resolution SEM images of (E) NS, (F) NS@Fe₃O₄, and (G) Nanocomplexes.

Low resolution scanning electron microscopy (SEM) images of NS with dimensions [r₁, r₂] = [60, 74] nm, where r₁ is the radius of the silica core and r₂ is the
radius of the Au shell, is shown in Figure 4.1B. A low resolution SEM image of NS coated with 10 ± 3 nm Fe3O4 nanoparticles is shown in Figure 4.1C and nanocomplexes with a 10 ± 4 nm SiO2 layer is shown in Figure 4.1D. The corresponding high resolution SEM images of the NS, NS@Fe3O4, and nanocomplexes are shown in Figure 4.1E-G. Crystallographic studies of the nanocomplexes using powder X-ray diffraction (XRD) show strong Au peaks as well as Fe3O4 peaks (Fig. 4.2A). The diffraction from Au dominates the pattern and the Fe3O4 peaks are relatively weaker, due to the heavy atom effect of Au. The Au peaks represent a cubic phase with cell parameters a = c = 4.0786 Å and space group Fm3m (225) (JCPDS card no. 98-000-0230). The Fe3O4 peaks observed in the XRD spectrum indicate a highly crystalline cubic phase of Fe3O4 with cell parameters a = c = 8.3969 Å and space group Fd-3m (227) (JCPDS card no. 98-000-0294). The corresponding XRD intensity profile of Au and Fe3O4 from the powder diffraction database is also included for reference. Transmission electron microscope image and XRD spectrum of the Fe3O4 nanoparticles is provided in Figure 4.3.

The extinction spectra of NS, NS@Fe3O4, and the nanocomplexes are shown in Figure 4.2B. The plasmon resonances of the nanocomplexes are tuned to match the emission wavelength of the fluorophore to maximize the fluorescence enhancement of ICG.105 The NS have a plasmon resonance at 770 nm, which redshifts to 815 nm when coated with Fe3O4. This significant redshift is due to the higher refractive index of Fe3O4 (n=3) relative to H2O (n=1.33).93 The extinction spectrum shifts to 822 nm when the NS@Fe3O4 nanoparticles are coated with additional silica. The corresponding emission spectrum of ICG doped within the silica layer of nanocomplexes is shown in Figure 4.2C-i. The fluorescence maximum was observed at 820 nm.
Figure 4.2. (A) Powder XRD patterns of nanocomplexes and corresponding XRD intensity profile of Au and Fe$_3$O$_4$ from powder diffraction database. (B) Extinction spectra of (i) NS, $\lambda_{\text{max}} \sim 770$ nm, (ii) NS@Fe$_3$O$_4$, $\lambda_{\text{max}} \sim 815$ nm, and (iii) nanocomplexes, $\lambda_{\text{max}} \sim 822$ nm. (C) Fluorescence (FL) spectra of (i) ICG doped within the silica layer of nanocomplexes, $\lambda_{\text{max-Em}} \sim 820$ nm, and (ii) control, ICG doped within SiO$_2$ nanospheres. Optical image of nanocomplexes dispersed in aqueous media and with magnet (shown with arrow) is provided as inset. Magnetization as a function of applied magnetic field of nanocomplexes at (C) 5 K, (D) 300 K, and (E) 300 K where external magnetic field is cycled between -70 kOe to 70 kOe.
ICG doped within 180 nm diameter silica nanospheres, following a similar procedure as the nanocomplexes, was used as a reference sample rather than ICG in aqueous solution, to ensure the molecules are in similar chemical environments for fluorescence quantification. The fluorescence spectra were collected in solution under identical excitation and detection conditions, allowing direct comparison of the nanocomplexes with the reference sample. Additionally, excess dye was removed by centrifuging both sample types, and the supernatant was monitored to quantify the concentration of fluorophore present. A maximum fluorescence enhancement of ~45X is achieved for ~500 ± 50 nM ICG doped within the silica layer of nanocomplexes relative to the reference sample. The enhancement of ICG by nanoshells observed in these complexes is comparable to that reported previously.\textsuperscript{105}

An optical image of the nanocomplexes dispersed in aqueous media is shown as an inset in Figure 4.2C. Upon placement of an Nd2Fe14B magnet next to the vial, the nanocomplexes collect near the magnet within ~2 hrs resulting in a transparent solution (shown in the adjacent optical image). The slower magnet-induced movement of the nanocomplexes compared to the as-fabricated Fe3O4 nanoparticles (30 minute collection time) is expected due to relatively large diamagnetic mass of Au present on the nanocomplexes.

In addition to their high quantum efficiency NIR fluorescence, the nanocomplexes also possess robust magnetic properties. The magnetization as a function of applied magnetic field at 5 K and 300 K is shown in Figure 4.2D-F. At 5 K, the thermal energy is insufficient to induce magnetic moment randomization, thus the nanoparticles show typical ferromagnetic hysteresis loops with remanence of 4.2 emu/g\textsuperscript{1} and coercivity of
385 ± 10.2 Oe. However, at 300K the thermal energy is enough to randomize the magnetic moments, leading to a decrease in magnetization, thus the nanoparticles show no remanence or coercivity.

Figure 4.3. (A) TEM image of 10 ± 3 nm diameter Fe₃O₄ nanoparticles. Scale bar is 100 nm. (B) Powder X-ray diffraction patterns of Fe₃O₄ nanoparticles and corresponding XRD intensity profile of Fe₃O₄ from powder diffraction database. Magnetization as a function of applied magnetic field of Fe₃O₄ nanoparticles at (C) 5 K, (D) 300 K, and (E) 300 K where external magnetic field is cycled between -70 kOe to 70 kOe. At 5K the Fe₃O₄ nanoparticles show a remanence of 18.3 emug⁻¹ and coercivity of 424.2 ± 1.6 Oe. At 300 K there is no remanence or coercivity which is typical superparamagnetic behavior. The saturation magnetization of the Fe₃O₄ nanoparticles is 58.2 emug⁻¹.

To evaluate the response of the nanocomplexes to an external magnetic field, the magnetization was measured at 300 K by cycling the field between -70 kOe and 70 kOe (Fig. 4.2F). The saturation magnetization, μsat, was determined to be 17.98 emug⁻¹ at 70 kOe, which is significantly higher than the μsat values reported for other Au-Fe₃O₄ or Au-
The saturation magnetization of the nanocomplexes was observed to be lower than that of the Fe$_3$O$_4$ nanoparticles, 58.2 emug$^{-1}$, (Fig. 4.3), which is possibly due to the large diamagnetic mass of Au contributing to the total mass of the nanocomplexes. Additionally, the presence of canted or noncollinear surface spins, which has been observed in ferromagnetic materials with substituted diamagnetic neighbor cations, may also contribute to a decrease in saturation magnetization, since saturation of these spins usually requires a higher magnetic field.$^{133,141,142}$

### 4.4 Magnetic Resonance Analysis and Relaxivity

MRI experiments were performed on a Bruker Avance Biospec, 9.4 T spectrometer, 21 cm bore horizontal imaging system (Bruker Biospin, Billerica, MA) with a 35 mm volume resonator. Two types of imaging experiments were carried out on the nanocomplexes dispersed in aqueous media with Fe$_3$O$_4$ concentration ranging from 0 mM to 0.2 mM. For each sample, a quantitative T$_2$ measurement scan was conducted which consisted of a spin echo sequence with 8 echoes and TR/TE/IE equal to 1000/10.29/10.29 ms with a Field of View (FOV) of 30x30 mm, slice thickness of 2 mm and an acquisition matrix of 256x256 yielding an in plane resolution of 120x120 μm. Additionally, a T$_2$ weighted image was acquired for all samples at the same position. The T$_2$ weighted scan had a spin echo sequence with a TR/TE equal to 2000/20 ms with 2 averages. Acquisition was same as above with a FOV of 30x30 mm, slice thickness of 2 mm and an acquisition matrix of 256x256 yielding an in plane resolution of 120x120 μm.
Figure 4.4. (A) T₂-weighted MR images of nanocomplexes in aqueous media at various concentrations. The [Fe] concentration (mM) in each sample is provided at the bottom of the respective images. (B) MR image intensity as a function of echo time of nanocomplexes in aqueous media at various concentrations. (C) Spin–Spin relaxation rate (T₂⁻¹) as a function of Fe concentration of nanocomplexes (color coordinated with reference to Figure 4.4B). r₂ is the relaxivity obtained from the slope.

Magnetic resonance (MR) images of the nanocomplexes were obtained at 9.4 Tesla and 400 MHz. From the MR images the value of T₂, the transverse, or spin-spin relaxation, was evaluated. The T₂-weighted MR images (echo time = 20 msec) of the nanocomplexes in aqueous media with Fe concentrations ranging from 0 mM – 0.2 mM were obtained (Fig. 4.4A). The Fe concentrations in our nanocomplexes were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). As the Fe concentration increases, the signal intensity of the MR images decreases, as expected for T₂ contrast agents. T₂ is determined from the slope of the normalized image intensity as a function of echo time (Fig. 4.4B). The increasing Fe concentration leads to a significant decrease in image intensity due to a shortening of the spin-spin relaxation time of water.
(Fig. 4.4A, B). The specific relaxivity, $r_2$, a measure of the change in spin-spin relaxation rate ($T_2^{-1}$) per unit concentration, was determined to be 390 mM$^{-1}$sec$^{-1}$ for the nanocomplexes (Fig. 4.4C). This high $r_2$ could be due to the large external magnetic field (9.4 T) applied to the nanocomplexes, as well as their magnetic properties. Based on the SEM images and the amine functionalization protocol, a nearly saturated coverage of the NS surface with Fe$_3$O$_4$ nanoparticles is estimated. Thus the interparticle distance between the Fe$_3$O$_4$ nanoparticles bound to the nanoshell surface is probably extremely small, resulting in an increased magnetic interaction among the nanoparticles and an enhanced specific relaxivity. Some aggregation of Fe$_3$O$_4$ nanoparticles on the nanoshell surface may have occurred which would contribute in enhancing the relaxivity. Additionally, the porous silica shell present on the nanocomplexes may increase the molecular motion of H$_2$O within the pores and enhance the proton relaxation rate. Enhanced proton relaxation rates within porous silica matrices have been previously reported.$^{143,144}$ These combined reasons could result in increased $T_2$ shortening and a consequent increase in specific relaxivity. The MR images and $r_2$ for the as-synthesized Fe$_3$O$_4$ nanoparticles is provided in Figure 4.5.

The $r_2$ values with increasing magnetic field strengths for superparamagnetic iron oxide (SPIO) and ultrasmall superparamagnetic iron oxide (USPIO) -based MRI contrast agents of various sizes is provided in Table 4.1 for comparison.$^{145-150}$ It is worth noting that at high magnetic field strengths the two contrast agents approved by the US Food and Drug Administration (FDA), AMI-25 and Resovist, have a considerably lower $r_2$ than the nanocomplexes we have synthesized. Some single modality contrast agents including
AMNP and USPIO (serial no.1) have been reported with relaxivities comparable to our nanocomplexes.

![Graph showing T2-weighted MR images of Fe3O4 nanoparticles in aqueous media at various concentrations.](image)

![Graph showing MR image intensity as a function of echo time of Fe3O4 nanoparticles in aqueous media at various concentrations.](image)

![Graph showing spin-spin relaxation rate (T2⁻¹) as a function of Fe concentration.](image)

**Figure 4.5.** (A) T₂-weighted MR images of Fe₃O₄ nanoparticles in aqueous media at various concentrations. The [Fe] concentration in each sample is provided at the bottom of the respective images. (B) MR image intensity as a function of echo time of Fe₃O₄ nanoparticles in aqueous media at various concentrations. (C) Spin – Spin relaxation rate (T₂⁻¹) as a function of Fe concentration (color coordinated with Fig. 4.5B).

**Table 4.1.** Comparison of size and T₂ relaxivity of nanocomplexes with various USPIO / SPIO agents at increasing magnetic field strengths

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Agent</th>
<th>Particle Size (nm)</th>
<th>r₂</th>
<th>Field (T)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>USPIO</td>
<td>20-100</td>
<td>410 mM⁻¹s⁻¹</td>
<td>4.7</td>
<td>145</td>
</tr>
<tr>
<td>2</td>
<td>AMI-25 (FDA)</td>
<td>120 – 180</td>
<td>132 mM⁻¹s⁻¹</td>
<td>7</td>
<td>146</td>
</tr>
<tr>
<td>3</td>
<td>USPIO</td>
<td>30</td>
<td>106 mM⁻¹s⁻¹</td>
<td>7</td>
<td>147</td>
</tr>
<tr>
<td>4</td>
<td>SPIO</td>
<td>50</td>
<td>247 mM⁻¹s⁻¹</td>
<td>7</td>
<td>148</td>
</tr>
<tr>
<td>5</td>
<td>AMNP</td>
<td>35</td>
<td>350 mM⁻¹s⁻¹</td>
<td>7</td>
<td>149</td>
</tr>
<tr>
<td>6</td>
<td>Resovist (FDA)</td>
<td>62</td>
<td>280 mM⁻¹s⁻¹</td>
<td>9.4</td>
<td>150</td>
</tr>
<tr>
<td>7</td>
<td>Nanocomplexes</td>
<td>185</td>
<td>390 mM⁻¹s⁻¹</td>
<td>9.4</td>
<td></td>
</tr>
</tbody>
</table>
4.5 Antibody Conjugation and Quantification of number of Antibodies

Figure 4.6. (A) Schematic diagram showing anti-HER2 conjugation to nanocomplexes via streptavidin-biotin binding. (B) ELISA results showing number of biotinylated-anti HER2 antibodies bound per nanocomplexes which are functionalized with streptavidin, relative to the control sample, nanocomplexes without streptavidin.

The efficacy of the nanocomplexes was also visualized in vitro by the molecular targeting of HER2+ breast cancer cells and performing MRI and FOI. As represented in the schematic diagram (Fig. 4.6A), the silica surface was terminated with thiols using a thiolated silane coupling agent, 3(mercaptopropyl) triethoxysilane, followed by covalent attachment of streptavidin maleimide. The maleimide group forms a thioester bond with the thiol on the silica surface. Anti-HER2 was initially biotinylated and then bound to the streptavidin conjugated nanocomplexes at physiological pH and 4 °C. The extraordinary affinity of avidin for biotin, \((K_a = 10^{15} \text{ M}^{-1})\) the strongest known noncovalent interaction of a protein and ligand, is a practical and widely used nanoparticle bioconjugation
The number of antibodies attached to the nanocomplexes was quantified by enzyme-linked immunosorbent assay (ELISA) shown in Figure 4.6B.

4.6 In vitro Magnetic Resonance Imaging

The nanocomplex-anti-HER2 conjugates and the control, unconjugated nanocomplexes, were incubated with HER2+ cells, SKBR3, at a concentration of $2 \times 10^9$ particles/mL, containing 0.18 mM Fe nanoparticles (determined by ICP-OES). MDA-MB-231 cells, which have been reported to have very low HER2 expression, was also incubated with nanocomplex-anti-HER2 conjugates and unconjugated nanocomplexes. MR images of the incubated SKBR3 cells embedded in 0.5% agarose gel, in a microcentrifuge tube, are shown in Figure 4.7Ai-ii. High resolution in vitro images were obtained on eppendorf 0.5 mL microcentrifuge tubes with cells suspended in 0.5% agarose using a 3D RARE (rapid acquisition with relaxation enhancement) sequence with a TR/TE equal to 2000/20 ms with a RARE factor of 8 leading to an effective TE of 60 ms. FOV was 20x20x10 mm with an acquisition matrix of 128x128x64 yielding an isotropic 156 μm resolution.

The cells labeled with nanocomplexes clearly appear as hypointense spots, indicated that the nanocomplex-anti-HER2 conjugates were bound to SKBR3 cells (Fig. 4.7Ai). Very few of the unconjugated nanocomplexes appeared to be bound to SKBR3 cells (Fig. 4.7Aii). MR images of the nanocomplex-anti-HER2 conjugates and the unconjugated nanocomplexes incubated with MDA-MB-231 cells showed significantly less MR contrast, as expected (Fig. 4.7Aiii-iv). Since the outer silica shell of the nanocomplexes possesses an overall negative charge it may permit nonspecific binding of the unconjugated complex to the positively charged proteins present in cells. This level of
nonspecific binding could be ameliorated by refinement of the surface chemistry of the nanocomplex.

Figure 4.7. (A) MR image of HER2 positive SKBR3 cells suspended in 0.5% agarose after incubating with (i) nanocomplexes-anti-HER2 conjugates with ~ 0.18 mM Fe₃O₄ and (ii) control, unconjugated nanocomplexes. MR images of HER2 negative MDA-MB-231 cells suspended in 0.5% agarose after incubating with (iii) nanocomplexes-anti-HER2 conjugates, and (iv) control. (B) Maximum intensity projection of 64x128x64 pixel threshold T₂ maps of the images corresponding to part B. (i) SKBR3 cells with nanocomplexes-anti-HER2 conjugates and (ii) control. (iii) MDA-MB-231 cells with nanocomplexes-anti-HER2 conjugates and (iv) control. The hypointense signals observed in parts B and C are the cells labeled with the nanocomplexes.

The maximum intensity projections (MIP) of 64x128x64 pixel threshold T₂ maps where each pixel represents the cubic volume of 156x156x156 μm are shown in Figure 4.7Bi-iv. The MIP were created from the 3D MRI data using a threshold segmentation approach in MATLAB® (2008a, The Mathworks, Natick, MA). The threshold was set at the average minus twice the standard deviation of the sample. Pixels under this value were considered to be hypointense and are labeled as black in the image. The surrounding
normointense agarose is labeled as a transparent pink. The hypointense pixels represent
the nanocomplex-labeled cells and the normointense pixels represent agarose. Each
hypointense pixel should contain a cluster of labeled cells since the scan resolution is
insufficient to identify individual cells. The number of hypointense pixels was quantified
to determine the specificity and selectivity of the nanocomplex-anti-HER2 conjugates in
targeting HER2+ carcinoma cells. Both the MDA-MB-231 and SKBR3 cells that had
been incubated with the unconjugated nanocomplexes showed approximately equal
counts of hypointense pixels. The MDA-MB-231 cells incubated with nanocomplex-anti-
HER2 had 6.5X the number of hypointense pixels relative to the cell samples incubated
with unconjugated nanocomplexes. In contrast, SKBR3 cells incubated with
nanocomplex-anti-HER2 conjugates had 26X the number of hypointense pixels relative
to SKBR3 cells incubated with the unconjugated nanocomplexes. SKBR3 cells incubated
with nanocomplex-anti-HER2 conjugates also had 4X the number of hypointense pixels
as the MDA-MB-231 cells incubated with the nanocomplex-anti-HER2 conjugates.

4.7 In vitro Fluorescence Optical Imaging
The targeting of the multimodality nanocomplex-antibody conjugates to tumor cells in
vitro was further confirmed by fluorescence optical imaging. Fluorescence images of
SKBR3 cells and MDA-MB-231 cells incubated with nanocomplex-anti-HER2
conjugates and unconjugated nanocomplexes were obtained, as shown in Figure 4.8. An
image of the DAPI-stained nuclei of SKBR3 cells is shown in Figure 4.8A (blue). The
cytoplasm marker, a secondary antibody conjugated with visible dye (Goat Anti-rabbit
IgG-Alexa Fluor 488) is shown in Figure 4.8B (green), and the NIR fluorescence image
of the nanocomplex-anti-HER2 conjugate sample, is shown in Figure 4.8C (red). After 2 hour incubation with SKBR3 cells, the nanocomplexes appear to be specifically bound to the cell membrane, as observed in the merged fluorescence image (Fig. 4.8).

**Figure 4.8.** Fluorescence images of SKBR3 cells showing (A) nuclei stained with DAPI (blue) (B) cytoplasm stained with secondary antibody-Alexa-fluor 488 (green), (C) NIR fluorescence by ICG doped in silica layer of nanocomplexes-anti-HER2 conjugates (red), and (D) A, B, C merged together showing the nanocomplexes binding outside the cells. (E) Control, unconjugated nanocomplexes. Some non-specific binding was observed for the control. Fluorescence images of HER2 negative MDA-MB-231 cells showing (F) nanocomplexes-anti-HER2 conjugates and (G) control. Some non-specific binding to the extracellular matrix was observed for both nanocomplexes-anti-HER2 conjugates and control.

A small amount of the unconjugated nanocomplexes also appeared to be attached to the SKBR3 cells (Fig. 4.8E). In this case, with the absence of the primary antibody (anti-HER2), the secondary antibody does not bind as well, resulting in no observable signal from the Alexa Fluor 488. Nanocomplex-anti-HER2 conjugates were also
incubated with MDA-MB-231 cells (Fig. 4.8F). In this case, only a small fraction of nanocomplex-anti-HER2 conjugates were observed to associate with the extracellular matrix, resulting in a very weak NIR signal. Again, without the presence of the primary antibody the secondary antibody did not bind significantly, resulting in a low Alexa Fluor 488 signal. The unconjugated nanocomplex incubated with MDA-MB-231 cells also did not associate appreciably with the cell membrane (Fig. 4.8G). This sequence of control experiments demonstrates that the nanocomplex-anti-HER2 conjugates target HER2+ cells with significant specificity.

### 4.8 In vitro Photothermal Therapy

![Figure 4.9](image)

**Figure 4.9.** Photothermal ablation and live/dead stain of SKBR3 cells incubated with (A) nanocomplexes-anti-HER2 conjugates and (B) control, unconjugated nanocomplexes and treated with NIR laser at 808 nm for 10 minutes at a power density of 3.72 W/cm² and spot size of ~ 1 mm diameter. Live cells are stained green with calcein and dead cells are stained red with propidium iodide. Similar staining procedure for MDA-MB-231 cells incubated with (C) nanocomplexes-anti-HER2 conjugates and (D) control, and treated with NIR laser as well.
In addition to HER2-specific targeting, MRI and fluorescence enhancement, the nanocomplexes also demonstrated the therapeutic function of photothermally induced ablation in vitro. SKBR3 cells and MDA-MB-231 cells were incubated with nanocomplex-anti-HER2 conjugates and the unconjugated nanocomplex for 2 hours. The nanocomplexes were suspended in media appropriate for the cell line prior to incubation at a concentration of $2 \times 10^9$ particles/mL. Photothermal therapy was performed using a 200 mW laser diode with a wavelength of 808 nm at a power density of 3.72 W/cm$^2$ and a spot size of ~1 mm for 10 minutes.

The cells were incubated with a solution mixture of calcein AM (live cell stain) and propidium iodide (PI) (dead cell stain) for 30 minutes prior to imaging. Calcein AM is a cell-permeant, non-fluorescent compound which enters the cells and is hydrolyzed by intracellular esterases in live cells into the green fluorescent calcein. PI is membrane impermeant for live cells and binds to the DNA (and nucleic acids) of dead cells by intrabase intercalation, generating red fluorescence. The cells stained with Calcein/PI after treatment with NIR laser are shown in Figure 4.9. The nanocomplex-anti-HER2 conjugates were specifically bound to the cell membrane of the SKBR3 cells; upon laser irradiation the nanocomplexes produced hyperthermia, resulting in cell death. The increased PI uptake by the dead cells within the laser spot is clearly observed (Fig. 4.9A). MDA-MB-231 cells incubated with nanocomplex-anti-HER2 conjugates were also exposed to the same NIR laser treatment for 10 minutes, and showed minimal cell death (Fig. 4.9C). A nominal amount of the unconjugated nanocomplex was nonspecifically bound to both the SKBR3 cells as well as the MDA-MB-231 cells, and resulted in some cell death after laser treatment (Fig. 4.9B, D). Nanocomplexes by themselves, without
photothermal ablation, were observed to be innocuous to cells and no cell cytotoxicity was observed (data not shown).

4.9 Conclusions

In conclusion, we have designed a multimodal theranostic with bright NIR fluorescence for fluorescence image enhancement, MR contrast enhancement, antibody targeting of HER+2 cells, and photothermal therapy of breast cancer cells in vitro. The NS enhance the fluorescence of ICG while efficiently integrating Fe₃O₄ nanoparticles into the requisite silica spacer layer between the metallic shell layer and the ICG fluorophore, resulting in a contrast agent with high fluorescence quantum yield as well as high specific relaxivity. These properties may potentially allow the use of lower nanoparticle doses for contrast enhancement both in vitro and in vivo. The high relaxivity observed for this nanocomplex may also be valuable for cell trafficking studies as well as detection of single magnetically labeled cells. Nanoparticles in the size range of 20–200 nm have been also shown to accumulate preferentially at tumor sites through an enhanced permeability and retention effect.¹⁵² Therefore these nanocomplexes may be practical prospects for theranostics of small tumor volumes in vivo. The bioconjugation techniques utilized in this study are straightforward and can be conveniently generalized to bind other antibodies and peptides which may be useful for targeting different cancer cell lines.
Chapter 5: A Molecularly Targeted Theranostic Probe for Ovarian Cancer

5.1 Introduction

The targeting, diagnostics and therapeutic capability of the nanocomplexes discussed in last chapter can be straightforwardly extended for the detection and treatment of other cancer cells. In this chapter, we have explored the theranostic capabilities of the nanocomplexes for targeting, multimodal imaging via MRI and FOI, and photothermal cancer therapy of HER2 expressing ovarian cancer cells at the cellular level. This study provides an important strategy for the diagnosis and treatment of ovary cancer which is specifically important due to the aggressiveness of ovarian cancer, lack of early detection, chemotherapeutic resistance as well as resistance to many clinically relevant drugs. Rizia Bardhan prepared the nanocomplexes, characterized them, and contributed in FOI and cancer therapy in vitro. Wenxue Chen performed cell culture, FOI, MRI and cancer therapy in vitro. Reproduced with permission from Wenxue Chen*, Rizia Bardhan*, Marc Bartels, Carlos Perez-Torres, Robia G. Pautler, Naomi J. Halas, and Amit Joshi, Mol. Cancer Therapeutics, 2010, in press (*equal contribution). Copyright 2010 American Association for Cancer Research.

Epithelial ovarian cancer is the most lethal gynecologic malignancy, and is the fourth most frequent cause of cancer-related death of women in Western countries. In 2008, there were 21,650 new cases of ovarian cancer and 15,520 deaths reported in the United States. However, the difficulty in detecting ovarian cancer at an early stage, aggressiveness, and the lack of effective therapy contribute to high mortality. Currently recognized prognostic factors in advanced ovarian cancer (AOC) are primarily clinical,
including patient performance status and characteristics of tumor volume, stage classified by the International Federation of Gynecology and Obstetrics (FIGO), residual tumor size after initial surgery, and presence of ascites. However, most prognostic models proposed in the literature do not include biological factors. In 2004, Camilleri-Broët and coworkers studied HER2 overexpression in ovarian cancer by using immunohistochemically paraffin-embedded tissues and analyzed the prognostic impact of HER2 protein level. They found an independent prediction with both overall and disease-free survival on multivariate analysis. HER2 is frequently overexpressed in ovarian cancer patients; the overexpression rate has been reported in a wide range from 8% to 66%. The 66% figure was reported by fluorescence in situ hybridization (FISH) study in 74 cases; 53% in 181 cases, 22% in 23 cases, and 16% in 95 cases. Dimova et al. analyzed multiple samples applying the highly reliable method of FISH on tissue microarray, containing 1006 ovarian tumors and reported 8% HER2 amplifications in ovarian malignant epithelial tumors. HER2 overexpression is associated with a poor prognosis due to acquired chemotherapy resistance. In this work, HER2 expressing cell line OVCAR3 was chosen because it was isolated from a malignant effusion, which is resistant to clinically relevant doses of cyclophosphamide, adriamycin, and cisplatin. Studies on these cells would hence be particularly promising for demonstrating molecularly targeting and photothermal therapy.

The design and development of novel nanoagents that synergistically incorporate multiple functionalities, including targeting, imaging and therapy, all within the same nanoprobe is emerging rapidly as an impending alternative to traditional therapeutic drugs and imaging agents. This promising new paradigm is hence termed as
“Theranostic” which entails the efficient integration of therapeutic and diagnostic moieties into a single nanoagent. Nanoparticles in the size range of 5-250 nm are effective interventional agents for cancer because of their unique size, which allows passive accumulation in tumors, and because of their ability to carry multiple diagnostic and therapeutic payloads. The majority of clinical trials involving nanoparticles focus on targeted chemotherapy delivery,\textsuperscript{163} because despite the emerging molecular medicine based shift towards cancer-specific cytostatic agents, cytotoxic chemotherapy is still considered more effective against broad patient populations. While nanocarrier-based chemotherapy can minimize traditional side effects, it is not externally controlled and in the case of liposomes, which have been approved since the 1990s, the inability to guarantee intracellular drug delivery often results in treatment failure.

Recently, some theranostic nanoprobes have been synthesized with complex geometries\textsuperscript{164-168} however, their capability has been mostly limited to a single imaging modality such as either MRI or optical imaging. We have designed and utilized a multifunctional Au nanoshell-based theranostic complex (nanocomplex) to actively target, image via MRI and FOI, and induce photothermal tumor ablation in breast cancer cells with NIR illumination\textsuperscript{1}. In this study, we demonstrate the efficacy of these nanocomplexes for simultaneous diagnosis and therapy of ovarian cancer cells \textit{in vitro} and further demonstrate the non-toxicity of NIR therapy and the nanocomplexes. The nanocomplex consist of a Au nanoshell encapsulated in a SiO\textsubscript{2} shell, which is doped with Fe\textsubscript{3}O\textsubscript{4} nanoparticles and a NIR emitting fluorophore ICG. Au nanoshells are optically tunable nanoparticles consisting of a SiO\textsubscript{2} core surrounded by a thin Au shell.\textsuperscript{12} Based on
the relative dimensions of the shell thickness and core radius, nanoshells can be designed to scatter and/or absorb light over a broad spectral range, including the NIR. The NIR wavelength region provides maximal penetration of light through soft tissue, including hypoxic regions in tumors, and irradiation of tumors with NIR laser light can lead to thermal ablation.\textsuperscript{15} ICG molecules, with a quantum yield of $\sim$ 1.3\% in aqueous media, when incorporated into the oxide layer just outside the gold shell, the nanoshell enhances the fluorescence quantum yield by nominally 4500\%, resulting in a very bright NIR fluorescent probe.\textsuperscript{105} The Fe\textsubscript{3}O\textsubscript{4} nanoparticle layer concurrently provides a high MR contrast thus enabling multimodal imaging with the same agent. Accumulation of nanoshells in tumor cells can be achieved via passive extravasation based on the EPR of small particles associated with the leaky tumor vasculature.\textsuperscript{152} However, when targeted using antibodies against oncoproteins overexpressed on cell surfaces, a higher concentration of nanoshells can be selectively bound to cell surfaces and enable extended periods of imaging as well as therapy.

The photothermal properties of nanoshells are attributed to their ability to absorb NIR light at their plasmon resonant wavelength, due to their large absorption cross section, efficiently converting the light energy to heat. The heat generated by the nanoshells raises the local temperature in their direct vicinity, resulting in the thermal ablation of cancer cells.\textsuperscript{17, 169} In particular, targeted nanoshells will accumulate at the specific tumor site, enabling photothermal therapy of cancer cells only, and greatly minimizing damage to adjacent healthy cells. In this study, we have demonstrated that NIR laser irradiation at low power densities, as well as nanoshells by themselves, are non-toxic and do not induce cytotoxicity. These molecularly targeted nanocomplexes are
highly promising and clinically relevant for providing molecule-specific diagnostic information that will enable the detection and treatment of cancer long before phenotypic changes occur. In practice, this will provide an efficient tool for the detection of tumors at an early stage and provide a benign therapeutic strategy for cancer treatment.

5.2 Experimental Methods

**Anti-Her2 conjugated Nanocomplexes:** Au nanoshells (NS) \([r_1, r_2] = [60, 74]\) nm were fabricated as described in chapter 1. The nanocomplexes were developed as described previously in chapter 4. Anti-HER2 (c-erbB-2) / HER-2 / neu epitope specific rabbit antibody 200 μg/mL, Thermo Scientific) was conjugated to the nanocomplexes similarly as described in chapter 4. The number of antibodies was quantified by ELISA.

**In Vitro Fluorescence Optical Imaging:** OVCAR3 Ovarian Adenocarcinoma Human (Homo sapiens) cells, and the control cell line, MDA-MB-231 Breast Adenocarcinoma Human (Homo sapiens) cells were grown in MEM/F-12 50/50 \(1\times\) (Dulbecco's Mod. of Eagle's Medium/Ham's F-12 50/50 mix with L-glutamine), 1 % antibiotics and 10 % fetal bovine serum (FBS). Cells were incubated at 37 °C in a 5 % CO2 environment and were detached from culture with trypsin (0.05 %) and EDTA (0.02 %) and resuspended in media for passaging to wells. \(3 \times 10^5\) cells of OVCAR3 and MDA-MB-231 were plated in each well of 4 well plates respectively, and allowed to incubate. Subsequently, cells were washed with \(1\times\)PBS twice and fixed with PFA (3.7 % paraformaldehyde in PBS). Cells were then quenched with Lysine-periodate and permeabilized with 0.2 % triton, following which they were washed twice with PBS. 10 % Normal Goat Serum (NGS) solution was added to each well plate and incubated for 15 min, following which excess
NGS was removed and the cells were incubated with nanocomplex-anti-HER2 conjugates and unconjugated nanocomplexes at particle concentration $2 \times 10^9$ particles/mL for 2 h at 4 °C. After 2 h, the cells were washed with PBS to remove unbound nanocomplexes, following which the secondary antibody, Goat Anti-Rabbit IgG-Alexa Fluor 488 (Invitrogen) was added to the wells and incubated for 1 h at 4 °C. The cells were again washed with PBS while protected from light for excess secondary antibody removal. The cell plates were then mounted on slides with mounting media containing DAPI (Invitrogen) and prepared for FOI.

**In Vitro MRI:** $1 \times 10^6$ cells of OVCAR3 and MDA-MB-231 were plated in each well of 60×15 mm Style cell culture dishes respectively, and allowed to incubate. A similar procedure was followed as described in section III. After 2 h incubation with the nanocomplexes, the cells were washed with PBS, followed by scraping the cells from the bottom of the petri dish, dispersed in 500 µL PBS, and centrifuged at 1100 rpm for 5 min. The supernatant was then removed leaving ~100 µL cells containing nanocomplex-anti-HER2 conjugates, and unconjugated nanocomplexes in the Eppendorf tubes respectively. 500 µL of 0.5 % agarose gel was added to each tube and the samples were left at 4 °C for 10 min to allow the agarose to solidify. The tubes containing the solidified agarose gel with OVCAR3 and MDA-MB-231 cells, with the nanocomplexes suspended within the gel were directly utilized for MR Imaging.

**In Vitro Photothermal Therapy and Cytotoxicity:** OVCAR3 and MDA-MB-231 cells were grown in 6 wells plate and incubated with either nanocomplex-anti-HER2 conjugates or unconjugated nanocomplexes, which were already suspended in media appropriate for the cell line at a concentration of $2 \times 10^9$ particles/mL. Cells were
incubated with nanocomplexes for 2 h and then washed with PBS. Laser ablation was performed using an NIR laser at 808 nm (L808P200, Thorlabs Inc.) for 10 min at a power density of 5.81 W/cm² and spot size of ~0.8 mm diameter. After irradiation, cells were rinsed gently with PBS and incubated with media for 4 h. Cell viability was assessed using Calcein to stain live cells and propidium iodide (PI) to stain dead cells. A dye solution mixture containing 3 \( \mu \text{M} \) PI and 2 \( \mu \text{M} \) Calcein was prepared and 150 \( \mu \text{L} \) was added to each well with nanocomplexes which were photothermally ablated. For cytotoxicity studies, cells were incubated with nanocomplexes-anti-HER2 conjugates, unconjugated nanocomplexes, and no nanocomplexes. These cells were not illuminated with the NIR laser. After incubating the cells with Calcein/PI mixture for 30 min at 37 °C, a cover slip was mounted and imaged. In this stain, calcein AM enters the cells and is cleaved by esterases in the live cells to yield cytoplasmic green fluorescence. Dead cells are determined by plasma membrane integrity. This can be assessed in two ways: The ability of a cell to prevent a fluorescent dye from entering it and the ability of a cell to retain a fluorescent dye within it. As a cell dies, its plasma membrane becomes permeable enabling fluorescent dyes enter the cell. This allows PI to enter and bind to nucleic acids generating red fluorescence. PI is membrane impermeant and excluded from viable cells.

**Instrumentation:** MRI experiments were performed on a Bruker Avance Biospec, 9.4 T spectrometer, 21 cm bore horizontal imaging system (Bruker Biospin, Billerica, MA) with a 35 mm volume resonator. Extinction spectra were obtained using a Cary 5000 UV/Vis/NIR spectrophotometer. The ICP-OES analysis were done using a Perkin Elmer inductively coupled plasma optical emission spectrometer the spectral range of 165-800 nm. Fluorescence emission spectra were obtained using Jobin Yvon Fluorolog 3 and the
samples were excited at 780 nm. To acquire the fluorescence images we used a Leica fluorescence microscope (DM6000 B; Leica Microsystems GmbH) with a 100 W xenon lamp and specific filters. The images were obtained using cutoff filters with appropriate excitation and emission wavelengths (chapter 4).

5.3 Properties of Nanocomplexes

Figure 5.1. (A) Schematic representation of anti-HER2 conjugated nanoshell contrast agents. (B) Extinction spectra of nanoshells (blue) resonant in the NIR at 777 nm, nanoshells after coating with Fe$_3$O$_4$ (red) resonant at 821 nm, and after encapsulating in a silica SiO$_2$ epilayer (green) resonant at 842 nm. (C) Fluorescence spectrum of ICG doped in the silica layer of the nanoshells at 833 nm.

A schematic illustration of the nanocomplexes conjugated with antibody is depicted in Figure 5.1A. Au Nanoshells (NS) were surrounded with thin (~ 10 – 15 nm) epilayers of SiO$_2$ doped with the fluorophore, ICG, and Fe$_3$O$_4$ nanoparticles. This was followed by streptavidin binding and biotinylated anti-HER conjugation of the nanocomplex. Normalized extinction spectra of the NIR resonant nanoshells, nanoshells
after coating with Fe$_3$O$_4$ and after encapsulating in a silica epilayer, are shown in Figure 5.1B. Fluorescence spectra of ICG doped in the silica layer of the nanocomplexes at 833 nm is shown in Figure 5.1C. These nanocomplex spectral characteristics provide a better understanding of their functions as imaging probes as well as a therapeutic agent.

5.4 In vitro Fluorescence Optical Imaging

The efficacy of the targeted nanocomplexes-anti-HER2 conjugates to provide image contrast for tumor cells in vitro was established by fluorescence optical imaging. Fluorescence images of OVCAR3 cells and MDA-MB-231 cells incubated with nanocomplex-anti-HER2 conjugates and unconjugated nanocomplexes were obtained. MDA-MB-231 Breast Adenocarcinoma Human (Homo sapiens) cells were chosen as a control due to the low levels of HER2 expression present in this cell line. Low levels of HER2 expression were used to assign the cell lines into previously clinically defined subtypes, which can be used to guide clinical trial design. The fluorescence optical images of HER2 positive OVCAR3 cells with nanocomplex-anti-HER2 conjugates are shown in Figure 5.2A where (i) depicts nuclei stained with DAPI (blue), (ii) cytoplasm stained with Alexa Fluor 488 conjugated secondary antibody Goat Anti-Rabbit IgG (green), (iii) NIR fluorescence from ICG doped in silica layer of nanocomplexes (red), and (iv) i, ii, iii merged together showing the nanocomplexes binding outside the cells membrane.
Figure 5.2. (A) Fluorescence optical images of HER2 overexpressing OVCAR3 cells with nanocomplex-anti-HER2 conjugates showing (i) nuclei stained with DAPI (blue) (ii) cytoplasm stained with secondary antibody-Alexa-fluor 488 (green), (iii) NIR fluorescence from ICG doped in silica layer of nanocomplexes (red), and (iv) i, ii, iii merged together showing the nanocomplex-anti-HER2 conjugates binding outside the cells membrane. (B) Fluorescence optical images of OVCAR3 cells with the control, unconjugated nanocomplexes. Fluorescence optical images of HER2 low expressing MDA-MB-231 cells with (C) nanocomplex-anti-HER2 conjugates and (D) control. Original magnification is ×400 and the scale bar is 10 μm for all panels.

After 2 hr incubation with OVCAR3 cells, the nanocomplex-anti-HER2 conjugates show specific binding to the cell membrane. Figure 5.2B shows the fluorescence images of OVCAR3 cells incubated with the control, unconjugated
nanocomplexes (without antibodies). Due to the absence of the primary antibody (anti-HER2), the secondary antibody did not bind and no signal was observed from the Alexa Fluor 488. Fluorescence images of low HER2 expressing MDA-MB-231 cells with nanocomplex-anti-HER2 conjugates and with the control are shown in Figure 5.2C and 5.2D. The nanocomplex-anti-HER2 conjugates showed minimal non-specific binding to the extracellular matrix of MDA-MB-231 cells, resulting in a very weak NIR signal from the ICG. Similarly, without the primary antibody, the secondary antibody did not bind as well leading to low fluorescence from Alexa Fluor. The unconjugated nanocomplex incubated with MDA-MB-231 cells also did not bind to the cell membrane. This sequence of experiments demonstrate that the nanocomplex-anti-HER2 conjugates significantly target HER2-overexpressing OVCAR3 cells membrane in comparison with MDA-MB-231 cells, which express low levels of HER2.

5.5 In vitro Magnetic Resonance Imaging

The nanocomplex-anti-HER2 conjugates and unconjugated nanocomplexes were incubated with OVCAR3 cells and MDA-MB-231 cells at a concentration of $2 \times 10^9$ particles/mL, containing 0.215 mM Fe nanoparticles (determined by ICP-OES). The schematic representation of sample preparation for in vitro MRI studies is shown in Figure 5.3Ai. Briefly, cells are incubated with nanocomplexes suspended in media for 2 hrs and subsequently centrifuged and redispersed in 0.5% agarose. Optical image of OVCAR3 cells bound to nanocomplex-anti-HER2 conjugates suspended in agarose, and OVCAR3 cells suspended with unconjugated nanocomplexes in agarose are shown in Figure 5.3Aii and 5.3Aiii.
Figure 5.3. (A) (i) Schematic representation of sample preparation for in vitro MRI studies. Magnetic nanocomplexes are incubated with cells, washed after 2 h, centrifuged and suspended in 0.5% agarose. Optical image of (ii) cells with nanocomplex-anti-HER2 conjugates suspended in agarose, and (iii) cells with control, unconjugated nanocomplexes suspended in agarose. MR image of OVCAR3 cells suspended in 0.5% agarose with (B) nanocomplex-anti-HER2 conjugates and (C) control, unconjugated nanocomplexes. MR images of MDA-MB-231 cells suspended in 0.5% agarose with (D) nanocomplex-anti-HER2 conjugates, and (E) control. Maximum intensity projection of 128x128x64 pixel threshold T2 maps of the images corresponding to part B-E. (F) OVCAR3 cells with nanocomplex-anti-HER2 conjugates and (G) control, (H) MDA-MB-231 cells with nanocomplex-anti-HER2 conjugates and (I) control.

In Figure 5.3B, MR images of OVCAR3 cells suspended in agarose with nanocomplex-anti-HER2 conjugates are shown. In vitro imaging of cells suspended in agarose was done using a 3D RARE (rapid acquisition with relaxation enhancement) sequence with a
TR/TE equal to 2000/20 ms with a RARE factor of 8 leading to an effective TE of 60 ms. FOV was 25.6 x 25.6 x 12.8 mm with an acquisition matrix of 128 x 128 x 64 yielding an isotropic 200 μm resolution. The cells labeled with nanocomplexes appear as hypointense signal (dark spots) or higher contrast, suggesting that the nanocomplex-anti-HER2 conjugates were bound to OVCAR3 cells. OVCAR3 cells incubated with unconjugated nanocomplexes (fig 5.3C) show a few hypointense signals, indicating minimal nonspecific binding. MR images of MDA-MB-231 cells suspended in agarose with nanocomplex-anti-HER2 conjugates (fig 5.3D) clearly show less binding in comparison to OVCAR3 cells. Additionally, MDA-MB-231 cells incubated with the control (fig 5.3E) also demonstrate nominal nonspecific binding.

The maximum intensity projections (MIP) of 128 x 128 x 64 pixel threshold T2 maps, where each pixel represents the cubic volume of 156 x 156 x 156 μm, are shown in Figure 5.3F-I. The MIP were created from the 3D MRI data using a threshold segmentation approach as described in section VI. The hypointense pixels (brown spots) represent the nanocomplex-labeled cells and the surrounding normointense pixels (blue) agarose. The OVCAR3 cells labeled with nanocomplex-anti-HER2 conjugates are shown in Figure 5.3F and the control, unconjugated nanocomplexes, are shown in Figure 5.3G.

MDA-MB-231 cells labeled with nanocomplex-anti-HER2 conjugates are shown in Figure 5.3H and the control is shown in Figure 5.3I. Each hypointense pixel represented here contains a cluster of labeled cells since the scan resolution is insufficient to identify individual cells. The number of hypointense pixels was quantified to determine the specificity and selectivity of the nanocomplex-anti-HER2 conjugates in targeting HER2 expressing cells. Both the MDA-MB-231 and OVCAR3 cells that had
been incubated with the unconjugated nanocomplexes showed approximately equal counts of hypointense pixels, which are nonspecific binding. The MDA-MB-231 cells incubated with nanocomplex-anti-HER2 had 2.3 times the number of hypointense pixels relative to the cell samples incubated with unconjugated nanocomplexes. On the contrary, OVCAR3 cells incubated with nanocomplex-anti-HER2 conjugates had 9.0 times the number of hypointense pixels relative to OVCAR3 cells incubated with the unconjugated nanocomplexes. OVCAR3 cells incubated with nanocomplex-anti-HER2 conjugates also had 3.1 times the number of hypointense pixels as the MDA-MB-231 cells incubated with the nanocomplex-anti-HER2 conjugates.

5.6 In vitro Photothermal Therapy and Cytotoxicity Studies

The photothermal ablation of OVCAR3 cells incubated with nanocomplex-anti-HER2 conjugates allowing targeted destruction is shown in Fig. 5.4. Both OVCAR3 and MDA-MB-231 cells incubated with the nanocomplexes-anti-HER2 conjugates and unconjugated nanocomplexes were illuminated with NIR laser light at 808 nm for 10 min at a power density of 5.81W/cm\(^2\) and a spot size of ~0.8 mm diameter. Following photothermal therapy, cells were stained with the Calcein/PI mixture. Live cells appeared green due to the calcein stain and dead cells appeared red due to the PI stain (Fig. 5.4). The nanocomplex-anti-HER2 conjugates, which were bound to the OVCAR3 cell membrane, produced hyperthermia upon laser irradiation, resulting in cell death (Fig. 5.4A). The increased PI uptake by the dead cells within the laser spot is clearly observable.
MDA-MB-231 cells incubated with nanocomplex-anti-HER2 conjugates were also exposed to the same NIR laser treatment, and showed minimal cell death (Fig. 5.4C). A small amount of the unconjugated nanocomplexes was nonspecifically bound to both the OVCAR3 cells and the MDA-MB-231 cells, and resulted in some cell death after laser treatment (Fig. 5.4B and D). Irradiation of OVCAR3 cells incubated with nanocomplex-anti-HER2 conjugates with NIR laser resulted in selective destruction of these cells. In contrast, MDA-MB-231 cells incubated with nanocomplex-anti-HER2 conjugates showed no observable effects on cell viability.
Figure 5.5. Cytotoxicity studies of OVCAR3 cells incubated with (A) nanocomplex-anti-HER2 conjugates, (B) unconjugated nanocomplexes, and (C) control, no nanocomplexes. Cytotoxicity studies of MDA-MB-231 cells incubated with (D) nanocomplex-anti-HER2 conjugates, (E) unconjugated nanocomplexes, and (F) control, no nanocomplexes. Live cells are stained green with calcein and dead cells are stained red with propidium iodide. Original magnification is ×200 and the scale bar is 100 μm for all panels.

Nanocomplexes by themselves, without photothermal ablation, were observed to be innocuous to cells, and nominal cell cytotoxicity was observed. OVCAR3 cells and MDA-MB-231 cells were incubated with nanocomplex-anti-HER2 conjugates and the unconjugated nanocomplexes for 2 h, followed by staining with Calcein/PI dye mixture. Cytotoxicity studies of OVCAR3 cells incubated with nanoshell-anti-HER2 conjugates, unconjugated nanoshells, and control, no nanocomplexes are shown in Fig. 5.5A-C. Similarly, cytotoxicity studies of MDA-MB-231 cells incubated with nanocomplex-anti-HER2 conjugates, unconjugated nanocomplexes, and control are shown in Fig. 5.5D-F. The results for both cell lines are comparable and minimal differences in cell viability and cell death were observed.
5.7 Discussion

Currently, the majority of ovarian cancer diagnoses are for rather advanced stages of the disease due to the lack of availability of early detection and treatment strategies. The standard protocols for the treatment of ovarian cancer have included conventional surgical approaches followed by chemotherapy or radiation therapy. These standard care treatments often require invasive surgical procedures or other therapies associated with significant side effects, high cost, and poor clinical outcome. Since HER2 receptor amplification occurs in ovarian cancers and is associated with short survival time and short time to relapse, it can be targeted with alternative nanoparticle based therapies. Furthermore, HER2 positive cell line OVCAR3 have also been found to be resistant to clinically relevant drugs including adriamycin, melphalan, and cisplatin, with survival rates of 43%, 45%, and 77%, respectively relative to untreated controls in vitro. The drug resistance of ovary cancer cells can be effectively addressed by image guided photothermal therapies.

The nanocomplexes utilized here provide a unique platform with targeting, diagnostic and therapeutic capabilities all within the same agent. There are several advantages offered by these nanoscale agents. First, the nanocomplexes effectively assimilate two imaging modalities, MRI and fluorescence imaging, which are non-invasive, safe, clinically relevant, and complementary techniques. While MRI has the advantage of 3D resolution and visualization of overall anatomical background, it lacks sensitivity. Optical imaging methods such as fluorescence provide high target sensitivity, although they lack 3D resolution. Hence combining these two imaging modalities synergistically integrates the advantages of the two techniques and overcomes the...
disadvantages simultaneously. Second, antibody targeting allows nanocomplex-anti-
HER2 conjugates to specifically bind to HER2-overexpressing cell surface receptors. Here, both immunofluorescence staining and MRI successfully demonstrate that nanocomplex-anti-HER2 conjugates bind to HER2-overexpressing OVCAR3 cells in contrast to MDA-MB-231 cells, which have low HER2 expression. Some nonspecific binding was observed for the unconjugated nanocomplexes. This could be attributed to the overall negative charge on the outer surface of the silica layer of the nanocomplex, as a consequence of the fabrication procedure, which interacts electrostatically with the proteins present in the cells. However, the non-specific binding can be reduced with improvised chemical modification of the nanocomplexes in future studies. Third, due to the unique plasmonic properties of the nanocomplexes and tunability in the NIR they can absorb resonant light; effectively convert light to heat, followed by photothermal therapeutic actuation resulting in tumor ablation with near 100% remission rates.\textsuperscript{17} Fourth, the nanocomplexes have low cytotoxicity and a particle size conducive to passive extravasation from the tumor vasculature.\textsuperscript{46, 47} This will allow future studies in animal models as these nanocomplexes will accumulate in the tumor enabling simultaneous comprehensive imaging and therapy.

5.8 Conclusions

In conclusion, this is the first demonstration of a successful integration of dual modal bioimaging with photothermal cancer therapy for the treatment of ovarian cancer cells, using a molecularly targeted gold nanoshell-based theranostic probe. Unlike conventional cancer therapy approaches such as radiation- or chemotherapy, which can have fatal side
effects, nanoshell-based photothermal ablation therapy is benign and safe. Moreover, due to their selective accumulation at cancer cells expressing the HER2 cell receptor, only cells retaining the nanoshells will heat up and ablate when illuminated with NIR laser, while neighboring healthy cells will not experience plasmonic heating. These nanocomplexes can potentially be used as a tool for evaluating transmembrane receptor number, cell viability and real-time monitoring of cell status. As cancer predominantly spreads through the blood and lymphatic system, the potential ability to molecularly image cancer cells in metastatic carcinoma without incision and with microdose amounts of safe, non-toxic, multifunctional nanoparticles will have a significant impact on the standard of diagnosis and therapy for many cancers. The low cytotoxicity of these nanocomplexes and their therapeutic efficacy could be potentially beneficial in the study of deep organ metastatic carcinoma in animal models.
6.1 Introduction

In the previous two chapters, chapter 4 and 5, the multifunctional nanocomplexes were explored for dual modal imaging and photothermal therapy in vitro. In this chapter we target and diagnose breast cancer cells in vivo by multimodal imaging modalities, MRI and FOI, with the nanocomplexes conjugated with antibodies. We studied biodistribution in animal models 72h post-injection of nanocomplexes. We also examined nanocomplexes structural integrity in vivo by analyzing tumor sections with TEM. Rizia Bardhan fabricated the nanocomplexes, characterized them, performed ICP-MS to analyze gold concentration in tissues, and assisted in tissue analysis by TEM and FOI in vivo. Wenxue Chen performed cell culture, xenografts, injected nanocomplexes in mice, sacrificed mice and collected organs 72h post-injection, assisted in TEM sample preparation and FOI in vivo. Manuscript in preparation: Wenxue Chen*, Rizia Bardhan*, Marc Bartels, Carlos Perez-Torres, Maria F. Botero, Robin Ward-McAninch, Rachel Schiff, Robia G. Pautler, Naomi J. Halas, Amit Joshi (*equal contribution).

The estrogen receptor (ER) and the HER (c-erbB) signaling pathways are the dominant drivers of cell proliferation and survival in the majority of human breast cancers and provide an ideal target for breast cancer theranostics. Targeting these pathways will, thus, provide the most effective therapies in appropriately selected patients. Endocrine therapy to target ER and trastuzumab to target HER2 provide prominent benefits in the adjuvant setting when micrometastatic disease is present (50% reductions in recurrence).176, 177 These treatments, while capable of inducing tumor
regression, are much less effective in patients with macroscopic metastatic tumor, indicating that other survival pathways must also be operative in this setting. The HER signaling pathway has been described in systems biology terms as a robust network with complex input signals (4 receptors, 11 ligands), a core processing unit (phosphorylation signaling cascade), a variety of output signals (transcription factors), and complicated system controls (+ve and -ve feedback circuits). These features provide redundancy that allow the cell to respond to “network damage” (targeted therapies) by evolving and switching to alternative pathways in order to maintain survival signals, resulting in clinical drug resistance. Therefore, de novo and acquired resistance remain major obstacles necessitating new therapeutic strategies for successful treatment.

Alternative cancer therapeutics based on the photothermal response of gold nanostructures designed to absorb NIR, tissue-penetrating light has exhibited near 100% efficacy in the remission of tumors, and stands as one of the most promising new technologies to emerge from nanoscience research in the past decade. Following the initially demonstrated therapeutic success of gold nanoshells, other gold nanoparticles such as gold nanorods, hollow gold nanospheres, and gold nanocages have also been used to demonstrate similar, highly promising therapeutic responses. Gold-based nanostructures show particular promise as theranostic agents, based on their straightforward adaptability to integrate targeting, diagnostic, and therapeutic functionalities into a single hybrid multifunctional nanoscale complex. Gold nanoshells resonant in the NIR effectively absorb NIR light and generate hyperthermia for externally controlled tumor cell death. Conventionally gold nanoshells and other gold nanostructures such as nanorods/cages are typically imaged optically by
exploiting their high absorption and scattering cross-section via microscopic methods and optical coherence tomography.\textsuperscript{14, 183-185} This has limited their visualization to tissue depths of \( \sim 1 \) mm. For imaging nanoshell distributions in tissue depths relevant to whole body imaging in nude mice, or for clinical applications, additional modalities need to be integrated, as native absorption/scattering contrast of nanoshells is not sufficient for sensitive photon migration measurements over multiple centimeters. Plasmon enhanced fluorescence of water-soluble and biocompatible NIR organic dyes can provide a potentially promising and sensitive optical detection route at centimeter scale tissue depths.\textsuperscript{105}

Highly sensitive, noninvasive NIR fluorescence optical imaging (FOI) is constrained by limited photon penetration in tissue and cases like breast cancer metastasis in deep axillary lymph nodes, and distant organs like lungs, liver, skeleton and brain.\textsuperscript{34, 35} While MRI does not have tissue depth constraints, SPIO particles require heavy tumor loading for imaging and therapy, and in case of surgery, they do not provide any intraoperative guidance for tumor margin determination.\textsuperscript{186} The complimentary capabilities of FOI and MRI imaging provide the motivation for combining NIR light and magnetic field based imaging and therapy in one nanoparticle. Gold nanostructures integrated with NIR emitters iron oxide nanoparticles can provide FOI and MRI enhancement and hence serve as simultaneous theranostic reporter-actuators.\textsuperscript{187} With antibody or peptide conjugation, these theranostic hybrid nanoparticles can be delivered to specific cells or tissues for therapy, with reporter functionalities providing tracking capabilities before, during and after treatment. This multimodality structure is ideal for
the next critical phase of cancer nanotechnology research: transitioning this potentially revolutionary technology into clinical practice.

Recently, we illustrated the design and theranostic capabilities of magneto-fluorescent gold nanoshells (nanocomplexes) for diagnosis and photothermal therapy of breast cancer cells *in vitro*.\textsuperscript{187} In this study, the efficacy of the nanocomplexes have been demonstrated *in vivo* for specifically targeting and diagnosing HER2 overexpressing breast cancer cells via MRI and FOI. The nanocomplexes were fabricated by encapsulating Au nanoshells in a SiO\textsubscript{2} epilayer which was doped with Fe\textsubscript{3}O\textsubscript{4} nanoparticles and NIR fluorophore ICG similar to that described in chapter 4.\textsuperscript{187} The plasmon response of nanoshells can be straightforwardly tuned to the NIR “water window” (670-900 nm), where hemoglobin, water and blood are minimally absorptive,\textsuperscript{38} by simply altering the dimensions of the core and shell.\textsuperscript{12} Gold nanoshells, due to their tunability in the NIR, the high intensity electromagnetic field on their surface, and strong absorption/scattering characteristics, have already been successfully demonstrated in a wide range of biomedical applications.\textsuperscript{13-16, 28} \textsuperscript{187} These plasmonic properties of nanoshells also contribute towards dramatic emission enhancements of weak NIR fluorophores in their vicinity.\textsuperscript{105, 130} We have shown that nanoshells enhance fluorescence of ICG molecules from a mere 1.3 % in aqueous media to nearly 80 % when positioned at an appropriate distance from the nanoshell surface.\textsuperscript{105, 187} To our best knowledge, this study is the first demonstration that exemplifies the difference between HER2 expression levels of two breast cancer cell xenografts via combined NIR fluorescence and MR imaging with gold nanoshell based agents.
6.2 Experimental methods

I. Nanocomplexes Fabrication: The nanocomplexes were fabricated similar to that described in chapter 4 and 5. The anti-HER2-biotin conjugate was mixed with the streptavidin attached nanocomplexes and gently stirred for 8 h at 4 °C. An additional step was included for the in vivo studies, 50 μM PEG-biotin (Nanocs, MW~5000) was finally added to block nonspecific adsorption sites and increase circulation time. Finally nanocomplexes were redispersed in phosphate buffer at pH 7.5.

II. Live animal studies: The protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine.

III. BT474AZ xenografts: The estrogen-dependent BT474AZ/ATCC breast carcinoma Human (in which HER2 is naturally amplified) cells were grown in RPMI 1640 medium, 1% Penicillin-Streptomycin and 10% fetal bovine serum (FBS). Cells were incubated at 37 °C in a 5% CO₂ environment and were detached from culture with trypsin (0.05%) and EDTA (0.02%) and resuspended in media for passaging to wells. Athymic Nude-Foxn1nu female mice (4-6wk of age, 20±3g, Harlan) were implanted s.c. with E2 pellets (0.2mg, 60d release; Innovative Research of America) on the dorsal flank. The next day, mice were injected s.c. on the right flank near 4th mammary gland with $1 \times 10^7$ BT474AZ/ATCC cells suspended in serum-free medium mixed with Matrigel (BD Biosciences) at 1:4 ratio as described. Tumors were allowed to grow to about 8 -10 mm in diameter before nanocomplexes injection and imaging.

IV. MDA-MB-231 xenografts: The estrogen-independent human breast adenocarcinoma (Homo sapiens) cell line MDA-MB-231 (which expresses basal levels of HER2 receptor) were grown in DMEM medium, 1% Penicillin-Streptomycin and 10% FBS. Cells were
incubated at 37 °C in a 5% CO2 environment and were detached from culture with trypsin (0.05%) and EDTA (0.02%) and resuspended in medium for passaging to wells. Athymic Nude-Foxn1nu female mice (4-6wk of age, 20±3 g, Harlan) were injected s.c. on the right flank near 4th mammary gland with 3×10⁶ cells/mouse, the cells suspended in serum-free medium. Tumors were allowed to grow to about 8-10 mm in diameter before nanocomplexes injection and imaging.

V. FOI Methodology: Tumor bearing mice were each placed on a dark platform and isoflurane was delivered in concentrations of 1-3% in oxygen (up to 5% for initial induction), using a precision vaporizer and ventilation. The body temperature of mice was maintained at 37 °C during anesthesia by employing a heating pad and temperature controller (FHC Bowdoin, ME, USA). The in vivo images were acquired using an optical imaging system in continuous wave (CW) mode with a charged-coupled device (CCD) camera (PhotonMax 512, Princeton Instruments) and a 28 mm Nikkor (Nikon) lense. Camera was custom-controlled by MatLab (The MathWorks, Inc.) software. Excitation light is generated with a 100 mW near infrared (NIR) diode at 785 nm (Thorlabs, Inc.) and diffused with the combination of an aspheric lense and a diffuser (both Thorlabs, Inc.). The excitation light was captured by using a neutral density filter with optical density (OD) 3 (Andover Corporation). The emission light was collected using a fluorescence band pass filter at (830 ± 20) nm (Andover Corporation) and a holographic notch filter (OD 6) at 785 nm (Kaiser Optical Systems, Inc.) while rejecting excitation leakage, as suggested by Hwang et. al.¹⁸⁹

VI. MRI Methodology: MR Imaging experiments were performed on a Bruker Avance Biospec, 9.4 T spectrometer, 21 cm bore horizontal imaging system (Bruker Biospin,
Billerica, MA) with a 35 mm volume resonator. Animals were initially anesthetized with gaseous isoflurane at 2-3% in oxygen (up to 5% for initial induction), and placed into a mouse holder within the magnet where they were subsequently maintained at 2% isoflurane in oxygen. During the imaging, the animal body temperature was maintained at 37 °C continually monitored with a rectal probe using an animal warmed air heating system (SA Instruments, Stony Brook, NY). Imaging was performed with a multislice RARE (rapid acquisition with relaxation enhancement) sequence with a repetition time (TR) of 2805 msec, an echo time (TE) of 20 msec (TR/TE equal to 2805/20 ms) with a RARE factor of 6 leading to an effective TE of 60 ms. the imaging sequence included a 5ms fat suppression pulse. FOV was 30×30 mm with 20 slices at 1mm thickness. The acquisition matrix of 256×256 yielded an in-plane isotropic 117 μm resolution.

VII. Sample Preparation for ICP-MS: The mice were sacrificed 72 h post-injection, tissues were collected and immediately frozen at -80 °C. The organs were lysed in trace-metal grade aqua regia, boiled to evaporate excess aqua regia and finally dispersed in 1% aqua regia. Gold standards were prepared in 1% aqua regia. The samples were measured on Perkin-Elmer Elan9000 inductively coupled plasma mass spectrometer.

VIII. Cell Preparation for TEM: After 72 h organs were collected and a small portion of BT474AZ tumor was immediately fixed in 2.5% glutaraldehyde and 2 mM CaCl₂ in 0.1 M cacodylate buffer (PH 7.39). Tissue was cut into 1 mm cube keeping them immersed in fixative solution. Specimens were stained for 1 hour in saturated uranyl acetate + 50% ethanol, then counter-stained for 4 minutes in Reynold's lead citrate. Sections were then cut at 70-75 nm (silver sections) on an RMC MT-6000XL ultramicrotome and used for TEM. A Hitachi H-7500 TEM at accelerating voltage 80 kv was used.
6.3 Properties of dual modal nanocomplexes

The multifunctional nanocomplexes were fabricated as described in chapter 4 and 5. The scanning electron microscope (SEM) image of nanocomplexes (Fig. 6.1A) and corresponding high resolution transmission electron microscope (TEM) image (Fig. 6.1B) demonstrates that these particles are nearly 185 nm in diameter. Since SPIO nanoparticles and SiO$_2$ epilayer have significantly less contrast than Au in TEM, the difference in the two layers may be difficult to visualize. However, the texture and surface roughness indicates the presence of these multiple layers. The extinction spectra of the nanocomplexes were tuned to the NIR demonstrating plasmon maximum at 825 nm in aqueous media (Fig. 6.1C). The nanocomplexes plasmon peak is tuned to match the emission wavelength of ICG molecules doped within the silica layer to maximize fluorescence enhancement.$^{105}$ Nearly 650 nM of ICG is doped in a particle concentration of $\sim9 \times 10^9$ particles/mL. The emission maximum is observed at 830 nm in aqueous media (Fig. 6.1D). NIR optical image of a syringe containing nanocomplexes prior to injection is also shown in Fig. 6.1D inset.

The silica epilayer encapsulating the SPIO coated nanoshells serve multiple functions simultaneously: (i) it provides an effective spacer between the Au nanoshell surface and ICG molecules which maximizes fluorescence enhancement as demonstrated previously,$^{105, 130}$ (ii) the doped ICG molecules in the silica layer are less prone to photobleaching due to interaction with aqueous media and tissue,$^{90}$ (iii) the porous silica layer increases the molecular motion of H$_2$O within the pores enhancing H$_2$O interaction with SPIO nanoparticles. This improves the proton relaxation rate and provides high T$_2$
relaxivity, 390 mM⁻¹sec⁻¹, and significant MR contrast and (iv) the silica surface is available for binding antibodies and PEG molecules via simple conjugation chemistry.

**Figure 6.1.** Characterization of the magneto-fluorescent nanocomplexes: (A) SEM image of several nanocomplexes with an overall radius of \( r = 92.5 \pm 4 \) nm. (B) High resolution TEM image of nanocomplexes clearly showing SPIO and silica layer on the surface. (C) Extinction spectra of nanocomplexes, plasmon maximum \( \lambda_{\text{max}} = 825 \) nm. (D) Fluorescence spectra of nanocomplexes, emission maximum \( \lambda_{\text{max}} = 830 \) nm. Fluorescence image of nanocomplexes in a syringe before injection is provided as inset.

### 6.4 Fluorescence optical imaging in vivo

The diagnostic capabilities of the nanocomplexes were visualized *in vivo* by targeting HER2 expressing breast cancer tumors in animal models. Molecular targeting was achieved by assembling anti-HER2 antibodies on silica surface via streptavidin-biotin binding procedure similar to that reported previously. This conjugation protocol yields \(~ 300-350\) antibodies per nanocomplex as quantified by enzyme-linked immunosorbent
PEG-biotin molecules were then conjugated to nanocomplexes to block nonspecific streptavidin adsorption sites, provide steric stability, improve circulation time in the body, reduce immunogenicity and in combination with antibodies increase nanoparticle accumulation in tumor. PEG, a nontoxic FDA approved biopolymer, is known to promote solubility in aqueous media, provide steric repulsion between colloidal nanoparticles reducing aggregation in tissue. For in vivo experiments, 9x10^9 particles/mL of nanocomplexes were injected systemically via the tail vein into nude mice (20±3 g), 12.5 µL/g body weight. The nude mice have HER2 overexpressing human breast cancer tumor, BT474AZ, and control, HER2 basal expressing MDAMB231 tumors on the right flank. NIR fluorescence images of mice with MDAMB231 xenografts (top) and BT474AZ xenografts (bottom) are shown (Fig. 6.2A) at 0.3 h, 2 h, 4 h, 24 h, 48 h and 72 h post-injection of nanocomplexes. The NIR images were obtained using a 100 mW, 785 nm diode laser focused uniformly over the entire mouse body. The fluorescence intensity of the tumor-to-body ratio of BT474AZ xenografts (n=6) and MDAMB231 xenografts (n=3) analyzed at different time points demonstrate maximum contrast at 4 h post injection (Fig. 6.2B).
Figure 6.2. NIR fluorescence images of mice with HER2 basal expressing MDAMB231 xenografts (top) and HER2 overexpressing BT474AZ xenografts (bottom) at 0.3 h, 2 h, 4 h, 24 h, 48 h and 72 h post-injection of nanocomplexes. (B) Fluorescence (Fl) intensity of tumor-to-body ratio at different time points of mice with BT474AZ xenografts (n=6) and MDAMB231 xenografts (n=3) showing maximum fluorescence at 4 h. (C) Fluorescence intensity comparison between tumors only of BT474AZ (n=6) and MDAMB231 (n=3) showing 71.5% increase in signal at 4 h in BT474AZ compared to MDAMB231 tumors.
Fluorescence intensities determined as tumor-to-body ratio represents the normalized signals and accounts for the total nanocomplexes distributed in the body relative to the tumor. Significant statistical variation as returned by unbalanced two-way ANOVA across tumor types, P value 0.007, and across time points, P value $3 \times 10^{-10}$, was observed suggesting high fluorescence contrast in tumor. Within 72 h nanocomplexes are cleared from the body indicated by the diminishing fluorescence intensity. Due to the higher binding affinity of the anti-HER2 conjugated nanocomplexes to the BT474AZ xenografts, more nanocomplexes are accumulated and retained in the BT474AZ tumors. A portion of the nanocomplexes are cleared by mononuclear phagocytes of the reticuloendothelial system comprising of the macrophages of liver and spleen. Over time some nanocomplexes are excreted via the kidneys which explain the noticeable fluorescence signal observed from the body of BT474AZ mice in the region of liver, spleen and kidneys.

While tumor-to-body ratio provides normalized signal intensities, to verify the specificity and sensitivity of nanocomplex-antibody conjugates in targeting HER2 overexpressing xenografts, fluorescence measurements of tumors only is needed. A comparison of the fluorescence intensities of tumors only at different time points (Fig. 6.2C) demonstrates a 71.5% increase in the BT474AZ tumor signal at 4 h compared to MDA MB231 tumors. This significant variation, P value 0.003 across tumor types and P value $1 \times 10^{-11}$ across time points, indicates that the antiHER2-nanocomplex conjugates accumulate in the tumor by specifically targeting the cell surface markers on the tumor in addition to the enhanced permeability and retention (EPR) effect.\textsuperscript{45}
6.5 Magnetic resonance imaging in vivo

Figure 6.3. T₂-weighted MR images of mice with BT474AZ xenografts (top) and MDAMB231 xenografts (bottom) pre-injection 0 h, and 4 h, 24 h, 48 h, and 72 h post-injection of nanocomplexes. The tumor is shown in red circle. (B) MR image intensity of tumor-to-body ratio at different time points of mice with MDAMB231 xenografts (n=3) and BT474AZ xenografts (n=3) showing T₂-weighted contrast for BT474AZ even at 72 h. (C) MR image intensity comparison between tumors only of BT474AZ (n=3) and MDAMB231 (n=3) showing 50.5% decrease in MR contrast at 24 h in BT474AZ tumors compared to MDAMB231 tumors.
The SPIO nanoparticles incorporated within the porous silica epilayer of nanocomplexes provide significant MR contrast enabling tumor diagnosis at considerable depths. The T2-weighted MR images of mice with BT474AZ xenografts (Fig. 6.3A top) and MDAMB231 xenografts (Fig. 6.3A bottom) pre-injection, 0 h, and 4 h, 24 h, 48 h, 72 h post-injection of nanocomplexes are represented. The tumor area is shown in red circle. As the nanocomplexes accumulate in the tumor, the intensity of the tumor decreases (darker), as expected for T2 contrast agents, and by 72 h the tumor intensity increases (brighter). The decrease in MR contrast over time was evaluated by analyzing the T2 values of tumor-to-body ratio. This accounts for variation in the performance of the MRI instrument over time as well as distribution of nanocomplexes in the tumor relative to the body. The MR image intensity of tumor-to-body ratio at different time points of mice with BT474AZ xenografts (n=3) and MDAMB231 xenografts (n=3) (Fig. 6.3B) shows T2-weighted contrast for BT474AZ tumors even at 72 h. The discrepancy between MR contrast and fluorescence in vivo is primarily attributed to the surface weighted characteristics of optical imaging, where as in MRI sections of the tumor core are visualized. It is not surprising that nanocomplexes take longer to distribute within the tumor core compared to accumulation in peripheral vasculature. Statistical analysis show significant variations only across tumor type, P value 0.002, but not across time points, P value 0.360. The low sensitivity of MRI and low signal-to-noise ratio can explain the high P value across time points which also contribute towards the discrepancy between MRI and FOI analysis.

Analogous to FOI analysis, comparison of the MR intensities of tumors only at different time points (Fig.6.3C) validates the specificity of the nanocomplexes in
targeting HER2 overexpressing breast cancer xenografts compared to HER2 basal expressing MDAMB231 xenografts. The comparison shows ~50.5% decrease in the BT474AZ tumor signal at 24 h compared to MDAMB231 tumors. Significant variation is observed among tumor types, P value 0.038, but not across time points, P value 0.118. The discrepancy in the results between the two diagnostic modalities, MRI and FOI, signifies the intrinsic differences between the two techniques. While MRI provides high spatial resolution and capability to achieve anatomical background information at significant depths, but it lacks sensitivity, FOI offers exceptional detection sensitivity but is limited by penetration depth and hence not suitable for deep tissue imaging. The integration of these complementary imaging techniques in a single functional contrast agent, nanocomplexes, is hence advantageous and allows their use in clinical procedures for pre- and post-operative MRI and then intra-operative FOI.128,192

6.6 Nanocomplex Biodistribution

Fluorescence and MR images in vivo provide significant information regarding time required for nanocomplexes to accumulate and retain in tumor, efficacy of nanocomplexes and their specificity in targeting breast cancer tumors. However, to determine the distribution of these nanocomplexes in different organs and clearance from the body, the individual tissues need to be analyzed. NIR fluorescence images of individual tissues, (Fig. 6.4A) retrieved from MDAMB231 (n=3) and BT474AZ (n=5) mice sacrificed 72 h post-injection of nanocomplexes, clearly demonstrate maximum accumulation in tumor compared to other tissues. Since a higher concentration of nanocomplexes were accumulated in the tumor of BT474AZ mice at all times points
measured (Fig. 6.2 and Fig. 6.3), consequently, more nanocomplexes are cleared from the body into liver, spleen and kidneys. This explains the higher fluorescence intensity observed in these organs of BT474AZ compared to MDAMB231 (Fig. 6.4A). A surface averaged fluorescence intensity analysis, where the fluorescence intensity was divided by the surface area of each tissue (Fig. 6.4B) demonstrate that tumors have maximum nanocomplex uptake, for both tumor types, followed by liver, kidneys, spleen, lungs, heart and brain. Significant variation, P value 0.005, is observed in fluorescence intensity across tumor types. Some nanocomplex accumulation observed in MDAMB231 xenografts may be due to basal HER2 expression in this cell line as well as the EPR effect. In the case of BT474AZ, it is surprising that kidneys and liver have similar surface averaged fluorescence intensity. This may be attributed to the surface weighted characteristics of optical images, and since liver is larger, nanocomplexes buried deeper into liver may not be observable by fluorescence. While FOI is an effective technique, a method that analyzes the atomic Au content accurately is required to verify the nanocomplex distribution in all organs. The Au content in each tissue was measured using inductively coupled plasma-mass spectrometry (ICP-MS).

Au distribution (µg) per mass of tissue (g) for each tissue type, for both BT474AZ and MDAMB231 mice, (Fig. 6.4C) corresponds well with the measured fluorescence intensities (Fig. 6.4B) with minor differences. In both tumor types, the tumors have maximum Au content followed by liver, kidneys, spleen, lungs, heart and brain. Tissues of BT474AZ have higher nanocomplex accumulation compared to MDAMB231 and it is also notable that BT474AZ mice livers have higher gold distribution than kidneys, as expected. The measured Au levels in lungs and heart may be due to residual blood left
behind in these highly perfused tissues. Both fluorescence and ICP-MS measurements show low Au concentration in brain, indicating that the nanocomplexes have low permeation through the blood-brain barrier. Statistically significant variation is observed across tumor types, P value 0.019.

After in vivo administration of nanoparticles, their biodistribution are largely based on the particle size and surface properties such as surface charge, hydrophobicity, and moieties bound to the surface.\textsuperscript{193} The mononuclear phagocytes of the reticuloendothelial system are known to rapidly scavenge nanoparticles from the blood stream, accumulate in these organs and subsequently eliminate via kidneys.\textsuperscript{194} However, PEG conjugated nanoparticles have been shown to minimize the recognition by the reticuloendothelial system, therefore prolonging blood circulation time.\textsuperscript{195, 196} The stealth character of PEG grafted surfaces can be attributed to the low interfacial free energy of PEG in aqueous media and steric repulsion arising from a loss of conformational entropy of PEG chains in close proximity to foreign materials.\textsuperscript{191} While PEG mediated passive targeting is effective for nanoparticle accumulation in the tumor by EPR effect, active targeting via antibodies have been shown to have higher specificity due to the direct recognition of the cell surface receptors.\textsuperscript{54, 197} The nanocomplexes utilized in this study simultaneously promote active and passive targeting via the attached PEG-biotin chains and the anti-HER2 antibodies. The high targeting capability of the nanocomplexes is attributable to their maximum accumulation in tumor and low uptake in liver and spleen.
Figure 6.4. (A) NIR fluoresce images of mice organs harvested from MDAMB231 (left) and BT474AZ (right) 72 h post-injection of nanocomplexes. (B) Surface averaged fluorescence intensity analysis of mice organs of BT474AZ (n=5) and MDAMB231 (n=3) showing maximum fluorescence in tumors. (C) Gold distribution per mass of tissue obtained from ICP-MS in various mice organs of BT474AZ (n=5) and MDAMB231 (n=3) showing maximum Au content in tumors.
6.7 TEM analysis of nanocomplexes in tumor sections

Figure 6.5. TEM images of BT474AZ tumor sections retrieved from mice 72 h post-injection. (A) Low resolution image showing nanocomplex bound to the cell surface (red) and nanocomplexes internalized in the cytoplasm (black). (B) High resolution image of the nanocomplex shown within red box in part A. (C) High resolution image of the nanocomplexes shown within black box in part A. (D) Low resolution image of a different area of the tumor section showing nanocomplex in cytoplasm. High resolution image of the nanocomplex provided as inset.

Tumor sections retrieved from BT474AZ mice 72 h post-injection of nanocomplexes were examined with TEM to verify the structural integrity of the nanocomplexes in vivo and tumor internalization (Fig. 6.5). Low resolution image (Fig. 6.5A) and corresponding high resolution image shows a nanocomplex bound to the cell surface (Fig. 6.5B) and nanocomplexes internalized in the cell (Fig. 6.5C). In Figure
6.5C, a few nanocomplexes are embedded in different planes of the tumor section appearing as dark gray spots in the image. A different area of the tumor section shows another nanocomplex internalized in the cell (Fig. 6.5D). Although, the SiO$_2$ epilayer and the tissue background have similar contrast in TEM, the high resolution images (Fig. 6.5B, 6.5C and 6.5D inset) clearly show the intact SiO$_2$ layer encapsulating the darker Au shell. This indicates that the nanocomplexes retained their topology and structural integrity \textit{in vivo}.

\textbf{6.8 Conclusions}

In summary, we have designed and developed non-toxic multifunctional nanocomplexes with enhanced fluorescence and MR contrast for effective diagnosis of breast carcinoma cells \textit{in vivo}. Additionally, we have demonstrated biodistribution 72 h post-injection showing nanocomplexes accumulation primarily in the tumor and verified their structural integrity \textit{in vivo}. For effective clinical translation, nanoparticle based diagnostic and therapeutic agents should essentially (i) be composed of well characterized non-toxic materials, (ii) be hydrophilic, (iii) have long blood circulation time, (iv) exhibit high targeting and uptake efficiency in diseased cells, (v) be less prone to aggregation \textit{in vivo} and retain the structural integrity for increased therapeutic benefits and (vi) have surface properties, which can maximize tumor accumulation evading the macrophages of the reticuloendothelial system.$^{54, 126, 198}$ The nanocomplexes utilized in this study efficiently integrate all these capabilities demonstrating their potential use as a theranostic agent for simultaneous detection and treatment of tumors. Ultimately these nanocomplexes will provide far more advantages than single modality conventional
imaging probes. Gold nanoshells have successfully demonstrated efficient light to heat conversion inducing hyperthermia and subsequent tumor remission.\textsuperscript{15, 27} As nanoshell-based photothermal cancer therapy transitions from bench to bedside,\textsuperscript{27} the application of these magneto-fluorescent nanoshells in clinical settings as theranostic agents is impending. Following exhaustive biodistribution studies and photothermal therapy \textit{in vivo} these nanocomplexes could potentially revolutionize early diagnosis of cancer followed with rapid treatment bringing us closer to an era of personalized medicine.
Chapter 7: Nanosphere-in-a-Nanoshell: A Simple Nanomatriushka

7.1 Introduction

Nanoshells have proven extremely effective as theranostic complexes for diagnosis and therapy of cancer, as described in the last 3 chapters. However, for treatment of specific types of cancer, for example brain cancer, development of smaller nanoparticles in the sub-100 nm size regime is essential for effective delivery into the brain. This chapter describes a simple strategy for designing sub-100 nm nanoshells resonant in the NIR by simply introducing a gold nanoparticle core inside a SiO$_2$/Au nanoshell. Multilayered Au/SiO$_2$/Au nanoshells resonant in the NIR have been fabricated in the sub-150 and sub-100 nm size regime to potentially expand the therapeutic capabilities of nanoshells. The nanostructures were fabricated, characterized and optical studies were performed by Rizia Bardhan. Shaunak Mukherjee contributed towards theoretical analysis and discussion. Reproduced with permission from Rizia Bardhan*, Shaunak Mukherjee*, Nikolay A. Mirin, Stephen D. Levit, Peter Nordlander, and Naomi J. Halas, *J.Phys.Chem.C*, 2009, in press (*equal contribution). Copyright 2009 American Chemical Society.

Metallodielectric layered nanoparticles and nanostructures form a unique and important class of nanomaterials linked by their ability to manipulate light in similar ways. Their optical properties arise from the surface plasmons supported at their metal-dielectric interfaces. Surface plasmons, whether on closely adjacent nanoparticles$^{199}$ or on different metal-dielectric interfaces$^{21,200}$ of the same nanoparticle, mix and hybridize in direct analogy with simple quantum systems. These nanostructures form a class of
plasmonic “artificial molecules” with hybridized plasmons that are highly dependent on nanoscale geometry. As nanostructures of this type are synthesized with increasingly complex geometries, this paradigm becomes even more important, providing a qualitative understanding of the optical resonances of the nanostructure prior to actual synthesis. The ability to control both near and far field properties from the dipole up has made this family of nanostructures interesting for a broad range of spectroscopic, biomedical and photonic applications.

Multilayered metallodielectric nanostructures, or “nanomatryushkas”, where nanoscale dielectric spacer layers separate concentric metallic layers, are of particular interest. Here the coupling between the surface plasmons is determined by the thickness of the dielectric spacer layers, which can be controlled in the nanoparticle synthesis. A theoretical model of a silver core/shell multilayered nanostructure has shown that large local field enhancements are characteristic of the void between the central metal core and the adjacent, innermost metal shell. Such multilayered shells can act as a collection of optical condensers that focus light toward the center of the structure multiplicatively, resulting in an exponential increase in the near-field enhancements as the number of metal shells increases. This structure is the spherical analog of the plasmonic “snowman”, a self-similar chain of adjacent nanoparticles of decreasing size, where the maximum near field focusing occurs not at the tip of the chain but in the junction between the smallest, terminal nanoparticle and its next-nearest, larger neighbor. In addition to these distinctive near-field properties, the far-field properties of concentric nanostructures are also controlled largely by the thickness of the intermediate dielectric layer. This geometric “tunability” of the plasmon resonances of the nanoparticle
into the NIR region of the spectrum is highly beneficial for light-based biomedical diagnostics and therapeutics.

Gold nanoshells, consisting of a silica nanoparticle core surrounded by a thin Au shell, have generated significant interest due to their applications in biotechnology and biomedicine. In therapeutic applications, tuning the nanoshell plasmon to the NIR "water window" of 700-1200 nm allows them to be strong optical absorbers or scatterers in a wavelength region where light penetrates several centimeters into the human body. In this regime, nanoshells can be used as contrast agents for enhancing optical imaging, as photothermal heat sources for cancer therapy, converting the light they absorb to heat for tumor ablation with near 100% remission rates, and as gene therapy vectors, where resonant light can release genetic cargo bound to their surfaces. Multiple diagnostic and therapeutic functions can even be designed into the same plasmonic nanoparticle theranostic complex. While many of these tasks can be achieved with nanoshell complexes in the 150 nm diameter size range, there are important reasons to develop nanocomplexes that maintain similar optical functionality in smaller size regimes. For the detection and treatment of specific types of tumors, such as brain tumors, nanoparticles in the sub-100 nm size regime are expected to have higher photothermal therapeutic efficacy since they can surpass the blood-brain barrier (BBB), allowing higher nanoparticle concentrations to accumulate in tumors. The BBB is a physical barrier in the form of tight junctions between epithelial cells which impede the transfer of injected agents in the bloodstream into brain tissue. Development of NIR resonant plasmonic nanostructures in the sub-100 nm size range will ultimately extend these powerful functionalities to this critical, and largely inaccessible, zone of treatment.
In this study we demonstrate the fabrication of sub-100 nm and sub-150 nm concentric nanostructures consisting of a solid Au nanoparticle immediately surrounded by a silica dielectric layer and then an outer Au shell. We show that the plasmon resonances of this nanoparticle result from the interaction between the essentially fixed-frequency plasmon response of the central nanosphere with the bonding and antibonding plasmons of the surrounding nanoshell. The coupling of nanosphere and nanoshell plasmons in this nanomatryushka particle provides even greater spectral tunability than for an individual Au nanoshell. We relate the optical spectra of the synthesized nanoparticles directly to the plasmon hybridization model of the nanoparticle. For larger nanomatryushkas of this geometry, we observe an additional quadrupolar mode due to phase retardation.

7.2 Experimental Methods

Materials Required: HAuCl₄.3H₂O (99%, Sigma), potassium carbonate (K₂CO₃, anhydrous, Fisher), CO gas (Matheson), tetraethyl orthosilicate (TEOS, Sigma), 30% NH₄OH (Fisher), 200 proof ethanol (Decon Labs Inc.), N-n-butyl-aza-2,2-dimethoxysilacyclopentane (Gelest, SIB1932.4), tetrakis(hydroxymethyl)phosphonium chloride (THPC, Sigma), 1M NaOH (Fisher).

Fabrication of Au Nanoparticles: Au nanoparticles were fabricated by reducing Au from a 1% HAuCl₄-K₂CO₃ solution with CO(g). Briefly, 25 mg K₂CO₃ was added to 100 mL H₂O in an amber glass bottle, and subsequently 1.5 mL 1% HAuCl₄ was added and the solution was allowed to age at room temperature, in the dark, for 24-72 hours. The 1% HAuCl₄ was aged for 14 days prior to adding to the K₂CO₃ solution. The 30 nm diameter
Au nanoparticles were fabricated by aging the 1% HAuCl₄- K₂CO₃ solution for 24 hours and bubbling CO(g) through the solution for 3 minutes under vigorous stirring. The 40 nm and 50 nm diameter Au nanoparticles were fabricated similarly, except the 1% HAuCl₄- K₂CO₃ solution was aged for 48 hours. The 80 nm diameter Au nanoparticles were also synthesized similarly, except the 1% HAuCl₄- K₂CO₃ solution was aged for 72 hours. The nanoparticles were centrifuged twice, 30 minutes each time, and redispersed in H₂O. The centrifuge speeds are mentioned below.

Fabrication of Nanoparticles coated with Silica: The 80 nm diameter Au nanoparticles were coated with silica following a similar procedure as reported previously. Briefly, 7 mL of a concentrated Au nanoparticle solution (~10¹⁰ particles/mL) was mixed with 40 mL of 200 proof fresh ethanol and 400 μL 30 % NH₄OH, and subsequently 4 μL tetraethyl orthosilicate (TEOS) was quickly added. The reaction was allowed to proceed for 45 minutes at room temperature under vigorous stirring and then stored in the refrigerator at 4 °C for 24 hours. The following day, the Au/SiO₂ nanoparticles are centrifuged and resuspended in 10 mL ethanol.

The 30, 40, and 50 nm diameter Au nanoparticles were coated with silica by following a slightly different procedure. Concentrated TEOS and NH₄OH (30%, pH~12) result in aggregation of the smaller nanoparticles due to charge destabilization at high pH and excess silane coupling agent. Hence, 2 mL of the smaller nanoparticle aqueous solution (5x10¹⁰ particles/mL) were mixed with 10 mL of 200 proof fresh ethanol and 70 μL of 0.4 % NH₄OH (pH ~ 8.5), and subsequently 50 -100 μL 10 mM ethanolic TEOS solution (11 μL TEOS in 5 mL ethanol) was quickly added to the reaction mixture. The reaction was allowed to proceed for 45 minutes at room temperature under vigorous
stirring and then stored in the refrigerator at 4 °C for 24 hours. The smaller nanoparticles were not centrifuged but directly used for silane functionalization.

**Cyclic Silane Functionalization of Nanoparticles:** The smaller Au/SiO₂ nanoparticles (30, 40, and 50 nm diameter) were functionalized with 200-400 μL of 1 mM ethanolic solution of cyclic silane (4 μL silane to 20 mL ethanol) for 24 hours. The 80 nm diameter Au/SiO₂ nanoparticles were functionalized with 1 mL of 1 mM ethanolic solution of cyclic silane for 24 hours. The silane-functionalized, silica-coated nanoparticles were used directly for the next step without further centrifugation. Excessive centrifugation resulted in aggregation of nanoparticles.

**Fabrication of Au/SiO₂/Au nanoshells:** The metal core nanoshells were fabricated by seed mediated electroless plating of Au onto the silica coated nanoparticles as previously reported.¹⁸ The precursor particles were prepared by decorating the silica coated nanoparticles with small gold colloid (2-3 nm) fabricated by the method reported by Duff et al.²⁰ Briefly, 10 mL of the silane functionalized silica coated nanoparticles were mixed with 40 mL of Duff gold colloid and 2 mL NaCl (1 M). The precursor particles were left unperturbed for 24 hours at room temperature, following which they were centrifuged and redispersed in 5 mL H₂O. The 1% HAuCl₄- K₂CO₃ solution previously prepared was used as the plating solution. A continuous gold shell was grown around the Au/SiO₂ nanoparticles by mixing 3 mL of the plating solution with different aliquots of the precursor particles and bubbling CO(g) for 10 seconds.²⁸ The reaction was scaled up to obtain appropriate volume, centrifuged and finally redispersed in H₂O.

**Instrumentation and Modeling:** Scanning electron microscope (SEM) images were obtained using a FEI Quanta 400 environmental SEM at an accelerating voltage of 25
kV. Extinction spectra were obtained using a Cary 5000 UV/Vis/NIR spectrophotometer. Simulated surface charge plots were performed using the RF module of the commercial Finite Element Method software (COMSOL Multiphysics 3.5a) using three-dimensional scattered harmonic propagation.

**Centrifuge Speeds for Nanoparticles of Different Sizes:** The following speeds were used:

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Au nanoparticles</th>
<th>Au/SiO₂ precursor</th>
<th>Au/SiO₂/Au nanoshells</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 nm</td>
<td>1500 rcf</td>
<td>390 rcf</td>
<td>150 rcf</td>
</tr>
<tr>
<td>50 nm</td>
<td>1600 rcf</td>
<td>700 rcf</td>
<td>250 rcf</td>
</tr>
<tr>
<td>40 nm</td>
<td>1700 rcf</td>
<td>800 rcf</td>
<td>300 rcf</td>
</tr>
<tr>
<td>30 nm</td>
<td>1850 rcf</td>
<td>920 rcf</td>
<td>370 rcf</td>
</tr>
</tbody>
</table>

### 7.3 Plasmon Hybridization of Au/SiO₂/Au Nanoshells

Plasmon Hybridization is a useful tool for interpreting the plasmon modes of complex metallic nanostructures. This approach has been used to explain the resonant modes of a nanomatriushka, where the plasmon modes of the inner and outer metallic shells hybridize, giving rise to symmetric and antisymmetric plasmon modes. The fabricated Au/SiO₂/Au nanoshell is a simple example of a nanomatriushka, where the inner solid gold nanosphere can be viewed as a gold nanoshell of zero aspect ratio. The response of this system can be interpreted as the interaction between the primitive plasmon mode of a solid Au sphere and the plasmon modes of the nanoshell.

For a nanomatriushka in the quasistatic regime, \([r_1, r_2, r_3] = [5, 7, 9] \text{ nm}\) (Fig. 7.1A), where \(r_1\) is the radius of the gold core, \(r_2\) the radius of the silica-coated core, and \(r_3\) the total nanoparticle radius, three dipolar plasmon modes are obtained. Two modes correspond to the hybridization of the low-energy bonding nanoshell plasmon with the nanosphere plasmon, giving rise to a low energy antisymmetric bonding mode \((|\omega^- >)_{(NS-NP)}\) and a symmetric antibonding mode \((|\omega^+ >)_{(NS-NP)}\).
Figure 7.1. Theoretical analysis of Au/SiO$_2$/Au nanoshells showing (A) an energy level diagram describing the interaction between the Au nanoparticle and Au nanoshell plasmon resonances resulting in three hybridized energy levels in the quasistatic regime $[r_1, r_2, r_3] = [5, 7, 9]$ nm in $n=1$ media in the dipole limit. (B) Surface charge distributions on the Au core, inner and outer Au shell interfaces qualitatively corresponding to the three energy eigenmodes. (C) Simulated far field extinction spectrum of the quasistatic Au/SiO$_2$/Au nanoshell using the Johnson & Christy dielectric function for gold; with plasmon peaks assigned as (a) hybridized bonding and (b) hybridized antibonding modes. (D) Energy level diagram describing plasmon modes of Au/SiO$_2$/Au nanoshell having dimensions $[r_1, r_2, r_3] = [40, 55, 65]$ nm showing the hybridized energy level for $l=1$ dipole (bottom) and $l=2$ quadrupole (top). (E) Simulated far field extinction spectrum of this larger Au/SiO$_2$/Au nanoshell using Johnson & Christy dielectric function for gold with appropriate peak assignments.

Because the interaction between the higher-energy antibonding nanoshell plasmon mode and the nanosphere plasmon is extremely weak, we designate this third mode
(|ω⁺ >₁ NB ) as a non-bonding mode. This mode is essentially dark, since it has a very small dipole moment due to its appreciable antibonding character. To determine the nature of the modes that couple to the incident plane wave, Finite Element Method based simulations were performed for this small quasistatic Au/SiO₂/Au nanoshell. The induced surface charge distribution (Fig. 7.1B) was calculated on Au core, inner and outer Au shell interfaces. These charge plots fully confirm the hybridization picture. The dipolar bonding |ω⁻ >₁ NS-NP mode is identified by its distinct regions of positive and negative charge density. The higher energy dipolar antibonding |ω⁺ >₁ NS-NP mode is recognized by the similar induced charges residing on each of the interfaces, and the higher energy nonbonding mode |ω⁺ >₁ NB possesses an alternating charge distribution for each successive interface of the nanoparticle.

The calculated far field extinction spectrum (Fig. 7.1C) shows two distinct plasmon peaks situated at 750 nm and 515 nm (labeled as ‘a’ and ‘b’) which corresponds to hybridized bonding |ω⁻ >₁ NS-NP and antibonding |ω⁺ >₁ NS-NP modes. This calculation was performed in vacuum (ε = 1) using the experimentally obtained Johnson and Christy dielectric function for gold\(^{115}\) and a constant dielectric value (ε = 2.04) for silica. The symmetric antibonding mode has a larger dipole moment relative to the antisymmetric bonding mode, which results in a strong coupling to the incident light. However, its overlap with the interband transitions of Au results in significant linewidth broadening and therefore a decrease in the peak amplitude.

The plasmon modes for a larger size Au/SiO₂/Au nanoshell [r₁, r₂, r₃] = [40, 55, 65] nm were calculated in the same manner and the modes are identified using the
plasmon hybridization model. (Fig. 7.1D). As for the small quasistatic shell in Figs. 7.1A-C, the same three dipolar hybridized modes are formed. The simulated far field extinction spectrum of this larger Au/SiO$_2$/Au nanoshell exhibit three peaks, positioned at 920 nm, 650 nm and 535 nm (labeled as $a \ (l = 1)$, $a \ (l = 2)$ and $b \ (l = 1)$ in Fig. 7.1E).

The peak at 920 nm is the dipolar bonding mode ($| \omega^- >_{NS-NP}^{(1)}$) and the peak at 535 nm is the dipolar antibonding mode ($| \omega^+ >_{NS-NP}^{(1)}$). The relative intensity of the antibonding mode is higher than for the small quasistatic nanoshell because here it is shifted further away from the interband transition threshold. The additional mode that appears as a weak shoulder at 650 nm is a quadrupolar bonding mode ($| \omega^+ >_{NS-NP}^{(2)}$), which can be excited because of phase retardation.

### 7.4 Fabrication and Characterization of Au/SiO$_2$/Au Nanoshells

The Au/SiO$_2$/Au nanoshells were experimentally fabricated as shown schematically in Figure 7.2A. The synthesis technique developed here is originally adapted and modified from one reported previously.$^{211}$ Au nanoparticles of various sizes are initially coated with an epilayer of amorphous SiO$_2$ by the condensation of tetraethyl orthosilicate in an alkaline medium. Typically NH$_4$OH (pH 8-12) is used to initiate this base-catalyzed reaction. The Au/SiO$_2$ nanoparticles were then functionalized with a cyclic silane (N-n-butyl-aza-2,2-dimethoxysilacyclopentane). The use of cyclic azasilanes has several advantages compared to the use of alkyl trialkoxy silane coupling agents such as 3-aminoproplytriethoxysilane (APTES) and 3-mercaptoproplytriethoxysilane (MPTES). Alkyl trialkoxy silanes undergo stepwise hydrolysis in alkaline aqueous media to form the corresponding silanol and subsequent
condensation to siloxanes. The hydrolysis is relatively rapid than the condensation reaction. During the condensation reaction, depending on the amount of H₂O present, polymerization of silanes may occur, which often results in nanoparticle agglomeration.

Figure 7.2. (A) Schematic representation of the fabrication procedure of Au/SiO₂/Au nanoshells. SEM images showing (B) Au nanoparticles of radius, r₁ = 40 ± 4 nm, (C) Au nanoparticles coated with 15 ± 2 nm SiO₂ epilayer, (D) Au/SiO₂ nanoparticles decorated with 2 nm Au colloid, (E) Au/SiO₂/Au nanoshells with dimensions [r₁, r₂, r₃] = [40, 55, 65] nm. The scale bar is 200 nm.

Cyclic azasilanes, alternatively, are particularly useful in surface modification of hydroxyl-containing nanoparticles, since they cause significantly less nanoparticle flocculation. Under basic conditions, cyclic azasilanes undergo ring opening by hydrolytic cleavage of the C-N bond which has a lower bond energy (320 kJmole⁻¹) than the Si-O bond (450 kJmole⁻¹). Due to this ring-opening mechanism, cyclic azasilanes relieve the ring strain and consequently hydrolyze faster. Therefore, unlike APTES and MPTES, cyclic azasilanes do not require water as a catalyst for hydrolysis, resulting in no reaction byproducts. Therefore self-polymerization of the silane is substantially reduced, resulting in less nanoparticle aggregation. Subsequent to silanization, small Au colloids (2-3 nm diameter) were attached to the silane functionalized Au/SiO₂ nanoparticles,
which serve as nucleation sites for the electroless plating of the outer Au shell layer onto the nanoparticle surface. A complete outer Au layer is formed by reducing Au from a 1% HAuCl₄ aqueous solution in the presence of gaseous CO as the reducing agent.¹²,¹⁸ This synthesis technique allows the fabrication of relatively monodisperse nanostructures with a complete and homogeneous outer Au layer. Scanning electron microscope (SEM) images of the nanostructures at various stages of the fabrication procedure are shown in Figure 7.2B-E. Au nanoparticles of radius 40 ± 4 nm (Fig. 7.2B) coated with 15 ± 2 nm layer of silica (Fig. 7.2C) were functionalized with silane and decorated with 2-3 nm diameter Au colloid (Fig. 7.2D). Completed Au/SiO₂/Au nanoshells with dimensions \[ r_1, r_2, r_3 \] = [40, 55, 65] nm are shown in Figure 7.2E.

7.5 Optical properties of Au/SiO₂/Au Nanoshells

The experimentally observed plasmon resonances of the Au/SiO₂/Au nanoshell solutions of varying dimensions, dispersed in aqueous media, are shown in Figure 7.3A. The extinction spectra of the core Au nanoparticles of radius ~ 40 nm and the Au/SiO₂ nanoparticles of radius ~ 55 nm are shown in Figure 7.3A i-ii. For these nanoparticles, the plasmon resonance of the core nanosphere redshifts from 550 nm (in aqueous solution) to 562 nm after coating with SiO₂, due to the higher refractive index of SiO₂ \((n = 1.46)\) compared to H₂O \((n = 1.33)\). Extinction spectra of Au/SiO₂/Au nanoshells with the same inner nanosphere radius and spacer layer but with increasing thickness of the outer core are shown in Figure 7.3A iii-vi. The observed optical spectra of these structures are dramatically different than those of the Au/SiO₂ nanoparticles. A new dipolar bonding resonance is observed in the NIR, which blueshifts across the 1000-800
nm wavelength range with increasing shell thickness. Specifically, as the outer Au shell thickness increases from 10 nm to 13 nm, 18 nm and 31 nm, the dipolar bonding resonance blueshifts from 990 nm to 930, 885, and 800 nm, respectively. In addition, a new broad resonance appears at 620 nm when the metallic shell is present (Fig. 7.3A iii), also shifting to shorter wavelengths with increasing shell thickness (Fig. 7.3A iv-vi).

The theoretical extinction spectra corresponding to the Au/SiO$_2$/Au nanoshells are shown in Figure 7.3B. The calculated optical spectra were obtained using Mie theory, assuming a symmetric nanostructure with a spherical Au core, and a spherically symmetric SiO$_2$ layer and outer Au shell using Johnson and Christy gold permittivity was used in these calculations. The maxima in each calculated extinction spectrum correspond well to those observed in the experimental spectra. The blueshifting resonances are the hybridized dipole bonding, $|\omega^>_{(1)NS-\text{NP}}$, and dipole antibonding, $|\omega^-_{(1)NS-\text{NP}}$ modes of the Au/SiO$_2$/Au nanoshells, (Fig. 7.1D). For increasing shell thickness, the interaction between the cavity and sphere plasmons in the nanoshell decreases, resulting in a blue shift of the bonding nanoshell plasmon resonance (Fig. 7.3B). The smaller narrow resonant feature observed in the theoretical spectra, which blue shifts from 685 nm to 600 nm as the outer Au shell thickness increases is the quadrupole bonding mode, $|\omega^-_{(2)NS-\text{NP}}$) indicated in Fig. 7.1E.
Figure 7.3. (A) Experimental extinction spectra of (i) Au nanoparticles of radius $r_1 = 40$ nm, (ii) Au/SiO$_2$ nanoparticles, $r_2 = 55$ nm, and Au/SiO$_2$/Au nanoshells where (iii) $r_3 = 65$ nm, (iv) $r_3 = 68$ nm, (v) $r_3 = 73$ nm, and (vi) $r_3 = 86$ nm. (B) Theoretical spectra corresponding to the experimental spectra from part A. The spectra are color coordinated and offset for clarity. (C) SEM images of Au/SiO$_2$/Au nanoshells corresponding to the spectra from part A. (i) $r_3 = 65$ nm, (ii) $r_3 = 68$ nm, (iii) $r_3 = 73$ nm, and (iv) $r_3 = 86$ nm. The scale bar is 100 nm.

Experimentally, this spectral feature is observed as a lineshape modulation, and in fact appears both broader and stronger in the experimental spectra than in the theoretical calculations. The broad linewidth of this feature is due to the inhomogeneous broadening characteristic of the optical spectroscopy of nanoparticle ensembles. The fact that this
feature is more clearly observable in the experimental spectra than in our calculated 
spectra we attribute to the reduced symmetry of the nanoparticles themselves, in other 
words, the irregularities of the synthesized nanoparticles. For structural defects that break 
the spherical symmetry of the system, such as for instance an outer shell of nonuniform 
thickness or a nonconcentric alignment of the shells, quadrupole modes will hybridize 
with dipolar modes, thus strongly enhancing their brightness. Scanning electron 
microscope (SEM) images of the Au/SiO2/Au nanoshells corresponding to the 
experimentally observed optical spectra are shown in Figure 7.3C i-iv, in the order of 
increasing Au shell thickness.

The optical extinction spectra of several Au/SiO2/Au nanoshells in the sub-100 nm 
diameter regime, and their corresponding SEM images, are shown in Figure 7.4. The 
optical spectra shown in Figure 7.4Ai-ii represent Au/SiO2/Au nanoshells equivalent in 
overall dimensions, but as the size of the Au core increases from \( r_f = 15 \) nm to 20 nm, the 
intermediate SiO2 layer thickness is decreased. This results in a redshift of the 
\( |\omega_r|_{NS-NP} \) plasmon resonance from 660 nm to 730 nm due to an increase in the 
interaction between the plasmons supported by the Au nanosphere core and the Au outer 
shell layer. The spectra in Fig. 7.4Aiii-iv shows redshifts of the \( |\omega_r|_{(1)NS-NP} \) mode from 
755 nm to 790 nm as the outer Au layer thickness decreases while all other dimensions 
remain constant. Theoretical Mie extinction spectra of the sub-100 nm Au/SiO2/Au 
nanosHELLS (Fig. 7.4B) quantitatively reproduce the experimental data (Fig. 7.4A). SEM 
images of Au nanoparticles of radius 25 ± 3 nm, and those coated with a homogeneous 
12 ± 2 nm layer of SiO2 are shown (Fig. 7.4C, D). The SEM images of the sub-100 nm 
Au/SiO2/Au nanoshells corresponding to the experimental extinction spectra are also
shown (Fig. 7.4E-H). The inner Au core is clearly visible when the silica layer thickness and outer Au shell layer are on the order of ~ 10 nm.

Figure 7.4. (A) Experimental extinction spectra of sub 100 nm Au/SiO$_2$/Au nanoshells with different dimensions (i) $[r_1, r_2, r_3] = [15, 30, 42]$ nm (ii) $[r_1, r_2, r_3] = [20, 30, 42]$ nm (iii) $[r_1, r_2, r_3] = [25, 37, 51]$ nm (iv) $[r_1, r_2, r_3] = [25, 37, 47]$ nm (B) Theoretical spectra corresponding to the experimental spectra in (A). The spectra are color coordinated and offset for clarity. SEM images of (C) Au nanoparticles of radius, $r_1 = 25 \pm 3$ nm, (D) and coated with $12 \pm 2$ nm SiO$_2$ epilayer. Sub 100 nm Au/SiO$_2$/Au nanoshells corresponding to the spectra from part A. (E) $[r_1, r_2, r_3] = [15, 30, 42]$ nm (F) $[r_1, r_2, r_3] = [20, 30, 42]$ nm (G) $[r_1, r_2, r_3] = [25, 37, 51]$ nm (H) $[r_1, r_2, r_3] = [25, 37, 47]$ nm. The scale bar is 100 nm.
7.6 Calculated Far-Field Properties of Au/SiO₂/Au Nanoshells

Upon illumination with resonant light, metallic nanoparticles will preferentially scatter or absorb light, or some combination of the two. In applications, scattering is useful for contrast enhancement in bioimaging. Absorption, and its subsequent photothermal heating, which occurs due to nonradiative plasmon decay, results in the therapeutic actuation of hyperthermic cell death or oligonucleotide release. For simple nanoparticles, the absorption and scattering efficiencies are mainly a function of nanoparticle size: for layered nanoparticles this relationship becomes more complex and geometry dependent. For multilayered nanomatrushkas, their relative absorption and scattering efficiencies can be modified, for a fixed nanoparticle size, by varying the internal geometry. The calculated absorption and scattering profiles of the sub-100 nm Au/SiO₂/Au nanoshells fabricated here are shown in Figure 7.5. For fixed nanoparticle size, an increase in the radius of the Au core and a decrease in the silica layer increase the coupling between the core and shell plasmons and results in an increase in absorption efficiency. As seen here, this dependence can be quite significant: by increasing core radius and reducing silica spacer layer thickness each by only 5 nm, the relative absorption efficiency increases from 46% to 63% for the same overall size nanoparticle (Fig. 7.5A, B). This suggests that for bioimaging applications, where a larger scattering cross section is preferable, Au/SiO₂/Au nanoshells with a thicker intermediate silica layer (> 10 nm) would be more efficient, while for therapeutic-actuation applications, where a larger absorption cross section is desired, a geometry with thinner silica layers (≤ 10 nm) would be preferred. The relative absorption and scattering efficiencies can also be modified by altering the outer Au shell thickness. A nanomatrushka with a thicker Au shell (> 10 nm) has greater
scattering efficiency, while the thinner Au shell (≤ 10 nm) has a greater absorption efficiency (Fig. 7.5C-D). Again, the higher absorption efficiency corresponds to the case where the constituent plasmons of the nanoparticle layers couple most strongly.

Figure 7.5. Calculated absorption (red) and scattering (black) efficiencies of Au/SiO$_2$/Au nanoshells of different sizes. (A) $[r_1, r_2, r_3] = [15, 30, 42]$ nm (B) $[r_1, r_2, r_3] = [20, 30, 42]$ nm (C) $[r_1, r_2, r_3] = [25, 37, 51]$ nm (D) $[r_1, r_2, r_3] = [25, 37, 47]$ nm.

7.7 Conclusions

The Au/SiO$_2$/Au nanoshells fabricated in this study are simple examples of nanomatryushkas and provide a high degree of plasmonic tunability. Their small size makes them extremely versatile and promising for a wide array of optical and biomedical
applications. The induced charges at the gold-dielectric interfaces govern both the near-field and far-field properties of the structure. The relative absorption and scattering efficiencies can be controlled by tuning the internal geometry for a fixed outer nanoparticle diameter. The plasmon hybridization model reveals that the spectral modes of this nanoparticle arise due to the coupling between the inner Au core and outer Au shell plasmon modes. Understanding precisely the plasmon response of concentric symmetric nanostructures is fundamentally interesting and may provide routes to a variety of technological applications, ranging from novel optical devices to biomedical imaging and the treatment of deadly diseases.
Chapter 8: Metallic Nanoshells with Semiconductor Cores: 
core media modification of absorption and scattering response

8.1 Introduction

In the previous chapter, we discussed a unique route to tuning the plasmon resonance of nanoshells to the NIR in sub-100 nm size regime by simply introducing Au nanoparticle core inside a SiO$_2$/Au nanoshell. In this chapter we investigate another approach for achieving sub-150 nm and sub-100 nm nanoshells resonant in the NIR, by changing the core material to a higher permittivity semiconductor oxide such as cuprous oxide (\(\varepsilon \sim 7.8\)). The high permittivity of the Cu$_2$O core induces a dramatic increase in absorption efficiency, a significant redshift of the plasmon energy, and a reduced plasmon linewidth, relative to nanoshells with lower permittivity dielectric cores. The Cu$_2$O core also results in a strong enhancement of the electromagnetic field inside the core, an important property which may enable new optical applications. Rizia Bardhan fabricated and characterized the Cu$_2$O/Au nanoshells, performed optical studies and calculated far field properties. Nathaniel Grady contributed in analysis and discussion. Manuscript in preparation: Rizia Bardhan, Nathaniel K. Grady, Tamer Ali, Naomi Halas.

Nanoshells, nanoparticles with dielectric cores and metallic shells, have elicited increasing scientific and technological interest due to their ability to manipulate light in unique ways. Their optical properties are governed primarily by the surface plasmons supported by this structure, whose resonance frequencies are tuned by varying the internal nanoparticle geometry. The surface plasmons give rise to intense local electromagnetic fields at the nanoshell surface, which have been harnessed for several photonic,$^{215, 216}$ spectroscopic$^{217, 218}$ and biomedical$^{27, 187}$ applications. At the plasmon
resonance, these nanoparticles also exhibit large far field scattering and absorption cross sections. Nanoshell plasmons have also been shown to dramatically affect radiative transitions in materials adjacent or inside of a metallic nanostructure.\textsuperscript{5, 130, 219} Analogous to the Purcell effect in cavities and waveguides, emission from an adjacent excited atom, molecule, or semiconductor can decay directly into a resonant plasmon, modifying the available photon density of states and changing its radiative decay rate.\textsuperscript{220} The plasmon then radiates the emission to the far field. This is essentially a nanoantenna effect, where the nanoparticle serves to couple the emission of the adjacent fluorescent medium to the far field. The scattering cross section of the nanoshell is critically important to the efficiency of this process.

The plasmon resonance frequencies of nanoshells is a function of the dimensions and permittivity of the core, the shell and the embedding medium.\textsuperscript{23, 25} In addition to tuning the resonance energy, the core and shell properties also dramatically influence nanoshell absorption and scattering efficiencies. For simpler nanoparticles like nanospheres or nanorods, the amount of light absorbed or scattered by the nanoparticle is a function of nanoparticle size. While smaller nanoparticles are predominantly absorptive, the absorption to scattering ratio decreases with increasing nanoparticle size, where ultimately larger particles are better light scatterers than light absorbers. With the layered nanoshell geometry, however, this relationship is more complex. Recently we showed that the absorption and scattering efficiencies of gold-silica-gold layered nanostructures can be modified, for a fixed nanostructure size, by tailoring the thickness of the intermediate dielectric layer.\textsuperscript{221} In this study we show that by changing the core material to a higher permittivity semiconductor cuprous oxide (Cu$_2$O), the absorption and
scattering cross sections of the nanoshell can be significantly altered. The unique ability to manipulate the optical properties of this nanoparticle by varying either internal geometry or material makes nanoshells extremely attractive for a wide variety of applications.

Cu$_2$O, a semiconductor with a bandgap of 2.17 eV, is an ideal material for studying exciton physics. In bulk Cu$_2$O, the lowest-energy "yellow" exciton series is a prototypical example of Wannier excitons, with energy levels following a simple hydrogenic model for $n \geq 2$ and oscillator strengths for $n \geq 3$. The 1s state is distinct because its excitonic Bohr radius (0.53 nm) is comparable to the lattice constant (0.43 nm), resulting in several interesting properties. Electron-hole exchange interactions split the 1s exciton into a triply degenerate orthoexciton with total orbital angular momentum $J = 1$, and a paraexciton 12 meV below the orthoexciton with a total orbital angular momentum $J = 0$. Due to the inversion symmetry of Cu$_2$O, direct creation of 1s excitons by one-photon optical transitions is only quadrupole allowed for the orthoexcitons and completely forbidden for the paraexcitons. In contrast, two-photon absorption processes may provide a route to populating these states. The small Bohr radius also results in a large binding energy, approximately 150 meV for the paraexciton and 140 meV for the orthoexciton, attributable to the lack of Coulomb screening by the lattice. Because these excitons are strongly bound, they retain their bosonic character up to a relatively high temperature and density, providing a promising system for the study of excitonic Bose-Einstein condensation (BEC). In addition to BEC, these excitons have also been well studied for numerous physical effects. The nearly ideal excitonic structure allows detailed studies of the manipulation of the band structure with magnetic,
electrical and optical fields. This can be exploited to populate and probe optically
forbidden exciton states,²²⁸-²³⁰ and has led to the observation of dynamical Stark shifts in
the exciton spectrum.²³¹ Exciton physics is not the only area in which Cu₂O has attracted
significant interest. Cu₂O is also a promising material for third harmonic generation in the
near- and mid-IR due to its significant non-resonant third order susceptibility (χ(3))
combined with a lack of competing second order processes and minimal absorption of the
generated light.²³²

By encapsulating Cu₂O in a nanoscale Au shell, the nanoshell plasmons generated
at the Cu₂O/Au interface can be used to effectively control the coupling of these excitons
to light. This can be achieved both by dramatically enhancing the excitation field in the
semiconductor, particularly important for two-photon absorption, and by tuning the
radiative decay rate. More directly, coupling between plasmons and excitons can lead to
the formation of hybrid plasmon-exciton, or plexciton, states. This has been previously
observed for Frenkel excitons in J-aggregates adsorbed to the surface of a nanoshell.²³³
The optically bright nanoshell plasmon modes can couple to the optically dark
paraexcitons of Cu₂O, providing a simple and feasible route for detecting these optically
forbidden exciton states. The nanoshell plasmon resonance also results in strong field
enhancements which can dramatically increase the nonlinear response of the Cu₂O core.

In this study, we experimentally fabricate nanoshells consisting of a Cu₂O core
coated with a thin Au shell, (Cu₂O/Au) and, in conjunction with theoretical models,
investigate the unique plasmonic properties of these nanoshells. The nanoshells were
fabricated in the sub-100 nm and sub-150 nm size regime and have tunable plasmon
resonances from the visible to the NIR region of the spectrum. Due to the high
permittivity of Cu$_2$O, the plasmon resonance of Cu$_2$O/Au nanoshells were observed to red shift by ~100-130 nm compared to nanoshells with a lower permittivity core material, such as SiO$_2$ core ($\varepsilon\sim2.04$) Au shell (SiO$_2$/Au) nanoshells of equivalent dimensions. Theoretical analysis reveals that the larger dielectric constant of Cu$_2$O results in a nanoshell with high absorption efficiency compared to dielectric core materials such as SiO$_2$. The presence of the Cu$_2$O core material results in a strong enhancement of the electromagnetic field inside the nanoparticle. Due to their compact size, tunability in the NIR and larger absorption cross-sections, Cu$_2$O/Au nanoshells should also be highly efficient nanoparticles for photothermal heating applications in biomedicine, where efficient conversion of absorbed light to heat is required.

8.2 Experimental Methods

Cu$_2$O nanoparticle synthesis: All materials were purchased from Sigma Aldrich and used without further purification. The Cu$_2$O nanoparticles were synthesized by adapting and modifying a protocol previously reported.$^{234}$ Briefly, 4 ml of 0.01 M aqueous CuSO$_4$ solution were mixed with 16 mL of PEG dithiol (MW 1530) at various concentrations under vigorous stirring at room temperature. Subsequently, 13 mL of 0.115 M NaOH and 10.5 mL of 0.005 M Ascorbic acid were mixed together in a separate vessel and quickly added to the CuSO$_4$ - PEG dithiol solution mixture. The reaction was proceeded for an additional 2 minutes under vigorous stirring following which it was quenched by blowing an inert gas (N$_2$ or Ar) into the reaction mixture for 20 minutes. Depending on the particle size, the solution turns from colorless to bright yellow (for smaller Cu$_2$O nanoparticles) or orange (for larger Cu$_2$O nanoparticles) in color. The nanoparticles were
purified by centrifuging the nanoparticles between 5500–7000 rpm depending on particle size. All reaction conditions are provided in Table 8.1.

**Cu₂O/Au nanoshells synthesis:** The Cu₂O/Au nanoshells were fabricated by seed mediated electroless plating of Au onto Cu₂O nanoparticles. Briefly, monodisperse Cu₂O nanoparticles were decorated with small gold colloid (2- 3 nm) prepared by the method reported by Duff et al.²⁰ A continuous gold shell was grown around the Cu₂O nanoparticles by reducing gold from an Au⁺ plating solution onto the attached small colloid in the presence of CO(g)²¹. The plating solution was prepared by mixing 50 mg of potassium carbonate with 200 mL of H₂O and 3 mL of a 1 % HAuCl₄ solution and allowed to age for 24 hours before using. The fabricated Cu₂O/Au nanoshells were centrifuged several times and finally redispersed in aqueous media to form desired particle concentrations.

**Formation of Cu₂O nanoparticle arrays:** Cu₂O nanoparticle arrays were formed by applying droplets of nanoparticle solution to a silicon or quartz substrate and allowing the solvent to evaporate under ambient conditions. The silicon and quartz substrate were thoroughly rinsed with acetone and dried in a stream of N₂ before applying the droplet of nanoparticle solution. The solvent evaporation resulted in the nanoparticle monolayer to self-assemble into ordered arrays. The pattern morphologies of the nanoparticle arrays were dependent on the particle concentration of the Cu₂O nanoparticle solutions. At very low particle concentrations, only isolated small monolayer domains of arrays with short-range order could be formed on the substrates. Increasing the particle concentration resulted in an increase in the domain size as the initially separated domains began to coalesce with each other to form continuous long-range-ordered monolayers.
Characterization: The nanoparticles were characterized by obtaining SEM images using a FEI Quanta 400 field emission SEM at an acceleration voltage of 25 kV, XRD patterns by using a Rigaku Ultima II vertical θ - θ powder diffractometer (Cu Kα, λ=1.5418 Å) and absorption measurements using a Varian Cary 5000 UV-Vis-NIR spectrophotometer.

8.3 Fabrication and Optical Properties of Cu₂O nanoparticles

Figure 8.1. SEM images of Cu₂O nanoparticles of different sizes. The radii of the nanoparticles are (A) 15 ± 0.5 nm, (B) 30 ± 1 nm, (C) 50 ± 2 nm, (D) 100 ± 5 nm, (E) 250 ± 30 nm, and (F) 350 ± 50 nm.
The Cu$_2$O/Au nanoshells were fabricated by a stepwise procedure by first synthesizing Cu$_2$O nanoparticles in the size range of 15 ± 0.5 nm to 350 ± 150 nm in radius in aqueous media at ambient temperature. The synthesis technique developed here is originally adapted and modified from one reported previously. The nanoparticles were fabricated by reducing copper (II) salts with ascorbic acid in the presence of sodium hydroxide and poly (ethylene) glycol dithiol (HS-PEG-SH). All the reaction conditions are provided in Table 8.1. Representative scanning electron microscope (SEM) images of the Cu$_2$O nanoparticles verify their morphology and dimensions (Fig. 8.1). X-ray powder diffraction spectra confirming the composition of the Cu$_2$O nanoparticles are shown in Figure 8.2. By simply varying the concentration of HS-PEG-SH, which acts as a stabilizing polymer, while keeping the concentration of the other reagents constant, the size of the Cu$_2$O nanoparticles were straightforwardly tuned. It is noticeable that the polydispersity is as low as ~ 3% for the nanoparticle sizes ≤ 200 nm; however, for the larger nanoparticles polydispersity increases beyond 10%. This observation indicates that at higher HS-PEG-SH concentrations, more nucleation sites are initiated leading to a higher rate of growth kinetics resulting in a larger size distribution. As the size of the nanoparticle increases, particularly for sizes ≥ 200 nm, more particles exhibit truncated cubic rather than near-spherical shapes (Fig. 8.1). The preferential adsorption of ions in a solution to different crystalline faces directs the development of nanoparticles into unusual shapes by controlling the growth kinetics along the different crystal axes. For Cu$_2$O, cubic shape is known to form when the crystalline growth is along the (111) plane. The observed shapes imply that a fraction of the PEG-dithiol molecules may be adsorbing on the (111) plane and hence assisting in the morphological development of
the Cu₂O nanoparticles along the <111> direction. In addition, the HS-PEG-SH molecules also terminate the Cu₂O nanoparticles with thiol groups which can attach to Au without the need of further functionalization for Au coating.

![XRD patterns of Cu₂O nanoparticles of different sizes.](image)

**Figure 8.2.** XRD patterns of Cu₂O nanoparticles of different sizes. The diameter of the nanoparticles corresponding to each pattern is provided on the right. The amorphous background observed on the lower two patterns is due to the glass substrate used for sample preparation.

**Table 8.1.** Experimental parameters for synthesizing Cu₂O nanoparticles of various sizes.

<table>
<thead>
<tr>
<th>CuSO₄ (4 mL)</th>
<th>PEG-dithiol (16 mL)</th>
<th>Ascorbic Acid (10.5 mL)</th>
<th>NaOH (13 mL)</th>
<th>Particle Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 M</td>
<td>0.005 M</td>
<td>0.005 M</td>
<td>0.115 M</td>
<td>700 ± 100</td>
</tr>
<tr>
<td>0.01 M</td>
<td>0.003 M</td>
<td>0.005 M</td>
<td>0.115 M</td>
<td>500 ± 60</td>
</tr>
<tr>
<td>0.01 M</td>
<td>0.0025 M</td>
<td>0.005 M</td>
<td>0.115 M</td>
<td>200 ± 10</td>
</tr>
<tr>
<td>0.01 M</td>
<td>0.0015 M</td>
<td>0.005 M</td>
<td>0.115 M</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>0.01 M</td>
<td>0.0008 M</td>
<td>0.005 M</td>
<td>0.115 M</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>0.01 M</td>
<td>0.0003 M</td>
<td>0.005 M</td>
<td>0.115 M</td>
<td>30 ± 1</td>
</tr>
</tbody>
</table>

The experimental and theoretical optical properties of the Cu₂O nanoparticles demonstrate the shape dependent optical response of these nanoparticles (Fig. 8.3).
Optical images (Fig. 8.3A) of the Cu$_2$O nanoparticles dispersed in aqueous media clearly shows as the nanoparticle sizes increase the solution changes from bright yellow to orange, similar to that reported previously for Cu$_2$O nanostructures.$^{234}$ Experimental extinction spectra of the Cu$_2$O nanoparticles of different sizes dispersed in aqueous media demonstrate the different peaks that arise as the size of the nanoparticles increase (Fig. 8.3B). The radius of the nanoparticles corresponding to each optical spectrum is indicated on the right. The extinction spectra of the smaller nanoparticles ($\leq 100$ nm) exhibit strong Rayleigh scattering and a small peak at 440 nm. As the nanoparticle sizes increase to 200 nm, new peaks are observed in the visible near 530 nm and in the near-infrared near 700 nm. With increasing size of the Cu$_2$O nanoparticles, the peaks in the visible and the near-infrared red shifts and additional features are observed in the near and mid-infrared. These additional extinction peaks observed for the larger nanocrystals are due to scattering at the well known Mie resonances of dielectric particles.$^{26,235}$

The theoretical extinction spectra of the Cu$_2$O nanoparticles (Fig. 8.3C) were obtained utilizing Mie theory assuming a spherical geometry with diameters matching the experimentally obtained nanoparticle sizes. The calculated extinction spectra correspond well to the measured spectra for all the nanoparticle sizes. Small disparities between calculated and experimental spectra are plausibly due to polydispersity, the combination of spherical and truncated cubic shapes in the nanoparticle solutions, and small variations in the dielectric function of the fabricated particles from the values for bulk Cu$_2$O obtained from literature.$^{236}$ Nanoparticles have been reported to have higher band gap energy compared to the bulk material which is attributed to impurities and defects in crystal structure.$^{237}$ The Cu$_2$O nanoparticles also form self-assembled ordered arrays
when a droplet of the nanoparticle solution is allowed to dry in ambient air at room
temperature on a silicon or quartz substrate as represented in the SEM images (Fig. 8.4).

**Figure 8.3.** (A) Optical image of Cu$_2$O nanoparticles dispersed in aqueous media of increasing sizes (indicated by arrow). (B) Experimental absorption spectra of the Cu$_2$O nanoparticles dispersed in aqueous media. The radius of the nanoparticles corresponding to the spectra is indicated on the right. Spectra are offset for clarity. (C) Calculated absorption spectra of the Cu$_2$O nanoparticles in aqueous media ($n = 1.33$). The spectra are color coordinated with part (B) and offset for clarity.
Figure 8.4. Self-assembled arrays of Cu$_2$O nanoparticles of increasing diameter (A) 30 nm, (B) 60 nm, (C) 100 nm and (D) 200 nm. The scale bar in (A) – (D) is 500 nm. (E) Low resolution SEM image of 100 nm diameter Cu$_2$O nanoparticle arrays.
8.4 Fabrication and Optical Properties of Cu₂O/Au Nanoshells

![Figure 8.5](image)

**Figure 8.5.** SEM images of Cu₂O/Au-NS of different sizes. (A) \([r_1, r_2] = [15, 32]\) nm, (B) \([r_1, r_2] = [30, 46]\) nm, (C) \([r_1, r_2] = [40, 54]\) nm, (D) \([r_1, r_2] = [50, 65]\) nm. The scale bar is 200 nm.

Cu₂O/Au nanoshells are fabricated by seed mediated electroless plating of Au onto the thiol terminated Cu₂O nanoparticles.\(^{12, 18}\) Small Au nanoparticles (~ 2 nm in diameter) are immobilized onto the surface of the Cu₂O cores. The immobilized Au nanoparticles act as nucleation sites for electroless plating of Au onto the surface of core particles, which gradually results in the formation of a continuous and complete metal shell layer upon reduction. Further metal deposition onto the nanostructure increases the thickness of the Au layer. SEM images of Cu₂O/Au nanoshells fabricated on cores ranging in size from a radius of 15 nm to 50 nm are shown in Figure 8.5. While Cu₂O nanoparticles can be synthesized in different shapes,\(^{234, 238}\) the growth of a uniform Au layer on the nanoparticles modifies the overall shape. The Au shell grows as a polycrystal on the Cu₂O nanoparticle cores and hence results in a spherical morphology.
with minor surface roughness. This facile synthesis route allows the fabrication of uniform, monodisperse, and non-flocculated core-shell nanostructures in sub-150 nm and sub-100 nm size regimes. The Au coating also allows dispersiblity in aqueous media and provides a biocompatible surface, which can be straightforwardly conjugated with functional molecules.

The experimental and calculated optical properties of the Cu$_2$O/Au nanoshells are represent the morphological development of the Cu$_2$O after Au shell growth (Fig. 8.6). The optical images of Cu$_2$O/Au nanoshells dispersed in aqueous media (Fig. 8.6A) indicates a change in the solution color from purple to blue as the size of the Cu$_2$O/Au nanoshells increases. The experimentally observed extinction spectra of the Cu$_2$O/Au nanoshells dispersed in aqueous media with different core/shell ratios corresponding to the SEM images (Fig. 8.5) are illustrated in Figure 8.6B. The extinction spectra spans from the visible to the NIR region of the optical spectrum with the smallest size $[r_1, r_2] = [15, 32]$ nm showing a plasmon resonance at 610 nm. With increasing nanoshell sizes, the plasmon resonance red shifts to 705 nm, 780 nm, and 830 nm respectively (Fig. 8.6A). In the quasistatic limit, where the size of the nanoshell is much smaller than the spatial wavelength of light corresponding to its plasmon resonance, the plasmon energies of a nanoshell depend on their aspect ratio $r_1/r_2$. As the aspect ratio increases the plasmon resonance shifts to longer wavelengths, which explains the redshifted plasmon resonance as the size of the Cu$_2$O/Au nanoshells increases. This behavior can be explained straightforwardly in terms of plasmon hybridization, an analytical tool useful for understanding the resonant modes of complex metal nanostructures.\textsuperscript{21}
Figure 8.6. (A) Optical image of Cu$_2$O/Au-NS of different sizes. (i) $[r_1, r_2] = [15, 32]$ nm, (ii) $[r_1, r_2] = [30, 46]$ nm, (iii) $[r_1, r_2] = [40, 54]$ nm, and (iv) $[r_1, r_2] = [50, 65]$ nm (B) Experimental extinction spectra of Cu$_2$O/Au-NS dispersed in aqueous media of different sizes: (i) $[r_1, r_2] = [15, 32]$ nm, $\lambda_{\text{max}} = 610$ nm, (ii) $[r_1, r_2] = [30, 46]$ nm, $\lambda_{\text{max}} = 705$ nm (iii) $[r_1, r_2] = [40, 54]$ nm, $\lambda_{\text{max}} = 780$ nm and (iv) $[r_1, r_2] = [50, 65]$ nm, $\lambda_{\text{max}} = 830$ nm. Spectra are offset for clarity. (C) Calculated extinction spectra of the Cu$_2$O/Au-NS in aqueous media. The spectra are color coordinated with part (B) and offset for clarity. The calculations were performed assuming spherical core and shell geometry.
For a spherical nanoshell where the dielectric constant of the core $\varepsilon_c \leq 8$, the low energy bonding nanoshell plasmon is optically active and is primarily observed in the extinction spectra.\textsuperscript{22, 23} For increasing shell thickness, the interaction between the cavity and sphere plasmons decreases, resulting in a blueshift of the bonding mode and a redshift of the antibonding mode. In addition, the plasmon resonance shifts to longer wavelengths with increasing overall size due to phase retardation effects.\textsuperscript{24, 25} The calculated extinction spectra of the Cu$_2$O/Au nanoshells corresponding to those observed experimentally were obtained using Mie theory assuming a spherical core and spherical shell geometry (Fig. 8.6C). The calculated spectra and peak positions correspond well with the experimental extinction spectra suggesting that Cu$_2$O nanoparticles $\leq$ 100 nm are mostly spherical and the small fraction of truncated cubic shapes present do not affect the ensemble plasmon line shape or peak position. For the smallest fabricated Cu$_2$O/Au nanoshells, with $[r_1, r_2] = [15, 32]$ nm, the observed plasmon line width is larger than those obtained theoretically, probably due to inhomogeneities in the Au layer thickness and some aggregation of the nanoshells after the growth of the Au layer.

The tunability of the plasmon resonance was also observed experimentally by varying the Au shell thickness for a fixed core size. The experimental extinction spectra of Cu$_2$O/Au nanoshells with the same core radius 40 ± 2 nm and varying the shell thicknesses for a total outer radius of 54 ± 5 nm, 57 ± 5 nm, 60 ± 7 nm, and 63 ± 11 nm are shown in Figure 8.7A. Spectra calculated using Mie theory corresponds well to the experimental extinction spectra (Fig. 8.7B). With increasing Au shell thickness the aspect ratio $r_1/r_2$ increases similar to that observed in Figure 8.6, the plasmon resonance peak blue shifts to shorter wavelengths from 780 nm to 710 nm.
Figure 8.7. (A) Experimental extinction spectra of Cu$_2$O/Au-NS for core radius $r_1 = 40 \pm 2$ nm with varying Au shell thicknesses: (i) $r_2 = 54 \pm 5$ nm, (ii) $57 \pm 5$ nm, (iii) $60 \pm 7$ nm and (iv) $63 \pm 11$ nm. (B) Calculated extinction spectra of Cu$_2$O/Au-NS of similar sizes as observed experimentally. The calculations were performed assuming a spherical core and shell geometry.

8.5 Far-field properties of Cu$_2$O/Au vs. SiO$_2$/Au nanoshells

Changing the core dielectric allows another important aspect of the optical response to be controlled: the relative contribution of absorption and scattering to the overall extinction cross section. The theoretical extinction, absorption, and scattering
spectra of Cu$_2$O/A nanoshells (solid) compared with SiO$_2$/Au nanoshells (dashed) of equivalent aspect ratios ($r_1/r_2$) demonstrates a significant difference in the optical response between the two materials (Fig. 8.8). The optical properties of 4 different sizes are examined here: $[r_1, r_2] = [30, 42]$ nm, $[40, 52]$ nm, $[50, 62]$ nm, and $[60, 72]$ nm. The extinction spectra of the Cu$_2$O/Au nanoshells are $\sim$ 100-130 nm red shifted compared to the SiO$_2$/Au nanoshells of equivalent dimensions for all the sizes calculated here due to the high dielectric constant of Cu$_2$O. The overall scattering efficiency of SiO$_2$/Au nanoshells is higher than Cu$_2$O/Au nanoshells for similar dimensions, despite the extinction being only slightly weaker. This is due to the high permittivity of the Cu$_2$O nanoparticle core which screens the induced charges more efficiently than the SiO$_2$ core. The line width is also reduced slightly in the case of Cu$_2$O/Au nanoshells. Scattering is essentially radiative decay of the plasmon; a reduction of the scattering cross section therefore corresponds to a reduction of the damping of the plasmon. Consequently, the corresponding plasmon line widths are significantly narrower for the Cu$_2$O/Au nanoshells. While the finite imaginary component of the Cu$_2$O dielectric function causes some additional damping, ohmic losses in the Au and radiation dominate the damping and the field in the core is fairly weak such that this is a minor effect.

The high absorption efficiencies of Cu$_2$O/Au nanoshells compared to SiO$_2$/Au nanoshells can be attributed to three possible reasons: (i) charge difference between the inner and outer surface of the Au shell, (ii) plasmon hybridization between the sphere and cavity plasmons and (iii) finite conductance of the Cu$_2$O cores which is absent for the SiO$_2$ cores. Due to the higher permittivity of the Cu$_2$O core, it polarizes more strongly reducing the charge on the inner surface of the Au shell. The increased imbalance of
charge between the inside and outside surfaces of the shell generates a larger field across the metal. Since the metal is highly absorptive, this results in higher absorption efficiencies. The stronger absorption can also be understood in terms of plasmon hybridization. The high permittivity core shifts the plasmon resonance of the cavity-like mode to lower energy resulting in an increase in the admixture of the cavity plasmons in the bonding mode since the energy of the sphere and cavity modes are closer. Hence, despite this mode maintaining a strong dipole moment due to interaction with the cavity plasmons, the energy primarily is lost to absorption because the cavity mode does not radiate effectively. This effect also occurs for the antibonding plasmon mode. Due to the larger admixture of sphere-like plasmon, the dipole moment increases allowing it to interact with light. This is particularly noticeable in Fig. 8.8L, where the scattering in Cu₂O/Au nanoshell exhibits peaks at wavelengths shorter than 600 nm, due to the increased admixture of the sphere-like plasmon in the anti-bonding modes. As the total size of the nanoshells increase, scattering dominates due to retardation effects and consequently the absorption cross-section decreases.

Thus far, the semiconducting nature of the core has been neglected. The plasmon response of semiconductor/Au nanoshells can be further understood by evaluating the wavelength dependence of the semiconductor dielectric function and examining plasmon-exciton coupling by overlap of plasmon wavelength with the semiconductor bandgap energy. In the case of Cu₂O, the real part of the dielectric function (Fig. 8.9) is \( \sim 8 \) in the near-infrared region of the spectrum and increases to \( \sim 9.5 \) near the band gap energy. Due to the conductance of Cu₂O, the imaginary part of the permittivity is finite and decreases monotonously to nearly 0 at long wavelengths.
Figure 8.8. Calculated extinction, absorption, and scattering efficiency spectra of Cu$_2$O/Au-NS (solid) compared with SiO$_2$/Au-NS (dash) nanostructures of different sizes. (A-C) $[r_1, r_2] = [30, 42]$ nm, (D-F) $[r_1, r_2] = [40, 52]$ nm, (G-I) $[r_1, r_2] = [50, 62]$ nm, (J-L) $[r_1, r_2] = [60, 72]$ nm. The calculations are performed assuming a spherical core encapsulated in a spherical shell.
Figure 8.9. Dielectric function of Cu$_2$O obtained from ref. 236.

Figure 8.10. Calculated extinction (blue), absorption (red) and scattering (black) spectra of Cu$_2$O/Au nanoshells of different sizes. Calculations were performed using Mie theory assuming a spherical core and shell with a constant permittivity of 8 for the cores.
Interestingly, neither the imaginary part of the dielectric function of Cu$_2$O nor the wavelength dependence of the dielectric function affects the nanoshell properties significantly. Calculated extinction, scattering and absorption efficiencies of Cu$_2$O/Au nanoshells using constant permittivity of 8 for the cores demonstrates that for similar sizes, the absorption and scattering efficiencies are comparable for both the constant dielectric (Fig. 8.10) and the frequency dependent dielectric function (Fig. 8.8). This observation is not surprising as below the bandgap of the semiconductor core the residual imaginary dielectric is not strong enough to affect the nanoshell properties. This is especially true for the Cu$_2$O/Au nanoshells fabricated here, since the observed plasmon resonances are at longer wavelengths beyond the Cu$_2$O bandgap energy.

Theoretically, the cases where the semiconductor band-gap is at the plasmon energy or at much lower energy than the plasmon resonance, can be investigated by considering Cadmium Telluride-core/Au-shell (CdTe/Au) nanoshells and Lead Sulfide core/Au-shell (PbS/Au) nanoshells, respectively. The band gap energy of CdTe is 1.49 eV and that of PbS is 0.37 eV. Calculated extinction, absorption and scattering spectra of Cu$_2$O/Au nanoshells are compared with CdTe/Au nanoshells and PbS/Au nanoshells of similar sizes (Fig. 8.11). In both cases, shifts in the plasmon resonance are essentially due to the increased dielectric constant rather than the frequency dependence or absorption in the core, similar to those discussed previously.$^{239}$ Unlike the case of Cu$_2$O, where the damping due to the imaginary part of the dielectric function is trivial, significant damping occurs due to the electronic transitions in the semiconductor core when the bandgap energy is at or below the plasmon resonance energy.
Figure 8.11. Calculated extinction (blue), absorption (red) and scattering (black) spectra of various semiconductor cores/Au shell nanoshells of size \([r_1, r_2] = [40, 54]\) nm. (A) Cu$_2$O core/Au shell (B) CdTe core/Au shell, (C) PbS core/Au shell.

Nonetheless, no plasmon-exciton coupling effects are observable. It may be surprising that the frequency dependence of the dielectric function of the core material has such a small effect. For most common semiconductors at room temperature, the excitons are sufficiently broad that they appear as fairly weak peaks over a strong polarizibility due to the one electron transitions. For quantum dots at room temperature or
bulk semiconductors at low temperature this may not be the case; however, a detailed discussion of plasmon-exciton coupling in these cases is beyond the scope of this paper.

8.6 Conclusions

In conclusion, we have engineered a plasmonic nanostructure, by utilizing straightforward fabrication technique, consisting of a high permittivity semiconductor core encapsulated in a thin Au shell. The plasmon resonance of the Cu$_2$O/Au nanoshells can be tuned from the visible to the NIR by simply modifying the core and shell dimensions. Compared to a dielectric core material such as SiO$_2$, the optical response of Cu$_2$O/Au nanoshells can be tuned farther into the NIR for equivalent nanoshell dimensions. This suggests that Cu$_2$O/Au nanoshells would span a broader range of the spectrum compared to SiO$_2$/Au nanoshells for smaller nanoparticle sizes. This property could be potentially useful in biomedical applications where smaller nanoparticle sizes resonant in the NIR are necessary. Altering the permittivity of the core material also allows optimization of the far field properties for specific applications. For photothermal applications such as cancer therapy and gene therapy Cu$_2$O/Au nanoshells with their enhanced absorption efficiency would be more practical. On the contrary, for photoemission applications such as bioimaging and biological sensing SiO$_2$/Au nanoshells with their high scattering efficiency would be more useful. In addition, the near field properties of Cu$_2$O/Au nanoshells may tremendously impact non-linear optical studies as well as BEC studies of Cu$_2$O. For Cu$_2$O, low temperature measurements are likely necessary to observe plasmon-exciton effects because the excitonic structure in Cu$_2$O is not observable at room temperature due to thermal broadening.
Chapter 9: Au Nanorice Assemble Electrolytically into Mesostars

9.1 Introduction

The assembly of materials and structures from constituent nanoparticles is currently an important topic of fundamental research, stimulated by the growth of numerous applications such as new optical devices, chemical sensing, composite materials, catalysis, and biomedicine. The design and synthesis of novel nanoparticles has been an important starting point, since nanoscale size, shape, and morphology determine the properties of larger materials assembled from nanoscale substituents. The hierarchical self-assembly of nanoscale building blocks can lead to the formation of complex supramolecular assemblies, frequently in striking similarity to structure formation in biological systems. In this chapter we report a surprising observation of the formation of mesoscale star-shaped particles with a hierarchical substructure when an aqueous suspension of nanorice particles are subjected to electrolysis. Rizia Bardhan performed nanorice fabrication, mesostar development, characterization, growth mechanism and optical studies. Oara Neumann contributed in electrolytic cell set-up, mesostar development, optical studies and discussion. Reproduced with permission from Rizia Bardhan, Oara Neumann, Nikolay Mirin, Hui Wang and Naomi J. Halas, ACS Nano, 2009, 3, 266-272. Copyright 2009 American Chemical Society.

The unusual electrolytically assisted assembly reported here is distinct from other electrochemically driven processes, such as the electrochemical synthesis of metal or metal oxide structures. In this case, electrolysis transforms both the shape and
composition of the nanorice particles. The nanorice particles consist of a prolate iron oxide (hematite, $\alpha$-Fe$_2$O$_3$) core coated with a thin Au shell, whereas the hyperbranched mesostars were composed of Au, iron oxide, and iron oxyhydroxide (goethite, $\alpha$-FeOOH). To the best of our knowledge, the process of electrolyzing an aqueous suspension to transform nanoparticles into mesoscale structures has not been previously reported. This discovery may provide a new approach for the development of new structures and materials from nanoscale constituents.

### 9.2 Mesostar Formation

**Materials Utilized:** Ferric chloride (FeCl$_3$·6H$_2$O), (3-aminopropyl) triethoxysilane (APTES, 99%), tetrachloroauric acid (HAuCl$_4$·3H$_2$O), tetrakis hydroxymethyl phosphonium chloride (THPC), polyvinyl pyridine (PVP) were purchased from Sigma-Aldrich (St. Louis, MO). 37% formaldehyde, sulfuric acid (H$_2$SO$_4$, 100%), hydrogen peroxide (H$_2$O$_2$, 30%), potassium dihydrogen phosphate (KH$_2$PO$_4$) and 200-proof ethanol were obtained from Fisher Scientific (Hampton, NH). All the chemicals were used as received without further purification. Platinum electrodes and platinum wire were purchased from Alfa Aesar. DC Power supply was purchased from Hewlett-Packard. Quartz slides were purchased from Technical Glass.

**Nanorice fabrication:** Monodisperse spindle-shaped $\alpha$-Fe$_2$O$_3$ nanoparticles and nanorice particles were fabricated as previously reported. Briefly, $\alpha$-Fe$_2$O$_3$ particles with an aspect ratio of 6.3 (340 nm x 54 nm) were prepared by forced hydrolysis of ferric chloride solutions by reacting 100 mL of an aqueous mixture containing $2.0 \times 10^{-2}$ M FeCl$_3$ and $4.0 \times 10^{-4}$ M KH$_2$PO$_4$ at 100 °C for 72 hours. The resulting precipitate was
centrifuged and washed several times with water and ethanol and finally redispersed in 20 mL ethanol. The surface of the $\alpha$-Fe$_2$O$_3$ particles was functionalized with organosilane molecules (APTES) to generate an amine terminated surface. This was achieved by mixing 500 $\mu$L of APTES with 5 mL of ethanolic solution of hematite particles for 12 hours under vigorous stirring. The resulting particles were centrifuged and redispersed in ethanol several times to remove excess APTES. These functionalized nanoparticles were decorated with small gold colloid (2-3 nm) prepared by the method reported by Duff et al.$^{20}$ Nanorice particles were fabricated via seed-mediated reduction of AuCl$_4^-$ ions onto the attached small colloid in the presence of formaldehyde.

Mesostar Formation: The electrolytic cell used to form the mesostars is shown (Scheme 9.1). Platinum electrodes 0.020 mm wide were placed 0.014 m apart in an aqueous suspension of nanorice particles ($10^9$ particles/mL) and connected to a DC power supply.

![Scheme 9.1](image)

Scheme 9.1. Schematic representation of experimental set-up showing a DC power supply connected between two Pt electrodes (gray bars), separated by 0.014 m, immersed in aqueous Au nanorice solution.

The nanorice particles utilized in these experiments consisted of prolate hematite cores with a longitudinal diameter of $340 \pm 25$ nm and a transverse diameter of $54 \pm 6$ nm,
coated with a 22 ± 3 nm Au shell. At ambient temperature and pressure, a potential difference of 5 V was applied to the cell, and the particles self-assembled to form mesostars within 24 hours.

**Nanoparticle films for optical measurements:** Nanoparticle films were obtained by immobilizing them on PVP functionalized quartz slides. Briefly, quartz slides were cleaned in piranha solution (H₂SO₄ (100%): H₂O₂ (30%) = 3:1) for 2 hours, rinsed with H₂O and 200 proof-ethanol and dried in a stream of N₂ gas. The slides were then immersed in a 1 wt% solution of PVP in ethanol for 24 hours, followed by rinsing thoroughly in ethanol and drying with N₂ gas. The PVP functionalized slides were immersed in aqueous solutions of nanorice, mesostars, hematite nanoparticles and pyramidal mesostructures for 1 hour. Upon removal from the individual solutions, the slides were rinsed with ethanol and dried with N₂ gas. This resulted in a monolayer of randomly oriented nanoparticles on the PVP functionalized slides.

**9.3 Mesostar Characterization**

The mesostars were characterized using several analytical techniques to elucidate their formation mechanism including TEM using a JEOL JEM-2010 TEM operated at 200 kV, SEM using a FEI Quanta 400 field emission SEM at an acceleration voltage of 20 kV, XRD by using a Rigaku Ultima II vertical θ - θ powder diffractometer (Cu Ka, λ=1.5418 Å) and absorption measurements using a Varian Cary 5000 UV-Vis-NIR spectrophotometer.

The mesostars were typically 650 ± 80 nm in length and width and ~380 ± 30 nm in height, as shown in the scanning electron microscope (SEM) image in Figure 9.1A. All the mesostars observed in solution had similar shape and morphology but varied in size.
distribution. Powder X-ray diffraction (XRD) studies (Fig. 9.1B) revealed a surprising transformation in the composition, and crystal structure of the mesostars relative to the nanorice particles. XRD spectrum of $\alpha$-Fe$_2$O$_3$ particles, which served as the core material of the nanorice is shown in Figure 9.1B-i.

**Figure 9.1.** Electron microscopy and crystallographic studies of mesostars prepared at 5V. (A) SEM image of Mesostars. (B) XRD spectra of (i) $\alpha$-Fe$_2$O$_3$ cores, (ii) nanorice (showing Au peaks), and (iii) mesostars. The spectra are offset for clarity.
These particles show a highly crystalline, hexagonal phase of $\alpha$-Fe$_2$O$_3$ with cell parameters $a = 5.035$ Å and $c = 13.747$ Å and space group R3c (167) (JCPDS card no. 98-000-0240). The XRD peak intensities correlate well with those from the powder diffraction intensity profile of $\alpha$-Fe$_2$O$_3$. However, a preferential orientation of the $\alpha$-Fe$_2$O$_3$ core particles is observed, a common feature among single crystalline nanoparticles. The XRD spectrum of nanorice (Fig. 9.1B-ii) clearly demonstrates the presence of a crystalline Au shell covering the $\alpha$-Fe$_2$O$_3$ cores. The crystal structure of the Au shell corresponds to the cubic phase of Au with cell parameters $a = 4.078$ Å and space group Fm3m (225) (JCPDS card no. 98-000-0230). The Au shell on the $\alpha$-Fe$_2$O$_3$ cores grows non-epitaxially as a polycrystal, which explains the absence of preferential orientation in the XRD spectrum of nanorice. The XRD spectrum of mesostars (Fig. 9.1B-iii) reveals a mixture of peaks corresponding to $\alpha$-FeOOH, $\alpha$-Fe$_2$O$_3$, and Au. The $\alpha$-FeOOH peaks suggest a partial transformation of $\alpha$-Fe$_2$O$_3$ into $\alpha$-FeOOH, which has an orthorhombic phase with lattice parameters $a = 4.937$ Å, $b = 4.432$ Å, and $c = 2.994$ Å, and space group P2$_1$nm (31) (JCPDS card no. 00-026-0792). The strongest peak corresponding to the $\alpha$-FeOOH (101) plane indicates that the mesostars are mostly composed of goethite, and also specifies a preferential orientation of the goethite crystalline domains.

The observed alteration in structure and composition of nanorice particles to form mesostars was further studied using transmission electron microscopy (TEM) and electron diffraction (ED). TEM images with corresponding ED and selected area electron diffraction (SAED) patterns of mesostars are shown in Figure 9.2. A smaller star-shaped structure obtained within 6 hours of electrolysis (Fig. 9.2A) was observed to be
polycrystalline as suggested by the ED ring pattern. The TEM micrograph and corresponding SAED pattern of a mesostar formed after 24 hours of electrolysis (Fig. 9.2B) demonstrates an orthorhombic single crystalline area that is oriented toward the (200) plane and corresponds to α-FeOOH. In addition to the mesostars, α-FeOOH nanocrystals were also observed in the solution mixture (Fig. 9.2C). These nanocrystals demonstrated the orthorhombic phase of α-FeOOH that is preferentially oriented towards the [001] direction. The tips of the mesostars (Fig. 9.2D) also consisted of single crystalline domains of α-FeOOH oriented along the (200) plane. Dark-field TEM, an effective tool in observing lattice defects, grain boundaries, and ordered domain structures, was utilized to image the mesostar tip as shown in Figure 9.2E. The highly crystalline α-FeOOH domains are clearly demarcated by the lighter areas in Figure 9.2E. The darker central area indicates that this region is relatively dense and does not allow the electron beam to penetrate through. The TEM micrographs and SAED patterns in Figure 9.2 (A-E) undoubtedly show that these mesostars have many crystalline domains of α-FeOOH, which explains the α-FeOOH peaks observed in the XRD spectrum of mesostars (Fig. 9.1B-iii). The chemical composition of the observed mesostars was generally identical, composed mostly of α-FeOOH crystalline domains. However, the mesostars are also composed of Au as indicated by the XRD spectrum. The ED pattern of a single mesostar (Fig. 9.2F, top inset) illustrates polycrystallinity, however, its SAED pattern (bottom inset) shows a face-centered cubic single crystalline domain of Au oriented along the (220) plane. Since these mesostructures were polycrystalline, individual crystallographic domains of Au, α-FeOOH, and α-Fe₂O₃ are difficult to
determine for each particle, however these representative TEM images confirm their composition.

**Figure 9.2.** TEM micrographs of a (A) star-shaped structure obtained after 6 hours of electrolysis and ED pattern showing polycrystalline rings provided as inset, (B) mesostar formed after 24 hours of electrolysis and corresponding SAED pattern representing orthorhombic \( \alpha \text{-FeOOH} \) single crystalline domain, (C) \( \alpha \text{-FeOOH} \) nanocrystals observed in solution and SAED pattern showing orthorhombic \( \alpha \text{-FeOOH} \) single crystalline domain, (D) tip of a mesostar and corresponding SAED pattern representing orthorhombic \( \alpha \text{-FeOOH} \) single crystalline area, (E) dark field image of mesostar tip corresponding to (D), and (F) mesostar obtained after 24 hours of electrolysis, ED pattern showing polycrystalline rings provided as top inset, and SAED pattern representing cubic Au single crystalline area provided as bottom inset. The selected area corresponding to the SAED patterns in (B), (C), (D) and (F) are shown in circle.

The phase transformation of \( \alpha \text{-FeOOH} \) to \( \alpha \text{-Fe}_2\text{O}_3 \) is known to occur via heat treatment by loss of \( \text{H}_2\text{O} \) molecules, \(^{251}\) or by liquid phase transformation.\(^{252}\) However, at present the reverse mechanism of \( \alpha \text{-Fe}_2\text{O}_3 \) conversion to \( \alpha \text{-FeOOH} \) is not well understood. The formation of \( \alpha \text{-FeOOH} \) nanocrystals is strongly influenced by the
The shape and size of $\alpha$-FeOOH nanocrystals influence many of their physical properties, which consequently determine the morphological development of larger-ordered structures. The formation mechanism discussed below explains the morphological modification as well as the alteration in the composition of $\alpha$-Fe$_2$O$_3$-Au nanorice particles to form mesostars composed of $\alpha$-Fe$_2$O$_3$, Au and $\alpha$-FeOOH.

![Diagram of mesostar formation mechanism](image)

**Figure 9.3.** (A) Schematic representation of mesostar formation mechanism. (B) SEM images supporting the mesostar formation mechanism starting with (i) nanorice, (ii) 2 hrs of electrolysis, (iii) 4 hrs, (iv) 6 hrs, (v) 8 hrs, (vi) 10 hrs, (vii) 12 hrs, and (viii) 24 hrs.

### 9.4 Formation Mechanism

A systematic and controlled study of mesostar formation revealed electrolysis-induced complex assembly of nanorice particles to form these hierarchical structures. A schematic representation of the mesostar formation mechanism (Fig. 9.3A) and representative SEM images taken after 0, 2, 4, 6, 8, 10, 12, and 24 hours of electrolysis (Fig. 9.3B) support
the proposed mesostar growth mechanism. Additional images showing an overview of the entire system at a given time period is shown in Figure 9.4. During electrolysis, the Au shell of nanorice particles (Fig. 9.3B-i) is etched away, leaving residual Au islands and small Au colloid in the reaction mixture (Fig. 3B-ii). The etching process of the Au shell may result from two plausible mechanisms. First, active O$_2$ is produced during electrolysis (eq. 2) which has been shown to etch Au.$^{255}$ Second, active hydroxyl groups, which are also produced during electrolysis (eq. 1), may react with the hematite core, altering its crystal structure. The subsequent re-crystallization of the hematite core may then induce strain on the Au shell, causing it to partially etch.$^{256}$

\[
\text{Cathode: } 2\text{H}_2\text{O} \rightarrow 2\text{H}_2 + 2\text{OH}^- \quad E_{\text{red}} = 0.00 \text{ V} \tag{1}
\]

\[
\text{Anode: } 4\text{OH}^- \rightarrow \text{O}_2 + 2\text{H}_2\text{O} + 4\text{e}^- \quad E_{\text{ox}} = -1.23 \text{ V}. \tag{2}
\]

Active hydroxyl anions produced during electrolysis could also be incorporated into the hematite crystal lattice as hydroxyl defects, forming a metastable phase termed “hydrohematite” (Fe$_2$O$_3$.H$_2$O).$^{257, 258}$ α-Fe$_2$O$_3$ possibly converts to α-FeOOH from the hydrohematite phase by replacement of oxygen atoms with hydroxyl ions:

\[
2\text{Fe}_2\text{O}_3.\text{H}_2\text{O} + 2\text{OH}^- + 2\text{H}^+ \rightarrow 4\text{FeOOH} + \text{O}_2 + 2\text{H}_2. \tag{3}
\]

In addition to partial removal of the Au shell, the electrolysis process partially disintegrates the α-Fe$_2$O$_3$ particles, converting them into α-FeOOH nanocrystals (see Figure 9.2C). Subsequent to Au shell disintegration, the partially etched nanorice particles were completely coated with α-FeOOH nanocrystals and colloidal Au particles. These structures then preferentially aligned along their centers to form a cross-shaped structure (Figure 9.3B-iii). In some cases, end-to-end assembly occurred and formed long chains, but these were produced in relatively low yield, Figure 9.5. After 4 hours of
electrolysis, α-FeOOH nanocrystals assembled on the central axis of the cross structures, which was possibly the most convenient polarizable axis in the DC-field. This transformed the cross structures into star-shaped structures (Figure 9.3B iv-v). These star-shaped mesostructures continued to grow and after 24 hours of electrolysis yielded hierarchical crystalline mesostars (Figure 9.3B vi-viii). The mesostars were formed in the bulk of solution, and were not observed on the electrodes. Representative SEM image of the cathode utilized in the electrolytic cell is shown in Figure 9.6 and high resolution image is provided as inset. α-FeOOH nanocrystals, which were also observed in the bulk solution, were present on the cathode, but mesostars were not observed. The anode is not shown as we did not observe any structures or particles on it. This suggests that mesostars were likely formed in the bulk of solution rather than on the electrodes. Colloidal Au particles that initially disintegrated from nanorice particles were also incorporated into these complex mesostructures. The conversion yield from nanorice particles to mesostars was ~ 50 %. Since these mesostructures are larger in size than the nanorice, and the formation of mesostars is distinct from solution phase synthesis or seeded growth method, a lower conversion yield is expected.

These observations elucidate the α-Fe₂O₃ and Au peaks observed in the XRD spectra of mesostars (Figure 9.1B-iii) as well as the Au SAED pattern shown in Figure 9.2F. The Au peaks in the XRD spectra could also be due to the residual colloidal Au colloidal particles in solution which disintegrated from the nanorice. These observations also explain the polycrystalline rings in the ED patterns (Fig. 9.2A, 9.2F), which could be a consequence of self-assembly and oriented attachment of many α-FeOOH nanoparticles to form the mesostars.
Figure 9.4. Additional SEM images supporting the mesostar formation mechanism starting with (A) 2 hrs of electrolysis, (B) 4 hrs, (C) 6 hrs, (D) 8 hrs, (E) 12 hrs, and (F) 24 hrs. The scale bar is 500 nm.

Figure 9.5. SEM image of long chain branched structures obtained both at 5 V and 10 V in addition to the mesostars.
Figure 9.6. SEM image of one electrode utilized in the electrolytic cell is shown. High resolution SEM image is provided as an inset, the scale bar is 100 nm.

The formation of the mesostars as a result of the specific oriented assembly of the α-FeOOH nanoparticles on the central axis of the cross structures could be due to defects in the α-FeOOH crystal structure and resulting magnetization in individual particles.\textsuperscript{258, 259} This magnetization results in weak interparticle magnetic interactions. Magnetism in α-FeOOH nanocrystals depends on several factors including the crystallinity of the particles, particle size, temperature, and surface defects. Bocquet et al.\textsuperscript{253, 254, 259} proposed that as the size of α-FeOOH particles decreases, the number of crystal defects increases facilitating magnetic cluster ordering and reduced Neel temperature ($T_N$). The $T_N$ of bulk α-FeOOH is $\sim$ 398 K. The saturation magnetization values found in nanoscale materials are usually smaller than the corresponding bulk phases, provided that no change in ionic configurations occurs. Bocquet et al.\textsuperscript{259} have reported a $T_N$ as low as $\sim$ 261 K for α-FeOOH nanocrystals, which indicates they have a net magnetic moment at ambient temperature. Structural defects in α-FeOOH nanocrystals, such as iron and hydrogen vacancies, results in unpaired chains at the surface, which also contributes to a net increase in magnetization.\textsuperscript{260, 261} Thus, the formation of mesostars could have occurred
due to the assembly of α-FeOOH nanocrystals along the most convenient polarizable axis in the DC-field as well as the weak interparticle magnetic interaction between the α-FeOOH nanocrystals.

The formation mechanism of these hierarchical mesostructures was further established by studying the electrolysis-assisted assembly of nanorice particles as a function of voltage. The voltage between the electrodes was varied between 0.5 V and 10 V, while the reaction time (24 hours) and the distance between the electrodes (0.014 m) remained constant. At both 0.5 V and 1 V, only aggregated nanorice particles were obtained, but mesostars were not observed (Figure 9.7A). This strongly indicates that below 1.23 V, the redox potential for H$_2$O electrolysis, mesostar formation is not initiated. At 2 V, very few micron-sized star-shaped structures were observed, while most of the nanorice particles remained in solution (Figure 9.7B). These were large fractal structures, measuring ~ 4 ± 0.2 μm in length and width, and 1.1 ± 0.2 μm in height. The small yield probably occurred because fewer nucleation sites for mesostar formation were initiated at lower voltage. This resulted in slower growth kinetics, which usually favors Ostwald ripening in order to minimize the overall surface free energy, resulting in the formation of larger but fewer stars. At 3 V, slightly smaller star-shaped structures with a length and width of 3.1 ± 0.7 μm and a height of 0.8 ± 0.3 μm were obtained. As the voltage was raised to 5 V, a higher yield of mesostars with a length and width of 650 ± 80 nm and a height of 380 ± 30 nm was observed. As the voltage was further increased to 10 V, a high yield of incomplete and deformed stars 450 ± 150 nm in length were obtained. These observations indicate that at higher voltage more nucleation sites are initiated.
leading to a higher rate of growth kinetics. This also results in the formation of distorted star-shaped morphologies with an overall decrease in size, and a larger size distribution.

Figure 9.7. SEM images of mesostructures obtained by varying the voltage while keeping electrolysis time constant at 24 hrs. (A) Aggregated nanorice formed at 0.5 V and 1 V and high resolution image provided as inset, (B) stars ~ 4 ± 0.2 μm in length and width, and 1.1 ± 0.2 μm in height formed at 2 V, (C) stars ~ 3.1 ± 0.7 μm in length and width, and 0.8 ± 0.3 μm in height obtained at 3 V, (D) mesostars 650 ± 80 nm in length and width, and 380 ± 30 nm in height observed at 5 V and, (E) distorted stars 450 ± 150 nm in length obtained at 10 V.

In addition to electrolysis, the resulting crystal structure of these hierarchical self-assembled mesostructures can also be interpreted in the context of nucleation, oriented attachment, and Ostwald ripening. Nucleation in the reaction mixture results in small
crystalline α-FeOOH primary particles which aggregate via oriented attachment to form secondary particles. Oriented attachment involves self-organization of adjoining particles such that a regular crystallographic orientation is achieved followed by the joining of these particles at a planar interface. Oriented assembly usually results in single crystals, which could explain the single crystalline domains found in the mesostars (ED and SAED patterns shown in Fig. 9.2). Several single crystalline domains then aggregate forming polycrystalline structures. The kinetics of crystal growth and coarsening strongly depend on various factors including the structure of the material, the surface chemistry of the particles resulting from the ions in the solution, and the interface between the crystals and surrounding solution. With increasing time, Ostwald ripening controls the crystal growth by the diffusion of ions along the matrix-particle boundary resulting in larger particle sizes. The observed morphology of the mesostars could be due to the simultaneous occurrence of adjacent particles in solution epitaxially assembling during the electrolysis process along the most convenient polarizable axis for DC-field as well as Ostwald ripening.

9.5 Optical Properties

Since the optical properties of materials are governed by shape, size, and chemical composition, the extinction spectra of the α-Fe₂O₃ cores, Au-nanorice, and mesostars were compared using UV-Vis spectroscopy (Fig. 9.8). The extinction spectra were obtained by immobilizing the particles on quartz slides. The α-Fe₂O₃ cores showed an absorption maximum at ~ 390 nm, while nanorice demonstrated two separate peaks at 650 nm and 1100 nm. The strong plasmon resonance feature at 1100 nm was due to the longitudinal plasmon mode of the nanorice while the weaker plasmon resonance at 650
nm was due to the transverse plasmon mode of the nanorice particles. The optical spectrum of the mesostars is strikingly different from nanorice and revealed a broad peak with absorption maximum at 430 nm, which was attributable to the presence of α-Fe₂O₃, α-FeOOH, and Au. In the control experiment, the optical properties of the pyramidal mesostructures obtained with the hematite cores did not show any well defined peak (see below) which additionally signifies the contribution of Au colloidal particles in the observed optical spectra of the mesostars.

Figure 9.8. Extinction spectra of (i) α-Fe₂O₃ cores with λ_{max}~390 nm, (ii) nanorice particles with longitudinal plasmon λ_{max}~1100 nm and transverse plasmon λ_{max}~650 nm, and (iii) mesostars with λ_{max}~430 nm. The samples measured are monolayers of nanoparticles immobilized on PVP-glass slides. The spectra are offset for clarity.

9.6 Control Experiment

A control experiment with the α-Fe₂O₃ cores without any Au shell was performed resulting in an alteration of the composition and morphology of the α-Fe₂O₃ nanoparticles. Very few pyramidal mesostructures were obtained, but mesostars were not
observed, as shown in Figure 9.9. The $\alpha$-Fe$_2$O$_3$ cores (Fig. 9.9A) assemble in a distinctive pattern after 10 hours of electrolysis at 5 V (Fig. 9.9B). After 14 hours of electrolysis, a low yield, ~ 5%, of pyramidal mesostructures 500 ± 50 nm in length and width, and 380 ± 30 nm in height (Fig. 9.9C) were observed. These pyramidal structures increased in size within 24 hours of electrolysis forming larger pyramidal mesostructures 650 ± 100 nm in length and width, and 370 ± 50 nm in height (Fig. 9.9D). Most of the $\alpha$-Fe$_2$O$_3$ core particles remained in solution. In addition to the pyramidal structures, $\alpha$-FeOOH nanocrystals were also observed in the reaction mixture. The formation mechanism of these structures is not completely understood, since a time-dependent study did not clearly reveal a step-by-step growth process.

Figure 9.9. SEM images of (A) $\alpha$-Fe$_2$O$_3$ cores, (B) assembled $\alpha$-Fe$_2$O$_3$ cores formed after 10 hrs of applying 5 V, (C) pyramidal mesostructures 500 ± 50 nm in length and width, and 380 ± 30 nm in height formed after 14 hrs, (D) larger pyramidal mesostructures 650 ± 100 nm in length and width, and 370 ± 50 nm in height formed after 24 hrs.
We propose a similar mechanism as the formation of mesostars, including electrolysis of H₂O leading to phase transformation of α-Fe₂O₃ to α-FeOOH. Subsequently, the nucleation of α-FeOOH nanoparticles, followed by oriented attachment of primary particles along specific crystallographic planes, and Ostwald ripening to form pyramidal mesostructures. It is evident that utilizing α-Fe₂O₃ particles instead of nanorice particles resulted in fewer nucleation sites, which consequently produced a poor yield of the pyramidal structures. The low yield of the pyramidal structures and absence of mesostars suggests that the Au shell may act as a direct agent in initiating nucleation sites for mesostar formation and facilitating shape evolution of these hierarchical mesostructures.

Figure 9.10. (A) XRD spectra of (i) α-Fe₂O₃ cores, and (ii) pyramidal mesostructures. B) Extinction spectra of (i) α-Fe₂O₃ cores, λ_{max} ~ 390 nm, and (ii) pyramidal mesostructures, λ_{max} < 300 nm. The samples measured are monolayers of nanoparticles immobilized on PVP-glass slides. The spectra are offset for clarity.
The XRD spectra of the $\alpha$-Fe$_2$O$_3$ cores and the pyramidal mesostructures (Fig. 9.10A) clearly reveal a crystalline morphology as well as conversion of $\alpha$-Fe$_2$O$_3$ to $\alpha$-FeOOH to form these structures. The strongest peak corresponding to the $\alpha$-FeOOH (101) plane in the XRD spectra of the pyramidal mesostructures suggest a preferential orientation of the goethite crystalline domains. The optical properties of the $\alpha$-Fe$_2$O$_3$ cores and pyramidal mesostructures are represented in Figure 9.10B. The $\alpha$-Fe$_2$O$_3$ core particles show an absorption peak at 390 nm. However, no well defined peak is observed in the optical spectra of the pyramidal mesostructures. A weak shoulder is observed between $\sim$ 450 – 600 nm which is probably characteristic to the pyramidal mesostructures.

9.7 Conclusions

In conclusion, we have observed the unusual formation of hierarchical mesostars via electrolysis of an aqueous suspension of nanorice particles. The Au-$\alpha$-Fe$_2$O$_3$ nanorice particles self-assembled into fractal mesostructures composed of $\alpha$-Fe$_2$O$_3$, $\alpha$-FeOOH, and Au. The remarkable differences in the geometry, composition, and properties between the nanorice particles and mesostars strongly indicate that electrolysis can significantly alter the morphology and constituent material of the nanostructures. A further examination of this striking assembly process could elucidate the unusual self-assembly processes observed in nature and biological systems. Further study of this system may be also promising in fundamental applications characteristic to hierarchical mesostructures.
Chapter 10: Facile Chemical Approach to ZnO Submicrometer Particles with Controllable Morphologies

10.1 Introduction

Development of semiconductor nanostructures has gained tremendous interest due to their technological importance in nanoelectronics, nanophotonics and as sensor components into single monolithic devices. In this chapter we report the fabrication, characterization, growth mechanism and emission properties of zinc oxide (ZnO) mesostructures with interesting geometries, controllable sizes and white light emissive properties. Rizia Bardhan fabricated the mesostructures, characterized them, performed the optical studies, and analyzed the growth mechanism. Hui Wang contributed in discussion and data interpretation and Felica Tam contributed in TEM studies. Reproduced with permission from Rizia Bardhan, Hui Wang, Felicia Tam, and Naomi J. Halas, Langmuir, 2007, 23, 5843-5847. Copyright 2009 American Chemical Society.

ZnO is currently one of the most attractive semiconducting materials for optical and electronic applications because of its direct wide bandgap (3.37 eV) and high exciton binding energy (60 meV). ZnO particles have been investigated extensively as active media for applications such as luminescent light emitters, solar cells, phosphors, and photocatalysts. A proliferation of ZnO nanostructure morphologies have been reported, such as nanobelts, nanowires, nanorods, nanotubes, nanohelices, tetrapods, nanosheets, and nanorings. The properties of ZnO particles are highly dependent upon particle size, shape and surface characteristics. Several fabrication techniques have been developed to prepare ZnO structures with well-defined shapes, such as vapor-phase evaporation, metal-organic vapor phase
epitaxy, template-based synthesis, and laser ablation. These fabrication procedures often require high temperature conditions, prolonged reaction times, and specifically designed reactors. The development of wet chemistry approaches to ZnO particles with controllable sizes and shapes is ultimately more desirable in terms of simplicity, efficiency, and low-cost.

ZnO is a useful and practical gain medium for solid-state UV lasers due to its high-quantum efficiency photoluminescence in the UV spectral region. ZnO particles have attracted recent attention as some of the smallest lasers yet reported. Meanwhile certain defects and impurities present in ZnO can also give rise to subbandgap states with strongly radiative decay channels. The broadband visible light emission reported in such systems has been correlated with specific dopants and defect morphologies. The subbandgap emission in ZnO-based materials has long been viewed as an adverse property, which can be reduced by various approaches, such as annealing or placing metallic films directly adjacent to the ZnO in various device structures. However, it has been recently realized that this visible light emission may be a potentially valuable property, rendering ZnO a promising prospective material for solid state lighting applications.

Here we describe a simple bottom-up approach to the selective fabrication of a series of ZnO particles with unique morphologies including rings, bowls, hemispheres, and disks of various aspect ratios. The size and morphology of the particles is systematically controlled by judiciously adjusting the concentration of the precursor, zinc acetate dihydrate, in the presence of NH₄OH. The pH of the reaction mixture is also found to be critical in the formation of these well-defined shapes. The as-fabricated ZnO
particles exhibit very strong broadband visible light emission upon UV excitation, far stronger than the interband emission.

### 10.2 Fabrication of ZnO Particles

**Materials Utilized:** Zinc acetate dihydrate (Zn(CH$_3$CO$_2$)$_2$.2H$_2$O) was purchased from Sigma-Aldrich (St. Louis, MO). 28.8% Ammonium hydroxide (NH$_4$OH) and 200-proof ethanol were obtained from Fisher Scientific (Hampton, NH). All the chemicals were used as received without further purification. Ultrapure water (18.2MΩ resistivity) was obtained from Milli-Q water purification system (Millipore, Billerica, MA).

**Fabrication:** ZnO rings were fabricated by heating 50 mL of 200-proof ethanol in a round bottom flask connected with a condenser at 60°C. While maintaining a constant temperature, 0.005 M Zn(CH$_3$CO$_2$)$_2$.2H$_2$O was introduced in the round bottom flask under vigorous stirring and allowed to dissolve in the ethanol. An appropriate amount of NH$_4$OH (2.8 wt %, pH ~ 8.2) was then added and the reaction was continued for 45 minutes under vigorous stirring. After completion of the reaction, the solution was cooled to room temperature. The resulting precipitate was centrifuged and washed several times and finally redispersed in 15 mL ethanol. The other ZnO particle morphologies were fabricated by varying the Zn(CH$_3$CO$_2$)$_2$.2H$_2$O concentration to 0.01 M, 0.02 M and 0.025 M to obtain bowls, hemispheres and disks, respectively, while the other experimental conditions were kept the same.
10.3 Characterization of ZnO Particles

The ZnO particles were characterized by obtaining scanning electron microscope (SEM) images on a Phillips FEI XL-30 environmental SEM at an acceleration voltage of 30 kV. Transmission electron microscope (TEM) micrographs were obtained using JEOL JEM-2010 TEM. Photoluminescence (PL) spectra were obtained using a JOBIN YVON UV-vis Fluorolog, excited at 360 nm. All the samples prepared for PL were maintained at the same concentration using the absorbance at 372 nm with a UV-vis-NIR spectrophotometer. Fluorescence images were obtained using Axioplan-2 Imaging-Zeiss Fluorescence microscope with a color camera (Hg, HBO 100 watt lamp and excitation filter between 340-380 nm). X-ray Diffraction (XRD) patterns were obtained using a Rigaku Ultima II vertical \( \theta - \theta \) powder diffractometer (Cu K\( \alpha \), \( \lambda = 1.5418 \) \( \text{Å} \)).

The SEM and TEM images of the different ZnO particles fabricated by this approach are shown in Figure 10.1. Different morphologies can be selectively obtained by simply varying precursor concentrations within a certain pH range (Table 10.1). When the concentration of Zn(CH\(_3\)CO\(_2\))\(_2\) <0.005 M, irregular ZnO nanoparticles are obtained (Fig. 10.2A). At 0.005 M Zn(CH\(_3\)CO\(_2\))\(_2\), ZnO rings with outer diameters of 460 ± 60 nm and inner diameters of 250 ± 80 nm are formed (Fig. 10.1A, B). A further increase in the concentration of the Zn precursor to 0.01 M results in submicrometer bowl shaped structures with an average size of 710 ± 80 nm and an inner diameter of 260 ± 50 nm (Fig. 10.1C, D). The inset of Figure 1C shows striations along the radial direction, suggesting the preferential growth along the radius of the ZnO crystals to form this unusual structure. Submicrometer hemispheres are formed when the concentration of precursor is increased to 0.02 M (Fig. 10.1E, F).
Figure 10.1. Low- and high-magnification (inset) SEM micrographs and corresponding TEM images of ZnO particles fabricated by varying the concentration of the Zn precursor. (A and B) Rings (0.005 M), (C and D) bowls (0.01 M), (E and F) hemispheres (0.02 M), and (G and H) disks (0.025 M). The scale bar for all of the high-magnification SEM images is 200 nm.

These particles are 790 ± 85 nm in diameter and clearly display a smooth outer surface but coarse inner surface with visible crystalline facets. As the concentration of Zn(CH$_3$CO$_2$)$_2$ is further increased to 0.025 M, disklike shapes are formed (Fig. 10.1G, H). Although increasing the precursor concentration from 0.02 - 0.025M did not radically change the morphology of the particles, the hemispheres appeared to increase radially resulting in a disk-like shape. These disks (average size ∼ 910 ± 70 nm) exhibit a
quasihexagonal cross section (Fig. 10.1H). At zinc acetate concentrations above 0.03 M, larger particles are formed with significant agglomeration and indefinite morphology (Fig. 10.2B). Therefore, it appears that only over a limited range of Zn\(^{2+}\) concentration are the fabrication conditions appropriate for the formation of ZnO particles with these well defined morphologies.

Figure 10.2. (A) Irregular ZnO nanoparticles obtained at 0.001 M of Zn precursor, and (B) agglomerated ZnO particles obtained at 0.04 M of Zn precursor.

Table 10.1. Synthesis parameters and morphology of different ZnO Structures

<table>
<thead>
<tr>
<th>Concentration of Zn(Ac)_2 (M)</th>
<th>pH (\text{NH}_4\text{OH} 2.8 %) Initial</th>
<th>Final</th>
<th>Morphology</th>
<th>Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>4.4</td>
<td>6.4</td>
<td>irregular nanoparticles</td>
<td>_</td>
</tr>
<tr>
<td>0.005</td>
<td>4.2</td>
<td>5.5</td>
<td>rings</td>
<td>460 ± 60</td>
</tr>
<tr>
<td>0.01</td>
<td>4.2</td>
<td>5.5</td>
<td>bowls</td>
<td>710 ± 80</td>
</tr>
<tr>
<td>0.02</td>
<td>4.0</td>
<td>5.5</td>
<td>hemispheres</td>
<td>790 ± 85</td>
</tr>
<tr>
<td>0.025</td>
<td>4.0</td>
<td>5.5</td>
<td>disks</td>
<td>910 ± 70</td>
</tr>
<tr>
<td>0.04</td>
<td>4.0</td>
<td>6.2</td>
<td>agglomerated particles</td>
<td>_</td>
</tr>
<tr>
<td>0.05</td>
<td>3.9</td>
<td>6.2</td>
<td>agglomerated particles</td>
<td>_</td>
</tr>
<tr>
<td>0.01*</td>
<td>4.3</td>
<td>5.6</td>
<td>inhomogeneous matrix</td>
<td>_</td>
</tr>
<tr>
<td>0.02*</td>
<td>4.1</td>
<td>5.5</td>
<td>inhomogeneous matrix</td>
<td>_</td>
</tr>
</tbody>
</table>

* We used KOH instead of NH\(_4\)OH maintained at similar pH
Figure 10.3. Crystallographic studies of the ZnO particles obtained at different concentrations of the Zn precursor. (A) XRD patterns of (i) rings (0.005 M), (ii) bowls (0.01 M), (iii) hemispheres 0.02 (M), and (iv) disks (0.025 M). (B) TEM image of a Submicrometer bowl with the corresponding ED pattern (top inset) and the SAED pattern (bottom inset, area shown in circle) characteristic of these structures. (C) TEM image of a polycrystalline ring. The SAED patterns (areas shown in circles) illustrate crystalline domains along different orientations.

The crystallinity of the ZnO particles obtained at different concentrations of Zn precursor was also investigated. Figure 10.3A shows the XRD patterns of all of the obtained morphologies rings, bowls, hemispheres, and disks. The XRD patterns exhibit sharp peaks, indicating that the particles possess large crystalline domains as well as a high degree of crystallinity. Figure 10.3B shows the TEM image of a submicrometer bowl and the corresponding electron diffraction (ED) pattern, revealing that the bowl structures are composed of several crystalline domains. Selected-area electron diffraction (SAED) (Figure 10.3B, bottom inset, area shown in circle) shows a single-crystalline domain with
hexagonal wurtzite structure along the [0001] zone axis in this region. The TEM image and corresponding SAED patterns of a submicrometer ring are illustrated in Fig. 10.3C. The two selected regions in the ring (areas shown in the circle) have crystalline domains with hexagonal wurtzite structure along the [0001] zone axis. However, the crystalline domains are oriented differently, verifying the polycrystallinity of the particles.

**10.4 Growth Mechanism of ZnO particles**

The growth mechanism and resulting crystal structure of these ZnO particles can be interpreted in the context of supersaturation, nucleation, Ostwald ripening, and oriented attachment.\(^{296}\) The kinetics of crystal growth and coarsening strongly depends on the structure of the material, the surface chemistry of the particles resulting from ions in the solution, and the interface between the crystals and surrounding solution.\(^{265}\) At Zn precursor concentration lower than 0.005 M, insufficient amount of solute is present to yield a supersaturated solution. At 0.005 M of Zn\(^{2+}\), nucleation occurs as a supersaturated solution is achieved. The ZnO nuclei further grow by diffusive mechanism forming primary particles.\(^{296}\) These small crystalline primary particles subsequently aggregate through oriented attachment forming secondary particles.\(^{265, 266, 297}\) Oriented attachment involves self-organization of adjoining particles such that a regular crystallographic orientation is achieved followed by joining of these particles at a planar interface.\(^{266}\) Oriented attachment usually results in single crystals, which could explain the single crystalline domains found in the bowls and rings (Fig 10.3B,C). However orientation adopted within one region is dissimilar to that in an adjacent region in the reaction mixture. Therefore, following aggregation process, polycrystalline structures composed
of several single crystalline domains are obtained. In solution phase synthesis, the formation of large polycrystalline particles with complex morphologies is not surprising. When the Zn precursor concentration is increased, Ostwald ripening controls the crystal growth by diffusion of ions along the matrix-particle boundary and results in larger particle sizes.\textsuperscript{298} The observed differences in morphology with increasing concentration of Zn(CH\textsubscript{3}CO\textsubscript{2})\textsubscript{2} could be due to the simultaneous occurrence of Ostwald ripening as well as oriented attachment during the growth of the crystals.\textsuperscript{297, 298} While Ostwald ripening leads to spherical/hemispherical shapes with smooth edge, oriented attachment forms irregular shapes with visible facets. The smooth outer surfaces of the bowls, hemispheres and disks with irregularly faceted inner surfaces verify this theory (Fig 10.1C, E, G).

In addition to the effect of precursor concentration on the particle morphologies, the pH of the reaction mixture also influences the shape evolution of the particles (Table 10.1). Although the exact mechanism of formation of these unique morphologies is debatable,\textsuperscript{263 299} we believe besides Zn(CH\textsubscript{3}CO\textsubscript{2})\textsubscript{2} concentration, the difference between the initial and final pH value of the reaction mixture may control the nucleation and growth event.\textsuperscript{286} Larger differences between the initial and final pH values of the reaction mixture results in a higher nucleation rate; consequently, ZnO particles are formed at a rapid rate leading to agglomerated particles with indefinite morphology. When the variation between initial and final pH values of the reaction mixture is small, the nucleation rate is slower, leading to development of well-defined particle shapes with different sizes. The ZnO submicron rings, bowls, hemispheres, and disks are formed when the difference between the initial and final pH values of the reaction were 1.3 – 1.5.
**Table 10.2.** Concentration of Ammonia and Morphology of ZnO Structures

<table>
<thead>
<tr>
<th>NH$_4$OH wt %</th>
<th>pH of Reaction</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>1.12</td>
<td>3.9</td>
<td>4.8</td>
</tr>
<tr>
<td>2.8</td>
<td>4.2</td>
<td>5.5</td>
</tr>
<tr>
<td>5.6</td>
<td>4.4</td>
<td>6.3</td>
</tr>
<tr>
<td>8.4</td>
<td>4.9</td>
<td>7</td>
</tr>
<tr>
<td>11.2</td>
<td>5.1</td>
<td>7.2</td>
</tr>
</tbody>
</table>

*Figure 10.4. (A) Distorted bowl-like shapes obtained at lower concentration of NH$_4$OH (1.12 wt %). (B) Inhomogeneous matrix obtained at 5.6 wt % of NH$_4$OH. (C) Matrix obtained at higher concentration of NH$_4$OH (8.4 wt % and 11.2 wt %)*

We have also investigated the precise pH range within which well-defined morphologies were obtained by varying the concentration of NH$_4$OH (Table 10.2) while maintaining the precursor concentration constant at 0.01 M. While distorted bowl-like shapes were observed, (Fig. 10.4A) at lower concentration of NH$_4$OH (1.12 wt %), inhomogeneous matrix was obtained at higher concentration of NH$_4^+$ (Fig. 10.4B, C). It appears that at a final pH value of $\sim$ 4.8, the reaction solution may be excessively acidic, forming distorted particles; additionally, at final pH values $>$ 6, the reaction conditions were unsuitable to form any distinctive structure. We also observed that at higher concentration of NH$_4$OH (5.6 wt % and above), the difference between the initial and final pH values of the reaction also increases, which explains the formation of matrices with no particular morphology. From these observations, it appears that in addition to the
Zn precursor, only a certain pH range is suitable for the formation of ZnO nuclei in solution, influencing the crystal growth rate and the resulting morphologies.

In addition to the precursor concentration and reaction pH, the effects of reaction temperature and formation of zinc-ammonia ligand on the particle size and morphologies were also investigated to gain a better insight into possible growth mechanisms. The particles reported here form only at ~ 60°C. At temperatures below 60°C, only amorphous ZnO nanoparticles were formed. At reaction temperatures higher than 60°C (70°C, 80°C), the formation of an extended ZnO matrix was observed. It thus appears that only at temperatures near 60°C does ZnO nucleate and aggregate into the observed morphologies. We also observed that the presence of ammonium cations was essential for the formation of the reported particles (rings, bowls, hemispheres and disks). Wang et al. have reported that NH₄⁺ ions may adsorb onto the surface of ZnO crystals due to the high surface energy associated with the nanophase materials, and thus affect crystal growth. This may also be occurring in the system reported here, and influence the shape of the particle morphologies. Alternatively, NH₄⁺ may participate in the reaction by forming zinc-ammonia ligand (Zn(NH₃)₄²⁺) which would then directly affect the morphology of the structures.

\[
\begin{align*}
\text{Zn}^{2+} + \text{NH}_3 & \rightarrow \text{Zn(NH}_3\text{)}_{4}^{2+} \\
\text{Zn(NH}_3\text{)}_{4}^{2+} + \text{OH}^- & \rightarrow \text{ZnO}
\end{align*}
\]

We confirmed the significance of the NH₄⁺ cations as a structure-directing agent by replacing NH₄OH with KOH, maintaining a similar pH (~ 8.2) as the original ammonium hydroxide reactant solution (Table 10.1). In this case, none of the reported morphologies were observed, but instead, large ZnO particles, which subsequently coalesced to form a
matrix, were achieved (Fig. 10.5A, B). Certain ligands have been known to exert thermodynamic control over chemical systems and influence crystal orientation through the metal-ligand bond length, ligand strength and the energy associated with the metal-ligand bond formation.\textsuperscript{301} So we infer that in addition to zinc acetate concentration, and reaction pH, the presence of NH\textsubscript{4}\textsuperscript{+} ion may also guide the structural formation of these ZnO sub-micron particles, possibly by forming the Zn(NH\textsubscript{3})\textsubscript{4}\textsuperscript{2+} ligand during the course of the reaction.

![Figure 10.5](image)

**Figure 10.5.** ZnO matrix obtained at different concentrations of Zn precursor and KOH (pH ~ 8.2). (A) 0.01 M, and (B) 0.02 M.

### 10.5 Optical Properties of ZnO Particles

These ZnO particles also possess a large concentration of surface defect states, which is directly related to the fabrication route and synthesis conditions. Optical studies of these particles reveal the presence of surface defects. The as-fabricated ZnO particles exhibit strong photoluminescence upon UV excitation. Photoluminescence (PL) spectra were obtained using a JOBIN YVON UV-vis Fluorolog, with excitation at 360 nm. All of the samples prepared for PL were maintained at the same concentration using the absorbance at 372 nm measured with a UV-vis-NIR spectrophotometer. Fluorescence
images were obtained using an Axioplan-2 Imaging Zeiss fluorescence microscope with a color camera (Hg, HBO 100 W lamp, and excitation filter between 340 and 380 nm). Figure 10.6A shows an image of white-light emission from a film of ZnO hemispheres. The rings, bowls, and disks all exhibit extremely similar light-emitting properties (Fig. 10.6B). The PL spectra of all particles show a weak interband emission at 370-389 nm (3.19-3.35 eV) and a broad visible band centered at ~ 558 nm (2.22 eV). The visible band spans from ~ 420 to 700 nm for all ZnO particle morphologies reported here.

Various explanations have been proposed for the white light emission from ZnO, based on oxygen or zinc defect states. The broadband visible light emission observed in these particles may be attributable to a combination of oxygen interstitial defects ($O_i$) and antisite oxygen defects ($O_{Zn}$). Oxygen interstitial defects have been reported to be responsible for yellow emission while antisite oxygen defects cause green emission as illustrated in the energy diagram of ZnO defect levels (Fig. 10.6C). A photogenerated hole is trapped at the surface by interstitial oxygen defects or antisite oxygen defects which then migrate to deeply trapped levels located above the valence band. A broad visible emission occurs when the photogenerated hole trapped in a deep level recombines with photogenerated electron trapped in a shallow level (levels 1 and 2). Depending on the temperature and the presence of oxygen during sample preparation, a mixture of oxygen defect states can be created, many of which can produce visible light. The precise origin of the broadband emission from these unique particles is still under investigation, yet the large ratio of the visible PL intensity to the interband PL intensity in these polycrystalline structures indicates the presence of a large number of surface defects.
Figure 10.6. (A) Fluorescence image of ZnO hemispheres emitting white light upon UV excitation. (B) PL spectra of the observed ZnO particles normalized relative to particle concentration: (a) rings, (b) bowls, (c) hemispheres, and (d) disks. Weak interband emission is observed at 370-389 nm, and strong visible emission centered at ~558 nm. The spectra are offset for clarity. (C) Energy diagram of defect states in ZnO.

10.6 Conclusions

In summary, by controlling the growth kinetics through precursor concentration, reaction temperature, pH, and the concentration of ions present in solution, we have fabricated
ZnO submicrometer particles of varying shapes and aspect ratio. The broad emission from the ZnO particles covering most of the visible region may be attributable to a combination of oxygen defect states resulting from the fabrication conditions. Future studies may be useful in advancing our understanding of the growth mechanism of hemispherical ZnO particles in comparison to rod like particles. In addition, future studies of the properties of these ZnO particles may lead to the development of economical, white-light-emitting materials for solid-state lighting applications.
11: Summary and Prospects

In this thesis novel plasmonic and photonic nanostructures are fabricated from fundamental building blocks and their optical properties are harnessed for several technological applications including plasmon enhanced fluorescence sensing, multimodal bioimaging and photothermal cancer therapy. The fluorescence enhancement of weak NIR fluorophore ICG, which is FDA approved for biomedical imaging, was examined as a function of distance from the surface of SiO₂-core/Au-shell nanoshells. The distance between the fluorophore and the nanoshells was manipulated by controllably growing silica epilayers of varying thicknesses. A 50X emission enhancement was obtained at a distance of 7 nm from the nanoshell surface and 7X enhancement was achieved at 42 nm from nanoshell surface. Due to the spherical symmetry of nanoshells Mie theory was utilized to theoretically analyze the electromagnetic interaction of nanoshells with light. Theoretical models correlated very well with experimental fluorescence enhancement, demonstrating nearly 90% calculated quantum yield for ICG molecules positioned 7 nm from the nanoshell surface.

The fluorescence enhancement of IR800, another clinically relevant NIR fluorophore, was then compared between nanoshells and gold nanorods to understand the detailed photophysics of plasmon enhanced fluorescence. The fluorescence enhancement is attributable to a combination of processes including absorption enhancement, scattering enhancement and radiative decay rate enhancement. The contribution of each of these processes was systematically investigated by examining the scattering efficiencies of the nanostructures both experimentally and theoretically as well as experimentally determining the quantum yield and lifetime of the fluorophore when
bound to the nanostructures at an appropriate distance. The quantum yield of IR800 was enhanced from a mere 7% to 86% when positioned in proximity to nanoshells and 74% near nanorods. The native lifetime of IR800 decreased from 564 ps to 121 ps when conjugated to nanorods and 68 ps for nanoshells. The experimental observations suggested that nanoshells, due to their larger scattering cross-section, improved the quantum yield of IR800 more effectively.

The plasmonically enhanced fluorescent nanoshells were then synergistically integrated with iron oxide nanoparticles enabling dual modal imaging including MRI and FOI of breast cancer cells. The multifunctional nanoshells (nanocomplexes) were conjugated with antibodies to actively target the cancer cells facilitating a higher concentration of nanoshells to accumulate in the tumor. The ability of nanoshells to efficiently convert absorbed light to heat allowed thermal ablation of tumor cells upon resonant light activation. The nanocomplexes had proven extremely effective in detecting and treating breast cancer cells in vitro. The theranostic capabilities of nanocomplexes were also extended to diagnosis and therapy of ovary cancer cells in vitro and the results were analogous to the breast cancer in vitro study. Cytotoxicity studies showed that nanocomplexes were innocuous to cells and only induced cell death when activated with a NIR laser. The in vitro studies clearly demonstrated that these versatile nanocomplexes could be practically employed for diagnosis and treatment of any type of cancer cells.

The efficacy of the nanocomplexes was then evaluated in vivo by examining breast cancer xenografts via MRI and FOI. Nude mice were injected with anti-HER2 conjugated nanocomplexes via the tail vein. Significant differences were observed between the HER2 overexpressing breast cancer tumors and control, HER2 low
expressing tumors. The biodistribution studies demonstrated maximum nanocomplex accumulation in the tumor relative to the organs of the reticuloendothelial system (RES) of liver and spleen. The nanocomplexes were also conjugated with PEG chains, in addition to antibodies, to provide a stealth character, increase circulation time in the body and reduce recognition by the macrophages of the RES.

While nanoshells in the sub-200 nm size regime are effective for cancer detection and treatment, for certain types of tumor, for example brain tumor, nanoparticle size must be sub-100 nm to effectively accumulate in the brain. Novel Au/SiO$_2$/Au multilayered nanoshells were wet-chemically fabricated in the sub-150 and sub-100 nm size regime and their plasmonic properties were experimentally and theoretically analyzed. These multilayered nanoshells are examples of a rudimentary nanomatryushka, concentric nanoshells previously fabricated in the Halas group, and provide a high degree of optical tunability. Plasmon hybridization theory was employed to assign the spectral modes resulting from the interaction of the Au core and Au shell. These structures are specifically interesting since the absorption and scattering properties can be tuned by simply altering the thickness of the intermediate silica layer for a fixed nanoparticle size.

Besides introducing a Au core embedded in a nanoshell geometry, the far field properties can also be controlled by modifying the core material to a high permittivity semiconductor core. Nanoshells in the sub-150 and sub-100 nm size regime consisting of a high permittivity Cu$_2$O core encapsulated in a thin Au shell were fabricated with tunable optical properties from the visible to the NIR. The high permittivity of Cu$_2$O resulted in increased polarizability of the core which contributed in higher absorption efficiencies and higher near field enhancements.
Finally photonic nanostructures of interesting geometries and optical properties are reported. Mesostars, composed of Au, α-FeOOH, and α-Fe₂O₃, were formed by electrolytically induced assembly of an aqueous suspension of nanorice particles. To the best of our knowledge, the process of electrolyzing an aqueous suspension to transform nanoparticles into mesoscale structures has not been previously reported. ZnO mesostructures are wet-chemically fabricated and their shape and size are controlled by manipulating the concentration of the Zn precursor molecules reduced in an alkaline media. The ZnO mesostructures exhibited broadband visible light emission which is attributable to a combination of oxygen interstitial defects and antisite oxygen defects.

The work described in this thesis has promising future prospects in biomedicine as well as in photonic devices. The Cu₂O/Au nanoshells and Au/SiO₂/Au nanoshells may be incorporated with fluorophores and MRI contrast agents enabling multimodal functionalities within a compact sub-100 nm size regime. This will expand the diagnostic and therapeutic capabilities of nanoshells to diseases which are largely inaccessible. In addition NIR fluorophores may be replaced by radionuclides facilitating dual modal positron emission tomography (PET) and MRI, both of which are clinically relevant diagnostic techniques.

Nanoshell based substrates may also be used for luminescence enhancement of weak NIR quantum dots, for example PbS, InAs, and utilize these nanoshell-quantum dot conjugates in photonic devices. The Au/SiO₂/Au nanoshells also provide an interesting geometry for examining fluorescence enhancement if the intermediate silica layer is doped with weak fluorophores of interest. In addition, an array of Au/SiO₂/Au nanoshells may be fabricated which will provide a robust substrate for sensing applications with
tunable absorption and scattering properties. These Au/SiO₂/Au nanoshell arrays can also act as a waveguide and could be useful in routing signals on a chip.

Plasmonic-molecular interface has huge technological importance and provides a foundation for several areas of research including biomedicine, molecular sensing, environmental remediation, optical interconnects, and energy efficient materials. As the need for improvising human health and environmental safety increases, I believe the interdisciplinary field of nanophotonics may ultimately provide routes to attaining these global objectives.
References


226

40. ACS. *American Cancer Society* 2009.


230


234


Appendix A: Publications related to the research described in this thesis


Also highlighted by:
Nanoparticle Could Allow Diagnosis and Treatment in One visit - The Medical News
Tracking New Cancer Killing Particles - Genetic Engineering & Biotechnology News
All-in-one cancer-killing nanoparticle can be tracked in real time with MRI - NanoWerk
New Nanoparticle Might Find, Treat cancer - United Press International
Multi-Tasking Nanoparticle Diagnoses and Fights Cancer Simultaneously - Popular Science
Can we diagnose and destroy Cancer in one sitting - CNET News
Nanoparticles + Imaging can Seek Out, Kill Cancer Cells - Health Imaging
Tracking nanoparticles in the human body - Physics Today
Rice, Baylor device cancer-busting nanoparticle - Small Times


Appendix B: Presentations related to the research described in this thesis


5. “Near-Infrared Fluorescence Enhancement via Gold Nanoshells and Biomedical Applications”, **R. Bardhan** and N. J. Halas, Rice Quantum Institute Research Colloquium (RQI), Houston, TX, August 2008 **Best Oral Presentation Award**


