Development of Luminescent Quantum Dot-Enabled Nano- and Microplatforms for Multiplex Detection of Biomarkers

Kristen S. Williams
University of New Orleans, keschexn@uno.edu

Follow this and additional works at: https://scholarworks.uno.edu/td

Part of the Analytical Chemistry Commons, and the Materials Chemistry Commons

Recommended Citation
https://scholarworks.uno.edu/td/2342

This Dissertation is brought to you for free and open access by the Dissertations and Theses at ScholarWorks@UNO. It has been accepted for inclusion in University of New Orleans Theses and Dissertations by an authorized administrator of ScholarWorks@UNO. The author is solely responsible for ensuring compliance with copyright. For more information, please contact scholarworks@uno.edu.
Development of Luminescent Quantum Dot-Enabled Nano- and Micropplatforms for Multiplex Detection of Biomarkers

A Dissertation

Submitted to the Graduate Faculty of the University of New Orleans in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Chemistry

by

Kristen Schexnayder Williams

B.S., Louisiana State University, 2010

May 2017
To my children: for making everything infinitely more meaningful. I hope your dreams and aspirations in life are limitless. I love you so much. I hope to make you proud.

To my mother: for being my biggest inspiration and the smartest and strongest person I know. Thank you for always truly believing that I am capable of accomplishing anything.
Acknowledgements

I must express my sincerest gratitude to my research advisor, Dr. Matthew A. Tarr, for everything he has done for me throughout this journey. His patience, support, and encouragement have been nothing short of incredible, and I’m truly grateful. I am appreciative of his overwhelmingly positive outlook, reassurance, and guidance. I wholeheartedly believe that he cares and advocates for his students, and I personally feel that he has gone above and beyond for me. My experience in Dr. Tarr’s research group has been invaluable, and I am glad to know him.

I would also like to acknowledge my committee members: Dr. John Wiley, Dr. Weilie Zhou, and Dr. Viktor Poltavets for their feedback and comments. Additionally, I would like to thank Mr. Harry Rees for always quickly and efficiently fixing computers and instrumentation when needed.

I am thankful for past and present research group members, especially, Arriel Wicks and Venkata Kethineedi for teaching me everything they knew about quantum dots, David Bwambok for his instruction and introduction to human serum albumin research, and Ujwal Patil for always being available to discuss research ideas. The undergraduate students who have worked with me have also been helpful on the different projects. I am particularly grateful for group members Donna Peralta and Parisa Pirani for their fellowship. Sharing an office with those ladies was full of laughter and support, and I’m glad to have formed lifelong friendships with them.

Over the years I have had many wonderful mentors who have directly contributed to my growth as a student and helped foster a love for research. Dr. Isiah Warner and Dr. Monica Sylvain from LSU have undoubtedly made a lasting, positive impact on my life. Dr. Warner pushed me to pursue a chemistry degree, and he sent me to Dr. Tarr. He and the LA-STEM
Research Scholars Program propelled me into undergraduate research and instilled in me that obtaining a PhD was not an option but a necessity. From the beginning, Dr. Warner made it clear that he had an unwavering certainty about my capabilities and repeatedly reminded me of this as well of his expectations of me. Dr. Monica Sylvain has also been instrumental in helping me overcome and grow as a person. Her wisdom and faith have pulled me through time and time again, and she has definitely been an inspiration to me.

My support system is unparalleled. I have been surrounded by people who have helped in any way possible to allow me to direct my focus on completing this program. My husband, Gabe Williams, is the most genuine person on the planet. He has been positive, encouraging, patient, and supportive this entire time. He always believed in me and was a constant outlet for stress. I am so fortunate to have him by my side. My parents, Lisa and Reed Schexnayder, are my biggest supporters and have been my entire life. Their unrelenting support is why I am even in any position to obtain a doctorate. I cannot count the ways they have helped and been there over these years. My success is a direct result of the foundation they have created for me. My sister, Adrienne, has spent countless hours as the world’s greatest aunt, and she has offered many encouraging words throughout this journey. My best friend, Dr. Christina Jones, understands this journey firsthand, and her support has made this experience much easier. My in-laws, Melanie and Greg Williams, Sr. have also been there: babysitting, cleaning, cooking, anything to allow me to focus on school. And I am tremendously grateful for my children, Natalie and Jacob, for bringing me joy constantly, especially when I needed it most. Earning a PhD would be significantly more difficult without these people, and I am truly grateful.

I am thankful for everyone who showed support and prayed, and of course, I am thankful to God for the opportunities, blessings, and life He has given me.
# Table of Contents

List of Tables .................................................................................................................. vii
List of Figures ................................................................................................................ viii
List of Schemes ............................................................................................................... xi
Abstract .......................................................................................................................... xii
Chapter 1 Introduction .................................................................................................... 1
  1.1 Objectives and Aims ............................................................................................... 1
  1.2 Significance ............................................................................................................ 2
  1.3 Fluorescence .......................................................................................................... 3
  1.4 Quantum Dots ....................................................................................................... 7
  1.5 Human Serum Albumin ........................................................................................ 12
  1.6 Immunoassay Development .................................................................................. 14
  1.7 Atherosclerosis .................................................................................................... 16
  1.8 References ............................................................................................................ 18
Chapter 2 Synthesis of Water Soluble Mesoporous Silica Quantum Dot Composite Particles ... 26
  2.1 Abstract .................................................................................................................. 26
  2.2 Introduction ............................................................................................................ 28
  2.3 Experimental Methods ........................................................................................ 30
  2.4 Results and Discussion ......................................................................................... 35
  2.5 Conclusions .......................................................................................................... 45
  2.6 References ............................................................................................................ 46
Chapter 3 Multiplex Detection of Atherosclerosis Biomarkers Using a Mesoporous Silica Quantum Dot Based Immunoassay ........................................................................ 49
  3.1 Abstract .................................................................................................................. 49
  3.2 Introduction ............................................................................................................ 51
  3.3 Experimental Methods ........................................................................................ 53
  3.4 Results and Discussion ......................................................................................... 60
  3.5 Conclusions .......................................................................................................... 75
  3.6 References ............................................................................................................ 76
Chapter 4 Synthesis of Quantum Dot Encapsulated Human Serum Albumin Nanocomposites . 79
4.2 Introduction ............................................................................................................ 80
4.3 Experimental Methods .......................................................................................... 81
4.4 Results and Discussion ......................................................................................... 86
4.5 Conclusions ............................................................................................................ 97
4.6 References ............................................................................................................. 98
Chapter 5 Multifunctional Human Serum Albumin Nanoparticles for Targeted Drug Delivery 101
5.1 Abstract ................................................................................................................ 101
5.2 Introduction ............................................................................................................ 103
Part I: Magnetically Triggered Release of Fluorescent Drug Analog from Human Serum Albumin Nanoparticles ......................................................................................... 106
5.3 Experimental Methods ......................................................................................... 106
5.4 Results and Discussion ......................................................................................... 108
Part II: Treatment of Renal Cell Carcinoma with Multifunctional Gold Nanorod Human Serum Albumin Nanoparticles ......................................................................................... 115
5.6 Experimental Methods ......................................................................................... 115
5.7 Results and Discussion ......................................................................................... 116
5.8 Conclusions ............................................................................................................ 120
5.9 Acknowledgments ................................................................................................ 121
5.10 References ........................................................................................................... 121
Chapter 6 Summary and Conclusions ........................................................................ 124
   References ............................................................................................................... 127
   Vita ......................................................................................................................... 129
List of Tables

Table 2.1 – Average ratios, standard deviation, and %RSD of SiQDs .............................................41

Table 2.2 – Dynamic Light Scattering (DLS) data of SiQD composites ..............................................45

Table 4.1 – Dynamic Light Scattering (DLS) data of HSAPs ..............................................................89

Table 5.1 – Dynamic Light Scattering (DLS) data of HSAPs ..............................................................109
List of Figures

Figure 1.1 – Jablonski Diagram ...........................................................................................................4

Figure 1.2 – Illustration of the light path through a filter cube of an inverted fluorescence microscope ..................................................................................................................7

Figure 1.3 – Illustration of quantum dot size due to the quantum confinement effect ...............8

Figure 2.1 – Fluorescence spectra ($\lambda_{ex} = 400$ nm) of CdSe/ZnS QDs emitting at (a) 475 nm, (b) 560 nm, (c) 565 nm, and (d) 620 nm .................................................................36

Figure 2.2 – Digital fluorescence microscope images and fluorescence spectra ($\lambda_{ex} = 400$ nm) of mesoporous silica beads embedded with QDs (a) 480 nm, (b) 560 nm, (c) 620 nm SiQDs ..........38

Figure 2.3 – Digital fluorescence microscope images and fluorescence spectra ($\lambda_{ex} = 400$ nm) of mesoporous silica beads embedded with green (565 nm) and red (620 nm) QDs at different ratios: (a) 1:1, (b) 1:2, (c) 3:1, (d) 1:4, (e) 4:1 ........................................39

Figure 2.4 – Fluorescence emission spectra of individual beads from a 1:4 green to red SiQD sample .........................................................................................................................40

Figure 2.5 – Average fluorescence emission spectra of 20 individual green to red SiQDs: (a) 1:1, (b) 1:2, (c) 3:1, (d) 1:4, (e) 4:1..................................................................................................................41

Figure 2.6 – SiQD with (a) no TEOS, (b) 25 $\mu$L TEOS, (c) 50 $\mu$L TEOS, (d) 100 $\mu$L TEOS, (e) 200 $\mu$L TEOS .................................................................................................................42

Figure 2.7 – Fluorescence emission spectra of SiQD composite particles coated with TEOS....43

Figure 2.8 – Fluorescence Imaging and Emission Spectra of SiQD-NH$_2$ in H$_2$O ......................44

Figure 2.9 – Fluorescence Imaging and Emission Spectra of SiQD-SH in H$_2$O .........................44

Figure 3.1 – Fluorescence microscopy imaging of 5 ng/mL of IL-15 from a (a) direct detection assay and (b) sandwich detection assay. The individual spots are the QD-antibody bioconjugates bound to the antigen present in the well. .................................................................62

Figure 3.2 – Fluorescence emission of 5 ng/mL IL-15 protein using a direct detection and sandwich detection immunoassay ..................................................................................................62

Figure 3.3 – Fluorescence microscopy imaging of MCP-1 using a (a) direct detection assay and (b) sandwich detection assay. The individual spots are the QD-antibody bioconjugates bound to the antigen present in the well. .................................................................63
Figure 3.4 – Fluorescence emission of 50 ng/mL MCP-1 protein using a direct detection and sandwich detection fluorescence immunoassay ............................................. 63

Figure 3.5 – Optimization of capture antibodies at 5 ng/mL of IL-15 (orange) and MCP-1 (green) proteins. $\lambda_{ex} = 400$ nm. ................................................................. 64

Figure 3.6 – Fluorescence microscopy imaging of IL-15: a) 5 ng/mL IL-15 with MCP-1 labeled SiQDs, b) 5 ng/mL MCP-1 antigen, c) 0 µg/mL, d) 1 pg/mL, e) 5 pg/mL, f) 50 pg/mL, g) 0.5 ng/mL, and h) 5 ng/mL .................................................................................. 67

Figure 3.7 – Fluorescence emission spectra of IL-15 ................................................................................. 68

Figure 3.8 – Fluorescence microscopy imaging of MCP-1: a) 5 ng/mL MCP-1 with IL-15 labeled SiQDs, b) 5 ng/mL IL-15 antigen, c) 0 µg/mL, d) 50 pg/mL, e) 0.5 ng/mL, f) 5 ng/mL, and g) 50 ng/mL ............................................................................... 69

Figure 3.9 – Fluorescence emission spectra of MCP-1 ................................................................................. 70

Figure 3.10 – Fluorescence microscopy imaging of IL-15: a) 5 ng/mL IL-15 with MCP-1 labeled SiQDs, b) 5 ng/mL MCP-1 antigen, c) 0 µg/mL, d) 1 pg/mL, e) 5 pg/mL, f) 50 pg/mL, g) 0.5 ng/mL, and h) 5 ng/mL .................................................................................. 72

Figure 3.11 – Fluorescence emission spectra of IL-15 ................................................................................. 72

Figure 3.12 – Fluorescence microscopy imaging of MCP-1: a) 5 ng/mL MCP-1 with IL-15 labeled SiQDs, b) 5 ng/mL IL-15 antigen, c) 0 µg/mL, d) 50 pg/mL, e) 0.5 ng/mL, f) 5 ng/mL, and g) 50 ng/mL ............................................................................... 73

Figure 3.13 – Fluorescence emission spectra of MCP-1 ................................................................................. 73

Figure 3.14 – Multiplexing of IL-15 and MCP-1: a) 50 ng/mL MCP-1 and 5 ng/mL IL-15, b) 50 ng/mL MCP-1 and 5 ng/mL IL-15, c) 5 ng/mL MCP-1 and 5 ng/mL IL-15 ............................................................................... 75

Figure 3.15 – Multiplex Immunoassay of IL-15 and MCP-1 ................................................................. 75

Figure 4.1 – Digital Fluorescence Microscopy Image and Emission Spectra of empty HSAPs .. 87

Figure 4.2 – Digital Fluorescence Microscopy Image and Emission Spectra of HSAPs loaded with Rhodamine 6G chloride ................................................................. 88

Figure 4.3 – Digital Fluorescence Microscopy Image and Emission Spectra of HSAP-QD (600 nm) ................................................................................................. 88

Figure 4.4 – Digital Fluorescence Microscopy Image and Emission Spectra of HSAP-QD (560 nm) ................................................................................................. 88
Figure 4.5 – Photostability measurements of HSAP-QDs and HSAP-Rhodamine dye .................90

Figure 4.6 – Fluorescence Intensity of Fluorophore Released from HSAPs over 10 Days...........92

Figure 4.7 – Digital Fluorescence Microscopy of IL-15 sandwich assay detection: a) 50 pg/mL, b) 0.5 ng/mL, and c) 5 ng/mL .........................................................................................................................94

Figure 4.8 – Fluorescence emission spectra of IL-15 using HSAP-QD (600 nm) .......................95

Figure 4.9 – Digital Fluorescence Microscopy of MCP-1 sandwich assay detection: a) 0.5 pg/mL, b) 5 ng/mL, and c) 50 ng/mL .................................................................................................................................95

Figure 4.10 – Fluorescence emission spectra of MCP-1 using HSAP-QD (560 nm) .................96

Figure 4.11 – Multiplex detection of IL-15 and MCP-1 using HSAP-QD (600 nm) conjugated to IL-15 and HSAP-QD (560 nm) conjugated to MCP-1 .................................................................97

Figure 5.1 – Electron microscopy images of multifunctional human serum albumin nanoparticles: (a) SEM image (10 kV), (b) TEM image (200 kV, room temperature), and (c) TEM image (80 kV, -78 °C) .........................................................................................................................108

Figure 5.2 – Field dependent magnetization of HSAP nanoparticles ........................................110

Figure 5.3 – Release of fluorescein-5-thiosemicarbazide from magnetic HSAP nanoparticles as a function of magnetic field strength ........................................................................................................111

Figure 5.4 – Release of fluorescein-5-thiosemicarbazide from magnetic HSAP nanoparticles as a function of time ..................................................................................................................112

Figure 5.5 – Fluorescence microscopy images of the release of fluorescein-5-thiosemicarbazide after magnetic treatment for (a) 0 min, (b) 10 min, and (c) 30 min ......................................................113

Figure 5.6 – Change in diameter of magnetic multifunctional HSAP nanoparticles over time after magnetic field treatment .............................................................................................................113

Figure 5.7 – TEM image of HSA-AuNR-TKI nanoparticle ................................................................116

Figure 5.8 – Percentage of tumor necrosis and average tumor size after treatment ..................117

Figure 5.9 – Histological samples of tumor after treatment: (a) control with viable tumor structure, (b) tumor after treatment with SRF, (c) tumor after treatment with HSA-AuNR-TKI, (d) laser treatment alone, (e) 20x image of HSA-AuNR tissue with silver staining showing AuNRs, and (f) tumor after treatment with laser irradiation and HSA-AuNR-TKI showing no viable tumor .........................................................................................................................118
List of Schemes

Scheme 1.1 - Schematic representation of a fluorescence sandwich immunoassay .....................15

Scheme 2.1 – Synthesis of CdSe/ZnS Quantum Dots..............................................................31

Scheme 2.2 – Schematic representation of the hydrophobic interactions between the hydrocarbon chain of the silica pore wall and the TOPO capping ligand on the quantum dot surface ..........37

Scheme 3.1 – Schematic representation of quantum dot fluorescence immunoassays ...............59

Scheme 3.2 – Reaction scheme for conjugating antibodies with SiQD-SH with Sulfo-SMCC. Silica spheres are represented by spheres with green dots (not to scale)..................................66

Scheme 3.3 – Reaction scheme for conjugating antibodies with SiQD-NH₂ composites with EGS. Silica spheres are represented by spheres with green dots (not to scale). .......................71

Scheme 4.1 – Reaction scheme for conjugating antibodies with HSAP-QD through EDC/NHS coupling..................................................................................................................93
Abstract

Luminescent semiconductor quantum dots (QDs) are extensively researched for use in biological applications. They have unique optical and physical properties that make them excellent candidates to replace conventional organic dyes for cellular labeling, multiplexing, nucleic acid detection, and as generalized probes. The primary focus of this dissertation was to utilize quantum dots for improvement in immunoassays. Specifically, atherosclerosis biomarkers were detected simultaneously in an effort to demonstrate advances in early detection diagnostics.

Quantum dot-antibody bioconjugates were prepared by encapsulation into mesoporous silica and functionalized with thiol and amine groups to enable bioconjugation. Functionalization of the mesoporous silica quantum dot composites facilitated biocompatibility for use with biological buffers in immunoassays. These bioconjugates were used in a sandwich immunoassay to detect atherosclerosis biomarkers IL-15 and MCP-1. Sandwich assays employ capture antibodies immobilized onto a well plate to bind as much of the antigen as possible. The capture antibodies increased binding by at least 4 times the amount of antigen bound to the surface of a direct detection assay. The sandwich immunoassay was able to detect 1 pg/mL of IL-15 and 50 pg/mL of MCP-1 biomarkers.

Human serum albumin nanoparticles (HSAPs) were synthesized via a desolvation and crosslinking method. Human serum albumin is a versatile protein being used in a variety of applications. Quantum dots were loaded into HSAPs as potential detection probes for immunoassays. Efficient loading was not achieved, and the assay was unable to improve current detection limits.
Controlled release studies were explored using HSAPs loaded with superparamagnetic iron oxide nanoparticles and a fluorescent drug analog. Exposure to a magnetic field resulted in degradation of the HSAPs. The fluorophore was released and measured to examine how cancer drugs might be controlled through a magnetic field. Gold nanorods and an anticancer drug, Sorafenib, were also encapsulated into HSAPs for treatment of renal cell carcinoma *in vivo*. Laser irradiation treatment combined with Sorafenib resulted in 100% tumor necrosis and total elimination of any viable tumor present. HSAPs have demonstrated remarkable potential as drug delivery nanocarriers.

Keywords: Quantum dots, fluorescence, silica, immunoassay, multiplexing, atherosclerosis, human serum albumin, drug delivery, biomarkers, biosensor
Chapter 1

Introduction

1.1 Objectives and Aims

The main objective of this research was to develop and optimize a sensitive multiplex bioanalytical assay utilizing luminescent semiconductor quantum dots to detect the presence of atherosclerosis biomarkers. Quantum dots have a wide array of advantages over conventional organic dye molecules used for analysis methods. Encapsulating quantum dots into mesoporous silica provides a multitude of barcodes for detection assays. Human serum albumin nanoparticles have also shown potential as a multifunctional analytical diagnostic and therapeutic tool. Employing quantum dots into human serum albumin nanoparticles can further enhance barcoding agents for theranostic applications. Additionally, the versatility of human serum albumin nanoparticles was explored as multimodal nanocarriers for targeted drug delivery utilizing combined methods of controlled release with nanoparticle materials. The specific aims of this research were 1) synthesis of water-soluble quantum dot loaded mesoporous silica composite particles as biosensors, 2) preparation of human serum albumin nanoparticles loaded with quantum dots, 3) multiplex detection of atherosclerosis biomarkers using a fluorescence-based immunoassay, and 4) exploration of targeted drug delivery using human serum albumin nanoparticles.
1.2 Significance

Early disease detection and prevention is critical across all arenas of healthcare worldwide. Rapid and effective diagnosis of many diseases could lead to earlier treatment assessments, improve prognosis, and increase lifetimes. Diagnostic tests and early screenings are only reliable when the tests are sensitive, precise, and specific.\textsuperscript{1} New biomarkers are constantly being discovered for different cancers and diseases, and the need for consistent and accurate means of detection is increasing.\textsuperscript{2-3} This research mostly focuses on detection of atherosclerosis biomarkers. Atherosclerosis is a main component of cardiovascular disease (CVD), which is the leading cause of death for men and women globally.\textsuperscript{4-6} Earlier detection of atherosclerosis is critical in order to reduce the astounding financial costs related to treatment and assessment as well as to reduce the likelihood of heart attack or stroke over an individual’s lifetime.\textsuperscript{7-8}

Bioanalytical immunoassays are one of the main tools for measuring the presence of elevated levels of biomarkers in biological fluids such as blood serum and urine.\textsuperscript{9-10} Immunoassays depend on specific, inherent antibody-antigen binding to indicate the presence of a given marker, however, many key factors affect the sensitivity and reproducibility of the assays.\textsuperscript{9,11-12} One component, in particular, is the detection reagent or probe used that produces the signal specifying the amount of analyte present.\textsuperscript{10} Many bioassays rely on fluorophores to measure the amount of a given protein because fluorescent molecules can be detected with high specificity, but they can be limited chemically and physically.\textsuperscript{9,13-18} Photobleaching, low fluorescence quantum yield, and broad emission spectra are a few of the limitations of conventional organic dyes.\textsuperscript{13,18} Luminescent semiconductor quantum dots (QDs) have advantages over organic dyes and have emerged as reliable alternatives.\textsuperscript{2,17,19-21}
This research primarily focused on exploiting the unique properties of semiconductor QDs to produce a simple, rapid, and reliable immunoassay capable of detecting multiple analytes at once while improving current detection limits. Encapsulating QDs into mesoporous silica enhances their brightness and provides easy surface functionalization for bioconjugation.\textsuperscript{22-28} QDs were also loaded into human serum albumin nanoparticles (HSAPs) to develop a new bioprobe with potential use as a detection agent in bioassays or to enhance other fluorescently labeled albumin probes currently used for cell tracking and imaging tools.\textsuperscript{29-30} Moreover, HSAPs were further explored to examine their multimodal functionality as targeted and controlled drug release agents. Encapsulating superparamagnetic iron oxide nanoparticles with a fluorescent drug analog provides insight on how a drug would be released from HSAPs via magnetic hyperthermia.\textsuperscript{31-32} Loading gold nanoparticles into HSAPs for degradation via laser irradiation shows definite promise for controlled release of anticancer drugs directly at tumor sites.\textsuperscript{33-35}

1.3 Fluorescence

1.3.1 The Fluorescence Process

Fluorescence is the emission of light by a substance due to absorption of a photon or other electromagnetic radiation.\textsuperscript{36} It is the result of a three-stage process: excitation, excited-state lifetime, and emission; this process is best illustrated by the Jablonksi diagram (Figure 1.1). The Jablonksi diagram depicts the processes that occur between absorption and emission. Fluorophores are typically polyaromatic hydrocarbons or heterocycles that can reach an excited state upon absorption of a photon and then emit light energy. The light energy emitted is at a lower energy than the photon absorbed due to vibrational relaxation.
During excitation, the fluorophore absorbs a photon usually from an incandescent lamp or laser, and an electron moves to an excited electronic state (S₁ or S₂). The excited state lasts for about $10^{-15}$ s, and during this time the fluorophore interacts with its molecular environment and can undergo conformational changes. Vibrational relaxation occurs and the electron moves to the ground level of the S₁ state, and some energy is lost as heat during the process. From here the electron can relax back to the ground state (S₀) and emit a photon of a lower energy and longer wavelength than the absorbed photon. This is the phenomenon of fluorescence. The electron can also relax back to the ground state through internal conversion, which is non-radiative, and a photon is not emitted, however, energy is lost through heat. If the electron in the S₁ state transitions to the triplet state (T₁), intersystem crossing occurs. Here, the electron can either relax to the S₀ ground state and emit a photon as phosphorescence, or it can go back to the S₁ state and then to the S₀ ground state emitting a photon as delayed fluorescence. Photons emitting via phosphorescence will have an even lower energy and longer wavelength than fluorescence. An electron can also travel from T₁ to S₀ in a non-radiative form not emitting a photon, but losing energy as heat. Fluorescence quantum yield is the ratio of the number of photons emitted relative to the number of photons absorbed.

Figure 1.1 Jablonski Diagram.
to the number of photons absorbed and is a measure of the extent to which the different processes may occur.

Fluorescence emission occurs when an excited electron relaxes back to the ground state and emits a photon. The energy is lower than that of the absorbed photon, and the difference in energy or wavelength \((\hbar \nu_{ex} - \hbar \nu_{em})\) is called the Stokes shift. The Stokes shift is important to fluorescence techniques because it allows emission to be detected against a low background.

### 1.3.2 Fluorescence Spectroscopy

Fluorescence emission can be analyzed through several different detection systems. Fluorescence spectrofluorometers and microplate readers are common instrumentation utilized for detection. Most fluorescence instruments have four basic items: 1) a light source, 2) a sample holder, 3) wavelength filters to isolate excitation photons and emission photons, and 4) a detector to read emission photons and produce a visual output of analysis.\(^{37}\) Fluorescence spectroscopy measurements performed in this work were measured primarily by a SpectraMax M2 microplate reader (Molecular Devices, Inc). This plate reader contains a Xenon flash lamp source and a photomultiplier (R-3896) detector. There is a built in excitation monochromator and emission monochromator that allows for detection of absorbance and fluorescence at multiple wavelengths. The light source is irradiated to the excitation monochromator allowing only certain wavelengths to pass through. The excited light passes through 1 mm fiber optic bundles to the microplate port where the light is directed to a focusing oval mirror. Incident light enters the sample from the top of the microplate, and the reflected light travels to the emission monochromator and photomultiplier tube prior for processing.
1.3.3 Digital Fluorescence Microscopy

Fluorescence microscopy is a technique for viewing and detecting fluorescence or phosphorescence in a substance. The microscope combines the magnifying properties of an optical light microscope with fluorescence technology allowing excitation and emission detection of the sample. Images are acquired based entirely on the fluorescence emission properties of the sample. This is particularly useful for live cell imaging within tissues, and proteins that are stained or exhibit a natural fluorescence can be imaged and tracked. An inverted fluorescence microscope is typically used for imaging. The components of the microscope are similar to that of a light microscope, but fluorescence microscopes include a powerful light source such as a xenon or mercury arc lamp that can emit light from UV-IR wavelengths. Filter cubes are another difference from traditional light microscopes; they are composed of an excitation filter, a dichroic mirror, and an emission filter (Figure 1.2). Light leaves the arc lamp and is directed through an excitation filter that selects the excitation wavelength. This light is reflected toward the sample by a dichroic mirror that only reflects light at the desired excitation wavelength. The light passes through to the objective and is focused onto the sample. The sample gives off its own fluorescence emission that passes back through the objective and is magnified. Epifluorescence is when the emission and excitation light pass through the same objective and can reduce the signal-to-noise ratio. The emission light passes through the dichroic mirror and through an emission bandpass filter. The bandpass filter only allows certain wavelengths to pass through thereby reducing the background, and the filtered light is sent to a detector such as a charge coupled device (CCD) camera where the image can be acquired digitally. The filtered emission light can also be directed to a spectrograph which can display the fluorescence emission spectrum of the sample. The fluorescent sample determines the excitation
and emission wavelengths used in the filter cube, and multiple combinations of filter cubes can be assembled for any given fluorescent sample type.

Figure 1.2 Illustration of the light path through a filter cube of an inverted fluorescence microscope.

1.4 Quantum Dots

1.4.1 Structural Properties

Quantum dots (QDs) are luminescent semiconductor nanocrystalline particles with unique chemical, structural, and photophysical properties. They are composed of elements from groups II-IV (CdSe, CdS, CdTe, ZnS, ZnO, ZnSe), III-V (InP, InAs, GaP, GaN, GaAs), and (PbS, PbSe, PbTe) of the periodic table. QDs average 2-10 nm in diameter and can contain hundreds to thousands of atoms. They approach the size of the exciton Bohr radius; the average distance in an electron-hole pair is confined in all three dimensions. Because both electrons and holes are confined to a small region, the mode of recombination is limited. QDs have characteristics in between bulk and discrete semiconductors; they differ
from bulk materials in that they are characterized by discrete atomic-like states.\(^\text{47}\) Bulk semiconductors have continuous energy levels with a fixed bandgap energy, but the bandgap energy of QDs is determined by its radius. QDs experience the quantum confinement effect, which is why they have discrete energy levels with finite separation between them (Figure 1.3).\(^\text{48}\) The addition or subtraction of a few atoms can alter the boundaries of the bandgap. Quantum confinement causes increased stress on the exciton resulting in increased energy of the emitted photon. Smaller QDs have less room for exciton separation and more energy is required to form the exciton so photons will be emitted at lower wavelengths. Adjusting the size of the bandgap changes emission frequency, and changes in surface geometry can affect the bandgap energy. These factors allow control over the output wavelength of QD emission.

**Figure 1.3** Illustration of quantum dot size due to the quantum confinement effect.

### 1.4.2 Optical Properties

The quantum confinement effect is essentially responsible for the unique optical properties exhibited by QDs that make them advantageous over organic fluorophores. When an
electron in the valence band is excited by photon absorption and moves to the conduction band, a hole is left in the valence band. When the electron relaxes back to the valence band, light is emitted as fluorescence. QDs have a broad absorption spectrum with narrow emission peaks, and relative to other common fluorophores, QDs are highly photostable. They have high emission quantum yield, a large molar extinction coefficient and are exceptionally bright. QDs also have a long fluorescence lifetime as well as a large Stokes shift and size dependent tunability. The surface of the QDs influences photoluminescence because of trap states that cause nonradiative electron transitions resulting in low quantum yield. Trap states usually occur due to dangling bonds or vacancies at the surface. Coating QDs, also known as surface passivation, with a shell of a higher bandgap semiconductor raises the quantum yield by reducing these effects. Zinc sulfide is commonly used as a coating shell because it enhances quantum yield, chemical stability, and photostability of the QDs.

1.4.3 Synthesis of Quantum Dots

QDs are synthesized through various physical or chemical methods. Physical processes include high-energy input through molecular beam epitaxy and metal organic chemical vapor deposition. More recently, however, chemical methods are typically used such as high temperature particle growth on solid substrates or by low energy input colloidal chemical precipitation. Colloidal semiconductor synthesis is the most successful method in terms of quality and monodispersity. The system is based on the pyrolysis of organometallic precursors in hot coordinating solvents. Changing the temperature, concentrations, time, and ligand molecules precisely controls size tuning of colloidal QDs. The most widely used and synthesized QDs are CdSe because it has tunable absorption energy throughout the UV-Vis-NIR spectrum and its precursors are inexpensive and readily available.
Synthesis of CdE (E = S, Se, Te) was first published by Murray et al. from MIT in 1993; they reacted dimethylcadmium Cd(CH₃)₂ in trioctylphosphine (TOP) with a solution of trioctylphosphine selenide (TOPSe) at high temperatures. Eventually, the procedure was modified by Peng et al. by replacing Cd(CH₃)₂ with CdO, Cd(AC)₂, and CdCO₃, and these cadmium precursors are typically used today. The mechanism for colloidal nanocrystal growth has been proposed to proceed from nucleation resulting from rapid injection of precursors into a hot coordinating solvent. The reagents undergo thermal decomposition and there is a supersaturation of monomers present. Monomers are added to the QD nuclei, and once the concentration is depleted, further growth proceeds by Ostwald ripening. The QD core can have low quantum yield due to surface defects, vacancies, and dangling bonds on the surface. When the excited electron or hole is trapped due to defects low quantum yield can result from nonradiative recombination. A shell of a higher bandgap semiconductor material is often added to overcome these defects and protect the QD core. ZnS is most often added to CdSe core QDs, and the ZnS shell reduces QD toxicity, improves quantum yield, and enhances photostability.

1.4.4 Bioconjugation of Quantum Dots

Inorganic quantum dots are synthesized in organic solvents, which is not suitable for biological applications. For QDs to be used as fluorescent labels they need to be soluble in aqueous solvents which requires either a surface ligand exchange or encapsulation in amphiphilic media for bioconjugation. Exchanging the trioctylphosphine oxide (TOPO) capping ligand for 16-mercaptohexadecanoic acid (MHDA) enables water solubility of the QDs; however, there is often a decrease in quantum yield and brightness after the ligand exchange. The ligands bind to the ZnS shell through thiol (-SH) groups on one end through metal affinity coordination while
the other enables hydrophilicity. Other ligands used to replace TOPO include dihydrolipoic acid (DHLA), dithiothreitol (DTT), mercaptoacetic acid (MAA), and 11-mercaptoundecanoic acid (MUA).\textsuperscript{59-62} The thiol bond to the ZnS shell is not strong enough, so QD encapsulation is a more appropriate method for water solubility.

Doping QDs into porous or mesoporous polystyrene and silica particles has shown great retention of luminescent properties.\textsuperscript{28,63-64} Polystyrene-encapsulated QDs are prepared through an emulsion polymerization method.\textsuperscript{65-66} Polystyrene beads are dissolved in an organic solution causing them to swell and enlarge their pores. The QDs penetrate the pores and adsorb to the hydrophobic walls. Upon removal of the solvent, the pores contract, and the QDs remain inside of the polystyrene beads.\textsuperscript{67} Silica encapsulation has also been shown to increase the brightness of QDs of a single probe by encapsulating thousands of QDs into one silica particle.\textsuperscript{28,58} Silica growth on the QD surface is possible through the Stöber synthesis method.\textsuperscript{27,68} This is a one-step method that does not utilize toxic solvents.\textsuperscript{69} Tetraethyl orthosilicate (TEOS) condenses in ethanol/water mixtures at room temperature.\textsuperscript{58,70-71} Multiple QDs of different sizes and emission wavelengths can be incorporated into the silica pores and remain stable due to strong hydrophobic interactions.\textsuperscript{28}

The main bioconjugation methods for attachment to QDs are covalent modification, direct attachment, and electrostatic interactions.\textsuperscript{13,72-73} Covalent modifications involve biomolecules attached to functional groups on the QD ligand surface. Amine (-NH\textsubscript{2}), thiol (-SH), and carboxyl (-COOH) groups all attach to QDs via covalent modifications. Direct attachment to QDs includes metal-affinity coordination to Zn atoms as well as dative thiol bonding. Proteins can also bind to QDs through electrostatic interactions. Quantum dot bioconjugates enable them for use in a multitude of applications.
1.4.5 Applications of Quantum Dots

The unique tunable properties of QDs make them useful in a wide variety of applications. Quantum computing, solar cells, LEDs, televisions, and diode lasers are a few of the applications currently under investigation for enhancement with QDs. There is ongoing research to replace incandescent light bulbs with red, green, and blue QDs.

QDs were first described in a biological context in 1998 by Nie and Alivisatos. Inorganic QDs are limited for use in in vivo applications, especially CdSe QDs, but they are widely utilized for in vitro biological applications. QDs are often used as biosensors for immunoassays, generalized probes, nucleic acid detection, and fluorescence resonance energy transfer (FRET). QDs have also been used for cellular labeling and imaging, and they have increased photoluminescence over conventional organic dyes. The greatest potential of quantum dot antibody bioconjugates for biosensing is multiplexing. Multiplexing is the simultaneous detection of multiple analytes in an immunoassay. QD-bioconjugates are constantly improving the sensitivity of clinical applications.

1.5 Human Serum Albumin

Human serum albumin (HSA) is the most abundant blood plasma protein comprising about 50% of the total protein present. It has extraordinary ligand binding capabilities that make it excellent for many bioapplications. HSA has a molecular weight of 65 kDa and contains 585 amino acids including 35 cysteine residues that form 17 disulfide bridges with one free thiol, and it also has one fluorescent tryptophan. HSA is naturally biocompatible, biodegradable, and non-toxic. The large amount of charged amino acids provide easy surface functionalization capabilities. HSA molecules are able to pass freely throughout the body and can pass through the blood brain barrier.
HSA molecules can form dimers and aggregate together for nanoparticle preparation. Human serum albumin nanoparticles (HSAPs) are typically prepared through an alcoholic desolvation method. The alcohols unfold or denature the tertiary structure of the albumin, and aggregates naturally coacervate or come together through electrostatic and hydrophobic interactions. A crosslinking agent such as glutaraldehyde then binds amine groups of lysine and arginine amino acids to one another forming albumin nanoparticles.

The diameter of HSAPs is dependent on experimental conditions, and modification of the different parameters gives control over the nanoparticle size. The rate at which ethanol is added to initiate desolvation affects the particle size and polydispersity. Adding ethanol at about 1 mL/min creates monodisperse HSAPs that average 200 nm in diameter. Furthermore, the pH prior to desolvation also regulates particle size. For HSAPs averaging 200 nm, the optimal pH is 7. Adjusting the pH to 8.2 decreases particle size, and significantly lowering the pH causes aggregation of initial aqueous albumin solution.

HSAPs have extraordinary potential for use in a wide range of bioapplications. They show promise particularly as vehicles for controlled release and targeted drug delivery, and they exhibit multimodal functionality. As nanocarriers, HSAPs can transport toxic and insoluble drugs throughout the body directly to tumor sites effectively improving current chemotherapeutic techniques. HSAPs covalently encapsulate and conjugate drugs with high loading efficiency for transport. Loading other metallic, organic, or inorganic materials with the chemotherapeutic drug allows for controlled release. Nanoparticle degradation and release of the drugs can be facilitated by magnetic nanoparticles or gold nanoparticles through exposure to a magnetic field or laser irradiation, respectively. Specifically directing
HSAPs to tumor sites also spares exposure of toxic chemotherapeutic agents to neighboring healthy cells.\textsuperscript{89, 103, 107-108}

1.6 Immunoassay Development

Many methods of protein detection are available and widely researched including: flow cytometry, western blots, immunoprecipitation, and immunohistochemistry. The best method, however, for quantifying the amount of a protein present is through a bioanalytical assay. Immunoassays are analytical tools for quantifying molecules based on selectivity and specificity of antigens and antibodies.\textsuperscript{9} They were first developed in the 1950s by Rosalyn Yalow and Solomon Berson who investigated radioisotope tracings to measure biological substances in human blood.\textsuperscript{109} The main types of immunoassays are: 1) competitive, 2) antigen-down, and 3) sandwich.\textsuperscript{9} Competitive binding assays are based upon competition of a labeled and unlabeled antigen for a limited number of binding sites on an antibody. A known amount of labeled, unbound antigen is added, and it is measured by its ability to compete with the unlabeled antigen. It gets blocked from binding to the antibody. The more analyte present in the sample, the more the labeled antigen is blocked from binding so the amount of labeled, unbound antigen is proportional to the amount of analyte present in the sample.\textsuperscript{110} Antigen-down assays involve binding an antigen to a solid surface for binding to antibodies present in a sample. Another labeled secondary antibody is added that binds to the antibodies in the sample. The signal from the labeled secondary antibody is proportional to the amount of primary antibody present.\textsuperscript{80} Sandwich assays are the most robust and sensitive.\textsuperscript{9, 13} They utilize a primary antibody and a secondary antibody to each bind to an antigen (Figure 1.4). The primary antibody is also known as the capture antibody. Monoclonal antibodies are often used as the primary antibody because they bind to one particular epitope of an antigen.\textsuperscript{11} This gives the best chances for positioning the
antigens in the same orientation on a well plate. After the antigen is added, a labeled, secondary detection antibody is added. This antibody is usually polyclonal and can bind to multiple epitopes on a given antigen, thereby increasing the antigen-antibody binding so that as much of the antigen as possible can be detected.\textsuperscript{12, 21, 111}

![Diagram of fluorescence sandwich immunoassay]

**Schematic 1.1** Schematic representation of a fluorescence sandwich immunoassay.

Selection of the detection label for the antibody is a critical factor in improving sensitivity of the immunoassay.\textsuperscript{9} Examples include radioactive isotopes, DNA reporters, and chemiluminescent tags.\textsuperscript{80, 112} The most popular labels are enzymes such as horseradish peroxidase and alkaline phosphatase. Enzyme labels are used in enzyme-linked immunosorbent assays (ELISAs), and they are currently the most commercially available assay type.\textsuperscript{9} In a sandwich ELISA, a capture antibody is bound to the surface, nonspecific binding sites are blocked, and the antigen is added for capture by the primary antibody. Another antibody is added forming the sandwich, and it binds specifically to the antigen. ELISAs require an additional secondary antibody that is linked to an enzyme; this antibody is used to bind to the antibody already bound to the antigen.\textsuperscript{110} A
chemical is added that converts the enzyme into a fluorescent or electrochemical signal.\textsuperscript{113} The antigen can be quantified by measuring the absorbance or fluorescence of the signal.\textsuperscript{113-114} Typically, the enzymatic reaction is short-term, and the signal must be measured immediately. Other limitations include false positives and negatives as well as altered enzymatic activity due to assay constituents. Replacing the label from an enzyme to quantum dots eliminates the need for a third antibody and reduces the chance for nonspecific binding.\textsuperscript{13, 20, 30, 79, 115-119}

1.7 Atherosclerosis

Cardiovascular disease (CVD) is the leading cause of death worldwide for men and women accounting for about 1 in 3 deaths.\textsuperscript{8} Atherosclerosis is a condition where the vascular arteries harden and increase in thickness, and it is a main contributor to CVD.\textsuperscript{5-6} An atherosclerotic plaque can lead to heart attack or stroke if it ruptures. Typically, cholesterol and other fatty materials or atheroma accumulate in an artery and lead to blockages that restrict blood flow, which can form blood clots.\textsuperscript{7} Atherosclerosis is a chronic disease, and it is not unusual for plaque formation to begin during childhood. The formation of an atherosclerotic plaque takes place over a series of steps. Low-density lipoproteins (LDL) transport cholesterol from the liver to the cells, and when it is oxidized it can cause damage to an artery wall.\textsuperscript{4, 7} Macrophages or white blood cells are then sent to the artery wall to repair and absorb the oxidized-LDL. This reaction forms foam cells, and accumulation of foam cells forms plaque in the artery wall.\textsuperscript{5} Two types of plaque can form: stable or unstable. Stable plaque formation is usually not harmful; the artery wall will increase in diameter and blood flow will not be restricted. They are asymptomatic and contain smooth muscle cells. Unstable plaques, however, have a tendency to rupture and increase the incidence of blood clots.\textsuperscript{7} They are composed of macrophages and foam cells. When blood flow is restricted due to blockages, the risk for heart attack or stroke rises.
Additionally, when plaques harden or calcify blood flow is also restricted, blood pressure is increased, and risk for clots rise.\textsuperscript{120}

Monitoring the progression of atherosclerosis could greatly improve prognosis and reduce the risk for plaque rupture. Detection for atherosclerosis is in dire need of enhancement in order to lower the number of fatal cardiovascular events. Unfortunately, diagnosis of atherosclerosis occurs typically after plaque formation has occurred. Angiograms, stress tests, cholesterol tests, X-rays, computed tomography (CT) scans, and electrocardiograms (EKG) are often ordered to examine abnormal behavior in the vascular system.\textsuperscript{8} Imaging techniques are beneficial, but they are used to search for atheroma and assess the condition of the artery walls. Angiograms can provide information on the lipid-rich plaques in an artery, but again, this is not ideal for early detection or monitoring of the progression of atherosclerosis. CT scans are effective at imaging calcification, but there must be substantial plaque present for imaging. Recently, clinical intravascular ultrasounds have been able to detect and measure atheroma, but it is invasive, extremely costly, and not widely utilized.

Several biomarkers have recently been identified as indicative of atherosclerotic plaques at various stages of plaque development from as early as the development of precursor legions to fatty acid streaks and complete lipid-rich plaques.\textsuperscript{4} Monocyte chemoattractant protein-1 (MCP-1) and interleukin 15 (IL-15) are two of the more recently discovered biomarkers.\textsuperscript{121-122} MCP-1 is a chemokine that rises in macrophage-rich areas of atherosclerotic plaques.\textsuperscript{123} Chemokines are released in response to signals from proinflammatory cytokines. IL-15 is a cytokine expressed in immune cells and atherosclerotic lesions.\textsuperscript{124} It is uniquely found in lipid-rich plaques in complex foam cells, and it stimulates T lymphocytes. High levels of IL-15 have also been found in
patients with coronary artery disease. Early detection of these biomarkers could lead to early treatment and assessment of atherosclerosis leading to improved prognosis for CVD.

1.8 References


23
114. Xiao, Y.; Isaacs, S. N., Enzyme-Linked Immunosorbent Assay (ELISA) and Blocking with Bovine Serum Albumin (BSA) - Not all BSAs are alike. *Journal of immunological methods* 2012, 384 (1-2), 148-151.


Chapter 2

Synthesis of Water-Soluble Mesoporous Silica Quantum Dot Composite
Particles

2.1 Abstract

Quantum dots are being exploited for their unique optical and physical properties to enhance many bioanalytical applications. Most fluorescent biosensors are composed of organic fluorophores, but they are limiting in their capabilities due to photobleaching and short fluorescence lifetime. Quantum dots are able to enhance numerous biosensing methods by overcoming many of the challenges organic fluorophores face. CdSe/ZnS quantum dots were used specifically to develop biosensors to improve immunoassays for biomarker detection. Quantum dots of different sizes were efficiently loaded into mesoporous silica particles ranging from 3-10 µm in diameter with 32 nm pores. In addition to silica composites containing single emission quantum dots, quantum dots of different sizes and emission wavelengths were precisely loaded into single silica composite particles. A wide selection of quantum dot loaded silica composite particles affords biological assays the ability to detect several analytes simultaneously. Incorporating CdSe/ZnS quantum dots into mesoporous silica enhances their brightness and provides a simple means for bioconjugation strategies.

Coupling the quantum dot mesoporous silica composites to antibodies is a key component for immunoassay optimization. Modifying them with amine or thiol groups allows for simple bioconjugation with antibodies. APTES and MPTMS were added to the quantum dot
silica composites to introduce amine and thiol groups, respectively. Tetraethyl orthosilicate (TEOS) was used to coat the SiQDs, effectively sealing the pores and preventing leakage of the QDs. Adding TEOS caused a shift of the emission wavelength of the SiQDs. The amount of TEOS added determined the change in emission wavelength. This modification allows for more spectral codes that can be used as biosensors without needing several different color quantum dots. These water soluble mesoporous silica quantum dot composites are used to develop immunoassays for biomarker detection.
2.2 Introduction

Bioanalytical assays commonly employ fluorescent probes as biosensors for detecting or quantitating other molecules.¹ Biosensors are often used for immunoassays, nucleic acid detection, fluorescence resonance energy transfer, and cellular labeling applications.¹ Fluorescent organic dyes and fluorescent proteins have been the most used as fluorophores for assays because of their high fluorescence intensity, but they face many photophysical challenges limiting their efficiency as reliable biosensors.² Quantum dots (QDs) have been found to be useful in bioanalytical applications.¹,³ They have many unique spectral and optical properties that are advantageous over conventional organic dyes.¹-²,⁴

Quantum dots were first identified in literature through a biological context in 1998.⁵-⁶ Organic fluorophores, while highly luminescent, have a short fluorescence lifetime, small Stokes shift, and are largely pH dependent.¹,⁷-⁸ Additionally, organic dyes and proteins have narrow absorption windows with broad emission spectra, and they are also susceptible to photobleaching and self-quenching.⁹-¹² Quantum dots are more robust than organic fluorophores and exhibit a multitude of distinct photophysical properties suitable for biosensing applications.⁸,¹³ These properties include: a large molar extinction coefficient, high quantum yield, broad absorption spectrum along with narrow size-tunable photoluminescent emission, and high resistance to photobleaching and chemical degradation.¹³-¹⁹ QDs also have a relatively long fluorescence lifetime, high photostability, size-dependent tunability and large Stokes shifts.¹,¹⁶,²⁰-²¹ A wide array of biological and chemical applications has utilized their extensive properties including: immunoassays, FRET, DNA targeting, cellular labeling and imaging, as well as pathogen and toxin detection.¹-²,¹¹,¹⁶,²²-²³
Proper fluorescent labels for biosensing functionalities are bright and soluble in buffers or culture media. They should also be stable, biocompatible, and easily functionalized for bioconjugation. QDs are usually stored in inorganic solvents making them unsuitable as fluorescent labels and solubility in aqueous solvents requires either a surface ligand exchange or encapsulation in an amphiphilic media to allow for bioconjugation. CdSe/ZnS core-shell QDs are most commonly used for bioanalytical applications, and they typically have a hydrophobic trioctylphosphine oxide (TOPO) capping ligand. Water soluble CdSe/ZnS QDs can be prepared by exchanging TOPO for mercaptohexadecanoic acid (MHDA), however, these QDs often display a substantial decrease in brightness and quantum yield compared to TOPO-capped QDs. QD encapsulation provides a more efficient means to enhance water solubility while maintaining the distinct, inherent properties QDs exhibit.

Doping QDs into porous or mesoporous polystyrene and silica particles has shown excellent retention of luminescent properties making them useful for spectral encoding. Polystyrene-encapsulated QDs can be prepared through an emulsion polymerization method whereby polystyrene beads are dissolved in an organic solution causing them to swell and enlarge their pores allowing QDs to penetrate the pores and adsorb to the hydrophobic walls. The pores contract upon removal of the solvent and the QDs remain inside the polystyrene beads. Silica encapsulation of fluorophores has shown to be a simple process that enables bioconjugation and increases photostability and emission quantum yield. Ruthenium diimine complexes have been successfully incorporated into mesoporous silica particles via the Stöber synthesis method. The Stöber method is a one-step synthesis involving the condensation of tetraethyl orthosilicate (TEOS) in ethanol/water mixtures at room temperature. This method
provides great control over the size of the resulting silica particles as well as a narrow size
distribution and smooth morphology.\textsuperscript{25,35}

2.3 Experimental Methods

2.3.1 Materials and Reagents

All chemicals were used as received without modification unless otherwise noted. Cadmium
oxide (99.99\%), lauric acid (98\%), technical grade (90\%) trioctylphosphine oxide (TOPO),
technical grade (90\%) hexadecylamine (HDA), technical grade (90\%) trioctylphosphine (TOP),
selenium powder (99.5\%), diethyl zinc solution 1.0 M in heptane, hexamethyldisilathiane,
methanol anhydrous (99.8\%), chloroform (99.5\%), tetraethyl orthosilicate (TEOS), (3-
aminopropyl)triethoxysilane (APTES), propanol, and 3-mercaptopropyltrimethoxysilane
(MPTMS) were purchased from Sigma-Aldrich (St. Louis, MO). Mesoporous silica particles
with 32 nm C\textsubscript{18} modified pores were obtained from Phenomenex (Torrance, CA). Ethanol, 200-
proof, ACS/USP grade was purchased from Pharmco-AAPER (Brookfield, CT). N-(trimethoxysilylpropyl)ethylenediaminetriacetate, trisodium salt, 35\% in water was purchased
from Gelest (Morrisville, PA). Nanopure deionized and distilled water (18.2 MΩ) purified with a
Barnstead Nanopure system was used for all experiments.

2.3.2 Synthesis of TOPO coated luminescent quantum dots

TOPO coated CdSe/ZnS quantum dots were prepared using a slightly modified version of
a method developed by Peng.\textsuperscript{30-31} Briefly, 12.5 mg of cadmium oxide (CdO) and 180 mg of
lauric acid were mixed in a three-neck flask under a nitrogen atmosphere. The mixture was
heated to 180-200 °C until the CdO fully dissolved in the lauric acid forming a clear, colorless
solution. Once fully dissolved, 1.94 g of trioctylphosphine oxide (TOPO) and 1.94 g of
hexadecylamine (HDA) were added to the solution under constant stirring. The temperature was increased to 280 °C for 10 minutes before being cooled down slowly. Meanwhile, a solution of 80 mg of selenium powder was dissolved in 2 mL of trioctylphosphine (TOP). This solution was rapidly injected into the mixture under vigorous stirring upon reaching the desired temperature during the cooling process. Size-tunable luminescent quantum dots are directly dependent upon temperature. The temperature at which the selenium component is injected determines the size of the quantum dots synthesized, corresponding to their emission wavelength. Typically, the temperature is slowly decreased from 280 °C to 180 °C. The higher the temperature upon injection, the larger the quantum dots will be in size.\textsuperscript{36} A precursor solution for a ZnS shell was prepared: 2 mL of TOP containing 1 mL of diethyl zinc (Zn(Et)\textsubscript{2}) and 250 µL of hexamethyldisilathiane ((TMS)\textsubscript{2}S). This shell was added dropwise into the solution after injection of the selenium. The reaction mixture was kept at 180 °C for 1 hour before cooling to room temperature. The resulting CdSe/ZnS quantum dots were washed 3 times with methanol by centrifugation at 4000 rpm for 10 minutes and redispersed in chloroform. The quantum dots were stored at room temperature in the dark.

Scheme 2.1 Synthesis of CdSe/ZnS Quantum Dots.
2.3.3 Preparation of Quantum Dot Mesoporous Silica Composite Particles (SiQDs) with Single and Multiple Quantum Dots at Controlled Ratios

Preparation of mesoporous fluorescent silica microbeads were based on previously reported methods. Briefly, 4 mg of mesoporous silica particles averaging 3, 5, or 10 µm in diameter with 32 nm C_{18}-modified pores were dispersed in 2 mL of butanol. Two hundred microliters of TOPO-CdSe/ZnS quantum dots were added to the solution under stirring. This mixture was incubated for 1 hour and washed 3 times with ethanol by centrifugation at 11,000 rpm for 20 minutes. The fluorescent silica microbeads were visualized using an Olympus IX-71 inverted microscope and stored at 4 °C.

For coding of single mesoporous silica beads with multiple quantum dots, a premixed solution of quantum dots varying in size was prepared. Controlled ratios are prepared by varying the volumes of the different quantum dot solutions. The premixed solution of multiple CdSe/ZnS quantum dots is added rapidly to the mesoporous silica in butanol and incubated with stirring for 1 hour followed by washing with ethanol.

2.3.4 Synthesis of TEOS-coated Fluorescent Mesoporous Silica Beads

TEOS coated fluorescent mesoporous silica beads were prepared by dispersing 2 mg of mesoporous fluorescent silica particles in 2 mL of ethanol and 500 µL of nanopure water. Upon stirring, 25, 50, 100, or 200 µL of tetraethyl orthosilicate (TEOS) was added dropwise. The mixture was incubated overnight and washed 3 times with ethanol for 20 min at 11,000 rpm. The resulting silica beads were resdispersed in 2 mL of ethanol and visualized using an Olympus IX-71 inverted fluorescence microscope. They were stored at 4 °C until further use.
2.3.5 Amine-modified TEOS capped SiQDs

TEOS-capped mesoporous silica beads were further modified by the addition of an amine group for bioconjugation. While stirring, 100 µL of (3-aminopropyl)triethoxysilane (APTES) was added to the TEOS-capped mesoporous silica beads. They were incubated overnight and washed with ethanol for 20 min at 11,000 rpm. The particles were resuspended in nanopure water via ultrasonication and visualized with an Olympus IX-71 inverted fluorescence microscope. These particles were used immediately for bioconjugation.

2.3.6 Thiol-modified SiQDs

Mesoporous silica beads were also modified with a thiol group for bioconjugation. Quantum dot loaded mesoporous silica beads were dispersed in 1 mL of propanol and 1 mL of water. To this solution 100 µL of 3-mercaptopropyltrimethoxysilane (MPTMS) was added while stirring. This mixture incubated overnight and was washed 3 times with ethanol for 20 min at 11,000 rpm. The thiol-capped mesoporous silica beads were resuspended in 1 mL of water and 1 mL of propanol and 50 µL of N-(trimethoxysilylpropyl)ethylenediaminetriacetate was added dropwise. The mixture stirred overnight and was washed 3 times with nanopure water. The resulting particles were dispersed in nanopure water and used immediately for fluorescence immunoassays.

2.3.7 Fluorescence Spectroscopy Measurements

Fluorescence emission spectra were obtained using a SpectraMax M2 spectrofluorometer microplate reader (Molecular Devices, Inc.) equipped with a 75 W continuous Xenon flash arc lamp as a light source and a photomultiplier (R-3896) detector. The excitation wavelength for all samples was 420 nm. Fluorescence emission was measured from 450-750 nm.
2.3.8 Digital Fluorescence Imaging Microscopy

Fluorescence images were taken using a digital fluorescence imaging microscopy system containing an Olympus IX-71 inverted fluorescence microscope equipped with a 100 X mercury lamp as a light source and a high performance color charge coupled device (CCD) camera (Olympus DP 70). Images were obtained through 10x, 20x, 40x, and 60x microscope objectives with numerical aperture = 0.5. A filter cube with a 425 20 nm band-pass excitation filter, a 465 dichroic mirror, and a 475 nm long pass emission filter was used for spectral imaging. Image analysis was done using DP Controller software. An exposure time of 10 ms was typically used for image acquisition. Fluorescence emission spectra and images of individual fluorescent silica microparticles in solution were taken using a digital fluorescence imaging microscopy system containing an Olympus IX-70 inverted fluorescence microscope coupled with a 0.300 m imaging triple grating monochromator/spectrograph Model SpectraPro 2300i. Spectral analysis of emission spectra was done using Roper Scientific software WinSpec/32. Emission spectra were collected using a filter cube with a 425 20 nm band-pass excitation filter, a 465 dichroic mirror, and a 475 nm long pass emission filter. An exposure time of 1000 ms was typically used for spectral acquisition. Emission spectra were taken for 20 different beads selected at random and averaged for analysis.

2.3.9 Dynamic Light Scattering (DLS) Measurements

Average particle size, zeta potential, and mobility of amine-modified fluorescent silica quantum dot composites were determined through dynamic light scattering. The values were obtained using a Mobius dynamic light scattering instrument (Wyatt Technology Corporation, Santa Barbara, CA) at 25 °C. Samples were dissolved in nanopure water prior to analysis.
2.4 Results and Discussion

2.4.1 Fluorescence Emission of CdSe/ZnS Quantum Dots

High-quality colloidal quantum dots are synthesized through pyrolysis of organometallic precursors in TOPO/TOP coordinating solvents.\textsuperscript{30} Size-tunable luminescent QDs are directly dependent on temperature.\textsuperscript{36-37} The temperature at which the selenium component is injected determines the size of the quantum dots, corresponding to their emission wavelength. The higher the temperature upon injection, the larger the resulting diameter of the QDs.\textsuperscript{38} Figure 2.1 depicts the fluorescence emission spectra of four different CdSe/ZnS QDs solutions; the only difference in preparation for each was the temperature at which the selenium in TOP component was added to the reaction mixture.
Figure 2.1 Fluorescence spectra ($\lambda_{\text{ex}} = 400$ nm) of CdSe/ZnS QDs emitting at (a) 475 nm, (b) 560 nm, (c) 565 nm, and (d) 620 nm.

CdSe/ZnS QDs fluoresce in the visible range, the lower the temperature at which selenium was added, the smaller the resulting QDs, the shorter their emission wavelength, and the bluer the QDs will fluoresce. The higher the temperature at which selenium is added, the longer their emission wavelength, and the redder the QDs will fluoresce. Blue QDs are about 2 nm in size, and red QDs are about 10 nm in size.

2.4.2 Fluorescence Emission of Mesoporous Silica Beads (SiQDs)

Encapsulating QDs into mesoporous silica enhances their brightness and retains the integrity of the CdSe/ZnS core-shell structure.\textsuperscript{39} The mesoporous silica has 32 nm pores which allow the QDs to penetrate into the silica where they remain stable because of strong
hydrophobic interactions between the C\textsubscript{18} hydrocarbon chain on the silica pore wall and the TOPO capping ligand on the surface of the CdSe/ZnS QDs (Scheme 2.2).

Scheme 2.2 Schematic representation of the hydrophobic interactions between the hydrocarbon chain of the silica pore wall and the TOPO capping ligand on the quantum dot surface.

Quantum dot fluorescence emission is directly related to size, and red QDs are the largest. These QDs will have a harder time competing with the smaller QDs for incorporation through the pores into the beads when loading multiple QDs of different sizes. Fluorescence microscopy images of blue, green, and red SiQDs are shown with corresponding overall emission spectra with max emission at $\lambda = 480$ nm, 560 nm, and 620 nm, respectively (Figure 2.2).
Figure 2.2 Digital fluorescence microscope images and fluorescence spectra ($\lambda_{ex} = 400$ nm) of mesoporous silica beads embedded with QDs (a) 480 nm, (b) 560 nm, (c) 620 nm SiQDs.

Multiple QDs of different sizes and emission wavelengths can be incorporated into single mesoporous silica beads. Microscopically, the SiQDs will appear as one color, a blend of the different color QDs in the individual bead, but spectrally, multiple emission peaks will be present for each color QD. A multitude of unique spectral codes can be produced when using multiple QDs in single mesoporous silica beads. The ratio of QDs is precisely controlled by using a premixed solution of QDs during preparation which limits the competition between the different size QDs into the silica pores. There is a homogenous distribution of color between the particles, and SiQDs with high amounts of green QDs appear greener and those with larger amounts of red QDs appear redder in color (Figure 2.3). The SiQDs appear yellow for samples with a 1:1 ratio of green to red QDs due to the combination of even loading distribution of green and red QDs. The 1:2 ratio of green to red QDs appear orange because there is twice the amount of red to green QDs. Samples with a 1:4 and 4:1 green to red combination appear almost entirely red or
green, respectively because of the minimal amount of green or red in each SiQD. The corresponding spectrum of each SiQD solution confirms that the beads do contain both red and green QDs. The ability to control these ratios allows for great expansion of possible biolabeling tools for bioanalytical applications.

**Figure 2.3** Digital fluorescence microscope images and fluorescence spectra ($\lambda_{ex} = 400$ nm) of mesoporous silica beads embedded with green (565 nm) and red (620 nm) QDs at different ratios: (a) 1:1, (b) 1:2, (c) 3:1, (d) 1:4, (e) 4:1.

**2.4.3 Spectral Analysis of Individual Fluorescent Mesoporous Silica Beads**

The fluorescence emission spectrum was taken for twenty different beads randomly selected from each sample. Their emission peak ratios were averaged and standard deviation for
each was calculated. Four beads selected from a sample of 1:4 green to red SiQDs are shown with their corresponding spectra (Figure 2.4). The spectrograph allows a single bead to be analyzed by collecting data from only a selected group of pixels.

![Fluorescence emission spectra of individual beads from a 1:4 green to red SiQD sample.](image)

**Figure 2.4** Fluorescence emission spectra of individual beads from a 1:4 green to red SiQD sample.

The average spectrum of twenty randomly selected beads for all green to red fluorescent mesoporous silica beads is shown (Figure 2.5) and the average ratios and standard deviation are depicted (Table 2.1).
Figure 2.5 Average fluorescence emission spectra of 20 individual green to red SiQDs: (a) 1:1, (b) 1:2, (c) 3:1, (d) 1:4, (e) 4:1.

Table 2.1 Average ratios, standard deviation, and %RSD of SiQDs.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Mean Ratio</th>
<th>σ</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>1.12611</td>
<td>0.31837</td>
<td>28%</td>
</tr>
<tr>
<td>1:2</td>
<td>1.99927</td>
<td>0.50158</td>
<td>25%</td>
</tr>
<tr>
<td>3:1</td>
<td>2.75091</td>
<td>0.39365</td>
<td>14%</td>
</tr>
<tr>
<td>1:4</td>
<td>3.85085</td>
<td>0.46017</td>
<td>11%</td>
</tr>
<tr>
<td>4:1</td>
<td>3.72310</td>
<td>0.46361</td>
<td>12%</td>
</tr>
</tbody>
</table>
2.4.4 TEOS-coated SiQDs

Although QDs are stable inside of the mesoporous silica beads, they should still be protected further to ensure minimal leakage of the QDs from the pores. The SiQDs must also be functionalized for bioconjugation. One approach is to coat the SiQDs with tetraethyl orthosilicate (TEOS). TEOS coats SiQDs through a sol-gel process. The SiQDs are dispersed in ethanol and various amounts of TEOS are added with water. TEOS hydrolyzes in the presence of water and condenses on the SiO$_2$ hydroxyl groups. A Si-O-Si siloxane bond is formed between the mesoporous silica surface and the TEOS. The concentration of TEOS determines the thickness of this silica layer. Coating with TEOS further increases stability of the particles, increases functionalization strategies, and enables solubility in aqueous solutions. Fluorescence microscopy and emission spectra reveal that increasing the amount of TEOS on the SiQD surface causes a blue shift of the emission wavelength. This is advantageous for barcoding because more spectral codes can be produced without the need for multiple quantum dots of different sizes.

![Figure 2.6 SiQD with (a) no TEOS, (b) 25 µL TEOS, (c) 50 µL TEOS, (d) 100 µL TEOS, (e) 200 µL TEOS.](image)
2.4.5 Fluorescence Emission and DLS Measurements of Water-Soluble Thiol and Amine-Modified SiQD Composite Particles

Mesoporous silica quantum dot composite particles were further functionalized with thiol (-SH) and amine (-NH₂) groups by the addition of 3-mercaptopropyltrimethoxysilane (MPTMS) and (3-aminopropyl)triethoxysilane (APTES), respectively. Primary amine groups are hydrophilic and positively charged at neutral pH. The presence of primary amines on the SiQDs surface will enhance water solubility and initialize them for bioconjugation to antibodies or other biological protein molecules. Thiol groups are well known for their coupling strategies to biological molecules, typically by binding to maleimide. To promote water solubility, N-(trimethoxysilylpropyl)ethylenediaminetriacetae trisodium salt was added in addition to MPTPS. It is a water soluble silane triol that drives SiQD-SH composites to stability in water. Fluorescence images and emission spectra were acquired (Figure 2.8). The SiQDs are well dispersed in water and the images portray little to no aggregation of the composite particles (Figure 2.9).
Dynamic light scattering (DLS) measurements were obtained for comparison of the SiQD composites. Each of the samples is relatively stable in solution based on the zeta potential. Typically, the larger the zeta potential the more stable the solution. Based on the polydispersity index, however, the samples are not considered very monodisperse; a PDI above 0.1 is considered to be moderately dispersed. Nonetheless, the final SiQD composites are soluble in water, easily functionalized, and have the potential to be a fluorescent biosensor in immunossays.
Table 2.2 Dynamic Light Scattering (DLS) data of SiQD composites.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Diameter (µm)</th>
<th>Polydispersity Index</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiQD</td>
<td>4.503</td>
<td>0.18</td>
<td>-24.32</td>
</tr>
<tr>
<td>SiQD-TEOS</td>
<td>4.512</td>
<td>0.19</td>
<td>-28.84</td>
</tr>
<tr>
<td>SiQD-NH₂</td>
<td>4.679</td>
<td>0.15</td>
<td>27.12</td>
</tr>
<tr>
<td>SiQD-SH</td>
<td>4.391</td>
<td>0.11</td>
<td>-25.96</td>
</tr>
</tbody>
</table>

2.5 Conclusions

The goal of this project was to prepare biocompatible mesoporous silica quantum dot composites to be used as detection probes for immunoassay development. Quantum dots are highly photostable nanocrystals with exceptional brightness, and they have the extraordinary ability to multiplex. Only one excitation source is needed to excite quantum dots of different sizes and emission wavelengths. Detection probes used for immunoassays should be soluble in water and assay buffers. Water solubility is usually promoted through a surface ligand exchange, however, this can decrease brightness and reduce quantum yield. Encapsulation is a more reliable route that retains quantum yield and does not diminish the inherent properties of the quantum dots.

Quantum dots of different emission wavelengths were successfully incorporated into mesoporous silica producing a set of spectral codes for use in a multiplex immunoassay. The quantum dots are stable inside of the silica due to strong hydrophobic interactions between the hydrocarbon chain and TOPO capping ligand. Multiple quantum dots of different sizes and different emission wavelengths can be incorporated into one silica composite particle and exhibit dual fluorescence emission. This greatly extends the amount of spectral coding available, which allows for several analytes to be detected simultaneously. In addition to dual emission silica quantum dot composites, adding tetraethyl orthosilicate to the composites causes a blue shift in
fluorescence emission. Silica quantum dot composites of different emission wavelengths can be prepared without the need for a multitude of quantum dots varying in size. Functionalization of the silica composites with thiol or amine groups enables them for bioconjugation with antibodies for use in an immunoassay. The functionalized quantum dot silica particles were soluble in water without the need for a ligand exchange of the quantum dots. These functionalized silica composite particles are the ideal candidate to replace fluorescent organic dyes as biosensing detection probes.

2.6 References


Chapter 3

Multiplex Detection of Atherosclerosis Biomarkers Using a Mesoporous Silica Quantum Dot Based Immunoassay

3.1 Abstract

Cardiovascular disease (CVD) is the leading cause of death in the U.S. for both men and women. CVD is mainly caused by hypertension and atherosclerosis. Atherosclerosis is the hardening and narrowing of arteries due to plaque buildup from fat and cholesterol accumulation. Plaque rupture can result in heart attack or stroke. Imaging techniques such as X-ray contrast angiography, magnetic resonance imaging (MRI), computed tomography (CT), and positron emission tomography (PET) have all been used for visualizing plaques, but they do not give information about inflammation suggestive of vulnerable plaques. Research has shown the presence of biomarkers at different stages of atherosclerosis. Specifically, monocyte chemoattractant protein-1 (MCP-1) and interleukin 15 (IL-15) have been identified as early markers indicative of atherosclerotic susceptibility. Detection of these biomarkers can provide early diagnosis and information for future incidence of the disease.

MCP-1 and IL-15 biomarkers were detected utilizing a quantum dot based fluorescence immunoassay. Quantum dot mesoporous silica bioconjugates were prepared by encapsulating CdSe/ZnS QDs into mesoporous silica and labeling them with antibodies. The fluorescence immunoassay was developed to detect both MCP-1 and IL-15 simultaneously. Simultaneous detection of biomarkers allows for more rapid diagnoses and can improve disease prognosis.
Antibodies were immobilized to a well plate to capture the specific biomarkers. Quantum dot-antibody labeled silica bioconjugates were bound to specific biomarkers and the fluorescence emission was measured. Multiplex immunoassays have great promise as a diagnostic tool for atherosclerosis.
3.2 Introduction

Cardiovascular disease (CVD) is the leading cause of death for men and women in the United States accounting for about 1 of every 3 deaths.\textsuperscript{1-4} Over 92 million Americans are living with some form of CVD, and related costs are estimated to be more than $316 billion for 2017.\textsuperscript{2} Plaque builds up in the arteries that supply oxygen-rich blood to the heart; the hardening of the plaque and narrowing of the arteries is known as atherosclerosis.\textsuperscript{5-6} Atherosclerosis can lead to heart attack or stroke.\textsuperscript{1,6} Plaque is composed of fat, cholesterol, calcium, and other substances in the blood. The hardening and narrowing of the arteries limits blood flow to the heart causing angina. Plaque rupture can form acute blood clots, which block blood flow through an artery leading to heart attack or stroke.\textsuperscript{1} Atherosclerotic plaques are either stable or susceptible, and susceptible plaques are prone to rupture.\textsuperscript{1} Susceptible plaques have a large lipid-rich core, a thin fibrous cap, and increased plaque inflammation.\textsuperscript{7} Hardened and narrowed arteries lead to heart attack, but researchers are realizing that vulnerable plaques are not always present on the artery wall causing blockage.\textsuperscript{1,4} Many heart attacks occur due to vulnerable atherosclerotic plaques inside the artery wall because of large degrees of inflammation.\textsuperscript{1,6} Fatty low density lipoprotein (LDL) particles are taken into the arterial cells, which release cytokines that lead to inflammation.\textsuperscript{4,6-7} The cytokines affect the surface of the artery wall which attracts monocytes that turn into macrophages. Macrophages engulf oxidized LDL particles, leading to fatty deposits.\textsuperscript{6,8} Over time, these deposits eventually form plaque within the artery walls.\textsuperscript{1} Bodily stresses can cause the thin covering to open and release the contents of the plaque into the bloodstream, and the cytokines capture platelets, rapidly forming a blood clot. Such clots can substantially or completely block blood flow, causing the surrounding tissue to become oxygen
starved. Heart muscle without sufficient oxygen supply cannot contract, resulting in a heart attack. Blockage in a brain artery can lead to death of brain cells (stroke).

Current atherosclerosis diagnostic methods include blood tests for abnormal levels of fats, cholesterol, sugars, and proteins in the blood.\textsuperscript{2, 8} Electrocardiograms (EKG) are also commonly used to monitor the heart’s electrical activity; however, an EKG typically shows signs of heart damage rather than providing information for future incidence of CVD. Chest x-rays, echocardiograms, computed tomography (CT) scans, angiographies, and stress tests are also used for diagnosis, but none of these methods provides early detection.\textsuperscript{2} These methods are used to confirm that atherosclerotic plaques are already present in the coronary arteries. Magnetic resonance imaging (MRI) and positron emission tomography (PET) are being explored to give more detailed information about the nature of the plaque buildup.

Early diagnosis of atherosclerotic plaque formation could lead to early treatment and improved prognosis for CVD. Several biomarkers have recently been identified as indicative of atherosclerotic plaques at various stages of plaque development.\textsuperscript{8-9} The biomarkers are present from development of precursor legions or fatty streaks to complete lipid-rich plaques.\textsuperscript{6, 8, 10} Cytokine interleukin 15 (IL-15) and chemokine monocyte chemoattractant protein-1 (MCP-1) have been recognized as two fundamental atherosclerotic biomarkers.\textsuperscript{9-12} IL-15 is a proinflammatory cytokine expressed in immune cells and atherosclerotic lesions and has shown involvement in the increase of macrophage content of plaques.\textsuperscript{3, 10, 13} MCP-1 is a chemokine that rises in macrophage-rich regions of atherosclerotic regions.\textsuperscript{9, 12, 14} Chemokines are released in response to signals from proinflammatory cytokines such as IL-15.\textsuperscript{11}
A quantum dot based fluorescence immunoassay was developed for detection of IL-15 and MCP-1 atherosclerosis biomarkers. Immunoassays are commonly used to measure and quantify the amount or presence of a given molecule primarily through antibody-antigen binding.\textsuperscript{15-17} Developing quantum dots in an immunoassay allow for simultaneous detection or multiplexing of multiple species.\textsuperscript{17-20} Detection antibodies labeled with quantum dots will exhibit fluorescence when the corresponding antigen or protein is present in the sample.\textsuperscript{21-22} Biomarkers can easily be identified through this immunoassay, and this capability could lead to earlier treatment options for those at risk for atherosclerosis development.

Previously, a quantum dot based immunoassay was proposed for detection of atherosclerosis biomarkers by directly conjugating quantum dots to the atherosclerosis antibodies. A ligand exchange was performed to replace the TOPO capping ligand of CdSe/ZnS quantum dots with 16-mercaptohexadecanoic acid (MHDA). This exchange permitted water solubility, and the quantum dots were covalently linked to the antibodies through EDC coupling. The immunoassay successfully detected two biomarkers, but it did not improve on current detection limits. Encapsulating quantum dots into mesoporous silica and modifications to the immunoassay show potential to improve sensitivity.

3.3 Experimental Methods

3.3.1 Materials and Reagents.

All chemicals were used without modification unless otherwise noted. Cadmium oxide (99.99\%), lauric acid (98\%), technical grade (90\%) trioctylphosphine oxide (TOPO), technical grade (90\%) hexadecylamine (HDA), technical grade (90\%) trioctylphosphine (TOP), selenium powder (99.5\%), diethyl zinc solution 1.0 M in heptane, hexamethyldisilathiane, anhydrous methanol
(99.8%), chloroform (99.5%), tetraethyl orthosilicate (TEOS), (3-aminopropyl)triethoxysilane (APTES), and anhydrous dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Mesoporous silica particles (5 µm in diameter) with 32 nm C\textsubscript{18}-modified pores were obtained from Phenomenex (Torrance, CA). Ethanol, 200-proof, ACS/USP grade was purchased from Pharmco-AAPER (Brookfield, CT). Ethylene glycolbis(succinimidylsuccinate) (EGS, 99±%) was purchased from ProteoChem (Loves Park, IL). Phosphate buffered saline (PBS) was obtained from Calbiochem (Billerica, MA). Mouse monoclonal IL-15 antibody, rabbit polyclonal IL-15 antibody, IL-15 protein, mouse monoclonal MCP-1 antibody, rabbit polyclonal MCP-1 antibody, and MCP-1 protein were purchased from Abcam (Cambridge, MA). Amine reactive maleic anhydride activated 96 well plates, sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (Sulfo-SMCC), and Super Block T20 PBS blocking buffer were purchased from ThermoFisher Scientific (Waltham, MA). N-(trimethoxysilylpropyl)ethylenediaminetriacetate, trisodium salt (35% in water) was purchased from Gelest. Nanopure deionized and distilled water (18.2 MΩ) purified with a Barnstead Nanopure system was used for all experiments.

### 3.3.2 Synthesis of TOPO-coated luminescent quantum dots

TOPO coated CdSe/ZnS quantum dots were prepared using a slightly modified version of a commonly used method developed by Peng.\textsuperscript{23-24} Briefly, 12-13 mg of cadmium oxide (CdO) and 180 mg of lauric acid were mixed in a three-neck flask under a nitrogen atmosphere. The mixture was heated to 180-200 °C until the CdO fully dissolved in the lauric acid forming a clear, colorless solution. Once fully dissolved, 1.94 g of trioctylphosphine oxide (TOPO) and 1.94 g of hexadecylamine (HDA) were added to the solution under constant stirring. The temperature was then increased to 280 °C for 10 minutes before being cooled slowly.
Meanwhile, a solution of 80 mg of selenium powder was dissolved in 2 mL of trioctylphosphine (TOP). This solution was rapidly injected into the mixture under vigorous stirring upon reaching the desired temperature during the cooling process. Size-tunable luminescent quantum dots are directly dependent on temperature. The temperature at which the selenium component is injected determines the size of the quantum dots synthesized, corresponding to their emission wavelength. Typically, the temperature is slowly decreased from 280 °C to 180 °C. The higher the temperature upon injection, the larger the quantum dots will be in size. A precursor solution for a ZnS shell was prepared: 2 mL of TOP containing 1 mL of diethyl zinc (Zn(Et$_2$)) and 250 µL of hexamethyldisilathiane ((TMS)$_2$S). This mixture was added dropwise into the solution after injection of the selenium. The resulting reaction mixture was kept at 180 °C for 1 h before cooling to room temperature. The resulting CdSe/ZnS quantum dots were washed 3 times with methanol by centrifugation at 4000 rpm for 10 minutes and redispersed in chloroform. The quantum dots were stored at room temperature in the dark.

3.3.3 Preparation of Fluorescent Mesoporous Silica Beads

Briefly, 4 mg of mesoporous silica particles averaging 3, 5, or 10 µm in diameter with 32 nm C$_{18}$-modified pores were dispersed in 2 mL of butanol. Two hundred microliters of TOPO-CdSe/ZnS quantum dots were added to the solution under constant stirring. This mixture was incubated for 1 hour and washed 3 times with ethanol by centrifugation at 11,000 rpm for 20 minutes. The fluorescent silica microbeads were visualized using an Olympus IX-71 inverted fluorescence microscope and stored at 4 °C.
3.3.4 Preparation of TEOS-capped fluorescent mesoporous silica beads

Silica coated fluorescent mesoporous silica beads were prepared by dispersing 2 mg of mesoporous fluorescent silica particles in 2 mL of ethanol and 500 µL of nanopure water. Upon stirring, 50 µL of tetraethyl orthosilicate (TEOS) was added. The mixture was incubated overnight, centrifuged for 20 minutes at 11,000 rpm and washed with ethanol. The resulting TEOS-capped fluorescent mesoporous silica beads were resuspended in nanopure water and stored at 4 °C.

3.3.5 Amine-Modified SiQDs

TEOS capped mesoporous silica beads were further modified by the addition of an amine group to the surface of the particles. Briefly, 2 mg of TEOS-capped fluorescent mesoporous silica beads were dispersed in 2 mL of ethanol. Under constant stirring, 50 µL of (3-aminopropyl)triethoxysilane (APTES) was added dropwise to the mixture and incubated overnight. The mixture was centrifuged for 20 minutes at 11,000 rpm and washed with ethanol. The amine-modified particles were resuspended in nanopure water via ultrasonication and stored at 4 °C until further use.

3.3.5 Thiol-Modified SiQDs

Mesoporous silica beads were also modified with a thiol group for bioconjugation. Quantum dot loaded mesoporous silica beads were dispersed in 1 mL of propanol and 1 mL of water. To this solution 100 µL of 3-mercaptopropyltrimethoxysilane (MPTMS) was added while stirring. This mixture was incubated overnight and was then washed 3 times with ethanol for 20 min at 11,000 rpm. The thiol-capped mesoporous silica beads were resuspended in 1 mL of water and 1 mL of propanol and 50 µL of N-(trimethoxysilylpropyl)ethylenediaminetriacetate
was added dropwise. The mixture was stirred overnight and was then washed 3 times with nanopure water. The resulting particles were dispersed in nanopure water and used immediately for bioconjugation.

3.3.6 Crosslinking of SiQDs to IL-15 and MCP-1 Antibodies

Amine modified fluorescent mesoporous silica composites were conjugated to atherosclerosis biomarkers via amine-to-amine crosslinking. The composites were air dried, and 2 mg were dispersed in 1 mL of nanopure water. A solution of ethylene glycolbis(succinimidylsuccinate) (EGS) in anhydrous dimethyl sulfoxide (DMSO) was prepared. An aliquot of IL-15 or MCP-1 polyclonal detection antibodies was diluted to 10 µg/mL in 500 µL of phosphate buffered saline (PBS). To the antibody solutions, 500 µL of SiQD-NH₂ composites was added, and 1 µL of EGS in DMSO was added to the mixture. The mixture was incubated at room temperature for 20 minutes and washed with nanopure water. The antibody-bead bioconjugates were redispersed in PBS buffer pH 7.4 and used immediately for fluorescence immunoassay.

Thiol-modified fluorescent mesoporous silica composites were conjugated to atherosclerosis biomarkers via thiol-to-amine crosslinking. SiQD-SH composites were dispersed in 1 mL of nanopure water. A solution of sulfosuccinimidyl-4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC) in water was prepared. An aliquot of IL-15 or MCP-1 polyclonal detection antibodies was diluted to 10 µg/mL in 500 µL of PBS. The sulfo-SMCC was added to the antibody solutions and incubated for 30 min at room temperature. Excess crosslinker was removed using a Zeba spin desalting column. Maleimide-activated antibodies were combined with SiQD-SH composites, incubated for 30 min at room
temperature, and washed with nanopure water. Resulting antibody labeled composites were redispersed in PBS pH 7.4 and used immediately for fluorescence immunoassays.

3.3.7 Individual IL-15 and MCP-1 Fluorescent Immunoassays

Amine-binding, clear microtiter well plates were washed three times with 200 µL of wash buffer (PBS pH 8.0 buffer containing 0.05% Tween 20). After removing the wash buffer, 100 µL of 25 µg/mL of IL-15 or MCP-1 monoclonal antibodies were added to the wells and incubated overnight at room temperature. The excess capture antibodies were removed from the wells and 200 µL of SuperBlock blocking buffer was added. The blocking buffer quenches the remaining active sites on the well surface. After 1 hour the blocking buffer was removed and the wells were washed three times with wash buffer. Varying concentrations of MCP-1 or IL-15 antigens were added to the wells to bind to the capture antibodies. The well plate was incubated at room temperature for 1 hour and washed three times with wash buffer. The detection antibody-fluorescent bead conjugates were added to the wells, and the plate was incubated at room temperature for 1 hour. Finally, the detection antibody-bead solutions were removed and the plate wells were washed with wash buffer three times (Scheme 3.1).
Scheme 3.1 Schematic representation of quantum dot fluorescence immunoassays.

3.3.8 Multiplex Fluorescence Immunoassays of IL-15 and MCP-1

Fluorescent immunoassays simultaneously detecting IL-15 and MCP-1 were performed similarly to individual immunoassays. Amine-binding clear microtiter well plates were washed three times with 200 µL of wash buffer. Upon removing the wash buffer, 50 µL of IL-15 antibodies and 50 µL MCP-1 antibodies at 25 µg/mL in PBS buffer pH 7.4 were added to the well plate and incubated overnight at room temperature. The capture antibody solutions were removed and the wells were blocked by adding 200 µL of SuperBlock blocking buffer. The plate was incubated with blocking buffer for 1 hour at room temperature. The blocking buffer was removed and varying concentrations of IL-15 and MCP-1 antigens were added to the well plate and incubated for 1 hour at room temperature. The plate was washed 3 times with wash buffer,
and 50 µL of IL-15 antibody-bead bioconjugates as well as 50 µL of MCP-1 antibody-bead bioconjugates were added to the wells. The antibody-bead bioconjugates were labeled with mesoporous silica beads containing quantum dots of different sizes, thereby each having a different fluorescence emission maximum. The well plate was incubated for 1 hour at room temperature and washed three times with wash buffer.

3.3.9 Fluorescence Spectroscopy Measurements

Fluorescence emission spectra were obtained using a SpectraMax M2 spectrofluorometer microplate reader (Molecular Devices, Inc.) equipped with a 75 W continuous Xenon flash arc lamp as a light source and a photomultiplier (R-3896) detector. The excitation wavelength for all samples was 400 nm. Fluorescence emission was measured from 500-750 nm.

3.3.10 Digital Fluorescence Imaging Microscopy

Fluorescence images were taken using a digital fluorescence imaging microscopy systems containing an Olympus IX-71 inverted fluorescence microscope equipped with a 100 W mercury lamp as a light source and a high performance color charge coupled device (CCD) camera (Olympus DP 70). Images were obtained through 10x, 20x, and 40x microscope objectives with numerical aperture = 0.5. A filter cube with a 425 ± 20 nm band-pass excitation filter, a 465 nm dichroic mirror, and a 475 nm long pass emission filter was used for spectral imaging. Image analysis was done using DP Controller software. An exposure time of 10 ms was typically used for image acquisition.

3.4 Results and Discussion
3.4.1 Direct Detection Assay of IL-15 and MCP-1 Biomarkers

IL-15 and MCP-1 atherosclerosis biomarkers were identified through a quantum dot bioconjugate based fluorescence immunoassay. The reported immunoassay detection limits for IL-15 and MCP-1 are 250 pg/mL and 0.2 ng/mL, respectively. The detection of the biomarkers was first measured through comparison of a direct detection assay and a sandwich detection assay. Direct detection assays do not use capture antibodies, the antigen is non-specifically bound directly to the surface of a 96-well plate, and detection antibodies labeled with quantum dots are added to bind to the antigens. Sandwich detection assays first bind a capture antibody to the surface of the well plate in order to capture more of the antigen protein and increase assay sensitivity (Scheme 3.1). Detection of 5 ng/mL IL-15 (Figure 3.1) and 50 ng/mL MCP-1 (Figure 3.2) were compared using a direct detection and sandwich detection immunoassay. For the sandwich immunoassays, a 100 µL solution of 25 µg/mL of monoclonal antibodies IL-15 and MCP-1 was used as capture antibodies. The fluorescence emission intensity of the sandwich detection assay is 4 times higher for each biomarker indicating that it is more sensitive than the direct detection assay. The direct assay was unable to detect biomarker concentrations at the current detection limits (data not shown). The biomarkers were nonspecifically adsorbed to the well plate surface in the direct assay. Utilization of a sandwich immunoassay provides a more sensitive means for detection of IL-15 and MCP-1 biomarkers.
Figure 3.1 Fluorescence microscopy imaging of 5 ng/mL of IL-15 from a (a) direct detection assay and (b) sandwich detection assay. The individual spots are the QD-antibody bioconjugates bound to the antigen present in the well.

Figure 3.2 Fluorescence emission of 5 ng/mL IL-15 protein using a direct detection and sandwich detection immunoassay.
Figure 3.3 Fluorescence microscopy imaging of MCP-1 using a (a) direct detection assay and (b) sandwich detection assay. The individual spots are the QD-antibody bioconjugates bound to the antigen present in the well.

![Fluorescence microscopy imaging of MCP-1](image)

Figure 3.4 Fluorescence emission of 50 ng/mL MCP-1 protein using a direct detection and sandwich detection fluorescence immunoassay.

3.4.2 Optimization of Capture Antibody Concentration

Capture antibody concentration is one of the most important parameters for immunoassay development because it determines the amount of antibody that adsorbs onto the well plate.
surface. Typically, monoclonal antibodies are used as the capture antibody in a sandwich assay complex to ensure specificity during the binding of the analyte. Monoclonal antibodies have an inherent monovalent affinity toward a single epitope that allows quantification of small differences in an antigen. Detection antibodies are usually polyclonal and bind multiple epitopes of a target antigen. This forms the “sandwich” with the antigen between two antibodies.

Capture antibody concentrations for each biomarker were tested between 0 and 50 µg/mL. The same amount of antigen was used for each assay to test the efficiency of the capture antibody immobilization, 5 ng/mL IL-15 and 50 ng/mL MCP-1 were used for investigation. The fluorescence intensity increased for both biomarkers from 0 to 25 µg/mL capture antibody concentration. Increasing to 50 µg/mL displayed no increase in fluorescence intensity indicating maximum loading efficiency at 25 µg/mL.

![Graph of fluorescence intensity vs. capture antibody concentration](image)

**Figure 3.5** Optimization of capture antibodies at 5 ng/mL of IL-15 (orange) and MCP-1 (green) proteins. $\lambda_{ex} = 400$ nm.
3.4.3 Individual Fluorescence Immunoassays of IL-15

Antibody-labeled-bioconjugates are key components to assay optimization. Mesoporous silica quantum dot composites were functionalized with thiols (-SH) and amines (-NH₂) to explore which crosslinking technique would be most suitable for quantum dot fluorescence immunoassays. Thiol groups were introduced to the SiQDs by the addition of (3-mercaptopropyl)trimethoxysilane (MPTMS) and the resulting SiQD-SH composites were labeled with detection antibodies via a sulfosuccinimidyl 4-[N-maleimidomethyl]cylcohexane-1-carboxylate (sulfo-SMCC) crosslinker (Scheme 3.2). Sulfo-SMCC is a heterobifunctional crosslinker containing a maleimide group and an N-hydroxysuccinimide (NHS) ester group that promotes covalent coupling of molecules containing amines and sulfhydryls. The maleimide group reacts with the thiol groups on the SiQD-SH surface forming stable thioether bonds, and the NHS ester on the opposite side of the crosslinker forms an amide bond with the primary amines of the antibodies.
Scheme 3.2 Reaction scheme for conjugating antibodies with SiQD-SH with Sulfo-SMCC. Silica spheres are represented by spheres with green dots (not to scale).

Individual fluorescence immunoassays were done using antibody labeled SiQD-SH composites. IL-15 detection antibodies were conjugated to SiQDs containing QDs ($\lambda_{EM} = 600$ nm), and MCP-1 detection antibodies were conjugated to SiQDs containing QDs ($\lambda_{EM} = 560$ nm). The capture antibodies were immobilized to the well plate and blocked with SuperBlock blocking buffer. The blocking buffer quenches any remaining active sites on the well plate surface. IL-15 antigens were added followed by the SiQD-antibody bioconjugates. The fluorescence intensity increased with increasing concentrations of IL-15 from 1 pg/mL to 5 ng/mL (Figure 3.4). The individual wells were visualized using a fluorescence microscope, and
the images show an increasing number of particles as the concentration of IL-15 increases. There were no particles attached to the wells at 0 µg/mL of IL-15. If there is no antigen present to bind to the immobilized capture antibodies then the fluorescent detection antibody conjugates will have nothing to bind to and should not exhibit any fluorescence. Additionally, MCP-1 antigens were added to wells prepared with IL-15 capture antibodies, and there was no fluorescence detected. IL-15 detection antibodies are not specific to MCP-1 and will not bind to them.

Figure 3.6 Fluorescence microscopy imaging of IL-15: a) 5 ng/mL IL-15 with MCP-1 labeled SiQDs, b) 5 ng/mL MCP-1 antigen, c) 0 µg/mL, d) 1 pg/mL, e) 5 pg/mL, f) 50 pg/mL, g) 0.5 ng/mL, and h) 5 ng/mL.
A similar assay was performed to quantify MCP-1 antigens using SiQD-SH detection antibody bioconjugates. MCP-1 capture antibodies were adsorbed to the well plate and blocked with blocking buffer. Different dilutions of MCP-1 antigens were added to the wells to bind to the capture antibodies. The detection antibodies bioconjugates were added to the wells and the fluorescence intensity was measured. Fluorescence intensity increased from 50 pg/mL to 50 ng/mL of the MCP-1 antigen (Figure 3.5). Microscopy images confirm the increasing number of particles bound to the well plate. Again, no particles were attached to the plate where no antigen was present or where IL-15 antigens were added. The IL-15 antigens will not attach to the MCP-1 capture antibodies nor will MCP-1 detection antibodies bind to any MCP-1 that may have non-specifically adsorbed to the well plate. Results indicate that the sandwich immunoassay is sensitive and specific which is ideal for diagnostic applications.
Figure 3.8 Fluorescence microscopy imaging of MCP-1: a) 5 ng/mL MCP-1 with IL-15 labeled SiQDs, b) 5 ng/mL IL-15 antigen, c) 0 µg/mL, d) 50 pg/mL, e) 0.5 ng/mL, f) 5 ng/mL, and g) 50 ng/mL.
Figure 3.9 Fluorescence emission spectra of MCP-1.

The individual immunoassays were also performed using detection antibodies conjugated to amine-modified SiQDs. Antibodies were coupled to SiQDs via amine-to-amine crosslinking using ethylene glycolbis[succinimidyIsuccinate] (EGS). Unlike sulfo-SMCC, EGS is a homobifunctional crosslinker containing two NHS ester reactive groups. Each NHS ester will bind to an amine group on the antibody as well as to an amine group on the SiQD (Scheme 3.3). NHS esters react with ε-amine groups present on lysine residues of antibodies as well as α-amine groups forming covalent amide bonds. N-hydroxysuccinimide is released during the reaction performed at neutral pH. NHS esters readily hydrolyze especially with increasing pH, so antibodies coupled via EGS should be used immediately for affinity immunoassays. IL-15 detection antibodies were coupled to SiQDs containing 600 nm QDs, and MCP-1 detection antibodies were coupled to SiQDs containing 560 nm QDs. The fluorescence intensity of IL-15 increased from 1 pg/mL to 5 ng/mL (Figure 3.6), and the fluorescence intensity of MCP-1 increased from 50 pg/mL to 50 ng/mL (Figure 3.7). Results were consistent with individual assays performed using antibody labeled SiQDs coupled using sulfo-SMCC.
Scheme 3.3 Reaction scheme for conjugating antibodies with SiQD-NH$_2$ composites with EGS. Silica spheres are represented by spheres with green dots (not to scale).
Figure 3.10 Fluorescence microscopy imaging of IL-15: a) 5 ng/mL IL-15 with MCP-1 labeled SiQDs, b) 5 ng/mL MCP-1 antigen, c) 0 µg/mL, d) 1 pg/mL, e) 5 pg/mL, f) 50 pg/mL, g) 0.5 ng/mL, and h) 5 ng/mL.

Figure 3.11 Fluorescence emission spectra of IL-15.
Figure 3.12 Fluorescence microscopy imaging of MCP-1: a) 5 ng/mL MCP-1 with IL-15 labeled SiQDs, b) 5 ng/mL IL-15 antigen, c) 0 µg/mL, d) 50 pg/mL, e) 0.5 ng/mL, f) 5 ng/mL, and g) 50 ng/mL.
3.4.5 Multiplex Immunoassay of IL-15 and MCP-1

IL-15 and MCP-1 biomarkers were detected simultaneously through a multiplex quantum dot fluorescence immunoassay. A mixture of IL-15 and MCP-1 capture antibodies at 25 µg/mL was added to the surface of the well plate. In this manner, the plate was then activated for capture of both antigens. Different combinations of IL-15 and MCP-1 biomarkers were added to bind to the capture antibodies. Antibody-labeled silica composite particles were added, and the well plate was analyzed. SiQDs labeled with IL-15 detection antibodies contained 600 nm emission quantum dots, and SiQDs labeled with MCP-1 detection antibodies contained 560 nm emission quantum dots. Both biomarkers were able detected simultaneously below the current limits of detection. Microscopy imaging clearly shows the presence of both biomarkers through the different colored particles bound to the well plate surface (Figure 3.14). The fluorescence emission spectra clearly show two peaks, one corresponding to each biomarker (Figure 3.15). Simultaneous detection of both atherosclerotic biomarkers using a sensitive sandwich immunoassay can allow for earlier and more rapid diagnostic and treatment assessments.
3.5 Conclusions

The goal of this study was to develop a quantum dot based fluorescence immunoassay using mesoporous silica quantum dot composite particles as the detection antibody label in order to detect atherosclerosis biomarkers IL-15 and MCP-1. Two different conjugation methods for coupling the detection antibodies to the SiQDs were explored. SiQDs were functionalized with
thiol and amine groups to enable them for bioconjugation to the antibodies. Coupling of the SiQD composites was achieved through crosslinking agents, sulfo-SMCC and EGS for the thiol and amine groups, respectively. Both conjugation methods yielded similar detection of the biomarkers. A direct detection assay was initially performed for comparison with a sandwich immunoassay. The direct assays for IL-15 and MCP-1 were unable to improve detection of the biomarkers. Direct detection assays nonspecifically adsorb the antigen directly to the surface of the well plate, and by adding a capture antibody, more of the antigen is bound, thereby improving assay sensitivity. Individual fluorescence sandwich immunoassays were performed, and detection limits of 1 pg/mL of IL-15 and 50 pg/mL of MCP-1 were achieved. These are an improvement from currently reported detection of IL-15 and MCP-1 biomarkers. Multiplex detection of the biomarkers was also attempted. Initial results show that both IL-15 and MCP-1 can be detected simultaneously using the quantum dot based fluorescence immunoassay, however, more optimization needs to be explored in order to reproducibly quantify the biomarker concentrations.

3.6 References


Chapter 4

Synthesis of Quantum Dot Encapsulated Human Serum Albumin Nanocomposites

4.1 Abstract

Human serum albumin nanoparticles (HSAPs) have been utilized for encapsulation of a variety of materials due to the versatile and unique ligand binding properties of HSA. A wide array of compounds can functionalize HSAPs for multimodal use. Drugs are commonly loaded into HSAPs for targeted drug delivery treatments. Organic fluorophores have also been used in HSAPs for imaging and tracking purposes. Quantum dots (QDs) exhibit many advantages over organic fluorophores and may be better tools for diagnostic imaging. QDs were loaded into HSAPs for exploration of immunoassay development. CdSe/ZnS QDs were added to a stock solution of HSA followed by ethanolic desolvation. Crosslinking of the nanoparticles was initiated by glutaraldehyde. The resulting QD-loaded human serum albumin nanoparticles (HSA-QDs) were employed in a fluorescence immunoassay to detect atherosclerosis biomarkers IL-15 and MCP-1.

HSA-QDs were conjugated to IL-15 and MCP-1 antibodies through EDC/NHS coupling which forms amide bonds between carboxylic acids and primary amine groups. The atherosclerosis biomarkers were detected individually, however, the detection limits did not improve based on previous immunoassays.
4.2 Introduction

Human serum albumin (HSA) is the most abundant protein in blood plasma and has unique ligand binding capabilities making it ideal for many bioapplications. HSA is biocompatible, biodegradable, and can conjugate drugs or other substances through attachment to functional groups. Formation of human serum albumin nanoparticles (HSAPs) through desolvation and crosslinking creates functional nanocarriers for encapsulating a multitude of molecules. Encapsulation of drug molecules into HSAPs has been shown to significantly improve drug delivery in vivo. Chemotherapeutic drug agents are often highly toxic, water insoluble, and unable to penetrate the blood brain barrier. Loading these drugs into HSAPs enables efficient transport directed to tumor sites because of how readily HSAPs are tolerated by the human body. HSAPs also offer precise control over release of chemotherapeutic agents making them ideal nanocarriers for disease treatment assessments and in clinical settings.

Fluorescent organic dyes have been employed as trackers in HSAPs to image cellular labeling and to mimic drug release for targeted delivery studies. Encapsulation of organic and inorganic materials into HSAPs through covalent bonding has shown to be robust and stable. Loading quantum dots into HSAPs could further enhance their utility range by extending their reach to applications where fluorescent dyes are limited. Fluorescent organic molecules are susceptible to photobleaching and have a short fluorescence lifetime. Incorporating quantum dots (QDs) into HSAPs may enhance their brightness and enable them as useful fluorescent probes. QDs are stable, have a long fluorescence lifetime, and resistant to photobleaching relative to organic dyes.
Imunoassay performance is measured by specificity, sensitivity, and reproducibility. Development of reliable immunoassays requires optimization of the influential factors that affect performance and consistency. The detection probes used to quantify analytes in immunoassays are crucial to improving sensitivity and detection limits. \(^{17, 21-22}\) HSAPs are highly biocompatible and easily functionalized due to the numerous charged amino acids on the protein surface. \(^{1, 8, 23}\) Their high specificity could enable them to be new and efficient tools for quantitative analysis. Early biomarker detection is critical for diagnostic assessment to improve disease prognosis. Encapsulating QDs into HSAPs will permit multiplexing which allows for simultaneous detection of antigens and can improve diagnostic capabilities.

### 4.3 Experimental Methods

#### 4.3.1 Materials and Reagents

All chemicals were used as received without modification unless otherwise noted. Cadmium oxide (99.99%), lauric acid (98%), technical grade (90%) trioctylphosphine oxide (TOPO), technical grade (90%) hexadecylamine (HDA), technical grade (90%) trioctylphosphine (TOP), selenium powder (99.5%), diethyl zinc solution 1.0 M in heptane, hexamethyldisilathiane, anhydrous methanol (99.8%), chloroform (99.5%), human serum albumin, lyophilized powder (≥ 97%), aqueous glutaraldehyde (8%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and sulfo-N-hydroxysulfosuccunimide (sulfo-NHS) were purchased from Sigma-Aldrich (St. Louis, MO). Ethanol, 200-proof, ACS/USP grade was purchased from Pharmco-AAPER (Brookfield, CT). Phosphate buffered saline (PBS) was obtained from Calbiochem (Billerica, MA). Mouse monoclonal IL-15 antibody, rabbit polyclonal IL-15 antibody, IL-15 protein, mouse monoclonal
MCP-1 antibody, rabbit polyclonal MCP-1 antibody, and MCP-1 protein were purchased from Abcam (Cambridge, MA). Amine reactive maleic anhydride activated 96 well plates and Super Block T20 PBS blocking buffer were purchased from ThermoFisher Scientific (Waltham, MA). Nanopure deionized and distilled water (18.2 MΩ) purified with a Barnstead Nanopure system was used for all experiments.

4.3.2 Synthesis of TOPO-capped CdSe/ZnS Quantum Dots

TOPO coated CdSe/ZnS quantum dots were prepared using a slightly modified version of a commonly used method developed by Peng et al.24-25 Briefly, 12-13 mg of cadmium oxide (CdO) and 180 mg of lauric acid were mixed in a three-neck flask under a nitrogen atmosphere. The mixture was heated to 180-200 °C until the CdO fully dissolved in the lauric acid forming a clear, colorless solution. Once fully dissolved, 1.94 g of trioctylphosphine oxide (TOPO) and 1.94 g of hexadecylamine (HDA) were added to the solution under constant stirring. The temperature was then increased to 280 °C for 10 minutes before being cooled slowly. Meanwhile, a solution of 80 mg of selenium powder was dissolved in 2 mL of trioctylphosphine (TOP). This solution was rapidly injected into the mixture under vigorous stirring upon reaching the desired temperature during the cooling process. Size-tunable luminescent quantum dots are directly dependent on temperature. The temperature at which the selenium component is injected determines the size of the quantum dots synthesized, corresponding to their emission wavelength. Typically, the temperature is slowly decreased from 280 °C to 180 °C. The higher the temperature upon injection, the larger the quantum dots will be in size. A ZnS shell was prepared: 2 mL of TOP containing 1 mL of diethyl zinc (Zn(Et₂)) and 250 µL of hexamethyldisilathiane ((TMS)₂S). The shell was added dropwise into the solution after injection of the selenium. The resulting reaction mixture was kept at 180 °C for 1 hour before cooling to
room temperature. The resulting CdSe/ZnS quantum dots were washed 3 times with methanol by centrifugation at 4000 rpm for 10 minutes and redispersed in chloroforms. The quantum dots were stored at room temperature in the dark.

4.3.3 Synthesis of Quantum Dot Loaded Human Serum Albumin Nanoparticles

Human serum albumin nanoparticles (HSAPs) were synthesized using a commonly used desolvation method. A stock solution of human serum albumin (HSA) in water was prepared by dissolving 100 mg of HSA into 1 mL of nanopure water. The pH of this solution was around 7. To a 20 mL scintillation vial, 200 µL of the HSA stock solution was added along with 500 µL of CdSe/ZnS quantum dots. The solution was stirred using a micro stir bar, and 3 mL of 100% ethanol was added using a peristaltic pump at a rate of 1 mL/min. To begin the crosslinking of the HSA, 4.7 µL of glutaraldehyde was added to the HSAP solution. After stirring for 24 hours, the particles were centrifuged for 25 minutes at 13,000 rpm and washed twice with a 1:3 water-to-ethanol mixture. The resulting quantum dot loaded human serum albumin nanoparticles (HSAP-QDs) were resuspended in nanopure water via ultrasonication and stored at 4 °C.

4.3.4 Crosslinking of HSAP-QDs to IL-15 or MCP-1 antibodies

Human serum albumin nanoparticles loaded with quantum dots (HSAP-QDs) were crosslinked to atherosclerosis biomarkers for fluorescence assay detection using classic EDC coupling methods. Carboxylic acid groups (−COOH) on the surface of the HSAP-QD particles are coupled with the amine groups (−NH₂) on the surface of the antibody. Typically, 500 µL of HSAP-QDs were mixed with 20 µL of 30 mg/mL EDC and 20 µL of 30 mg/mL sulfo-NHS. The mixture was vortexed for 20 minutes at room temperature in order to activate the free carboxylic acid groups on the HSAP-QD nanoparticles. Next, 500 µL of IL-15 or MCP-1 antibodies were
added and the mixture was gently stirred for 2 hours. The resulting conjugate particles were separated by centrifugation at 13,000 rpm for 10 minutes, and they were washed twice with PBS buffer at pH 7.4. They were redispersed in PBS buffer at pH 7.4 via ultrasonication and used immediately for fluorescence assay detection.

4.3.5 Individual Fluorescence Immunoassays of IL-15 and MCP-1

Fluorescent immunoassays of atherosclerosis biomarkers IL-15 and MCP-1 were performed similarly to previous methods. Amine-binding, clear microtiter well plates were washed three times with 200 µL of wash buffer (PBS pH 8.0 buffer containing 0.05% Tween 20). After removing the wash buffer, 100 µL of 25 µg/mL concentrations of IL-15 or MCP-1 capture antibodies were added to the wells and incubated overnight at room temperature. The capture antibodies were removed from the wells and 200 µL of Super Block blocking buffer was added and incubated at room temperature for 1 hour. The wells were washed three times with wash buffer after removing the blocking buffer. Varying concentrations of MCP-1 or IL-15 antigens were added to the wells for binding to the capture antibodies. The plate was incubated at room temperature for 1 hour and then washed three times with wash buffer. The detection antibody-fluorescent HSAP-QD conjugates were added to the wells, and the plate was incubated at room temperature for 1 hour. Finally, the detection antibody-HSAP-QD solutions were removed and the plate wells were washed with wash buffer 3 times.

4.3.6 Multiplex Fluorescence Immunoassays of IL-15 and MCP-1

Fluorescent immunoassays simultaneously detecting IL-15 and MCP-1 were performed similarly to individual immunoassays with slight modifications. Amine-binding, clear microtiter well plates were washed three times with 200 µL of wash buffer (PBS pH 8.0 buffer containing
0.05% Tween 20). Upon removing the wash buffer, 50 µL of IL-15 and 50 µL of MCP-1 capture antibodies at 25 µg/mL in PBS buffer pH 7.4 were added to the well plate and incubated overnight at room temperature. The capture antibody solutions were removed and the wells were blocked by adding 200 µL of Super Block blocking buffer. The plate was incubated with blocking buffer for 1 hour at room temperature. The blocking buffer was removed and varying concentrations of IL-15 and MCP-1 antigens were added to the well plate and incubated for 1 hour at room temperature. The plate was washed three times with wash buffer, and 50 µL of IL-15 antibody-HSAP-QD (λ<sub>EX</sub> = 600 nm) composites as well as 50 µL of MCP-1 antibody-HSAP-QD (λ<sub>EX</sub> = 560 nm) composites were added to the wells. The antibody-HSAP-QD composites contained quantum dots of different sizes, thereby each having a different fluorescence emission maximum which allows for multiplexing. The well plate was incubated for 1 hour at room temperature and washed three times with wash buffer.

4.3.6 Fluorescence Spectroscopy Measurements

Fluorescence emission spectra were obtained using a SpectraMax M2 spectrofluorometer microplate reader (Molecular Devices, Inc.) equipped with a 75 W continuous Xenon flash arc lamp as a light source and photomultiplier (R-3896) detector. The excitation wavelength for all samples was 420 nm. Fluorescence emission was measured from 450-750 nm.

4.3.7 Digital Fluorescence Imaging Microscopy

Fluorescence images were taken using a digital fluorescence imaging microscopy system containing an Olympus IX-71 inverted fluorescence microscope equipped with a 100 W mercury lamp as a light source and a high performance color charge coupled device camera (CCD) (Olympus DP 70). Images were obtained through 10x, 20x, 40x, and 60x microscope objectives
with numerical aperture = 0.5. A filter cube with a 425 ± 20 nm band-pass excitation filter, a 465 nm dichroic mirror, and a 475 nm long pass emission filter was used for spectral imaging. Image analysis was done using DP Controller software. An exposure time of 10 ms was typically used for image acquisition.

4.3.8 Dynamic Light Scattering Measurements

Average particle size, zeta potential, and mobility of HSAP-QD composites were determined through dynamic light scattering. The values were obtained using a Mobius dynamic light scattering instrument (Wyatt Technology Corporation, Santa Barbara, CA) at 25 °C. Samples were dissolved and diluted in nanopure water prior to analysis.

4.4 Results and Discussion

4.4.1 Fluorescence Emission of HSA-QDs

Human serum albumin nanoparticles (HSAPs) were loaded with CdSe/ZnS QDs through ethanolic desolvation. The QDs were mixed with a stock solution of HSA in water. Inorganic QDs are water-insoluble, however, upon addition of ethanol, the albumin matrix was denatured and aggregates of HSA and QDs were formed. A glutaraldehyde crosslinking agent was added to bind amines from lysine and arginine residues on the albumin surface. The QDs were encapsulated inside the nanoparticle during formation due to hydrogen bonding with the side chains of the hydrophobic amino acids and the TOPO capping ligand on the QD surface.1,26-27

Fluorescence emission of HSAP-QDs was measured using a SpectraMax Pro M2 spectrometer, and fluorescence microscopy images were taken using an inverted fluorescence microscopy, Olympus IX71. HSAPs range from 100-200 nm in diameter, which is too small to
be visualized individually via fluorescence microscopy. However, aggregates are visible, and overall fluorescence emission can be observed in the digital images. In addition to the QD loading, a fluorescent dye, rhodamine 6G chloride, was encapsulated into the HSAPs for comparison. Rhodamine 6G chloride is commonly used as a fluorescent tracer, and it exhibits a yellow-green fluorescence emission. Empty HSAPs without any dye or QD loaded exhibited no fluorescence emission, while HSAPs loaded with rhodamine, QD-600 and QD-560 displayed their respective fluorescence emission wavelengths. Human serum albumin has a natural weak green fluorescence (Figure 4.1). Loading the HSAPs with QD-560 or rhodamine demonstrated a considerable difference through microscopy. After several washings of the encapsulating HSAPs, the supernatants of the HSAPs showed no fluorescence. It appears there may be some background or free QDs in the solution, but this is actually due to aggregation of the HSAPs. Both QD-560 nm and rhodamine 6G chloride appear green when fluorescing, and HSAPs loaded with 600 nm QDs should appear orange-reddish in color. However, in samples of HSAP-QD-600, there are green aggregates dispersed throughout the solution (Figure 4.3). This indicates that not all of the HSAPs contain QDs and the loading is not evenly distributed during synthesis. Inefficient loading is a major limitation for multiplexing, as aggregates will not be distinguishable microscopically. The green aggregates seen throughout are unloaded HSAPs.

**Figure 4.1** Digital Fluorescence Microscopy Image and Emission Spectra of empty HSAPs.
Dynamic light scattering (DLS) measurements were taken to observe any changes in the sizes of the HSAPs after loading with a fluorophore or with quantum dots (Table 4.1). The
expected diameter range of QDs synthesized is 100 – 200 nm. All samples exhibited particle diameter within this range, however, only the unloaded HSAPs exhibited true monodispersity. QD-loaded and dye-loaded HSAPs were relatively monodisperse, but there is some aggregation present within those samples. The zeta potential (mV) for all of the samples indicates that they nanoparticle systems are stable. Larger zeta potentials are characteristic of stable colloidal systems.\textsuperscript{7,28}

**Table 4.1** Dynamic Light Scattering (DLS) data for HSAPs.

<table>
<thead>
<tr>
<th></th>
<th>HSAP</th>
<th>HSA-Rhodamine</th>
<th>HSA-QD (560 nm)</th>
<th>HSA-QD (600 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Particle Diameter (nm)</strong></td>
<td>194.73 ± 2</td>
<td>143.10 ± 3</td>
<td>162.79 ± 1</td>
<td>201.19 ± 1</td>
</tr>
<tr>
<td><strong>Polydispersity Index (PDI)</strong></td>
<td>0.0943</td>
<td>0.1261</td>
<td>0.1243</td>
<td>0.1316</td>
</tr>
<tr>
<td><strong>Zeta Potential (mV)</strong></td>
<td>-24</td>
<td>-24</td>
<td>-22</td>
<td>-26</td>
</tr>
</tbody>
</table>

### 4.4.2 Photostability

Conventional organic dyes are known for not being very photostable and having susceptibility to photobleaching.\textsuperscript{15,29} Photobleaching occurs when a fluorophore loses the ability to fluoresce due to the cleaving of covalent bonds and interactions with the surrounding environment.\textsuperscript{16,30} When electrons transition from an excited singlet state to an excited triplet state, the fluorophore can interact with other molecules and undergo chemical modification.\textsuperscript{16} Excited electrons remain in the triplet state longer than in the singlet state, thus giving more time to interact with the environment. The rate at which a molecule experiences photobleaching is dependent on its molecular structure and the surrounding environment, but in many cases for organic dyes, the number of excitation and emission cycles is known. Photobleaching is a major limitation for fluorescence microscopy, and fluorescent materials that do not lose their ability to
fluoresce are ideal for labeling and imaging. A typical organic dye may experience photobleaching after absorption of $10^5$ photons while CdSe/ZnS QDs have been reported to absorb $10^8$ photons prior to any photobleaching.\textsuperscript{15} QDs are inherently brighter than organic dyes with high molar extinction coefficients and comparable quantum yields, yet they have more stability, which accounts for the decrease in photobleaching susceptibility.\textsuperscript{15,31}

Quantum dot loaded HSAPs were compared with HSAPs loaded with rhodamine 6G chloride. The nanoparticle solutions were illuminated for 2 hours using a 100 W mercury lamp. Fluorescence emission was measured every 15 minutes, and it is evident that HSAPs loaded with QDs have higher photostability than rhodamine 6G chloride (Figure 4.5). The fluorescence emission intensity decreased about 10\% for HSAP solutions containing 600 nm or 560 nm QDs. Particle solutions containing rhodamine 6G chloride decreased 40\%.

![Figure 4.5 Photostability measurements of HSA-QDs and HSA-Rhodamine dye.](image)
4.4.3 HSAP Leakage and Stability

Fluorescence intensity of QD-loaded HSAPs and rhodamine loaded HSAPs was monitored for 10 days to examine if there was any leakage of QDs or fluorophore from the HSAP core (Figure 4.6). HSAPs have shown capabilities of encapsulating inorganic materials, but the limited availability of hydrophobic amino acids causes the loading efficiency to be lower than hydrophilic materials. Aggregation of QDs inside of the HSAPs may also affect the integrity of the albumin matrix that could lead to some leakage. Nanomaterials with significant leakage are not suitable for *in vivo* applications as nanocarriers especially if leakage will release toxic materials. Significant leakage from nanoparticle also presents limitations to long-term labeling or monitoring *in vitro* applications. The supernatant of the HSAP solutions was measured and any fluorescence emission was recorded. There was relatively little to no leakage of dye or QDs from the HSAPs over the first couple of days, but after day 3, the intensity of rhodamine in the supernatant started to increase. QD fluorescence was evident by day 5, so even though they lasted longer, some leakage still occurred. The fluorescence intensity in the supernatants continued to rise steadily through day 10 for all HSAP samples. After HSAP preparation, the nanoparticles should be used immediately in order to ensure optimal brightness, stability, and minimal leakage of the fluorophore.
4.4.4 Fluorescence Immunoassays of IL-15 and MCP-1

QD-loaded HSAPs were used in fluorescence immunoassays to measure the presence of atherosclerosis biomarkers IL-15 and MCP-1. Sandwich immunoassays were utilized in order to increase the opportunity for antigen-antibody binding and improve assay sensitivity (Scheme 4.1). IL-15 or MCP-1 antibodies were immobilized on a well plate surface and incubated at 37 °C for 1 hour. The well plate was blocked with SuperBlock blocking buffer to quench remaining active sites on the well plate surface. Different concentrations of each antigen were then added to the well plate for binding by the capture antibodies.

Meanwhile, detection antibodies were conjugated to the HSAP-QDs via EDC coupling of carboxylic acids (-COOH) and amines (-NH₂) on the HSAP and antibody surfaces. EDC
coupling is a classic bioconjugation reaction for biomolecules.\textsuperscript{32-33} EDC reacts with carboxylic acids forming an amine-reactive O-acylisourea intermediate.\textsuperscript{34-35} The intermediate is highly susceptible to hydrolysis, unstable, and short-lived.\textsuperscript{15,31} The targeted amine group must quickly find the active carboxylate prior to hydrolysis or the coupling reaction will not occur. Adding sulfo-NHS overcomes this issue by converting the intermediate to an amine-reactive sulfo-NHS ester intermediate and increasing its stability. The sulfo-NHS ester intermediate reacts with the amine groups on the detection antibody and forms the HSAP-QD-antibody bioconjugates. The resulting bioconjugates are stored in PBS buffer and used immediately for assay detection.

\textbf{Scheme 4.1} Reaction scheme for conjugating antibodies with HSAP-QD through EDC/NHS coupling.
HSAP-QDs containing 600 nm emission QDs were coupled with IL-15 detection antibodies. The QD-antibody bioconjugates were added to the well plate and allowed to bind to the IL-15 antigens for 1 hour before washing. The plate was then visualized through fluorescence microscopy, and the emission was measured using the microplate reader. Microscopy images show an increasing amount of orange-reddish HSAP-QDs as the concentration of IL-15 is increased. The particles in the images are not individual HSAPs; because HSAPs are too small to be seen individually via fluorescence microscopy, aggregates are displayed in the images. There does not appear to be an even distribution of color throughout the images again indicating that the QDs are not evenly distributed throughout the HSAP solution. The assay was able to detect as low as 50 pg/mL IL-15, but this is not as sensitive as other reported detection methods (Figure 4.8). The antigen-antibody binding is specific as expected; HSAP-QDs conjugated to MCP-1 detection antibodies did not show fluorescence emission when added to wells containing IL-15 antigens (data not shown).

Figure 4.7 Digital Fluorescence Microscopy of IL-15 sandwich assay detection: a) 50 pg/mL, b) 0.5 ng/mL, and c) 5 ng/mL.
Figure 4.8 Fluorescence emission spectra of IL-15 using HSAP-QD (600 nm).

HSAP-QDs containing 560 nm emission QDs were coupled to MCP-1 antibodies, and results were similar to the IL-15 assays. Microscopy imaging shows an increase in the number of HSAP-QD aggregates as the concentration of MCP-1 is increased (Figure 4.9). There is likely inefficient loading of QDs in these samples as well, but unloaded HSAPs are not distinguishable from 560 nm QD-HSAPs. Fluorescence intensity measurements revealed consistent data with imaging, the intensity increased as concentration increased (Figure 4.10). This assay had a limit of detection of 0.5 ng/mL MCP-1, which is not an improvement over current detection methods.

Figure 4.9 Digital Fluorescence Microscopy of MCP-1 sandwich assay detection: a) 0.5 pg/mL, b) 5 ng/mL, and c) 50 ng/mL.
A multiplex immunoassay was performed using HSAP-QDs containing 600 nm and 560 QDs conjugated to IL-15 and MCP-1 detection antibodies, respectively. Capture antibody solutions containing both IL-15 and MCP-1 antibodies were added to the wells for immobilization. The well plate was blocked, and both IL-15 and MCP-1 antigen solutions were added to the same wells. The QD-antibody bioconjugates were finally added to measure the presence of the antigens. Upon washing, the well plate was visualized via fluorescence microscopy, and the fluorescence emission was measured (Figure 4.11). The fluorescence intensity clearly shows two peaks: one at 560 nm and one at 600 nm indicating that both biomarkers were present and detected simultaneously. This shows potential in the use of an HSA based immunoassay; however, the different particles are not discernable through fluorescence microscopy. If HSAP-QDs with 600 nm QDs are not efficiently loaded and samples contain significant empty HSAPs, these particles could not be distinguished from HSAP-QDs containing 560 nm QDs. In order to utilize QDs in this assay, QD loading would have to be evaluated further if there is any potential use for detection of biomarkers.
4.5 Conclusions

In summary, CdSe/ZnS quantum dots were used for encapsulation into human serum albumin nanoparticles. HSAPs have tremendous versatility throughout a wide array of biological applications. They have extraordinary ligand binding properties that enable them to encapsulate different types of nanomaterials including inorganic, water-insoluble materials. This feature provides opportunities for QDs and other inorganic materials to be more useful in bioanalytical and clinical settings. HSAPs contain a plethora of amino acids that encourage binding to the different materials. Traditionally, the amine and carboxylic acid groups of the albumin matrix are exploited for binding to hydrophilic molecules. However, there are some hydrophobic amino acid residues present that are capable of binding to inorganic QDs. There was inefficient loading of the QDs inside the HSAPs, and this could be overcome by a surface ligand exchange of the QDs. Replacing the TOPO capping ligand with a hydrophilic ligand such as 16-mercaptophexadecanoic acid (MHDA) would make them water soluble and biocompatible. Loading would be much more efficient, however, assay sensitivity might not improve due to lower quantum yield and brightness of MHDA-capped QDs. The current assay was able to detect...
50 pg/mL of IL-15 and 0.5 ng/mL of MCP-1. While this shows potential for albumin-based assay development, these detection limits are not better than currently available detection methods.

### 4.6 References


98


Chapter 5

Multifunctional Human Serum Albumin Nanoparticles for Targeted Drug Release

This chapter presents collaborative work with Dr. David Bwambok (Part I) and Connor Carry, James Liu, and Dr. Benjamin Lee of Tulane University School of Medicine (Part II). Images in Part II were reprinted by permission of John Wiley & Sons, Inc. © 2016

5.1 Abstract

Human serum albumin nanoparticles (HSAPs) were used for targeted drug release studies. HSAPs are monodisperse, stable, and biocompatible. These robust nanoparticles are capable of encapsulating various materials and can be functionalized for use in a multitude of therapeutic applications. HSAPs loaded with superparamagnetic iron oxide nanoparticles (SPIONs) and fluorescein-5-thiosemicarbazide were examined for their potential as drug release agents. An external magnetic field was applied to the nanocomposites to monitor the amount of fluorophore released upon exposure to the magnetic field. Increasing the strength of the magnetic field correlated to an increase in fluorescence intensity of the HSAPs supernatant. Dynamic light scattering (DLS) showed an overall decrease in particle size of the nanocomposites over time. Results indicate that magnetic HSAP nanoparticles may be excellent drug carriers with the ability to control release from the albumin matrix using an external magnetic field.
HSAPs were also used in conjunction with photothermal laser ablation to treat a renal cell carcinoma mouse model *in vivo*. Gold nanorods (AuNRs) and sorafenib (SRF), a tyrosine kinase inhibitor, were encapsulated into HSAP nanoparticles (HSA-AuNR-SRFs) and used for treatment of the tumors. The nanocomposites were injected into the tumor sites and examined for tumor necrosis. Injection of HSA-AuNR-SRFs alone and treatment with the laser alone showed minimal tumor shrinkage and low necrosis. The combination of laser ablation and HSA-AuNR-SRFs resulted in 100% tumor necrosis and total elimination of any visible tumor after treatment. These results show great promise to utilize HSAPs as multimodal systems for theranostic treatment applications.
5.2 Introduction

Efforts to use nanotechnology for targeted drug delivery in cancer treatment methods may revolutionize cancer therapies.\textsuperscript{1} Improvements in disease diagnosis, treatment, and prevention are all moving toward nanoscale systems.\textsuperscript{2} Controlled release of anticancer drugs from nanoparticle carriers has gained attention as a new method for targeted drug delivery. The most common and effective anticancer drugs tend to kill healthy cells as well as cancerous cells.\textsuperscript{3} Nanocarriers are able to deliver anticancer drugs directly to a tumor site reducing the likelihood of affecting healthy cells.\textsuperscript{2,3} Presently, drug delivery systems are dominated by synthetic materials because there is precise control over synthesis and exploitation of their properties. Examples of common synthetic nanocarriers for targeted drug delivery include polymeric nanoconstructs, carbon nanotubes, and gold nanocages.\textsuperscript{1,4} Natural materials such as lipids, nucleic acids, proteins, and peptides have been explored as more biocompatible alternates for nanocarrier development.\textsuperscript{4} Mechanisms of controlled release from nanocarriers have been reported through changes in different stimuli including pH, enzymes, temperature, and laser irradiation.\textsuperscript{2,4-6}

Human serum albumin (HSA) is the most common protein in blood plasma, and it avoids rejection when used \textit{in vivo}.\textsuperscript{7} Development of human serum albumin nanoparticles (HSAPs) as a natural material for targeted drug delivery has become a platform of interest.\textsuperscript{8} HSAPs are biodegradable, and naturally biocompatible for functionalization because of an abundance of charged amino acids.\textsuperscript{7} Their versatility allows them to encapsulate drugs and other nanomaterials.\textsuperscript{9} HSAPs are also easily bioconjugated for surface labeling of antibodies or other molecules through interactions primarily with the carboxylic acids and amines of the amino
acids. Controlled release studies using HSAPs for treatment of neuroblastomas and breast cancer cells show that HSA has great promise as an anticancer drug nanocarrier.

Magnetically-triggered release from nanocarriers utilizes an external magnetic field to stimulate magnetic nanoparticles which degrades the nanocarrier and releases the anticancer drug. Studies using magnetic fields to release drugs from magnetically labeled materials have shown potential for their use therapeutically. Using an external magnetic field improves their possibility as nanocarriers because drug release can be triggered non-invasively. Reports using magnetically labeled silica, collagen gels, polymersomes, and polymeric nanocomposites have shown release of fluorescent materials. Superparamagnetic iron oxide nanoparticles (SPIONs) are ideal for magnetically enabled nanocarriers. SPIONs range between 10 – 100 nm in diameter and are coated with biocompatible polymers that enable them for bioconjugation. Not only can they be used as drug delivery vehicles, but they can also be used to monitor materials via magnetic resonance imaging (MRI).

The main feature of the magnetic nanoparticles is their superparamagnetism. This property is due to their small size, and when an external magnetic field is applied, the nanoparticles become magnetized. When the magnetic field is removed there is no residual magnetization. Unlike larger magnets, SPIONS do not exhibit multiple domains. There is a uniform, single spin that has high magnetic susceptibility not seen in bulk magnets. SPIONs have a stronger and faster magnetic response with low remanence and nearly zero coercivity. These features make them excellent candidates for targeted drug delivery. SPIONs have already shown use in targeted hypothermia where cancer cells are treated by localized, internal heating. When exposed to a high frequency magnetic field they become heated due to Neel and Brown relaxation. Encapsulating SPIONs in human serum albumin (HSA) can allow for efficient
cellular uptake and localization within tumor sites. Degrading the HSA matrix from external magnetic field application can effectively and precisely release an anticancer drug.\textsuperscript{20}

Gold nanorods (AuNRs) are another avenue through which multifunctional drug delivery HSA vehicles can be developed.\textsuperscript{21} They have been of interest as biosensors for disease diagnosis and monitoring. AuNRs absorb energy from near-infrared light and emit it as heat.\textsuperscript{22} This can directly destroy tumors without affecting nearby tissues.\textsuperscript{23} Encapsulation in HSAPs along with an anticancer drug forms an ultrasensitive nanomaterial capable of \textit{in vivo} diagnostic applications. Photothermal laser ablation therapy provides irradiation to the AuNR encapsulated HSAPs (HSAP-AuNRs) which effectively heats and destroys tumor cells.\textsuperscript{24}
Part I: Magnetically Triggered Release of Fluorescent Drug Analog from Human Serum Albumin Nanoparticles

5.3 Experimental Methods

5.3.1 Materials and Reagents

All chemicals were used as received without modification unless otherwise noted. Human serum albumin (HSA), lyophilized powder (≥ 97%), aqueous glutaraldehyde (8%), sodium hydroxide (≥97%), and fluorescein-5-thiosemicarbazide (80%) were purchased from Sigma-Aldrich (St. Louis, MO). Polyethylene glycol (PEG) coated iron oxide magnetic nanoparticles (10 nm, 1 mg/mL) were purchased from Ocean Nanotech (San Diego, CA). Phosphate buffered saline (PBS) was obtained from Invitrogen (Waltham, MA). Ethanol, 200-proof, ACS/USP grade was purchased from Pharmco-AAPER (Brookfield, CT). Nanopure deionized and distilled water (18.2 MΩ) purified with a Barnstead Nanopure system was used for all experiments.

5.3.2. Synthesis of Fluorescent and Magnetic Multifunctional Human Serum Albumin Nanoparticles

Multifunctional human serum albumin nanoparticles loaded with a fluorescent drug analog and magnetic nanoparticles were prepared using a modified common desolvation method. A stock solution of HSA was prepared by dissolving 100 mg of HSA into 1 mL of nanopure water. The pH was adjusted to 8.2 using 0.01 M NaOH. A 500 µL aliquot of the HSA stock solution was mixed with 200 µL of fluorescein-5-thiosemicarbazide (1 mg/mL), 30 µL of PEG-coated iron oxide nanoparticles (10 mg/mL) in water, and 270 µL of nanopure water. The mixture stirred for 2 hours at room temperature. Desolvation of the HSAPs solution began upon the addition of 3 mL of ethanol. The ethanol was added using a peristaltic pump at a rate of 1
mL/min. To begin the HSA crosslinking, 4.7 µL of 8% glutaraldehyde was added to the HSAP solution. After stirring for 24 hours, the HSAPs were centrifuged for 30 minutes at 13,000 rpm and washed with ethanol. The resulting fluorescent and magnetic HSAPs were resuspended in PBS buffer via sonication and stored at 4 °C.

5.3.3 Characterization of Fluorescent and Magnetic Multifunctional Human Serum Albumin Nanoparticles

Average particle size, size distribution, zeta potential, and morphology of the HSAP nanoparticles were acquired using dynamic light scattering (DLS), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Field emission SEM (FESEM) images were taken using a LEO 1540VP (LEO Elektronenmikroskpie GmbH, Oberkochen, Germany) FESEM electron microscope. The HSAP nanoparticles were dried on a glass slide and sputter coated with gold. TEM images were taken using a JEOL 2010 TEM electron microscope. The samples were prepared by drop casting the HSAP nanoparticle solution onto a carbon coated copper grid and allowing it to air dry. The DLS used was a Desla S Nano system (Beckman Coulter Instruments). The HSAP nanoparticle solution was diluted with PBS buffer (pH 7.4) prior to analysis. A Superconducting Quantum Interference Device (SQUID) magnetometer was used to determine the magnetic properties of the HSAPs. The samples were isolated by centrifugation and air dried.
5.3.4 Magnetically-triggered Fluorophore Release from HSAPs

To employ the magnetic nanoparticles for fluorophore release from the HSAPs, 150 µL of the HSAPs were placed in a glass container 3 mm in diameter. The sample was situated between a ferrite ring wrapped with a copper coil to provide an AC voltage. The maximum frequency was 28 kHz and the maximum field was 900 Oe. The HSAP nanoparticles were centrifuged for 10 minutes at 10,000g after exposure to the magnetic field and the supernatant was measured using a microplate reader (Molecular Devices, SpectraMax M2) to determine the amount of fluorophore released. The excitation and emission wavelengths used for the released fluorescein-5-thiosemicarbazide were 495 nm and 525 nm, respectively. An Olympus IX71 inverted fluorescence microscope with a digital camera was used for imaging of the HSAP before and after exposure to the magnetic field. The Delsa Nano S system (Beckman Coulter Instruments) was used to monitor change in particle size of the HSAP after magnetic field exposure. HSAP nanoparticles were measured using DLS after magnetic field exposure for 0, 30, 40, 45, and 55 minutes.

5.4 Results and Discussion

5.4.1 Characterization of Multifunctional Fluorescent and Magnetic Human Serum Albumin Nanoparticles

Human serum albumin nanoparticles were loaded with a fluorescent drug analog and superparamagnetic iron oxide nanoparticles (SPIONs) to evaluate degradation of HSAPs and release of the fluorophore. HSAP nanoparticles are formed primarily by a desolvation process which has been repeatedly reported to be a robust and reproducible technique for synthesis. Ethanol is added to the HSA solution causing the albumin to precipitate followed by stabilization.
of the protein nanoparticles with a glutaraldehyde crosslinker.\textsuperscript{8, 25-26} SEM imaging has shown that the resulting HSAP nanoparticles are uniform and spherical (Figure 5.1a). The TEM imaging clearly shows the magnetic nanoparticles embedded within the HSAP nanoparticles (Figure 5.1b); however, the high energy electron beam degrades or melts the protein during imaging. Using a lower voltage while cooling the sample stage with liquid nitrogen preserved the integrity of the HSAP nanoparticles and revealed the spherical nanoparticles consistent with the SEM imaging (Figure 5.1c).

![Figure 5.1](image)

**Figure 5.1** Electron microscopy images of multifunctional human serum albumin nanoparticles: (a) SEM image (10 kV), (b) TEM image (200 kV, room temperature), and (c) TEM image (80 kV, -78 °C).

DLS was used to measure particle size, zeta potential, and polydispersity of the HSAP nanoparticles. The average diameter of the nanoparticles was $158 \pm 19$ nm with a zeta potential of $-48$ mV. Zeta potential is a measure of electrostatic potential of a particle surface and is measured through electrophoretic mobility.\textsuperscript{27} It is a fundamental parameter of colloidal stability and can provide insight into particle dispersion or aggregation. The stability of a particle dispersion can be determined by the zeta potential, and typically, the larger the value whether positive or negative, the more stable the system. The polydispersity index (PDI) of the HSAP nanoparticles was 0.065 which indicates the nanoparticle solution is monodisperse. A perfectly uniform sample as determined by DLS has a PDI of 0.0, and a sample in the range 0.05-0.1
indicates a narrow, monodisperse system. These values demonstrate that the multifunctional fluorescent and magnetic HSAP nanoparticles are excellent candidates for targeted drug delivery applications.

**Table 5.1** Dynamic Light Scattering (DLS) data for HSAPs.

<table>
<thead>
<tr>
<th>Human Serum Albumin Nanoparticles Loaded with magnetic iron oxide nanoparticles and fluorescein-5-thiosemicarbazide</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle diameter (nm)</td>
<td>158 ± 19</td>
</tr>
<tr>
<td>Polydispersity Index (PDI)</td>
<td>0.065 ± 0.010</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>-48</td>
</tr>
</tbody>
</table>

Paramagnetic materials are attracted to an external magnetic field. In the presence of a magnetic field, the materials form internal magnetic moments in the direction of the external magnetic field and produce a net positive magnetization. When a material is small enough (diameter between 3-50 nm) to change rotation under the influence of temperature it becomes superparamagnetic. The rotational changes of superparamagnetic materials from the thermal energy cause them to flip the direction of magnetization. The magnetic properties of the HSAP nanoparticles were measured using a SQUID magnetometer with a field sweep between -15000 to 15000 Oe.
The magnetization curve of the magnetic HSAP nanoparticles does not have a hysteresis loop which is consistent with superparamagnetic nanoparticles (Figure 5.2).\textsuperscript{30} When a magnetic field is removed from a ferromagnetic material, it does not relax to zero magnetization unless a magnetic field of the opposite direction is applied.\textsuperscript{14-15} Applying alternate magnetic fields results in a retraceable hysteresis loop often referred to as the magnetic “memory” of a ferromagnetic material.\textsuperscript{30} The absence of a hysteresis loop indicates zero coercivity which is another prominent feature of superparamagnetic particles. Coercivity is a measure of the magnetic field strength needed to demagnetize a ferromagnetic material.\textsuperscript{14,30} The superparamagnetic HSAP nanoparticles are enabled only in the presence of a magnetic field further presenting them as strong candidates for controlled drug delivery tools.

\textbf{5.4.2 Magnetized fluorophore release from multifunctional HSAP nanoparticles}

An applied magnetic field was applied to the HSAP nanoparticles to release fluorescein-5-thisemicarbazide. The fluorophore acts as a fluorescent drug analog and gives insight into how the HSAP nanoparticles might behave as drug delivery carriers.\textsuperscript{31} The HSAP nanoparticles in
PBS buffer were placed in a glass container situated between a ferrite ring wrapped with a copper coil. The nanoparticles were exposed to a maximum frequency of 28 kHz and the maximum field strength was 900 Oe. The particles were separated by centrifugation after exposure to the magnetic field, and the fluorescence intensity of the supernatant was measured to evaluate the amount of fluorophore released. An increase in the amount of fluorophore released directly correlated to an increase in magnetic field strength (Figure 5.3). This can provide a means for control of targeted drug release from the nanoparticles.

Figure 5.3 Release of fluorescein-5-thiosemicarbazide from magnetic HSAP nanoparticles as a function of magnetic field strength.

The duration of the magnetic field also plays a factor in the amount of fluorophore released from the nanoparticles. More fluorophore was released the longer the magnetic field was applied (Figure 5.4). The same magnitude was applied for each exposure time. A small amount of fluorescence was detected even without application of the magnetic field. This could be background fluorescence from fluorophore not entirely removed during the washing as well as some leakage from the HSAP nanoparticles over time. There was no fluorescence intensity increase from unloaded HSAP nanoparticles without iron oxide nanoparticles indicating the
observed fluorophore release resulted from the magnetic field. After 1 hour of exposure there
was still some fluorophore that was not released indicating the magnetic field did not completely
degrade the HSAP nanoparticle matrix.

![Fluorophore release graph](image)

**Figure 5.4** Release of fluorescein-5-thiosemicarbazide from magnetic HSAP nanoparticles as a function of time.

Fluorescence microscopy images of the HSAP nanoparticles before and after magnetic-
triggered released were acquired (Figure 5.5). The resolving power of the fluorescence
microscope is not high enough to visualize individual HSAP nanoparticles, however, aggregates
are visible. Prior to exposure to the magnetic field (Figure 5.5a), groups of aggregates are
noticeable, while after 10 and 30 minutes of magnetic field treatment, fewer aggregates are
observed (Figures 5.5 b,c). The magnetic field exposure caused localized heating of the
superparamagnetic iron oxide nanoparticles which is responsible for the breakdown of the
albumin matrix over time.
Figure 5.5 Fluorescence microscopy images of the release of fluorescein-5-thiosemicarbazide after magnetic treatment for (a) 0 min, (b) 10 min, and (c) 30 min.

The DLS results show that there is an overall decrease in size of the HSAP nanoparticles over time under r.f. magnetic field exposure (Figure 5.6). The PDI of the HSAP nanoparticles also increased over this time indicating that the nanoparticle system is no longer monodisperse. The change in polydispersity supports the nanoparticle degradation as a result of magnetic field treatment.

Figure 5.6 Change in diameter of magnetic multifunctional HSAP nanoparticles over time after magnetic field treatment.
Part II: Treatment of Renal Cell Carcinoma with Multifunctional Gold Nanorod Human Serum Albumin Nanoparticles

5.6 Experimental Methods

5.6.1 Materials and Reagents

All chemicals were used as received without modification unless otherwise noted. Human serum albumin (HSA), lyophilized powder (≥ 97%) and aqueous glutaraldehyde (8%), were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) was obtained from Invitrogen (Waltham, MA). Ethanol, 200-proof, ACS/USP grade was purchased from Pharmco-AAPER (Brookfield, CT). Bare gold nanorods (AuNRs) were purchased from Nanopartz Inc. (Loveland, CO). Sorafenib (SRF) was supplied by Tulane University Medical Center. Nanopure deionized and distilled water (18.2 MΩ) purified with a Barnstead Nanopure system was used for all experiments.

5.6.2 Synthesis of HSAP, HSAP-AuNR, HSAP-AuNR-SRF Nanoparticles

The HSAP-AuNR-SRFs were prepared similarly to the magnetic HSAPs via ethanolic desolvation. For unloaded, empty HSAPs, 1 mL of water was added to a 20 µL aliquot of the HSA stock solution. HSAP-AuNRs were prepared by adding of 1 mL of AuNR stock solution (5.9 x 10^{11} AuNRs) to a 20 µL aliquot of the HSA stock solution. HSAPs containing both AuNRs and a tyrosine kinase inhibitor (TKI) were prepared by adding sorafenib (SRF) in addition to the 1 mL AuNR stock solution. A 0.5 mL aliquot of SRF stock solution (1 mg/mL DMSO/EtOH) was added dropwise at a rate of 1 mL/min. To all samples, 3 mL of 100% ethanol was added at 1 mL/min using a peristaltic pump to initiate desolvation. Crosslinking of the HSAP samples was initiated by the addition of 4.7 µL of 8% glutaraldehyde. The nanoparticle
solutions were stirred for 24 hours followed by centrifugation for 30 minutes at 13,000 rpm and washing using a 1:3 water/EtOH solution. The samples were resuspended in nanopure water via ultrasonication using a F60 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) at 2 W root mean square. The resulting HSAP samples were stored in nanopure water at 4 °C prior to use.

5.6.3 Photothermal Laser Ablation Therapy Using HSA-AuNR-SRF Nanoparticles

Foxn1nu athymic nude mice were subcutaneously injected with a 200 µL solution containing a 1:1 ratio of 100 µL of RCC 786-0 cells (2.5 x 10^6 cells) and 100 µL of Matrigel basement membrane matrix. Each mouse grew bilateral tumors, one as a control, and the other for treatment. Once the tumors reached 1 cm in their longest direction, treatment with HSA-AuNR-SRF nanoparticles began. The tumors were injected with 0.1 mL SRF 10 mM SRF solution in PBS, 0.1 mL suspension of HSA-AuNR stock or 0.1 mL HSA-AuNR-SRF stock. Cellular uptake of the particles occurred over 24 h. Laser ablation was performed using a Laserglow Technology collimated diode laser system (Toronto, Canada) for continuous wave irradiation at 2100 mW and 808 nm. The laser was placed 14 cm directly above the tumor, covering an area 1 cm in diameter and applied for 6 min. The tumors were harvested and haematoxylin and eosin (H&E) slides were prepared for examination.

5.7 Results and Discussion

5.7.1 Characterization of Human Serum Albumin Nanoparticles Loaded with Gold Nanorods and a Tyrosine Kinase Inhibitor

The desolvation technique for human serum albumin nanoparticle synthesis was also used to encapsulate AuNRs and sorafenib (SRF), a tyrosine kinase inhibitor (TKI) approved for renal cell carcinoma treatment. The HSAP nanoparticles containing AuNRs and SRF (HSA-
AuNR-TKI) coupled with photothermal ablation were used to assess treatment in a renal cell carcinoma (RCC) mouse model. The human serum albumin nanocomposites were synthesized in a similar manner as those loaded with iron oxide and fluorescein-5-thiosemicarbazide. To a stock solution of HSA (100 mg/mL), AuNR was added to form HSA-AuNR nanocomposites. AuNR and SRF were added to the HSA stock solution to form the HSA-AuNR-TKI nanocomposites. Ethanol was added for denaturation and to initiate aggregation. Glutaraldehyde was then added for crosslinking of the amine groups on the albumin. After stirring overnight, the HSAPs were washed and purified via centrifugation and ultasonication. The resulting HSAPs were resdispersed in nanopure water. Spherical HSA-AuNR-TKI nanoparticles were imaged using TEM (Figure 5.7). The AuNRs are clearly encapsulated within the HSA nanoparticles. There was some melding of the nanoparticles due to heat from the high energy electron beam.

**Figure 5.7** TEM image of HSA-AuNR-TKI nanoparticle.

**5.7.2 Renal Cell Carcinoma Tumor Treatment Using HSA-AuNR-TKIs with Laser Ablation**
Human metastatic RCC cell line RCC 786-0 cells were cultured and injected into Foxn1

athymic nude mice subcutaneously for in vivo tumor establishment. Treatment with HSA nanoparticles commenced once the tumor size reached 1 cm in the longest direction. Each mouse grew bilateral tumors: one for treatment and one as a control. Marked tumors were injected with 10 mM SRF, HSA-AnNR stock, or HSA-AuNR-TKI stock solutions. After 24 hours of cellular uptake, the tumors were treated with laser ablation (~2100 mW at λ = 808 nm) using a collimated diode laser system. The control and treated tumors were harvested and used to create haematoxylin and eosin (H&E) slides, and they were also silver stained for imaging.

**Figure 5.8** Percentage of tumor necrosis and average tumor size after treatment.

The average tumor size of the controls was 9.38 mm with 1% tumor necrosis. Tumors treated with SRF and HSA-SRF without laser ablation showed no statistical difference in tumor necrosis with 4.2% and 11%, respectively. There was also only a slight decrease in tumor size with 9.2 mm and 8.25 mm after treatment. Utilizing laser ablation alone and with HSA-AuNR nanoparticles exhibited 62% and 63.4% tumor necrosis, respectively as well as an average tumor...
size of 3.9 mm and 12.2 mm. Combining laser ablation with HSA-AuNR-SRF resulted in 100% tumor necrosis and <0.01 mm average tumor size (Figure 5.8).

![Histological samples of tumor after treatment](image)

**Figure 5.9** Histological samples of tumor after treatment: (a) control with viable tumor structure, (b) tumor after treatment with SRF, (c) tumor after treatment with HSA-AuNR-TKI, (d) laser treatment alone, (e) 20x image of HSA-AuNR tissue with silver staining showing AuNRs, and (f) tumor after treatment with laser irradiation and HSA-AuNR-TKI showing no viable tumor.

Histological tissue samples show how each treatment type affected tumor viability (Figure 5.9). The control tumor without any treatment displayed viable tumor structure as indicated by the H&E staining throughout the tissue. Tissue samples after treatment with SRF alone or HSA-AuNR-SRFs alone presented mild coagulative necrosis. Using laser ablation photothermal therapy alone resulted in major coagulative necrotic tissue. The AuNRs are visible in the histology tissue samples after magnification and silver staining. Combining laser irradiation and HSA-AuNR-SRFs showed no viable tumor visible in the histological tissues which is consistent with the total shrinkage in tumor size.
5.8 Conclusions

Multifunctional HSAPs encapsulated with superparamagnetic iron oxide nanoparticles or gold nanorods were explored for targeted drug delivery. The HSAPs were successfully prepared through simple desolvation and crosslinking with glutaraldehyde. HSAPs have been shown to be versatile and non-invasive nanocarriers. Their broad functionality enables them to be useful in chemotherapeutic applications. New drug therapies can utilize the effectiveness and robustness of HSAPs. Exposure to an external magnetic field or laser ablation photothermal therapy creates a direct and controlled environment for drug release minimizing effects to neighboring healthy cells.

Fluorescence microscopy and DLS analysis revealed that the applied magnetic field caused the HSAPs to degrade and release the fluorescent drug analog. The amount of fluorophore release was dependent on the strength of the magnetic field as well as on the duration of exposure. Combining magnetically triggered release with an anticancer drug could result in significant cell death as seen through laser irradiation. Laser irradiation of HSA-AuNRs has been shown to promote NIR-induced hyperthermia. Total cell death and complete elimination of tumor sites was achieved solely by the combination therapy of laser irradiation with the HSA-AuNR-TKI nanoparticles. Both methods demonstrate that human serum albumin nanoparticles are excellent and ideal tools for controlled release of anticancer drugs as well as for drug delivery vehicles.
5.9 Acknowledgments

I would like to thank Dr. David Bwambok, Dr. Leszek Malkinski, Connor Carry, Cameron Callaghan, Dr. Donna Peralta, and Dr. Benjamin Lee for their assistance and collaboration with this work.

5.10 References


Chapter 6

Summary and Conclusions

Luminescent semiconductor nanocrystals or quantum dots (QDs) have demonstrated amazing potential for use in biological and bioanalytical applications. They possess unique optical and photophysical properties that give them tremendous advantages over conventional fluorescent organic dyes. CdSe/ZnS QDs are currently the most commonly used QDs for biosensing applications because of their exceptional tunability and brightness. CdSe/ZnS are tunable through the UV-Vis-NIR spectrum enabling them to be useful in the largest range of applications. Some of these properties include: broad absorption spectra, narrow emission spectra, a large Stokes shift, and a large molar extinction coefficient. They also exhibit excellent photostability, high quantum yield, high chemical stability, and size-dependent luminescence.

This work primarily focused on utilizing QDs in multiplex immunoassays. The size dependent luminescence, broad absorption spectra, and narrow emission are responsible for the distinctive multiplexing feature. Multiple analytes can be detected simultaneously using one excitation light source. QDs of different sizes and fluorescence emission wavelengths can be excited together and produce narrow emission peaks allowing the different analytes to be distinguished from one another.

A key feature of immunoassay development is the specificity of the detection probe. Developing an appropriate bioconjugate for use as the detection probe is fundamental to immunoassay optimization. The first project focused on enabling CdSe/ZnS QDs to be biocompatible with biological buffers commonly used in assays yet retain their inherent brightness and high quantum yield. Encapsulating QDs into mesoporous silica facilitated
bioconjugation while preserving the unique properties of CdSe/ZnS. The QDs remained stable in the mesoporous silica particles due to strong hydrophobic interactions between a C\textsubscript{18} chain and TOPO capping ligand on the surface of the QDs.\textsuperscript{2,15} The quantum dot silica composite particles (SiQDs) were further modified with thiol (-SH) and amine (-NH\textsubscript{2}) groups through (3-mercaptopropyl)trimethoxysilane (MPTMS) and (3-aminopropyl)triethoxysilane (APTES), respectively. Addition of the silanes enables bioconjugation for coupling with antibodies. The bioconjugates are formed through the use of a crosslinking agent. Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) and ethylene glycolbis(succinimidyl succinate) (EGS) were used to crosslink the thiol and amine groups, respectively. Addition of the amine group on the surface of the SiQDs creates an overall net positive charge on the surface and enables water solubility. To promote water solubility of the thiol modified SiQDs, (trimethoxysilylpropyl) ethylenediamine triacetic acid (TMS-EDTA) was added to the surface as well.

These bioconjugates were utilized in a quantum dot based fluorescence immunoassay to detect atherosclerosis biomarkers IL-15 and MCP-1. Direct detection assays were compared with sandwich detection assays, and using a sandwich detection immunoassay significantly increased detection limits of the biomarkers. In a sandwich immunoassay, a capture antibody is immobilized on the surface of a well plate. The well plate is activated with maleic anhydride groups, which readily bind to antibodies. The antigen is added to the well to bind to the capture antibodies. The presence of the capture antibody pulls down more antigens and provides specific binding sites rather than the nonspecific adsorption that occurs in a direct detection assay. The quantum dot-antibody bioconjugates are added to the well for binding to the antigen. Biomarker presence is quantified by measuring the fluorescence of the quantum dots present on the well
plate. Fluorescence intensity correlates to the amount of antigen present. The detection limits of this immunoassay were 1 pg/mL for IL-15 and 50 pg/mL for MCP-1, which is an improvement from currently, reported detection methods.

The focus of this dissertation shifted to exploration of human serum albumin nanoparticles (HSAPs). Human serum albumin (HSA) is the most abundant protein in blood plasma, and it is extremely versatile and capable of being utilized in numerous biological applications.\textsuperscript{16-17} HSAPs can freely transport throughout the body and they are excellent candidates as drug nanocarriers.\textsuperscript{18} The binding properties of HSAPs allow them to encapsulate various substances including insoluble chemotherapeutic drugs.\textsuperscript{19-20} They can transport these drugs directly to a tumor site and spare neighboring healthy cells exposure to toxicity.\textsuperscript{21} HSAPs are also multimodal: they can transport materials and they can non-invasively control the release of a given substance. Their multifunctionality enables them useful for assessment and treatment purposes, and they can also track and monitor drug release.

Quantum dot loading of HSAPs was examined to explore their use in an immunoassay. QDs were loaded into HSAPs via desolvation and crosslinking, but the loading was inefficient, and further studies are required to enhance loading of QDs into HSAPs. The HSAP-QD-antibody bioconjugates was able to detect IL-15 and MCP-1; however, distinguishing between the two biomarkers during multiplexing was not achieved.

The last two projects reported were collaborative efforts examining HSAPs for targeted drug delivery applications. First, HSAPs were loaded with superparamagnetic iron oxide nanoparticles and fluorescein-5-thiosemicarbazide as a fluorescent drug analog. A magnetic field was applied to the HSAPs to learn how the fluorophore was released upon albumin degradation as this could give insight on how drug molecules are released from HSAPs. Only in the presence
of the magnetic field was the fluorescein dye released from the albumin matrix. The controlled release potential of HSAPs was directly observed magnetically. And secondly, gold nanorods and a tyrosine kinase inhibitor (Sorafenib) were loaded into HSAPs for treatment of a renal cell carcinoma mouse model. The HSAPs were injected directly into tumor sites, and the gold nanorods were activated by laser irradiation. This allows direct control of the breakdown of the HSAPs to release the drug into tumor. Laser ablation causes the gold nanorods to heat up resulting in hyperthermia.\textsuperscript{22-23} As the integrity of the HSAPs degrade, the drugs inside are released to the tumor site. Utilizing both Sorafenib and the gold nanorods together in the mouse model resulted in 100\% tumor necrosis with no viable tumor present.\textsuperscript{24}

References

The author was born in Metairie, Louisiana and raised in Destrehan, Louisiana. She obtained her Bachelor of Science degree in biochemistry from Louisiana State University in 2010. She then joined the University of New Orleans chemistry graduate program and became a member of Professor Matthew A. Tarr’s research group.