Quantum Dots: Applications as Bioimaging and Biosensing Agents

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THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Chemistry
in the Graduate College of the
University of Illinois at Chicago, 2016

Chicago, Illinois

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This Thesis is dedicated to my family, for their endless love, support and encouragement, and also to all my teachers who have shaped my mind and made all the difference in my life.

"Success is the result of perfection, hard work, learning from failure, loyalty, and persistence."

~Colin Powell
ACKNOWLEDGMENTS

I would like to thank my thesis committee Preston T. Snee, Scott A. Shippy, Jordi Cabana, and Stephen J. Kron for their assistance and guidance. I would also like to thank all my friends, lab members in the Snee group, and all the UIC Chemistry Department faculty and staff for their endless support.
Contribution of Authors

Chapter 1 is a brief introduction of QD’s synthesis, properties, characterization and functionalization. Chapter 2 represents a published manuscript (A Toolkit for Bioimaging Using Near-Infrared AgInS$_2$/ZnS Quantum Dots”, A. Shamirian, O. Appelbe, Q. Zhang, B. Ganesh, S. J. Kron and P. T. Snee, Journal of Materials Chemistry B, 2015, 3 (41), 8188-8196) for which I was the primary author and major driver of the research. Oliver Appelbe and Qingbei Zhang generated Figure 13. Balaji Ganesh assisted me in the QD’s cytotoxicity studies. My research mentor, Dr. Preston Snee and our collaborator Dr. Stephen Kron contributed to the writing of the manuscript. Chapter 3 represents a published manuscript (In Vitro Detection of Hypoxia Using a Ratiometric Quantum Dot-based Oxygen Sensor”, A. Shamirian, H. Samareh Afsari, A. Hassan, L. W. Miller and P. T. Snee, ACS Sensors, 2016, DOI: 10.1021/acssensors.6b00452) for which I was the primary author and major driver of the research. Hamid Samareh Afsari generated Figures 22 and 25. Asra Hassan assisted by helpful discussions. My research mentor, Dr. Preston Snee and our collaborator Dr. Lawrence Miller contributed to the writing of the manuscript. Chapter 4 represents a published manuscript (Ratiometric QD-FRET Sensing of Aqueous H$_2$S in Vitro”, A. Shamirian, H. Samareh Afsari, D. Wu, L. W. Miller and P. T. Snee, Anal. Chem., 2016, 88 (11), 6050-6056) for which I was the primary author and major driver of the research. Hamid Samareh Afsari generated Figure 40. Donghui Wu generated figure 41. My research mentor, Dr. Preston Snee and our collaborator Dr. Lawrence Miller contributed to the writing of the manuscript. Chapter 5 represents a series of my own unpublished experiments directed towards calcium and
sodium ions sensing. I anticipate that this line of research will be continued in the laboratory after I leave and that this work might ultimately be published as part of a co-authored manuscript. Chapter 6 represents highlights and comprehensive conclusion of the previous chapters.
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List of Abbreviations

AAS  Atomic absorption spectroscopy
ATP  Adenosine triphosphate
BALB Bagg Albino (inbred research mouse strain)
CCD Charge-coupled device
DCC $N,N'$-dicyclohexylcarbodiimide
DCE 1,2-Dichloroethane
DCM Dichloromethane
DLS Dynamic light scattering
DMAP 4-(Dimethylamino) pyridine
DMEM Dulbecco’s modified Eagle’s medium
DSC $N,N'$-disuccinimidyl carbonate
EDC 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA Ethylene-diamine-tetraacetic acid
EtOAc Ethyl acetate
FBS Fetal bovine serum
FRET Förster resonance energy transfer
FWHM Full width half maximum
GC Gas chromatography
HEPES $N$-2-hydroxyethylpiperazine-$N'$-2-ethanesulfonic acid
IUPAC International union of pure and applied chemistry
JCPDS Joint committee on powder diffraction standards
LED Light emitting diode
MMTV Mouse mammary tumor virus
MPEG CD Methoxypolyethylene glycol carbodiimide
MRI Magnetic resonance imaging
MTT Methylthiazolyl diphenyl-tetrazolium bromide
MWCO
Molecular weight cut-off
NC
Nanocrystal
NHS
N-hydroxysuccinimide
NMR
Nuclear magnetic resonance
ODE
1-Octadecene
PAA
Poly(acrylic acid)
PAH
Polycyclic aromatic hydrocarbon
PL
Photoluminescence
PVC
Poly (vinyl chloride)
QD
Quantum dot
QY
Quantum yield
Sulfo-SMCC
Sulfo-succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate
TEA
Trimethylamine
TEM
Transmission electron microscopy
THF
Tetrahydrofuran
TODGA
N, N,N',N'-tetraoctyl-3-oxapentane-1,5-diamide
TOP
Trioctylphosphine
TOPO
Trioctylphosphate oxide
XPS
X-Ray Photoelectron Spectroscopy
XRD
X-Ray diffraction
SUMMARY

This thesis presents the synthesis and functionalization of quantum dots (QDs) for bioimaging and biosensing applications. QDs are very small (≤ 100 nm) inorganic crystalline semiconductors that possess size-tunable physical and optical properties. QDs provide a versatile platform for biosensing probes due to their strong absorption and efficient emission, as well as the many facile strategies for water solubilization and functionalization. Despite many remarkable demonstrations of their use in biosensing, there are concerns for the future application of these dots in clinical trials as most reported systems contain cadmium. Therefore, the development of cadmium free QDs has become topical; however, it should be noted that the toxicity of cadmium materials does not appear to be an issue even in animal studies.

In this thesis I present the synthesis of novel non-cadmium near-IR emissive AgInS$_2$/ZnS QDs. They were characterized using analytical techniques such as XPS, XRD, TEM, DLS, fluorescence and UV/Vis spectroscopy. The QDs were water-solubilized and functionalized to construct an oxygen sensing probe by conjugating water-soluble perylene-PEG-amine dye to the surface of the QDs. The response to the low oxygen levels (hypoxia) was investigated using an enzymatic oxygen scavenging system. In vitro studies were also conducted to demonstrate the sensor efficiency within a biological milieu.

A biosensor comprising of green emitting CdSe/ZnS QDs and rhodamine B dye linked by a disulfide bond was constructed to detect biological levels of H$_2$S, which is a gasotransmitter that is involved in many biological pathologies. The response mechanism is based on termination of FRET due to the reduction of the disulfide bond by the analyte.
The ratiometric response that was observed in HeLa cells is one of the best reported demonstrations of QD-based ratiometric sensing.

Two versions of a novel QD-based nano-optodes are reported for the detection of sodium and calcium ions based on a traditional bulk optode response mechanism. Use of QDs improved the sensitivity and the response time significantly, but the selectivity and the reproducibility of the methods were not suitable for further investigations.
1 Introduction

1.1 Introduction

Nanoscience and nanotechnology represent fascinating research topics that have attracted attention from different fields such as chemistry, physics, biology, and engineering. Among the many different nano-products that exist, nanostructured inorganic materials are of interest because of their extraordinary properties that bridge the gap between molecular properties and the macroscopic features of bulk materials. Nanostructures generally have a size in one or more dimensions of \( \leq 100 \) nm and are categorized as 1) two-dimensional (quantum wells), 2) one-dimensional (quantum wires), 3) zero-dimensional (quantum dots). Since their discovery in 1983,\(^1\) semiconductor quantum dots (QDs) have been in the spotlight due to their size-dependent optoelectronic properties and promising applications in various fields such as renewable energy, electronics, and bioimaging. Presently, the main topics of QD research include manipulation of physical and optical properties, the synthesis of different structures and types, the development of water solubilization methods and subsequent functionalization, and the demonstration of possible applications. These topics are discussed in this chapter.

1.2 Quantum Dots

Quantum dots are colloidal inorganic semiconductor nanocrystals with diameters less than 10 nm. They are capped with organic surfactant molecules (ligands), which
dictate their miscibility in a particular medium and prevent their agglomeration. Due to their smaller size compared to the bulk material, QDs have interesting physical properties. One of the most important is the ability to alter the band gap with size.\(^2\) In a bulk semiconductor, the absorption of a photon causes excitation of an electron (e\(^-\)) from the valence to the conduction band, leaving a hole (h\(^+\)) in the valence band. Since the Coulomb attraction prevents them from independent motion, they form e\(^-\)-h\(^+\) bound pair called an exciton. The radius of an exciton is determined by the charge carriers’ effective masses and the dielectric constant of the host, and varies from \(\sim 1 – 50\) nm.\(^3\) When the size of the exciton exceeds the size of the semiconductor crystal, an unusual phenomenon called “quantum confinement” occurs where charge carriers acquire higher kinetic energy that results in an increase in the band gap.\(^4\) Furthermore, the bulk bands are replaced by discrete levels that are more atomic-like. This phenomenon can be explained by quantum mechanical particle-in-a-sphere model.\(^5\)

1.3 Basic properties of quantum dots

1.3.1 Absorption

Quantum Dots absorb a photon if the energy exceeds the QD’s band gap. Their small size also increases the exciton binding energy. As a result, QDs have a relatively sharp absorption feature at the position of the 1\(^{st}\) excitonic transition, the position of which depends on the band gap and thus the size of the particles (Figure 1). Smaller particles have blue-shifted absorption features due to the quantum confinement effect.\(^6\) More information may be extracted from the absorption spectra such as the size distribution,
shape, and stoichiometry of the QDs. The first absorption feature is characterized by the Full Width Half Maximum (FWHM), which is influenced by the size distribution. Monodisperse samples exhibit a sharp and narrow feature with small FWHM while polydisperse samples have a broad absorption spectrum with a large FWHM. The relationship between a QD absorption and the concentration is complex. Unlike other compounds, the concentration of QDs cannot be determined by gravimetric methods due to the presence of an unknown number of organic ligands on their surface.\textsuperscript{7} Experimental determination of the size-dependent extinction coefficient of QDs is not a trivial task as it involves the use of transmission electron microscopy (TEM) for size measurements, and elemental analysis such as atomic absorption spectroscopy (AAS) for concentration determination. The correlation between the 1\textsuperscript{st} absorption feature’s wavelength, size, and the extinction coefficient ($\varepsilon$) for different size of QDs have been reported.\textsuperscript{8-10} Empirical mathematical functions are often derived to relate QD size and ($\varepsilon$) to a reasonable level of approximation.

1.3.2 Photoluminescence

Luminescence from photoexcited QDs occurs when both charge carriers relax to their lowest excited states, and subsequently recombine resulting in band-edge emission. The emission of QDs can be tuned by changing the particle size (Figure 1), since band gap of the QDs is size-dependent. The shape and width of the emission indicate the quality of the QDs since only samples with proper surface passivation with a narrow size distribution emit narrow and symmetrical peaks with high efficiency. The presence of
Figure 1. Absorbance and fluorescence emission of QDs.
impurities and defective sites on the surface of the QDs can create energy levels usually referred as “deep-levels” that trap charge carriers and result in non-radiative recombination.\textsuperscript{11} The FWHM of the emission band is indicative of size distribution of the QDs, where for the best CdSe samples the FWHM lies in the range of 15-25 nm. The emission peak maxima of the QDs are 10-20 nm red-shifted from the first absorption feature, which is described as the Stokes-shift and occurs due to the fine electronic structure of the exciton energy levels of QDs.

1.3.3 Fluorescence quantum yield (QY)

Quantum yield is a measurement of the emission efficiency that characterizes the ratio between the number of emitted photons to the number of absorbed photons. Usually, QD cores do not have high QYs, which is the consequence of quenching by surface traps. Therefore the quality of the QDs’ surface plays a major role in this case. The QY is improved significantly after overcoating by an inorganic shell that removes the trap sites by passivating the surface. The type of organic ligands that are capping the QDs also affect the photoluminescence (PL) properties of the QDs. More details on overcoating QDs are discussed in the synthesis section. The QY can be determined by comparing the integrated emission intensity of the QDs to that of a dye standard. The absorbance of both the QDs and the standard should be kept below 0.1 at the excitation wavelength. The QY can be calculated using the equation provided by IUPAC.\textsuperscript{12}

\[
QY = QY_s \frac{1 - 10^{-A_s}}{1 - 10^{-A}} \times \frac{n^2}{n_s^2} \times \frac{I}{I_s}
\]

Eq. 1.1
where $QY$ and $QY_s$ are quantum yields ($s$: standard), $A$ and $A_s$ are absorbances at the same excitation wavelength, $n$ and $n_s$ are the refractive indices of the solvents, and $I$ and $I_s$ are integrated emission areas of the QDs and standards. Inconsistency in QY measurements can occur due to the use of different approaches for the measurement, concentrations and the absorbances of samples and standards that are too high, the use of different excitation wavelengths, lack of overlap between the emission of the QDs and standard dyes, or instrumental errors such as light source fluctuations and wavelength shifts.

### 1.3.4 Core/shell structure and types

Surface engineering plays an important role in improving the optical properties of QDs. Passivation of QDs improves the fluorescence QY and enhances their photostability against oxidation. The most popular strategy in this regard is to grow an inorganic shell of another semiconductor over the core. The shell function is not always the same, depending on the band gap and electronic energy levels of the semiconductors used for the synthesis of cores and shells (Figure 2).\textsuperscript{13} If the band gap of material used for the shell synthesis is larger than the core, then both $e^-$ and $h^+$ are confined in the core resulting in a type-I QD.\textsuperscript{14} In type I QDs the shell’s role is to passivate the surface of the cores and to improve the optical properties by physically separating the cores from the surrounding medium. The shell also reduces the number of surface dangling bonds that would erstwhile act as traps for electrons and holes. Fluorescence QY and photostability of type-I QDs are generally improved due to overcoating. A red shift of both the first absorption
feature and the PL emission is expected due to partial leakage of the exciton into the shell material.

In type-II QDs the valence or conduction band of the shell is located in the band gap of the core resulting in a staggered band alignment that leads to spatial separation of the e\(^-\) and h\(^+\) in different regions of the core or shell (Figure 2).\(^{15}\) The net result of this structure is a smaller effective band gap for the QD than either the core or the shell materials. By controlling the shell thickness in type-II QDs a significant red shift in the PL emission can be achieved. Type-II QDs have longer fluorescence lifetimes due to the spatial separation of the charge carriers.

1.4 **Synthesis of QDs**

1.4.1 **Synthesis methods**

QDs were first discovered in a glass melt by Russian physicist Ekimov in 1981.\(^{16}\) However, systematic studies and advancements in the technology of QDs were driven after 1984 when Louis Bruce derived a relationship between the size and bandgap of QDs by application of a particle-in-a-sphere model.\(^3,17\) Since then numerous efforts have been made to synthesis QDs. Historically, QDs were prepared by chemical precipitation of nanoparticles within inverse micelles in aqueous media at room temperature.\(^{18-20}\) Although aqueous techniques were the first reported, their use results in samples with large size distributions. Overall, aqueous QDs are of low quality. It nearly took a decade for Murray *et al.* to introduce the “hot injection” method where precursors are rapidly added into a hot organic solvent (Figure 3).\(^{21}\) In general, the synthesis can be separated
Figure 2. Electronic properties of QDs. (A) Exciton and $e^-h^+$ recombination. (B) Energy diagram of different types of QDs. Reprinted with permission from Ref. 22 Copyright 2014 The Royal Society of Chemistry.
into nucleation and growth steps. Initially, a supersaturated solution of semiconductor precursors is generated upon injection into the hot organic solvent. At this point, the precursor’s concentration is above the nucleation threshold resulting in fast nucleation.$^2^3$ As the concentration of precursors drops due to consumption induced by nucleation and some subsequent growth, nucleation stops and the particles continue to grow as long as the system has precursors. Nucleation is also quenched by the fast cooling of the reaction mixture due to injection of the precursors and removal from the heat source. If particles growth continues under conditions of low (or no) precursor concentration, Ostwald ripening will occur.$^2^4$ In this process, small particles dissolve and redeposit on to larger ones due to the smaller particles' higher surface energy. Ostwald ripening usually widens the size distribution significantly, and therefore should be avoided.

1.4.2 Core/shell synthesis

Core surface passivation via an inorganic shell overcoating can reduce surface defects significantly and improve the QD’s photoluminescence properties.$^{2^5-2^7}$ The core/shell structure also improves the stability of the QDs and reduces toxicity by preventing the leaching of toxic elements, generally cadmium, from the cores. Generally, the shell material is chosen based on the crystal structure of the core and desirable energy level alignments. Epitaxial growth with minimum lattice mismatch between the core and shell materials is preferred.$^2^8$ Non-epitaxial shell growth can cause strain and defect formation at the core/shell interface, which will act as deep-trap states and reduce the QY.$^2^9$ The crystal structure and lattice parameters of selected semiconductor materials are listed in Table I.$^{3^0}$
Figure 3. Hot injection method for QD synthesis.
1.4.3 Doped and alloyed QDs

Doping and alloying QDs are alternative ways of altering the band gap without changing the size of the QDs. Alloying resulting ternary systems in which a fraction of the cations or anions is substituted resulting in a band gap in-between those of the binary systems.\(^{31-33}\) Doping is another approach in which small amount of “impurities” are introduced into the crystal structure.\(^{34}\) These impurities introduce local quantum states that perturb the band gap.\(^{35, 36}\) Both n-type or p-type doped QDs can be obtained depending on the type of the element, and opto-physical properties maybe affected by the quantity and position of the dopants.\(^{37}\)

1.5 Water-solubilization and functionalization of QDs

Although the aqueous synthesis of QDs results in instantly water soluble materials, the crystalline quality, quantum yield, and the emission bandwidth of the dots are sub-optimal. On the other hand, the synthesis of hydrophobic QDs in organic solvents imparts a higher quantum yield, enhanced monodispersity, and a narrower emission bandwidth.\(^{21}\) As such, I focus on hydrophobic as-prepared dots. One of the fundamental aspects of manufacturing QD-based sensors and biomarkers is water solubilizing the organic capped QDs and functionalizing them for conjugation to biologicals or organics such as dyes.

Transferring organic capped QDs into an aqueous solution and functionalizing them was a significant challenge for many years.\(^{38}\) Several water solubilizing methods have been developed over the two past decades (Scheme 1), with the majority falling under
<table>
<thead>
<tr>
<th>Material</th>
<th>Structure (300 K)</th>
<th>Type</th>
<th>$E_{\text{gap}}$ (eV)</th>
<th>Lattice parameter (Å)</th>
<th>Density (kg/m$^3$)</th>
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<td>4.136/6.714</td>
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<tr>
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Table I. Structural parameters of some bulk semiconductors. Reprinted with permission from Ref. 30 Copyright 2009 John Wiley & Sons, Inc.
the categories of micelle encapsulation, ligand exchange, or silanization. The water-soluble capping layer on the surface of the QDs usually possesses reactive groups that can be used for conjugation of target molecules using a variety of available chemistries. In most water solubilizing schemes, carboxylic acid groups are the part of the transfer agent that renders as-prepared hydrophobic QDs soluble in water at a physiological pH of 7.4 and engenders biological functionalization with proteins via amide bond formation. Generally, commercially-available carbodiimide crosslinking reagents such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) are used. Coupling efficiencies may be enhanced with the addition of N-hydroxysuccinimide (NHS) or sulfo-NHS. However, charge cancellation of anionic QDs with cationic species such as EDC can render the colloidal dots unstable, causing them to precipitate from water. This effect is mollified by either canceling out the electrostatics of either the dots or the reagents using poly(ethylene glycol), which prevents the loss of products and an enhancement of reaction yields. Sulfhydryl coated QDs may be functionalized using a reagent such as sulfosuccinimidyl-4-((N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) which is an amine-to-thiol cross-linker. Other conjugation approaches are based on biotin-streptavidin interactions or self-assembly of polyhistidine-tagged molecules onto the zinc-rich surface of the QDs.

1.6 Applications of QDs

During the last two decades, QDs have gained a lot of popularity in multidisciplinary areas of research such as light emitting diodes (LEDs), QD-based lasers, solar
cells, bioimaging, biosensing and drug delivery systems. The vast variety of applications are made possible due to the unique physico-chemical and optical properties of QDs such as good thermal and photostability, high fluorescence QYs, emission tunability, and easy functionalization via surface modification that offers advantages over traditional chromophores such as organic dyes.

In this thesis, I focus on the application of QDs as bioimaging and biosensing agents. I have developed protocols for the synthesis of high-quality non-toxic AgInS$_2$/ZnS near infrared (IR) QDs that were used to create an oxygen sensing probe. I also report a unique QD-based ratiometric sensor for detection of aqueous H$_2$S based on the modulation of Förster resonance energy transfer (FRET). The potential for use in biological applications was demonstrated by measuring the response from these QD-based ratiometric sensors after microinjection into live HeLa cells. I also demonstrate a novel design of ratiometric ion-selective nano-optode for the detection of sodium and calcium ions. This device is essentially a miniaturized version of a traditional ion-selective polymer matrix containing a pH indicator dye (chromoionophore) and an ion-binding molecule (ionophore) immobilized on the QDs.
Scheme 1. Cap-exchange and encapsulation methods for water-solubilization of QDs. (Adapted from Ref. 72 with permission, and reprinted with permission from Ref. 73, Copyright 2014 The Royal Society of Chemistry).
2 A Toolkit for Bioimaging Using Near--Infrared AgInS$_2$/ZnS Quantum Dots

The content of this chapter has been published as "A Toolkit for Bioimaging Using Near-Infrared AgInS$_2$/ZnS Quantum Dots", A. Shamirian, O. Appelbe, Q. Zhang, B. Ganesh, S. J. Kron and P. T. Snee, Journal of Materials Chemistry B, 2015, 3 (41), 8188-8196. Reproduced with permission from The Royal Society of Chemistry. Animal imaging was performed by Kron Group at The University of Chicago.

2.1 Introduction

Semiconductor quantum dots (QDs, or nanocrystals) have a large number of applications, especially for renewable energy, display technology, and biological imaging and sensing. Quantum dots have strong and broad absorption spectra and narrow size--tunable emission. As a result, QDs of different sizes can be excited by a single wavelength and emit in separate spectral regions to allow for the realization of multiplexed experiments within a biological milieu. Unlike organic fluorescent dyes, quantum dots are highly photostable due to their inorganic composition and surface passivation which allows for long--term imaging.

The first use of the now ubiquitous high quality CdSe/ZnS QDs as bioimaging tools was reported in 1998. Since then many types of colloidal quantum dots composed from groups II–VI (CdSe, CdTe) and III–V (InP, InAs) have been synthesized and applied for the same purpose. Due to potential toxicity issues for the most well--developed cadmium containing materials, there are concerns for the future application of these dots in clinical trials. An obvious solution is to develop less toxic QDs for biological applications. Among such alternatives, ternary I–III–VI quantum dots are of significant interest due to their long PL lifetimes and near--infrared emissions. They have high extinction coefficients and are overall excellent for in vivo imaging; furthermore, their long
emission lifetimes may be exploited to minimize background from tissue autofluorescence using time-gated imaging.\textsuperscript{88-90} As a result, the synthesis of several I–III–VI semiconductor nanocrystals, such as CuInS\textsubscript{2},\textsuperscript{91-95} CuInSe\textsubscript{2},\textsuperscript{96, 97} CuInGaSe\textsubscript{2},\textsuperscript{98} and AgInS\textsubscript{2}\textsuperscript{99-102} have been reported. These studies have revealed that the main challenge in the synthesis of I–III–VI semiconductors nanocrystals is to impart the correct stoichiometric ratio between the three elements by controlling the reactivity of the two cationic precursors.\textsuperscript{103} Xie \textit{et al.} balanced the reactivity of the precursors using alkane thiols ligands to control the reaction rates of group IB ions to obtain ternary quantum dots.\textsuperscript{103} As a result, they were able to synthesize high quality CuInS\textsubscript{2}/ZnS with a PL quantum yield of 30\% over a 500 nm to 950 nm range of emission. Preliminary results on the synthesis of AgInS\textsubscript{2} quantum dots were presented using the same strategy.

Among the I–III–VI semiconductors, AgInS\textsubscript{2} quantum dots are interesting materials due to reduced toxicity issues and near–IR band gap that ranges from 1.87 to 1.98 eV, depending on the underlying crystal structure.\textsuperscript{104-106} AgInS\textsubscript{2} quantum dots are direct band gap semiconductors with broad PL emission and large Stokes shifts, which could be explained by the “donor–acceptor pairs” model proposed by Krustok \textit{et al.}\textsuperscript{107} There are two major approaches for synthesis of AgInS\textsubscript{2} ternary QDs. One is the thermolysis of various metal–sulfur complexes or the direct reaction of metal cationic and sulfur precursors. Torimoto \textit{et al.} reported a method for the preparation of ZnS–AgInS\textsubscript{2} QDs based on pyrolysis of (AgIn)\textsubscript{x}Zn\textsubscript{2(1-x)}[S\textsubscript{2}CN(C\textsubscript{2}H\textsubscript{5})\textsubscript{2}]\textsubscript{4}, which resulted in QDs with an emission that can be tuned from 540 nm to 720 nm and a maximum quantum yield of 24\%.\textsuperscript{108} Another method is the direct reaction of metal precursors with elemental sulfur in the presence of dodecanthiol as demonstrated by Burda and Xie.\textsuperscript{103,109} They studied how
the surface and intrinsic trap states contribute to the emission of the dots. Chang et al. measured the effect of different variables such as the molar ratios of metal precursors (Ag:In), the concentration of dodecanthiol capping ligands, the reaction temperature, and the ZnS surface passivation on the luminescence properties of AgInS$_2$ QDs.$^{110}$ Tang et al. reported the diffusion of Zn in the crystal structure of preformed AgInS$_2$ QDs, which can be used as a strategy for tuning the emission wavelength.$^{111}$ They blue shifted the emission by increasing the temperature in the process of overcoating with ZnS. Burda and co–workers studied the mechanism of Ag to Zn cation exchange occurring during the shell growth.$^{112}$

The reports on ZnS coated AgInS$_2$ have shown that the addition of a higher bandgap shell results in a significant blue shift in the emission, which removes one of the motivations for synthesizing these intrinsically infrared-emissive materials to begin with. Burda and co-workers$^{101}$ tried to resolve this issue via manipulation of temperature and zinc concentration in the synthesis of core/shell of AgInS$_2$/ZnS. While successful, the quantum yield was not as high as reported for other synthetic methods that result in blue–shifted emission. As such, an efficient synthesis of high quantum yield yet near–IR AgInS$_2$ / ZnS QDs was developed as reported here. Furthermore, many known methods for water solubilizing and functionalizing CdSe/ZnS QDs were examined with AgInS$_2$ / ZnS QDs. This was done as we do not believe it is safe to assume that previous methods can be applied to new materials even if they share the same zinc sulfide surface. However, in the present study, I can demonstrate that both cap exchange and encapsulation render aqueous dispersions of AgInS$_2$ / ZnS QDs. Additional surface functionalization with PEG and organic dyes are reported, as well as results from in vivo imaging studies.
2.2 Experimental

2.2.1 Materials

Technical grade 1–octadecene (ODE, 90%), indium acetate (99.99%), silver nitrate (>99.8%), zinc chloride (≥98%), cesium carbonate (99%), (3-mercaptopropyl)trimethoxysilane (95%), oleic acid (90%), dodecylamine (≥98%), sulfur powder (99.98%), trioctylphosphine oxide (TOPO) (90%), and 1–dodecanthiol (≥98%) were purchased from Sigma–Aldrich. Trioctylphosphine (TOP, 97%) was purchased from Strem. Zinc acetate (99%) and n–decylamine (99%) were purchased from Acros. Stearic acid (≥97%) was purchased from Fluka. 4–(N–Maleimidomethyl)cyclohexane–1–carboxylic acid 3–sulfo–N–hydroxysuccinimide ester sodium salt (Sulfo–SMCC) was purchased from Thermo Scientific–Pierce (≥90%). Oleic acid was purified for all syntheses discussed below using the protocol of Ref. 113, see also Ref. 114 for a video tutorial. All the other chemicals were used without further purification. 40% octylamine–modified poly(acrylic acid) (PAA) was prepared according to Refs. 115 and 116. Methoxypolyethylene glycol 750 amine was prepared according to Ref. 117. Methoxypolyethylene glycol 350 carbodiimide (MPEG 350 CD) was prepared according to Ref. 118. AminoPEG–pyrene chromophores were prepared according to Ref. 119. Rhodamine B piperazine was prepared according to Ref. 120. Chloroform was dried using activated molecular sieves (3 Å, 3.2 mm pellets) from Sigma–Aldrich.
2.2.2 **Synthesis of AgInS$_2$ nanocrystals**

This procedure is adapted from Ref. 103. A 3–neck 25 mL glass round–bottom flask was loaded with 0.085 g (0.5 mmol) silver nitrate, 0.146 g (0.5 mmol) indium (III) acetate, 0.5 mL (1.58 mmol) oleic acid, 0.5 g (1.75 mmol) stearic acid, and 10 mL of 1–octadecene. The solution was degassed at 90˚C, and then 1.2 mL 1–dodecanthiol was added under N$_2$ flow. The solution was heated to 125˚C, after which a small portion of TOP (~ 0.2 mL) was added into the solution. This was found to improve the solubility of the resulting AgInS$_2$ quantum dots. A sulfur precursor was prepared by dissolving 0.048 g (1.5 mmol) of sulfur powder in 1.21 g of dodecylamine and degassing under vacuum. This was swiftly injected into the 3–neck flask while rapidly stirring, which was maintained at ~125 ºC for 20 min. Afterward, the heating mantle was removed and the solution was allowed to cool to room temperature. The samples were stored under ambient conditions.

2.2.3 **Preparation of a zinc precursor for ZnS overcoating**

A 0.04 M zinc acetate stock solution was prepared by the procedure of Zhu et al.$^{121}$ First, 0.0734 g (0.4 mmol) of zinc acetate was mixed with 0.5 g of TOPO, 2 mL of TOP, 0.2 mL of oleic acid, and 8 mL of ODE. This mixture was heated to 225˚C to obtain a clear solution, which remained transparent after cooling to room temperature.

2.2.4 **Synthesis of AgInS$_2$/ZnS nanocrystals**

Half of a batch of AgInS$_2$ dots prepared as described above were processed by precipitation via the addition of ethanol. The supernatant was discarded and the core QDs were dispersed in hexane. A 4–neck 50 mL glass round–bottom flask was loaded with 5 mL of ODE and 4 mL of TOP. The solution was vacuum degassed at 80 ºC and then
flushed with N$_2$. The core QDs were added and hexane was subsequently removed under vacuum over the course of 1 h. A sulfur precursor was prepared by dissolving 0.005 g (0.15 mmol) of sulfur powder in 2.8 mL of n–decylamine. This solution was injected into the 4–neck flask and stirred for 30 min while maintaining an 80 °C temperature. The temperature was increased to 140 °C and 1 mL of the zinc precursor solution diluted with 2 mL of ODE added slowly through an addition funnel over the course of ~20 min.

### 2.2.5 Water solubilizing using 40% octylamine–modified poly(acrylic acid) polymer

Excess ethanol was added to 0.5 g of the as–prepared AgInS$_2$/ZnS QDs to induce flocculation. The supernatant was discarded, and the precipitate was dried under vacuum. Next, 30 mg of 40% octylamine–modified PAA was added to the QDs followed by ~3 mL chloroform. The resulting solution was sonicated for several minutes to dissolve the polymer completely. The solvent was removed under vacuum and 0.1 M NaOH was added to disperse the QDs into water. The excess amount of polymer was removed by dialysis using Amicon Ultra centrifugal filters (100,000 MWCO) from Millipore. D.I. water was used as the diluent.

### 2.2.6 Water solubilizing by silane coating of QDs

As–prepared AgInS$_2$/ZnS QDs were processed following the same procedure as described above. After drying the precipitated materials, ~2 mL of dichloromethane was added to dissolve the QDs followed by 50 µL (0.27 mmol) of (3–mercaptopropyl)trimethoxysilane, 9 mg (0.067 mmol) of zinc chloride, and 88 mg (0.27 mmol) of cesium carbonate. The mixture was stirred overnight. The next day the insoluble
cesium carbonate and other by-products were removed by centrifugation. Hexane was then added to the colored yet clear supernatant to induce precipitation. The precipitate collected by centrifuge and dried under ambient conditions. Two drops of methanol were added to the dried precipitate, and then 0.1 M aqueous NaOH was added. The sample was stirred overnight which generally resulted in QDs fully dispersed in basic water. The sample was dialyzed and diluted by D.I. water using a dialysis tube (Float–A–Lyzer G2, 100 kD MWCO, from Spectrum Labs) to a neutral state.

2.2.7 **PEGylation of water solubilized QDs**

Approximately 1 mL of polymer–encapsulated water solubilized AgInS$_2$/ZnS QDs was mixed with 10 mg of MPEG 350 CD and was stirred for 15 min. Next, 15 mg of MPEG 750 amine was dissolved in D.I. water and was subsequently acidified to pH 8 with additional phosphate buffers. This solution was added to the quantum dots, and the subsequent pH was adjusted to ~8 by addition of phosphate buffers. Note the solution should never be allowed to have a pH > 9. The mixture was stirred overnight and the next day the solution was dialyzed to remove excess amount of MPEG 750 amine and MPEG 350 CD using Amicon Ultra centrifugal filters (100,000 MWCO) from Millipore.

2.2.8 **Reaction yield for functionalizing water soluble QDs**

As the reaction yield of PEGylation is difficult to determine accurately, AgInS$_2$/ZnS QDs were functionalized with organic dyes to evaluate the efficiency of coupling organic species to their surface. In this endeavor, 10 mg of MPEG 350 CD was added to 1 mL of polymer–encapsulated water solubilized AgInS$_2$/ZnS QDs and was stirred for 15 min. A solution of an amine functional dye, either pyrene-PEG-amine or Rhodamine B
piperazine, in pH 8 phosphate buffer was prepared and was added to the activated QDs solution. Generally, dye was added until a relatively equal emission was observed from the dye and quantum dot under excitation with a black light. The pH of the solution was adjusted to 8 and was stirred overnight. The next day the solution was dialyzed using Amicon Ultra centrifugal filters (100,000 MWCO) from Millipore.

Silane–coated AgInS$_2$/ZnS QDs were activated using sulfo–SMCC by dissolving 1 mg of this activator in 0.5 mL of pH 6 phosphate buffer followed by adding 1 mL of silane–coated water soluble QDs. The pH of the solution was adjusted to 7 by adding 0.1 M NaOH and was stirred for 30 min. Next, the solution was run through desalting column to remove excess sulfo–SMCC. A solution of amine functional dye (e.g. Rhodamine B piperazine)$^{120}$ in pH 8 phosphate buffer was added to the activated QDs and was stirred overnight. The next day the solution was dialyzed using a dialysis tube (Float–A–Lyzer G2, 100 kD MWCO, from Spectrum Labs).

2.2.9 In vivo studies

BALB-neuT is a transgenic mouse line bearing the rat Her2/neu gene under control of an MMTV promoter$^{43}$ that serves as a model for sporadic breast cancer. BALB-neuT mice develop multifocal mammary tumors in all ten mammary glands by 30 weeks of age. For these studies, one tumor-bearing mammary gland was irradiated with 15 Gy X-irradiation three days prior to imaging to enhance vascular permeability. Fluorescein-conjugated tomato lectin was purchased from Vector Laboratories. For imaging studies, mice were anesthetized by inhalation of isoflurane gas or i.p. injection of ketamine (90 mg/kg) and xylazine (10 mg kg$^{-1}$). A Leica SP5 Tandem Scanner Spectral 2-Photon
confocal microscope was used for intravital imaging. Skin flap surgery was used to expose tumor tissue and blood vessels while also avoiding any autofluorescence from the skin. Exposed tumors were placed in a glass–bottom petri dish filled with 1× PBS and the dish was placed on the microscope stage for imaging. Fluorescence was imaged with a 20× objective lens plus digital zoom using the 458 nm excitation laser and differing emission spectrum for fluorescein (500-541 nm) and QDs (680-781 nm). All animal studies were performed in compliance and with the approval of the University of Chicago Institutional Animal Care and Use Committee, ACUP# 70931 and 72354.

2.2.10 Characterization

Optical spectra (absorbance) of the samples were measured using a Varian Cary 300 Bio UV/vis spectrophotometer and photoluminescence spectra were obtained using a custom–designed Fluorolog from HORIBA Jobin–Yvon. Transmission electron microscopy (TEM) measurements were obtained using a JEOL JEM–3010 operating at 300 keV. X–ray analyses were performed on D8 Advance ECO Bruker XRD diffractometer using monochromatized Cu Kα (λ=1.54056 Å) radiation. FisherBiotech™ FB –SB –710 horizontal electrophoresis system with FB300 power supply has been used for gel electrophoresis studies. XPS analyses were performed on a monochromatic Al Kα source instrument (Kratos, Axis 165, England) operating at 12 kV and 10 mA for an X–ray power of 120 W. Spectra were collected with a photoelectron takeoff angle of 90° from the sample surface plane, energy steps of 0.1 eV, and a pass energy of 20 eV for all elements. All spectra were referenced to the C 1s binding energy at 284.8 eV.
2.3 Results and discussion

2.3.1 Synthesis and characterization of AgInS$_2$ cores

AgInS$_2$ QDs have a ternary composition. Unfortunately, this adds difficulty in their synthesis as binary QDs will form if the reactivities of two cationic precursors are not balanced. Concerning AgInS$_2$, silver is generally the more reactive reagent and Ag$_2$S QDs may be exclusively synthesized upon injection of sulfur. Based on the work of Xie et al., 1-dodecanethiol ligands were employed to suppress the reactivity of Ag$^+$ while fatty acids were used to control the reactivity of In$^{3+}$. The metal and ligands were mixed and degased, after which a sulfur solution in dodecylamine was injected. The dots were grown briefly at 120 °C and were stored under ambient conditions for characterization and subsequent overcoating.

Several methods of characterization were employed. The atomic ratio was found to be Ag$_{1.0}$In$_{1.0}$S$_{2.1}$ by XPS as shown in Figure 4, which is in good agreement with the expected formula. I conjecture that the slightly higher ratio for sulfur is due to bonding of excess thiol ligands to the surface of QDs. The powder X–ray diffraction shown in Figure 5 confirms that the sample has a pure orthorhombic crystal structure (JCPDS 00–025–1328). Transmission electron microscopy (TEM) micrographs show that QDs are mostly spherical and single–crystalline particles with a size distribution of 3.9 ± 0.8 nm in diameter. The UV–Vis absorption spectrum of AgInS$_2$ cores shown in Figure 6 does not display excitonic features commonly seen in type II–VI and III–V quantum confined materials. This is generally observed for ternary QDs. The optical band gap of the synthesized AgInS$_2$ cores was calculated to be 1.86 eV by extrapolation of the linear
portion of the Tauc plot shown in Figure 7. AgInS$_2$ core QDs have broad emission that could not be narrowed by our attempts at size selection.

![Figure 4. XPS survey spectrum of core AgInS$_2$ and core/shell AgInS$_2$/ZnS QDs. Reprinted with permission from Ref. 122 Copyright 2015 The Royal Society of Chemistry.](image)
Figure 5. XRD patterns of AgInS$_2$ cores and overcoated AgInS$_2$/ZnS quantum dots. Reprinted with permission from Ref. 122 Copyright 2015 The Royal Society of Chemistry.

Figure 6. Absorption and emission spectra of core and core/shell QDs. Inset: A photograph of AgInS$_2$/ZnS in hexane under UV excitation. The camera exposure time makes the sample appear brighter than observed visually. Reprinted with permission from Ref. 122 Copyright 2015 The Royal Society of Chemistry.
This is likely due to the fact that the broad emission is intrinsic to the material as discussed by Hamanaka et al. They proposed that broad emission spectrum is due to donor–acceptor pair recombination as evident from the excitation intensity dependence of the emission spectrum and the observed shift in the time–resolved photoluminescence spectrum. The emission spectrum of the AgInS$_2$ cores followed by a tail off that I attribute to surface defects. As a result, the red tailing portion of the emission spectrum was removed using multiple Gaussian function fitting for quantum yield measurements, see Figure 8 a,b. Quantum yields of the core AgInS$_2$ quantum dots have been measured using Rhodamine 101 and IRDye®800CW dyes as standards, see Figure 8 c,d. Over the course of this study, the range of quantum yield of different batches of core QDs in hexane varied over 4–10%.
2.3.2 Synthesis and characterization of AgInS$_2$/ZnS QDs

The challenge with overcoating near–IR emissive AgInS$_2$ cores with zinc sulfide is to prevent the significant blue shift in emission that results from diffusion of Zn from the shell structure into the core and cation exchange of silver by zinc. Tang et al. showed that the emission of the AgInS$_2$ QDs can be tuned by incorporation of different amounts of zinc at different temperatures.$^{111}$ Burda and co-workers studied the partial Ag–to Zn cation exchange in AgInS$_2$/ZnS QDs.$^{112}$ They showed that the extent of zinc diffusion and consequently blue shift in the emission of the QDs depends on the zinc concentration, temperature, and duration of the overcoating process. Our observations are consistent with these conclusions in that at overcoating at higher temperatures (>180˚C) results in green emitting (525 nm) materials. Thus, a lower temperature needs to be employed; however, coating a core QD often requires elevated temperatures because of the low solubility of the precursors. Colvin and co-workers showed that ZnS shell can be generated at low temperature by improving the solubility of reactive precursors.$^{121}$ They successfully overcoated CdSe QDs by ZnS at temperature range of 65–180˚C. I modified Colvin’s method for overcoating AgInS$_2$ QDs at 140˚C using a n-decylamine / sulfur solution and a mixture of TOP, TOPO, oleic acid, and zinc acetate. Subsequent characterization demonstrated the success of this procedure. The emission spectrum of overcoated QDs is only slightly blue shifted compared to the core, but remains in the near IR region as shown in Figure 6. Specifically, the band gap of the overcoated QDs was calculated to be 1.83 eV (see Figure 7), slightly red-shifted compared to the core. Most telling is the fact that the quantum yield of the QDs improved after overcoating, with a variance of 11–28% for samples produced during this study.
Figure 8. Quantum yield calculations and fittings. (a, b) QY measurements were calculated by Gaussian decomposition of the spectrum. The red tail of the emission was excluded when calculating the quantum yields. (c) An example of the emission spectra of samples and Rhodamine 101 dye standard used to determine the quantum yields. (d) An example of the emission spectra of AgInS$_2$ and IRDye® 800CW dye used to determine the quantum yield. This dye is not a good standard for AgInS$_2$/ZnS samples. Reprinted with permission from Ref. 122 Copyright 2015 The Royal Society of Chemistry.
XPS analysis shown in Figure 4 revealed the ratio of atoms in the overcoated QDs to be $\text{Ag}_{1.0}\text{In}_{1.0}\text{S}_{2.8}\text{Zn}_{0.2}$. The XRD pattern (see Figure 5) for overcoated QDs is almost the same as cores likely as the orthorhombic AgInS$_2$ spectrum is very similar to cubic ZnS (JCPDS 00–005–0566). The TEM image shown in Figure 9 reveals that size distribution for overcoated QDs is $4.0 \pm 0.4$ nm. As this is only slightly larger than the cores, it is possible that zinc diffusion and alloying still occurs in the procedure described here.

Figure 9. TEM image and size distribution. (a) A TEM image of AgInS$_2$/ZnS QDs reveals round, crystalline materials and (b) the distribution of particle diameters fitted with a Gaussian function show that the average size is $4.0 \pm 0.4$ nm. Reprinted with permission from Ref. 122 Copyright 2015 The Royal Society of Chemistry.
2.3.3 Water solubilization of quantum dots

There are two major approaches to water solubilize QDs. One simple method is to encapsulate the QDs into amphiphilic phospholipid or polymer micelles by mixing them with these surfactants in a common solvent. Encapsulation occurs because of the hydrophobic interaction between the polymer and organic caps of the QDs. This method has several advantages, including water solubilization of QDs without (or minimal) damage to the original surface ligands. This usually results in a better retention of the photoluminescence properties after transferring to water. In our experience, the chemical stability in an aqueous environment is such that the dots may stay soluble indefinitely. Last, functionalizing QDs may be enhanced by adding linkable groups to the polymer before coating the nanocrystals. There are different types of modified polymers for encapsulating QDs, and for the purposes of this study, 40% octylamine modified polyacrylic acid was used.\textsuperscript{124} It was found that there was no observable loss of materials in the phase transfer process although the quantum yields of the dots decreased by ~60\% after solubilization. The range of quantum yields of water soluble QDs has been measured to be 4–10\% for polymer encapsulated QDs. At the time of this writing, no materials have been observed to precipitate (~36 months).

Another water solubilization method is based on ligand exchange on the surface of the QDs, in which the original caps have been replaced by chemical species that impart new solubilities. There are different examples of ligand exchange methods in the literature,\textsuperscript{125-127} but many suffer from lack of long term stability and loss of photoluminescence.\textsuperscript{128} Recently our group developed a new ligand–exchange method which produces a monolayer of silane coated QDs.\textsuperscript{129} These cap exchanged dots can be
transferred into water and may remain soluble for ~3 months under ambient conditions. The photoluminescence properties can be comparable and sometimes better than that observed with polymer encapsulated QDs. Quantum yields of the water–soluble QDs have been measured to 4.5–11% for silane–coated QDs.

2.3.4 PEGylation of QDs

Water solubilization is the first step to realize biological applications with QDs. Often imaging studies use QDs coated with polyethylene glycol (PEG) to minimize QD aggregation, enhance salt stability, passivate against protein binding and increase solubility in serum or blood.\textsuperscript{40,130-132} Generally less negatively charged dots are preferred. In this study AgInS\textsubscript{2}/ZnS QDs were water solubilized using 40% octylamine–modified polyacrylic acid to make anionic dots as confirmed by gel electrophoresis. Next, they were conjugated PEG 750 amine\textsuperscript{133} to the surface of the QDs using a polyethylene glycol carbodiimide crosslinking agent.\textsuperscript{118} To investigate the effect of PEG conjugation on the surface charge of the QDs, gel electrophoresis studies were conducted to compare the zeta potential of the QDs before and after conjugation with PEG. The electrophoretic mobility can be calculated by dividing the electrophoretic velocity (migration distance/time) by the electrophoretic potential (voltage applied/gel distance). The zeta potential can be estimated from the electrophoretic mobility using the equation derived by Henry:\textsuperscript{134}

\begin{equation}
U = \frac{v \times L}{V}
\end{equation}

\begin{equation}
\xi = \frac{4\pi\eta U}{\varepsilon}
\end{equation}
where $U$ is electrophoretic mobility, $v$ is speed of the particle, $V$ is the applied voltage, $L$ is the gel distance, $\varepsilon$ is dielectric constant, and $\eta$ is the viscosity of the medium, and $\zeta$ is the zeta potential. As a result, the zeta potential is proportional to the length that a particle travels in a gel. In the present study, the PEGylated QDs migrated less distance compared to the unmodified dots as shown in Figure 10, which allows us to determine that the zeta potential of PEGylated QDs is 36.4% less than non–PEGylated version. Although somewhat indirect, this is evidence of the successful coupling of PEG to the surface of the dots. These materials were also used in biological imaging studies as discussed below.

2.3.5 **Functionalizing QDs**

QDs can be used in analytical applications for chemical and biological sensing, when their photoluminescence properties can be modulated in response to the presence of a specific analyte. Generally, this requires linking the dots to a second, analyte-responsive chromophore, to observe fluorescence resonance energy transfer (FRET), FRET efficiency modulation may be used for sensing purposes. This requires further surface functionalization of the dots usually with organic dyes as energy transfer–based sensing necessitates close proximity of the coupled chromophores. There are multiple crosslinking chemistries available that can be exploited to do so, and different types of bonds can be formed as a result. This is generally dependent on the water solubilization method and the chemical functionalities available in the sensing chromophore. I employed two strategies to create organic dye–water soluble AgInS$_2$/ZnS QDs coupled chromophores. These experiments also allowed me to characterize the reaction yields
Figure 10. Gel electrophoresis in 0.5% agar gel using pH 8 phosphate buffer at an applied voltage of 80 ± 2 volts across a gel distance of 10 cm for 45 min. Reprinted with permission from Ref. 122 Copyright 2015 The Royal Society of Chemistry.

Figure 11. Dye conjugation yield calculation based on absorbance spectra before and after dialysis. (a) Pyrene-PEG-amine conjugated to polymer encapsulated QDs. (b) Rhodamine B piperazine conjugated to silane coated QDs. Reprinted with permission from Ref. 122 Copyright 2015 The Royal Society of Chemistry.
which I was not able to do with the PEGylation experiments. First, polymer encapsulated water soluble QDs with carboxylic acid surface groups were conjugated to amine functional organic dyes using polyethylene glycol carbodiimide. Specifically, a NH$_2$–PEG–pyrene chromophore was synthesized and conjugated to 40% octylamine modified polyacrylic acid encapsulated AgInS$_2$/ZnS dots. The reaction yield can be calculated by comparing absorbance spectra before and after dialysis, which was found to be 60% as shown in Figure 11a. Dual emission from simultaneous direct excitation of the coupled chromophores is observed in Figure 12a. This system may also function as a ratiometric sensor for oxygen as pyrene dye is O$_2$ sensitive.$^{137}$

As silane–coated water soluble AgInS$_2$/ZnS QDs have thiol surface functional groups, the thiol–to–amine crosslinker sulfo–SMCC was used to attach an amine functional dye. Rhodamine B piperazine dye$^{138}$ was conjugated to the silane–coated AgInS$_2$/ZnS QDs a model for this type of the chemistry. A 95% reaction yield was determined from the absorbance spectra before and after dialysis as seen in Figure 11b. Dual emission is also observed in this coupled chromophore (see Figure 12b), which may be further functionalized to create quantum–dot based protein sensors as we recently demonstrated.$^{139}$

2.3.6 In vivo studies

To examine the potential capabilities of encapsulated and PEGylated AgInS$_2$/ZnS QDs as tools for in vivo imaging, BALB-neuT mice bearing spontaneous mammary tumors were injected with QDs along with fluorescein-conjugated tomato lectin by tail-
Figure 12. Emission spectra of the dye-linked QDs. (a) Emission spectrum of pyrene–functionalized polymer encapsulated AgInS₂/ZnS QDs. (b) Emission spectrum of Rhodamine B piperazine attached to cap exchanged AgInS₂/ZnS QDs. Insets: cartoons of the dot–dye coupled chromophores. Reprinted with permission from Ref. 122 Copyright 2015 The Royal Society of Chemistry.

Figure 13. *In vivo* studies. Quantum dot circulation in microcirculation in BALB-neuT mouse spontaneous mammary tumors at different time intervals. Reprinted with permission from Ref. 122 Copyright 2015 The Royal Society of Chemistry.
vein injection, 3 days after irradiation of one mammary gland tumor. Using the tomato lectin to visualize endothelial cells lining the tumor microvasculature, distribution of QD's in the irradiated tumor was monitored at intervals by intravital fluorescence imaging as shown in Figure 13. QDs were observed flowing freely through tumor blood vessels at 20 min. Within 40 min, QDs could be observed extravasating into the tumor tissue, consistent with a loss of microvascular permeability induced by radiation. By 80 min, fluorescence in the circulating blood decreased, likely reflecting elimination via kidney excretion and/or uptake by the reticuloendothelial system, but fluorescence in the tumor persisted. These data demonstrate the potential of tracking of perfusion and delivery of QDs as reporters of macromolecule/nanoparticle delivery to tumors.

2.4 Conclusions

Near infrared AgInS$_2$ QDs were synthesized by balancing the reactivity of silver and indium using 1–dodecanethiol and oleic acid. Core dots were overcoated with ZnS using precursors which are reactive at lower temperatures. Overcoating the QDs in this way resulted in significantly less blue shifting in emission compared to previously reported methods, which is likely due to suppression of zinc diffusion as a result of the lower temperatures employed. A three–fold improvement in quantum yield was observed after overcoating which confirms the passivation of the surface of the QDs. Two water solubilizing methods were successfully employed to transfer the QDs into aqueous solution. Different functionalizing approaches were demonstrated, and amine–functional dyes were attached to QDs that may act as ratiometric sensors and also stand in as
models for protein conjugation. *In vivo* imaging studies were conducted to determine the optimum conditions for bioimaging with PEGylated dots, which reveal that the photostability of the water soluble QDs is such that NIR emission is observable over the course of several days. Furthermore, the dots have distinctive behavior compared to other known imaging agents in terms of their localization within murine models.

I hope that this report will help guide researchers in the use of II–III–V NIR emitting semiconductor quantum dots for biological imaging and chemical sensing purposes. A method to circumvent the problem while retaining NIR emission in AgInS$_2$/ZnS nanocrystals is presented, as well as their use for biological imaging and possible chemical sensing. Fortunately, known methods for water solubilization and functionalization of CdSe/ZnS QDs were found to be effective in the case of AgInS$_2$/ZnS dots. However, one motivation for this study is that it is not wise to assume that previously developed procedures will translate to new systems, especially for nanomaterials.
3 In vitro Detection of Hypoxia using a Ratiometric Quantum Dot-based Oxygen Sensor

The content of this chapter has been published as “In Vitro Detecion of Hypoxia Using a Ratiometric Quantum Dot-based Oxygen Sensor”, A. Shamirian, H. Samareh Afsari, A. Hassan, L. W. Miller and P. T. Snee, ACS Sensors, 2016, DOI: 10.1021/acssensors.6b00452. Reproduced with permission from The American Chemical Society.

3.1 Introduction

Molecular oxygen plays a vital role in a broad range of chemical and biological reactions. While reactive oxygen species are toxic to all organisms, O2 is none-the-less necessary for ATP generation in aerobic cells and for several other physiological functions. Low O2 concentrations results in hypoxia, which is observed in tumor tissues due to the fact that cancer cells rapidly consume oxygen as they produce energy via a high rate glycolysis (i.e. the “Warburg Effect”). Significant efforts have been devoted to develop of reliable techniques for the detection of oxygen as O2 levels are indicative of the physiological behavior of living systems. Furthermore, the quantification of oxygen is an indispensable part of environmental analysis, food packaging, and industrial monitoring.

Oxygen has a high diffusion rate and a limited temperature-dependent solubility in aqueous solutions. These properties complicate sampling and necessitate real-time measurements. Various oxygen detection techniques have been reported based on chemical (Winkler titration), electrochemical (Clark electrode), and instrumental (GC or MRI) methods. Many of these suffer from drawbacks such as relatively long response times or a lack of consistency, accuracy, or reversibility. Worst
of all is the potential consumption of oxygen during the detection process. Recently, optical methods have attracted attention since they offer reversible responses with no electrical interferences and generally do not annihilate the analyte. Furthermore, they are inexpensive and easy to miniaturize, and may be used in a relatively noninvasive manner.\textsuperscript{153-155}

In this chapter, I report a probe for the \textit{in vitro} detection of oxygen based on non-toxic near IR-emissive quantum dots (QDs) coupled to a novel perylene dye derivative. This platform offers several advantages over other optical reporters. Specially, the use of near-IR emissive quantum dots reduces photobleaching and enhances the signal for through-tissue imaging. I have also avoided incorporating ubiquitous CdSe/ZnS QDs in favor of cadmium-free AgInS\textsubscript{2}/ZnS nanocrystals to mitigate issues with toxicity. As the supermolecular size and surface passivation of core/shell quantum dots removes their environmental sensitivity, the nanocrystals were conjugated to an oxygen-sensitive perylene dye derivative. The result is an O\textsubscript{2} sensor that has a ratiometric fluorescent response that is fully quantitative and eliminates problems with light source fluctuations and emission scattering that are issues with single-response “turn-on” or “turn-off” probes. Finally, I demonstrate that the sensor may be used to detect hypoxia in the cytosol of live HeLA cells.
3.2 Experimental section

3.2.1 Materials and instrumentation

Perylene (≥99.5%), N,N'-dicyclohexylcarbodiimide (DCC, 99%), N-hydroxysuccinimide (98%), triphenylphosphine (99%), hydrazine monohydrate (≥98%), 1,2-dichloroethane (DCE, ≥99%), titanium (IV) chloride (TiCl₄, ≥99%), xylenes (≥98.5%), diethylene glycol (≥99%), trimethylamine (TEA, ≥99%), ethyl acetate (EtOAc, 99.8%), dichloromethane (DCM, ≥99.8%) and hydrochloric acid (HCl, 37%) were purchased from Sigma-Aldrich. Sodium sulfate (anhydrous, >99%), potassium hydroxide (≥85%) and tetrahydrofuran (THF, 99.9%) were purchased from Fisher Chemicals. Succinic anhydride (99%) was purchased from Acros Organics. Where indicated, solvents were dried using activated molecular sieves (3 Å, Sigma-Aldrich). The QD-functionalization reagent methoxypolyethylene glycol 350 carbodiimide (MPEG 350 CD), and 40% octylamine-modified poly(acrylic acid) (PAA) were prepared according to previously published protocols.⁵²,¹⁵⁶ The precursor N₃-PEG₄₀₀-amine was prepared according to Ref. 157. NMR spectra (¹H and ¹³C) were recorded on a Bruker Avance DRX 400 NMR spectrometer. UV/vis absorbance spectra were measured using a Varian Cary 300 Bio UV/vis spectrophotometer, and fluorescence emission spectra were obtained using a custom-designed Horiba Jobin Yvon FluoroLog spectrophotometer. Oxygen levels in aqueous solutions were measured using a SevenGo pro™ dissolved oxygen meter SG6 with inLab® 605 O₂ sensor from Mettler Toledo. HeLa cells (CCL-2) were purchased from American Type Culture Collection. Dulbecco’s modified eagle medium (DMEM, 10-014 CV) and 0.25% trypsin/2.21 mM EDTA were purchased from Corning Cellgro®. MEM non-essential amino acids (11140) and HEPES (15630-080) were purchased from
Gibco®. Micropipette preparation glass bottom culture dishes (P50G-1.5-14-F) were purchased from Matek Corporation (Ashlan, MA). XenoworksTM Microinjection Systems, P-1000 pipette puller and borosilicate glass tubes (BF100-78-10) were used for microinjections (Sutter Instruments, Novato, CA).

3.2.2 Perylene-PEGamine synthesis

The synthesis of water-soluble perylene-PEGamine dye is outlined in Scheme 2 and is detailed below.

3.2.3 4-oxo-4-(perylene-3-yl)butanoic acid (1)

Perylene (0.252 g, 1 mmol) and succinic anhydride (0.152 g, 1.5 mmol) were dissolved in dry DCE (5 mL). The solution was kept stirring under a nitrogen atmosphere and was immersed in an ice bath. A solution of TiCl₄ (0.15 mL, 1.3 mmol) in 1 mL of DCE was added dropwise to the solution, and the mixture was stirred overnight at room temperature. The next day the product hydrolyzed by adding dilute HCl and the precipitate was collected by filtration. The product was extracted into EtOAc (20 mL) and washed with DI water (3×15 mL). The organic layer was dried over anhydrous Na₂SO₄ and the solvent was removed under reduced pressure. The solid brown substance was recrystallized from 25 mL of xylene after heating to 140 °C. The brown powder was separated by centrifuge and dried under reduced pressure (61% yield). NMR is shown in Figure 14.
Scheme 2. Perylene-PEGamine synthesis and conjugation to QDs. Reprinted with permission from Ref. 158 Copyright 2016 American Chemical Society.
3.2.4 **4-(perylene-3-yl)butanoic acid (2)**

4-oxo-4-(perylene-3-yl)butanoic acid (0.177 g, 0.5 mmol), KOH (0.224 g, 4 mmol), and hydrazine monohydrate 98% (0.24 mL, 5 mmol) were dissolved in 5 mL of diethylene glycol, followed by 90 minutes reflux at 180°C. After 1.5 h the excess water and hydrazine were drained from condenser allowing the temperature to rise to 200°C, and the refluxing was continued overnight. The next day the solution was poured into ice bath and acidified to pH 6 using 3 M HCl. Then the product was extracted with 20 mL EtOAc, and washed with DI water (3×15 mL). The organic layer was dried over anhydrous Na₂SO₄ and the solvent was removed under reduced pressure. The product was used for the next step without further purification (70% yield). The ¹H NMR is shown in Figure 15.

3.2.5 **2,5-dioxopyrrolidin-1-yl 4-(perylene-3-yl) butanoate (3)**

4-(perylene-3-yl)butanoic acid (0.102 g, 0.3 mmol) and N-hydroxysuccinimide (34.52 mg, 0.3 mmol) were dissolved in dry THF (10 mL). The mixture was placed in an ice bath and a THF solution (2 mL) of DCC (61.90 mg, 0.3 mmol) was added dropwise, followed by stirring overnight at room temperature. The next day a white precipitate (1,3-dicyclohexylurea) was filtered out and the filtrate was subjected to reduced pressure to remove THF, resulting in a solid brown powder. The pure product was recrystallized from EtOH (62% yield). The ¹H NMR is shown in Figure 16.

3.2.6 **N-poly(ethylene) glycol amine-4-(perylene-3-yl)butanamide (4)**

2,5-dioxopyrrolidin-1-yl 4-(perylene-3-yl) butanoate (44.85 mg, 0.1 mmol) and N₃-PEG₄₀₀-amine (42.40 mg, 0.1 mmol) were dissolved in 5 mL of dry DCM. 14 µL of TEA
Figure 14. $^1$H NMR spectrum of 4-oxo-4-(perylen-3-yl)butanoic acid (structure shown in the inset). Reprinted with permission from Ref. 158 Copyright 2016 American Chemical Society.
Figure 15. $^1$H NMR spectrum of 4-(perylene-3-yl)butanoic acid (structure shown in the inset). Reprinted with permission from Ref. 158 Copyright 2016 American Chemical Society.
(0.1 mmol) was added to the solution and it was stirred under nitrogen at room
temperature overnight. The next day solvent was removed under reduced pressure and
triphenylphosphine (26.23 mg, 0.1 mmol) was added, followed by 10 mL of THF. The
mixture was stirred 4 h at room temperature, after which 0.5 mL of DI water was added
to the mixture which was kept stirring at room temperature overnight. The next day the
solvent was removed by reduced pressure and 15 mL of 1 M HCl was added to the
substance. The aqueous dye solution was filtered to separate the insoluble
triphenylphosphin oxide. The filtrate was washed with Et2O (3×15 mL) and then 3 g of
KOH was added to the solution, followed by extraction of the product with DCM (6×10
mL). The organic layer was dried over anhydrous Na2SO4 and the solvent was removed
under reduced pressure to obtain pure product in the form of an intense yellow viscous
oily liquid (60% yield). The 1H NMR is shown in Figure 17 and Figure 18.

3.2.7 AgInS2/ZnS QDs synthesis and water solubilization

AgInS2/ZnS core/shell QDs were synthesized according to our previously published
protocol. Approximately 0.5 g of the crude sample was processed by addition of excess
ethanol to induce flocculation. The supernatant was discarded and the precipitate was
dried under reduced pressure. Next, 30 mg of amphiphilic 40% octylamine-modified PAA
was added to the QDs followed by ~3 mL of dry chloroform. The solution was sonicated
for several minutes to dissolve the polymer completely. The solvent was removed under
reduced pressure and 0.1 M NaOH was added to disperse the QDs into water. To remove
the excess polymer, the solution was dialyzed to neutrality using Amicon Ultra centrifugal
filters (100,000 MWCO) from Millipore.
Figure 16. $^1$H NMR 2,5-dioxopyrrolidin-1-yl 4-(perylene-3-yl) butanoate (structure shown in the inset). Reprinted with permission from Ref. 158 Copyright 2016 American Chemical Society.
Figure 17. $^1$H NMR N-poly(ethylene)glycol amine-4-(perylene-3-yl)butanamide (structure shown in the inset). Reprinted with permission from Ref. 158 Copyright 2016 American Chemical Society.
Figure 18. $^{13}$C NMR N-poly(ethylene)glycol amine-4-(perylene-3-yl)butanamide (structure shown in the inset). Reprinted with permission from Ref. 158 Copyright 2016 American Chemical Society.
3.2.8 Perylene-PEG-amine conjugation to QDs

AgInS$_2$/ZnS QDs were activated by adding 10 mg of MPEG 350 carbodiimide in 0.5 mL of water-solubilized QDs and stirring for 20 min. A solution of perylene-PEG-amine (4) dissolved in pH 8 phosphate buffer was prepared and was added dropwise to the activated QDs solution until desired dye emission was achieved. The pH of the solution was adjusted at 8 by adding phosphate buffer, and it was stirred overnight. The next day the solution was dialyzed using a dialysis tube (Float-A-Lyzer G2, 20 kD MWCO, from Spectrum Labs) until all the unconjugated dye eluted out.

3.2.9 Enzymatic oxygen scavenging titration system

Two separate experiments were conducted to measure the probe’s response to varying O$_2$ levels. In the first, the emission of the probe in Tris-HCL pH 7.4 buffer was measured and compared to the same in a screw top cuvette containing an enzyme mixture composed of glucose (10 mg/mL), glucose oxidase (0.5 mg/mL), catalase (0.08 mg/mL), and ATP (3 mM), that was allowed to equilibrate for 20 min. The O$_2$ levels were measured using a Mettler Toledo SG6-SevenGo pro™ dissolved oxygen meter with InLab® 605 O$_2$ sensor. The oxygen concentration dropped from 6.8 ppm to 0.01 ppm, which confirms the efficiency of the oxygen scavenging system. A second experiment was conducted under identical enzymatic conditions with the glucose initially absent. Next, glucose was added in small portions in 20 min intervals, after which time the emission spectrum was recorded. Unfortunately, absolute O$_2$ concentrations are not certain, although I assume that they still span the ~6.8 to 0.01 ppm range. The excitation wavelength of 425 nm was chosen to co-excite both chromophores simultaneously in
these experiments. The fluorescence responses were quantified by dividing the
integrated emission in visible range (originating from perylene) over the integrated
emission in the NIR range (originating from the QD).

3.2.10 Cell culture

HeLa cells were maintained in DMEM (+) (DMEM supplemented with 10% FBS, 1×
MEM non-essential amino acids and 15 mM HEPES) at 37 °C and 5% CO₂. The cells
were passaged with 0.25% trypsin/2.21 mM EDTA.

3.2.11 Microinjection

HeLa cells were grown to 60-70% confluency in a glass bottom dish before
microinjection and kept in 19.8% O₂ and 6.9% O₂ for studies under ambient and low level
oxygen conditions. Micropipettes were prepared by the following parameters: heat, ramp-
27; pull, 95; velocity 27; time 250; and pressure, 250. Micropipettes were loaded with the
sensor solution by capillary action and the solution was injected into the cytosol by using
the following microinjector parameters: transfer pressure, 17 hPa; injection width, 0.1 sec;
injection pressure, 200 hPa.

3.2.12 Fluorescence microscopy

Fluorescence images of the injected cells were acquired after 10 min using an epi-
fluorescence microscope (Axiovert 200, Carl Zeiss, Inc.) equipped with an intensified
CCD camera (XR Mega 10-S30CL ICCD, Stanford Photonic Inc.) modified with two LEDs
for UV and white light (Prizmatix, Ltd.). All images were obtained with a 63×/1.25 N.A. EC
Plan Neofluar oil-immersion objective (Carl Zeiss, Inc.). G365 nm and 427 nm (± 5)
excitation filters were used to excite quantum dots and dye respectively. Emission filters 
(775 \pm 70 \text{ nm} \text{ and } 472 \pm 15 \text{ nm}) were used to separately image and measure quantum 
dots and dye emission intensities, respectively. The same settings were used to acquire 
images under low and ambient levels of oxygen. All images are dark current corrected. 
Signal intensity was determined from ROIs in the cytoplasm using the Image J software 
(v 1.47) package.\textsuperscript{159} Note that the magnitudes of the dye/QD emission ratios from the 
fluorimeter and microscope experiments do not span the same range of values. This is 
due to differences in the methods of collection, and the wavelength-dependent response 
of the CCD camera.

3.3 **Results and discussion**

There have been several reports on the development of QD-organic dye coupled 
chromophore sensors to create synergistic systems with the robust qualities of quantum 
dots and the analytical capabilities of organic dyes. As large core/shell QDs are passive 
to their environment, they must be conjugated to a reporting chromophore to engender a 
quantifiable optical response to targeted analytes. Species of interest include proteins 
such as proteases,\textsuperscript{160} biological thiols,\textsuperscript{161} as well as pH,\textsuperscript{162} and O\textsubscript{2} levels as these analytes 
are associated with cancer. Recently, McLaurin et al. developed a QD-based fluorescent 
sensor for O\textsubscript{2} by conjugating oxygen-sensitive osmium complexes to CdSe/ZnS 
nanocrystals.\textsuperscript{163} They characterized FRET energy transfer from the QD to these ligands 
and demonstrated oxygen-dependent ratiometric emission. The benefits of these 
systems include high two-photon absorption cross sections and QD-to-chromophore
FRET. However, osmium-containing compounds can be expensive and toxic, have low emission quantum yields (~1%), and the probes did not respond to physiological O_2 levels. As such, the same group later used CdSe/ZnS-palladium(II) porphyrin coupled chromophores to profile oxygen levels in vivo, although the gas was quantified via modulation of the Pd(II)-porphyrin luminescence lifetime. Amelia et al. demonstrated oxygen sensing using CdSe/ZnS QDs functionalized with pyrene, an organic chromophore with a well-characterized O_2 sensitivity and reduced toxicity. However, the design of the system resulted in significant QD quenching and was reported to function only in an organic solvent; clearly this precludes applications in many biological systems.

I sought to demonstrate oxygen quantification in a biological system using a fully fluorescence-based approach with a singular non-toxic platform. Analyte-dependent ratiometric emission provides a simple output that can be realized using a coupled chromophore. To this end, I employed AgInS_2/ZnS quantum dots due to their enhanced photostability, high brightness, and broad absorption spectra that allows for co-excitation of both chromophores (Figure 19). Furthermore, the use of a cadmium-free AgInS_2/ZnS mitigates toxicity issues and offers advantages such as near-IR photoluminescence and a large Stokes shift that is desirable for in vivo imaging.

The choice of an O_2-sensitive chromophore must take into account the dye’s photophysical properties that engender oxygen-dependent emission efficiency. I will briefly review the many possibilities here although more detailed information can be found in recently published review papers. Optical sensors for O_2 can either be colorometric (absorptive), although fluorescence-based reporters are preferred for
Figure 19. Absorbance and emission spectra of AgInSe/ZnS QDs and perylene-PEGamine dye. Reprinted with permission from Ref. 158 Copyright 2016 American Chemical Society.

numerous reasons. They function by collisional quenching of the chromophore’s excited state(s) by molecular oxygen\textsuperscript{171, 172} as depicted by the Jablonski diagram in Figure 20. Absorption of a photon eventually places the chromophore in the lowest singlet excited state $S_1$. Next, many O$_2$-sensitive dyes undergo non-radiative intersystem crossing to the lowest excited triplet state $T_1$ followed by phosphorescence emission to the ground state. However, ground triplet state molecular oxygen ($^3\Sigma_g^-$) can act as an energy acceptor to non-radiatively quench the phosphor’s excited triplet state,\textsuperscript{173, 174} resulting in an O$_2$-sensitive phosphorescence intensity. This mechanism is commonly observed in organometallic complexes such as Pd porphyrins\textsuperscript{175} that have been commercialized for oxygen sensing.\textsuperscript{176} As I wish to avoid such metal complexes, I have examined the use of a chromophore that undergoes oxygen-assisted fluorescence quenching from the excited
S\textsubscript{1} to the T\textsubscript{1} state\textsuperscript{177}. This mechanism of fluorescence sensing is commonly observed in polycyclic aromatic hydrocarbons (PAHs) due to their long-lived singlet excited states, which is necessary to observe diffusional quenching by O\textsubscript{2}\textsuperscript{178}.

![Figure 20. Jablonski diagram of fluorescence and phosphorescence quenching by molecular O\textsubscript{2}. This study employs a perylene dye that undergoes fluorescence quenching by oxygen.](image)

While pyrene is an attractive candidate in this regard\textsuperscript{166}, I wish to avoid UV excitation in cell studies. As such, a larger PAH (perylene) was employed\textsuperscript{179}. Perylene dyes can be excited in visible range and have high emission quantum yields (0.89-0.99) in the blue-green region\textsuperscript{180}. Unfortunately the commercial availability of perylene dye derivatives is limited. As such, I developed a protocol to synthesize a water-soluble perylene-PEGamine that can be conjugation to aqueous QDs encapsulated with 40% octylamine-
modified poly(acrylic acid). The synthetic protocol is shown in Scheme 2. The quantum yield of the water-soluble perylene-PEGamine has been measured to be 0.68 ± 0.03 (Figure 21). The chromophore was also found to be non-toxic as shown in the results of a MTT assay (Figure 22). It was also found to permeate live cells, which engenders the possibility of using it as a “turn-on” sensor for cellular O₂ levels. However, such systems cannot ever be fully quantitative as one cannot distinguish between, for example, low O₂ levels in the nucleus of a live cell vs. the inability of the chromophore to bypass the nuclear envelope. This issue is addressed by conjugating the perylene derivative to AgInS₂/ZnS QDs to allow for the oxygen-insensitive dot emission to act as an internal calibration standard. As such, I can ascertain whether minimal perylene emission from the interior of a live cell is due to high O₂ content vs. a low density of delivered coupled chromophores.

The ratiometric QD-dye probe was prepared using neutral carbodiimide coupling reagents and purified by dialysis to remove any free perylene dye. Next, the probe was titrated with oxygen to quantify its response to O₂ levels. This was attempted by a variety of methods, including sparging the solution with nitrogen and the freeze-pump-thaw technique. However, the results were never consistent, likely due to perturbation of the coupled chromophore as QD precipitation was occasionally observed with sparging. As such, I investigated the use of an enzymatic oxygen scavenging system that offers rapid O₂ removal and the ability to maintain the anoxia for a long period of time. One of the most well-established systems is based on the oxidation of glucose with glucose oxidase.
Figure 21. Quantum yield data on perylene-PEGamine dye vs. coumarin 102 reference standard. Reprinted with permission from Ref. 158 Copyright 2016 American Chemical Society.

Figure 22. MTT assay demonstrates no *in vitro* toxic effect due to exposure perylene-PEGamine dye. The bar chart shows the mean absorbance at 550 nm as a percentage of positive control (DMEM only) following incubation with the dye (30 min) at indicated concentrations (three replicates for each condition). Error bars are the relative standard deviations. Reprinted with permission from Ref. 158 Copyright 2016 American Chemical Society.
In this ATP-driven process glucose oxidase oxidizes glucose to gluconolactone, which spontaneously hydrolyzes to gluconic acid and hydrogen peroxide. Catalase removes the H$_2$O$_2$ by decomposing it into water and oxygen; note that there is an overall net loss of O$_2$ as shown in equations 1 & 2:

\[
\text{glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + \text{H}_2\text{O}_2 \quad (1)
\]

\[
\text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} \text{H}_2\text{O} + \frac{1}{2} \text{O}_2 \quad (2)
\]

A solution composed of the QD-based oxygen probe, glucose oxidase, catalyse, and ATP was titrated with increasing quantities of glucose in 20 min intervals to progressively lower the O$_2$ concentration. The fluorescent response of the sensor shown in Figure 23A was quantified by fitting multiple Gaussian functions to calculate the integrated dye/QD emission ratio. As can be seen in Figure 23B, the dye/QD ratio increased by adding glucose until complete deoxygenation occurred at which point the response was saturated. The overall alteration of the emission ratio is >100% over a measured oxygen concentration range of 6.8 to 0.01 ppm as shown in Figure 24. As such, the coupled perylene-QD chromophore can ratiometrically respond to physiologically relevant O$_2$ levels in the visible-to-NIR region of the spectrum.

I investigated the probe’s response to alterations of oxygen concentration in vitro. As discussed previously, the water soluble perylene-PEGamine dye is cell permeable; however, the QD-dye conjugate isn’t and needed to be microinjected into cells. With this issue resolved, I next investigated several methods to control the oxygen levels in HeLa cells. First, incubation of the cells in the presence of the enzymatic oxygen scavenger failed due to cell death. As such, cells were imaged immediately after exposure to the O$_2$
scavenging system. However, no effect was observed, which we believe is due to the fact that equilibrium in the gas levels was not achieved over a short time interval. Despite these failures, I was able to demonstrate \textit{in vitro} \( O_2 \) quantification from two sets of HeLa cells that were cultured overnight under 6.9\% and 19.8\% \( O_2 \) concentrations. Next, they were microinjected with the QD-perylene dye, and wavelength-selective microscopic images were obtained of the cells using separate bandpass filters for the QD and dye channels. As shown in Figure 25, there was a 30\% enhancement in the intensity of the dye/QD signal in the hypoxic cells; the detailed changes in the intensities of the QD and dye signals are quantified in Table II. As such, these control experiments demonstrate the potential use of a QD-perylene dye conjugate for the ratiometric measurement of hypoxia in live cells, which is a biological indicator of cancer.

### 3.4 Conclusions

I have developed a fluorescent ratiometric oxygen probe based on \( O_2 \) modulation of the fluorescence of perylene dye conjugated to oxygen-insensitive near-IR emitting AgInS\(_2\)/ZnS QDs. An enzymatic system composed of glucose, glucose oxidase, and catalase was used to deoxygenate the samples which proved to be highly robust and reproducible compared to sparging the solution or the freeze-pump-thaw technique. A >100\% increase in dye:QD ratiometric signal was observed upon deoxygenation from 6.8 ppm to 0.01 ppm. \textit{In vitro} measurements were conducted by microinjection of the probe into live HeLa cells incubated in different oxygen levels overnight, which demonstrates the possibility of using this \( O_2 \) sensor for cancer detection and research.
Figure 23. Response of the sensor to oxygen scavenging. (A) Emission of the sensor in response to decreasing oxygenation of the solution due to increasing glucose concentration (inset) that is the substrate of an enzymatic oxygen scavenging system. (B) Dye/QD integrated emission ratio in response to the addition of glucose to the enzymatic oxygen scavenging system. Reprinted with permission from Ref. 158 Copyright 2016 American Chemical Society.
Figure 24. Emission of AgInS$_2$/ZnS-perylene dye conjugate before and after saturation with the oxygen scavenging solution. O$_2$ measurements with a Mettler Toledo meter reveal an ambient 6.8 ppm O$_2$ level that drops to 0.1 ppm upon ATP-driven oxidation of glucose by glucose oxidase in the same solution. The enhancement on the dye:QD integrated emission ratio is 118%. Reprinted with permission from Ref. 158 Copyright 2016 American Chemical Society.

Figure 25. Epifluorescence microscopy images reveal a ratiometric response of the sensor to hypoxia in HeLa cells incubated under 19.8% O$_2$ (top row) and hypoxic 6.9% O$_2$ (bottom row) levels overnight. Reprinted with permission from Ref. 158 Copyright 2016 American Chemical Society.
Table II. Dye/QD signal intensity in Hela cells for two different oxygen concentrations. Reprinted with permission from Ref. 158 Copyright 2016 American Chemical Society.

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4 Ratiometric QD-FRET Sensing of Aqueous H$_2$S in Vitro

The content of this chapter has been published as “Ratiometric QD-FRET Sensing of Aqueous H$_2$S in Vitro”, A. Shamirian, H. Samareh Afsari, D. Wu, L. W. Miller and P. T. Snee, Anal. Chem., 2016, 88 (11), 6050-6056. Reproduced with permission from The American Chemical Society.

4.1 Introduction

H$_2$S is a colorless, noxious, and toxic gas that is mainly produced by the decomposition of organic compounds or as a byproduct from various industries.$^{182}$ Studies have shown that H$_2$S is damaging to biological systems due to its high permeability through lipid membranes.$^{183,184}$ Regardless, the gas has many significant biological roles. For example, H$_2$S has recently been recognized as an endogenously generated gaseous transmitter.$^{185}$ Biological production of H$_2$S can take place via enzymatic or non-enzymatic pathways. In mammalian systems, physiological H$_2$S levels are regulated by enzymes$^{186,187}$ found in the brain and liver as well as in nervous system and vasculature tissues.$^{188,189}$ The regulation of the gas has biologically beneficial effects. For example, it has been shown that H$_2$S has antioxidant effects$^{190}$ and is an anti-inflammatory agent.$^{191}$ H$_2$S levels are also associated with cardioprotective and anti-apoptotic effects,$^{192-194}$ the regulation of vascular tension, and controlling blood pressure.$^{195,196}$ However, recent studies have indicated that H$_2$S levels are related to diseases such as cancer,$^{197}$ Down syndrome,$^{198}$ and Alzheimer’s disease.$^{199}$

There is an ongoing debate concerning the concentration of hydrogen sulfide in cells, blood, and in tissues. A wide range of 2-300 μM has been reported by different groups,$^{200-204}$ which is likely the result of the use of different sampling techniques and detection methods. Concentrations in the nanomolar range have also been reported.$^{205}$
This is unfortunate as the biological effects of H$_2$S depend on its concentration, as the gas is cytoprotective at lower levels while it is cytotoxic and causes apoptosis in human cells at higher concentrations. Furthermore, to function as a gaseous transmitter the intracellular concentration of H$_2$S has to be high to activate the signaling mechanism, but it is consumed quickly to maintain the whole tissue concentration at safe lower levels.$^{205}$

The development of a reliable and efficient method for the detection of the H$_2$S in biological environments has a crucial importance to understand its role in many pathologies. Methods of H$_2$S detection such as chromatography, colorimetry, and electrochemical assays,$^{206,207}$ suffer from poor biological compatibility, and require complicated sample preparation processes. One strategy to address these issues is based on the fact that H$_2$S dissociates in aqueous solution to form an equilibrium between H$_2$S $\leftrightarrow$ HS$^{-}$ $\leftrightarrow$ S$^{2-}$, where bisulfide (HS$^{-}$, or hydrogen sulfide) is favored and is the target analyte “stand-in” for H$_2$S. As such, the design of fluorescent probes for bisulfide have attracted significant attention due to the convenience, compatibility, and sensitivity of fluorescence methods that facilitate the real-time detection of the analyte within biological environments. Sulfide-reactive fluorescent probes have been designed based on different strategies such as metal-sulfide interaction, reduction of azide and nitro groups, and nucleophilic addition; detailed mechanisms and discussion of these systems can be found in recent reviews.$^{208-210}$ Most of the reported probes are single-emission turn-on sensors that can be difficult to quantify in a complex biological environment. A ratiometrically responsive (i.e. color-changing and thus self-calibrating) sensor addresses this problem, although there are few reports of such.$^{211-213}$ In this chapter, we report a novel design of a QD-based energy-transfer sensor for detection of bisulfide ion in solution. The response
mechanism is based on the reducing ability of the ion that can cleave the disulfide bond connecting the QD to dye. The action of the ion results in a loss of FRET between a green emitting QD donor and a rhodamine dye acceptor that produces a ratiometric fluorescence response to the presence of HS\textsuperscript{-.}

4.2 Experimental section

4.2.1 Materials and instrumentation

1,2,4-benzenetricarboxylic anhydride (97%), 3-diethylaminophenol (97%), 4-(dimethylamino) pyridine (DMAP, ≥98%), DL-dithiothreitol (>98%), D-(+)-glucose (>99.5%), L-cysteine (97%), L-glycine (>98.5%), L-lysine (>98%), potassium bisulfate (≥99%), trimethylamine (TEA, ≥99%), zinc chloride (≥98%), and high molecular weight polyvinyl chloride were purchased from Sigma-Aldrich. Glutathione (reduced, 98%), sodium sulfide nonahydrate (>98%), and N,N'-disuccinimidyl carbonate (DSC, 98%) were purchased from Acros. Sodium sulfate (anhydrous, >99%), sodium sulfite (anhydrous, >98%), and sodium thiosulfate pentahydrate (≥ 99.5%) were purchased from Fisher Chemicals. Sodium thiocyanate (98%) was purchased from Alfa Aesar. Mono-t-boc-cystamine·HCl was purchased from Fisher Scientific. Bis(2-ethylhexyl)sebacate (≥98%) was purchased from TCI America. Methoxypolyethylene glycol 350 carbodiimide (MPEG 350 CD),\textsuperscript{52} Rhodamine B piperazine,\textsuperscript{120} and 40% octylamine-modified poly(acrylic acid) (PAA)\textsuperscript{39} were prepared according to previously published protocols. \textsuperscript{1}H NMR spectra were recorded on a Bruker Avance DRX 400 NMR spectrometer. UV/vis absorbance spectra of the samples were taken using a Varian Cary 300 Bio UV/vis spectrophotometer, and fluorescence emission spectra were obtained using a custom-designed Horiba Jobin
Yvon FluoroLog spectrophotometer. Dynamic light scattering (DLS) experiments were performed on a Malvern Zetasizer Nano. HeLa cells (CCL-2) were purchased from American Type Culture Collection. Dulbecco’s modified eagle medium (DMEM, 10-014 CV) and 0.25% trypsin/2.21 mM EDTA were purchased from Corning Cellgro®. MEM non-essential amino acids (11140) and HEPES (15630-080) were purchased from Gibco®. Micropipette preparation glass bottom culture dishes (P50G-1.5-14-F) were purchased from Matek Corporation (Ashlan, MA). XenoworksTM Microinjection Systems, P-1000 pipette puller and borosilicate glass tubes (BF100-78-10) were used for microinjections (Sutter Instruments, Novato, CA).

4.2.2 Bisulfide-reactive carboxyrhodamine B synthesis

The bisulfide-reactive rhodamine B derivative used in this study was synthesized following the outline in Scheme 3.

4.2.3 Synthesis of carboxyrhodamine B (3)

A mixture of 3-diethylaminophenol 1 (1.03g, 6.05 mmol), and 1,2,4-benzenetricarboxylic anhydride 2 (0.6 g, 3.02 mmol) was heated to 195 °C in the presence of a catalytic amount of ZnCl₂ under a nitrogen atmosphere for 1 h. The resulting red mixture was cooled to room temperature, and dissolved in 5% NaOH solution. The mixed isomers of carboxyrhodamine B were precipitated out of the solution by acidification using HCl (pH=1). 5- and 6- carboxyrhodamine B 4 isomers were separated as TEA salts by flash chromatography (DCM:MeOH:TEA 4:1:0.5). Solvents were evaporated under reduced pressure. Each isomer was dissolved in ETOAc (40 mL), and washed with 1 M KHSO₄ (3×30 mL), brine (30 mL), and dried over anhydrous Na₂SO₄ to
Scheme 3. Synthesis of the bisulfide-reactive carboxyrhodamine B dye, and subsequent conjugation to water-soluble QDs. Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.
obtain pure 5-carboxyrhodamine B (575 mg, 39%), and 6-carboxyrhodamine B (452 mg, 30%). $^1$H NMR data are shown in Figure 26.

### 4.2.4 Synthesis of 6-carboxytetraethylrhodamine N-hydroxysuccinimide ester (6)

6-carboxyrhodamine B 5 (100 mg, 0.2 mmol), DMAP (122 mg, 1 mmol), and TEA (140 µL, 1 mmol) were dissolved in dry DCM (10 mL). DSC (105 mg, 0.4 mmol) was added, and the mixture was stirred under nitrogen atmosphere at room temperature for 1 h. After 1 h the reaction was quenched by addition of AcOH (120 µL, 2 mmol), and the
The final product was purified by flash chromatography using 1% AcOH in acetone followed by MeOH:DCM:AcOH (9:89:2) as eluent (65% yield). $^1$H NMR data are shown in Figure 27.

Figure 27. $^1$H NMR spectrum of 6-carboxytetraethylrhodamine N-hydroxysuccinimide ester (structure shown in the inset). Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.
4.2.5 Synthesis of N-((2-aminoethyl)disulfanyl)ethyl)-3',6'-bis(diethylamino)-3-oxo-3H-spiro [isobenzofuran-1,9'-xanthene]-6-carboxamide (8)

6-carboxytetraethylrhodamine N-hydroxysuccinimide ester 6 (58.4 mg, 0.1 mmol), mono-t-boc-cystamine-HCl (28.81 mg, 0.1 mmol), and TEA (14 µL, 0.1 mmol) were dissolved in dry DCM, and stirred under nitrogen atmosphere at room temperature overnight. The next day the solvent was evaporated under reduced pressure, and the resulting residue was purified by flash chromatography (DCM:MeOH 85:15) to obtain compound 7. For boc deprotection, the resulting compound was dissolved in DCM (4 mL), and then CF₃COOH (2 mL) was added. After the mixture was stirred for 2 h under nitrogen atmosphere at room temperature, the solvent was evaporated under reduced pressure. The product was purified by flash chromatography (DCM:MeOH:TEA 4:1:0.5) to obtain compound 8 (81% yield). Optical and ¹H NMR data are shown in Figure 28a and Figure 29.

4.2.6 QDs synthesis, PVC modification, and water solubilization

CdSe/CdZnS core/shell QDs were synthesized according to previously published protocols.²⁷ Approximately 0.5 g of the crude sample was processed by addition of a small amount of isopropanol followed by methanol to induce flocculation. The supernatant was discarded, and the precipitate was dried under vacuum. The QDs were dissolved in 2 mL of THF, and 50 µL of a PVC coating solution¹⁵⁷ (50 mg high molecular weight polyvinyl chloride and 100 mg of bis(2-ethylhexyl) sebacate dissolved in 5 mL of THF) was added. The mixture was stirred gently overnight, after which the solvent was evaporated under reduced pressure. Next, 65 mg of amphiphilic 40% octylamine-modified PAA was added
Figure 28. Absorbance, emission, and QY of the dye. (a) Absorbance and emission spectra of the bisulfide-reactive carboxyrhodamine dye (compound 8). (b) The water-soluble CdSe/ZnS QDs used for the cell imaging / sensing studies have a quantum yield of 0.54 ± 0.05. Fluorescein in 0.1 M NaOH was used as a standard (Ex. at 470 nm QY 0.91 ± 0.05). Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.

to the QDs followed by ~3 mL of dry THF. The solution was sonicated for several minutes to dissolve the polymer completely. It is hypothesized that the hydrophobic portions of the polymer coordinate to pores in the PVC coating, which is why the QDs dispersed into 0.1 M NaOH solution after the solvent was evaporated under reduced pressure. The solution was dialyzed to neutrality to remove excess polymer. The solution was then filtered through a 0.2 µm syringe filter to yield a monodisperse plastic-coated water-soluble QD solution. The fact that the materials passed through the filter and remained colloidally stable indicated that the materials were not agglomerated. Samples without PVC modification were prepared by repeating the procedure above without the addition of the PVC coating solution. These materials were used in cell imaging studies. As such, their quantum yield was measured to be 0.54 vs. fluorescein, see Figure 28b.
Figure 29. $^1$H NMR spectrum of compound 8 in the aromatic (top) and aliphatic (bottom) regions (structure shown in the inset). Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.
4.2.7 **Compound 8 dye conjugation to QDs**

A solution of ~5 mg of MPEG 350 CD dissolved in 0.5 mL of water-solubilized CdSe/CdZnS QDs (1.24 × 10⁻⁶ M) was stirred for 30 min. Next, a sub-milligram quantity of compound 8 was dissolved in pH 8 phosphate buffer and was added dropwise to the activated QD solution until the dye emission appeared to slightly dominate the dots under illumination with a black light. Next, 2 mL of pH 8 phosphate buffer was added, and the reaction was allowed to stir overnight. The next day, dialysis was performed using centrifugation filters to remove excess unreacted dye. The QD sulfide sensor solution was then diluted to working concentration of 3.43 × 10⁻⁸ M using pH 7.4 Tris-HCl buffer. The conjugation yield for the unmodified (PVC free) dots was calculated to be 42% by comparing absorbance spectra before and after dialysis, and the number of the dye per QD was determined to be 1.3 based on the absorptivity of the dye component. These data are shown in Figure 30. The characteristic FRET distance (R₀) was determined to be 5.95 nm using the methods outlined in Ref. 27. Furthermore, the FRET efficiency was determined to be >70% for both plastic coated and unmodified water-soluble QD/dye conjugates based on the quenching of the donor fluorescence intensity shown in Figure 31. The high efficiency observed in the PVC modified QDs is surprising given the hydrodynamic radius determined from DLS measurements. We believe that the porous nature of the PVC coating, due to the addition of the plasticizer,²¹⁵ allows the dye to interpolate within the coating such that FRET efficiency is enhanced.
Figure 30. The dye conjugation reaction yield. (a) The dye conjugation reaction yield (42%) was calculated from the ratio of the integrated dye absorption of the QD/bisulfide-reactive dye conjugate system before and after dialysis. (b) Subtraction of the blank QD from the QD-dye conjugate absorption reveals Rhodamine B dye-like features that were used to calculate of number of the dyes per QD. Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.

Figure 31. The FRET efficiency of the QD/dye conjugates. (a) Unmodified QDs and (b) PVC modified QDs (“plastic coated”). The efficiency is determined by measuring the quenching of the donor-only fluorescence and after conjugation to the energy-accepting dye. Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.
4.2.8 **Cell culture**

HeLa cells were maintained in DMEM (+) (DMEM supplemented with 10% FBS, 1× MEM non-essential amino acids and 15 mM HEPES) at 37 °C and 5% CO₂. The cells were passaged with 0.25% trypsin/2.21 mM EDTA.

4.2.9 **Microinjection and fluorescence microscopy**

HeLa cells were grown to 60-70% confluency in a glass bottom dish before microinjection. Micropipettes were prepared by the following parameters: heat, ramp-42; pull, 80; velocity 30; time 100; and pressure, 400. Micropipettes were loaded with the sensor solution by capillary action and the solution was injected into the cytosol by using the following microinjector parameters: transfer pressure: 15 hPa; injection width, 0.2 sec; injection pressure 3000 hPa. Fluorescence images of the injected cells were acquired after 20 min using an epi-fluorescence microscope (Axiovert 200, equipped with AxioCam MRm CCD camera and Axiovision software (V 4.6.1.0), Carl Zeiss, Inc.) modified with a UV LED emitting at 365 nm (UV-LED-365, Prizmatix, Ltd.). All images were obtained with a 63×/1.25 N.A. EC Plan Neofluar oil-immersion objective (Carl Zeiss, Inc.) and G365 excitation filter. Emission filters (535 ± 25 nm and 610 ± 37.5 nm) were used to separately image and measure the quantum dot and dye emission intensities. The same settings were used to acquire images before and after the addition of sodium sulfide solution. All images are dark current corrected. Signal intensity was determined from ROIs in the cytoplasm using the Image J software (v 1.47) package.¹⁵⁹
4.3 Results and discussion

Considering the equilibrium $\text{pK}_a \approx 7.0$ for $\text{H}_2\text{S} \leftrightarrow \text{HS}^- \text{ and the significantly higher $\text{pK}_a2$ of HS}^- \leftrightarrow \text{S}^2^-$, it is not surprising that the $\text{H}_2\text{S} / \text{HS}^-$ ratio within the cells is believed to be equal, and that almost 80% of the $\text{H}_2\text{S}$ is in the form of anionic state in extracellular fluid and plasma. Furthermore, HS$^-$ is a stronger nucleophile compared to cysteine, glutathione, and other anions such as chloride and hydroxide. This feature can be utilized for selective sensing of $\text{H}_2\text{S}$ via a nucleophilic displacement mechanism of HS$^-$ under biological conditions.

The wide variation in reported concentration levels discussed above is likely due to the choice of technique and approach. The employment of harsh chemical conditions for analyte extraction and sampling may alter the quantity of sulfur containing species. Moreover, technical limitations involved in some methods result in indirect detection of $\text{H}_2\text{S}$. Regardless, the employment of reactive fluorescent probes has advantages, including minimal sample preparation, direct visualization of the analyte, high sensitivity and (possibly) low detection limit, and non-destructive detection in biological environments. Concerning fluorescence-based sensing, activation of a chromophore by an analyte provides relatively background-free signal that is easy to interpret. However, one cannot ascertain the effect of analyte concentration within a complex environment such as a cell. For example, if no emission is observed from a cell nucleus, is that because no analyte is present or due to the fact that the probe did not diffuse through the nuclear membrane?
These issues are addressed by the use of ratiometrically reporting chromophores, which change color in the presence of the analyte. As a result, the spectrum of the probe provides the analytical metric, rather than the fluorescent probe intensity. Ratiometric fluorescent sensors can be prepared by conjoining a donor / acceptor pair of chromophores that engage in FRET, the efficiency of which is altered by the action of the analyte. Such sensitivity can be imparted by cleavage of the donor-acceptor link by the activity of the analyte that results in the termination of the energy transfer. As such, the emission of the donor becomes more dominant, which alters the integrated emission ratio.\textsuperscript{219,220} Because cleavage of a disulfide bond by HS\textsuperscript{-} is facile, linking a dye to a QD via a disulfide bond can be incorporated within a ratiometric fluorescent sensing strategy. To this end I have synthesized a carboxyrhodamine B derivative with an amine-terminated linker containing a disulfide bond. This chromophore can act as an acceptor for a green-emitting water-soluble quantum dot donor after carbodiimide coupling the two together (Scheme 4); see Figure 31 & Figure 32 for spectroscopic data. The disulfide bond cleaves upon exposure to HS\textsuperscript{-}, causing the dye to diffuse away from the QD and terminating FRET. This was confirmed by measuring the filtrate of samples that were exposed to Na\textsubscript{2}S solution after dialysis, see Figure 33.

The system was tested using freshly-prepared sodium sulfide in pH 7.4 Tris-HCl buffer solution to represent an H\textsubscript{2}S source. The fluorescent response of the sensor shown in Figure 34a was quantified by fitting multiple Gaussian functions to the sulfide-dependent emission spectra to separate the QD and dye components. The integrated emission ratio of the QD/dye was plotted as a function of the HS\textsuperscript{-} concentration.
Scheme 4. Mechanism of the ratiometric response of the sensor based on FRET modulation. Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.

Figure 32. Photoluminescence excitation spectra of (a) water-soluble and (b) PVC modified water-soluble QDs (blue solid) and the same conjugated to the bisulfide-reactive carboxyrhodamine dye (red dash) measured by the intensity of emission at 620 nm, near the maximum of the dye fluorescence. The mixture of QD and dye-like features in the coupled chromophore and the relatively weak intensity of the neat QD sample are indicative of energy transfer from the QD to the dye. Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.
(Figure 34a), which shows a linear correlation. The detection limit was determined to be 21.6 ± 0.4 μM using the bootstrap method. Note that the detection limit of ratiometric sensors is scalable with concentration to within reasonable limits as determined by the detection efficiency. As such, the sensor was diluted by 10×, reanalyzed, and was found to have a detection limit of 1.36 ± 0.03 μM using the data shown in Figure 34b. For studying the selectivity, the ratiometric response of the QD-dye complex was measured after exposure to various thiols and amino acids in pH 7.4 Tris-HCl buffer (Figure 35). As can be seen, the sensor has a stronger response towards the HS− anion compared to other thiols such as glutathione and cysteine, which we believe is a result of the smaller size and greater nucleophilicity of the analyte.

Figure 33. The absorption spectra of the filtrate from a QD/sulfide reactive dye coupled chromophore before and after exposure to Na2S reveals the presence of rhodamine dye in the filtrate. This must be due to reduction of the S-S bond that links the quantum dot and dye. Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.
Figure 34. Ratiometric response of the sensor to Na₂S solution. (a) Normalized emission of the coupled QD/bisulfide-reactive carboxyrhodamine dye (compound 8) sensor (3.4 × 10⁻⁸ M) upon exposure to HS⁻. Also the ratio of the integrated emission of the QD donor over the dye acceptor as a function of HS⁻ concentration reveals a 21.6 ± 0.4 μM detection limit. (b) The same for the QD/bisulfide-reactive carboxyrhodamine dye sensor at 10× dilution (3.4 × 10⁻⁹ M) reveals a lower detection limit of 1.36 ± 0.03 μM. Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.
Several control experiments were conducted to investigate the mechanism of sensing and identify possible pitfalls of the method. For example, a Rhodamine B derivative was used as the organic chromophore as it was believed to have minimal intrinsic sensitivity to environmental factors. However, an amide bond-coupled (and thus non-sulfide reactive) QD/Rhodamine B piperazine chromophore responded to bisulfide ion in a nearly-identical manner to that observed in the QD/bisulfide-reactive dye system (see Figure 36). Further investigation revealed that the absorption of Rhodamine B piperazine is suppressed when titrated with HS⁻ (Figure 37a), which would result in a ratiometric response in the coupled chromophore due to a modulation of the FRET efficiency. The response shows a linear correlation between the integrated QD/dye emissions ratio versus HS⁻ concentration to 150 µM of bisulfide under the conditions
employed. The response saturated above this range, which was not true for the reduction-sensitive FRET system discussed above. This is likely due to the difference in analyte recognition mechanisms; regardless, the amide-bonded coupled chromophore was not used in further studies due to this limitation.

Given that Rhodamine B was found to have an intrinsic response to the presence of the analyte, I also investigated the absorption of the bisulfide-reactive carboxyrhodamine dye due to exposure to Na$_2$S and found an even stronger response as shown in Figure 37b. These data suggest that the ratiometric response of the sensor shown in Figure 34 may be due to both dye cleavage and suppression of the dye’s absorptivity. However, cleavage of the QD-dye linker must ultimately be responsible for the observations due to the lack of saturation behavior and the irreversibility of the reaction.

Although Figure 34a shows that the emission of the QD/bisulfide-reactive carboxyrhodamine B dye has a clean response to bisulfide exposure with a clear isosbestic point appearing at ~570 nm, the absolute intensity of the sensor’s emission was reduced as seen in the unnormalized spectra in Figure 38a. It was determined that the overall loss of fluorescence efficiency was due to bisulfide quenching of the QD donor component. As such, several avenues were explored to protect the QD from quenching by the analyte, one of which was to coat the QDs with polyvinyl chloride (PVC) before water solubilization with 40% octylamine-modified poly(acrylic acid). These water soluble, “plastic-coated” QDs were conjugated to the bisulfide-reactive carboxyrhodamine B dye and were then titrated with bisulfide solution. The emission spectra of this sensor are
Figure 36. Ratiometric response of the QD-RB piperazine to Na$_2$S solution. (a) Emission spectra of QD/rhodamine B piperazine conjugated chromophore in the presence of increasing concentrations of Na$_2$S. (b) Calibration data from the ratio of the integrated emission of the QD donor over the dye acceptor as a function of HS$^-$ concentration reveal a roll-off of the response at higher levels of bisulfide that is not observed in the QD/bisulfide-reactive carboxyrhodamine dye conjugate. Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.

Figure 37. Absorbance changes of the RB disulfide dye and RB piperazine in response to Na$_2$S solution. (a) Absorbance changes of Rhodamine B piperazine due to increasing exposure to Na$_2$S solution. (b) Absorbance changes of compound 8 due to increasing exposure to Na$_2$S solution. Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.
shown in Figure 38b, where it can be seen that the plastic coating reduced quenching of the QDs due to HS\textsuperscript{−} exposure. The ratiometric response to bisulfide was linear (Figure 39) with a detection limit of 41.9 ± 0.3 μM; in fact these data appear nearly identical to that shown in Figure 34. However, DLS results shown in Figure 41 reveal that the plastic-coated QDs are ~ 150 nm in diameter which is a significant increase from ~ 8.5 nm observed in un-modified water-soluble dots. Furthermore, these dots did not diffuse freely and distribute themselves evenly in live cells after microinjection, most likely due to their large size. As a result, these samples were not examined further, and all experiments with sulfide sensing in live cells were performed using sensors that were not modified with a PVC coating.

The efficacy of the sensor in live cells was investigated. HeLa cells were microinjected with ~1.5 μmol of the QD/bisulfide-reactive carboxyrhodamine dye and were incubated for 5 min. Wavelength-selective microscopic images were obtained using 535 ± 25 nm and 610 ± 37.5 nm bandpass filters for the QD and dye channels, respectively. Next, the cells were treated with a 200 μM bisulfide solution for an additional 20 min, after which time they were re-imaged as shown in Figure 40. The detailed changes in the intensities of the QD and dye signals are quantified in Table III. The average enhancement in the intensity of the QD signal (+17%) and quenching of dye intensity (-29%) confirms the potential use of the ratiometric sensor for the detection of bisulfide in biological environments. To verify that the enhancement in the QDs’ emission was due to FRET modulation, HeLa cells were microinjected with water-soluble QDs and were imaged before and after sulfide solution exposure in the same manner as discussed above (Figure 42). The results showed drop in intensity of the QD emission (-9%).
Figure 38. Effect of PVC coating on QD emission. Raw emission spectra of (a) QD/sulfide reactive dye coupled chromophore, the normalized data from which appear in Figure 34a, and (b) PVC modified QD/sulfide reactive dye in the presence of increasing concentrations of Na$_2$S. The data show that the quantum dot emission is less perturbed in the plastic coated sample. Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.

Figure 39. Ratiometric response of PVC modified sensor to Na$_2$S. (a) Normalized emission of the PVC modified QD/bisulfide reactive dye sensor (3.4 × 10$^{-8}$ M) upon exposure to HS$^-$ (raw data appear in Figure 38b). (b) Ratio of the integrated emission of the QD donor over the dye acceptor as a function of HS$^-$ concentration. The detection limit was determined to be 41.9 ± 0.3 μM for a QD sensor concentration of 3.4 × 10$^{-8}$ M. Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.
Figure 40. Two sets of fluorescence images of HS detection in HeLa cells. The cells are imaged with optical filters to separately measure the donor QD vs. dye acceptor emissions as labeled in the figures. Before: Image prior to sodium sulfide addition. After: The same after exposure to sodium sulfide solution. Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.
Figure 41. DLS data show that the PVC modified QDs are significantly larger than the unmodified QD samples in water. Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.
This confirms that the ratiometric response observed in the cells was not due to a singular, intrinsic response of QDs due to Na$_2$S exposure.

We attempted to quantify the \textit{in vitro} data by comparing them to the calibration curve shown in Figure 43 generated by measuring the emission of the sensor in liquid drops of bisulfide standards using the same microscope system. Unfortunately, the \textit{in vitro} data is not sensibly consistent with this calibration with regard to the observed QD/dye emission ratios and the magnitude of response due to exposure to 200 µM of the analyte. This result was not entirely unexpected as previous attempts to quantitatively measure the pH in tumor microenvironments using QD-based ratiometric sensors was found to be similarly problematic.$^{216}$ As such, it is likely that quantitative biological measurements require the use of calibration standards that have similar chemical and biological composition as the sample.

\textbf{4.4 Conclusion}

In conclusion, a QD-based FRET platform has been developed for sensing aqueous bisulfide ions within biological environments. The probe has a detection limit of 1.36 ± 0.03 µM in buffer and is selective towards HS$^-$ compared to the other thiols including amino acids. The self-calibrating response of the sensor, in addition to the positive qualities of QDs such as high quantum yield and photostability, make this probe a unique tool for the qualitative detection of the H$_2$S (via aqueous bisulfide) concentration changes in complex biological matrices.
Figure 42. Effect of Na$_2$S solution addition on bare QDs emission in HeLa cells. Fluorescence images of water-soluble QDs microinjected into live HeLa cells in the QD channel (535 ± 25 nm) before after treatment with Na$_2$S solution (200 μM) reveal a slight loss of QD intensity. Relatively little QD emission is observed to bleed through the dye channel (610 ± 37.5 nm). Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.

Figure 43. Microscope calibration curve. Calibration data from QD/bisulfide-reactive dye conjugates diluted in HS$^-$ standards imaged using the fluorescence microscope, where the ratio is determined from the intensity of the QD channel divided by the intensity of the dye channel. Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.
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Table III. QD-Dye signal intensity change after treatment with Na₂S (200 μM). Reprinted with permission from Ref. 181 Copyright 2016 American Chemical Society.
5 Ion-selective Fluorescent Nano-optodes for Detection of Calcium and Sodium

5.1 Introduction

We have successfully developed QD-based sensors for the detection of dissolved oxygen and bisulfide ion. We have also designed QD-based FRET probes for sensing aqueous ions such as sodium and calcium. The detection mechanism is based on that employed for ion-selective optical sensors (optodes),\textsuperscript{167} which has been thoroughly characterized.\textsuperscript{168} Furthermore, optodes have been successfully miniaturized.\textsuperscript{222-225} An optode consists of a membrane that is usually composed of a polymer matrix containing a pH sensitive dye that has protonated and deprotonated forms with correspondingly different absorption spectra. This species is referred as the “chromoionophore”. In addition, there is an ion receptor that can selectively bind to the analyte cation and transport it across the membrane. This compound is referred to as the “ionophore”. Depending on the type and charge of the chromoionophore and ionophore, sometimes lipophilic anionic sites are introduced to maintain the charge balance in the membrane. This is due to the fact that the membrane is kept electrostatically neutral. If cationic analytes diffuse into the membrane by action of the ionophore, a concomitant deprotonation of the chromoionophore occurs to maintain the charge neutrality in the membrane. Since the number of protons released into the solution is directly proportional to the amount of the cation extracted from it, the absorbance change of the chromoionophore due to deprotonation can be quantitatively correlated to the concentration of the cation. Since ions in the membrane and in solution should be
maintained at equilibrium for every measurement, the thickness of the membrane and thus the diffusion time through it are the factors that limit the optode response time. This response time can be described by Fick’s law of diffusion, which stipulates that the time needed to achieve 95% equilibrium is: \(^{169}\)

\[
t_{95\%} = 1.13 \frac{d^2}{D_m} \quad \text{Eq. 5.1}
\]

where \(d\) and \(D_m\) are the membrane thickness and the mean diffusion coefficient, respectively. Since minimizing the membrane thickness and miniaturizing the system can improve the response time, numerous efforts have been made to design nano-optodes. In our nano-optode design, a polyvinyl chloride (PVC) membrane was used which contained the appropriate ionophore and fluorescein, which was used as the chromoionophore. This membrane was immobilized over the surface of blue emitting CdZnS/ZnS QDs that emit in the same region that fluorescein absorbs. It was hypothesized that energy transfer efficiency between the QD and fluorescein would be modulated in response to exposure of the analyte. The first successful implementation of nano-optodes incorporating QDs was reported by Dubach et al.\(^{157}\) In their system, an absorptive chromoionophore was used to modulate the emission of a single quantum dot emitter, resulting in a "turn-off" type of sensor. To achieve a ratiometric response the group incorporated a second quantum dot into the design. However, it is not clear (and is rather unlikely) that each optode was composed of a single QD reporter / QD reference. As such, this design lacks finesse as each nano-optode is not a stand-alone system, an issue that we address here by developing a very different motif for ratiometric reporting of analytes. Considering the small size of the QDs and the thin layer of the membrane coating, a fast response to analyte concentration changes can be anticipated. As
discussed previously, a ratiometric response offers several advantages including self-calibration, which is especially useful in complex biological matrices.

5.2 Experimental section

5.2.1 Materials and instrumentation

Cadmium oxide (≥99.99), zinc oxide (≥99%), sulfur (≥99.5%), dodecylamine (≥99%), (diglycolyl chloride (95%), dioctylamine (97%), trioctylphosphine (97%), oleylamine (≥98%), diethylzinc (≥52 wt. % Zn basis), bis(trimethylsilyl) sulfide (synthesis grade), trimethylamine (TEA, ≥99%), oleic acid (90%), 1-octadecene (90%), 6-aminofluorescein (95%), sodium ionophore VI, sodium tetrakis[3,5-bis(trifluoromethyl)phenyl] borate and high molecular weight polyvinyl chloride were purchased from Sigma-Aldrich. Sodium bicarbonate (99.5%) was purchased from Acros. Sodium sulfate (anhydrous, >99%) and ethyl ether anhydrous were purchased from Fisher Scientific. Bis(2-ethylhexyl)sebacate (≥98%) was purchased from TCI America. The QD solubilizing agent 40% octylamine-modified poly(acrylic acid) (PAA) was prepared according to previously published protocols, and oleic acid was purified using the protocol of Ref 113. ²H NMR spectra were recorded on a Bruker Avance DRX 400 NMR spectrometer. UV–vis absorbance spectra of the samples were taken using a Varian Cary 300 Bio UV/vis spectrophotometer, and fluorescence emission spectra were obtained using a custom-designed Horiba Jobin Yvon FluoroLog spectrophotometer.

5.2.2 \( N,N,N',N'-\text{tetraoctyl-3-oxapentane-1,5-diamide} \) (TODGA)

A solution of diglycolyl chloride (1.03 g, 6 mmol) in 20 mL of diethyl ether was added dropwise to a mixture of dioctylamine (1.6 g, 12 mmol) and triethylamine (1.22 g, 12 mmol)
in 30 mL of diethyl ether at 0°C and stirred at room temperature overnight. Next the mixture was washed with HCl (0.1 M), followed by saturated NaHCO₃ solution and brine. Next, the organic phase was dried over anhydrous Na₂SO₄, and the solvent was removed under reduced pressure to obtain the product in a form of an amber oil. The ¹H NMR (Figure 44) confirmed the structure of the product. The reaction yield was 85%.

Figure 44. ¹H NMR spectrum of TODGA
5.2.3 **CdZnS/ZnS quantum dots synthesis**

A mixture of CdO (0.032 g, 0.25 mmol), ZnO (0.040 g, 0.50 mmol), purified oleic acid (1 g, 3.54 mmol) and octadecene (5 g, 19.80 mmol) in 3-neck round bottom flask was heated to 80°C under reduced pressure, and continued heating to 310 °C under nitrogen flow. A degassed solution of sulfur (25 mg, 0.78 mmol) in dodecylamine (1 g, 5.40 mmol) was swiftly injected into the hot pot, which was kept stirring at 300 °C for 30 min. Half of the batch of QDs prepared as described above was processed by precipitation via the addition of a small amount of isopropanol followed by methanol. The supernatant was discarded and the core QDs were dispersed in hexane. A 4-neck 50 mL glass round-bottom flask was loaded with 4 mL of oleylamine and 5 mL of TOP. The solution was degassed at 80°C and then flushed with N₂. The core QDs in hexane were added and the hexane was subsequently removed under vacuum over the course of 1 h. The temperature was increased to 180 °C and a mixture of diethyl zinc (80 mg, 0.65 mmol) and bis(trimethylsilyl) sulfide (160 mg, 0.90 mmol) in 4 mL of TOP was added slowly through an addition funnel over the course of 1 h, and was kept stirring for 30 min afterward (Figure 45).

5.2.4 **Aminofluorescein-PAA**

0.3 mg of 40% octylamine-modified PAA and 30 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were dissolved in 5 mL of DMF. Next, 1 mg of 6-aminofluoresceine was added to the mixture which was stirred overnight. The following day the aminofluorescein-PAA was precipitated from the solution by adding slightly acidic water, and dried under reduced pressure to obtain a yellowish powder.
5.2.5 Nano-optode synthesis

Approximately 0.5 g of the CdZnS/ZnS QDs were processed by addition of a small amount of isopropanol followed by methanol to induce flocculation. The supernatant was discarded, and the precipitate was dried under vacuum. The QDs were dissolved in 2 mL of THF, and 50 μL of a PVC coating solution (50 mg of high molecular weight polyvinyl chloride and 100 mg of bis(2-ethylhexyl) sebacate, 0.1 mg of sodium tetrakis[3,5-bis(trifluoromethyl)phenyl] borate and 0.25 mg of ionophore (TODGA or sodium ionophore VI) dissolved in 5 mL of THF) was added. The concentration ratio of the QD/ionophore was adjusted to 1:75. The mixture was stirred gently overnight, after which the solvent was evaporated under reduced pressure. Next, 65 mg of amphiphilic 40%
octylamine-modified PAA and 5 mg of pre-conjugated 6-aminofluoresceine-PAA were added to the QDs followed by ∼3 mL of dry THF. The solution was sonicated for several minutes to dissolve the polymer completely. The QDs were dispersed into 0.1 M NaOH solution after the solvent was evaporated under reduced pressure. The solution was dialyzed to neutrality to remove excess polymer. The solution was then filtered through a 0.2 μm syringe filter to yield a monodisperse ion-selective nano-optode solution.

5.3 Results and discussion

The chromoionophore used for this study is pH-sensitive aminofluorescein, which we established is effective for synthesizing a QD-based pH sensor. The absorbance of aminofluorescein in the 460-500 nm region changes under different pH conditions as shown in Figure 46a. By conjugating this dye to a blue-emitting CdS/ZnS quantum dot, FRET can be established from the QD to the fluorescein dye. The emission is ratiometrically pH sensitive as shown in Figure 46b. The goal of our study is to turn this pH sensor to an ion-selective sensor by introducing an ionophore and other traditional optode components into the design (Figure 47). As discussed previously there are different designs possible, depending on the type and charge of the chromoionophore and the ionophore. Since the uncomplexed ionophores used in our study are neutral, they have the same charge as the analyte when bound together. As such, to ensure the permselectivity (the preferential permeation of certain ionic species) of the membrane, the incorporation of lipophilic ions of opposite charge is required. It is believed that the membrane matrix poly(vinyl chloride) already contains some anionic impurities that play the same role. Furthermore, fluorescein dye is anionic at pH 7.4. Regardless, we added additional ionic sites to the membrane matrix to optimize the selectivity.
Figure 46. pH sensing by a QD/fluorescein conjugate. (a) The aminofluorescein absorbance change under different pH conditions. (b) A ratiometric response to change in pH are observed from a CdZnS/ZnS QD-fluorescein conjugate due to modulation of FRET efficiency.
Figure 47. Nano-optode design. (L: ionophore, R: organic anion)
Sodium tetrakis[3,5-bis(trifluoromethyl)phenyl] borate was added as lipophilic ion exchanger to the membrane matrix.

5.3.1 **Nano-optode for detection of sodium**

A novel nano-optode for detection of sodium was constructed using the aforementioned design with blue-emitting QDs, fluorescein, and sodium ionophore VI in the PVC membrane. This nano-optode was water solubilized using 40% octylamine modified polyacrylic acid to obtain a homogenous and clear solution. The nano-optode shows a significant response to the addition of sodium as shown in Figure 48a,c. However, interference tests demonstrated that the presence of the other cations, such as K⁺, Li⁺, NH₄⁺, can result in a ratiometric response as shown in Figure 48b. The selectivity of the nano-optode can be improved by replacing the ionophore with a more selective one, but there are limitations due to solubility issues.

5.3.2 **Nano-optode for detection of calcium**

A calcium nano-optode was constructed using the same paradigm with $N,N,N',N'$-tetraoctyl-3-oxapentane-1,5-diamide (TODGA) acting as the ionophore. The response of the nano-optode to Ca²⁺ was significantly greater than observed in the control, which was comprised of the same materials minus the TODGA ionophore. These data are shown in Figure 49. Due to the irreproducibility of the response, further investigations on this system were halted.
Figure 48. Ratiometric response of the sodium nano-optode. (a) The integrated dye/QD ratio as a function of sodium concentration. (b) The response of the system to similar analytes. (c) Ratiometric response of the nano-optode to different concentrations of sodium.
Figure 49. Ratiometric response of the calcium nano-optode. (a) dye/QD ratio change in response to calcium. (b) Blank nano-optode with no ionophore incorporated ratio change in response to calcium. (c) Ratiometric response of the nano-optode to different concentrations of calcium.
5.4 Conclusion

Our group has previously developed a ratiometric pH sensor by conjugating aminofluorescein to blue-emitting CdZnS/ZnS QDs. The pH probe was re-cast into a nano-optode by incorporation of an ionophore into a PVC coating on the QDs. Two types of ionophores were used to construct the nano-optodes for the detection of sodium or calcium ions. The system comprised of sodium ionophore VI showed a significant response to the analyte; however, interference tests revealed a lack of selectivity. The TODGA ionophore was synthesized and added to the PVC coating to create a functioning ratiometric calcium probe. The results were significantly better than previous reports, but, unfortunately they were not reproducible, and further investigations were not pursued.
6 Conclusion

Quantum dots have numerous potential applications. In this regard, the use of QDs for bioimaging and biosensing probes will likely have a significant impact. During my studies in the Snee group my focus as an analytical chemist was to design fluorescent diagnostic tools using novel QDs that I also developed. QD synthesis, characterization, and functionalization were discussed in this thesis. A novel protocol for the synthesis of near IR emitting AgInS$_2$/ZnS as bioimaging probes was introduced. The most prominent water-solubilization and functionalizing methods were tested on these QDs, and the potential use of these QDs as bioimaging agents was demonstrated with *in vivo* studies. These QDs were also used in a platform to construct oxygen sensing probes. A novel oxygen sensing dye (perylene-PEG-amino) was created and conjugated to AgInS$_2$/ZnS to achieve a self-calibrating ratiometric response that offers advantages over traditional turn-on probes, especially in complicated biological matrices. *In vitro* studies in HeLa cells also showed significant response that can be used to detect hypoxia conditions.

A ratiometric QD sensor was designed for the detection of H$_2$S. The system responds to bisulfide ion due to an alteration of the Förster resonance energy transfer efficiency from a green-emitting CdSe/ZnS QDs to a modified rhodamine B that is linked via a disulfide bond. The bond can be reduced in the presence of the HS$^-$, which results in a change in emission color from the probe. The sensor showed a strong response to the analyte with high selectivity. *In vitro* studies also confirmed the ratiometric sensing paradigm functions in a biological environment.
Finally, nano-optodes were created for the detection of the sodium and calcium ions based on traditional bulk optode design. Blue-emitting CdZnS/ZnS QDs were synthesized successfully, and a PVC membrane containing both an ionophore and a chromoionophore (aminofluorescein) was added to create a nano-optode structure. Deprotonation of the chromoionophore in response to the presence of the analyte ions created a ratiometric response. Due to the lack of the selectivity and reproducibility the results were not published in the form of a scientific manuscript.
CITED LITERATURE

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