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Development of Multifunctional Nanoparticles: From Synthesis to Theranostic Applications

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by

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Abstract

The objective of my research is to develop multifunctional nanoparticles for sensing and photodynamic therapy applications.

Ethylenediaminetriacetic acid (EDTA)-functionalized silica nanoparticles were synthesized by microemulsion method. The EDTA groups on the nanoparticle surface have the role to chelate with metal ions to form paramagnetic nanoparticles in aqueous solution, which would reduce the relaxation rate of water protons. In this study, SiO$_2$@TMS-EDTA@Fe$^{3+}$ NPs and SiO$_2$@TMS-EDTA@Gd$^{3+}$ NPs were used for detection of dopamine and phosphate ion, respectively. The results demonstrate that paramagnetic nanoparticles can be integrated into relaxation based detection schemes while avoiding the aggregation problem commonly associated with more widely used superparamagnetic nanoparticles.

Lanthanide-based NaYF$_4$:Yb$^{3+}$,Tm$^{3+}$ upconversion nanoparticles were synthesized in the presence of polyacrylic acid (PAA) via solvothermal method. This is an one step synthesis method to get uniform, reproducible and biocompatible NaYF$_4$:Yb,Tm upconversion nanoparticles with –COOH surface functional groups. In this study, we designed a ligase-assisted signal-amplifiable DNA biosensor based on NaYF$_4$:Yb$^{3+}$,Tm$^{3+}$ UCNPs with high sensitivity and specificity.

Silver nanoparticles captured by mesoporous silica nanoparticles with photosensitizer (Ag@MS@HPIX) were synthesized using silver nitrate as the silver source, formaldehyde as the reducing agent, CTAB as the template/stabilizer, TEOS/TMS-EDTA as the silane source, sodium hydroxide as the catalyst, and HPIX as the loading photosensitizer. Ag@MS@HPIX NPs show strong enhanced singlet oxygen
generation because of the strong resonance coupling between surface plasmon Ag nanoparticles and the photosensitizing molecules, consequently demonstrating highly efficient photodynamic inactivation (PDI) efficacy against both gram-positive and gram-negative bacteria. In this study, PDI efficacy of Ag@MS@HPIX NPs was tested against a multidrug-resistant strain of *Staphylococcus aureus*.

Theranostic silver nanoparticles were synthesized via a new, robust, simple, and reproducible microwave-assisted method. The EDTA groups on the nanoparticle surface have the role to chelate with Gd$^{3+}$ ions to form paramagnetic nanoparticles in aqueous solution, which would reduce the relaxation rate of water protons. In addition, the surface of Ag@TMS-EDTA@Gd$^{3+}$ NPs has the potential to form a lanthanide-porphyrin complex, serving as photosensitizers in photodynamic therapy (PDT). This nanoplatform combines functions of magnetic resonance imaging (MRI) contrast agents and PDI agents in a single nanostructure as theranostics. In this study, we performed a systematic study of multifunctional Ag@TMS-EDTA@Gd$^{3+}$@HPIX NPs to assess the PDI efficacy against the MRSA, which is a multidrug-resistant strain of *Staphylococcus aureus*.

In summary, multifunctional nanoparticles, including silica, upconversion and silver nanoparticles were successfully synthesized and their potential applications in sensing and photodynamic therapy were explored.
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TABLE OF CONTENTS

ABSTRACT .......................................................................................................................... ii

ACKNOWLEDGMENTS ....................................................................................................... v

TABLE OF CONTENTS ..................................................................................................... vi

LIST OF FIGURES .............................................................................................................. viii

LIST OF TABLES ................................................................................................................. xiii

Chapter 1. Introduction to Nanotechnology ........................................................................ 1

1.1 Overview ......................................................................................................................... 2

1.2 Application of Nanoparticles ......................................................................................... 2

1.2.1 Sensors ....................................................................................................................... 2

1.2.2 Photodynamic Therapy ............................................................................................ 6

1.3 Objective of the Research ............................................................................................. 7

1.4 References .................................................................................................................... 9

Chapter 2. Development of Nanoparticle-based Sensors .................................................... 12

2.1 Paramagnetic Relaxation-based Biosensor for Selective Dopamine Detection .......... 13

2.1.1 Introduction .............................................................................................................. 13

2.1.2 Materials and Methods .......................................................................................... 16

2.1.3 Results and Discussion ......................................................................................... 18

2.1.4 References ............................................................................................................. 25

2.2 Magnetic Relaxation-based Sensing of Phosphate Ion ................................................. 29

2.2.1 Introduction ............................................................................................................. 29

2.2.2 Materials and Methods .......................................................................................... 32
Chapter 2. Results and Discussion

2.2.3 Results and Discussion ................................................................. 34

2.2.4 References .............................................................................. 38

Chapter 2.3. Luminescence Resonance Energy Transfer based DNA Biosensor

2.3.1 Introduction ............................................................................. 42

2.3.2 Materials and Methods ............................................................. 43

2.3.3 Results and Discussion ............................................................ 45

2.3.4 References ............................................................................... 53

Chapter 3. Development of Nanoparticle based Photosensitizers for Photodynamic Therapy

3.1 Silver Nanoparticle-enhanced Photosensitizer Hybrids for Photoinactivation of Multidrug-resistant Bacterium *Staphylococcus aureus* (MRSA) .................................. 56

3.1.1 Introduction ............................................................................. 56

3.1.2 Materials and Methods ............................................................. 57

3.1.3 Results and Discussion ............................................................ 60

3.1.4 References ............................................................................... 65

3.2 Multifunctional Nanoparticles as MRI Contrast Agents and Photosensitizers against Multidrug-resistant Bacterium .......................................................... 68

3.2.1 Introduction ............................................................................. 68

3.2.2 Materials and Methods ............................................................. 69

3.2.3 Results and Discussion ............................................................ 72

3.2.4 References ............................................................................... 77

Chapter 4. Summary and Future Efforts .................................................. 82
LIST OF FIGURES

Figure 1.1. Schematic illustration of a typical sensor (a), MRS sensor (b)........................................3

Figure 1.2. Schematic illustration of detection mechanism of MRS sensor in the presence of target........................................................................................................................................4

Figure 1.3. Advantages of MRS sensor........................................................................................................4

Figure 1.4. Upconversion mechanism of UCNPs doped with Yb\(^{3+}\)/Er\(^{3+}\) or Yb\(^{3+}\)/Tm\(^{3+}\)........................................................................................................................................5

Figure 1.5. The Yb\(^{3+}\)-rich shell (red) absorbs the 980 nm excitation radiation and subsequently transfers energy to the Er\(^{3+}\)/Yb\(^{3+}\)-doped core (green), generating blue, green, and red emissions................................................................................................................................6

Figure 1.6. PDT needs a PS, light with appropriate wavelength, and oxygen.................7

Figure 1.7. Design of magnetic relaxation sensing method based the target-binding process........................................................................................................................................8

Figure 2.1.1. Schematic illustration of the detection mechanism of dopamine biosensor. Upon the addition of dopamine, SiO\(_2\)@TMS-EDTA@Fe\(^{3+}\) nanoparticles bind to dopamine, leading to increased T\(_2\) relaxation time of the surrounding water protons........................................................................................................................................15

Figure 2.1.2. (a) TEM image of the SiO\(_2\)@TMS-EDTA@Fe\(^{3+}\) nanoparticles. (b) R\(_2\) relaxivity measurement of SiO\(_2\)@TMS-EDTA@Fe\(^{3+}\) nanoparticles in acetate buffer........................................................................................................................................19

Figure 2.1.3. (a) UV-vis spectra of the SiO\(_2\)@TMS-EDTA and SiO\(_2\)@TMS-EDTA@Fe\(^{3+}\) nanoparticles with dopamine. (b) A distinct color of SiO\(_2\)@TMS-EDTA@Fe\(^{3+}\)@dopamine sample........................................................................................................................................20
**Figure 2.1.4.** FT-IR spectra of the SiO$_2$@TMS-EDTA and SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles with dopamine.................................................................21

**Figure 2.1.5.** $\Delta T_2$ % as a function of dopamine concentration with samples in (a) acetate buffer, and (b) aCSF........................................................................................................22

**Figure 2.1.6.** $\Delta T_2$% as a function of analyte concentration for samples in acetate buffer containing dopamine, sucrose, and glucose, respectively................................................23

**Figure 2.2.1.** Schematic representation of the detection mechanism. Phosphate binding to SiO$_2$@TMS-EDTA@Gd$^{3+}$ NPs leads to the increase of T$_2$ relaxation time of water protons...................................................................................................................31

**Figure 2.2.2.** (a) TEM images of the SiO$_2$@TMS-EDTA@Gd$^{3+}$ nanoparticles. Scale bar is 100 nm (b) $R_2$ relaxivity measurement of SiO$_2$@TMS-EDTA@Gd$^{3+}$ nanoparticles........................................................................................................35

**Figure 2.2.3.** $\Delta T_2$ % as a function of phosphate concentration in tris buffer.........................................................................................................................36

**Figure 2.2.4.** $\Delta T_2$ % as a function phosphate concentration of fertilizer in tris buffer.........................................................................................................................36

**Figure 2.2.5.** The specificity of the phosphate sensor. $\Delta T_2$ % after adding various anions including control (water), CO$_3$–, acetate, Cl–, F–, NO$_3$–, H$_2$PO$_4$– and SO$_4$$_2$– to the SiO$_2$@TMS-EDTA@Gd$^{3+}$ nanoparticle solution at pH 7.5.........................................................37

**Figure 2.3.1.** Schematic illustration of DNA detection based on UCNPs..................45

**Figure 2.3.2.** (a) TEM picture of UCNPs (Scale bar: 500 nm), (b) FTIR spectrum of UCNPs with surface –COOH groups.................................................................47
Figure 2.3.3. SG1 fluorescence emission excited at 490 nm with different amounts of DNA_seg1 (a), and a calibration curve (b)...

Figure 2.3.4. Normalized excitation and emission spectra of SG1 and emission spectrum of NaYF4:Yb3+,Tm3+ upconversion nanoparticles excited at 980 nm...

Figure 2.3.5. (a) Luminescence emission spectra under 980 nm excitation after 0, 5, 10, 20 thermal cycles with 1 pmol DNA_tar, and with water as control. (b) I_{530}/I_{477} ratio vs. number of thermal cycle...

Figure 2.3.6. (a) Luminescence emission spectra under 980 nm excitation with different amounts of DNA_tar after 20 thermal cycles. (b) I_{530}/I_{477} ratio vs. amount of initial DNA_tar...

Figure 2.3.7. Luminescence emission spectra under 980 nm excitation with 1 pmol of DNA_tar and 1 pmol of DNA_mut, respectively...

Figure 3.1.1. (a) Schematic illustration of synthesis of Ag@MS@HPIX hybrid and its singlet oxygen generation. (b) Chemical structure of HPIX. (c) TEM image of Ag@MS nanoparticle. Scale bar is 20 nm. (d) Zeta potential and hydrodynamic size of Ag@MS nanoparticles and Ag@MS@HPIX hybrids...

Figure 3.1.2. (a) UV-vis absorption spectra of Ag@MS, Ag@MS@HPIX, MS@HPIX, and HPIX, (b) Fluorescence emission spectra of Ag@MS@HPIX, Ag@MS, and free HPIX...

Figure 3.1.3. Singlet oxygen (a) emission and (b) excitation spectra of Ag@MS@HPIX hybrids, Ag@MS nanoparticles, and pure HPIX...

Figure 3.1.4. Antibacterial assays of Ag@MS@HPIX hybrids (a), Ag@MS nanoparticles (b), and HPIX (c) against MRSA with or without white light illumination.
Ag@MS@HPIX and Ag@MS contain the same amount of Ag. Results are expressed as mean±SD (n=3, p<0.05). (d) Bacterial killing efficacy of Ag@MS@HPIX hybrids, Ag@MS nanoparticles, and HPIX against MRSA under white light illumination. Enhancement in bacterial killing efficacy is defined as: 

$$\log_{10}(\text{enhancement killing}) = \log_{10}(\text{Ag@MS@HPIX killing}) - \log_{10}(\text{Ag@MS killing}) - \log_{10}(\text{HPIX killing})$$

Figure 3.2.1. Schematic illustration of the synthesis of Ag@TMS-EDTA@Gd$^{3+}$@HPIX NP and its singlet oxygen generation under light illumination.

Figure 3.2.2. UV-vis absorption spectra of Ag@TMS-EDTA@Gd$^{3+}$@HPIX NPs, Ag@TMS-EDTA@Gd$^{3+}$ NPs, TMS-EDTA@Gd$^{3+}$@HPIX, Ag@TMS-EDTA NPs, and free HPIX.

Figure 3.2.3. (a) TEM image of Ag@TMS-EDTA NPs. Scale bar is 20 nm. (b) $R_1$ and $R_2$ relaxivities of Ag@TMS-EDTA@Gd$^{3+}$@HPIX NPs.

Figure 3.2.4. Singlet oxygen (a) emission and (b) excitation spectra of Ag@TMS-EDTA@Gd$^{3+}$@HPIX NPs, Ag@TMS-EDTA@Gd$^{3+}$ NPs, and HPIX.

Figure 3.2.5. Antibacterial assays of Ag@TMS-EDTA@Gd$^{3+}$@HPIX NPs (a), Ag@TMS-EDTA@Gd$^{3+}$ NPs (b), and HPIX (c) against MRSA with or without white light illumination. Results are expressed as mean±SD (n=3, p<0.05). (d) Bacterial killing efficacy of Ag@TMS-EDTA@Gd$^{3+}$@HPIX NPs, Ag@TMS-EDTA@Gd$^{3+}$ NPs, and HPIX against MRSA under white light illumination. Enhancement in bacterial killing efficacy is defined as: 

$$\log_{10}(\text{enhancement killing}) = \log_{10}(\text{Ag@TMS-EDTA@Gd$^{3+}$@HPIX killing}) - \log_{10}(\text{Ag@TMS-EDTA@Gd$^{3+}$ killing}) - \log_{10}(\text{HPIX killing})$$
**Figure 4.1.** A general scheme can be used for sensing purpose: SiO$_2$@TMS-EDTA @paramagnetic NPs. Surface –COOH functional groups can be used for conjugation of different recognition elements to detect other targets.........................................................83

**Figure 4.2.** TMS-EDTA@Gd$^{3+}$ complex has the key role in attracting porphyrin unit of HPIX..................................................................................................................................................84
LIST OF TABLES

Table 2.1.1. Zeta potential and size of the nanoparticles measured by particle size analyzer........................................................................................................................................19

Table 2.3.1. Probes and target DNA sequences.................................................................................................................................46
Chapter 1

Introduction to Nanotechnology
1.1 Overview

Nanotechnology, the science of building small features, is defined as the understanding and controlling of matter at the nanometer scale, which is at the dimensions of approximately 1 to 100 nanometers [1,2]. At the nanoscale, the physical, chemical, and biological properties of materials differ from the properties of matter either at smaller scales, such as single atoms or molecules, or at larger scales such as bulk materials in millimeters [3,4]. Unique properties of nanomaterials are highly dependent on their size, shape, unique surface chemistry and high surface area [5,6]. Because of these unique and tunable properties, nanomaterial-based systems have great potential to enhance current analytical techniques, and advantages of enabling the design of easy to use, robust, and portable detections with high sensitivity/specificity and cost-effectiveness [7]. They have a broad and great impact in research and clinical applications [8].

1.2 Applications of Nanoparticles

1.2.1 Sensors

Sensors are devices that measure biological, chemical or physical change and convert it to a detectable signal [9]. One of the important components of sensor is recognition element that can selectively sense the target. Another main component of a sensor is the transducer that converts the observed change into a detectable signal. And the last component of sensor is signal processor that collects and displays the signal [10] (Figure 1.1a). Nanotechnology allows researchers to build new sensing devices that are small, fast in response time and cost-efficient, giving them the opportunity to be more sensitive and more specific than current techniques. Various nanomaterials, such as silica
[11], gold [12], silver [13], upconversion [14], magnetic nanomaterials [15], and quantum
dots [16], have been extensively explored in sensor design with high sensitivity and
selectivity.

**Magnetic Nanoparticle-based Magnetic Relaxation Switch Sensor**

Magnetic nanoparticle-based magnetic relaxation switch (MRS) methods have
great potential to provide fast and reliable results using benchtop nuclear magnetic
resonance (NMR) relaxometer [17] (Figure 1.1b).

![Figure 1.1](image)

**Figure 1.1.** Schematic illustration of a typical sensor (a), MRS sensor (b).

The principle of detection mechanism of MRS methods is based on their ability to
switch between a disperse and clustered state upon target interaction resulting in a change
in the spin-spin relaxation time ($T_2$) of their surrounding water protons [18] (Figure 1.2).
Figure 1.2. Schematic illustration of detection mechanism of MRS sensor in the presence of target.

In addition, change in $T_2 (\Delta T_2)$ is detectable for any target in a variety of complex, opaque samples without any sample preparation and washing steps [19] (Figure 1.3).

Figure 1.3. Advantages of MRS sensor.
Upconversion Nanoparticle-based Sensor

Lanthanide-doped upconversion nanoparticles (UCNPs) have drawn increasing attention because of their unique capability of converting low energy photons to high energy photons through consecutive excitation of multiple photons via an anti-Stokes emission process [20] (Figure 1.4).

![Figure 1.4. Upconversion mechanism of UCNPs doped with Yb\(^{3+}\)/Er\(^{3+}\) or Yb\(^{3+}\)/Tm\(^{3+}\) [20].](image)

UCNPs are usually comprised of an inorganic crystalline host lattice, embedded with low concentration of dopants as lanthanide ions like Yb\(^{3+}\), Er\(^{3+}\), and Tm\(^{3+}\) [20] (Figure 1.5). UCNPs have multiple unique properties, including the absence of auto-fluorescence behavior, cross-excitation, and deep light penetration through the tissues [21]. Moreover, their optical properties can be controllable to enhance upconversion at a selected wavelength, which make them popular for biomedical applications especially in sensing, imaging, drug delivery and photodynamic therapy [22].
Figure 1.5. The Yb$^{3+}$-rich shell (red) absorbs the 980 nm excitation radiation and subsequently transfers energy to the Er$^{3+}$/Yb$^{3+}$-doped core (green), generating blue, green, and red emissions [20].

1.2.2 Photodynamic Therapy

Photodynamic therapy (PDT) of cancer cells and microorganisms is one of the most promising and innovative approaches, which generates singlet oxygen to kill cancer cells and microorganisms by combining photosensitizer (PS), light illumination of appropriate wavelength, and oxygen [23]. When the photosensitizer is exposed to specific wavelengths of light, it becomes activated from the ground state to an excited state. As it returns to the ground state, it releases energy, which is transferred to nearby oxygen to generate singlet oxygen (Figure 1.6). However, PDT has some limitations due to the delivery of PSs and light into tissues [24]. In addition, most of the PSs are hydrophobic and tend to aggregate easily in aqueous solutions, resulting in reduced singlet oxygen generation [25]. Therefore there is a great interest in developing photosensitizers based on nanoplatorms incorporating PSs into nanoparticles to overcome these limitations [26].
1.3 Objective of the Research

Development of Nanoparticle-based Sensors

Magnetic Relaxation-based Sensor Design

Superparamagnetic iron oxide nanoparticles (SPIONs) are commonly used in MRS based sensing. Nevertheless, there are some challenges associated with the target-induced clustering detection schemes, e.g., over-clustering of nanoparticles will lead to their precipitation, undermining the reliability of $T_2$ measurement results [27]. Our aim is to develop magnetic relaxation-based detection system based on the target binding process, instead of the target-induced clustering process (Figure 1.7).
Figure 1.7. Design of magnetic relaxation sensing method based the target-binding process.

Upconversion Nanoparticles-based Sensor Design

Lanthanide based upconversion nanoparticles are commonly used for sensing, imaging and photodynamic therapy [28]. However it remains a big challenge to synthesize uniform, reproducible and biocompatible upconversion nanoparticles with hydrophilic surface [29]. Our aim is to synthesize NaYF₄:Yb,Tm upconversion nanoparticles with –COOH surface functional groups, and design an upconversion nanoparticle-based sensor to detect DNA with high sensitivity/specificity.

Development of Nanoparticle-based Photosensitizers for Photoinactivation of a Multidrug-resistant strain of *Staphylococcus aureus* (MRSA)

We recently reported that Ag@PS hybrid nanoparticles exhibit enhanced singlet oxygen generation because of the strong resonance coupling between surface plasmon of Ag nanoparticles and the photosensitizing molecules, leading to highly efficient photodynamic inactivation against both gram-positive and gram-negative bacteria [30].
Our aim is to design theranostic Ag@PS hybrid nanostructures, which not only produce enhanced PDI effect against a multidrug-resistant strain of *Staphylococcus aureus*, but also provide MR imaging capability.

1.4 References


Chapter 2

Development of Nanoparticle-based Sensors

The study in chapter 2.1 has been published in *Chemical Communications*:


The study in chapter 2.3 is a collaborative effort with Peng Wang. I mainly worked on the development of synthesis method for upconversion nanoparticles and their characterization. It has been published in *RSC Advances*:

Chapter 2.1

Paramagnetic Relaxation-based Biosensor for Selective Dopamine Detection

2.1.1 Introduction

One of the major challenges in biosensing is the accurate, selective and rapid detection of important targets, such as disease biomarkers, pathogens and cancer cells in biological samples, preferably with little or no pretreatment. Over the years different biosensing systems have been developed to detect such targets with good selectivity and sensitivity, enabling early disease diagnosis [1-3]. Among them, magnetic nanoparticles (MNPs) based biosensing has received considerable attention recently [4-6]. Magnetic relaxation switch (MRS) based sensing methods have shown good promise to provide fast and reliable results using benchtop nuclear magnetic resonance (NMR) relaxometer [7-11]. These methods are based on the use of surface-functionalized MNPs to recognize specific targets. The switch between a dispersed and clustered state of the MNPs upon interaction with the target lead to a change in the transverse relaxation time ($T_2$) of the water protons in the solution [12,13].

Superparamagnetic iron oxide nanoparticles (SPIONs) are commonly used in MRS based sensing. Nevertheless, there are some challenges associated with the target-induced clustering detection schemes, e.g., over-clustering of nanoparticles will lead to their precipitation, undermining the reliability of $T_2$ measurement results. Thus, improvement in the stability of MNPs prior to and after target addition is crucial to the relaxation-based detection.
Paramagnetic metal ions, such as Gd$^{3+}$, Fe$^{3+}$, Mn$^{2+}$, Mn$^{3+}$ and Cu$^{2+}$, can also affect the transverse ($T_2$) and longitudinal ($T_1$) relaxation times of the surrounding water protons, and serve as contrast agents in magnetic resonance imaging (MRI) [1,14-17]. Due to the toxicological concerns on the free metal aqua ions, chelating ligands are commonly used to reduce the toxicity of the metal ions [18-21]. However, complete coordination of metal ions decreases the access of water protons to the metal ions, and the overall effectiveness of the metal ions as contrast agents decreases [22,23]. Therefore it is important to develop metal chelates with both strong magnetic relaxivity to act as good contrast agents and good stability to ensure low toxicity [24,25]. Gd$^{3+}$ chelates have been the most frequently used contrast agents commercially because of their strong paramagnetic properties. Other more biocompatible paramagnetic metal chelates have also been investigated of viability as contrast agents [26,27]. Fe$^{3+}$ ion has five unpaired electrons with high magnetic moment, thus is a good candidate for acting as contrast agent [28]. However, Fe$^{3+}$ ions tend to form insoluble oxides at pH $>$ 4. On the other hand, complete coordination of Fe$^{3+}$ ions by ligands would greatly interfere with the interaction between Fe$^{3+}$ ions and water protons, which largely reduces the efficiency of Fe$^{3+}$-based contrast agents [28]. Hence, one of the major challenges in developing Fe$^{3+}$-based contrast agents is to improve the stability and dispersibility of Fe$^{3+}$ complexes in the aqueous media while maintaining the accessibility of the Fe$^{3+}$ ions by water protons [20,29].

In this study, we developed a $T_2$-based biosensor to detect dopamine, which is important neurotransmitter associated with various brain functions including learning and memory. Currently, there have been a number of methods reported on the detection of
dopamine, such as, surface-enhanced Raman spectroscopy (SERS) [30-32],
electrochemistry [33], UV-vis spectroscopy [34] and fluorescent spectroscopy [35].
While these methods have shown varying degrees of success in detecting dopamine, there
is ongoing need to develop alternative methods that are less labor-intensive, less time-
consuming or expensive, and with little sample pretreatment. The detection described
here can be carried out on a benchtop NMR relaxometer within minutes, and does not
require sample pretreatment.

Figure 2.1.1. Schematic illustration of the detection mechanism of dopamine biosensor. Upon the
addition of dopamine, SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles bind to dopamine, leading to
increased T$_2$ relaxation time of the surrounding water protons.
The principle of the sensing scheme is shown in Figure 2.1.1. Ethylenediaminetriacetic acid groups (EDTA) are first grafted onto the surface of silica nanoparticles (SiO$_2$ NPs) through the use of EDTA-functionalized silane. As strong chelating agents for many metal ions, the EDTA groups on the nanoparticle surface can readily capture and stabilize Fe$^{3+}$ ions in aqueous solution, which would reduce the relaxation rate of water protons. Meanwhile, dopamine would bind to Fe$^{3+}$ ions selectively due to the high affinity of its catechol groups to Fe$^{3+}$ [32,36], and interfere with the interaction between the Fe$^{3+}$ ions and water protons, subsequently affecting the relaxation rate of water protons in the solution [19,37]. Experimentally, by monitoring the T$_2$ of water protons, which is directly associated with the relaxation rate of water protons, we can detect the presence of dopamine.

2.1.2 Materials and Methods

Materials

(Trimethoxysilylpropyl)ethyldiaminetriacetic acid trisodium salt (TMS-EDTA) (35 wt% solution in water) was purchased from Gelest. Tetraethylorthosilicate (TEOS), Triton X-100 (TX-100), n-hexanol, cyclohexane, dopamine hydrochloride, ferric chloride, ammonium hydroxide (30wt%) and glacial acetic acid were from Sigma-Aldrich. All chemicals were used without further purification. Deionized (DI) water was used for the preparation of all solutions.

Synthesis of Fe$^{3+}$-chelated SiO$_2$ nanoparticles (SiO$_2$@TMS-EDTA@Fe$^{3+}$)

Paramagnetic SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles were prepared in two steps using a modified version of the previously published protocol [38]. First, SiO$_2$@TMS-EDTA nanoparticles with EDTA groups on surface were synthesized by mixing 1.77 g
Triton X-100, 7.5 mL cyclohexane, 1.6 mL n-hexanol, and 480 µL DI water in a glass vial and stirring for 5 min. Next, 60 µL of NH₄OH was added to the microemulsion and stirred for 20 min, followed by the addition of 50 µL TEOS. The mixture was stirred at room temperature for 24 h. Then, 50 µL of TEOS was added to the microemulsion and stirred for 30 min, and finally 25 µL of TMS-EDTA was added, followed by another 24 h stirring. Subsequently, approximately 20 mL of acetone was added to break down the microemulsion system. SiO₂@TMS-EDTA nanoparticles were recovered by centrifuging at 14000 rpm for 20 min, and then washed three times with acetone, ethanol and DI water, respectively. The resulting SiO₂@TMS-EDTA nanoparticles were dispersed in DI water.

Paramagnetic property was introduced to the SiO₂@TMS-EDTA nanoparticles by mixing excess amount of 0.1 M FeCl₃ solution and stirring overnight. Afterwards, nanoparticles were washed three times with DI water and then dispersed in acetate buffer (pH 4) for storage.

**ICP-MS measurements**

SiO₂@TMS-EDTA@Fe³⁺ nanoparticle samples were prepared in 2% trace grade nitric acid, which can release Fe³⁺ into the solution. A standard addition plot was made for SiO₂@TMS-EDTA@Fe³⁺ nanoparticle samples and acetate buffer (as control). The released Fe³⁺ concentration of each sample was measured by a triple quadruple ICP-MS (ICP-QQQ, Agilent Technologies). Signal from the control was subtracted from that of each sample.
Paramagnetic Relaxation Assays

For the relaxation measurements, Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequences were used to limit the effect of magnetic inhomogeneity because of instrument [1]. Transverse relaxation times ($T_2$) were measured at 1.41 T by a Bruker Minispec mq60 relaxometer operating at 40°C without any washing step [9]. Briefly, fresh dopamine stock solution was prepared and serially diluted. In a typical run, $T_2$ measurements of the SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles in acetate buffer were done before target addition. Diluted dopamine solutions were added to the SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticle solution, and $T_2$ measurements were conducted after ~4 hr of incubation at 40°C. The change of $T_2$ ($\Delta T_2$) was obtained. In the case of using aCSF as solvent matrix, aCSF solutions containing dopamine and the SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles were prepared separately. Then calculated amounts of dopamine/aCSF solution were added into SiO$_2$@TMS-EDTA@Fe$^{3+}$/aCSF solution to obtain different concentrations of dopamine, and incubated for ~4 hr at 40°C before $T_2$ measurement.

2.1.3 Results and Discussion

SiO$_2$@TMS-EDTA nanoparticles were synthesized following a modified version of the previously published protocol while introducing the EDTA-functionalized silane (TMS-EDTA) to the microemulsion system [38]. Fe$^{3+}$ ions can bind to the SiO$_2$@TMS-EDTA nanoparticles at low pH, which greatly simplifies the preparation process and eliminates the insoluble oxides formed by Fe$^{3+}$ ions.

The synthesized SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles were characterized by a number of techniques. The diameters of the SiO$_2$@TMS-EDTA nanoparticles were confirmed by transmission electron microscopy (TEM), which show an average diameter
of ~60 nm (Figure 2.1.2a). Results of dynamic light scattering (DLS) measurement (Table 2.1.1) are consistent with that of TEM. Chelation of SiO$_2$@TMS-EDTA with Fe$^{3+}$ ions results in the change of zeta potential of SiO$_2$@TMS-EDTA nanoparticles. Nevertheless, zeta potentials of both SiO$_2$@TMS-EDTA and SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles indicate fairly good stability of the nanoparticles at pH 4 (Table 2.1.1). The amount of Fe$^{3+}$ species in the SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticle solution was quantified using ICP-MS and determined to be 0.228 µg/mL.

**Table 2.1.1.** Zeta potential and size of the nanoparticles measured by particle size analyzer.

<table>
<thead>
<tr>
<th></th>
<th>Zeta potential (mV)</th>
<th>Size by DLS (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO$_2$@TMS-EDTA</td>
<td>-53±5</td>
<td>68.1±0.7</td>
</tr>
<tr>
<td>SiO$_2$@TMS-EDTA@Fe$^{3+}$</td>
<td>-31±3</td>
<td>65.5±0.5</td>
</tr>
</tbody>
</table>

**Figure 2.1.2.** (a) TEM image of the SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles. (b) $R_2$ relaxivity measurement of SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles in acetate buffer.
The binding of dopamine to the SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles is reflected by the change in the absorption spectrum (Figure 2.1.3a). It has been reported that Fe$^{3+}$-dopamine complex in solution displays characteristic absorption peaks depending on the number and type of ligands. Additional chelation to Fe$^{3+}$ ion by ligands results in peak shift in the absorption spectrum [32]. In this study, the SiO$_2$@TMS-EDTA@Fe$^{3+}$-dopamine complex has a dark green-blue color with a broad absorption band at ~650 nm. The color develops very quickly with high concentration of dopamine (Figure 2.1.3b).

![Absorption Spectrum](image)

**Figure 2.1.3.** (a) UV-vis spectra of the SiO$_2$@TMS-EDTA and SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles with dopamine. (b) A distinct color of SiO$_2$@TMS-EDTA@Fe$^{3+}$+dopamine sample.

FTIR spectra of SiO$_2$@TMS-EDTA, and SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles were obtained to verify the surface functional groups by TMS-EDTA silane and the subsequent chelation with Fe$^{3+}$. As shown in Figure 2.1.4, a strong absorption band between 1000 and 1200 cm$^{-1}$ indicate the presence of Si-O bands [39]. The absorption
peaks at 1396 and 1633 cm\(^{-1}\) are attributed to the symmetric C–O stretching and the antisymmetric C–O stretching vibrations after the TMS-EDTA treatment [39]. A weak asymmetric CH\(_2\) stretching band can be observed at 2981 cm\(^{-1}\). The broad band at 3100-3600 cm\(^{-1}\) comes from the O-H stretching vibrations.

![FT-IR spectra of the SiO\(_2\)@TMS-EDTA and SiO\(_2\)@TMS-EDTA@Fe\(^{3+}\) nanoparticles with dopamine.](image)

Figure 2.1.4. FT-IR spectra of the SiO\(_2\)@TMS-EDTA and SiO\(_2\)@TMS-EDTA@Fe\(^{3+}\) nanoparticles with dopamine.

Transverse relaxation times (T\(_2\)) of water protons were measured by a Bruker Minispec mq60 relaxometer operating at 1.41 Tesla and 40°C without any washing step. The T\(_2\) values of the SiO\(_2\)@TMS-EDTA@Fe\(^{3+}\) nanoparticles in aqueous solution result in the transverse relaxivity of the nanoparticles, R\(_2\) = 11.19 mM\(^{-1}\)s\(^{-1}\), as plotted in Figure 2.1.2b. This R\(_2\) value is comparable to that of Fe\(^{3+}\) ions reported in the literature [28].

The percentage change of T\(_2\) relaxation time (ΔT\(_2\)\%) upon the binding of dopamine to SiO\(_2\)@TMS-EDTA@Fe\(^{3+}\) nanoparticles is calculated according to the following equation [40,41]:

\[
\Delta T_2\% = \frac{(T_2\text{ target} - T_2\text{ blank}) \times 100}{T_2\text{ blank}}
\]

where T\(_2\) blank and T\(_2\) target is the average T\(_2\) relaxation time of three replicates of
We first tested the detection of dopamine in acetate buffer. As shown in Figure 2.1.5a, a continuous increase in $\Delta T_2\%$ is observed as the dopamine concentration rises up to 80 µM. A linear relationship between $\Delta T_2\%$ and dopamine concentration exists in the range of 1-10 µM with a correlation coefficient ($R^2$) of 0.995 and a detection limit of 0.2 µM.

![Figure 2.1.5](image)

**Figure 2.1.5.** $\Delta T_2\%$ as a function of dopamine concentration with samples in (a) acetate buffer, and (b) aCSF.

To assess the specificity of this detection scheme, comparison experiments using glucose and sucrose as potential dopamine competitors of comparable concentrations were carried out. Results shown in Figure 2.1.6 demonstrate that these sugar molecules do not significantly affect the detection of dopamine. Furthermore, dopamine present in a complex matrix, an Artificial Cerebrospinal Fluid (aCSF), was used as the sample. The aCSF is a complex mixture simulating the easily accessible fluid in the ventricular system. It contains several salts and sugars: 124 mM NaCl, 2.5 mM KCl, 2.0 mM
MgSO₄, 1.25 mM KH₂PO₄, 26 mM NaHCO₃, 10 mM glucose, 4 mM sucrose, and 2.5 mM CaCl₂ [30]. Results shown in Figure 5b demonstrate that, even at the relatively high concentrations of various salts and sugars, the SiO₂@TMS-EDTA@Fe³⁺ nanoparticles can still detect dopamine quantitatively. A linear relationship between dopamine concentration in aCSF and ΔT₂% is observed for the same dynamic range of 1-10 µM and a detection limit of 0.5 µM. Since the aCSF contains millimolar concentrations of sugars, it is expected that the slight increase in the uncertainty of ΔT₂% measurement by the presence of sugars in samples would be reduced with aCSF as the solvent matrix, as compared to the case of using acetate buffer as the solvent matrix (Figure 2.1.5b). The results illustrate the capability of detecting dopamine in the complex matrix quantitatively, without the need of sample pretreatment.

![Figure 2.1.6](image)  

**Figure 2.1.6.** ΔT₂% as a function of analyte concentration for samples in acetate buffer containing dopamine, sucrose, and glucose, respectively.
It is worthwhile to point out a couple of important features in this nanoparticle-based design of sensing. The SiO$_2$ component of the SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles appears to be critical in creating a change of the T$_2$ value of water protons before and after the dopamine binding. It is well known that free Fe$^{3+}$ ions can bind to dopamine in solution. Yet the T$_2$ value of water protons in solution containing only free Fe$^{3+}$ ions is unstable, which cannot be used to signal the presence of dopamine. In contrast, the T$_2$ value of water protons in solution containing SiO$_2$@TMS-EDTA nanoparticles is very stable. The synthesized SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles have excellent water dispersibility and colloidal stability in the acidic buffer. The presence of carboxyl and hydroxyl groups on the surface of SiO$_2$@TMS-EDTA nanoparticles prevents their aggregation, which tends to be a common issue with superparamagnetic nanoparticles, and provides good stability over weeks without any precipitation. To the other end, in many Fe$^{3+}$-ligand complexes the Fe$^{3+}$ ion usually have a coordination number of 6, which prevents either the binding of dopamine to the Fe$^{3+}$-center or the direct interaction between water protons and the Fe$^{3+}$-center [42]. Thus the T$_2$ value of water protons in solution containing only Fe$^{3+}$-ligand complexes, such as Fe-EDTA, barely changes upon the dopamine addition. TMS-EDTA is a pentadentate ligand [43], and has less coordination sites for metal center at low pH, resulting in more available coordination sites on the Fe$^{3+}$ ion to interact with dopamine and water protons [44]. The SiO$_2$ nanoparticle and TMS-EDTA combine to serve well as a platform for the paramagnetic relaxation based sensing.

The Fe$^{3+}$ species of the SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles in this scheme has two functions: 1) it is the paramagnetic agent that directly causes change to the T$_2$
relaxation time of the surrounding water protons; 2) it is the target recognition element that selectively binds to dopamine. Moreover, by immobilizing other target recognition elements on the nanoparticle surface, the SiO$_2$@TMS-EDTA@Fe$^{3+}$ nanoparticles can be adapted to detect other targets that do not directly bind to the Fe$^{3+}$ species.

In summary, we have demonstrated a T$_2$-based detection method for dopamine using paramagnetic nanoparticles. Detection limits of sub-micromolar concentration are achieved for dopamine in both acetate buffer and aCSF matrix without sample pretreatment.

2.1.4 References


Chapter 2.2

Magnetic Relaxation-based Sensing of Phosphate Ion

2.2.1 Introduction

Phosphorous is one of the most abundant elements of the earth and living systems, which is most commonly found as phosphate ion [1]. It is beneficial for many biological processes in the environment, but high amount of phosphate can disrupt the balance of the ecosystem [2,3]. Uses of phosphate containing fertilizers and detergents are the main source of increase of the phosphate concentration in the environment [4-6]. Pollution from phosphate and phosphorylated compounds is one of the main reasons for the eutrophication of natural water sources, which has led to the dangerous increase in toxic algal blooms [7]. According to EPA report in 1992, accelerated eutrophication was one of the major problems facing the Nation's lakes and reservoirs [8]. Depletion of dissolved oxygen level as a result of eutrophication caused by the overabundance of phosphate in water can subsequently cause a variety of problems, including poor water quality and death of aquatic life [9-11].

The increasing need of phosphate ion sensors in the field of environmental, industrial and medicinal analysis is pushing scientists to develop new sensors for the fast, accurate, reproducible and selective detection of assessing phosphate over a wide range of concentrations in various samples with sufficient sensitivity [12-15]. Thus far, there have been many kinds of methods for the determination of phosphate, including electrochemistry [16], UV-vis spectroscopy [17], chromatography [18], fluorescence spectroscopy [19], and enzymatic biosensors [20]. Yet most of them use organic solvents
as the media. The detection of phosphate in aqueous solution, hence, remains a big challenge [21].

Magnetic relaxation (MR) based sensing have been one of the popular methods that promise fast and reliable results for detection of various target using nuclear magnetic resonance (NMR) relaxometer [22-25]. These sensors are based on the use of surface-functionalized magnetic nanoparticles (MNPs) to detect specific targets in a variety of complex, opaque samples without any sample preparation steps [26]. Interaction of target with MNPs leads to a significant change in the transverse relaxation time ($T_2$) of water protons in the solution [27-30].

Paramagnetic metal ions, such as Gd$^{3+}$, Fe$^{3+}$, Mn$^{2+}$, Mn$^{3+}$ and Cu$^{2+}$, can affect the transverse ($T_2$) and longitudinal ($T_1$) relaxation times of the surrounding water protons, and their metal chelates serve as contrast agents in magnetic resonance imaging (MRI) [31-33]. In our previous study, we showed that paramagnetic metal chelated nanoparticles could be used for MR based sensing, similar to the more commonly used superparamagnetic iron oxide nanoparticles [34].

We report herein a MR based sensing method that can detect phosphate ions in aqueous solution based on the same principle as our previous study [34]. We designed our sensing method using Gd$^{3+}$ ion, which have been most commonly used in the development of contrast agents because of its strong paramagnetic property with seven unpaired electrons and the stability at physiological pH [35-36]. In addition, after chelation of Gd$^{3+}$ ions with the ethylenediaminetriacetic acid (EDTA) functional groups on the surface of nanoparticles, the access of water protons to the metal surface increases
because of its high coordination number. Consequently, the overall effectiveness of the metal chelate as MR contrast agent increases.

The principle of the sensing scheme is illustrated in Figure 2.2.1. EDTA groups grafted onto the surface of silica nanoparticles (SiO₂ NPs) can readily capture and stabilize Gd³⁺ ions, which would affect the relaxation rate of the surrounding water protons. Phosphate ions would bind to the silica nanoparticle surface due to the high affinity of phosphate ions to Gd³⁺ [37], and exchange with the water molecules surrounding the Gd³⁺ ions, subsequently affecting the T₂ relaxation time of water protons in the solution [34]. We can then quantitatively detect phosphate by monitoring the T₂ change of water protons [28].

![Schematic representation of the detection mechanism](image)

**Figure 2.2.1.** Schematic representation of the detection mechanism. Phosphate binding to SiO₂@TMS-EDTA@Gd³⁺ NPs leads to the increase of T₂ relaxation time of water protons.
2.2.2 Materials and Methods

Materials

(Trimethoxysilylpropyl)ethyldiaminetriacetic acid trisodium salt (TMS-EDTA) (35 wt% solution in water) was purchased from Gelest. Tetraethylorthosilicate (TEOS), Triton X-100 (TX-100), n-hexanol, cyclohexane, gadolinium (III) chloride hexahydrate, ammonium hydroxide (30 wt%), KH$_2$PO$_4$ and glacial acetic acid were from Sigma-Aldrich. Fertilizer was purchased from grocery store. All chemicals were used without further purification. Deionized (DI) water was used for the preparation of all solutions.

Synthesis of Gd$^{3+}$-chelated SiO$_2$ nanoparticles (SiO$_2$@TMS-EDTA@Gd$^{3+}$)

Paramagnetic SiO$_2$@TMS-EDTA@Gd$^{3+}$ nanoparticles were prepared in two steps using a modified version of the previously published protocol [34]. First, SiO$_2$@TMS-EDTA nanoparticles with EDTA groups on surface were synthesized by mixing 1.77 g Triton X-100, 7.5 mL cyclohexane, 1.6 mL n-hexanol, and 480 µL DI water in a glass vial and stirring for 5 min. Next, 60 µL of NH$_4$OH was added to the microemulsion and stirred for 20 min, followed by the addition of 50 µL TEOS. The mixture was stirred at room temperature for 24 h. Then, 50 µL of TEOS was added to the microemulsion and stirred for 30 min, and finally 25 µL of TMS-EDTA was added, followed by another 24 h stirring. Subsequently, approximately 20 mL of acetone was added to break down the microemulsion system. SiO$_2$@TMS-EDTA nanoparticles were recovered by centrifuging at 14000 rpm for 20 min, and then washed three times with acetone, ethanol and DI water, respectively. The resulting SiO$_2$@TMS-EDTA nanoparticles were dispersed in DI water.
Paramagnetic property was introduced to the SiO$_2$@TMS-EDTA nanoparticles by mixing excess amount of 0.1 M GdCl$_3$ solution and stirring overnight at 40°C. Afterwards, nanoparticles were washed three times with DI water and then dispersed in 20 mM of tris buffer (pH 7.5) for storage.

**ICP-MS measurements**

SiO$_2$@TMS-EDTA@Gd$^{3+}$ nanoparticle samples were prepared in 2% trace grade nitric acid, which can release Gd$^{3+}$ into the solution. A standard addition plot was made for SiO$_2$@TMS-EDTA@Gd$^{3+}$ nanoparticle samples and tris buffer (as control). The released Gd$^{3+}$ concentration of each sample was measured by a triple quadruple ICP-MS (ICP-QQQ, Agilent Technologies). Signal from the control was subtracted from that of each sample.

**Paramagnetic Relaxation Assays**

For the relaxation measurements, Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequences were used to limit the effect of magnetic inhomogeneity of instrument [31]. Transverse relaxation times ($T_2$) were measured at 1.41 T by a Bruker Minispec mq60 relaxometer operating at 40°C without any washing step.

**Detection of Phosphate ions**

Firstly, KH$_2$PO$_4$ stock solution was prepared, and serially diluted. In a typical run, $T_2$ measurements of the SiO$_2$@TMS-EDTA@Gd$^{3+}$ nanoparticles in tris buffer were first taken at 40°C. Then, KH$_2$PO$_4$ samples were added to the SiO$_2$@TMS-EDTA@Gd$^{3+}$ nanoparticle solution and incubated for 6 hours at 40°C before another round of $T_2$ measurements were taken.
In the case of detecting phosphate ions in fertilizer, one gram of fertilizer was dissolved in DI water and then serially diluted. Fertilizer samples were introduced to the SiO$_2$@TMS-EDTA@Gd$^{3+}$ NPs in tris buffer. After incubation for 6 hr, T$_2$ measurements were taken.

To test the specificity of the sensing method, several SiO$_2$@TMS-EDTA@Gd$^{3+}$ nanoparticle solutions were prepared in tris buffer. Then 1 mL solutions of 0.1 M CO$_3^-$, acetate, Cl$^-$, F$^-$, NO$_3^-$, H$_2$PO$_4^-$ and SO$_4^{2-}$ were prepared and added to each of the above SiO$_2$@TMS-EDTA@Gd$^{3+}$ nanoparticle solutions to reach an analyte concentration of 4 mM in each sample. T$_2$ measurements were taken after 6 hr incubation.

### 2.2.3 Results and Discussion

SiO$_2$@TMS-EDTA nanoparticles were synthesized following a modified version of the previously published protocol while introducing Gd$^{3+}$ ions to chelate with surface EDTA functional groups \[34\]. Gd$^{3+}$ ions can easily chelate with SiO$_2$@TMS-EDTA nanoparticles around physiological pH, which eliminates the insoluble Gd$^{3+}$ oxides formation.

The synthesized SiO$_2$@TMS-EDTA@Gd$^{3+}$ nanoparticles were characterized by a number of techniques. The average diameters of the SiO$_2$@TMS-EDTA nanoparticles are ~60 nm according to transmission electron microscopy (TEM) as shown in Figure 2.2.2a. The amount of Gd$^{3+}$ ions in the SiO$_2$@TMS-EDTA@Gd$^{3+}$ nanoparticles was quantified using ICP-MS and determined to be 0.7 mg/mL.

Transverse relaxation times (T$_2$) of water protons were measured by a Bruker Minispec mq60 relaxometer operating at 1.41 Tesla and 40°C. As shown in Figure 2.2.2b, transverse relaxivity ($R_2$) of the SiO$_2$@TMS-EDTA@Gd$^{3+}$ nanoparticles is 96.98 mM$^{-1}$s$^{-1}$. 
which is much higher than clinically used Gd-DTPA (4.6 mM⁻¹s⁻¹) [38]. This is likely attributed to that the chelation with TMS-EDTA, a pentadentate ligand [39], leaves Gd³⁺ ion, which tends to form complexes with a coordination number of nine [40], with more available coordination sites to interact with water protons [41], as compared to the chelation with DTPA.

Figure 2.2.2. (a) TEM images of the SiO₂@TMS-EDTA@Gd³⁺ nanoparticles. Scale bar is 100 nm (b) R₂ relaxivity measurement of SiO₂@TMS-EDTA@Gd³⁺ nanoparticles.

The percentage change of T₂ relaxation time (ΔT₂%) upon the binding of phosphate ions to SiO₂@TMS-EDTA@Gd³⁺ nanoparticles is calculated according to the following equation [40,42]:

\[
\Delta T_2\% = (T_2,\text{target} - T_2,\text{blank}) \times 100 / T_2,\text{blank}
\]

where T₂,blank and T₂,target is the average T₂ relaxation time of three replicates of nanoparticle solution before and after the addition of phosphate ions, respectively.

We first tested the working principle of our sensing method using pure KH₂PO₄ as a phosphate source. As shown in Figure 2.2.3, a significant increase in ΔT₂% is observed as a result of phosphate addition to the nanoparticle solution in tris buffer. A linear relationship between ΔT₂% and phosphate concentration is observed in the range
of 3.9-31.2 µM with a correlation coefficient ($R^2$) of 0.9993 and a detection limit of 0.1 µM.

Figure 2.2.3. $\Delta T_2$ % as a function of phosphate concentration in tris buffer.

To test the selectivity and utility of this sensing method in a complex matrix for actual applications, commercial phosphate-containing fertilizer was used in this study. The fertilizer contains several ingredients: 0.05% chelated Zn, 0.05% chelated Mn, 0.1% chelated Fe, 20% $K_2O$, 30% $P_2O_5$ and 20% N. Results shown in Figure 2.2.4 demonstrate that $\text{SiO}_2@\text{TMS-EDTA}@\text{Gd}^{3+}$ nanoparticles can detect phosphate in the fertilizer quantitatively. A linear relationship between the concentration of phosphate in fertilizer and $\Delta T_2$% is observed in the range of 16-132 µM with a correlation coefficient ($R^2$) of 0.9824 and a detection limit of 0.5 µM.

Figure 2.2.4. $\Delta T_2$ % as a function phosphate concentration of fertilizer in tris buffer.
To examine the specificity of this phosphate sensing method, the change in T2 relaxation time of water protons, as a result of interaction of SiO2@TMS-EDTA@Gd3+ nanoparticles with various ions including CO3−, acetate, Cl−, F−, NO3−, H2PO4− and SO42− was examined under the same conditions. The final concentration of the anions was 4 mM. As shown in Figure 2.2.5, it can be seen that only phosphate ions cause a significant increase in ΔT2% while F− ions can slightly affect the ΔT2%. All other anions have negligible effect.

Figure 2.2.5. The specificity of the phosphate sensor. ΔT2% after adding various anions including control (water), CO3−, acetate, Cl−, F−, NO3−, H2PO4− and SO42− to the SiO2@TMS-EDTA@Gd3+ nanoparticle solution at pH 7.5.

In summary, we report a sensitive and specific sensing method to detect phosphate ions using the SiO2@TMS-EDTA@Gd3+ nanoparticles. The SiO2@TMS-EDTA@Gd3+ nanoparticles have excellent water dispersibility and colloidal stability.
even at physiological pH, which broadens the usage of the nanoparticles in biological systems. The method can detect phosphate ions at physiological pH, with high selectivity towards phosphate ions over many other anions. It can also detect the phosphate in fertilizer quantitatively with a detection limit of 0.5 µM without any sample pretreatment. The method has a good potential to be adapted for the detection of other targets. The Gd\(^{3+}\) ion in the SiO\(_2\)@TMS-EDTA@Gd\(^{3+}\) nanoparticles can serve as both the contrast agent and the phosphate recognition element for magnetic relaxation based detection systems. The results pave way for further development and usage of SiO\(_2\)@TMS-EDTA@metal nanostructures for desirable sensing applications.

### References


Chapter 2.3

Luminescence Resonance Energy Transfer based DNA Biosensor

2.3.1 Introduction

DNA biosensors have attracted extensive attention due to their broad range of potential applications, including gene analysis, clinical diagnostics and forensic applications [1,2] In general, targets were amplified and labeled with fluorophore using polymerase chain reaction (PCR), which amplifies target sequence by using short target primers and thermally stable polymerase with multiple thermal cycles, to detect DNA [3]. However, PCR reactions are time-consuming, suffer from contamination of non-template DNAs, and induce errors during the polymerase reaction [4].

Nanomaterials such as metal nanoparticles, quantum dots, upconversion nanoparticles, and carbon nanotubes have been used to design DNA biosensors to achieve extremely sensitive detection of DNA owing to their unique size dependent properties and large specific surface areas [5-17]. But so far most of the reported DNA sensors have been used to detect selected DNA sequences after PCR amplification.

In recent years, upconversion nanoparticles (UCNPs), which can emit higher energy photons after absorbing lower energy photons, have drawn increasing attention because their unique optical characteristics of anti-Stokes shift [18]. They have been considered as especially attractive nanomaterials for biomedical applications such as biosensing, bioimaging and photodynamic therapy [19-22].
UCNPs have been used to design multifunctional detection platforms and benefit from their absence of autofluorescence behavior and cross-excitation, and deeper light penetration compare to over conventional fluorophores and quantum dots. In this study, we designed a ligase-assisted signal-amplifiable DNA biosensor based on NaYF₄:Yb³⁺,Tm³⁺ UCNPs with high sensitivity and specificity.

2.3.2 Materials and Methods

Materials

Y(NO₃)₃·6H₂O, Yb(NO₃)₃·5H₂O, Tm(NO₃)₃·5H₂O, polyacrylic acid (PAA, MW ~15000), NaOH, NH₄F, ethylenediaminetetraacetic acid (EDTA) and acetone were purchased from Sigma. SYBR Green I was purchased from Life Technology. Ethylene glycol (EG), 1-Ethyl-3-[3-dimethylamonopropyl]carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Thermo Scientific.

Single-stranded DNA fragments were ordered from IDT DNA (Coralville, IA). The sequences were DNA_seg1 (5’ amino-CAG TAA CGG CAG A-3’) amine-modified at the 5’-end, DNA_seg2 (5’-CTT CTC CAC AGG AGC CGT TAC TG-3’) phosphorylated at the 5’-end, DNA_tar (5’-TCC TGT GGA GAA GTC TGC CGT TAC TG-3’), and DNA_mut (5’-TCC TGA GGA GAA GTC TGC CGT TAC TG- 3’). Thermostable DNA ligase (Ampligase®) and 10x Ampligase® reaction buffer were purchased from Epicentre (Madison, WI). The melting points of various DNA strands were calculated using the OligoAnalyzer 3.1 program available at the vendor website (www.idtdna.com).
**Synthesis of NaYF₄;Yb⁺⁺,Tm⁺⁺ UCNPs**

PAA (0.3 g), NaCl (23.4 mg), YCl₃ (48.5 mg), YbCl₃ (13.9 mg), and TmCl₃ (1.1 mg) were added into 3 ml EG, using vortex and sonicator to make the mixture homogenous (Solution A). Separately, 0.03 g of NH₄F was added to 2 mL of EG in a Teflon container (Solution B). Solution A was added drop wise into Solution B under stirring. The Teflon container was then placed in a sealed, stainless-steel capsule, and incubated for 2 hr at 200 °C. The resulting solution was clear with light yellow in color. The nanoparticles were collected after centrifuging the solution for 1 hr at 15,000 rpm to remove the supernatant. They were washed 3 times by ethanol and twice by DI water, before dispersed in 2 ml DI water for storage.

**Conjugation of DNA_seg1 to UCNPs**

Two mL of washed UCNP aqueous solution was pre-treated with 10 µL of EDC (0.2 M) and 10 µL of NHS (0.05 M) for 5 min under stirring at 8000 rpm. Then, 30 µL of 100 µM DNA_seg1 was added into the reaction mixture and stirred at 8000 rpm overnight. The resulting nanoparticles were washed 3 times by DI water, before dispersed in 500 µL DI water.

**Ligation and hairpin loop amplification in thermal cycles**

Fifty µL of DNA_seg1 conjugated UCNP solution was mixed with 10 µL of 10× Ampligase® reaction buffer, 20 µL of 1 µM DNA_seg2 and 1 µL of Ampligase with different amounts of 0.1 µM DNA_tar. The solution of a total volume of 100 µL was then treated in 90°C for 30 second. Then the solution was cooled down to 72°C and maintained for 1 min. At last the solution was cooled down to 45°C and maintained for 3 min. This thermal cycle was repeated up to 20 times.
2.3.3 Results and Discussion

Figure 2.3.1. Schematic illustration of DNA detection based on UCNPs.

The detection scheme is shown in Figure 2.3.1. Two single-stranded DNA segments are designed as probes to detect target DNA, each complementary to the juxtaposed section of the target DNA. The sequence of the DNA probes are so designed that, when they are joined into one long sequence, a hairpin loop structure will be formed under ambient temperatures. One of the DNA probes is covalently attached to the UCNPs. In the presence of the target DNA and ligase, the two DNA probes are joined into a longer hairpin-forming strand, where an intercalating dye, SYBR Green I (SG1), can be trapped in the double-stranded stem portion. The underlying principle for the detection is luminescence resonance energy transfer (LRET) between an UCNP, NaYF₄ co-doped with Yb³⁺ and Tm³⁺, and SG1. Upon 980 nm excitation, NaYF₄:Yb³⁺,Tm³⁺ nanoparticles emit at 477 nm, overlapping with the excitation band of SG1. LRET occurs between the UCNPs and the intercalated SG1 when hairpin-forming strands formed.
Essentially, the target DNA serves as a template for the formation of the hairpin-forming DNA strands (the joined probes) on the UCNP surface, the number of which can be increased by thermal cycling between ligation at a lower temperature and dehybridization at a higher temperature. The signal from LRET is therefore amplified.

To demonstrate the effectiveness of the detection scheme, we use several single-stranded DNAs with nucleotide sequences as listed in Table 2.3.1. DNA_seg1 (13mer, amine-modified at the 5’-end) and DNA_seg2 (23mer, phosphorylated at the 5’-end) are complementary to the juxtaposed sections of DNA_tar (26mer). In the presence of DNA_tar, ligase and the reaction buffer, DNA_seg1 and DNA_seg2 are joined together into a hairpin-forming DNA strand with sequence of

5’CAGTAACGGCAGACTTCTCCACAGGAGCCGTTACTG-3’, where the underlined bases indicate the stem portion of the hairpin structure. DNA_mut is a single-base mutation of DNA_tar.

<table>
<thead>
<tr>
<th>DNA_seg1</th>
<th>5’-(C6-amino)-CAGTAACGGCAGA-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA_seg2</td>
<td>5’-pho-CTTCTCCACAGGAGCCGTTACTG-3’</td>
</tr>
<tr>
<td>DNA_tar</td>
<td>5’-TCCTGTGAGAAGTCTGCGTTACTG-3’</td>
</tr>
<tr>
<td>DNA_mut</td>
<td>5’-TCCTGAAGGAGAAGTCTGCGTTACTG-3’</td>
</tr>
</tbody>
</table>

**Table 2.3.1.** Probes and target DNA sequences.

The NaYF₄:Yb³⁺,Tm³⁺ nanoparticles were synthesized by a hydrothermal method modified from procedures described previously [22]. They have an average diameter of ~143 nm, as indicated by TEM image (Figure 2.3.2a). They are very uniform and well dispersed in aqueous solution. DNA_seg1 is covalently attached to these nanoparticles
through the EDC/NHS conjugation chemistry. FTIR results of the DNA-conjugated UCNPs confirm the existence of carboxylic acid groups with the typical broad band at ~3500 cm\(^{-1}\) region (Figure 2.3.2b).

**Figure 2.3.2.** (a) TEM picture of UCNPs (Scale bar: 500 nm), (b) FTIR spectrum of UCNPs with surface –COOH groups.

The amount of conjugated DNA_seg1 on the surface of UCNPs can be determined experimentally (Figure 2.3.3). The concentration of DNA_seg1 in the UCNP-conjugated DNA_seg1 working solution was calculated to be 4.4 µM and the yield of EDC/NHS conjugation between UCNPs and DNA_seg1 to be ~73%.

**Figure 2.3.3.** SG1 fluorescence emission excited at 490 nm with different amounts of DNA_seg1 (a), and a calibration curve (b).
The emission band of the UCNPs upon 980 nm excitation overlaps well with the excitation band of SG1, as shown in the Figure 2.3.4, allowing energy transfer to occur between the two when they are in proximity. The intensity of SG1 emission under the 980 nm excitation via LRET is generally weak, due to the low efficiencies of upconversion and LRET processes. We thus use the ratio of SG1 emission to that of UCNPs ($I_{530}/I_{477}$) as the indicator to monitor the degree of LRET.

![Figure 2.3.4](image)

**Figure 2.3.4.** Normalized excitation and emission spectra of SG1 and emission spectrum of NaYF$_4$:$\text{Yb}^{3+},\text{Tm}^{3+}$ upconversion nanoparticles excited at 980 nm.

Different amounts of DNA_tar were added, respectively, into the mixture containing DNA_seg1 functionalized UCNPs, DNA_seg2, and ligase in reaction buffer. For each thermal cycle, the mixture was first heated to 90 °C for 30 sec, then cooled to 72 °C and maintaining for 5 min, and lastly to 45 °C and maintaining for 3 min. By the end of the last thermal cycle, EDTA solution was quickly added to the mixture to stop the ligation. The nanoparticles were centrifuged and washed, and redispersed in phosphate buffer containing SG1 before spectroscopic measurements were taken on a spectrofluorometer equipped with a customized excitation source of a 980-nm laser.
Results are shown in Figure 2.3.5a, where emission spectra of UCNPs with 1 pmol of DNA_tar after different numbers of cycle are plotted. The ratio of 477 nm peak to 530 nm peak, $I_{530}/I_{477}$, is plotted against different numbers of cycle in Figure 2.3.5b, which displays a good linear relationship between the two, confirming that the detection scheme works as designed. The use of the $I_{530}/I_{477}$ ratio is used as the signal indicator since it is less influenced by experimental factors affecting optical measurements.

**Figure 2.3.5.** (a) Luminescence emission spectra under 980 nm excitation after 0, 5, 10, 20 thermal cycles with 1 pmol DNA_tar, and with water as control. (b) $I_{530}/I_{477}$ ratio vs. number of thermal cycle.

The $I_{530}/I_{477}$ ratio vs. different initial amounts of DNA_tar all after 20 thermal cycles is plotted in Figure 2.3.6. Again, a good linear relationship is observed between $I_{530}/I_{477}$ and the initial amounts of DNA_tar, allowing for quantitative detection of the initial amount of DNA_tar. The results show that 0.25 pmole of initial DNA_tar can be readily detected. Note that the detection limit can be further improved by increasing the number of thermal cycles, and the dynamic range of detection can also be widened by starting with a larger amount of DNA_seg1 functionalized UCNPs and DNA_seg2.
Figure 2.3.6. (a) Luminescence emission spectra under 980 nm excitation with different amounts of DNA_tar after 20 thermal cycles. (b) $I_{530}/I_{477}$ ratio vs. amount of initial DNA_tar.

A number of factors need to be considered for this detection scheme to work properly. Notice that the target DNA itself is not replicated in this scheme, which is a key difference between this scheme and PCR. The target DNA simply serves as a template to repetitively link the two DNA probes into the joined DNA_seg1-DNA_seg2, which are the actual targets to be detected via LRET. Thus the sequences of the two DNA probes should be carefully designed. Firstly, the two probe strands should be juxtaposed when hybridized to the target DNA, to allow the ligation to occur. Secondly, the two probe sequences each contain a segment complementary to each other to allow the hairpin loop formation after they are joined together. Also, the joined DNA_seg1-DNA_seg2 should maintain its hairpin loop formation, i.e., detached from the template (DNA_tar), at the temperature the ligation takes place. This last requirement is important to achieve amplification of the joined probes. Note that these principles of probe design can be applied to detect other target DNAs, and for a given target more than one pair of probes can be used.
The selection of the high and low temperatures in the thermal cycle takes into consideration the melting points of the double-stranded DNA_tar/DNA_seg1-DNA_seg2 and that of the hairpin loop structure derived from DNA_seg1-DNA_seg2. At 90°C, all double-stranded DNAs will dehybridize. When lowered to 72°C, the hairpin loop structure is more stable than the double-stranded DNA_tar/DNA_seg1-DNA_seg2, and DNA_seg1-DNA_seg2 would opt to form the hairpin loop. At 45°C, DNA_tar will hybridize with DNA_seg1 and DNA_seg2 simultaneously, and allow the ligation to proceed. The availability of a ligase that can endure multiple thermal cycles at relatively high temperature is important to facilitate this scheme [23]. The thermostable ligase, Ampligase, derived from a thermophilic bacterium, can maintain its activity at rather high temperature (94/80°C) over extended period of time [24] and is used in this study.

The adoption of the intercalating dye, SG1, allows this detection scheme to be label-free. In the presence of single-stranded DNAs, SG1 emits weakly under direct excitation at ~490 nm [25] which offer a means to determine the amount of DNA_seg1 conjugated to the UCNPs. When excited at ~980 nm via LRET from the UCNPs, the emission from SG1 in the solution is so weak that it is negligible. This allows the SG1 concentration in the assay mixture to be relatively high without increasing the background noise in the detection. In the presence of double-stranded DNAs, SG1 emission increases markedly. Still, only those SG1 molecules that are intercalated in the stem portion of the hairpin loop structure and thus in proximity to the UCNPs can be excited by the 980 nm laser via LRET. Therefore, any free SG1 molecules in the mixture would not interfere with the detection. Furthermore, SG1 is not susceptible to photobleaching since it is not directly excited by the laser.
This detection scheme displays good specificity. To demonstrate, 1 pmole of DNA_mut, a single-base mutation of DNA_tar, was added in place of the DNA_tar, and subject to the same thermal treatment as DNA_tar, with results shown Figure 2.3.7. The high specificity of the detection most likely results from (1) the built-in ligation specificity, and (2) the requirement of a hairpin loop formation in the joined DNA sequence.

![Figure 2.3.7. Luminescence emission spectra under 980 nm excitation with 1 pmol of DNA_tar and 1 pmol of DNA_mut, respectively.](image)

Unlike PCR, where the number of target DNAs increases exponentially, the amplification scheme described here only increases the number of the hairpin loop DNA structures linearly after each cycle. The initial target DNAs are repetitively being used as the template for ligation. While this limits the rate of the signal amplification, it reduces the possibility of generating errors during the amplification. This may also contribute to the high specificity of the detection.

In conclusion, we have demonstrated a new ligase-assisted signal-amplifiable DNA detection scheme with high sensitivity and specificity, based on UCNPs via LRET.
The scheme requires only the presence of intercalating dyes prior to the final measurements. It is not susceptible to photobleaching because of the adoption of UCNPs. We believe this method to be very useful for many DNA detection applications.

2.3.4 References


Chapter 3

Development of Nanoparticle-based Photosensitizers for Photodynamic Therapy
Chapter 3.1

Silver Nanoparticle-enhanced Photosensitizer Hybrids for Photoinactivation of Multidrug-Resistant bacterium

Staphylococcus aureus (MRSA)

3.1.1 Introduction

Multidrug-resistant Staphylococcus aureus (MRSA) is resistant to numerous antibiotics including methicillin, amoxicillin, penicillin, etc [1-4], which can cause mild infections or life-threatening infections [5-7]. According to the Centers for Disease Control and Prevention (CDC&P), at least 2 million people are infected with antibiotic resistant bacteria, leading to >23,000 deaths each year in the US [8]. As the drug resistance grows, finding alternative antibacterial therapies that can circumvent the mechanisms for resistance presents a big challenge.

Photodynamic inactivation (PDI) is one of the most promising and innovative approaches, which generates singlet oxygen to kill bacteria by combining photosensitizer (PS), light illumination of appropriate wavelength, and oxygen [9-12]. The main advantages of PDI are that bacteria can be killed in a short period of time and will not readily develop resistance to PDI [13,14]. However, PDI has some limitations due to the delivery of PSs and light into the tissue [15-18]. Many of the PSs cannot be excited by long-wavelength light sources, which have deep tissue penetration [19]. In addition, most of the PSs are hydrophobic and tend to aggregate easily in aqueous solutions, resulting in reduced singlet oxygen generation [20]. Therefore there is a great interest in developing photosensitizers based on nanoplatfroms incorporating PSs into nanoparticles to
overcome these limitations [21-25]. There have been studies highlighting various approaches for utilizing nanoparticles for PDI, mostly using the nanoparticles carrier for PS [26]. Nanoparticles with hydrophilic surface modification can hold hydrophobic PSs and prevent their aggregation in aqueous environment [27]. Besides carrier functionality, nanoparticles with their unique optical absorption and emission properties that can significantly enhance the PDI efficiency [28-30].

We recently reported that hybrid Ag@PS nanoparticles exhibit enhanced singlet oxygen generation because of the strong resonance coupling between surface plasmon and photosensitizing molecules, leading to highly efficient PDI against both gram-positive and gram-negative bacteria [30-32]. In this study, we report that this type of hybrid Ag@PS nanoparticles demonstrate high PDI efficacy against a multidrug-resistant strain of *Staphylococcus aureus*.

### 3.1.2 Materials and Methods

**Materials**

Cetyltrimethylammonium bromide (CTAB), formaldehyde (37 %), ammonium nitrate, sodium hydroxide, and sodium cyanide were purchased from Thermo Fisher. Hematoporphyrin IX dihydrochloride (HPIX) was purchased from Frontier Scientific. Tetraethyl orthosilicate (TEOS), 3-(trihydroxysilyl) propyl methylphosphonate (THMP), and silver nitrate were from Sigma Aldrich. All chemicals were used as received. PBS buffer was used for the preparation of samples for PDI assays. Multidrug-resistant *Staphylococcus aureus* (ATCC BAA-44) was purchased from ATCC.
Synthesis of Ag@MS@HPIX nanoparticles

Silver core and mesoporous silica shell nanoparticles containing HPIX (Ag@MS@HPIX) were prepared in two steps based on a modified version of the previously published procedure [30]. Firstly, Ag@MS nanoparticles were synthesized as follows. 20 mg of CTAB was dissolved in a solution containing 9.6 mL of water and 70 µL of 2M NaOH. After stirring at 80°C for 10 min, 60 µL of 1.0 M formaldehyde and 240 µL of 0.1 M silver nitrate were added. Then 100 µL of TEOS was added into the mixture under stirring. After 15 min, 50 µL of 3-(trihydroxysilyl)propyl methylphosphonate (THMP) was added into the mixture, followed by another 2h of stirring at 80°C. Ag@MS nanoparticles were pelleted by centrifugation (14,800 rpm) for 20 min. CTAB on Ag@MS nanoparticles was removed by extraction in ethanol solution containing ammonium nitrate (6 g/L) at 60°C for 30 min three times. Ag@MS nanoparticles were washed three times with water and then dispersed in water for storage.

Secondly, HPIX was loaded onto the Ag@MS nanoparticles. In brief, 1mL of 1mM HPIX in ethanol was mixed with 1mL of Ag@MS nanoparticle solution, and stirred overnight at room temperature. The products were centrifuged at (14,800 rpm) for 20 min, and washed three times with ethanol/water mixture (50:50) to remove any excess HPIX. The as-synthesized Ag@MS@HPIX hybrids were then dispersed in DI water for later use.

The loading amount of HPIX was determined by UV-vis absorbance spectrum, which was recorded with a spectrophotometer (USB4000-ISS, Ocean Optics). A series of different concentrations of HPIX in ethanol/water (50/50) mixture were first used to establish a calibration curve. Excess amount of sodium cyanide solution was applied to a
known amount of Ag@MS@HPIX hybrids to dissolve the Ag core (noted as MS@HPIX hereafter). Absorbance of the MS@HPIX solution was compared to the calibration curve to calculate the loading amount of HPIX in the hybrids.

The morphology of the Ag@MS nanoparticles was characterized by a Phillips Biotwin 12 transmission electron microscope (TEM). Nanotrac particle size analyzer was used to measure hydrodynamic size and surface zeta potential of the Ag@MS and Ag@MS@HPIX nanoparticles.

Fluorescence and phosphorescence measurements were carried out on a QM-40 spectrofluorometer (PTI Inc.) equipped with a high performance InGaAs photodiode and a lock-in amplifier (model 410, Scitec Instruments Ltd.). The singlet oxygen generation was detected by monitoring its phosphorescence emission at ~1280 nm. The light source was a Xenon arc lamp. A long-pass filter (850 nm cut-off) was placed in the front of the detector to remove any possible higher-order artifact signals. All measurements were done in ethanol/water (50/50) solvent. The excitation wavelength for fluorescence and singlet oxygen generation of HPIX is 403 nm.

**Photodynamic inactivation (PDI) assays**

*Staphylococcus aureus* (ATCC BAA-44) was used as a model multidrug-resistant bacterium in this study. Typically, overnight culture of *S. aureus* was inoculated in PBS buffer solution (pH=7.4) and mixed with a series of diluted concentrations of Ag@MS@HPIX hybrids, Ag@MS nanoparticles, and HPIX. All bacterial mixtures, including the non-treated bacterial controls, were then transferred to the wells of 96-well plates (200 µL/well). The final cell concentration of the mixtures was ~10⁶-10⁷ colony-forming units per mL (CFU/mL). The wells were illuminated by a non-coherent, white
light source with interchangeable fiber bundle (model LC-122, LumaCare). Intensity of the white light was measured as 408 mW/cm² by a laser power meter (model 840011, SPER Scientific) and the irradiation fluence was 73 J/cm². After illumination, a plate count method was used to determine the viable bacterial numbers (CFU/mL) in mixtures. Dark control experiments were run in parallel. Three independent runs were carried out for each experiment. Each experiment was performed in triplicate. The data are presented as the means with standard deviations. Two-sample t-test was used to analyze the results and the probability values of <5% are considered significant.

3.1.3 Results and Discussion

MRSA, one of the most common multidrug-resistant bacteria, has become a public health problem. It is associated with serious disease in both hospitals and community [1-4]. It has also been reported that MRSA has rapidly developed resistance to most antibiotics [5-7]. While several reports have shown that under proper light illumination free photosensitizing molecules could inactivate various bacteria including MRSA [33-35], to our best knowledge this is the first report on the PDI against MRSA by silver nanoparticle-enhanced photosensitizer hybrids.

The synthesis of Ag@MS@HPIX hybrids was similar to the previously published protocol with slight modification. THMP was introduced to increase the stability of the Ag@MS nanoparticles in aqueous solution. The mesoporous silica shell is thinner, compared to that from our previous method. The schematic illustration of synthesis of Ag@MS@HPIX hybrid and its singlet oxygen generation is shown in Figure 3.1.1a.
Figure 3.1.1. (a) Schematic illustration of synthesis of Ag@MS@HPIX hybrid and its singlet oxygen generation. (b) Chemical structure of HPIX. (c) TEM image of Ag@MS nanoparticle. Scale bar is 20 nm. (d) Zeta potential and hydrodynamic size of Ag@MS nanoparticles and Ag@MS@HPIX hybrids.

TEM image Ag@MS nanoparticle shows an average diameter of ~42 nm, where the thickness of the mesoporous silica shell is ~2 nm and the diameter of Ag core ~42 nm (Figure 3.1.1c). HPIX is adsorbed into the thin mesoporous silica shell, in close proximity to the Ag core. The zeta potential of the Ag@MS nanoparticles changed slightly after the HPIX loading. Still, zeta potentials of both Ag@MS and Ag@MS@HPIX nanoparticles indicate fairly good stability (Figure 3.1.1d).

UV-vis absorption spectra of Ag@MS, Ag@MS@HPIX, MS@HPIX, and HPIX are shown in Figure 3.1.2a. Ag@MS nanoparticles have a strong plasmon peak at 414
nm. HPIX has the typical high-energy Soret band at 396 nm, and low-energy quasi-allowed bands at 498, 535, 566, and 618 nm [30]. There is a good overlap between the absorption of HPIX and the surface plasmon of Ag@MS nanoparticles. MS@HPIX has the same absorption bands as pure HPIX, indicating the successful removal of Ag core by cyanide.

**Figure 3.1.2.** (a) UV-vis absorption spectra of Ag@MS, Ag@MS@HPIX, MS@HPIX, and HPIX, (b) Fluorescence emission spectra of Ag@MS@HPIX, Ag@MS, and free HPIX.

The fluorescence spectra of Ag@MS@HPIX, free HPIX, and Ag@MS are shown in Figure 3.1.2b. The HPIX concentrations in Ag@MS@HPIX and free HPIX are both 2.4 μM, while Ag@MS@HPIX and Ag@MS contain the same amount of Ag. The fluorescence intensity of HPIX in the hybrid is much lower than that of the free HPIX at the same concentration, probably due to the self-quenching of localized high concentration of HPIX on the nanoparticles and the quenching of HPIX by the Ag core.

Singlet oxygen generation is directly monitored by measuring its phosphorescence emission at ~1280 nm. As shown in Figure 3.1.3a, Ag@MS@HPIX hybrids have much higher singlet oxygen generation than Ag@MS nanoparticles and free
HPIX under 403 nm excitation. Note that in these measurements HPIX concentrations in Ag@MS@HPIX and free HPIX are both 9.5 µM, and Ag@MS@HPIX and Ag@MS have the same amount of Ag. While singlet oxygen generation from the free HPIX at such concentration is insignificant, there is appreciable amount of singlet oxygen generated by the Ag@MS nanoparticles due to the Ag core, which is consistent with previous report in the literature [30]. Compared to our previous studies [30,32], where Ag@MS nanoparticles were synthesized by a slightly different procedure and were smaller in size, singlet oxygen generation by Ag@MS nanoparticles in this work is more pronounced, most likely attributed to the larger Ag core. Regardless of the Ag core size, the Ag@MS@HPIX hybrids display synergistic effect in singlet oxygen generation, consistent with our previous studies. The singlet oxygen excitation spectra (Figures 3b) show that Ag@MS@HPIX hybrids can be excited in a much broader spectral region than free HPIX, which improves their utility under near infrared light excitation for applications requiring deep tissue penetration [30-32].

Figure 3.1.3. Singlet oxygen (a) emission and (b) excitation spectra of Ag@MS@HPIX hybrids, Ag@MS nanoparticles, and pure HPIX.
We evaluated the antibacterial efficacy of Ag@MS@HPIX hybrids, Ag@MS nanoparticles, and HPIX against the multidrug-resistant S. aureus (ATCC BAA-44). Results are shown in Figure 3.1.4. While free HPIX and Ag@MS nanoparticles under these conditions display almost negligible antibacterial activities, Ag@MS@HPIX hybrids demonstrate significant inactivation capability of MRSA under white light illumination. Bacterial killing of up to ~6-log is observed. The hybrids display synergistic effect in PDI efficiency, compared to the Ag@MS nanoparticles and free HPIX (Figure 3.1.4d). This is consistent with the results from the spectroscopic measurements on singlet oxygen generation.

**Figure 3.1.4.** Antibacterial assays of Ag@MS@HPIX hybrids (a), Ag@MS nanoparticles (b), and HPIX (c) against MRSA with or without white light illumination. Ag@MS@HPIX and Ag@MS contain the same amount of Ag. Results are expressed as mean±SD (n=3, p<0.05). (d)
Bacterial killing efficacy of Ag@MS@HPIX hybrids, Ag@MS nanoparticles, and HPIX against MRSA under white light illumination. Enhancement in bacterial killing efficacy is defined as:

$$\log_{10}(\text{enhancement killing}) = \log_{10}(\text{Ag@MS@HPIX killing}) - \log_{10}(\text{Ag@MS killing}) - \log_{10}(\text{HPIX killing}).$$

In summary, we report that Ag@MS@HPIX hybrid photosensitizers display significant singlet oxygen generation with broadened excitation profile. Subsequently, they demonstrate very high PDI efficacy against *S. aureus* (ATCC BAA-44), a multidrug-resistant strain, under the condition where the free HPIX and Ag@MS of equivalent amounts show little antibacterial activity. These results support that such hybrid photosensitizers can be used for photodynamic inactivation of a broad spectrum of bacteria, including the drug-resistant strains, without involving antibiotics.

### 3.1.4 References


Chapter 3.2

Multifunctional Nanoparticles as MRI Contrast Agents and Photosensitizers against Multidrug-resistant Bacterium

3.2.1 Introduction

Nanoparticles with combined diagnostic and therapeutic functions, known as theranostic functions, show great promise towards diverse biomedical applications in bioimaging [1-3], gene therapy [4-6], biosensing [7,8], and drug delivery [9-12]. A variety of nanoparticles, including superparamagnetic iron oxide [13,14], quantum dots [15-16], upconversion [17-20], silver [21,22], gold [23,24], liposomes [25-26], and polymeric micelles [27-28], have been proposed to be used as theranostic multifunctional probes for magnetic resonance imaging (MRI) contrast agents, fluorescence imaging, photodynamic and photothermal therapy. Although promising, these nanoparticles reported thus far have limitations regarding complexity of fabrication, biocompatibility and limited ability to deliver drugs or imaging agents efficiently to target tissues [29-31]. Additional efforts are usually required to attach or load both therapeutic and diagnostic agents [32].

Here we report on a robust, simple and reproducible method that can be used to synthesize Ag@TMS-EDTA nanoparticles using only a single organic silane and metal source. The paramagnetic property was introduced to the Ag nanoparticles through the chelation of Gd$^{3+}$ ions with surface ethylenediaminetriacetic acid (EDTA) groups, which would reduce the relaxation rate of water protons in the solution [33-35]. In addition, the surface of Ag@TMS-EDTA@Gd$^{3+}$ NPs has the potential to form a lanthanide-porphyrin
complex, serving as photosensitizers in photodynamic therapy (PDT) [36,37]. This nanoplatfor

This nanoplatfor integrates functions of magnetic resonance imaging (MRI) contrast agents and PDI agents in a single nanostructure. In this study, we performed a systematic study of multifunctional Ag@TMS-EDTA@Gd^{3+}@HPIX NPs to assess how efficiently they generate singlet oxygen under light illumination for photodynamic inactivation of MRSA, which is a multidrug-resistant strain of Staphylococcus aureus.

3.2.2 Materials and Methods

Materials

(Trimethoxysilylpropyl)ethylenediamine triacetic acid trisodium salt (TMS-EDTA) (35 wt%) was purchased from Gelest. Silver nitrate was from Sigma-Aldrich. Hematoporphyrin IX dihydrochloride (HPIX) was purchased from Frontier Scientific. All chemicals were used as received. PBS buffer was used for the preparation of samples for PDI assays. Multidrug-resistant Staphylococcus aureus (ATCC BAA-44) was purchased from ATCC.

Synthesis of Ag@TMS-EDTA NPs

Ag@TMS-EDTA NPs were prepared using a newly developed protocol. First, Ag NPs with EDTA groups on the surface were synthesized by adding 80 µL of 0.1 M of AgNO₃ into 2 mL of DI water in a sealed vessel and stirring for 5 min, followed by the addition of 100 µL TMS-EDTA silane. The mixture was heated to 150 °C in a microwave and maintained at this temperature for 30s under stirring. Subsequently, acetone was added to recover the Ag@TMS-EDTA NPs by centrifugation at 14000 rpm for 10 min, and then washed twice with 1:1 acetone/water mixture. The resulting Ag@TMS-EDTA NPs were dispersed in DI water.
Chelation of Ag@TMS-EDTA NPs with Gd$^{3+}$ions and HPIX

Paramagnetic property was introduced to the Ag NPs by mixing excess amount of 0.05 M GdCl$_3$ solution and stirring overnight at 40 $^\circ$C. Afterwards, NPs were washed three times with 1:1 acetone/water mixture and dispersed in DI water. Then excess amount of 1 mM HPIX in ethanol was mixed with Ag@TMS-EDTA@Gd$^{3+}$ NPs solution under stirring overnight at 40$^0$C, to form a complex with Gd$^{3+}$ on the nanoparticle surface. The resulting NPs were again washed three times to remove any excessive HPIX, and dispersed in DI water for later use.

The amount of HPIX on NPs was determined by its UV-vis absorbance spectrum, which was recorded with a spectrophotometer (USB4000-ISS, Ocean Optics). A series of different concentrations of HPIX in ethanol/water (50/50) mixture were first measured to establish a calibration curve. Separately, an excess amount of sodium cyanide solution was applied to a known amount of Ag@TMS-EDTA@Gd$^{3+}$@HPIX NPs to dissolve the Ag core (noted as of TMS-EDTA@Gd$^{3+}$@HPIX NPs hereafter). Absorbance of the TMS-EDTA@Gd$^{3+}$@HPIX nanoparticle solution was compared to the calibration curve to calculate the loading amount of HPIX in the NP structure. The morphology of the Ag@TMS-EDTA nanoparticles was characterized by a Phillips Biotwin 12 transmission electron microscope (TEM).

Fluorescence and phosphorescence measurements were carried out on a QM-40 spectrofluorometer (PTI Inc.) equipped with a high performance InGaAs photodiode and a lock-in amplifier (model 410, Scitec Instruments Ltd.). The singlet oxygen generation was detected by monitoring its phosphorescence emission at ~1280 nm. The light source was a Xenon arc lamp. A long-pass filter (850 nm cut-off) was placed in front of the
detector to remove any possible higher-order artifact signals. All measurements were done in ethanol/water (50/50) solvent. The excitation wavelength for fluorescence and singlet oxygen generation of HPIX is 403 nm.

**Photodynamic inactivation (PDI) assays**

*Staphylococcus aureus* (ATCC BAA-44) was used as a model multidrug-resistant bacterium in this study. Typically, overnight culture of *S. aureus* was inoculated in PBS buffer solution (pH 7.4) and mixed with a series of diluted concentrations of Ag@TMS-EDTA@Gd<sup>3+</sup>@HPIX NPs, Ag@TMS-EDTA@Gd<sup>3+</sup> NPs, or HPIX. All bacterial mixtures, including the non-treated bacterial controls, were then transferred to the wells of 96-well plates (200 µL/well). The final cell concentration of the mixtures was ~10<sup>6</sup>-10<sup>7</sup> colony-forming units per mL (CFU/mL). The wells were illuminated by a non-coherent, white light source with interchangeable fiber bundle (model LC-122, LumaCare). Intensity of the white light was measured as 408 mW/cm<sup>2</sup> by a laser power meter (model 840011, SPER Scientific) and the irradiation fluence was 73 J/cm<sup>2</sup>. After illumination, a plate count method was used to determine the viable bacterial numbers (CFU/mL) in mixtures. Dark control experiments were run in parallel. Three independent runs were carried out for each experiment. Each experiment was performed in triplicate. The data are presented as the means with standard deviations. Two-sample t-test was used to analyze the results and the probability values of <5% are considered significant.
3.2.3 Results and Discussions

We developed a new protocol to synthesize Ag@TMS-EDTA NPs using only TMS-EDTA as a reducing and stabilizing agent. After chelation of Gd$^{3+}$ with the EDTA functional groups on the surface of nanoparticles, the porphyrin unit of HPIX can also chelate with Gd$^{3+}$ because of its high coordination number. The Gd$^{3+}$ ion in the Ag@TMS-EDTA@Gd$^{3+}$@HPIX nanostructure has two functions: (1) it forms a paramagnetic complex with EDTA functional groups that directly causes change to the T$_2$ relaxation time of the surrounding water protons; (2) it plays the key role in attracting HPIX to nanoparticle surface through coordination with its porphyrin unit. The schematic illustration of the synthesis of Ag@TMS-EDTA@Gd$^{3+}$@HPIX NP and its singlet oxygen generation under light illumination.

Figure 3.2.1. Schematic illustration of the synthesis of Ag@TMS-EDTA@Gd$^{3+}$@HPIX NP and its singlet oxygen generation under light illumination.
generation under light illumination is shown in Figure 3.2.1.

UV-vis absorption spectra of Ag@TMS-EDTA NPs, Ag@TMS-EDTA@Gd\(^{3+}\) NPs, Ag@TMS-EDTA@Gd\(^{3+}\)@HPIX NPs, TMS-EDTA@Gd\(^{3+}\)@HPIX, Ag@TMS-EDTA NPs and HPIX have shown that they have strong peaks at 408 nm, 404 nm, 398 nm, 396 nm and 396 nm, respectively (Figure 3.2.2). HPIX has the typical high-energy Soret band at 396 nm, and low-energy quasi-allowed bands at 498, 535, 566, and 618 nm [38]. There is a good overlap between the absorption of HPIX and the surface plasmon of Ag@TMS-EDTA@Gd\(^{3+}\) NPs. The TMS-EDTA@Gd\(^{3+}\)@HPIX solution has the same absorption bands as pure HPIX, indicating the successful removal of Ag core by cyanide. The HPIX concentration in Ag@TMS-EDTA@Gd\(^{3+}\)@HPIX NP solution was determined to be 2.8 \(\mu\)M.

![Figure 3.2.2. UV-vis absorption spectra of Ag@TMS-EDTA@Gd\(^{3+}\)@HPIX NPs, Ag@TMS-EDTA@Gd\(^{3+}\) NPs, TMS-EDTA@Gd\(^{3+}\)@HPIX, Ag@TMS-EDTA NPs, and free HPIX.](image)

TEM image Ag@TMS-EDTA NPs shows an average diameter of \(~\)20 nm (Figure 3.2.3a). Transverse (\(T_2\)) and longitudinal (\(T_1\)) relaxation times of water protons were
measured by a 1.41 T Bruker Minispec mq60 relaxometer operating at 40°C. The Ag@TMS-EDTA@Gd^{3+}@HPIX NPs have been demonstrated to be potential contrast agents for magnetic resonance imaging. The longitudinal relaxivity (R₁) and transverse (R₂) relaxivity, a measure of MRI contrast agent efficiency, were higher than the clinically used Magnevist with R₁ of 7.6 mM⁻¹ s⁻¹ and R₂ of 13.2 mM⁻¹ s⁻¹ compared to 4.1 and 4.6 mM⁻¹ s⁻¹, respectively, for Magnevist (Figure 3.2.3b) [39].

**Figure 3.2.3.** (a) TEM image of Ag@TMS-EDTA NPs. Scale bar is 20 nm. (b) R₁ and R₂ relaxivities of Ag@TMS-EDTA@Gd^{3+}@HPIX NPs.

Singlet oxygen generation is directly monitored by measuring its phosphorescence emission at ~1280 nm. As shown in Figure 3.2.4a, Ag@TMS-EDTA@Gd^{3+}@HPIX NPs have much higher singlet oxygen generation than Ag@TMS-EDTA@Gd^{3+} NPs and free HPIX under 403 nm excitation. Note that in these measurements HPIX concentrations in Ag@TMS-EDTA@Gd^{3+}@HPIX and free HPIX are both 0.85 µM, and Ag@TMS-EDTA@Gd^{3+}@HPIX and Ag@TMS-EDTA@Gd^{3+} have the same amount of Ag. While singlet oxygen generation from the free HPIX at such concentration is insignificant, there is appreciable amount of singlet oxygen generated by the Ag@TMS-EDTA@Gd^{3+} nanoparticles due to the Ag core, which is
consistent with previous report in the literature [40]. Regardless of the Ag core, the Ag@TMS-EDTA@Gd$^{3+}$@HPIX NPs display synergistic effect in singlet oxygen generation, consistent with our previous studies [38,41,42]. The singlet oxygen excitation spectra (Figures 3.2.4b) show that Ag@TMS-EDTA@Gd$^{3+}$@HPIX NPs can be excited in a much broader spectral region than free HPIX, which improves their utility under near infrared (NIR) light excitation for applications requiring deep tissue penetration [38,41,42].

![Figure 3.2.4. Singlet oxygen (a) emission and (b) excitation spectra of Ag@TMS-EDTA@Gd$^{3+}$@HPIX NPs, Ag@TMS-EDTA@Gd$^{3+}$ NPs, and HPIX.](image)

We tested the antibacterial efficacy of Ag@TMS-EDTA@Gd$^{3+}$@HPIX NPs, Ag@TMS-EDTA@Gd$^{3+}$ NPs, and HPIX against the multidrug-resistant MRSA. Results are shown in Figure 3.2.5. While free HPIX and Ag@TMS-EDTA@Gd$^{3+}$ NPs under these conditions display almost negligible antibacterial activities, Ag@TMS-EDTA@Gd$^{3+}$@HPIX NPs demonstrate significant inactivation capability of MRSA under white light illumination. Bacterial killing of up to ~3-log is observed at 0.06 µM of HPIX. The Ag@TMS-EDTA@Gd$^{3+}$@HPIX NPs display synergistic effect in PDI efficiency, compared to the Ag@TMS-EDTA@Gd$^{3+}$ NPs nanoparticles and free HPIX.
(Figure 3.2.5d). This is consistent with the results from the spectroscopic measurements on singlet oxygen generation.

Figure 3.2.5. Antibacterial assays of Ag@TMS-EDTA@Gd\textsuperscript{3+}@HPIX NPs (a), Ag@TMS-EDTA@Gd\textsuperscript{3+} NPs (b), and HPIX (c) against MRSA with or without white light illumination. Results are expressed as mean±SD (n=3, p<0.05). (d) Bacterial killing efficacy of Ag@TMS-EDTA@Gd\textsuperscript{3+}@HPIX NPs, Ag@TMS-EDTA@Gd\textsuperscript{3+} NPs, and HPIX against MRSA under white light illumination. Enhancement in bacterial killing efficacy is defined as: log\textsubscript{10}(enhancement killing) = log\textsubscript{10}(Ag@TMS-EDTA@Gd\textsuperscript{3+}@HPIX killing) – log\textsubscript{10}(Ag@TMS-EDTA@Gd\textsuperscript{3+} killing) – log\textsubscript{10}(HPIX killing).
In summary, we report that Ag@TMS-EDTA@@Gd$^{3+}$@HPIX nanoparticles show significant singlet oxygen generation with broadened excitation profile. They demonstrate very high PDI efficacy against a multidrug-resistant *Staphylococcus aureus* (MRSA), under the condition where the free HPIX and Ag@TMS-EDTA@@Gd$^{3+}$ nanoparticles show insignificant antibacterial activity. In conclusion, Ag@PS hybrid nanoparticles, which have demonstrated promising enhancement at singlet oxygen generation based on the surface plasmon-resonance coupling, currently being studied as NIR active nanoprobes for PDI [38,41,42]. Moreover, theranostic nanoplatforms incorporating NIR excitable Ag@Gd$^{3+}$@photosensitizer (porphyrin based) have been developed in this study, which not only produce enhanced PDI effect, but also provide MR imaging capability.

### 3.2.4 References


Chapter 4

Summary and Future Efforts
We have demonstrated a novel paramagnetic relaxation based sensing system using SiO$_2$@TMS-EDTA@Fe$^{3+}$ and SiO$_2$@TMS-EDTA@Gd$^{3+}$ nanoparticles. The Fe$^{3+}$-EDTA or Gd$^{3+}$-EDTA complex on the SiO$_2$ nanoparticles in this scheme has two functions: 1) it is the paramagnetic agent that directly causes change to the T$_2$ relaxation time of the surrounding water protons; 2) it is the target recognition element that selectively binds to dopamine and phosphate ion, respectively. Moreover, by immobilizing other target recognition elements on the nanoparticle surface, the SiO$_2$@TMS-EDTA@Fe$^{3+}$/Gd$^{3+}$ nanoparticles can be adapted to detect other targets that do not directly bind to the Fe$^{3+}$, or Gd$^{3+}$. The results pave way for further development and usage of SiO$_2$@TMS-EDTA@metal nanostructures for desirable sensing applications (Figure 4.1).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.1.png}
\caption{A general scheme can be used for sensing purpose: SiO$_2$@TMS-EDTA @paramagnetic NPs Surface –COOH functional groups can be used for conjugation of different recognition elements to detect other targets.}
\end{figure}
We also developed a new protocol to synthesize Ag@TMS-EDTA@Gd\(^{3+}\)@HPIX NPs. The Gd\(^{3+}\) ion in the Ag@TMS-EDTA@Gd\(^{3+}\)@HPIX nanostructure has two functions: (1) it forms a paramagnetic complex with EDTA functional groups that directly causes change to the T\(_2\) relaxation time of the surrounding water protons; (2) it plays the key role in attracting HPIX to nanoparticle surface through coordination with its porphyrin unit (Figure 4.2). So this nanoplatform integrates functions of magnetic resonance imaging (MRI) contrast agents and PDI agents in a single nanostructure as theranostics. Moreover, Gd-porphyrin complex can be adapted to other porphyrin-based PS, which promising a general nanoplatform to be able to test efficiency of singlet oxygen generation or PDI of different porphyrin-based PSs.

![Figure 4.2](image)

**Figure 4.2.** TMS-EDTA@Gd\(^{3+}\) complex has the key role in attracting porphyrin unit of HPIX.