Small organic molecule-functionalized upconversion nanoparticles for detection and bioimaging

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NANYANG
TECHNOLOGICAL
UNIVERSITY

**SMALL ORGANIC MOLECULE-FUNCTIONALIZED
UPCONVERSION NANOPARTICLES FOR DETECTION AND
BIOIMAGING**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2017

ORGANIC SMALL MOLECULE-FUNCTIONALIZED UCNPS FOR DETECTION AND BIOIMAGING

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2017

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

2017-Aug-18

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Date

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Gu Bin

Abstract

In recent years the bio-detection and bio-imaging are hot research areas, more and more probes have been reported to monitor intracellular and intercellular substances, which are highly sensitive, high-resolution and low-cost. Though they have many advantages, these probes have some main limitations such as short lifetime, small penetration depth, damage to the samples and autofluorescence. Therefore new probes should be developed to overcome these shortcomings. On the other hand, upconversion nanoparticles (UCNPs) have got considerable interest and have many potential applications in this field. Compared with conventional fluorescent probes, UCNPs have many advantages: chemical stability, non-autofluorescence from bio-samples, large light penetration depth, long lifetime (millisecond scale) and less damage to samples.

To better understand the development of UCNPs, this thesis introduces basic principles and applications of the UCNPs, followed by some experimental methodologies such as synthetic methods, purification methods, basic characterization techniques, spectroscopy and electrochemical techniques and bio-applications.

This thesis focused on developing new UCNP-based probes, using Luminescence Resonance Energy Transfer (LRET) process for bio-detection and bio-imaging, and this thesis contains two parts.

In the first part, a Hg^{2+} UCNP-based probe is elaborated. Hg^{2+} is an extremely toxic ion, which will accumulate in human bodies and cause severe nervous system damage. Therefore, the sensitive and efficient monitoring of Hg^{2+} is of great importance. In this part, a thiazole-derivative-functionalized NaYF_4 probe is demonstrated, and upconversion emission intensity ratio of 540 nm to 803 nm (I_{540}/I_{803}) is employed as a ratiometric signal to detect Hg^{2+} in living cells and showed excellent photostability and high

selectivity, with detection limit to be 0.21 μM . The structure of nano-probe was characterized by transmission electron microscopy (TEM), Powder X-ray diffraction (Powder XRD) and the low cytotoxicity was confirmed by an MTT assay. The UCL (upconversion luminescence) test in Hela cells was carried out by confocal microscopy. The results demonstrated that organic-dye-functionalized UCNPs should be a good strategy to detect toxic metal ions when studying cellular bio-systems.

In the second part, an improved Cu^{2+} - NaYF_4 -based probe is demonstrated. Cu^{2+} plays a key role in metabolism. Its disequilibrium in human body will cause several severe diseases. Therefore, sensitively and efficiently monitoring Cu^{2+} in both environment and human body is of great importance. In addition, developing an inorganic-organic hybrid probe with multi-channel responses to more accurately detect ions in living systems is very challenging but highly desirable. In this part, for the first time, electrically-active ferrocene group was attached onto the framework of optically-active Rhodamine to form a new dye (RB-FC), which could be further loaded onto the surface of silica-coated UCNPs to construct a novel nano probe (RB-FC-UCNPs) to detect Cu^{2+} through both electrochemical method and optical ways. Upon the interaction with Cu^{2+} , this as-prepared nano-probe showed obvious changes in absorption, emission and electrochemical parameters. The detection employed absorption intensity at 558 nm (A_{558}), emission intensity ratio of 540 nm to 654 nm (I_{540}/I_{654}) as spectral signals, and oxidation peak of cyclic voltammetry curve as electrochemical signal, demonstrating high stability and low detection limit of this novel probe. In addition, bio-imaging test revealed that this probe could be applied in detecting and visualizing Cu^{2+} in A549 cells with low cytotoxicity.

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Abbreviations

CD	Cyclodextrin
CLSM	Confocal Laser Scanning Microscopy
CV	Cyclic Voltammetry
Cys	Cysteine
EDG	Electron Donating Group
ESA	Excited State Absorption
ETU	Energy Transfer Upconversion
EWG	Electron Withdrawing Group
FRET	Förster Resonance Energy Transfer
FTIR	Fourier Transform Infrared Spectroscopy
GSH	Glutathione
Hcy	Homocysteine
HOMO	Highest Occupied Molecule Orbital
ICT	Intramolecular Charge Transfer
LRET	Luminescence Resonance Energy Transfer
LUMO	Lowest Unoccupied Molecule Orbital
MS	Mass Spectrum
NIR	Near Infrared Range
NMR	Nuclear Magnetic Resonance
OA	Oleic Acids
PA	Photon Avalanche
PET	Photoinduced Electron Transfer
TEM	Transmission Electron Microscopy
UCL	Upconversion Luminescence
UCNP	Upconversion Nanoparticles
UV-Vis	Ultraviolet-Visible Spectroscopy
XRD	X-ray Diffraction

Chapter 1

Introduction

In this chapter, the background and importance of bio-detection and bio-imaging are described. Due to the limitations of conventional fluorescent probes, novel probes based on UCNPs are highly required because UCNPs have many benefits. The hypothesis is based on the idea that taking advantage of the benefits of UCNPs, and combining with organic dyes, new probes with excellent performance should be developed. In addition, the structure of this thesis is briefly exhibited as follows: introduction, literature reviews, experimental methodology, main discoveries of these years, summaries and future work. Finally, the main outcomes are elaborated.

1.1 Hypothesis/Problem Statement

The development of biological science is highly related to new technologies. Now people are more and more interested in the detection of intracellular and intercellular substances, and scientists are continually working on new analysis technologies. Because many molecules only have weak signals, it is necessary to introduce bio-labels to get sensitive signals, and therefore developing new bio-labels has become one of the hottest areas.

Among all the technologies, photon-signal-based bio-sensors are the most commonly used ones. These sensors are easier to manipulate, and will not damage samples, therefore these sensors have bright prospects and related studies have got significant progress.

Now bio-imaging and detection techniques are highly sensitive, high-resolution, low-cost, but could not avoid the absorption and scattering of bio-samples. In addition, autofluorescence produced by bio-samples will also interfere with the detection and bio-imaging. To overcome these shortcomings, new bio-sensors should be developed.

With the application of some advanced technologies such as wide-field fluorescence microscopy (EFM), confocal laser scanning microscopy (CLSM) and total internal reflection fluorescent microscope (TIRFM) in modern medicine and molecular biology, luminescence-based bio-labeling has got increased attention. These probes provide a unique approach to visualize details of living cells and animals, and have become a powerful tool to study these bio-samples. Because of the development of material science and nano technology, many luminescent probes such as organic dyes,¹⁻⁴ metal complexes,⁵⁻¹⁰ semiconductor nanocrystals¹¹ have been reported.

However these bio-labels have some limitations. Usually the emission lifetimes of organic dyes are less than 100 ns,¹² which is hard to differentiate from other short-lived signals. Quantum dots have high toxicity and short circulation half time,¹³ and could not be widely used in bio-samples. On the other hand, another material upconversion

nanoparticles (UCNPs) have got considerable interests and have many potential applications in this field. Compared with conventional fluorescence probes, UCNPs have many advantages: chemical stability,^{14,15} non-autofluorescence from bio-samples,¹⁶⁻¹⁸ large light penetration depth,¹⁹⁻²¹ long lifetime (millisecond scale)^{22,23} and less damage to samples.²⁴⁻²⁸

Therefore, the research in this thesis is based on the following hypothesis:

(1) Bio-detection and bio-imaging are becoming more and more important because they could reflect some crucial physiological processes. For normal fluorescence probes, their usage is limited because of the autofluorescence from the sample. Compared with conventional probes, UCNPs have many benefits, especially no autofluorescence, which is suitable for bio-detection and bio-imaging. Considering the fact that Hg^{2+} is extreme toxic to human bodies and will cause severe nervous system damage, in this thesis a novel UCNP-based probe for Hg^{2+} detection is developed.

(2) Several UCNP-modified probes have been reported. Though they are effective, they are only based on spectra change. These single-channel probes may be affected by different chemical conditions. To overcome this problem, a new multi-channel probe is demonstrated in this thesis. In addition to spectra change, this new probe employs electrochemical signals, which improves stability and adaptability.

1.2 Objectives and Scope

The motivation of this project is to develop novel probes for bio-detection and bio-imaging based on UCNPs. To extend the further application of UCNP-based probes, the properties are observed and compared with conventional probes. Moreover, developing an inorganic-organic hybrid probe with multi-channel responses to more accurately detect ions in living systems is very challenging but highly desirable.

The specific objectives are:

- (1) To design, synthesize and characterize the novel UCNP-based probe for Hg^{2+} detection and study the detection and bio-imaging properties of this probe.
- (2) To develop new UCNP-based probe with multi-channel detection for Cu^{2+} and investigate the effect of introduced electrochemical signal.

1.3 Dissertation Overview

The thesis addresses the issue to develop novel UCNP-based probes for bio-detection and bio-imaging. To further explore this strategy, new technique is introduced into the detection. Therefore the advantages of multi-channel probe are also studied.

Chapter 1 provides a rationale for the research, explaining the reason why UCNP-based probes are chosen and then the hypothesis is proposed. Second, the objectives and scope for the project are listed. Third, the outline of the thesis is briefly introduced. Finally, the findings and outcomes are elaborated.

Chapter 2 reviews the literature concerning the development of UCNPs. This chapter is divided into two parts. The first part elaborates basic conceptions of UCNPs, including mechanisms of upconversion, component of UCNPs, color tuning, surface modification and related mechanisms of organic fluorescent probes. The second part demonstrated the development of UCNP-based bio-detection and bio-imaging.

Chapter 3 introduces synthetic methods and procedures to get final probes, some useful purified methods, basic characterization techniques for structure confirmation, spectroscopy and electrochemistry characterization methods to further study the properties of the probe and some biological related techniques for bio-imaging. For all methods, their principles are explained and for all techniques, the main parts of the

instruments are listed.

Chapter 4 demonstrates a thiazole-derivative-functionalized UCNP probe. The structure of the nano-probe is characterized by TEM, powder XRD, FT-IR, and the low cytotoxicity of the probe is confirmed by MTT assay. Upconversion emission intensity ratio of 540 nm to 803 nm (I_{540}/I_{803}) is employed as a ratiometric signal to detect Hg^{2+} in living cells with excellent photostability and high selectivity, and the UCL test in HeLa cells shows that this probe is effective in living cells.

Chapter 5 describes a novel inorganic-organic hybrid probe with multi-channel detection for Cu^{2+} . The probe contains electrically-active ferrocene group, optically-active Rhodamine and silica-coated UCNPs. The structure is confirmed by TEM, powder XRD, and low cytotoxicity of the probe is confirmed by MTT assay. Employing absorption intensity at 558 nm (A_{558}), emission intensity ratio of 540 nm to 654 nm (I_{540}/I_{654}) as spectral signals, and oxidation peak of cyclic voltammetry curve as electrochemical signal, this probe shows high stability and low detection limit. In addition, bio-imaging test demonstrates that this probe is suitable for bio-detection and bio-imaging in A549 cells.

Chapter 6 includes three parts. The first part summarizes the whole work in this dissertation. The second part discusses whether the hypothesis is proved. In the third part, some ideas and plans for the future work are proposed.

1.4 Findings and Outcomes

This research led to several novel outcomes by:

1. Applying new UCNP-based probe for bio-detection and bio-imaging of Hg^{2+} . The designed strategy and experimental procedures could be transferred to other ions or small molecules detection.

2. Developing multi-channel UCNP-based probe for Cu^{2+} detection and bio-imaging. Improving the strategy by introducing electrochemical signals into the detection, and increasing stability and adaptability. The properties of the new probe is deeply studied. This new strategy is not only effective for Cu^{2+} , but also for other analytes. And it also could be further improved by combination with other techniques, depending on the properties of the analytes.

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Chapter 2

Literature Review

To better understand the development of upconversion nanoparticles (UCNPs), this chapter introduces basic principles and applications of the UCNPs. In the first part, some basic conceptions are elaborated including upconversion mechanisms, components, surface modification and color tuning. In the second part, some new probes for bio-detection and bio-imaging are demonstrated, including ion probes, gas molecule probes and small molecule probes. The detection is based on the interaction between chromophore and UCNPs, which is called luminescence resonance energy-transfer (LRET) process. The mechanisms of organic probes and LRET process are also discussed.

2.1 Basic Principles and Applications of UCNPs

Upconversion (UC) refers to nonlinear optical process, which absorbs two or more low energy photons through intermediate long-lived energy states, followed by emitting a high energy photon. For normal fluorescence, the wavelength of excitation photon is shorter, energy level is higher than that of emission photon. While upconversion is on the opposite, the energy level of emission photon is higher. Low energy photon is converted into high energy photon, and that is the reason why this process is called “upconversion”. The phenomenon of upconversion has been known since 1960s,¹ but the use of this effect was limited only to bulk glass and crystalline materials.^{2,3} Until late 1990s, because of the development of nanotechnology and nanoscience, upconversion nanoparticles (UCNPs) underwent a significant expansion, and had become one of the most attractive research fields within nanoscience community. The electron configuration of lanthanide ions is $4f^n 5s^2 5p^6$, $n = 0-14$. The partly filled 4f shell is responsible for the multiple energy states in the NIR, visible (Vis) and ultraviolet (UV) spectrum range. Because the 4f inner electrons are shielded by the outer 5s and 5p electronic, the electronic transitions are almost not affected by the surrounding environment. Energy levels keep constant, and therefore each lanthanide ion has a group of unique emission peaks like spectroscopic fingerprints, and these peaks are very sharp.

2.1.1 Upconversion Mechanisms

As shown in **Fig. 2.1.1.1**,⁴ there are three main upconversion mechanisms: (a) excited-state absorption (ESA), (b) energy transfer upconversion (ETU) and (c) photon avalanche (PA).⁵⁻⁷

2.1.1.1 Excited-state absorption (ESA)

Excited-state absorption (ESA) refers to the process that a ground level ion successively absorbs two pump photons to E2 level, followed by emission and come back to ground

Fig. 2.1.1.1 Mechanisms of upconversion (Reprinted with permission from ref. 4. Copyright 2009, Royal Society of Chemistry.)

level again (**Fig. 2.1.1.1**). Two factors play key roles in this process: similar energy gap from G to E1 and from E1 to E2, as well as long lifetime of the intermediate level E1. When an ion is excited to the E1 level, it is still probably to accept another photon with same wavelength, and be promoted to the E2 level. To increase efficiency of ESA, lanthanide ions with ladder-like energy states are required. Only a few lanthanide ions such as Er^{3+} , Ho^{3+} and Tm^{3+} have such energy level distribution.¹

2.1.1.2 Energy transfer upconversion (ETU)

Energy transfer upconversion (ETU) is quite different from ESA, because ESA is operated on only one single ion, while ETU involves two neighbor ions (**Fig. 2.1.1.1**). In an ETU process, ion 1 that is acted as a sensitizer, which could absorb a pump photon, and is excited to intermediate level E1. Due to the fact that level E1 is not very stable, ion 1 transfers the energy to a neighbor ion 2 that is acted as an activator. Ion 1 comes back to ground state, while ion 2 is excited to E1 level. The energy transfer process happens again and ion 2 is excited to its upper emitting state E2, followed by emission of a converted photon. The upconversion efficiency of ETU is highly related to the average

distance from sensitizer to neighboring activator, which depends on the concentrations of these dopants. The ETU process is of great importance for UCNPs because most efficient UCNPs to date are based on ion pairs of sensitizers and activators such as $\text{Yb}^{3+}/\text{Tm}^{3+}$, $\text{Yb}^{3+}/\text{Er}^{3+}$, and $\text{Yb}^{3+}/\text{Ho}^{3+}$.

2.1.1.3 Photon avalanche (PA)

For PA process, the excited energy should be above a certain threshold value ($E_2 - E_1$). Actually, the PA is a looping process begins with the promotion of ion from level E_1 to level E_2 by ESA process. Then an efficient cross-relaxation process happens between E_2 level ion and another ground state ion, and both ions fill into intermediate E_1 state: ion 1 (E_2) + ion 2 (G) $\rightarrow$ ion 1 (E_1) + ion 2 (E_1). The net effect of the loop is that one ion at level E_1 produces two ions at level E_1 , and these two ions are readily to fill into E_2 for further looping process. When the loop ensues, two ions will produce four, and four will produce eight, producing level E_2 ions exponentially, like an avalanche (**Fig. 2.1.1.1**). In addition, it is easy to identify PA process, because it usually requires excitation threshold and a long time (seconds) to build up.

2.1.2 Component of UCNPs

It has been proved that most lanthanide-doped materials can exhibit upconversion, but efficient upconversion only occurs for particular dopant-host combinations. Most efficient UCNPs are based on energy transfer process between ion pairs. The ion that donates energy is called sensitizer, while the ion that accepts the energy and emits photons is called activator. Here the selection criteria of activator, sensitizer and host materials are provided.

2.1.2.1 Activators

Most lanthanide ions have more than one excited 4f energy level. While to generate

practically useful upconversion emission, the energy gap between E2 to E1 and E1 to G

Fig. 2.1.2.1.1 Three main activators (Reprinted with permission from ref. 4. Copyright 2009, Royal Society of Chemistry.)

should be close enough to facilitate ions to absorb photons with same wavelength. Er^{3+} , Tm^{3+} and Ho^{3+} typically have this ladder-like energy levels distribution (**Fig. 2.1.2.1.1**), and they are frequently used as activators. For Er^{3+} , the energy gap (10350 cm^{-1}) between $4I_{11/2}$ and $4I_{15/2}$ levels is similar to the gap (10370 cm^{-1}) between the $4F_{7/2}$ and $4I_{11/2}$ levels. Therefore the ladder-like distributed levels of $4I_{15/2}$, $4I_{11/2}$, and $4F_{7/2}$ could be used to generate upconversion emission. In addition, the energy gap law implies that the multiphonon relaxation rate constant decreases exponentially when increasing energy gap. Er^{3+} and Tm^{3+} demonstrate relatively large energy gaps, indicating low probabilities of non-radiative transitions among various excited energy levels, which is in agreement with the fact that the most efficient UCNPs to date are obtained based on Er^{3+} and Tm^{3+} .

2.1.2.2 Sensitizers

For single-doped nanoparticles, two factors are highly related to the upconversion efficiency: two neighbor activator ions' distance and absorption cross-section. Unfortunately, most lanthanide activator ions have low absorption cross-sections. To decrease distance between two neighbor activators, doping concentration should be increased. However, high concentration will lead to deleterious cross-relaxation, resulting in the quench of upconversion efficiency. Thus the concentration of activator ions should

be kept low to avoid quench effect. Usually the overall upconversion efficiency of single-doped nanoparticles is relatively low.

Fig. 2.1.2.2.1 Energy transfer from sensitizer to activator (Reprinted with permission from ref. 4. Copyright 2009, Royal Society of Chemistry.)

To increase efficiency, another ion should be co-doped with activator. This sensitizer should have large absorption cross-section in the near-infrared region, to take advantage of the sensitizer and activator pairs through efficient ETU process. Yb^{3+} possesses an extremely simple energy level distribution (**Fig. 2.1.2.2.1**) with only one excited 4f level $^2\text{F}_{5/2}$. The absorption band of Yb^{3+} locates at 980 nm, corresponding to the $^2\text{F}_{7/2} \rightarrow ^2\text{F}_{5/2}$ transition that also matches energy gaps of Er^{3+} , Tm^{3+} and Ho^{3+} , facilitating efficient energy transfer from Yb^{3+} to Er^{3+} , Tm^{3+} and Ho^{3+} . These optical properties make Yb^{3+} very suitable as sensitizer. The sensitizer concentration is normally kept high (around 20 mol%) in double-doped or triple-doped nanoparticles, while the activator concentration is very low (around 2 mol%).

2.1.2.3 Host Materials

The host materials should have low lattice phonon energies, chemical stability and close lattice matches dopant ions. Since all lanthanide ions have similar chemical properties and ionic size, their inorganic compounds are suitable for host materials. Low lattice phonon energies could maximize radiative emission and minimize non-radiative energy loss. Heavy halides such as chlorides, bromides and iodides have low phonon energies of less than 300 cm^{-1} . However, they are hygroscopic, which limits their usage. Oxides show excellent chemical stability, but their phonon energies are relatively high. On the other hand, fluorides usually exhibit high chemical stability and relatively low phonon energies (around 350 cm^{-1}), therefore they are typical component for UCNPs.

In addition, the crystal structure of the host materials also could affect the optical properties of UCNPs. For example, hexagonal-phase $\text{NaYF}_4\text{:Yb/Er}$ shows much higher upconversion efficiency compared with its cubic-phase counterpart.⁸ This difference could be ascribed to different crystal-fields around lanthanide ions in matrices of various symmetries. Compared with high symmetry host materials, low symmetry counterparts produce a crystal-field with more uneven components around the dopant ions. These uneven components enhance electronic coupling of 4f energy levels and increase f-f transition probabilities.

2.1.3 Upconversion Multicolor Tuning

The manipulation of UCNPs' output color is crucial in biological labeling. Ideal UCNP-based labels should have high photochemical stability, strong absorption under particular excitation and sharp emission peaks in spectrums. Several strategies have been developed to meet these requirements.

2.1.3.1 Controlling Dopant-Host Combination

Because each lanthanide ion has a group of unique energy levels, different ions will show distinct sharp emission peaks, which cover a broad spectrum, from NIR to UV. For a given lanthanide ion, its luminescence may change a little in different host materials, because of different site symmetries in host crystals. Therefore changing combination of different dopant ions and host materials is a direct way to effectively modulate output colors.

The most common activators are Er^{3+} , Tm^{3+} and Ho^{3+} , because of their ladder-like energy level distributions, and all three activators could accept energy from sensitizer Yb^{3+} . This fact enables single-wavelength excitation (around 980 nm) to modulate emissions of UCNP s with different activators. Er^{3+} has three main emission bands: two green bands around 525 nm and 545 nm, one red band around 660 nm, corresponding to $^2\text{H}_{11/2} \rightarrow ^4\text{I}_{15/2}$, $^4\text{S}_{3/2} \rightarrow ^4\text{I}_{15/2}$ and $^4\text{F}_{9/2} \rightarrow ^4\text{I}_{15/2}$ transitions respectively.^{9,10} Ho^{3+} has two main emission bands: green emission around 540 nm, red emission around 645 nm, attributed to $^5\text{S}_2 \rightarrow ^5\text{I}_8$ and $^5\text{F}_5 \rightarrow ^5\text{I}_8$ transitions¹¹. Tm^{3+} has one main emission band within NIR range at 800 nm, originated from $^3\text{H}_4 \rightarrow ^3\text{H}_6$ transition. Scientists demonstrated various colors based on different dopants combinations:¹² (**Fig. 2.1.3.1.1**) $\text{NaYbF}_4\text{:Tm}$ (blue); (b) $\text{NaYbF}_4\text{:Ho}$ (green); (c) $\text{NaYbF}_4\text{:Er}$ (red); (d) $\text{NaYF}_4\text{:Yb}$ (magenta). Under 980 nm excitation, the UCNP s solutions show four different colors. Table lists some typical dopant-host combinations to get multicolor UCNP s. For specific dopant ions, changing host materials may affect emission peaks.

Fig. 2.1.3.1.1 Colors of UCNP s with different dopants combinations (Reprinted with permission from ref. 12. Copyright 2008, American Chemical Society.)

Table 2.1.3.1.1 Different combinations of UCNP

Dopant Yb ³⁺	Host	Major emissions ^a (nm)		
		Blue	Green	Red
Tm ³⁺	α -NaYF ₄ ⁸	450, 475 (S)		647 (W)
	β -NaYF ₄ ¹³	450, 475 (S)		
	LaF ₃ ¹⁴	475 (S)		
	LuPO ₄ ¹⁵	475 (S)		649 (S)
Er ³⁺	α -NaYF ₄ ⁸	411 (W)	540 (M)	660 (S)
	β -NaYF ₄ ¹³		523, 542 (S)	656 (M)
	LaF ₃ ¹⁴		520, 545 (S)	659 (S)
	YbPO ₄ ¹⁵		526, 550 (S)	657, 667 (S)
	Y ₂ O ₃ ¹⁶		524, 549 (W)	663, 673 (S)
Ho ³⁺	α -NaYbF ₄ ¹²		540 (S)	
	LaF ₃ ¹⁴		542 (S)	645, 658 (M)
	Y ₂ O ₃ ¹⁶		543 (S)	665 (M)

^a S, M and W refer to strong, moderate and weak emission intensities

2.1.3.2 Controlling Dopant Concentration

The emission color also changes with the concentration of dopant ions. The concentration determines average distance between neighboring dopant ions, which plays a key role in ETU process, and exhibits a strong influence on optical properties of UCNP.

A general method was developed¹⁷ to modulate output color based on α -NaYF₄ with different concentration of Yb³⁺, Tm³⁺ and Er³⁺. When increasing Yb³⁺ concentration from 18% to 60%, the output color changed from yellow to red (**Fig. 2.1.3.2.1**), and both green bands around 525 nm and 545 nm decreased. It was because the increased amount of Yb³⁺ would facilitate back-energy-transfer from Er³⁺ to Yb³⁺. When the concentration of Er³⁺ increased from 0.2% to 1.5%, both green bands and red bands increased, and the color changed from blue to white. Since dopant ions and host materials combinations cover a broad range, this strategy should produce a large library of emission spectrums.

Fig. 2.1.3.2.1 Colors of UCNP with different dopants concentrations (Reprinted with permission from ref. 17. Copyright 2008, American Chemical Society.)

2.1.3.3 Controlling Nanoparticles Size

Emission color of UCNP also could be modified by changing nanoparticles' size, which should be ascribed to surface effects. As UCNP grow smaller, the amount of dopant ions on the surface is increased, and surface-induced effects become more prominent, leading to multiphonon-assisted non-radiative relaxations, and changing output color.¹⁸ Several groups have studied size-dependent UCNP emission. For example, α -NaYF₄:Yb/Er with different size from 5.1 nm to 8 nm are dominated by red band ($^4F_{9/2} \rightarrow ^4I_{15/2}$) and green

bands ($^2H_{11/2} \rightarrow ^4I_{15/2}$, $^4S_{3/2} \rightarrow ^4I_{15/2}$), demonstrate various colors (**Fig. 2.1.3.3.1**).¹⁹

Fig. 2.1.3.3.1 Colors of UCNP with different crystal sizes (Reprinted with permission from ref. 19. Copyright 2007, American Chemical Society.)

2.1.3.4 Controlling Core-Shell Structures

Using core-shell structure is another typical strategy to modulate emission colors.²⁰ The core and the shell could employ different dopant ions and host materials combinations to produce more kinds of UCNP. The shell of UCNP suppresses deleterious cross relaxation between dopant ions, and decreases quenching of emission caused by surface effect, therefore yielding a variety of emission colors, and still keep high efficiency. To improve this strategy, scientist developed a core-shell-shell structure (**Fig. 2.1.3.4.1**),²¹ further exploring the possibility to modulate output colors.

Fig. 2.1.3.4.1 Colors of UCNP with different core-shell structures (Reprinted with permission from ref. 21. Copyright 2013, Elsevier B.V.)

2.1.4 Surface Modification

Surface modification not only improves photo-stability, but also provides a platform for various kinds of functionalizations. For biological applications, UCNPs should be compatible with bio-samples. Typical preparation process of UCNPs is through high temperature routes, which prefer oil solvents rather than aqueous solution, therefore UCNPs are covered by oleic acids (OA), showing hydrophobic properties. Surface modification with hydrophilic ligands is required to improve bio-compatibility. Several groups developed some typical strategies to convert UCNPs from hydrophobic to hydrophilic.

2.1.4.1 Ligand Exchange

Ligand exchange refers to replacing hydrophobic ligands with hydrophilic ligands (**Fig. 2.1.4.1.1**), which is the most popular strategy to modify UCNPs' surface. Most UCNPs are capped with oleic acids (OA). The carboxyl group (-COOH) of OA coordinates with lanthanide ions on the surface, while the hydrophobic long-chain hydrocarbon faces outside. A group of ligands have been developed, including poly(acrylic acid) (PAA),²² poly(ethyleneglycol) (PEG)-phosphate,²³ mercaptopropionic acid (MPA),²⁴ 1, 10-decanedicarboxylic (DDA),²⁵ hexanedioic acid (HDA),²⁶ 3-dimercaptosuccinic acid (DMSA),²⁷ mercaptosuccinic acid (MSA),²⁸ citrate,²⁹ mercaptonodecanoic acid (MUA),²⁵ and poly-(amidoamine) (PAMAM).³⁰ After ligand exchange, UCNPs are easier to be dispersed in aqueous solution.

Fig. 2.1.4.1.1 Illustration of ligand exchange (Reprinted with permission from ref. 4. Copyright 2009, Royal Society of Chemistry.)

2.1.4.2 Ligand Oxidation

Ligand oxidation is another effective method to increase water solubility of UCNPs (**Fig. 2.1.4.2.1**). This process is based on oxidation of carbon-carbon double bonds ($R-CH=CH-R'$). OA contains double bond in the long-chain, which is suitable for ligand oxidation process, and get azelaic acids ($HOOC(CH_2)_7COOH$). This method has disadvantages of low yield and long reaction time, but it is very straightforward to get a free carboxylic acid group after oxidation, allowing further functionalization.

Fig. 2.1.4.2.1 Illustration of ligand oxidation (Reprinted with permission from ref. 4. Copyright 2009, Royal Society of Chemistry.)

2.1.4.3 Layer-by-Layer Assembly

Layer-by-layer assembly refers to sequential electrostatic absorption of oppositely charged polymers on the surface of UCNPs (**Fig. 2.1.4.3.1**). The layer thickness could be modulated precisely. Another advantage is that this strategy helps to prepare coated nanoparticles with various shapes and sizes. More importantly, this strategy could control the sign and magnitude of the surface, which helps to be applied for bio-targeting and other bio-applications. Scientists have generated water-soluble UCNPs based on this strategy.³¹ After adsorption of positively charged poly(allylamine hydrochloride) (PAH) and negatively charged poly(sodium 4-styrenesulfonate) (PSS), they modified surface of $NaYF_4:Yb/Er$ with amino-rich shell.

Fig. 2.1.4.3.1 Illustration of layer-by-layer assembly (Reprinted with permission from ref. 4. Copyright 2009, Royal Society of Chemistry.)

2.1.4.4 Surface Silanization

Surface Silanization refers to coating an amorphous silica shell on the core of UCNPs through hydrolysis and condensation of siloxane monomers (**Fig. 2.1.4.4.1**). Silica is highly stable, biocompatible and optically transparent. After coating, the UCNPs will be easier to be dispersed in aqueous solution, and suitable to be introduced to bio-samples. Silanes have abundant functional groups such as $-\text{COOH}$, $-\text{NH}_2$, $-\text{SH}$, and could combine with a variety of bio-molecules. In addition, both hydrophobic and hydrophilic UCNPs could employ this coating strategy based on tetraethoxysilane (TEOS).

Fig. 2.1.4.4.1 Illustration of surface silanization (Reprinted with permission from ref. 4. Copyright 2009, Royal Society of Chemistry.)

2.1.5 Organic Probes Mechanisms

In recent years, it has become a main target to develop chemical sensors toward ions and small molecules for biological applications. Metal ions like iron, zinc and copper play a key role in metabolism, while some other ions like mercury, lead, cadmium are extremely harmful to human bodies. To keep physiological functions normal, the concentrations of these ions should be within a particular range. For this purpose, great amount of probes have been designed and synthesized based on fluorescence technology.

For biological application, luminescence-based bio-imaging has attracted significant attention. It provides a unique method to visualize tissues with high resolution, and it is an effective technique to manipulate and investigate subcellular species in living cells. Because of high selectivity, high sensitivity, short response time, effective turn-on mode and relatively long lifetime, this technique has attracted increasing attention and becomes a hot research area and many kinds of probes have been reported for bio-imaging. They need two functional parts: one part is called reaction site, which can react with target molecule; the other one is called luminophore, which has a spectrum signal that is related to the reaction with the analyte (**Fig. 2.1.5.1**).³² There are also two basic strategies. In first approach, the analyte reacts with the probe and forms a new compound after reaction, leading to a spectrum change. In second method, the analyte catalyzes a reaction, changing the structure of probe, also leading to a spectrum change.

Though numerous fluorophores have been reported, there are still some challenges: first, water solubility of these probes should be improved; second, excitation wavelength of probes had better lay in deep red and near-infrared range; third, understanding of cellular uptake behavior is also important.

A probe should show proper fluorescent properties (including wavelength, emission intensity and lifetime) that can be differentiated between before and after the reaction with analyte. As a result, all factors having an effect on fluorescent properties can be designed as probes. Here several mechanisms are described.

Fig. 2.1.5.1 Two basic strategies for analyte detection (Reprinted with permission from ref. 32. Copyright 2012, American Chemical Society.)

2.1.5.1 π -Electron System

Most of probes are aromatic compounds. Their spectrum properties are related to the degree of delocalization, so changing the degree of π -electron system will also modify absorption and emission characteristics. For example, the compound shown in **Fig. 2.1.5.1.1** can be used as a cyanide probe. After the addition of cyanide, the ion can react with the probe through Michael Addition,³² largely changing the conjugation system, resulting in a 14-fold decrease of fluorescence intensity.³³

Fig. 2.1.5.1.1 Detection of cyanide ion by changing π -electron system (Reprinted with permission from ref. 33. Copyright 2006, American Chemical Society.)

2.1.5.2 EDG or EWG substitution

The effect of substitutions of a compound is not easy to analyze, but generally speaking, the introduction of electron-withdrawing group (EWG) or electron-donating group (EDG) will cause a change of maximum absorption peak, as well as maximum fluorescence peak. According to the probes that have been reported, main EDGs include aromatic amines and phenols, and main EWGs include aldehyde, ketone, amide, nitrile and nitro group.³² The amino group of following compound is exposed because of the oxidation of iron ion (Fig. 2.1.5.2.1). The maximum absorption peak has a blue-shift and the emission has a dramatical increase.³⁴

Fig. 2.1.5.2.1 Detection of iron ion by substitution of EDG (Reprinted with permission from ref. 34. Copyright 2010, Royal Society of Chemistry.)

2.1.5.3 Intramolecular Charge Transfer

If a π -electron system conjugates with an electron-withdrawing group and an electron-donating group at the same time, a donor- π -system-acceptor structure (or D- π -A structure) is generated. This new structure could trigger a process called intramolecular charge transfer (ICT) that will change charge distribution of the whole molecule. The ICT process may also cause a fluorescent change due to the polarity change. Most of the reported probes employ the ICT-based strategy by modifying π -system, electron-withdrawing group or electron-donating group of the fluorophores when reacting with the analyte. In Fig. 2.1.5.3.1, an ICT process happens from the amine part to the positive charged indolium part with weak blue fluorescence. After the nucleophilic addition reaction with cyanide, the ICT is disrupted, and causes a significant fluorescence change

to green.³⁵

Fig. 2.1.5.3.1 Detection of cyanide by ICT process (Reprinted with permission from ref. 35. Copyright 2011, Royal Society of Chemistry.)

2.1.5.4 Photoinduced Electron Transfer

Photoinduced electron transfer (PET) is a widely-used mechanism in fluorescence probes design. Usually a PET probe contains a fluorophore and a receptor. When the fluorophore is excited, electron transition will take place from highest occupied molecule orbital (HOMO) to lowest unoccupied molecule orbital (LUMO). If a nearby part has an electron at an energy level between HOMO and LUMO, it will fill into the HOMO of the fluorophore, and quench the fluorescence (**Fig. 2.1.5.4.1**). Upon reacting with analyte, the energy level of the donor is lower than HOMO of the fluorophore, which means the electron transition can't happen, and the fluorescence is recovered (**Fig. 2.1.5.4.1**). In **Fig. 2.1.5.4.2**, fluorescence of the probe is quenched by PET process. However, the oxidation process of peroxide lowers the energy level of triarylphosphine, blocks PET, and turns on the fluorescence.³⁶

2.1.5.5 Forster Resonance Energy Transfer

Förster resonance energy transfer (FRET) is a non-radiative process that an excited energy donor transfers energy to an energy acceptor in ground state by long range interaction. Probes should meet two requirements for FRET. First, the emission spectrum of the donor should have a substantial overlap with the absorption spectrum of the

Fig. 2.1.5.4.1 Mechanism of PET process³⁷ (Reprinted with permission from ref. 37. Copyright 2010, Royal Society of Chemistry.)

Fig. 2.1.5.4.2 Detection of peroxide by blocking PET process (Reprinted with permission from ref. 36. Copyright 2005, Elsevier B.V.)

acceptor. Second, distance between donor and acceptor needs to lie in a certain range (1 to 10 nm).³⁸ There are mainly three strategies to use FRET mechanism (shown in **Fig. 2.1.5.5.1**): (a) Modulating the absorption coefficient of acceptor; (b) Changing donor-acceptor distance; and (c) Modifying overlap degree between donor emission spectrum and acceptor absorption spectrum.³⁹ As shown in **Fig. 2.1.5.5.2**, the probe has FRET process between BODIPY (donor) and rhodamine (acceptor). Emission peak of rhodamine at 590 nm could be observed. The addition of Cys (analyte) causes the cleavage of BODIPY part and rhodamine part, switching off the FRET process, and emission of BODIPY at 510 appears.⁴⁰

Fig. 2.1.5.5.1 Main detection strategies of FRET process (Reprinted with permission from ref. 39. Copyright 2014, Royal Society of Chemistry.)

Fig. 2.1.5.5.2 Detection of Cys by blocking FRET process (Reprinted with permission from ref. 40. Copyright 2011, Royal Society of Chemistry.)

2.1.5.6 Challenges of Probes Design

Based on the above mechanisms, a variety of fluorophores have been designed, such as rhodamine, fluorescein, 1,8-naphthalimide, cyanine, coumarin, thiazole, pyrene,

porphyrin, BODIPY and squaraine. Though so many kinds of fluorophores have been reported, there are still some challenges. First, for real application of these probes, water solubility is a key factor. Most existed probes can perform well in common organic solvents, but do not perform well in water, which will limit their further applications in biological systems. Second, the optimal excitation wavelength lies in deep red and near-infrared range, which could penetrate the tissue deeper and lower autofluorescence of tissues. Third, in addition to molecules design, understanding of cellular uptake behavior is of the same importance.

2.1.6 Bio-Detection and Bio-Imaging

Optical sensing and assay are of great importance because of the capability to detect crucial molecules, as well as to accurately monitor particular physiological processes. Compared with conventional fluorescent probes, UCNPs have many benefits such as chemical stability, large penetration depth and less damage to samples. The most important factor is that the excitation light of UCNPs is usually within NIR range, and will not be absorbed by bio-samples, therefore there is almost no autofluorescence produced by the samples. Thus UCNPs are promising for bio-detection and bio-imaging. The luminescent properties are not directly related to the biological conditions, so UCNPs should be combined with suitable recognition elements to be applied in detection. After the recognition process, the optical signals produced by UCNPs will change through luminescence resonance energy-transfer (LRET) mechanism. In a LRET system, UCNPs (donor) can transfer the energy to chromophores (acceptor), which will result the change of upconversion luminescence (UCL).

To trigger the LRET process in detection, a chromophore should meet several requirements.

- (1) The chromophore is responsive to an analyte;
- (2) Before and after the reaction with the analyte, the absorption spectra of the chromophore should be different;

- (3) The absorption band of the chromophore overlaps with the emission band of UCNP.
- (4) The chromophore should be close enough to UCNP.

For example, in the following probe for MeHg^+ (**Fig. 2.1.6.1**),⁴¹ before the addition of the analyte, the absorption of chromophore overlaps with 660 nm band of UCNP's emission, and this band is almost quenched. After the reaction with MeHg^+ , the absorption of chromophore overlaps with 800 nm band of UCNP's emission, therefore the 660 nm band is gradually recovered and 800 nm band is quenched (**Fig. 2.1.6.2**).

Fig. 2.1.6.1 Illustration of LRET process (Reprinted with permission from ref. 41. Copyright 2013, American Chemical Society.)

Fig. 2.1.6.2 Spectrum change of the detection (Reprinted with permission from ref. 41. Copyright 2013, American Chemical Society.)

These years many UCNP-based probes have been reported for ions (Ag^+ , Ca^{2+} , Fe^{3+} , Zn^{2+} , Cu^{2+} , Cr^{3+} , Hg^{2+} , Cd^{2+} , Pb^{2+} , CN^- , F^- , NO_2^-), reactive species (O_2 , H_2O_2 , $\bullet\text{OH}$, HClO , NO), thiols (Cys, GSH, H_2S). Most of these probes employed organic dye as energy acceptors, here some typical examples are listed.

2.1.6.1 Bio-Detection of Ions

Hg^{2+} is extremely toxic to mammals, therefore the bio-detection of Hg^{2+} is very important. Zhang *et al*⁴² reported a UCNP-modified probe for Hg^{2+} detection in aqueous media based on energy transfer mechanism (**Fig. 2.1.6.1.1**). A single-stranded DNA that contains organic dye SYBR Green I was covalently attached to $\text{NaYF}_4:\text{Yb/Tm}$ nanoparticles, to be used as the Hg^{2+} -capturing element. Under the illumination of 980 nm laser, $\text{NaYF}_4:\text{Yb/Tm}$ acts as donor to transfer energy to the acceptor SYBR Green I. By monitoring the spectra change before and after the addition of Hg^{2+} , the concentration of Hg^{2+} was quantitatively detected, and the limit of detection was measured to be 0.06 nM.

Fig. 2.1.6.1.1 Illustration of Hg^{2+} detection based on LRET (Reprinted with permission from ref. 42. Copyright 2010, Elsevier B.V.)

Li *et al*⁴³ developed a chromophoric ruthenium complex-assembled UCNP probe to detect and bio-image intracellular Hg^{2+} (**Fig. 2.1.6.1.2**). This probe was also based on energy transfer mechanism and using the UCL intensity ratio as detection signal, the limit of detection was measured to be 1.95 ppb, which is lower than the standard of Environmental Protection Agency of US. One of the highlights is that the probe was capable of monitoring Hg^{2+} distribution changes in living cells.

Fig. 2.1.6.1.2 Illustration of Hg^{2+} detection based on LRET (Reprinted with permission from ref. 43. Copyright 2011, American Chemical Society.)

Jiang *et al*⁴⁴ reported a UCNP probe containing RB-hydrazide dye for Cu^{2+} detection (**Fig. 2.1.6.1.3**). This probe employed FRET process and was the first optical sensor for Cu^{2+} detection based on UCNP.

Fig. 2.1.6.1.3 Illustration of Cu^{2+} detection based on LRET (Reprinted with permission from ref. 44. Copyright 2012, Royal Society of Chemistry.)

Chang *et al.*⁴⁵ rationally designed and synthesized a UCNPs-based probe for Zn^{2+} (**Fig. 2.1.6.1.4**). The absorption of chromophore on UCNPs surface overlapped with the emission of UCNPs, therefore after combination with chromophores, the UCL would be effectively quenched, and subsequently recovered with the addition of Zn^{2+} , allowing the quantitative monitoring of Zn^{2+} . This probe was capable of in vitro and in vivo detection and imaging of Zn^{2+} in mouse brain slice with Alzheimers disease and zebrafish, showing high sensitivity and selectivity.

Fig. 2.1.6.1.4 Illustration of Zn^{2+} detection based on LRET (Reprinted with permission from ref. 45. Copyright 2015, American Chemical Society.)

The detection of some other metal ions such as Pb^{2+} , Cd^{2+} and Co^{2+} were also developed based on UCNPs.⁴⁶ Not only metal ions, probes for non-metal ions were also reported.

The first pH probe based on UCL signals was described by Wolfbeis *et al.*⁴⁷ They obtained lanthanide-ions-doped upconversion nanorods, and combined with a pH probe BTB. The absorption of BTB overlaps with emission of upconversion nanorods (**Fig. 2.1.6.1.5**), and the detection was based on energy transfer. It should be noticed that both emission bands would change after modulating the pH value, therefore there was no reference in this probe.

Chen *et al.*⁴⁸ developed a UCNP-based probe for pH detection (**Fig. 2.1.6.1.6**).

Fig. 2.1.6.1.5 Illustration of pH detection based on LRET (Reprinted with permission from ref. 47. Copyright 2009, Royal Society of Chemistry.)

They synthesized a chromophore ETH 5418 that had different absorption spectra of protonated and unprotonated forms. These two absorption bands overlap with two emission bands of UCNPs, and the detection mechanism was based on the energy transfer between UCNPs and chromophore. In addition, they applied this probe in blood samples.

Because of the extreme toxicity of CN^- , a great deal of effort has been invested on developing probes to detect CN^- . Li *et al*⁴⁹ fabricated a hybrid probe composed of iridium complex and UCNPs to detect CN^- in pure water (**Fig. 2.1.6.1.7**). After reaction with CN^- , the chromophore showed different absorption spectrum, and detection was based on the different emission spectrum.

Fig. 2.1.6.1.6 Illustration of pH detection based on LRET (Reprinted with permission from ref. 48. Copyright 2012, American Chemical Society.)

Fig. 2.1.6.1.7 Illustration of CN^- detection based on LRET (Reprinted with permission from ref. 49. Copyright 2012, Wiley-VCH Verlag GmbH & Co. KGaA.)

2.1.6.2 Bio-Detection of Gas Molecules

Detection of gas molecules such as oxygen, carbon dioxide and ammonia has great importance in biochemistry and clinical medical diagnosis. The detection using UCNP for gas molecules has attracted much attention because of the excellent performance. Wolfbeis *et al*⁵⁰ reported first oxygen sensor based on UCNP. They synthesized a thin film that containing both UCNP and Ir complex. The emission bands of UCNP overlap with absorption band of Ir complex (**Fig. 2.1.6.2.1**) and the oxygen content was quantified through the LRET process. However, the detection of oxygen is still limited to gas environment.

Fig. 2.1.6.2.1 Illustration of O₂ detection based on LRET (Reprinted with permission from ref. 50. Copyright 2011, Wiley-VCH Verlag GmbH & Co. KGaA.)

Carbon dioxide and ammonia are toxic to many aquatic organisms even at low concentrations, thus the detection of them is crucial. Because they can dissolve in water and change the pH value of biological condition, this is an opportunity to quantify the concentration through pH variations. Wolfbeis *et al*⁵¹ reported a carbon dioxide probe based on UCNPs using this strategy (**Fig. 2.1.6.2.2**). The limit of detection was measured to be 0.11%.

Fig. 2.1.6.2.2 Illustration of CO₂ detection based on LRET (Reprinted with permission from ref. 51. Copyright 2010, Elsevier B.V.)

According to similar idea, Wolfbeis *et al* also developed an ammonia sensor (**Fig.**

2.1.6.2.3).⁵² A pH probe phenol red was combined with UCNP. The intensity ratio of green channel to red channel could measure the limit of detection to be 400 μM .

Fig. 2.1.6.2.3 Illustration of NH_3 detection based on LRET (Reprinted with permission from ref. 52. Copyright 2010, American Chemical Society.)

2.1.6.3 Bio-Detection of Small-Molecules

Endogenous H_2O_2 is an important signal molecule and involved in many physiological processes. Monitoring this molecule in living systems is essential but challenging because most of H_2O_2 probes are using UV/Vis excitation light and not practical for bio-imaging. Zhang *et al*⁵³ reported a novel probe based on organic chromophore-attached UCNP (**Fig. 2.1.6.3.1**). This probe contains a UCNP core, covered by cyclodextrin to improve water solubility, followed by combining with organic chromophore. The absorption spectrum of the chromophore overlapped with the emission spectrum of UCNP, and the detection mechanism was to employ LRET process.

Fig. 2.1.6.3.1 Illustration of H_2O_2 detection based on LRET (Reprinted with permission from ref. 53. Copyright 2015, Elsevier B.V.)

H_2S is another important signaling molecule involving in many physiological processes and diseases. To study its biological signaling mechanism, much effort has been spent on developing probes for bio-imaging. However, conventional probes are not suitable for bioimaging because of the damage to the sample and the short tissue penetration depth. To overcome these limitations, Zhang *et al*⁵⁴ for the first time demonstrated a new UCNP-based probe that contained the coumarin-hemicyanine (CHC1). This inorganic-organic hybrid probe displayed a fast response time employing LRET process (**Fig. 2.1.6.3.2**), and could be used in plasma.

Fig. 2.1.6.3.2 Illustration of H_2S detection based on LRET (Reprinted with permission from ref. 54. Copyright 2014, Wiley-VCH Verlag GmbH & Co. KGaA.)

Glutathione (GSH) is the most abundant thiolated tripeptide in human bodies and plays a key role in cellular defense against free radicals and toxins. GSH level is typically associated with cancer, aging, or heart problems, therefore it is crucial to monitor GSH concentration in real time. Liu *et al*⁵⁵ reported a new UCNP-based probe combining with manganese dioxide nanosheets (**Fig. 2.1.6.3.3**). The manganese dioxide on the UCNP surface will quench the emission of UCNP. After the addition of GSH, the manganese dioxide will be reduced to Mn^{2+} and dissolve into solution, the energy transfer process is blocked and turn on the emission. This probe could be applied in aqueous solutions and living cells.

Fig. 2.1.6.3.3 Illustration of GSH detection based on LRET (Reprinted with permission from ref. 55. Copyright 2011, American Chemical Society.)

2.2 Questions to Answer Based on Literature

UCNP-based bio-detection and bio-imaging are emerging as a new thrust. The unique optical properties remarkably decrease the limit of detection compared with conventional probes. However, the efficiency of UCNPs is one of main limitations. Developing high efficiency UCNPs still has room for improvement.

In addition, multi-channel detection will be a hot area in the future. Multi-channel refers to the detection that includes more than one technique such as optical change as well as electrochemical change. Conventional probes are usually based on single-channel, which will be affected by chemical conditions, while multi-channel detection improves the stability and adaptability.

2.3 PhD in Context of Literature

For literature review, the first part is about the basic conceptions of UCNPs, including upconversion mechanisms, components, surface modification and color tuning. The second part describes several new probes based on UCNPs. The literature review explains how the bio-detection and bio-imaging happen and the key point is LRET process. If the emission bands of UCNPs overlaps with absorption bands of chromophore, the energy transfer process happens between UCNPs and chromophore. The probes elaborated are based on this strategy, and new probes are also designed and synthesized to improve this strategy.

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Chapter 3

Experimental Methodology

In this chapter, the synthetic method of nano probe is introduced first, including the synthesis of upconversion nanoparticles, silica-coated nanoparticles and Knoevenagel Condensation. Second, some useful purification methods to obtain the final probe are described, including recrystallization, filtration and centrifugation. Third, some basic characterization techniques to get structure information of target molecules are elaborated, including Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS), Powder X-ray Diffraction (Powder XRD), Transmission Electron Microscopy (TEM) and Infrared Spectroscopy (IR). Among them NMR plays a key role in confirming structure of organic compound, while Powder XRD and TEM are of great importance to confirm nanoparticles' structure. Next, some spectroscopy and electrochemistry characterization methods are listed to further study the properties of the nano probe, including Ultraviolet-Visible Spectroscopy (UV-Vis), Fluorescence Spectroscopy, Upconversion Luminescence Spectroscopy (UCL) and Cyclic Voltammetry (CV). Finally some biological related techniques are elaborated to explore the application of the nano probe in bio-samples, including MTT assay and confocal laser scanning microscopy (CLSM). For all these methods and techniques, their basic principles are explained.

3.1 Rationale for Selection

The selection of the following parts: synthetic method, purification method, basic characterization, spectroscopy and electrochemistry characterization and biological characterization are based on the following principles: how to get the final probe, how to purify them, how to confirm their structure and how to characterize their properties.

For the synthetic methods, the reactions and procedures should be reliable and repeatable with high yields. In this section, the synthesis of upconversion nanoparticles, silica-coated nanoparticles to obtain water-soluble nano probe and Knoevenagel Condensation to get organic dyes that could be loaded onto the probe are explained.

For the purification methods, various useful methods are described. Recrystallization and filtration are used for removing impurities of an organic compound. Centrifugation is used for separating nano particles from solid-liquid mixture. Because the size of nanoparticle is so small that normal filtration is not effective and costs much time, while centrifugation could accelerate the separation process.

In basic characterization techniques part, several most common used techniques are elaborated. Generally speaking, Nuclear Magnetic Resonance (NMR) is the most frequently used for organic compounds because it provides the most detailed structure information. Mass Spectrometry (MS) and Infrared Spectroscopy (IR) are used as complementary techniques. Powder X-ray Diffraction (Powder XRD) and Transmission Electron Microscopy (TEM) are suitable for nanoparticles. Powder XRD could give the crystal structure information of the particles, while TEM could show the morphology and aggregation of the particles.

In the next part, to further characterize the properties of the nano probe, some spectroscopy and electrochemistry techniques are listed. Ultraviolet-Visible Spectroscopy

(UV-Vis) reflects the electron transition from ground state to excited state. In contrast, Fluorescence Spectroscopy and Upconversion Luminescence Spectroscopy (UCL) reflect the electron transition from excited state to ground state. To compare with fluorescence, UCL requires the excitation wavelength to match the energy gap of the sample. Cyclic Voltammetry (CV) reflects the oxidation and reduction trend of a compound, and also indicating the intrinsic chemical properties.

In the final section, two biological-related techniques are introduced. To apply the probe in bio-samples, its cell toxicity should be first investigated. MTT assay is widely used to study cell toxicity. To prove that the probe has potential application in bio-samples, confocal laser scanning microscopy (CLSM) is elaborated to study the bio-detection and bio-imaging properties.

3.2 Synthesis Method

3.2.1 Upconversion Nanoparticles (UCNPs) Synthesis

In thermolysis process, with the help of surfactants, organometallic compounds that act as precursors will decompose in organic solvent with high boiling point at a threshold temperature. The whole process is shown in **Fig. 3.2.1.1**.¹ The commonly used solvent is 1-octadecene (ODE) while the surfactants could be oleic acid (OA), oleylamine (OM) or trioctylphosphine oxide (TOPO). These surfactants contain a functional group to coordinate with the metallic elements and a long hydrocarbon chain to prevent nanoparticles aggregation. It also should be noticed that the rapid decomposition of metallic compounds creates a burst of nucleation, which is important to control size distribution of nanoparticles. The conception of “burst nucleation” was raised by LaMer and coworker in 1940s, when they were studying the preparation of uniform colloidal particles.² In this theory, many nuclei are generated simultaneously, followed by growing without new nucleation. The separation of nucleation and growth is the key point to control size distribution of particles. Otherwise, if nuclei are generated during the whole

process, the growing time of particles would differ largely from each other, and final size distribution is hard to control. By studying different experimental variables such as metal compounds concentration, the properties of the solvents, reaction time and temperature, thermolysis process could produce high-quality UCNPs with a narrow size distribution, excellent optical properties and good crystallinity.

Fig. 3.2.1.1 Illustration of UCNPs synthesis (Reprinted with permission from ref. 1. Copyright 2014, American Chemical Society.)

3.2.2 Silica-Coated UCNPs Synthesis

For UCNPs with hydrophobic capping ligands, a reverse micro-emulsion method should be employed to coat a silica layer on UCNPs surface.³ A homogeneous mixture of cyclohexane, ammonia, surfactant (Igepal CO-520) and TEOS generates hydrophilic cavities, facilitating the hydrolysis of TEOS, forming a silica shell with varying thickness. The hydrolysis mechanism of TEOS is shown in **Fig. 3.2.2.1**, including hydrolysis and condensation.

Fig. 3.2.2.1 Illustration of TEOS hydrolysis

3.2.3 Knoevenagel Condensation

Compound containing active methylene group could react with aldehyde or ketone under weak base catalysis, forming C=C bond (**Fig. 3.2.3.1**). In this condensation reaction, catalyst is usually a weak basic amine, and Z is an electron withdrawing group. Two Z groups could be either same or different, and should be powerful enough to activate the hydrogen atoms. In addition, a strong base catalyst in this reaction would result in self-condensation of the aldehyde or ketone.

Fig. 3.2.3.1 Illustration of Knoevenagel Condensation

3.3 Purification Method

3.3.1 Recrystallization

Recrystallization is a technique to purify compounds with small amount impurities. Dissolve compound A and impurity B in one solvent, then add another solvent that either A or B could dissolve in, while the other could not (**Fig. 3.3.1.1**). One component will remain in the solution, while the other one will precipitate. It should be noticed that the amount of two solvents is critical. Usually compound A and impurity B should fully dissolve in the smallest amount of first solvent, forming a saturated solution, while the

second solvent should be added slowly to the solution to get more precipitation. In addition, some compounds have very different solubility at different temperature. Heating and cooling could also increase purification efficiency in this technique.

Fig. 3.3.1.1 Illustration of recrystallization

3.3.2 Filtration

Filtration is a technique that separates solid from solid-liquid mixture. The key point is filter paper, a semi-permeable paper. Liquid could permeate the filter paper, while solid could not (**Fig. 3.3.2.1**). The parameters of filter paper such as porosity, particle retention, flow rate and capacity should be selected by the properties and amount of liquid and solid.

Fig. 3.3.2.1 Illustration of filtration

3.3.3 Centrifugation

Centrifugation is another technique to get solid from solid-liquid mixture. Under high rotate speed, components are separated because of centrifugal force. High-density

component moves to the bottom of centrifugal tube and forms sedimentation, while low-density component moves upwards (**Fig. 3.3.3.1**). There is a correlation between particle's density, size and the separation rate. The higher the density and the larger the size of particles, the faster they are separated from the mixture. Because of the large centrifugal force to the mixture, the separation of the particles is remarkably accelerated. Therefore compared with filtration, centrifugation is suitable to separate particles with smaller size.

Fig. 3.3.3.1 Illustration of centrifugation

3.4 Basic Characterization

3.4.1 Nuclear Magnetic Resonance (NMR)

Proton nuclear magnetic resonance (^1H -NMR) is a technique that collects structure information of ^1H nuclei in a molecule through nuclear magnetic resonance (**Fig. 3.4.1.1**). This method is a basic characterization to confirm the structure of a compound. The structure information mainly includes two parts: chemical-shift and spin-spin coupling.

Chemical shift value reflects the electron density of a hydrogen nucleus. Under outside magnetic field, electrons around a nucleus will create a secondary smaller magnetic field that has opposite direction to the outside field, according to Lenz's law. This secondary field leads to the fact that the actual magnetic field of the nucleus is smaller than the outside magnetic field, and this effect is called shield. The higher the electron density is,

Fig. 3.4.1.1 Example of ^1H -NMR spectrum

the stronger the shield effect will be. Adding tetramethylsilane (TMS) as an internal standard, the decrease of magnetic field could be calculated as chemical shift value to indicate electron density of hydrogen atom. The exact value of chemical shift is highly related to solvent, hybridization type and neighboring functional groups. If neighboring group is electron-withdrawing, such as $-\text{COOH}$, the chemical shift value will be large. While for electron donating group, such as $-\text{CH}_3$, the chemical shift value will be small. For example, the green hydrogen atoms (**Fig. 3.4.1.1**) near carbonyl group have higher chemical shift value around 2, while the blue hydrogen atoms (**Fig. 3.4.1.1**) near methylene group shows lower chemical shift value around 1.

In addition to chemical shift value, spin-spin coupling also reveals much information. Because nuclei themselves have small magnetic field when spinning and will affect each other, energy and actual magnetic field of a nucleus is related to nearby nuclei. This spin-caused resonance is called spin-spin coupling. The coupling shows effect through chemical bonds, and could be found in three bonds away. For example, in $\text{CH}-\text{CH}$ group, two hydrogen atoms are linked through three bonds. Suppose that chemical shift value of one hydrogen atom is δ , and another hydrogen atom causes the signal to split into two peaks, where one peak is a little higher than δ , while the other is a little lower than δ . The

difference of two peaks' frequency is called coupling constant. It should be noticed that the coupling constant is caused by the magnetic field of nearby nuclei, therefore it is independent of spectrometer magnetic field, which indicates that coupling constant could provide more structure information. In **Fig. 3.4.1.1**, the green hydrogen atoms in $-\text{CH}_3$ group show only one peak, because there are no other hydrogen atoms within three bonds, while both red and blue hydrogen atoms show split peaks, because of spin-spin coupling. Similar to ^1H -NMR, carbon nuclear magnetic resonance (^{13}C -NMR) is another useful technique based on ^{13}C nuclei. Because the spin of ^{12}C is 0, which could not be used in NMR, ^{13}C is typically used. One disadvantage of ^{13}C -NMR is that the sensitivity is much weaker than ^1H -NMR because the amount ratio of isotope ^{13}C is only 1.1%.

3.4.2 Mass Spectrometry (MS)

Mass spectrometry (MS) is a technique that collects molecular mass information through different mass-to-charge ratio (m/z). High energy electrons beam is used in ion source to hit compound, producing fragments with positive charge, and then these ions are accelerated in mass analyzer. The mass analyzer could sort ions by different m/z value. Finally the detector measures ions' amount and provides their intensity. **Fig. 3.4.2.1** is a typical mass spectrum. The X-axis represents the ratio of an ion's mass to the charges that it carries, and Y-axis represents the signal intensity of the ion. The molecular mass of toluene is $M = 92$, refers to C_7H_8^+ , while the highest peak at $M = 91$ refers to C_7H_7^+ . MS is a very useful complementary technique to NMR to decipher compound structure. In addition, some separation techniques are combined with MS to enhance mass determining capabilities. Common tandem combinations are gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS).

3.4.3 Powder X-ray Diffraction (Powder XRD)

Powder X-ray diffraction (Powder XRD) is a rapid analytical technique for crystal structure information. The wavelength of X-ray is from 0.001-10 nm, similar to the

Fig. 3.4.2.1 Example of MS spectrum

spacing of planes in crystal lattice, thus X-ray diffraction (XRD) could be used to study molecule structure. XRD is based on constructive interference of single-wavelength X-rays and a crystalline sample. According to Bragg equation, when conditions satisfy $n\lambda = 2d\sin\theta$, the interaction between X-ray and the sample produces constructive interference (**Fig. 3.4.3.1**). In this formula, λ refers to wavelength of X-ray, d refers to inter-planar distance of the sample, θ refers to scattering angle and n is a positive integer.

Fig. 3.4.3.1 Illustration of Bragg equation

For typical powder samples, the scanning range is from 5 to 70 degrees (**Fig. 3.4.3.2**). If parameters satisfy Bragg equation, the detector will record diffraction peaks intensity. All materials have a set of unique diffraction peaks, comparing the sample's diffraction peaks with these standard reference patterns could identify the sample.

Fig. 3.4.3.2 Example of powder XRD spectrum

X-ray diffractometers include three parts: an X-ray tube, a sample holder and an X-ray detector. In X-ray tube, a filament is heated to produce electrons, after that the electrons are accelerated by applying a voltage, and bombarding a target material. If electrons have sufficient energy to remove inner shell electron, outside electron will transit into inner shell and emit high energy photon, forming X-ray. These X-rays are directed onto the sample, and the reflect X-ray signal is recorded when the detector is rotating. To collect data, the sample rotates with an angle θ on the pathway of X-ray beam, while the detector rotates with an angle of 2θ to collect diffracted X-ray data. By scanning the powder sample through a range of angles, all possible diffractions of the crystal could be recorded due to the random orientation of the crystals.

3.4.4 Transmission Electron Microscopy (TEM)

TEM is a microscopy technique to provide surface information of ultra-thin sample with high resolution. A beam of accelerated and gathered electrons are transmitted to the sample, and directions are changed after colliding with atoms of the sample. Different scattering angles are related to the sample's density and thickness, forming images with different brightness. Then the image is magnified and focused on an imaging device.

Because the electrons have small de Broglie wavelength, TEM is capable to image at a much higher resolution than conventional light microscopes, reaches to 0.1 nm. Therefore

TEM is suitable to capture fine details of sample, even as small as a column of atoms.

A TEM is composed of several parts, including an electron emission source to generate electron beam, a vacuum system for electrons moving, a series of electromagnetic lenses, electrostatic plates, a device allowing the insertion and removal of sample from the beam path and an imaging device to create an image based on the electrons that exit the system.

The electron emission source may be a tungsten filament, a needle or a lanthanum hexaboride (LaB_6) single crystal. The gun is connected with a high voltage source (around 100-300 kV), and under sufficient current, the gun will emit electrons into the vacuum system. Once the electron beam is generated, the upper lenses will focus the beam to the target location.

Usually a vacuum system is evacuated to low pressures, typically 10^{-4} Pa. There are two reasons for such low pressure. First, low pressure could increase the voltage between the cathode and the ground without generating an arc. Second, low pressure could decrease the collision frequency of electrons and gas atoms to a negligible level, which is characterized by the mean free path. TEM components such as specimen holders and film cartridges must be routinely inserted or replaced requiring a system with the ability to re-evacuate on a regular basis. As such, TEMs are equipped with multiple pumping systems and airlocks and are not permanently vacuum sealed.

To manipulate the electron beam path, two physical effects are used. The moving electrons in a magnetic field will be affected by Lorentz force, therefore the use of magnetic field could manipulate the electron beam with various focusing power. In addition, electrostatic fields could cause the electron beam to be deflected a certain angle. Two electrostatic fields with opposite directions will lead to a shift in the beam path. Under these two effects, electron beam path could be sufficiently controlled. Unlike the optical microscope, the optical configuration of TEM could be rapidly changed, because the lenses on the beam path could be changed simply through electrical switching.

TEM specimen stage should minimize the loss of vacuum when inserting the specimen holder into the vacuum system. The specimen holders support a standard sample grid that is usually made of copper, molybdenum, gold or platinum, with 3.05 mm in diameter. Once inserted into the TEM, the sample should be moved to the particular region of the beam. To achieve this goal, the sample could be moved in XY plane, Z axis and be rotated in at least one direction. As TEM could be operated on different magnification, the specimen stage should be highly resistant to movement. Early designs of TEM contained a complex set of mechanical devices to control the motion of the stage, while modern designs may use electrical devices, providing the operator with a computer-based stage input.

3.4.5 Infrared Spectroscopy (IR)

Infrared spectroscopy (IR) is a common used technique, and it reveals the vibration information of a molecule.

Chemical bonds have different types of vibration modes, and each mode corresponds to a frequency that is affected by bond strength and mass of atoms. The infrared will be absorbed if the frequency matches the vibrational frequency. Because different functional groups have their own absorption range, IR spectroscopy is often used to get structure information through intensity and position (frequency).

Based on IR spectroscopy, Fourier transform infrared (FTIR) spectroscopy is developed. FTIR spectroscopy collects high resolution data over a wide spectral range simultaneously, which is much better than measuring intensity of a single wavelength each time. FTIR achieves this goal through a less intuitive way. Each time FTIR spectrometer emits a beam containing many frequencies of light and records the absorption of the sample. Then the FTIR spectrometer changes the frequencies combination and records second data point. This process is repeated many times and a computer takes all these data and calculates the absorption at each wavelength. **Fig.**

3.4.5.1 is a typical FTIR spectrum indicating infrared light transmission at a range of frequency.

Fig. 3.4.5.1 Example of powder FTIR spectrum

3.5 Spectroscopy and Electrochemistry Characterization

3.5.1 Ultraviolet-Visible Spectroscopy (UV-Vis)

UV-Vis spectroscopy is a routinely used technique, referring to absorption spectrum ranging from ultraviolet to visible region. Within this region, atoms and molecules undergo electronic transitions, reflecting transitions from the ground state to the excited state. This technique has various applications such as quantitatively determining highly conjugated compounds in analytical chemistry, and measuring bandgap of organic compound in organic chemistry. The principle is shown in **Fig. 3.5.1.1**.

The electron in a molecule should be in one of three kinds of orbitals: namely σ (single-bond), π (multiple-bond), or n (non-bond). When the molecule absorbs exciting photons, the electron will be excited to a higher energy orbital, and this excited state is called anti-bonding state.

Fig. 3.5.1.1 Energy level diagram of UV-Vis spectrum

σ electron is the most stable among these three types. It has the lowest energy level and transition of σ electron requires high energy photon like ultraviolet light. π electron has much higher energy level than σ electron, therefore it is relatively unstable and easier to be excited. n electron has the highest energy level in three types and also could be excited by ultraviolet and visible light. It should be noticed that most UV-Vis absorption is due to π -electron transitions and n -electron transitions.

When a compound is exposed to excitation light, if the photon energy matches the energy difference of two levels, the photon will be absorbed and the electron will be promoted to a higher level. A spectrometer is designed to measure the degree of absorption at different wavelength and form a spectrum of absorbance (A) versus wavelength (λ). The **Fig. 3.5.1.2** below is spectrum of isoprene. At $\lambda = 222$ nm, isoprene has maximum absorption.

A spectrophotometer consists of light source, diffraction grating, sample holder and detector (**Fig. 3.5.1.3**). The light source is usually Tungsten filament, deuterium arc lamp or Xenon arc lamp, providing ultraviolet to visible wavelength excitation. The diffraction

Fig. 3.5.1.2 Example of UV-Vis spectrum

grating make sure that only photons with single wavelength reach the sample at one time, and absorption intensity is measured as a function of wavelength. After diffraction grating, the output light is split into two beams. One beam passes through reference, and the other passes through the sample. The reference beam intensity is set as 100% transmission, and the instrument will calculate the ratio of two beam intensities. In different systems, two beams intensity could be measured by one detector together or by two detectors separately.

Fig. 3.5.1.3 Illustration of UV-Vis structure

3.5.2 Fluorescence Spectroscopy and Upconversion Luminescence Spectroscopy (UCL)

As a complementary technique to UV-Vis, fluorescence spectroscopy is a commonly used technique that analyzes fluorescence of a sample. It involves using a beam of light, usually ultraviolet light, that excites the electrons in molecules of certain compounds and causes them to emit light.

In fluorescence, the sample molecule absorbs a high energy photon (usually ultraviolet range), and the outer layer electron is promoted to an excited state. After collision with other molecules, the electron reaches the lowest vibrational state of the excited state, followed by emitting a low energy photon and the electron returns to ground state. This process is demonstrated with a Jablonski diagram (**Fig. 3.5.2.1**).

Fig. 3.5.2.1 Jablonski diagram

A fluorometer includes several main parts: light source, monochromator, sample holder and detector. The light path is shown in **Fig. 3.5.2.2**.

Light source provides excitation to promote the electron of sample from ground state to excited state. There are two types of light sources to be used. A mercury vapor lamp emits light at a very narrow wavelength interval, make the excitation filter unnecessary,

Fig. 3.5.2.2 Illustration of fluorometer structure

while the disadvantage is that the wavelength could not be changed too much. On the other hand, a xenon arc shows continuous emission spectrum from 300 nm to 800 nm with almost same intensity.

Monochromator is used to provide light with a narrow range of wavelength. To get different spectra, two monochromators should be placed after light source and before detector. The most common used monochromator is based on diffraction grating. The light gets out of the grating with different angles depending on wavelength, and could be selected to pass through the sample or detector.

Detector is the component to detect emission light intensity and transfer into digital signal. It could be either single-channel or multi-channel, both have advantages and disadvantages. In addition, though the incident light absorbed by the sample is from one direction, the fluorescence is emitted to all directions. To minimize the effect of transmitted or reflected light, the detector is placed vertical to the excitation light.

Conventional fluorescence absorbs high energy photon and emits low energy photon,

while UCL absorbs two low energy photons and emit one high energy photon. The mechanism is shown in **Fig. 3.5.2.3**. Another difference is that UCL requires the excitation to be particular wavelength that matches the energy gap of doping ions in the sample. For example, 980 nm excitation is suitable for NaYF₄: Yb/Er. For the instrument, UCL and conventional fluorescence is almost the same.

Fig. 3.5.2.3 Illustration of upconversion mechanisms (Reprinted with permission from ref. 4. Copyright 2009, Royal Society of Chemistry.)

3.5.3 Cyclic Voltammetry (CV)

Cyclic voltammetry (CV) is a potentiodynamic electrochemical technique. In a CV test, the working electrode's potential is scanned from initial position to final position with a constant rate, and then change the direction to return the initial potential. This cycle could be repeated several times. During the whole process, the current of working electrode is recorded and plotted versus potential, and output a CV curve to indicate electrochemical properties of a sample in solution.

A standard CV experiment requires three electrodes (**Fig. 3.5.3.1**): working electrode (WE), reference electrode (RE) and counter electrode (CE). Platinum is commonly used as working electrode, while counter electrode could be any material that is stable enough and has good conductivity. A voltage is applied between WE and RE, and at the same

Fig. 3.5.3.1 Illustration of CV structure

time, the current between WE and CE is recorded. Three electrodes are immersed into the solution and electrolyte is also added to provide sufficient conductivity.

Fig. 3.5.3.2 Example of CV result

Fig. 3.5.3.2 is a typical cyclic voltammogram, where E_{pc} and i_{pc} refer to the potential and current of oxidation peak, and E_{pa} and i_{pa} refer to the potential and current of reduction peak.

3.6 Biological Characterization

3.6.1 MTT Assay

The MTT assay is a colorimetric technique to assess cell viability and metabolic activity.

The number of viable cells could be reflected by a special enzyme that could also reduce the tetrazolium dye MTT to form a purple insoluble formazan (**Fig. 3.6.1.1**). Dissolving the formazan in DMSO and measuring the amount of formazan through UV-Vis could calculate the cell viability. Therefore this technique is usually employed to study cytotoxicity of a compound.

Fig. 3.6.1.1 Illustration of MTT reduction

3.6.2 Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) is an optical imaging technique that increases optical resolution and contrast. Conventional microscope only shows structure information as far as the light could penetrate into the sample, while CLSM could image same depth level at a time. CLSM scans a sample point by point using a focused laser beam, and forms three-dimensional reconstruction based on images obtained at different depths. This technique has gained popularity from scientific and industrial communities and has potential applications in life science and material science.

The mechanism is shown in **Fig. 3.6.2.1**. A laser beam passes through beam splitter and lens, focusing on focal plane. Then the sample is excited and as-produced fluorescence travels backwards through the same path that laser travels. Because the wavelength has been changed, the fluorescence could not pass through the beam splitter and be reflected toward the detection system. A pinhole is added before the detector to eliminate noise

signal. It allows only a small part of fluorescence be received by the detector. Therefore only the light of the targeted point that is under observation will be recorded, while other signals will be blocked. There is an emission filter before the detector to select particular color channel for detection. Though noise signal is eliminated, the fluorescence recorded by detector has very low intensity. Thus photomultiplier tube (PMT) is introduced to detect and amplify the light signal, followed by transferring the light signal into electrical signal and displaying on the screen. All these steps happen very quickly, and as the laser beam scans the sample, it seems like a real-time imaging of the sample.

Fig. 3.6.2.1 Illustration of CLSM structure

Fig. 3.6.2.2 is a typical confocal laser scanning result.⁴ These pictures are obtained through red and blue channel.

Fig. 3.6.2.2 Example of CLSM result (Reprinted with permission from ref. 4. Copyright 2015, American Chemical Society.)

A CLSM consists of several main parts: light source, beam splitter, objective lens, scanner, Z-control, pinhole, photomultiplier tube and detector.

Light source should provide laser with single wavelength. And the wavelength could be changed depends on the fluorophores of the sample.

Beam splitter could separate the emission light of the sample from the laser and reflect to the detector.

Objective lens is a key part of the microscope and mainly determines the resolution of the system.

Scanner guides the laser beam to scan the sample point by point, which is based on two or more mirrors.

Z-control allows operator to select different focal plane within the sample. The motorised Z-stepper allows movement along Z direction by small steps (>10 nm) with high precision.

Pinhole is an adjustable iris in the intermediate image plane. It blocks most of the out-of-focus light, only let small portion of light pass through. It also determines the thickness of the optical slice and is highly related to the properties of objective lens.

Photomultiplier tube (PMT) detects photons emitted by the sample and amplify the signal, followed by transferring into electrical signal.

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Chapter 4

Thiazole derivative-modified upconversion nanoparticles for Hg^{2+} detection in living cells*

Mercury ion (Hg^{2+}) is an extremely toxic ion, which will accumulate in human bodies and cause severe nervous system damage. Therefore, the sensitive and efficient monitoring of Hg^{2+} in human bodies is of great importance. Upconversion nanoparticles (UCNP) based nano-probes exhibit no autofluorescence, deep penetration depth and chemical stability in biological samples, as well as a large anti-Stokes shift. In this study, a thiazole-derivative-functionalized UCNPs was developed, and upconversion emission intensity ratio of 540 nm to 803 nm (I_{540}/I_{803}) was employed as a ratiometric signal to detect Hg^{2+} in living cells and showed excellent photostability and high selectivity. The nano-probe was characterized by transmission electron microscopy (TEM) and powder X-ray diffraction (Powder XRD). The low cytotoxicity of the probe was confirmed by an MTT assay and the UCL test in Hela cells was carried out by confocal microscopy. The results demonstrated that organic-dye-functionalized UCNPs should be a good strategy to detect toxic metal ions when studying cellular bio-systems.

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4.1 Introduction

Detection of the structural and functional properties in living systems is a perennial key challenge for a long time.¹⁻⁸ For instance, diagnosis and treatment of cancer necessitate highly contrasted real-time bio-imaging.⁹⁻¹¹ Conventional luminescent materials such as organic dyes and quantum dots¹² (QDs) have been explored in the past decades. However, these bio-labels have some limitations. Usually the emission lifetimes of organic dyes are short (less than 100 ns),¹³ which are difficult to differentiate from other short-lived autofluorescence from biological samples. The QD has high toxicity and short circulation half time,¹⁴ which will limit its bio-application. Therefore, lanthanide doped upconversion nanoparticles (UCNP) have been suggested as a promising bio-label.

Usually the energy of fluorescent photons are higher than that of excitation ones. However, lanthanide-doped UCNPs can convert near-infrared (NIR) excitation into visible emission of different colors,¹⁵⁻¹⁷ with a large anti-Stokes shift of hundreds nanometers.¹⁸⁻²⁰ UCNPs have many benefits such as chemical stability,^{21,22} non-autofluorescence from biological samples,²¹⁻²⁵ remarkable light penetration depth,^{23,26-28} long lifetime (millisecond time scale)^{19,29} and less damage to samples,³⁰⁻³⁴ thus it can replace conventional organic dyes or quantum dots, and has evoked considerable interest for biological applications.

As molecular or ionic probes, UCNPs should combine with other chromophores through luminescence resonance energy-transfer (LRET) process. In a LRET system, UCNPs (donor) can transfer the energy to chromophores (acceptor), which will result the change of upconversion luminescence (UCL). Nowadays, several LRET-based sensing and imaging systems have been reported. Li and co-workers developed a cyanine-modified UCNPs for the detection of methylmercury;³⁵ Liu and co-workers discovered manganese dioxide-loaded nanosheets for glutathione detection;³⁶ Chang and co-workers created dye-assembled UCNPs for sensing zinc

ion *in vitro* and *in vivo*.³⁷ Chen and co-workers used a sensitive time-resolved bio-probe to detect avidin through lanthanide-doped zirconia nanoparticles.³⁸ Liu and co-worker demonstrated a NaYF₄ sandwich structure for calcium ion detection³⁹ and silver nanoclusters modified UCNPs for biothiols detection.⁴⁰ This year Qu and co-workers designed a hyaluronic acid modified UCNPs for reactive oxygen species detection and bio-imaging.⁴¹ Zhang *et al* also reported the progress to detect hydrogen peroxide,⁴² hypochlorous acid⁴³ and hydrogen sulfide³⁰ in living cells. Continuing on the research in this direction, this thesis focused on employing this method to detect mercury ion in bio-system because mercury ion, a strong neurotoxin, accumulates in human bodies and cause severe nervous system damage.⁴⁴ In the present study an inorganic-organic hybrid probe was designed and fabricated for the detection of mercury ion. The as-prepared nano-probe has excellent biological applications based on the LRET system that is composed of nano-phosphors (NaYF₄: 20% Yb, 1.8% Er, 0.5% Tm) and a Hg²⁺-responsive thiazole derivative dye (Scheme 1). Hexagonal NaYF₄ was chosen because of its higher upconversion efficiency, compared with its cubic counterpart.⁴⁵ Using the ratio of UCL intensity at 540 nm to 803 nm (I_{540}/I_{803}) as the detection signal, this thesis also demonstrated that the intensity of 540 nm would recover gradually with the increased amount of Hg²⁺. Moreover, the as-prepared UCNPs could monitor Hg²⁺ in living cells.

4.2 Experimental Methods

Fig. 4.2.1 is a schematic illustration of synthesis route to get final probe 2-UCNP. The OA-capped UCNPs were prepared by a modified solvothermal method,⁴⁶⁻⁵² which are hydrophobic. In order to combine the UCNP and compound 2 into one nano-system in aqueous solution, α -cyclodextrin (α -CD) was employed to convert the hydrophobic UCNP into the hydrophilic form by self-assembly of host (α -CD) and guest molecules (OA).^{53,54} The surface of the as-converted nano-probes will be

much easier to be modified with compound 2, forming three-layer nanostructure 2-UCNP with good water solubility.

Fig. 4.2.1 Schematic illustration of the structure design of 2-UCNP (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

4.2.1 Synthesis of Target Molecule

Our target molecule compound 2, containing a thiazole electron withdrawing group and a diethylamine electron donating group, was synthesized by a two-step reaction with an overall yield of 6.3% (**Fig. 4.2.1.1**).

Fig. 4.2.1.1 Synthesis route of compound 2 (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

Compound 1: 2-methylbenzo[d]thiazole (1.511 g, 10.1 mmol), 2-bromoacetic acid (2.184 g, 15.7 mmol) and 50 ml toluene were added into a one-neck flask. After refluxing for 20 hours, the solution was cooled down and the resultant precipitate was filtered and washed four times with 2 ml methanol/methylene chloride (1:9, v:v) to give a light grey solid (0.358 g): Yield 12.2%; ^1H NMR (400 MHz, CDCl_3 , TMS): δ =3.18 (s, 3H, Me), 5.74 (s, 2H, NCH_2), 7.79-7.83 (m, 1H, Ph), 7.87-7.91 (m, 1H, Ph), 8.27 (d, 1H, Ph), 8.45 (d, 1H, Ph).

Fig. 4.2.1.2 ^1H -NMR spectrum of compounds 1 in DMSO (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

Compound 2: Compound 1 (0.337 g, 1.17 mmol) and 4-(diethylamino)-2-hydroxybenzaldehyde (0.231 g, 1.20 mmol) were dissolved in 40 ml ethanol, and 40 μl piperidine was added as catalyst. After refluxing for 24 hours, the mixture was cooled down and the resulting precipitate was filtered and washed with 2 ml methylene chloride/ethyl ether (1:9, v:v) mixture for four times to produce a dark green solid (280 mg): Yield 51.7%; ^1H NMR (400 MHz, CD_3OD , TMS): δ =1.23

We can't get ^{13}C -NMR of compound 2 because of its poor solubility in organic solvents.

OA-UCNP was synthesized by a modified procedure according to a previous report.⁴⁶⁻⁵² YCl₃·6H₂O (235.7 mg, 0.777 mmol), YbCl₃·6H₂O (77.5 mg, 0.20 mmol), ErCl₃·6H₂O (6.9 mg, 0.018 mmol) and TmCl₃·6H₂O (1.9 mg, 0.005 mmol)

Fig. 4.2.1.4 ESI-MS spectrum of compound 2 (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

were dissolved in 10 ml methanol by sonication. After removing the methanol, 7 ml oleic acid and 15 ml 1-octadecene were added. The mixture was heated up to 160°C for 30 minutes and a homogeneous solution was formed. After cooling down to room temperature, 10 ml methanol solution containing NaOH (100 mg, 2.5 mmol) and NH₄F (148 mg, 4 mmol) were added. In an Argon environment, the resulting colloidal mixture was slowly heated up to 140°C for 10 minutes to remove methanol, and then increased to 300°C, and maintained for 1.5 hours. After cooling down the solution naturally, the nanoparticles were obtained by adding ethanol, followed by centrifugation and washed with ethanol for three times.

Powder X-ray diffraction (Powder XRD) peaks of UCNP correlated very well with

Fig. 4.2.2.1 Powder XRD of UCNPs and standard pattern of β -NaYF₄ (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

the hexagonal structure of NaYF₄ (**Fig. 4.2.2.1**), and all the diffraction peaks in UCNPs could be indexed to the standard pattern of β -NaYF₄, indicating the high purity of UCNPs.

4.2.3 Synthesis of α -CD Functionalized UCNPs

CD-UCNPs were synthesized by a modified procedure.⁵⁴ 12 ml ethanol/water (2:1, v:v) solution that contains 60 mg OA-UCNPs was mixed with 12 ml α -Cyclodextrin (α -CD) aqueous solution (20 mg ml⁻¹). After vigorously stirring the mixture under room temperature, a transparent solution resulted. After stirring for 20 hours at room temperature, the solution was centrifuged (10000 rpm, 20 minutes) and the resulting particles were washed with deionized water for three times.

4.2.4 Synthesis of 2-UCNP

20 mg compound 2 was dissolved in 4 ml ethanol, and mixed with 10 mg α -CD functionalized UCNPs. After heating at 70°C for 24 hours, the mixture was cooled down and the solution was centrifuged (9000 rpm, 10 minutes). The as-obtained particles were washed with ethanol for three times and dried in a fume hood.

Fig. 4.2.4.1 FTIR spectrum of OA-UCNP, CD-UCNP, compound 2 and 2-UCNP (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

The surface modification was confirmed by Fourier-transform infrared (FTIR) spectroscopy (**Fig. 4.2.4.1**). For OA-UCNP, the band at 3433 cm⁻¹ is attributed to the stretching vibration of the OH group, while two peaks at 2925 and 2854 cm⁻¹ belong to both symmetric and asymmetric C–H stretching vibrations, indicating the presence of OA on UCNPs surface. For α -CD functionalized UCNPs, the band at 1077 cm⁻¹ is attributed to C–O–C stretching vibration, confirming that α -CD has

attached on UCNP. Compared with OA-UCNP, 2-UCNP showed three new peaks at 1427, 1568, 1624 cm^{-1} and one new peak at 756 cm^{-1} , which could be assigned to the C=C stretching vibration and C-H bending vibration of benzene of compound 2, respectively. These facts indicated that compound 2 was successfully assembled on the surface of UCNP.

Fig. 4.2.4.2 TEM (A) and HR-TEM (B) of OA-UCNP, TEM (C) and HR-TEM (D) of 2-UCNP (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

Transmission electron microscopy (TEM) images (**Fig. 4.2.4.2**) show that there were no significant changes in size, shape and crystallinity after modification with compound 2. These images also indicated that both OA-UCNP and 2-UCNP had an average diameter of about 25 nm, and no evident aggregation was observed.

4.3 Principle Outcomes

4.3.1 Design Strategy and Synthesis Route

Our design strategy was based on the fact that UCNP could transfer energy to the chromophore by LRET process (**Fig. 4.3.1.1**). To achieve this goal, the emission band of UCNP should overlap with the absorption band of the chromophore. The target compound **2** has a maximum absorption peak at 546 nm, which perfectly matches the UCL emission of $^2H_{11/2} \rightarrow ^4I_{15/2}$ and $^4S_{3/2} \rightarrow ^4I_{15/2}$ transitions of Er^{3+} (**Fig. 4.3.1.2**). As previously reported,⁵⁵ the addition of Hg^{2+} to the solution of as-prepared nano-probes will cause a blue-shift of maximum absorption peak to 465 nm, forming **2'** and leading to the recovery of these UCL emission bands. Thus it is reasonable to suggest that the degree of energy transfer can be modulated by the concentration of Hg^{2+} .

Fig. 4.3.1.1 Illustration of LRET process (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

4.3.2 Sensing Properties of compound **2**

In the present study, the sensing ability of compound **2** for Hg^{2+} was investigated

Fig. 4.3.1.2 Absorption of compound 2, 2' and emission of UCNPs (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

in DMSO/HEPES (1:9, v:v). The absorption spectra of compound 2 with and without Hg^{2+} are shown in **Fig. 4.3.2.1**. In the absence of Hg^{2+} , compound 2 had a maximum absorption peak at 546 nm (**Fig. 4.3.2.2**, $\epsilon=1.51 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$). With the increase of Hg^{2+} concentration, the peak at 546 nm gradually decreased, and the absorption peak at 465 nm increased with an isosbestic point at 481 nm, leading to an evident color change from red to green. This color change is attributed to the formation of a relatively stable metal complex.⁵⁵⁻⁵⁷ Both the sulfur atom in benzothiazolium moiety and the oxygen atom in the phenolic moiety may combine with Hg^{2+} , forcing the thiazole derivative to adopt a cis configuration, thus breaking the trans-cis dynamic equilibrium.

4.3.3 Sensing Properties of 2-UCNP

After the dye-loading process, the sensing property of 2-UCNP was also investigated by both UV-Vis absorption spectrum and UCL spectrum.

Fig. 4.3.2.1 Absorption spectrum of compound 2 with the addition of Hg^{2+} from 0 to 1 eq in DMSO/HEPES (1:9, v:v). The inset is the color change from red to green and absorption ratio of 546 nm to 465 nm decreases with the addition of Hg^{2+} . (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

Fig. 4.3.2.2 Molar Absorptivity of Compound 2 in DMSO/HEPES (1:9, v:v) (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

Fig. 4.3.3.1 Absorption and emission of 2-UCNP with Hg^{2+} (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

(A) Absorption spectrum of 0.005 mg ml^{-1} 2-UCNP in DMSO/HEPES (0.5:99.5, v:v) with different Hg^{2+} concentration from 0-8.1 μM . Inset: the photo shows the color change from red to green. (B) UCL spectrum of 1 mg ml^{-1} 2-UCNP in DMSO with different Hg^{2+} concentration from 0.8 to 5.0 mM. Inset: the photo shows the emission change from red to green. (C) The ratio of absorption at 540 nm to 463 nm decreased when the Hg^{2+} concentration increased. (D) The ratio of UCL emission at 540 nm to 654 nm increased when the Hg^{2+} concentration increased. (E) The ratio of UCL emission at 540 nm to 803 nm increased when the Hg^{2+} concentration increased.

For absorption spectrum, 2-UCNP showed a broad band with the maximum absorption at 540 nm. After the addition of Hg^{2+} , the absorption peaks had a blue-shift, changing from 540 nm to 463 nm, corresponding to the color change from red to green (**Fig. 4.3.3.1**). This blue-shift (77 nm) was in agreement with the change of the pure compound 2, which indicated that a reaction between Hg^{2+} and

the compound 2 existed at the 2-UCNP surface. In addition, using A_{540}/A_{463} as detection signal, the limit of detection was measured to be 0.063 μM (**Fig. 4.3.3.2**).

Fig. 4.3.3.2 The analysis of detection limit of Hg^{2+} through absorption data (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

As shown in **Fig. 4.3.1.2**, under excitation of 980 nm, the OA-UCNP showed four UCL emission bands at 514–534 nm, 534–560 nm, 635–680 nm and 775–825 nm, attributed to $^2\text{H}_{11/2} \rightarrow ^4\text{I}_{15/2}$, $^4\text{S}_{3/2} \rightarrow ^4\text{I}_{15/2}$ and $^4\text{F}_{9/2} \rightarrow ^4\text{I}_{15/2}$ transitions of Er^{3+} , and $^3\text{H}_4 \rightarrow ^3\text{H}_6$ of Tm^{3+} , respectively. The dye-loaded UCNPs only showed 635–680 nm and 775–825 nm emission bands, and another two bands were almost quenched through LRET process because they had overlapped with the absorption band of compound 2.

After the addition of Hg^{2+} , the spectral overlap between the green emission band (514–560 nm) of UCNPs and the absorption band (540 nm) of compound 2 was reduced, causing a decrease of LRET process, thus the emission at 514–560 nm was recovered gradually (**Fig. 4.3.3.1**). Note that another two emission bands at 635–680 nm and 775–825 nm were not involved in the LRET process, and their intensity would not be affected before and after the addition of Hg^{2+} , which means these two bands could be used as a reference standard. Herein the ratio of UCL

intensity at 540 nm and 803 nm (I_{540}/I_{803}) was employed as the detection signal, and the limit of detection was measured to be 0.21 μM (**Fig. 4.3.3.3**). I_{540}/I_{654} was also chosen to improve the signal stability. As shown in **Fig. 4.3.3.1**, the values of both I_{540}/I_{803} and I_{540}/I_{654} increased with the addition of Hg^{2+} .

Fig. 4.3.3.3 The analysis of detection limit of Hg^{2+} through UCL data (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

Compared with 2-UCNP, simply physical mixing of OA-UCNP with compound 2 did not show obvious quench of 540 nm band. For I_{540}/I_{654} and I_{540}/I_{803} values, physical mixing only led to a 4% and 8% decrease, respectively, indicating that the quench effect of 2-UCNP was mainly ascribed to LRET process. The UCL intensity change was tested after the physical mixing and found that the 540 nm band intensity decreased very fast (**Fig. 4.3.3.4**). In 6 minutes at room temperature, the intensity will decrease about 45% (**Fig. 4.3.3.5**). These results indicated that compound 2 was easily combined with UCNP.

Fig. 4.3.3.4 UCL intensity change after physical mixing of OA-UCNP with compound 2 (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

The 540 nm band decreased about 45% in 6 minutes after the physical mixing at room temperature

Fig. 4.3.3.5 UCL intensity ratio of 540 nm to 654 nm and 540 nm to 803 nm (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

For an excellent ion probe, high selectivity plays a key role. To validate the selectivity of 2-UCNP, some other metal ions such as alkali (K^+ , Na^+), alkali earth (Ca^{2+} , Mg^{2+}) and some transition-metal ions (Mn^{2+}) were tested under the same condition for both absorption and emission (**Fig. 4.3.3.6**). For absorption, the absorbance ratio of 463 nm to 540 nm was calculated. Because the addition of Hg^{2+} would cause significant decrease at 540 nm and increase at 463 nm, the ratio of 463 nm to 540 nm would remarkably increase. Other metal ions do not show this effect, which led to small value of absorption ratio. For UCL spectrum, the intensity ratio of 540 nm to 803 nm was calculated. Hg^{2+} would recover the 540 nm band, increasing the ratio value, while other ions do not. Compared with absorption spectrum, the UCL intensity at 803 nm will not change, which means that the distinction between Hg^{2+} and other ions seems not as obvious as absorption spectrum. In addition, to study if the existence of other ions would interfere with the detection of Hg^{2+} , the UCL intensity of 2-UCNP containing both Hg^{2+} and other ions was tested. With or without other ions, the UCL intensity ratios only changed a little, indicating that the co-existence of other ions would not interfere with the detection of Hg^{2+} .

Furthermore, the photostability of 2-UCNP was also investigated under exposure of 980 nm and 365 nm light. For 980 nm exposure, after 3 hours, the absorbance of 2-UCNP began to show slight decrease. After 9 hours, the absorbance decreased 2%. For 365 nm exposure, the absorbance decreased obvious after 1 hour and showed 27% decrease after 9 hours illumination (**Fig. 4.3.3.7**). Therefore, the nano-system is more stable by using UCL emission as detection signal, and this significant improvement shows potential in practical applications.

4.3.4 Monitoring the Presence of Hg^{2+} in the Living Cell

Before the application of 2-UCNP in bio-imaging, the cytotoxicity was investigated using the methyl thiazolyl tetrazolium (MTT) assay (**Fig. 4.3.4.1**).

Fig. 4.3.3.6 UCL and absorption intensity ratio of 2-UCNP with different metal ions (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

Fig. 4.3.3.7 Photostability of 2-UCNP (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

Following the incubation of 2-UCNP for 24h, the HeLa cells exhibited only minimal cytotoxicity. The cellular viability of HeLa cells was still higher than 80% even at a high concentration of $800\ \mu\text{g ml}^{-1}$, indicating that the 2-UCNP is a bio-compatible nano-probe, and is suitable for UCL bio-imaging applications.

Fig. 4.3.4.1 Cell viability was quantified by the MTT assay (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

To demonstrate the applicability of 2-UCNP in monitoring intracellular Hg^{2+} , a laser-scanning upconversion luminescence microscopy (LSUCLM) test was conducted (**Fig. 4.3.4.2**). Both the control (A to C) and test rows (D to F) were incubated with $0.5\ \text{mg ml}^{-1}$ 2-UCNP for 180 minutes, and the test row was followed by incubated with $200\ \mu\text{M}$ Hg^{2+} . Under 980 nm excitation, the control row cells only showed a weak UCL emission at 540 nm, indicating the LRET process still happened in living cells. After the addition of Hg^{2+} , an enhancement of green emission was observed, which meant that the LRET process was blocked. Moreover, the intensity of red emission almost did not change. As a result, the ratio of green to red emission showed an enhancement. These results suggested that 2-UCNP could be used for monitoring intracellular Hg^{2+} through the ratiometric UCL method.

Fig. 4.3.4.2 Ratiometric UCL images in HeLa cells (top, A to C) and 200 μM Hg^{2+} treated HeLa cells (bottom, D to F) incubated with 0.5 mg ml^{-1} 2-UCNP in 180 minutes. Emission was collected by both the green channel at 500-560 nm (A and D) and red channel at 600-700 nm (B and E). (C and F) Ratiometric UCL images with ratio of green to red channels. (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

4.4 Conclusion

In summary, the surface of UCNPs was successfully modified with compound 2 and demonstrated a new nano-system for UCL detection and bio-imaging of Hg^{2+} in living cells. The sensing mechanism was based on blocking the LRET process and recovering the green emission band after the addition of Hg^{2+} . The emission intensity ratio of 540 nm to 803 nm (I_{540}/I_{803}) was also employed as signal to confirm that the green emission band increased with the addition of Hg^{2+} . More importantly, this as-fabricated nano-system was capable of monitoring Hg^{2+} in living cells with low cytotoxicity. This method could provide a promising strategy for further detection and bio-imaging probes.

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Chapter 5

Organic Dye-Modified Upconversion Nanoparticle as A Multi-Channel Probe to Detect Cu^{2+} in Living Cells*

Developing an inorganic-organic hybrid probe with multi-channel responses to more accurately detect ions in living systems is very challenging but highly desirable. In this research, for the first time, electrically-active ferrocene group was attached onto the framework of optically-active Rhodamine to form a new dye (RB-FC), which can be further loaded onto the surface of silica-coated upconversion nanoparticles (UCNPs) to construct a novel nano probe (RB-FC-UCNPs) to detect copper ion through both electrochemical method and optical ways. Upon the interaction with Cu^{2+} , this as-prepared nano-probe showed obvious changes in absorption, emission and electrochemical parameters. The detection employed absorption intensity at 558 nm (A_{558}), emission intensity ratio of 540 nm to 654 nm (I_{540}/I_{654}) as spectral signals, and oxidation peak of cyclic voltammetry curve as electrochemical signal, demonstrating high stability and low detection limit of this novel probe. In addition, bio-imaging test revealed that this probe could be applied in detecting and visualizing Cu^{2+} in A549 cells with low cytotoxicity.

5.1 Introduction

Copper ion is one of main trace metal cations in human body, which plays a key role in metabolism. Its disequilibrium in human body would cause several severe diseases such as Menkes disease¹ and Wilson's disease.² Therefore, sensitively and efficiently monitoring copper ions in both environment and human body is of great importance.

Recently, lanthanide doped upconversion nanoparticles (UCNPs) have been demonstrated to be a promising probe because UCNPs can successively absorb two or more low energy photons via intermediate long-lived energy states, and emit a high energy photon, which is known as the conversion of near-infrared (NIR) excitation into visible emission,³⁻⁵ with a large anti-Stokes shift on spectrum.⁶⁻⁸ The reported research results have already proved that UCNPs possess many advantages including no auto-fluorescence from bio-tissue,⁹⁻¹³ deep penetration depth,^{11,14,15} chemical stability,^{9,10} long lifetime (milliseconds)^{7,16} and less harmful to bio-samples.¹⁷⁻²¹ Continuing to work on this research direction, scientists have successfully developed a novel detection strategy, namely, loading chromophore on the UCNPs to detect ions or bio-signals through luminescence resonance energy transfer (LRET) process. In LRET systems, UCNPs can absorb long-wavelength photons and emit short-wavelength photons (visible range), which can be further absorbed by specific chromophores. In such cases, the emission from UCNPs would become invisible. If targeted ions or bio-signals can interact with chromophores to cause the change of their absorption, the emission from UCNPs can be turned on or off, which can be used to detect ions or bio-signals. In fact, several impressed probes based on LRET process have been reported recently. Li *et al* reported cyanine-modified UCNPs for methylmercury detection,²² Chang *et al* discovered dye-assembled UCNPs for zinc ion detection in vitro and in vivo,²³ Liu *et al* created manganese-dioxide-loaded nano-sheets for intracellular glutathione detection,²⁴ Chen *et al* demonstrated a time-resolved bio-probe to detect avidin,²⁵ Liu *et al* used NaYF₄-based sandwich structure for the detection of calcium ion²⁶ and silver nano-cluster based UCNPs for bio-thiol detection,²⁷ Qu *et al* designed hyaluronic-acid-based UCNPs for the

detection and imaging of reactive oxygen species (ROS).²⁸ Zhang et al also published some progress based on similar strategy, including cyanine modified UCNPs for sensing endogenous hydrogen peroxide signaling in vivo,²⁹ Rhodamine-modified UCNPs for the detection of hypochlorous acid in living cells,³⁰ organic hybrid UCNPs for the detection and bio-imaging of hydrogen sulfide in mouse model,¹⁷ and thiazole-derivative-modified UCNPs for the recognition of mercury ion.³¹

Though these probes are effective, they are all single-channel-based and easy to be affected by environment. In order to address this issue, a new type of probes was developed with multi-channel responses because these probes provide higher sensitivity, better selectivity and self-calibration ability. Here, a new multi-channel copper ion probe was demonstrated by integrating the signals from absorption, emission and electrochemistry into one system. This as-prepared probe shows the improved stability and adaptability. To the best of knowledge, this is the first time to develop a multi-channel Cu^{2+} probe based on UCNPs. This as-designed probe consists of a lanthanide-doped UCNP (NaYF_4 : 20%Yb, 1.8%Er, 0.5%Tm) core with silica as a hydrophilic shell, followed by loading copper-ion-responsive chromophores to form the final probe RB-FC-UCNPs.

5.2 Experimental Methods

Fig. 5.2.1 is a schematic illustration of synthesis route to get final probe RB-FC-UCNP. The OA-capped UCNPs were prepared by a modified solvothermal method,³²⁻³⁸ which are hydrophobic. In order to disperse the UCNP and RB-FC together into aqueous solution, a silica layer was coated onto the UCNP according to a reported literature.³⁵

5.2.1 Synthesis of Target Molecule

Our target molecule RB-FC is based on Rhodamine-derivative and contains a ferrocene group. The synthesis route and procedure is according to a reported method.³⁹ Two

intermediates Rhodamine B Hydrazide and 5-Ethynylferrocene-Salicylaldehyde were synthesized first, then combined them through C=N bond. Some characterization results of these two compounds are listed (Fig. 5.2.1.1 to Fig. 5.2.1.6).

Fig. 5.2.1 Schematic illustration of the synthesis route of RB-FC-UCNP

5.2.2 Synthesis of OA-UCNP

OA-UCNP was synthesized by a modified procedure according to a previous report.³²⁻³⁸ YCl₃·6H₂O (235.7 mg, 0.777 mmol), YbCl₃·6H₂O (77.5 mg, 0.20 mmol), ErCl₃·6H₂O (6.9 mg, 0.018 mmol) and TmCl₃·6H₂O (1.9 mg, 0.005 mmol) were dissolved in 10 ml methanol by sonication. After removing the methanol, 7 ml oleic acid and 15 ml 1-octadecene were added. The mixture was heated up to 160°C for 30 minutes and a homogeneous solution was formed. After cooling down to room temperature, 10 ml methanol solution containing NaOH (100 mg, 2.5 mmol) and NH₄F (148 mg, 4 mmol) were added. In an Argon environment, the resulting colloidal mixture was slowly heated

Fig. 5.2.1.1 ^1H -NMR spectrum of RB-FC Fig. 5.2.1.2 ^{13}C -NMR spectrum of RB-FC

RB FC#1 RT: 0.01 AV: 1 NL: 6.34E5
T: + p Full ms [150.00-900.00]

Fig. 5.2.1.3 Mass spectrum of RB-FC

Fig. 5.2.1.4 ¹H-NMR spectrum of Rhodamine B Hydrazide

Fig. 5.2.1.5 ¹H-NMR spectrum of 5-Ethynylferrocene-Salicylaldehyde

Fig. 5.2.1.6 ¹³C-NMR spectrum of 5-Ethynylferrocene-Salicylaldehyde

up to 140°C for 10 minutes to remove methanol, and then increased to 300°C, and maintained for 1.5 hours. After cooling down the solution naturally, the nanoparticles were obtained by adding ethanol, followed by centrifugation and washed with ethanol for three times.

Fig. 5.2.2.1 Powder XRD of UCNPs and standard pattern of β -NaYF₄

Powder X-ray diffraction (Powder XRD) peaks of UCNPs correlated very well with the hexagonal structure of NaYF₄ (**Fig. 5.2.2.1**), and all the diffraction peaks in UCNPs could be indexed to the standard pattern of β -NaYF₄, indicating the high purity of UCNPs.

5.2.3 Synthesis of RB-FC-UCNPs

RB-FC-UCNPs were synthesized by a modified procedure according to a previous report.³⁵ Igepal CO-520 (0.2 ml) was dispersed into cyclohexane (8.0 ml). Then OA-UCNPs were dispersed into cyclohexane (4.0 ml, 10.0 mg ml⁻¹), and this solution was added into the Igepal CO-520 solution. After stirring the mixture vigorously until the solution becomes transparent, ammonium hydroxide (29.4% (wt/wt), 80 μ l) was added

into the mixture, and stirred the mixture vigorously again to become transparent. Followed by adding TEOS (40 μ l) to the mixture, and stirred the mixture gently for 48 h at room temperature. Acetone (20 ml) was added into the resulting nanocomposites, and then collected the precipitates by centrifugation (7500 rpm, 15 min, 25 °C). Silica coated UCNPs were obtained after washing the collected nanocomposites with ethanol three times. Silica coated UCNPs and RB-FC were dissolved in DMSO, stirred and heated under 70 °C for 24 h. The final solution was RB-FC-UCNPs.

Transmission electron microscopy (TEM) images (**Fig. 5.2.3.1**) show that there were no significant changes in shape and crystallinity after modification with silica layer. Both OA-UCNP and silica-coated-UCNP had an average diameter within 15-20 nm, and no evident aggregation was observed.

5.3 Principle Outcomes

5.3.1 Design Strategy and Synthesis Route

Our target chromophore RB-FC has a weak absorbance in solvent, however, after the

Fig. 5.2.3.1 TEM (A) and HR-TEM (B) of OA-UCNP, TEM (C) and HR-TEM (D) of silica-coated-UCNP

addition of Cu^{2+} , the as-formed complex has a maximum absorption peak at 558 nm, which perfectly matches the upconversion luminescence (UCL) emission of $^2\text{H}_{11/2} \rightarrow ^4\text{I}_{15/2}$, $^4\text{S}_{3/2} \rightarrow ^4\text{I}_{15/2}$ transitions of Er^{3+} (**Fig. 5.3.1.1**). The emission bands of UCNP overlap with the absorption band of the chromophore, indicating that UCNP could transfer energy to RB-FC, and suggesting that RB-FC-UCNP is suitable for triggering LRET process (**Fig. 5.3.1.1**). A ferrocene group was also imported into RB-FC because electrochemical signal produced by $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ redox couple could be combined with optical signal.

In this study, the detection employed the absorption peak at 558 nm (A_{558}) as one detection channel (**Fig. 5.3.1.2**), the UCL intensity ratio of 540 nm to 654 nm (I_{540}/I_{654}) as second one, and electrically-active ferrocene as third channel.

Fig. 5.3.1.1 Spectrum overlap and LRET process

(A) Emission of UCNP and absorption of RB-FC + Cu^{2+} overlapped at 540 nm band. (B) Structure of RB-FC-UCNP and Illustration of LRET process from UCNPs to RB-FC.

5.3.2 Sensing Properties of RB-FC-UCNPs

RB-FC-UCNPs did not show strong absorption peak, while with the presence of Cu^{2+} , a maximum absorption peak at 558 nm was observed (**Fig. 5.3.2.1**), corresponding to a

color change to red, which was in agreement with pure RB-FC. Using A_{558} as a detection signal, the limit of detection was measured to be $0.11 \mu\text{M}$ (**Fig. 5.3.2.1**).

Fig. 5.3.1.2 Illustration of multi-channel probe for Cu^{2+} detection

Fig. 5.3.2.1 Absorption and emission of RB-FC-UCNPs with Cu^{2+}

(A) Absorption spectrum of 0.12 mg ml⁻¹ RB-FC-UCNPs in DMSO with increasing Cu²⁺ concentration from 4-20 μM. (B) UCL spectrum of 1.2 mg ml⁻¹ RB-FC-UCNPs in DMSO with increasing Cu²⁺ concentration from 0.0-0.4 mM. (C) The absorption at 558 nm increased with the addition of Cu²⁺. (D) The ratio of UCL intensity using 654 nm as reference standard decreased with the addition of Cu²⁺.

For UCL test, under 980 nm excitation, RB-FC-UCNPs show four emission bands at 514–534 nm, 534–560 nm, 635–680 nm and 775–825 nm, attributed to ²H_{11/2}→⁴I_{15/2}, ⁴S_{3/2}→⁴I_{15/2} and ⁴F_{9/2}→⁴I_{15/2} transitions of Er³⁺, and ³H₄→³H₆ of Tm³⁺, respectively. After the addition of Cu²⁺, the RB-FC-Cu²⁺ complex has strong absorption at 558 nm and the LRET process will be turn on. Two emission bands were gradually quenched, and could only find the other two: 635–680 nm and 775–825 nm (**Fig. 5.3.2.1**). Therefore, it is reasonable to suggest that the degree of quenching could be used to calculate the concentration of Cu²⁺. It should be noticed that when the emission intensity gradually decreased, the band at 534–560 nm decreased much faster than the band at 514–534 nm (**Fig. 5.3.2.1**). This was because the absorption spectrum of RB-FC-Cu²⁺ complex overlapped more with the band at 534–560 nm than with the band at 514–534 nm, and the emitted photons within the band at 534–560 nm were much easier to be absorbed by RB-FC-Cu²⁺ complex. This result would further confirm that the quench was caused by LRET process. Since the other two emission bands (635–680 nm and 775–825 nm) were not involved in the LRET process and their intensity should not be affected by the addition of Cu²⁺, these two bands could be employed as internal reference standards, and the density of the detected channel can be compared with these references, which could make the result more accurate. Here UCL intensity ratio of 540 nm to 654 nm (I₅₄₀/I₆₅₄) was employed as detection signal, and the limit of detection was measured to be 5.95 μM (**Fig. 5.3.2.2**).

As an ion probe, high selectivity plays a key role. To verify the selectivity of RB-FC-UCNPs, some other metal ions were tested such as alkali metal (K⁺, Na⁺), alkali earth metal (Ca²⁺, Mg²⁺) and some transition metal (Ag⁺, Mn²⁺). The intensity ratio of 540 nm

Fig. 5.3.2.2 Linear relationship between UCL intensity ratio and Cu^{2+} concentration

to 654 nm was calculated. With the presence of Cu^{2+} , the intensity ratio was much lower than other metal ions (**Fig. 5.3.2.3**), suggesting excellent selectivity.

Fig. 5.3.2.3 Selectivity test of RB-FC-UCNPs to Cu^{2+} over other metal ions

Probes containing ferrocene groups will show remarkable potential shift of $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ redox couple before and after the interaction with analyte. Here electrochemical test was conducted based on ferrocene group (**Fig. 5.3.2.4**). Without Cu^{2+} , the oxidation peak of RB-FC-UCNPs is 0.548V. When gradually increasing the concentration of Cu^{2+} to 140 μM , both oxidation peak and reduction peak decreased due to the formation of RB-FC- Cu^{2+} complex, and the oxidation peak decreased to 0.377V. The oxidation peak had a linear relationship with the concentration of Cu^{2+} , and the limit of detection was measured to be 10.0 μM .

Fig. 5.3.2.4 Cyclic voltammogram of RB-FC-UCNPs and Cu^{2+} and their linear relationship

(A) Cyclic voltammogram of RB-FC-UCNPs before (upper) and after (bottom) the addition of Cu^{2+} . (B) Oxidation peak values decreased when adding Cu^{2+} .

5.3.3 Monitoring the Cu^{2+} in the Living Cell

Before conducting the bio-imaging test in living cells, the cytotoxicity has been first investigated using the methyl thiazolyl tetrazolium (MTT) assay (**Fig. 5.3.3.1**). Following the incubation of RB-FC-UCNPs for 24h, the probe exhibited small cytotoxicity to A549 cells. The cellular viability of A549 cells was still higher than 80% even at a high concentration of $500 \mu\text{g ml}^{-1}$, proving that the RB-FC-UCNPs is a biocompatible probe suitable for bio-imaging applications.

Fig. 5.3.3.1 Cell viability was quantified by MTT assay (A549 cells, 24h)

To monitor intracellular Cu^{2+} through RB-FC-UCNPs, a laser-scanning upconversion luminescence microscopy (LSUCLM) test was conducted (**Fig. 5.3.3.2**). Under 980 nm laser, ratiometric (G/R) of control group was 10.82 ± 0.45 , and LRET process did not happen yet. After the addition of Cu^{2+} , the ratiometric (G/R) of test group decreased to 5.96 ± 0.37 , indicating that LRET process was triggered, and green emission band had

been largely quenched. This result suggested that the probe could be applied to monitor Cu^{2+} in living cells.

Fig. 5.3.3.2 Ratiometric UCL images in A549 cells

0.5 mg ml^{-1} RB-FC-UCNPs were added to control group (A to C) and test group (D to F). After 45 minutes, test group were treated with $200 \text{ } \mu\text{M Cu}^{2+}$. Emission was collected by both the green channel at 500-560 nm (A and D) and red channel at 600-700 nm (B and E). (C and F) Ratiometric UCL images with ratio of green to red channels.

5.4 Conclusion

In summary, a multi-channel UCNP-based nanoprobe that contains Rhodamine and ferrocene derivative for Cu^{2+} detection was first designed and synthesized. Through absorption signal, emission signal, and electrochemical signal, this multi-channel detection system is the first time to be reported to detect Cu^{2+} with low limit of detection and high selectivity. In addition, this strategy has been applied in bio-samples and Cu^{2+} was monitored in A549 cells with low cytotoxicity. This multi-channel strategy should be

helpful to illuminate the important roles of Cu^{2+} in human health and environment, as well as to provide a promising future for further detection and bio-imaging of other ions and molecules.

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Chapter 6

Summaries/Hypothesis Discussion/Future Work

First, the contents of this thesis are summarized. A new probe based on UCNP is designed and synthesized to detect and monitor Hg^{2+} in living cells. Its spectral properties have been studied in depth and this probe demonstrates high sensitivity and selectivity with low limit of detection and low cytotoxicity. This strategy paves the way for other analytes detection and bio-imaging. To further explore this strategy, a new probe for Cu^{2+} has been developed. This new probe contains a UCNP core and is coated with silica layer to improve water solubility, followed by the combination with chromophore that is responsive to Cu^{2+} . The chromophore contains a ferrocene group that could produce electrochemical signals. This probe employs multi-channel detection based on absorption, emission and cyclic voltammogram signals, improves stability and adaptability. Second, whether the hypothesis has been proved is discussed. All these results demonstrate that the probe based on UCNP could be applied in detection and bio-imaging with high sensitivity and low limit of detection, and also could be combined with other detection signal to improve stability. Finally, the ideas for future work are proposed. For example, dual-ions detection and detection combine with more channels should be promising directions and more effort could be focused on these fields.

6.1 Summary

This thesis mainly focused on nano-probes for metal ions based on LRET process. The research includes two parts.

First, a thiazole-derivative-functionalized UCNPs probe was designed and synthesized to detect Hg^{2+} in living cells. The probe contains a UCNP core and is covered with α -cyclodextrin (α -CD) layer to be converted into hydrophilic form, followed by loading chromophore onto the surface (**Fig. 6.1.1**). The structure was confirmed by TEM, powder XRD and FTIR and low cytotoxicity was confirmed by MTT assay. The UCL intensity ratio of 540 nm to 803 nm (I_{540}/I_{803}) was employed as one signal, absorption intensity ratio of 540 nm to 463 nm (A_{540}/A_{463}) as another signal to measure the limit of detection to be 0.21 μM and 0.063 μM respectively. In addition, UCL test in Hela cells that was carried out by confocal microscopy demonstrated that this probe was suitable for monitoring intracellular Hg^{2+} .

Fig. 6.1.1 Illustration of detection mechanism (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

Second, to improve this strategy, a multi-channel probe for Cu^{2+} detection in living cells was designed and obtained. This probe also contains a UCNP core and is coated with silica layer to increase water solubility, followed by loading chromophore (**Fig. 6.1.2**). The chromophore contains a ferrocene group that could produce electrochemical signals. The structure was confirmed by TEM and powder XRD and low cytotoxicity was confirmed by MTT assay.

Fig. 6.1.2 Illustration of structure design of the probe

For this probe, the detection employed absorption intensity at 558 nm (A_{558}), emission intensity ratio of 540 nm to 654 nm (I_{540}/I_{654}) as spectral signals, and oxidation peak of cyclic voltammetry curve as electrochemical signal to form multi-channel detection and the limit of detection was measured to be 0.11 μM , 5.95 μM and 10.0 μM . Confocal microscopy test in A549 cells showed that this new probe could be applied in detection of Cu^{2+} in bio-samples.

6.2 Hypothesis Discussion

Compared with conventional probes, UCNP probe has many advantages such as high chemical stability, non-autofluorescence from biological samples, large light penetration depth, long lifetime and less damage to samples. The key point in the detection is LRET

process (**Fig. 6.2.1**). To trigger the LRET process in detection, a chromophore should meet several requirements.

- (1) The chromophore is responsive to an analyte;
- (2) Before and after the reaction with the analyte, the absorption spectra of the chromophore should be different;
- (3) The absorption band of the chromophore overlaps with the emission band of UCNPs (**Fig. 6.2.1**).
- (4) The chromophore should be close enough to UCNPs

Fig. 6.2.1 LRET process and spectrum overlap (Reprinted with permission. Copyright 2016, Royal Society of Chemistry.)

The purpose of this study was to develop a probe to detect Hg^{2+} in bio-samples because of its extreme toxicity. Considering that Hg^{2+} has strong affinity to sulfur atom, a thiazole-derivative was synthesized as a chromophore to form a new UCNP probe using this strategy. It should be noticed that this strategy is effective not only for Hg^{2+} or metal ions, but also for other ions and small molecules. Therefore the importance of this research is more than developing a Hg^{2+} probe, other analytes could also be detected and monitored in bio-samples using this strategy.

Several probes based on UCNPs have been reported. Though they are effective, all of them are single-channel-based and easy to be affected by environment. In this part a new Cu^{2+} probe with multi-channel responses was designed and synthesized (**Fig. 6.2.2**).

Compared with the above Hg^{2+} probe, this Cu^{2+} probe employed electrochemical method. In addition to the advantages of the above-mentioned Hg^{2+} probe, there are several highlights for this research.

- (1) To the best of the knowledge, this is the first time to develop a multi-channel Cu^{2+} probe based on UCNP.
- (2) The limit of detection measured by both electrochemical method and optical ways were similar to each other, which increased stability of this probe.
- (3) This probe has high adaptability and is more suitable in different conditions.

Fig. 6.2.2 Illustration of multi-channel detection of Cu^{2+}

This multi-channel probe should be helpful to illuminate the important roles of Cu^{2+} in human health and environment, and one more important thing is to introduce other technique into the detection with high stability and adaptability, which provides a promising future for further bio-detection and bio-imaging of other ions and molecules.

6.3 Future Work

6.3.1 Dual-Responsive Probe

The above studies focused on single-analyte detection. Researchers need to design and synthesize different probes for each analyte, which is not efficient. Therefore in the future work part, this thesis will focus on developing more complex dual-responsive probes.

First part is to detect Cys and Hcy at the same time.

Fig. 6.3.1.1 Synthesis route of target molecule of Cys and Hcy probe

Biothiols such as Cysteine (Cys) and Homocysteine (Hcy) play important roles in various physiological processes. But the discrimination of Cys from Hcy is still a challenging problem because of their similar structures. One promising method is to take advantage of the unique nucleophilicity of the thiol groups. In this part, a target molecule that

contains α , β -unsaturated carbonyl group is designed. The target molecule could react with thiol group through Michael Addition, followed by a cyclization reaction (**Fig. 6.3.1.1**). The thiol groups of Cys and Hcy have different reaction activities, and show different reaction time. Therefore Cys and Hcy could be differentiated through the time that the spectrum is changed. This target molecule will be combined with UCNP and be dispersed into bio-samples to detect Cys and Hcy at the same time.

In the second part, this thesis focuses on developing a probe that is responsive to pH value change and oxidation/reduction condition.

The following target molecule is designed to detect pH value (**Fig. 6.3.1.2**). In alkaline solution, the target molecule will be deprotonated. The negative charge on oxygen atom will lead to color change.

Fig. 6.3.1.2 Illustration of pH value detection

In addition, Liu et al reported a MnO_2 -nanosheet-modified UCNP probe to detect intracellular Glutathione.¹ In this research, MnO_2 -nanosheets formed on the surface of UCNPs act as an efficient quencher for UCL emission, as well as an oxidant. After the addition of GSH, MnO_2 will be reduced to Mn^{2+} and dissolve in solution, and therefore the UCL emission will be recovered (**Fig. 2.1.6.3.3**). This probe is not only effective for GSH, but also for other reductants in bio-samples.

If the MnO_2 -nanosheet-modified UCNP is combined with the pH probe, the as-prepared new probe should reflect pH value change and reductant, which is a dual-responsive probe. One promising application is to monitor physiological processes in living cells.

Because chemical substances are not equally distributed in cells, this probe will help to visualize different distribution. There is one prerequisite to form the final probe: the MnO_2 -nanosheet should not oxidize the pH probe. Otherwise the MnO_2 -nanosheet needs to be replaced with other materials.

6.3.2 Keypad Lock Device

Another direction of the future work is to develop a new device that is also based on UCNP detection, which is keypad lock.

Keypad lock is a molecular device that is capable of differentiating different chemical sequences. The output signal not only depends on the correct inputs but also depends on the correct sequence of the inputs. The development of a molecular-scale keypad lock is an attractive goal because it is a new approach to detect and protect information at the molecular scale.

First target is to develop a F^- and CN^- keypad lock. An effective way to detect F^- is to take advantage of the cleavage of silicon-oxygen bond while the key point to detect CN^- is to change the π -electron system. Based on these two strategies, a molecule that can detect both F^- and CN^- is designed (**Fig. 6.3.2.1**). This target molecule contains two parts. The indolium part can be synthesized by heating 2,3,3-trimethyl-3H-indole and bromo-acid. The 4-(tert-butyldiphenylsilyloxy)benzaldehyde part can be synthesized by nucleophilic substitution in the presence of imidazole as alkali. Then two parts form target molecule through Knoevenagel condensation. tert-butyldiphenylsilane (TBDPS) group is chosen because it is an excellent leaving group and is easier to combine with F^- , which makes this group a common fluoride-sensing group. The displacement of fluoride atom at the silyl ether will produce a phenolate anion, and causes the color change (expect to change into yellow). Besides, CN^- also can react with this probe on carbon-nitrogen double bond. When changing from indole to indolium, the positive charge on nitrogen atom enhances the electrophilicity of sp^2 -carbon. In the absence of CN^- , there

will be an ICT process occurs from benzene group to the positive indolium part. The addition of CN^- will disrupt the ICT process through nucleophilic addition to the carbon-nitrogen double bond. The solution is expected to change to colorless.

Fig. 6.3.2.1 Synthesis route of target molecule of anion keypad lock device

After combination with UCNP, this target molecule will be much easier to be dispersed into water and detect F^- and CN^- in aqueous solution. If adding fluoride ion, followed by CN^- , after the reaction with F^- , the probe still can react with CN^- because F^- will not affect the carbon-nitrogen double bond. So the color is expected to change to yellow, then turn to colorless. If change the addition sequence, add CN^- first, then F^- , the probe will not show color change upon the addition of F^- because CN^- will attack the carbon-nitrogen double bond, and the color will directly change to colorless. Though the silicon-oxygen bond will cleave when adding F^- , no color change will be observed. If adding both ions simultaneously, because F^- react with the probe faster, the color is expected to change to yellow first, then change to colorless in a short time. Therefore this device acts as a keypad lock.

Second keypad lock device is designed for H_2O_2 and H_2S . The key point of H_2O_2

detection is boric acid ester, and an effective way to detect H_2S is to employ nucleophilic addition. a target molecule is designed according to these two strategies (**Fig. 6.3.2.2**). The synthesis route is also based on Knoevenagel condensation of indolium part and boric acid ester part. The indolium part is elaborated above, and the boric acid ester part is commercially available.

Fig. 6.3.2.2 Synthesis route of target molecule of signaling molecule keypad lock device

After combination with UCNP, this target molecule will be dispersed into aqueous solution. The detection mechanism is similar to the above F^- and CN^- device. If adding H_2O_2 first, then H_2S , the color is expected to be changed from red to yellow. While if change the sequence, the color will directly change to colorless. It should be noticed that one important difference is that this device will be used in bio-samples. Both H_2O_2 and H_2S are signaling molecules and they play key roles in metabolism, therefore the detection and bio-imaging of them will give much information about some important physiological processes.

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