Synthesis and surface modification of quantum dots for biophotonics applications

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**SYNTHESIS AND SURFACE MODIFICATION OF
QUANTUM DOTS
FOR BIOPHOTONICS APPLICATIONS**

ZHANG BUTIAN

SCHOOL OF ELECTRICAL AND ELECTRONIC ENGINEERING

2016

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Abstract

In the last two decades, quantum dots (QDs) have demonstrated great potential for biophotonics applications such as optical imaging and analytes sensing. For these purposes, cadmium-based (Cd-based) QDs are most commonly used because of their excellent optical properties, while cadmium-free (Cd-free) QDs are attracting increasing attention due to the low toxicity of their components. Generally, for use in biological fluids, QDs are either prepared using the aqueous synthesis method or the hot colloidal synthesis method followed by a phase transfer process. This thesis studies the preparation of water-dispersible Cd-based and Cd-free QD by using these two methods and explores their potential for biophotonics applications.

First, this thesis presents a facile aqueous synthesis method for preparing the biofunctionalized Cd-based QDs. The CdTe QD nanocrystals were fabricated in a microfluidic chip by using protein molecules as coordination ligands. Compared to the conventional bench-top method, the microfluidic synthesis approach produced the CdTe QDs with enhanced stability and improved biofunctionalization efficiency. Another method of preparing the aqueous QDs, phase transfer of the QDs synthesized in organic solvents, was also examined. Hydrophilic ligands with different chain lengths and functional groups were applied to render the QDs water-dispersible by using the ligand exchange method. Each set of QDs capped with specific surface ligands displayed strengths and limitations in certain aspects of their physicochemical properties, and induced different cellular responses.

To mitigate toxicity concerns in biophotonics applications, Zn-Cu-In-S QDs, which are quaternary and Cd-free QDs, were prepared using a facile aqueous synthesis method. The composition effect on their optical properties is demonstrated and correlated with the

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evolution of their structure and energy band gap. Using the same strategy of composition control, Zn-Ag-In-S QDs with different emission wavelengths were synthesized in the organic phase and then transferred into the aqueous phase via the encapsulation method. The resulting water-dispersible ZAIS QDs exhibited bright photoluminescence and long fluorescence lifetime and were applied in temporally multiplexed imaging of cells. Finally, ZnO QDs, a type of Cd-free QDs made from wide-band-gap oxides, were prepared using the sol-gel method. Their optical properties were modified via chemical doping and photodoping techniques. Their redox-sensitive photoluminescence and plasmonic properties make them promising candidates for biosensing applications.

In summary, this thesis covers the manipulation of optical properties, surface chemistry and biocompatibility of Cd-based and Cd-free QDs using versatile strategies in synthesis and surface modification processes, and demonstrates the potential of using these QDs in biophotonics applications.

Chapter 1 Introduction

Since the discovery in the 1980s,^{1, 2} quantum dots (QDs) have enabled new advances in photovoltaics,^{3, 4} display technologies,⁵ computing,^{6, 7} and found their most extensive applications in biomedical field in the last two decades.⁸⁻¹⁰ Up to now, researchers have investigated using QDs to address many challenging biophotonics problems such as cellular and molecular imaging,¹¹⁻¹³ analytes sensing,¹⁴ early disease diagnosis,¹⁵ etc. Although commercial organic fluorophores are widely used in ordinary biophotonics applications, the susceptibility of these compounds to photobleaching and degradation has limited their use for long-term imaging and accurate analysis in complex physiological fluids.^{16, 17} By comparison, the excellent photochemical stability of the colloidal QDs allows their use in harsh conditions under long-term or intense excitation. Other important properties of QDs are high quantum yield (QY), narrow spectral width, and widely tunable photoluminescence (PL) emission. PL emission wavelengths are mainly determined by the chemical composition of the QDs and can be further adjusted by altering the composition ratio, size, shape, and surface conditions¹⁸⁻²⁰ to cover the ultraviolet (e.g., ZnO, ZnS), visible (e.g., CdSe, InP), and near-infrared (e.g., PbSe, Ag₂S) spectral regions. The best-quality QDs could achieve a QY comparable to that of the organic dyes in the visible range, and a QY higher than that of the organic dyes in the near-infrared range.^{21, 22} Moreover, the broad absorption and narrow photoluminescence (PL) profiles of QDs enables simultaneous detection of multiple agents using a single excitation wavelength.²³⁻²⁵ Recent studies of various types of QDs have revealed many novel optical features that are promising for biophotonics purposes, such as long fluorescence lifetime, large multiphoton absorption cross section, tunable infrared plasmonic absorption, and efficient upconversion emission.²⁶⁻²⁹

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To fully realize the potential of QDs for biophotonics applications, the QD preparation methods need to be carefully selected and optimized to obtain high quality QDs. For applications in biological fluids, QDs are either obtained by the aqueous synthesis or the hot colloidal synthesis methods. The QDs synthesized by the hot colloidal method require a phase transfer process to achieve water dispersibility. Afterwards, the water-dispersible QDs are conjugated with the desired biomolecules (e.g., genes, proteins) according to the application purpose. Different synthesis or surface modification methods can make a difference in the optical properties, stability, cellular uptake and cytotoxicity the QDs, thereby influencing the effectiveness of using these QDs for biophotonics applications. This thesis evaluates the effects of the QD preparation methods on their physicochemical properties, and addresses some of the challenging problems in synthesis and surface modification of QDs.

In Chapter 2, the fundamentals of the QDs are briefly introduced and the current status on preparation and application of QDs are reviewed. Among a variety of QDs, the Cd-based QDs are most commonly used owing to their excellent optical properties and the rich chemistry of their nanocrystal growth. Although standard and reproducible synthesis methods have been established, challenges still remain in simplifying the synthesis process for obtaining the biofunctionalized QDs. In Chapter 3, a facile one-step synthesis method is proposed to produce the protein-functionalized Cd-based QDs in aqueous phase. The microfluidic synthesis approach was used to obtain a rapid and small-scale production, and the synthetic parameters were optimized via modelling and experiments. Currently, the majority of the Cd-based QDs are still obtained by the hot colloidal synthesis method. The obtained hydrophobic QDs require surface modification using hydrophilic or amphiphilic ligands to achieve dispersibility in biological fluids. Because a variety of surface ligands have been developed for biomedical applications, a comprehensive understanding is required

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regarding the effects of the surface ligands on the physicochemical properties of QDs. In Chapter 4 of this thesis, ligands with different functional groups and chain lengths were used to acquire the Cd-based QDs with different hydrodynamic diameters and surface charges. Next, a systematic study was performed to evaluate the effect of the surface ligands on the colloidal stability, photostability, cellular uptake, and cytotoxicity of the QDs.

Figure 1.1 Cytotoxicity of the elements used for synthesis of the QDs in this thesis. The cytotoxicity of the corresponding salts of the cations were analyzed by MTT assay on MiaPaCa-2 cells (n=4).

In recent years, Cd-free QDs are attracting increasing attention from biomedical fields due to their intrinsic low toxicity. Moreover, these Cd-free QDs exhibit diverse and novel optical properties such as long fluorescence lifetime, upconversion PL, and strong visible absorbance. In this thesis, the quaternary Cu-In-Zn-S and Ag-In-Zn-S QDs were synthesized by aqueous synthesis and hot colloidal synthesis methods, which will be discussed in Chapter 5 and 6. To examine the potential of using these materials to reduce the toxic concern, an MTT assay was performed on the cytotoxicity of the cation ions that will be used for synthesizing the QDs (**Figure 1.1**). The MiaPaCa-2 cells treated with ZnCl₂, AgNO₃, CuCl₂,

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and InCl_3 salts exhibited higher viability compared to those treated with the same concentration of CdCl_2 salts. After examining their intrinsic toxicity, the aqueous synthesis of the Zn-Cu-In-S QDs were optimized by adjusting their composition ratio at a relatively low temperature. The effects of the composition control and shell coating on their optical properties were analyzed by correlating with the energy band gap, elements distribution, and crystal structure. Also by using the composition control strategy, Zn-Ag-In-S QDs with a wide tunability of emission wavelength were obtained via hot colloidal synthesis approach. The hydrophobic QDs were subsequently transferred into the aqueous phase by encapsulation method. Owing to their long fluorescence lifetime, their emissions can be easily distinguished from the emissions of the CdSe QDs and commonly used organic dye by fluorescence lifetime imaging system. By using the ZAIS QDs, CdSe QDs and dye, we examined the potential of using the QDs with different fluorescence lifetimes for multiplexed temporally imaging. The selection of optical set-ups and the data analysis algorithms were investigated and discussed. Another Cd-free QDs, ZnO QDs, were prepared using sol-gel method and their optical properties were manipulated by chemical doping of trivalent ions and photodoping using UV irradiation (Chapter 7). The effect of these two doping strategies on their PL properties and plasmonic properties were monitored and analyzed. Finally, Chapter 8 concludes and compares the results of this thesis work.

Chapter 2 Literature Review on Colloidal Quantum Dots (QDs)

This chapter reviews the literature on the physics, preparation methods, applications, and toxicity of the QDs for biophotonics applications. To elucidate the origin of QDs optical properties, the fundamental aspects related to the physics of QDs is introduced in section 2.1. This section includes a discussion of the mechanism behind the photoluminescence process and the construction of the core/shell QD architectures. Inspired by the extraordinary optical properties of QDs, tremendous effort has been invested into engineering the QDs to improve their QY, increase their emission tunability, and enhance their colloidal and photochemical stability. Section 2.2 reviews the key progress in optimizing the synthesis of various types of QDs, including the conventional Cd-based QDs and the newly developed Cd-free QDs. To achieve the full potential of QDs in biological fluids, researchers developed surface modification strategies to endow the QDs with water dispersibility, to passivate them for enhanced colloidal stability, and to functionalize them with desired biomolecules. The related techniques are discussed in section 2.3. Section 2.4 summarizes recent developments in using engineered QDs in biophotonics applications. These applications demonstrate the capability and potential of QDs in the biomedical field and provide guidelines for improving the performance of QDs during the synthesis and surface modification processes. Despite these promising applications, toxicity is a major concern that limits the use of QDs for in vivo and clinical applications. Section 2.5 reviews the studies addressing the toxicity of QDs using cell and animal models, and summarizes the factors that influence the toxicity of the QDs.

2.1 Fundamentals of Colloidal QDs

2.1.1 Quantum Confinement and Photoluminescence Properties

QDs are semiconductor nanocrystals that consist of hundreds or thousands of atoms. Their sizes typically range from 1 to 10 nm, which is an intermediate scale between bulk materials and small molecules. Therefore, the origin of QD's electronic and optical properties can be elucidated from two perspectives: scaling down from bulk semiconductors and scaling up from molecules. From the perspective of scaling down from bulk, one can see different effects on the motions of electrons and holes in different sized materials. The physical boundaries of a bulk semiconductor have little effect on the motions of its electrons and holes. The electrons and holes in QDs are spatially confined in all three dimensions to the same magnitude as the exciton Bohr radius (a_B), which is the average distance between the electron and hole in an exciton in bulk semiconductors. It can be computed from the following equation:

$$a_B = \frac{\hbar^2 \epsilon}{e^2} \left(\frac{1}{m_e} + \frac{1}{m_h} \right) \quad (2.1)$$

where $\hbar$ is the reduced Planck constant, ϵ is the dielectric constant, e is the charge of an electron, m_e is the effective mass of the electron, and m_h is the effective mass of the hole.

As the dielectric constant (ϵ) and the effective masses (m_e and m_h) are material-dependent, the exciton Bohr radius could vary substantially between different semiconductors (e.g., 2.5 nm for ZnS, 5.6 nm for CdSe, and 18 nm for PbS). Within this size regime, the motions of electrons and holes in QDs are strongly constrained by their physical boundaries. As a result, the QDs exhibit discrete energy levels and enlarged band gap, which is substantially different from the electronic structures of their macroscopic counterparts (**Figure 2.1a**). From the

perspective of scaling up from molecules, the energy structures of the QDs could be regarded as a result of molecular orbital hybridization. Upon increasing the number of the molecules in a cluster, the gap between the discrete energy levels of the molecule will be gradually decrease due to the Pauli exclusion principle until they form quasi-continuous states in the bulk materials. QDs are an intermediate stage in this process, and they exhibit discrete energy band structures due to the hybridization of finite molecular orbitals.

Figure 2.1 Quantum confinement and photoluminescence properties of QDs. (a) Energy band diagram of macroscopic crystals and QDs.³⁰ The macroscopic crystals exhibit a quasi-continuous electronic structure, and the QDs exhibit discrete energy levels with enlarged band gaps. (b) Schematic illustration of one-photon band-edge photoluminescence process: formation of exciton by absorption of a photon (green line); nonradiative thermal relaxation (cyan line); radiative exciton recombination with emission of a photon (red line).³¹ (c) Size dependence of the absorption and emission wavelength of CdSe QDs.³²

The most commonly observed type of photoluminescence (PL) originating from QDs is one-photon band-edge PL. Like their bulk counterparts, the mechanism behind the PL emission of the QDs is a photon-electron-photon interaction process (Figure 2.1b). Typically, upon absorption of a photon with energy above its band gap, an electron of the QD is excited to the conduction band and forms an exciton with the hole it left at the valence band (Figure 2.1b, green line). At certain probability, the recombination of the electron and hole pair gives out energy in the form of a photon (Figure 2.1b, red line). The PL emission we observed contains a large number of photons emitted by an ensemble of QDs. This PL emission usually has the following characteristics:

- (1) The energy of PL peak is lower than that of the absorption peak, and the difference is defined as Stokes shift, which is mainly caused by a nonradiative thermal relaxation of the electron before the radiative recombination (Figure 2.1b, cyan line);
- (2) The wavelengths of the absorptions and PL emissions are size dependent (i.e., smaller QDs exhibit shorter wavelength and vice versa; see Figure 2.1c). This phenomenon is attributed to the increased confinement effect in smaller QDs and the consequent band gap widening;
- (3) The PL emission exhibits a symmetric spectral band with a narrow spectral width, represented by full-width at half-maximum (FWHM). The spectral width can be affected by the variation of crystal size and shape, lattice distortion, homogeneity of the composition, and the crystal phase purity, etc.;
- (4) The number of photons emitted is smaller than the number of the photons absorbed in a PL process. Their ratio is calculated as quantum yield (QY). The QY of QDs is affected by many factors such as their crystalline quality, surface passivation condition, and the solvent polarity;

(5) The PL lifetimes can vary from nanoseconds to microseconds and are determined by the various radiative and non-radiative recombination rates and their proportion.

Except for one-photon band-edge PL, multi-photon PL and deep level PL are also observed in QDs. In the multi-photon PL process, QDs simultaneously ($<10^{-15}$ second) absorb two or more photons, which elevates one electron to the conduction band.³³ In this case, QDs could emit photons with energies higher than those of the excitation photons. Therefore, one can obtain up-conversion emission by using a long wavelength excitation, usually NIR light. To increase the probability of simultaneously absorbing multi-photons, researchers often use ultrafast lasers to provide a high flux of photons. Deep-level PL emissions involve the energy levels in between the conduction and valence band, which are formed by dopants or by internal or surface defects in the QD crystal lattice. In this process, the radiative recombination is accomplished by a transition of electrons from the conduction band to the deep level, from one deep level to another one, or from a deep level to the valence band. As a result, the deep-level PL usually exhibits a longer wavelength than the band-edge PL. Other features of deep-level PL include broad emission spectra and prolonged fluorescence lifetime.

2.1.2 Core/Shell Architectures

Core/shell QDs are obtained by depositing a shell of a semiconductor material different from the core material on the core-only QDs. Core/shell QDs can be categorized into three types of band alignment: type-I, type-I^{1/2} (quasi-type-II), and type-II (**Figure 2.2**). The core/shell architecture exhibits several advantages. First, by adjusting the band alignment, one can confine the wave functions of the charge carriers in either core or shell and thus manipulate the exciton binding and the optical properties. Second, the additional shell can passivate the core and isolate it from the surface defects or solvent molecules, thereby reducing surface-related quenching and improving the QY. Third, appropriate shell passivation may

enhance the chemical stability and biocompatibility of the QDs. Finally, the same type of shell provides a unified interface for the subsequent surface modification in spite of different core materials.

Figure 2.2 (Upper) schematic band diagrams of type-I, type-I^{1/2} (or quasi-type-II), and type-II core-shell QDs. (Lower) schematic depictions of electron (blue) and hole (red) ground-state wave functions in corresponding core/shell structures.³⁴

Type-I (e.g., CdSe/ZnS, CuInS₂/ZnS) and type-II QDs (e.g., CdTe/CdSe, ZnTe/ZnSe) are the most commonly used core/shell architectures. In energy diagram of type-I core/shell QDs, the conduction band of the shell lies above that of the core, and the valence band of the shell lies below that of the core. As a result, both holes and electrons are confined within the QD core. The shell isolation can inhibit the interaction of the excitons with the surface defects or solvent molecules, thereby reducing the nonradiative recombination. Therefore, Type-I architecture has been widely used in QDs to enhance their PL QY.^{35, 36 37} Wide-band-gap ZnS semiconductors are the most commonly used shell material for Type-I QDs. Owing to their high chemical stability, the constructed type-I core/shell QDs usually exhibit improved stability and prolonged shelf life compared to core-only QDs. In the case of type-II QDs, the

conduction and valence band of the shell material are either both higher or both lower than those of the core. When they are both higher, electrons are primarily confined within the core and the holes are primarily confined within the shell (and vice versa). This spatial separation of the electrons and holes results in novel QD optical properties. In Type-II CdTe/CdSe QDs, NIR emissions can be tuned from 700 to 1000 nm by adjusting the core size and shell thickness.³⁸ The energies of these emissions are even lower than the bulk band gap of their composite materials, which are hardly accessible in type-I QDs. This wavelength extension is attributed to the recombination of the electrons and holes across the core-shell interface. More recently, simply by adjusting the shell thickness, Bang et al. obtained type-II ZnTe/ZnSe QDs with emissions ranging from blue to amber (Figure 2.3).³⁹ The high dependence of the emission wavelength on the shell thickness was commonly observed in type-II but not in type-I QDs. Another distinctive characteristic of the type-II QDs is the prolonged PL lifetime, which results from the low recombination rate of the spatially separated charge carriers.

Figure 2.3 (a) Absorption and (b) PL emission spectra of the ZnTe core QDs (black), ZnTe/ZnSe core/shell QDs that have the same ZnTe cores and 1 to 5 monolayers of ZnSe shells. The inset in panel (b) shows the photograph of ZnTe/ZnSe (core/shell) QDs illuminated by UV light.³⁹

2.2 Synthesis of Cadmium-Based and Cadmium-Free QDs

Roughly, the fabrication methods of QDs can be categorized into two approaches: the top-down approach using the lithography or etching technique and the bottom-up approach using vapor-phase or solution-phase synthesis. Cadmium-based (Cd-based) QDs are the most commonly used QDs for biomedical applications owing to their excellent optical performance and well-developed synthesis methods. In recent years, cadmium-free (Cd-free) QDs have been attracting increasing attention from the QD community because of the low toxicity of their components. Intensive efforts have been devoted to developing synthesis methods for Cd-free QDs, optimizing their optical properties (PL QY, PL tunability, FWHM, etc.), and increasing their stability. In this section, the most commonly used methods for synthesizing Cd-based QDs are summarized. Next, the synthesis of Cd-free QDs is introduced. **Table 2.1** presents the representative studies on the synthesis of Cd-based and Cd-free of QDs. It also summarizes the exciton Bohr radius and the bulk band gap of the semiconductors used for synthesizing QDs, which provide a guideline for selecting the appropriate materials to obtain the desired optical properties. Generally, the bulk band gap of the composite materials used for making QDs determines the accessible range of PL wavelength. The degree to which the PL emission can be tuned is affected by the available size range of the synthesized QDs and the exciton Bohr radius.

Table 2.1 Fundamentals and synthesis of QDs.

QD Type ^a	Exciton Bohr Radius [nm]	Bulk Band Gap [eV]	Synthesis Method	Size ^b [nm]	Emission Wavelength [nm]	Quantum Yield	Ref
CdTe	7.5	1.5	hot colloidal	2.5-7	550-650	30-65%	⁴⁰
			hydrothermal	3-4	517-618	24-45%	⁴¹

Chapter 2 Literature Review on Colloidal QDs

CdTe/ZnS	—	1.5	hydrothermal	5.3	624-735	45-62%	42
CdSe	5.6	1.7	hot colloidal	~2-5	460-625	5-15%	43
CdSe/ZnS	—	1.7	hot colloidal	2.3-5.5	470-625	30-50%	43
CdSe/CdS	—	1.7	hot colloidal	5.2-6.8	~600	up to 97%	44
CdSe/CdS/ ZnS	—	1.7	hot colloidal	9.2±0.34	~610	up to 80%	45
CdSe/ZnSe/ZnS	—	1.7	hot colloidal	~4-6	~590	up to 70-85%	46
CdS	3.0	2.4	hydrothermal	3.2	560	6%	47
CdS/ZnS	—	2.4	hot colloidal	5.8	460-480	20-30%	48
InP	20	1.35	hot colloidal	2.8	480-750	1-30%	49
InP/ZnS	—	1.35	hot colloidal	4.5	480-735	up to 60%	49
CuInS₂	4.1	1.5	hot colloidal	6	510-630	up to 80%	37
			hot colloidal	2-4	630-780	5-10%	50
			hot colloidal	2.5	620-870	up to 18%	51
			hydrothermal	2.2	660	3.3%	52
CuInS₂/ZnS and ZnS-CuInS₂	—	1.5-3.7	hot colloidal	7	550-815	up to 60%	53
			hot colloidal	4	550-800	20-80%	51
			hydrothermal	3.8±0.3	543-625	20-38%	54
			microwave	3.3	540-680	up to 24%	55
AgInS₂	5.5	1.87	hot colloidal	2.5-4.3	770-855	36-63%	56, 57
AgInS₂/ZnS and ZnS-AgInS₂	—	1.87-3.7	hot colloidal	1.4-5.2	620-815	1.5 ±0.1%	58
			thermal decomposition	4.1-4.4	540-720	up to 24%	59
			sonochemical	12 ± 1.3	480-700	57.6-336% relative to CdSe/ZnS	60
Cu₂S	3-5	1.2	sono-electrochemical	12.1-18.1	NIR Abs peak >1100 nm		61
			hot colloidal	2.5-6	NIR Abs peak >1796 nm		62

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CuInSe₂	10.6	1.04	hot colloidal	2-3.5	650-975	up to 25%	63
Ag₂S	2.2	0.9-1.1	hot colloidal	5.6	1200	16%	64
			hot colloidal	1.5-4.6	690-1227	0.18%	65
			hydrothermal	2.6-3.7	687-1096	14.2-16.4%	73
			hydrothermal	1.5-6.3	510-1221	up to 2.1%	66
Ag₂Se	2.9	0.15	aqueous at room temperature	3.4-5.9	966-1228	11.1-12.2%	67
			hot colloidal	3.4	1300	29.4% after phase transfer	68
ZnS-AgInSe₂	—	1.2-3.7	hot colloidal	5	660-800	up to 50%	69
Mn:ZnSe	4.5	2.7	hydrothermal	3.8	410,480,570	2.4%	70
			hydrothermal	7.0	400,480,590	up to 17%	71
Mn:ZnS	2.5	3.7	hot colloidal	3.8 ± 0.2	585	51.3%	72
Cu:ZnS	2.5	3.7	hydrothermal	—	482	4.2%	73
Silicon	4	1.1	pulsed laser ablation	4	450	0.4%	74
			microemulsion	3.5-4.3	420-280	5.2%	75
			gas-phase	2.8-5.2	700-1000	0.3-10.2%	76
ZnO	2.3	3.3	sol-gel	2.85-12.1 6	450-550	23-61%	77
			sol-gel	3-4	520-550	20-50%	78
Graphene	Infinite	0	hydrothermal	5-25	377-493 (Exc: 300-400)	28%	79
			pyrolysis	4.66± 1.24	415-580 and 800-850 (Exc: 320-540)	up to 54.5%	80

^a Ideal chemical formulae are provided despite the off-stoichiometry of the practically obtained QDs, *e.g.*, Cu₂S can represent Cu_xS and AgInS₂ can represent Ag_xIn_yS.

^b Particle sizes are obtained from TEM or calculated from excitonic absorption peak.

2.2.1 Synthesis of Cd-Based QDs

Solution-phase synthesis is the most commonly used method for fabricating Cd-based QDs, which can be further classified as hot colloidal route and hydrothermal route.^{81, 82}

Hot colloidal synthesis is performed in non-aqueous media by using hydrophobic coordination ligands, such as oleic acid, oleylamine, trioctylphosphine (TOP), and trioctylphosphine oxide (TOPO). In a typical hot colloidal synthesis of Cd-based QDs, the Cd precursors (CdMe_2 , CdO , or CdCl_2) and chalcogenide precursors (TOPSe, TOPS, or TOPTe) are injected into the TOP/TOPO mixtures at an elevated temperature (e.g., 300°C). The rapid injection results in a solution highly supersaturated over the critical point for nucleation, leading to the spontaneous formation of the nuclei (**Figure 2.4**). Subsequently, due to the decrease of the precursors' concentration upon nucleation, the supersaturation drops below the critical

Figure 2.4 (a) Schematic illustration on hot colloidal synthesis for Cd-based QDs. (b) The temporal change in degree of supersaturation during different reaction stages of QDs synthesis: (I) injection of precursors, (II) nucleation of QDs and (III) growth of QDs.⁸³

point, and the nucleation terminates. Also, the mixture is cooled down to a temperature (e.g., 250°C) to avoid the generation of new nuclei and maintain the nanocrystal growth.⁸⁴ During this process, the TOP and TOPO ligands can coordinate with the unsaturated metal atoms on the crystal surface and limit the growth rate of the QDs to avoid the formation of bulk

semiconductors.⁸⁵ Also, they function as surface ligands to stabilize the QDs and prevent the formation of aggregates. By adjusting the precursor concentrations and collecting the QDs at different growth time, QDs of the desired particle size can be obtained. The last two decades have seen remarkable developments in hot colloidal synthesis by using lower-cost and safer precursors, applying mild reaction conditions, and precisely controlling the growth kinetic of the QDs.⁸⁶

The hydrothermal synthesis is performed in aqueous solvents using hydrophilic coordination ligands. Like hot colloidal synthesis, hydrothermal synthesis consists of nucleation and limited growth processes. The coordination ligands are usually thiol ligands such as mercaptopropionic acid (MPA), mercaptoundecanoic acid (MUA), cysteine, etc. Recently, more complex ligands, such as proteins,⁸⁷ which have an affinity for QD's surfaces, were also used in the aqueous synthesis of QDs. These ligands can interact with cadmium ions and control the reactivity of the precursors at the desired pH and temperature. Also, they make QDs water-dispersible through ionization of their functional groups and keep the QDs separate from each other via electrostatic repulsion between similarly charged surfaces. Compared to hot colloidal synthesis, hydrothermal synthesis is usually cheaper and less toxic, and can be done at a relatively low-temperature. The as-synthesized QDs are readily dispersed in water, which makes them appropriate for biomedical applications. However, conventional aqueous-prepared Cd-based QDs usually have lower QY than non-aqueous-synthesized QDs. In recent years, several groups have proposed aqueous-synthesized QDs with QYs comparable to non-aqueous-synthesized QDs.^{88, 89 42}

A common approach used to improve the optical performance of QDs is to passivate the nanocrystal core using a wide-band-gap semiconductor shell, as discussed in section 2.1.2. By injecting Zn and S precursors into the reaction mixtures containing core QDs, CdTe/ZnS, CdSe/ZnS, and CdS/ZnS core/shell QDs were obtained, and they exhibited enhanced PL

intensity compared to the core-only QDs.^{42 43 48} When synthesizing these core/shell QDs, the lattice mismatch between the core and shell materials should be carefully considered. Large lattice mismatch makes it difficult to deposit a shell epitaxially on the core or would result in interface defects, which degrade the PL intensity. A general solution is to grow an intermediate layer as “lattice adapter” between the core and shell to form core/shell/shell QDs. For example, Dmitri et al. introduced a layer of CdS or ZnSe between the CdSe core and ZnS shell to synthesize the CdSe/CdS/ZnS QDs or CdSe/ZnSe/ZnS QDs. The reason for selecting CdS and ZnSe as the intermediate shell materials was that their lattice constants lie between those of CdSe and ZnS. This architecture helped to reduce the interface lattice strain and yielded almost defect-free ZnS shell, which was seldom achieved in CdSe/ZnS QDs. The resulting sandwiched QDs exhibited improved PL QY (up to 70%–85%) and enhanced photostability compared to the CdSe/ZnS QDs.

Although the solution-phase synthesis of Cd-based QDs appears to have reached a mature stage, researchers are continually seeking improvements in their performance. Very recently, Bawendi’s group synthesized monodisperse CdSe/CdS QDs with high QY (97%), minimal blinking (~94% on time), and narrow spectral width (~20 nm FWHM). The shell growth rate was slowed down by using cadmium oleate and octanethiol as precursors. This slow shell-growth process yielded a high-quality CdS shell, which could passivate the CdSe core sufficiently even when it is a thin layer.

2.2.2 Synthesis of Cd-Free QDs

More recently, Cd-free QDs have received great attention from researchers in biomedical fields because their components are less toxic than Cd²⁺ ions. In the last decade, researchers have developed a variety of Cd-free QDs, such as III-V QDs (InP), II-VI QDs (doped Zn chalcogenide), I-III-VI QDs (CuInS₂, AgInS₂), I-VI QDs (Ag₂S, Cu₂S), and Group IV QDs

(Silicon, Graphene). The hot colloidal and hydrothermal methods used for synthesizing Cd-based QDs could be adapted to fabricate the Cd-free QDs by carefully selecting the precursors, coordinating ligands, and solvents. However, the fabrication of high-quality Cd-free QDs is still challenging, and researchers have made many attempts to optimize the synthesis methods.

InP QDs are expected to be structurally stable because they are made from the robust III-V semiconductors, which are more covalent than the II-VI materials.⁹⁰⁻⁹⁵ Peng's group obtained monodispersed InP QDs by injecting tris (trimethylsilyl)phosphine/amine) solution into a mixture of indium acetate, octadecene, and myristic acid at 190°C.⁹⁶ The InP QDs were grown at a lower temperature of 178°C for different reaction times. The challenge of this synthesis process mainly lies in the use of the tris (trimethylsilyl)phosphine, an expensive and pyrophoric precursor. To overcome this challenge, Li et al. developed an economic and safe method by using low-cost and air-stable calcium phosphide as phosphor precursors.⁹⁷ Similar to the synthesis of Cd-based QDs, a shell of a wide-band-gap semiconductor (e.g., ZnS, ZnSe) were deposited on InP core QDs to improve their PL intensity and colloidal stability.⁹⁸⁻¹⁰¹ As an analogy to the core/shell/shell Cd-based QDs, Ippen et al. developed sandwiched InP/ZnSe/ZnS QDs to relax the lattice mismatch between the core and the outer shell.¹⁰² The InP/ZnSe/ZnS QDs exhibited improved PL intensity (50%–70% QY) compared to the conventional InP/ZnS QDs.

For preparing the I-III-VI CuInS₂ and AgInS₂ QDs, indium stearate, copper acetate (or silver nitrate), and sulfur powder were used as the precursors in a hot colloidal synthesis.¹⁰³ To balance the reactivity of two cationic precursors and avoid the formation of Cu_xS, soft Lewis base ligands (alkane thiols) were adopted to suppress the reactivity of copper. By adjusting the Cu:In or Ag:In ratio, the PL of CuInS₂ and AgInS₂ QDs could be tuned to cover the visible to NIR region. Furthermore, a Cu-poor and In-rich composition was found to be

Chapter 2 Literature Review on Colloidal QDs

favorable for PL enhancement.⁵¹ Later, researchers incorporated Zn into the CuInS₂ and AgInS₂ by cation exchange at an elevated temperature to obtain Zn-Cu(Ag)-In-S QDs (**Figure 2.5**).^{19, 104} These quaternary QDs exhibit enhanced PL emission intensity at shorter wavelengths compared the ternary QDs without Zn incorporation. Recently, several groups also attempted to employ hydrothermal synthesis methods to obtain CuInS₂ and AgInS₂ QDs, but the QYs were relatively low.⁵² By applying the soft-hard base-acid theory in aqueous synthesis, Chen et al. carefully controlled the reactivity of the Cu and In precursors by using the 1-Glutathione and sodium citrate as coordination ligands and obtained QDs with improved QY.⁵²

Figure 2.5 Synthesis of CuInS₂ QDs and formation Zn-Cu-In-S QDs by cation exchange.

By doping the transition ions (e.g. Cu²⁺, Mn²⁺, Co²⁺, Gd³⁺) into the ZnS or ZnSe QDs, one can obtain the doped Zn chalcogenide QDs with PL and paramagnetic properties.^{71, 105, 106} In the doped QDs, the excitons are usually generated in the large-gap host materials and preferentially recombine with electrons or holes in the dopant level. As a result, the doped QDs exhibit many extraordinary optical features, including the large Stokes shift due to the large energy gap between the host and the dopant, and the long fluorescence lifetime due to the slow recombination of the excitons in the dopant level. Early in 2005, Peng's group developed growth- and nucleation-doping strategies, to synthesize highly fluorescent Cu- and Mn-doped ZnSe QDs.¹⁰⁷ Because the optical properties of the doped QDs is strongly dependent on the distribution and concentration of the dopants within the

nanocrystal-matrix,^{108, 109} controllable doping is important in the preparation of high-quality doped QDs. In the designed strategies, the precursors were carefully selected to decouple the doping from nucleation/growth process. As a result, the QDs exhibited an enhanced dopant PL with suppressed band-edge PL. More recently, the hydrothermal method has been developed to obtain doped Zn chalcogenide QDs. In the aqueous phase, Zeng et al. fabricated monodispersed Cu-doped ZnSeS/ZnS QDs with 40% QY and excellent photostability (Figure 2.6).¹¹⁰ The QY reached a maximum at 0.43% Cu⁺ content and then decreased upon further increasing the Cu⁺ concentration. This PL deterioration was associated with the nonradiative recombination due to the interaction between the high concentrated dopants.

Figure 2.6 Synthesis of Cu:ZnSeS/ZnS QDs using the hydrothermal method.¹¹⁰ (a) Absorption, PL spectra and (b) XRD patterns of Cu:ZnSeS and Cu:ZnSeS/ZnS QDs. TEM images of (c) Cu:ZnSeS; and (d) Cu:ZnSeS/ZnS QDs; and (e) digital photograph of Cu:ZnSeS/ZnS QDs under a 365 nm excitation.

Ag_2S and Ag_2Se QDs are based on I-VI semiconductor materials with narrow band gaps of 0.9–1.1 eV and 0.15 eV.⁶⁶ Their emission can be extended to a NIR-II window (1000–1350 nm), where the light attenuation by biological media is minimal and thus appropriate for deep-tissue imaging.^{111, 112} By thermal decomposition of $\text{Ag}(\text{DDTC})$ precursors in the presence of 1-dodecanethiol (DT), Zhang et al. obtained fluorescent Ag_2S QDs with an NIR-II emission peak at 1200 nm.¹¹¹ In this synthesis method, DT simultaneously functioned as the solvent, the coordination ligands, the surfactant, and part of the sulfur source. Interestingly, the PL emission wavelength of the synthesized QDs is size-independent (**Figure 2.7**), which was thought to be a result of the DT-related PL mechanism. Except for DT, other organothiols are also applied as surfactants in the formation of Ag-based QDs. Zhu et al.¹¹² evaluated the effect of 1-octanethiol, tetradecylphosphonic acid (TDPA), and oleylamine (OAm) on the synthesis of Ag_2Se QDs and found the 1-octanethiol to be the most effective for producing the monodisperse and photostable Ag_2Se QDs. The reason was that the organothiol is soft Lewis-base ligand and has favorable interaction with the $\text{Ag}(\text{I})$ precursor, a soft Lewis acid. In addition to non-aqueous synthesis methods, hydrothermal synthesis was developed for obtaining Ag_2S QDs with tunable emission wavelength.^{66, 113} Aqueous synthesis of Ag_2Se QDs was demonstrated to proceed at room temperature by using a multidentate polymer as capping ligands.⁶⁷

Figure 2.7 (A-C) TEM images of the DT-capped Ag_2S QDs with different sizes: (A) 5.4 nm; (B) 7 nm; (C) 10 nm and (D) their PL spectra.

Silicon and graphene are privileged materials for making QDs due to their good biocompatibility and high abundance in nature. Compared to other Cd-free QDs, the methods for fabricating these Group IV QDs vary a lot in their starting materials and synthesis methods. For example, silicon (Si) QDs can be prepared by plasma decomposition of silane,¹¹⁴ thermal decomposition of organosilane in supercritical solvents,¹¹⁵ and high-temperature aerosol reaction.¹¹⁶ Graphene QDs can be fabricated by hydrothermal reduction of graphene oxide,⁷⁹ cage-opening of fullerene,¹¹⁷ and electrochemical reduction of carbon nanotubes.¹¹⁸ Recently, considerable efforts have been invested in synthesizing these QDs in order to improve the PL efficiency,^{79, 119} extending their emission to longer wavelength region,⁷⁶ and using inexpensive and plentiful raw materials for synthesis.

2.3 Surface Modification of QDs

To prepare QDs for biophotonics applications, the surface modification of QDs can be performed in three stages: synthesis, phase transfer, and stabilization or bioconjugation of water-dispersible QDs. For QDs synthesized in the aqueous phase, the surfactants (e.g., short thiols, proteins) used in synthesis naturally provide water solubility and a certain level of stability. For QDs synthesized in nonpolar solvents capped with aliphatic chains (e.g., TOP, oleic acid),¹²⁰ the phase transfer process is performed to render the QDs water-dispersible, which is a prerequisite for applications in biological fluids. The water-dispersible QDs obtained from aqueous-phase synthesis or phase transfer could be further modified to improve their colloidal stability by cross-linking or polymerization. Finally, the bioconjugation process could functionalize QDs with desired biomolecules (e.g., cell-specific targeting ligands, genes, drugs, proteins) for specific biomedical purposes.

2.3.1 Phase Transfer of QDs

Two methods, ligand exchange and encapsulation (**Table 2.2**), were used to transfer the hydrophobic QDs from the oil-phase to the aqueous-phase. Ligand exchange replaces the hydrophobic ligands on the surface of QDs with hydrophilic ligands. Encapsulation caps the QDs with another amphiphilic layer while retaining the original hydrophobic capping ligands. Usually, ligand exchange yields QDs with smaller hydrodynamic diameters, while encapsulation provides QDs with better colloidal stability.

The ligand exchange process is primarily driven by mass action and thus requires a large excess of replacing ligands.¹²¹ The ligands should contain the metal affinity groups (e.g., thiol groups, amino groups, or phosphine groups) for attaching on the surface of QDs, and they should possess hydrophilic groups (e.g., carboxyl groups, amino groups, or hydroxyl groups) to achieve water compatibility. The compact mono-mercapto ligands, such as mercaptoacetic acid and cysteamine, are the ones with the simplest structure that meet these criteria. However, the resulting QDs usually have short shelf lives. This is mainly due to two reasons: the thiol-Zn or thiol-Cd bonds are weaker than the Au-thiol bond and are very dynamic in an aqueous environment;¹²² the complex interactions of carboxyl/amino groups in physiological fluids will cause aggregation of QDs. To avoid the aggregation induced by the desorption of surfactant, dithiol ligands^{123, 124} are used to achieve stronger anchoring onto the QDs surface. On the other hand, ligands with hydroxyl groups as ending groups are used to improve the colloidal stability of QDs in a wide range of pH compared to those with carboxyl or amino groups.⁸⁸ Except for small thiol ligands, large molecules such as peptides, proteins, and polymers, are used as surfactants in ligand exchange to provide a better isolation between the QD surface and the external environment. The end-functionalized polymers, such as multidentate thiol-polyethylene glycol (PEG-SH), hyperbranched amine-containing polymers, are examples which can endow QDs with good colloidal stability and only a slight increase

Table 2.2 Phase transfer methods of QDs.

Phase Transfer of Quantum dots

Ligand Exchange			Encapsulation		
Ligand Types	Examples	Interaction Mechanisms	Ligand Types	Examples	Interaction Mechanisms
Thiolate alkyl ligands	MPA	Metal-thiol bonds	Synthetic amphiphilic polymers	Polyacrylic acids	Micelles Direct hydrophobic interaction
	Cysteine			Poly(maleic anhydride)	
	DHLA			Di/tri-block copolymers	
	Thiolate dendrons				
Peptides or proteins	TAT	Mainly metal-thiol bonds	Natural polymers	Phospholipids	Liposomes Micelles
	Phytochelatin peptides			Chitosan	
	Streptavidin			Salep-g-PAA	
	BSA			Agar	
End-functionalized polymers	DHLA-PEG	Metal-thiol bonds	Host molecules	Calixarenes	Complementary architectures
	Poly(thiophenes)	Metal-amine bonds		Cyclodextrins	
	Amine-containing dendrimers	Metal-phosphine bonds		Crown ethers	
	Oligomeric phosphines	Metal-carboxyl bonds			
	Poly(maleic anhydride)				
Silanization (e.g. Silica, Titania)					
Polymerization (e.g. PEG)					

in size.¹²⁵ Specific polymers are selected for QDs used in different biomedical applications. For example, highly cationic polyethyleneimine (PEI) polymers are used to promote cell-penetrating efficiency, and PEG molecules are adopted to resist the nonspecific binding to proteins and cells. Biomolecules such as peptides or proteins could render QDs water-dispersible and simultaneously provide biological functions. For example, bovine serum albumin (BSA) and denatured BSA could anchor to the surface of the QDs and serve as the carrier for molecules with low water solubility.¹²⁶

Encapsulation strategy can isolate inner QDs with the external environment and leave the QD surface undisturbed. Small host molecules and large amphiphilic polymer molecules are commonly used for this purpose. Host molecules are cyclic molecules consisting of several subunits and can enclose spatially fitted structures by noncovalent interactions.¹²⁷ For example, cyclodextrins are cyclic oligosaccharides with lipophilic central pockets, which could enclose the hydrophobic ligands on the QD surface and allow the phase transfer of QDs without size increase.¹²⁸ Amphiphilic polymers, such as poly(maleic anhydride) and polyacrylic acids, are commercially available and usually served as the backbone for encapsulating QDs by direct hydrophobic interaction. Other than these synthetic copolymers, the natural biomolecules, such as phospholipids, denatured proteins, and polysaccharides were also employed to endow QDs with water dispersibility and biocompatibility. For synthetic and natural polymers, the formation of micelles is a common way to encapsulate QDs. Micelles are ordered assemblies of amphiphiles in water, which consist of a hydrophilic outer shell to achieve water compatibility and a hydrophobic inner core to entrap “oil-like” components.¹²⁹ The amphiphilic polymer molecules associate at critical association concentration (CAC) and form micelles above critical micelle concentration (CMC).^{129, 130} The hydrophobic/hydrophilic ratio of polymers, the reaction temperature, and solvent

composition can affect the CAC and CMC values and thus should be carefully designed when preparing the micelle for encapsulating QDs.¹³⁰⁻¹³³

Besides these organic ligands, inorganic materials have also been applied as the shell material to passivate the QDs. Silica is an ideal matrix for enclosing QDs owing to its optical transparency and chemical inertness. Surface silanization can significantly reduce the toxicity of QDs and permit further conjugation. So far, researchers have developed the sol-gel method (Stöber method), reverse microemulsion (water-in-oil, W/O) method, and normal microemulsion (oil-in-water, O/W) method to control the silica shell growth on QD surface. In the commonly used reverse microemulsion method, silica shell were allowed to grow at the water-in-oil interface.¹³⁴ Therefore, before the silica growth, a ligand-exchange process for hydrophobic QDs was required to enable their entry into the water droplets,¹³⁵ which usually compromised the QY of QDs. To avoid this problem, the normal microemulsion method was favored because it directly coats the hydrophobic QDs in the oil-in-water system without ligand exchange. However, it is relatively difficult to control the silica growth on the oil-in-water interface and only a handful of reports have addressed this issue. Barcova's groups selected three different silica precursors for stepwise silica growth and obtained silica-coated QDs of small size (~18 nm), high QY (50–70% relative to hydrophobic QDs), and high product yield (up to 92%).¹³⁶ Recently, Wang's group used triblock copolymer (PEO-PPO-PEO) to template silica growth on the PPO/PEO interface.¹³⁷ The resulting shell provided a micelle/silica dual-protection for the internal QDs, and the QDs exhibited excellent resistance to strong acids/alkalines and concentrated salt solutions.

After phase transfer of QDs, cross-linking and PEGylation are commonly used strategies to enhance the stability of QDs. Cross-linking of the monodentate ligand shell can yield a polymer-like multidentate shell. For example, vinyl-functionalized dendrons can be crosslinked by using the ring-closing metathesis (RCM) reaction,¹²⁵ and the carboxyl groups

can be crosslinked by using amino-terminated lysines.¹³⁸ QDs capped with liposomes, silica shells, and many other coatings have limitations in specific targeting applications due to their nonspecific adsorption onto cellular components. The PEGylation can reduce the nonspecific adsorption of QDs and prolonged their circulation time in vivo.¹³⁹ Furthermore, with plenty of ether groups, PEG molecules can form a large number of hydrogen bonds with solvents and improve the stability of QDs in a wide pH range and at high salt concentrations.

2.3.2 Bioconjugation of QDs

Compared to small fluorophores, QDs provide spacious and flexible surfaces for bioconjugation. A variety of biomolecules, including antibodies, peptides, aptamers, nucleic acids, liposomes, and small molecules, have been conjugated with QDs according to different application purposes (e.g., cell targeting, biosensing, drug/gene delivery). **Figure 2.8** illustrates representative methods for preparing the QD bioconjugates, which can be classified as covalent and noncovalent conjugation routes.¹⁴⁰ A series of covalent conjugation methods have been applied based on the functional groups of biomolecules and the QD surface. Carbodiimide chemistry is often used to couple the carboxyl and amino groups on QD surfaces and biomolecules (Figure 2.8 ②). Reactions between amino groups can take place by using the compounds containing two N-hydroxysulfosuccinimide (NHS) esters (Figure 2.8 ①). Linkage to sulfhydryl groups can be achieved by the assistance of maleimide-terminal ligands (Figure 2.8 ③, ⑥). Copper-free click chemistry is designed for conjugating QDs with azido-biomolecules.¹⁴¹ The resulting covalent conjugates are relatively stable in terms of dissociation, which provides an advantage for cell and tissue targeting in complex physiological conditions. However, the covalent conjugation may affect the structures and biofunctions of the conjugated molecules (e.g., cause the denaturation of proteins). To circumvent this problem, oligo (ethylene glycol) spacers were inserted between

QDs and biomolecules.¹⁴² On the other hand, noncovalent conjugates can be formed via the electrostatic interaction between oppositely charged molecules (Figure 2.8 ⑤), the hydrophobic interactions between QDs and lipids (Figure 2.8 ④), and the strong affinity between avidin and biotin terminated molecules. These noncovalent interactions are mostly reversible and are thus appropriate for biosensing and therapeutic agent delivery applications.

Figure 2.8 Schematic illustration of representative bioconjugation methods of QDs.¹⁴⁰

2.4 Biophotonics Applications of QDs

QD technologies have found wide-ranging applications in biophotonics, which include optical imaging, biosensing, and multimodal imaging. Targeting ligands can be attached to QDs for imaging specific biological sites from tissue to molecular level. The obtained information may facilitate a better understanding of the physiological processes such as disease progression. By combining QDs with other contrast agents or using the fluorescent QDs with magnetic doping, one can achieve multimodal imaging in vitro and in vivo.^{143, 144} In addition to optical imaging, because many QDs have optical properties that are sensitive to external stimulus, they can serve as indicators for local levels of pH, temperature,

biomolecules, and specific ions.¹⁴⁵⁻¹⁴⁸ The potential uses of QDs in optical imaging, multimodal imaging, and biosensing make them promising candidates for diagnosing a variety of diseases (e.g., cancer, epidemic disease, neural disease) via an integrated approach.¹⁴⁹⁻¹⁵¹

2.4.1 Optical Imaging

Optical imaging is a non-invasive technique for visualizing biological structures and physiological processes in vitro and in vivo.^{152, 153} The main advantages of optical imaging include high scanning speed, low cost, high spatial resolution, and reduced exposure to ionizing radiation. Progress in optical imaging has enabled the monitoring of the distribution of drug molecules¹⁵⁴⁻¹⁵⁶, disease progression,^{157, 158 159} and tumor metastasis.¹⁶⁰⁻¹⁶³ Optical contrast agents for these applications should have high fluorescence efficiency, good colloidal stability, and superior photostability. Benefiting from their high QY and excellent stability, QDs can outperform organic fluorophores as optical probes in a wide range of optical imaging applications. In addition, their large surface area and modifiable surface properties allow the attachment of multiple functionalities for highly selective bioimaging. This section reviews the research progress of using QDs in the diagnosis and treatment of cancer and in understanding of neural processes. New developments in applying the extraordinary optical properties of QDs for multiplexed imaging, near-infrared imaging, and multi-photon imaging are highlighted.

The burden of cancer continues to increase as the world's population ages.¹⁶⁴ QD technology provides a useful tool for cancer detection and image-guided surgical removal of tumors. For cancer detection, QDs with the size-tunable and well-resolved emission spectra can facilitate the identification of the types and stages of cancer by using multiplexed imaging. For example, Liu et al. demonstrated the molecular mapping of human prostate

cancer tissue specimens by using multi-color QDs.¹⁶⁵ Conjugated with the secondary antibodies of four protein biomarkers, the multi-color QDs demonstrated distinct staining patterns for benign and malignant tumor cells, which revealed significant tumor heterogeneity at the molecular and cellular levels. Based on this result, researchers monitored the structural transitions of prostate glands during the cancer's progression. The transitions were observed to start with a double layer of basal and luminal cells, go through an intermediate stage with partially benign glands,

Figure 2.9 Multiplexed imaging of human prostatectomy specimens using multicolor QDs conjugated with antibodies.¹⁶⁵ (a) Benign prostate gland. (b) Largely benign prostate gland with a premalignant or malignant cell in the luminal layer (yellow arrow). (c) Prostate gland with a malignant segment (yellow arrows), a benign segment (red) and intermediate structures. (d) Malignant gland (yellow arrows) next to a benign gland. (e) Complex prostate gland in which one portion is malignant (yellow arrows) and the rest is largely benign. (f) Malignant gland. Scale bar=10 nm.

and finally lead to a single luminal cell layer of malignant cells (**Figure 2.9**). This study demonstrated the possibility of correlating the morphological and molecular information for cancer diagnosis, which has not been achieved using traditional approaches such as H&E staining and molecular profiling. As for cancer detection in vivo, unconjugated QDs were found to selectively accumulate at tumors due to the enhanced permeability and retention (EPR) effect¹⁶⁶ and thus were used for passive targeting of tumors.¹⁶⁷ To further enhance their targeting efficiency and selectivity, QDs are conjugated with small targeting ligands or antibodies for tumor-specific targeting. Tumor proliferation requires a large amount of certain vitamins (e.g., folate, biotin (vitamin H), and vitamin B12) and leads to the overexpression of the receptors involved in vitamin uptake on tumor cells,¹⁶⁸ which can be selected as the target for designing biofunctionalized QDs. For example, many types of aggressive and undifferentiated tumor cells at an advanced stage show an increase in folate receptor (FR) density and thus folic acid (FA, the synthetic form of the folate) conjugated QDs can enhance the internalization in these tumor cells.¹⁶⁹ Antibody-mediated delivery provides another efficient route for tumor-specific cell imaging. Yong et al. showed the specific targeting of pancreatic cancer cells by using InP QDs conjugated with either monoclonal antibody (anticlaudin-4) or polyclonal antibody (anti-PSCA), whereas the reduced uptake of the same QDs were observed in receptor-negative cells.¹⁷⁰ For in vivo studies, Wang et al. reported the preparation of the anti-vascular endothelial growth factor (antiVEGF) conjugated Ag₂S QDs for targeted imaging of tumors. Compared to unconjugated Ag₂S QDs, the antiVEGF conjugated Ag₂S QDs showed longer retention time at the tumor site.¹⁷¹ The visualization of the sentinel lymph nodes can benefit cancer metastasis prediction and facilitate surgical resection. Kobayashi et al. observed multi-color fluorescence lymphangiography in a mouse model by intracutaneously injecting the multi-color QDs into five sites in the ears, the upper extremity, and the chin.¹⁷² Primary

draining lymph nodes with different fluorescent colors were simultaneously visualized through the skin of the mice.

To investigate the complicated neural processes, the fluorescent imaging, especially single-molecule imaging provides a straightforward and effective technique. The reason for using single-molecule imaging is that the discrete changes of the neural cells (e.g., state transition of the channels, stepping of the molecular motors) can only be monitored by tracking single molecules.¹⁷³ For this purpose, fluorescent dyes or proteins are limited by their insufficient photostability or variable expressions. In comparison, QDs, with superior photostability and modifiable surface, are thus appropriate for long-term molecular tracking and specific targeting. Moreover, the blinking behavior of QDs could be used to identify individual QDs in a temporal image series, from which one can distinguish the attached single molecule from the molecule clusters.^{173, 174} By comparing the blinking behavior of a single QD and the fluorescent fluctuations of QD-TrkA complexes in cells, Vu's group determined that the single receptor was conjugated with the discrete single QD. Subsequently, they visualized the trafficking of individual QD-TrkA complexes in neural cells.¹⁷⁴ TrkA receptors play critical roles in the growth and regeneration of the nervous system by shuttling to different subcellular sites and propagating biochemical signalling cascades. To observe their activities, the researchers stimulated the rapid sprouting of new neural growth using nerve growth factor (NGF). In the spatiotemporal images, QD-TrkA complexes were observed to shuttle actively in a way consistent with the one reported for TrkA, in the anterograde, retrograde, as well as bidirectional directions (**Figure 2.10**). Finally, they were found in the newly formed cone tips. More recently, Kovtun et al. investigated the trafficking of dopamine transporter (DAT) using the QD-based labelling approach.¹⁷⁵ Dopamine (DA) is the neurotransmitter that modulates physiological functions in the central nervous system. A number of neural disorders (e.g., bipolar disorder, major depression, Parkinson's disease)

have shown associations with impaired DA and DAT. Based on the high affinity between the DAT to cocaine, researchers functionalized QDs ligands with a cocaine analogue for targeting DAT. The QDs showed superior photostability compared to FITC dye and are thus more appropriate for monitoring the DAT expression changes in long-term study. For in vivo imaging of the central nervous of system, the effect of using optical probes is mainly compromised by the low blood-brain barrier (BBB) permeability. To overcome this limitation, Gao et al. conjugated QD-entrapped PEG-PLA nanoparticles with a type of lectin for brain imaging in mice.¹⁷⁶ The lectin, wheat germ agglutinin, demonstrated the ability of assisting the transmigration of the nanoparticles across the BBB. The lectin-QD nanoparticles showed the brain targeting property after intranasal administration in mice.

Figure 2.10 Actively shuttled QD-TrkA receptors in neural processes.¹⁷⁴ Left panel is the DIC image of a live PC12 cell showing a junction of three neurites. The fluorescent images at right are sequential frames of a time-lapse video showing movement of QD-TrkA complexes within neurites. Red arrows indicate the anterograde shuttling and blue arrows indicate the retrograde shuttling.

Generally, the fluorescence lifetimes of QDs are longer than those of the typical dyes and the cell autofluorescence. As a result, in addition to wavelength-resolved imaging, QDs can be used for time-resolved imaging such as lifetime imaging and time-gated imaging. Heyes's

group used the CuInS₂/ZnS QDs with a PL lifetime of 21 ns for time-gated imaging.¹⁷⁷ Compared to the AlexaFluor 488 dyes, which have a lifetime slightly longer than the cell autofluorescence, the CuInS₂/ZnS QDs achieved a clearer contrast between their fluorescence and the cell autofluorescence. Therefore, QDs with a long lifetime can outperform typical dyes for suppression of cell autofluorescence in time-gated imaging. Similarly, Wang et al. used the Si QDs with 10.2 ns fluorescence lifetime for cell imaging under the fluorescence lifetime imaging (FLIM) microscope.¹⁷⁸ The fluorescence from the Si QDs could be easily distinguished from the cell autofluorescence by settling the gating periods at 5 to 12 ns and 1 to 5 ns.

A major challenge for optical imaging is to increase the penetration depth for deep tissue imaging. In the near-infrared (NIR) region, there are two optical transparent biological windows, NIR-I (650-950 nm) and NIR-II (1,000-1,350 nm) windows, which exhibit reduced attenuation of transmitted light, minimal autofluorescence, and reduced tissue scattering.¹⁷⁹⁻¹⁸¹ Therefore, NIR-emitting QDs hold great potential for deep-tissue imaging. The AgInS₂ QDs and CuInS₂ QDs can be used for imaging in the NIR-I region, and the Ag₂S QDs and Ag₂Se QDs, made from the narrower band gap materials, can be used for NIR-II imaging. For example, Deng et al. encapsulated CuInS₂/ZnS QDs in FA-modified chitosan micelles for in vivo cancer imaging in the NIR-I window.⁵¹ The fluorescent imaging showed the tumor-targeting capability of QD-chitosan-FA complexes. Multiplexed imaging in the NIR-I region was performed by simultaneously injecting QDs with 720 nm and 800 nm emissions. Two NIR signals from different QDs were well separated by using the optical wavelength filter. Using Ag₂Se QDs with emission centered at 1,300 nm, Dong et al. performed an in vivo deep imaging study in mice. The Ag₂Se QDs were modified with C18-PMH-PEG to obtain water dispersibility with high colloidal stability. The NIR-I fluorescent dye, indocyanine green (ICG), provided fuzzy images of the vasculature due to

the tissue scattering and absorbance of the transmitted light. On the other hand, the Ag₂Se QDs with NIR-II emissions provided high-resolution images of the vasculature and organs at a depth down to ~100 μ m, demonstrating the potential of using these NIR-II-emitting Ag₂Se QDs for deep tissue imaging applications (**Figure 2.11**).⁶⁸

Figure 2.11 In vivo imaging of live mice in supine position by tail injection of (a) NIR dyes, ICG and (b) Ag₂Se QDs.⁶⁸ (c) A zoom-in image of the selected zone in (b). (d) A cross-sectional intensity profile measured along the red-dashed line in (c).

Another approach to increase the imaging depth is to use the QDs with two-photon PL and excite the samples using NIR excitation. The NIR excitation has longer penetration length and can minimize photodamage to the biological tissues. Nitrogen-doped graphene QDs have been developed as contrast agents for two-photon imaging.²⁷ The cross-section of their two-photon absorption reached a high value of 48,000 Göppert-Mayer units, which far exceeds that of the conventional organic dyes. By using these QDs, researchers acquired the fluorescent images at a depth of 1,800 μ m in tissue phantom. Owing to the large two-photon absorption cross-section, good biocompatibility, and excellent photostability, these graphene QDs can be used as valuable optical probes for long-term two-photon imaging.

2.4.2 Biosensing

The sensitivity of the optical properties of QDs to the external environment can be used to identify biological species or indicate physiological parameters. The variations of the pH, temperature, the concentration of the chemical ions, and biomolecules may have an impact on the surface conditions of the QDs, which may in turn increase or decrease their PL intensity. For example, Zhu et al. developed the highly luminescent AgInS₂/ZnS QDs for monitoring the concentration of intracellular Cu²⁺ ions.¹⁸² When Cu²⁺ ions were introduced, a CuS shell layer formed on the surface of AgInS₂/ZnS QDs by displacing the original Zn²⁺ ions, which resulted in the quenching of PL. The selectivity of AgInS₂/ZnS QDs for detecting Cu²⁺ ions was examined by comparing their PL intensity change upon the addition of 17 cations. Only the addition of Cu²⁺ ions led to a significant PL quenching. Based on this result, these AgInS₂/ZnS QDs were applied to the HeLa cells and monitored the intracellular Cu²⁺ concentration by confocal microscopy. Another approach is to functionalize the surface of the QDs with the ligands sensitive to analytes. Su's group developed the fibrinogen conjugated CuInS₂ QDs to detect the thrombin in biological environments.¹⁸³ The thrombin catalyzed the polymerization of the fibrinogen on QDs surface to form an insoluble fibrin clot. As a result, the PL intensity of the supernatants decreased upon increasing the concentration of thrombin in the QD solution. In human serum samples, this work achieved a lower detection limit for thrombin than electrochemical and other fluorometry methods.

Compared to molecular fluorophores, simultaneous assays of multiple analytes by using QDs offer the advantage of providing more resolvable colors by using a single excitation wavelength. Giri et al. presented nine-plex QD-barcode assay panels for identifying the genetic biomarkers of the bloodborne pathogens HIV, hepatitis B/C, malaria, and syphilis (**Figure 2.12**).¹⁸⁴ A series of oligonucleotides were synthesized as the target strands by

Figure 2.12 Schematic illustration of QD barcode assay. (a) The basic principles for identifying the genetic biomarkers. (b) The flow chart of sequential steps involved in the assay.

mimicking the short genetic sequences of the pathogens. QD barcodes were prepared by encapsulating a mixture of different ratios of green and red emitting QDs. The resulted nanocomplexes were then conjugated with multiple capture-strands, each of which has a sequence complementary to part of the target sequences. A set of such QD barcodes were mixed with the secondary oligonucleotide conjugated fluorescent dyes and the emission of the mixture was analyzed by using the flow cytometry technique. The simultaneous detection of one type of QD barcodes and the dyes was regarded as the positive detection of the respective target sequence. Also by using flow cytometry analysis, Chattopadhyay et al. used QDs and dyes for multiplex phenotyping of the antigen-specific T-cell populations.¹⁸⁵ The minimal overlap between the emission spectra of the QDs allowed the use of seventeen

colors to analyze the proteins derived from HIV, cytomegalovirus (CMV), and Epstein-Barr virus (EBV). The above two studies provide representative examples of using multiplex QDs for diagnosis and therapy of epidemic diseases.

Another common biosensing strategy is to measure the fluorescence resonance energy transfer (FRET) between the QDs and other energy donors/acceptors such as dyes, luciferase protein, and gold nanoparticles.¹⁸⁶⁻¹⁸⁸ The FRET efficiency is critically dependent on the distance between the energy donor and acceptors. Based on this mechanism, researchers have designed many detection systems in which the analytes either function as linkers for connecting the donors and acceptors or serve as scissors for cutting off the connections between the existed donor-acceptor pairs. Recently, Xue et al. designed a detection probe for fluoride anions (F^-) based on the FRET between QDs and gold nanoparticles (AuNPs).¹⁸⁹ The diol ligands on the surface of CdTe QDs can react with the phenylborinic acid on the surface AuNPs to form boronate esters, which results in the formation of QD-AuNP assemblies. In these assemblies, the spectral overlap between the emission of the QDs and absorbance of the AuNPs allowed the FRET from QDs to AuNPs, thereby causing the PL quenching. The addition of the F^- could break apart the QDs-AuNPs assemblies by converting the cyclic boronate ester to the trifluoro borate and result in the recovery of PL. A linear correlation was found between the concentration of F^- and the PL intensity of the QDs, which enabled an accurate determination of the F^- concentration.

2.4.3 Multimodal Imaging

In addition to optical imaging, a variety of other imaging methods (e.g., computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), photoacoustic imaging) have been developed for visualizing biological structures and physiological processes in the preclinical and clinical phases. Each imaging method has its

strengths and limitations in terms of resolution, sensitivity, cost, scanning rate, and potential toxicity effects. Combining two or more imaging methods (i.e., using a multimodal imaging method) provides comprehensive information that is difficult to obtain using a single imaging method.

In multimodal imaging, QDs are commonly integrated with other contrast agents (e.g., gold nanoparticles, Raman tags, radioactive labels) to create a multifunctional nanocomplex. To integrate fluorescent and plasmonic imaging, Jin et al. combined QDs with the plasmonic gold nanostructures by using a QD core-gold-shell structure (**Figure 2.13**).¹⁹⁰ In the synthesis process, an organic layer was attached to the surface of the QDs to immobilize Au^{3+} ions for gold shell growth and to control the spacing between the QD core and gold shell to avoid PL quenching due to energy transfer. The resulting dual-modality probes exhibited overlapping dark field and fluorescent signals. Another method of integrating contrast agents is to encapsulate them in a single nanocontainer. Tan et al. developed a dual-modal nanoprobe for optical and MRI imaging by co-encapsulating QDs and iron oxides into polymer nanoparticles.¹⁹¹ The resulting nanoprobes can provide images with long penetration depth, good sensitivity, and high resolution, which combines the advantages of both methods.

Another way to endow QDs with magnetic properties is to insert paramagnetic ions into them to produce doped QDs. Typical paramagnetic ions used for preparing doped QDs include Mn^{2+} ions and Gd^{3+} ions.^{192, 193} For example, Wang et al. prepared Mn:ZnSe/ZnS/ZnMnS sandwiched QDs for combined MRI and optical imaging.⁷¹ Paramagnetic Mn^{2+} ions were doped in the core and the outer shell of the QDs to produce the dopant-related PL emissions and provide magnetic resonance properties. After encapsulating these QDs within liposomes and conjugating them with folic acids, they were successfully used as the optical probes to produce fluorescent signals and MRI contrast agents to provide

magnetic resonance signals from treated tumor cells. Similarly, $\text{CuInS}_2@\text{ZnS:Mn}$ QDs¹⁹⁴ and Gd-doped ZnO QDs¹⁹⁵ have been engineered for optical/MRI dual-modal imaging.

Figure 2.13 Gold-shell-encapsulated QDs as dual modality probes.¹⁹⁰ (a) Schematic illustration of growing a layer of gold shell on the polymeric ligand of the QD core. (b-f) Fluorescent, dark field, and merged images of the QDs with and without the gold shell.

Recently, QDs made from Cu_{2-x}Se and Cu_{2-x}S materials have demonstrated potential for multimodal imaging. Liu et al. developed a heterodimer composed of plasmonic gold nanoparticles and Cu_{2-x}Se QDs for dark-field optical imaging and photoacoustic (PA) imaging.¹⁹⁶ The Cu-deficient QDs had a large number of free holes that acted as charge carriers for generating the localized surface plasmon resonance (LSPR), which provided enhanced contrast for PA imaging. Using this heterodimer, researchers achieved a large imaging depth of 17 mm when visualizing the sentinel lymph node of a rat, thus demonstrating the clinical relevance of these heterogeneous nanoparticles. To obtain the MRI/PA dual-modality probes, Mou et al. prepared aqueous Cu_{2-x}S contrast agents through thermal decomposition followed by phospholipid encapsulation.¹⁹⁷ The nanocomplexes demonstrated passive targeting capability of tumors through the EPR effect and enhanced the MRI contrast both in vitro and in vivo. Also, they exhibited strong NIR optical absorption due to their LSPR properties, which allowed them to be used as contrast agents in PA imaging. The use of the single-component Cu_{2-x}S QDs for multimodal imaging may benefit disease diagnosis and therapy.

2.5 Toxicity of Quantum Dots

The potential toxicity effect of QDs is a key to assess the feasibility of using them in preclinical and clinical studies. Therefore, considerable effort has been devoted to understanding the underlying mechanisms of QDs toxicity using a wide range of cell and animal models. Broadly speaking, toxicity of QDs is governed by their physicochemical properties and their interaction with biological components.

The major toxicity concern arises from the highly toxic components of the commonly used Cd-based QDs. Some studies have demonstrated that Cd-based QDs can cause damage to the cells and tissues when they degrade and release Cd^{2+} ions.¹⁹⁸ To mitigate this toxicity

concern, two general methods are proposed. One is to passivate the surface of Cd-based QDs with a robust ligand shell to enhance their stability and prevent degradation.¹⁹⁹ The other method is to synthesize Cd-free QDs to avoid the use of highly toxic components. Multidentate polymers,²⁰⁰ compacted silica,^{136, 201} and large biomolecules¹⁹⁹ can be used to grow robust ligand shells. On the other hand, as mentioned in section 2.2.2, Cd-free QDs, such as InP, CuInS₂, Ag₂S, Silicon, Graphene, and doped Zn chalcogenide QDs, have been developed to minimize the potential toxicity effects in biophotonics applications. Numerous studies have examined the in vitro and in vivo toxicity of these Cd-free QDs via cell viability assay, inflammation monitoring,¹⁶⁷ blood assay,²⁰² and histological analysis.²⁰³ Most of these reports suggest a lower cytotoxicity of Cd-free QDs compared to Cd-based QDs. For example, Pons et al. observed a reduced inflammatory response in mice treated with CuInS₂/ZnS QDs compared to those treated with the same concentration of Cd-based QDs (**Figure 2.14**).²⁰⁴ On the other hand, some studies show no significant toxicity difference between Cd-free QDs and well-passivated Cd-based QDs.²⁰⁵

Figure 2.14 Regional lymph nodes resected from mice 7 days after administration of a saline buffer solution, 20 pmol of CuInS₂/ZnS QDs, and 20 pmol of CdTeSe/CdZnS QDs (left to right).²⁰⁴ The arrows indicate inflammation sites. Scale bar: 1 mm.

The size of QDs has a great impact in determining their toxicity.²⁰⁶ Size influences the uptake rate of QDs by cells²⁰⁷ and QDs biodistribution in vivo.¹³⁹ The large surface-to-volume ratio of QDs can significantly enhance surface-initiated processes. More important, the size of QDs is a major determinant for the long-term fate of QDs in the body.

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QDs with a hydrodynamic size below 5.5 nm were cleared rapidly from the body through renal excretion, but larger QDs cannot be excreted through this pathway.²⁰⁸

Surface coatings of QDs play a critical role in the induced toxicological response and can be considered from several perspectives. First, as mentioned above, robust surface coatings can prevent the degradation of QDs and thus minimize the toxicity caused by the released Cd^{2+} ions. Second, using the surface ligand molecules of different sizes can result in a variation in the hydrodynamic size of the QDs, which in turn affects the biodistribution and excretion of QDs. Third, the variation of the surface charge and surface functional groups may affect the cell toxicity response. By using CdSe/CdZnS QDs as a model, Park et al. evaluated the toxicity of QDs using positively charged, zwitterionic, and negatively charged lipoic acids as surface coatings.²⁰⁹ The QDs capped with zwitterionic ligands were found to cause minimal toxicity in cells. Also, QDs taken up by cells and tissues can interact with biological components and cause the conformational change and dysfunction of biomolecules, which may induce a toxicological response.²¹⁰ Surface coatings using neutrally charged polymers (e.g., PEG) are anti-fouling and can reduce the nonspecific adsorption of biological components.

In addition, QDs used in biological fluids may cause cytotoxicity due to the generation of reactive oxygen species (ROS). As efficient energy donors, QDs can transfer absorbed energy to surrounding molecules and generate a number of ROS species such as singlet oxygen, radical superoxide, and hydroxide. These ROS species may trigger oxidative stress, induce damage to cell organelles, and lead to cell death.²¹¹ Using the illuminated InP/ZnS QDs, Chibli et al. studied cytotoxicity induced by the generation of ROS species.²¹² Upon visible irradiation, the QDs in solution were found to generate ROS species, including superoxide and hydroxyl radical but no singlet oxygen. When applied in cells, the InP/ZnS QDs caused a more significant cytotoxicity in the presence of light.

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Finally, the toxicological response is highly dependent on the applied cell lines and animal models,^{213 214} which make it difficult to compare the toxicity of different QDs formulations. Therefore, a set of generalized standards needs to be established to assess and compare the toxicity of QDs.

Chapter 3 Microfluidic Synthesis of Biofunctionalized CdTe QDs

As introduced in Chapter 2, biofunctionalized QDs provide a useful tool for cell labelling, drug/gene delivery and bioanalytical applications.²¹⁵ Currently, the preparation of biofunctionalized QDs is often laborious and needs multiple steps including synthesis, phase transfer and bioconjugation. A facile preparation approach is required to drive forward the progress of employing the biofunctionalized QDs for clinical diagnostics such as immunoassay. In this chapter, a one-step microfluidic synthesis method was proposed for fabricating the bovine serum albumin (BSA) functionalized CdTe QDs. Compared to the QDs obtained by conventional bench-top method, the microfluidic synthesized BSA-QDs exhibited an improved functionalization efficiency and enhanced stability. The microfluidic synthesis approach also offers additional advantages of shortened reaction time, minimal chemical waste and low cost.

3.1 Introduction

Up to now, cadmium (Cd)-based QDs are the most extensively used QDs in biophotonics applications owing to their excellent optical properties and relatively matured synthesis technology.²¹⁶⁻²¹⁸ CdTe QDs is a representative example which exhibit emissions ranging from visible to near-infrared (NIR) region and thus can be applied for multiplexed sensing and imaging.^{219, 220} For specific biological applications, these QDs are required to be modified with functionalities (e.g. vitamins, proteins, genes) for targeting desired molecules, cells and tissues.^{221, 222} The commonly adopted method for preparing the functionalized Cd-based QDs needs multiple steps including organic phase synthesis, organic-aqueous phase transfer, biomolecule conjugation, and purification procedures after each step.^{10, 11} This multi-step preparation method provides flexibility for versatile applications but increases the

difficulty of obtaining the reproducible biofunctionalized QDs. Researchers attempt to simplify the preparation procedures, but challenges remain in synthesizing the biofunctionalized QDs in a single step because the reaction conditions for yielding the high-quality QDs are unfavorable for retaining the bioactivity of the biomolecules. In the commonly used bench-top synthesis, continuous heating at a high temperature are required to obtain QDs with good crystallinity, high PL efficiency and superior stability. However, this long-term heating usually results in the degradation of biomolecules. On the other hand, shortening the reaction time and lowering the temperature may compromise the PL property and colloidal stability of the QDs. Therefore, new approaches need to be developed to synthesize the biofunctionalized QDs with simplified procedures and retained bioactivity of the biomolecules.

Compared to the bench-top (BT) synthesis in macroscale reaction vessels, the microfluidic (MF) method enables the synthesis of nanoparticles in a volume ranging from microliter to picoliter by adjusting the dimensions of the MF channels.²²³ The miniaturized synthesis can shorten the reaction time for producing the crystalline QDs and thus are more promising to retain the activity of the biomolecules. Other advantages of using MF synthesis for preparing QDs include: (i) the miniaturized synthesis system can avoid the generation of large amount of chemical wastes during the trial-and-error stage; (ii) the shortened reaction time of synthesizing QDs allows one to rapidly evaluate a series of recipes to optimize the synthesis parameters; (iii) the rapid heat transfer rate and the controllable fluid mixing will facilitate the production of homogeneous nanocrystals;^{157 224} (iv) the MF devices are customizable and can be integrated with other modules for preparing multifunctional nanoparticles for desired applications.^{225, 226 227, 228} In 2002, Mello's group reported the first MF synthesis of nanocrystals.²²⁹ The CdS nanoparticles synthesized by using the MF chip showed improved monodispersity compared to those synthesized by using BT method. Since

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then, a variety of metallic, metal oxide and semiconductor nanocrystals such as Ag,^{230, 231} Au,^{232, 233} Fe₃O₄,²³⁴ SiO₂,²³⁵ CdSe,²³⁶⁻²³⁸ CdTe,²²⁴ ZnSe,²³⁹ and InP²⁴⁰ nanoparticles have been successfully prepared using MF synthesis method.²⁴¹ The highly controllable reaction conditions in MF synthesis allow the reproducible production of nanoparticles with almost identical properties, which facilitate the quantitative assay in clinical diagnosis applications.²⁴² Despite all the above advantages, the MF method also shows some limitations. Due to the limited volume of the microfluidic channel, the MF synthesis is suitable for small-volume production of QDs but is tedious for mass production. Compared to BT method, it also requires an additional preparation step of fabricating microfluidic chips. Therefore, the suitability of the MF and BT method for the QD synthesis need to be determined by the application requirements.

In this chapter, a MF synthesis method was proposed for preparing the protein-functionalized CdTe QDs. The parameters of MF channels were optimized using finite element simulation as references for designing the microfluidic chips. Accordingly, the MF chips were fabricated and used to prepare the bovine serum albumin- (BSA-) passivated CdTe QDs. The optical properties, photochemical stability, and the surface functionality of the synthesized QDs were characterized and compared with those of the QDs synthesized via the BT method. The MF synthesis resulted in higher protein functionalization efficiency, improved colloidal stability, and enhanced photostability of the QDs. By using the same MF synthesis method, the folic acid- (FA-) functionalized QDs were obtained and they demonstrated the potential for targeted cell labelling.

3.2 Experimental Methods

3.2.1 Materials

Cadmium perchlorate hydrate ($\text{Cd}(\text{ClO}_4)_2 \cdot x\text{H}_2\text{O}$, 99.999%), tellurium powder (Te, 99.8%), 3-Mercaptopropionic acid (MPA, $\geq 99\%$), sodium borohydride (NaBH_4 , 99.99%), bovine serum albumin (BSA, $\geq 98\%$) and folic acid (FA, $\geq 97\%$) were purchased from Sigma-Aldrich. Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were obtained from Sinopharm Chemical Reagent Co., Ltd. The Dulbecco's modified Eagle's medium (DMEM medium) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT). Penicillin and streptomycin were obtained from Gibco Laboratories (NY, USA). All chemicals were used as received. 18.2 M Ω •cm deionized (DI) water was used through the experiments.

3.2.2 Simulation Study of the QD Synthesis in Microfluidic (MF) Channels

The MF synthesis of CdTe QDs was simulated by using the software COMSOL Multiphysics 4.3a to study the effect of reaction temperature and MF channel dimensions. The MF channel was modeled as shown in **Figure 3.1**. The laminar flow were simulated by solving the Navier–Stokes equation (Equation 3.1), the continuity equation (Equation 3.2) and the mass balance equation (Equation 3.3),

$$-\nabla \cdot \mu \left(\nabla u + (\nabla u)^T \right) + \rho(u \cdot \nabla u) + \nabla p = F \quad (3.1)$$

$$\nabla \cdot u = 0 \quad (3.2)$$

$$\nabla \cdot (-D\nabla c + cu) = 0 \quad (3.3)$$

where μ is the viscosity, u is the velocity, T is the reaction temperature, ρ is the density, p is the pressure, F represents the sum of the body forces, D represents the diffusion coefficient, and c is the concentration. The viscosity (μ) and density (ρ) of the fluid were set as the values

of water that relative to temperature (T). Diffusion coefficient (D) was set to be $5 \times 10^{-7} e^{-2000/T}$. At the inlet of the channel, the flow rate was set to be $500 \mu\text{l min}^{-1}$ and the concentration of Cd^{2+} and Te^{2-} reactive species was set to be 10 mM and 3.3 mM, respectively. The velocity is set to be zero at the channel walls based on the boundary conditions.

3.2.3 MF and Bench-Top (BT) Synthesis of CdTe QDs

The nucleation process for synthesizing MPA-CdTe QDs were performed by following the previous reports^{220, 243} Prior to the synthesis, Te precursors were prepared by reducing 4 mg of Te powder using 10 mg of NaBH_4 in 1ml of deoxygenated DI water. 31 mg of $\text{Cd}(\text{ClO}_4)_2$ and 0-17 μl of MPA were mixed in 20 ml of deoxygenated water in the flask. The pH of the mixture was adjusted to 9 by drop addition of the NaOH solution. Afterwards, the colorless (or light pink) Te precursors were injected below the surface of the mixture. The reaction temperature was slowly elevated to 98°C under N_2 flow. For the MF synthesis, 10 ml of the reaction mixture was collected as the nucleates of CdTe QDs 2 min later. The nucleate solution were unstable and should be used freshly prepared. For the BT synthesis, the reaction mixture was kept at 98°C under stirring for another 4 hours.

For nucleation of BSA-CdTe QDs, 2 ml of PBS solutions of BSA ($0\text{-}5.0 \text{ mg mL}^{-1}$) were mixed with 31 mg of $\text{Cd}(\text{ClO}_4)_2$, 0-17 μl of MPA in 20 ml of deoxygenated water at room temperature. The pH of the solution was adjusted to 9 by using NaOH solution. Under vigorous stirring, the freshly prepared Te precursors were rapidly injected into the mixture and the reaction temperature was elevated to 65°C under N_2 atmosphere. For the BT synthesis, the reaction mixture was stirred at 65°C for 4 h. For the MF synthesis, after 2 min of injection, 10 ml of the mixture solution was collected as nucleates of BSA-CdTe QDs. The nucleates of folic acid (FA)-CdTe QDs were prepared by following the same procedure except using the PBS solutions of $0\text{-}6.4 \text{ mg mL}^{-1}$ FA instead of BSA solutions.

For MF synthesis of QDs, the MF chips with different channel widths and lengths were fabricated by using the soft-lithography and replica-molding techniques.^{226, 244} The nucleate solutions of MPA-, BSA- and FA-CdTe QDs were respectively injected into the MF channels at a flow rate of 500 $\mu\text{l min}^{-1}$ by using a syringe pump. The MF chips were preheated on a hot plate with temperatures setting at 50, 65, 85, 100°C for preparing BSA- and FA-CdTe QDs. The QDs were collected at the outlet and washed with ethanol by centrifugation.

3.2.4 Characterization of CdTe QDs

According to the Lambert-Beer's law, the molar concentration of CdTe nanocrystals can be estimated by using the equation,

$$C = \frac{A}{\epsilon L} \quad (3.4)$$

where C is the molar concentration of the QDs, A is the absorbance of the first exciton peak, ϵ is the molar extinction coefficient, L is the optical path length and was fixed at 10 mm here. Here the ϵ value was calculated based on an empirical function of the size of the CdTe nanocrystals (Equation 3.5).²⁴⁵ Since the prepared QD products exhibited emissions mostly centered at 460 nm, the diameter (D) of the CdTe nanocrystals was estimated to be 1.5 nm.

$$\epsilon = 10043 (D)^{2.12} \quad (3.5)$$

Then, the particle number of CdTe QDs per liter was then calculated by using Equation 3.6,

$$n_p = CN_A \quad (3.6)$$

where N_A is the Avogadro constant.

The UV-vis absorption spectra of the QD solution samples were obtained by using a UV 2450 spectrophotometer (Shimadzu, Japan). The PL spectra were collected by a Fluorolog-3 Fluorometer (HORIBA Jobin Yvon, Edison, NJ USA) under 400 nm excitation. For Fourier

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transform infrared (FT-IR) spectroscopy, the powder form of the QDs were mixed with potassium bromide (KBr) and compressed into pellets. The FT-IR spectra were obtained using a Shimadzu infrared spectrometer. For photostability test, QDs solution were continuously irradiated under a G10T8 UV lamp (Watts: 10 W, Spectral Output: 254 nm) and the PL spectra were collected at a time interval of 1 h. For evaluation of the colloidal stability, the purified QDs were dispersed in aqueous solutions with pH levels of 5-12. The PL spectra were recorded once every 12 hours and the hydrodynamic diameter were monitored by a 90Plus particle size analyzer (Brookhaven Instruments, NY USA). The functionalization efficiency of the BSA-QDs was studied using the agarose gel electrophoresis. 10 μ l of the purified QDs solution was mixed with 30 μ l of NaOH solution (pH=12). The mixtures were subjected to electrophoresis in 1% agarose gel buffered with 1 $\times$ Tris-Acetate-EDTA solution at a voltage of 110 V for 15 min.

3.2.5 Cell Studies of MF-BSA-QDs and MF-FA-QDs

RAW264.7 macrophage cells and Panc-1 pancreatic cancer cells were cultured in DMEM medium supplemented with 10% FBS, 100 μ g ml⁻¹ of penicillin and 100 μ g ml⁻¹ of streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. Prior to treatment with QDs, cells were seeded onto cover glasses in a 6-well plate and cultured in DMEM medium. The QDs solutions were diluted with PBS buffer (pH=7.2) and added to the culture wells at a final concentration of 10-20 μ g ml⁻¹. After 4 h of incubation, the treated cells were washed with PBS buffer for three times. Nikon Eclipse Ti inverted Microscope (Nikon, Japan) equipped with a mercury illuminator was used for fluorescence imaging study.

3.3 Results and Discussion

3.3.1 Simulation and Experimental Study on MF Synthesis of CdTe QDs

In the MF synthesis of the BSA-CdTe QDs, the MPA and BSA molecules serve as the coordination ligands to control the growth of the QDs and surface ligands to stabilize the QDs in aqueous dispersion. The thiol groups of the MPA molecules and the amino acid residues of the BSA molecules were assumed to coordinate with the Cd^{2+} ions.^{246, 247} The presence of multiple functional groups on BSA molecule allows the multivalent anchoring of BSA onto the QD surface, which improves the stability of QDs in aqueous solvent. An additional reason for selecting BSA is its good biocompatibility.^{248, 249}

For the MF synthesis, the effect of temperature on the growth rate of the QDs was firstly studied using simulation approach. The Reaction Engineering (*re*) model in COMSOL Multiphysics 4.3a was applied to predict the evolution of the concentration of the synthesized QDs over reaction time at a specified temperature. Using a MF channel model with $200\ \mu\text{m} \times 200\ \mu\text{m}$ channel cross section, the distribution profiles of the concentration of BSA-QDs in the MF channels under reaction temperature of 50°C , 65°C , 85°C and 100°C were calculated and plotted (**Figure 3.1a**). With the increase of the reaction temperature from 50 to 100°C , the concentration of the QDs was predicted to increase from 1.6 mM to 2.4 mM at 5 min of reaction time (Figure 3.1b). Experimental results showed a similar trend on the effect of reaction temperature. Calculated from the absorbance measurements data and Lambert-Beer's law,²⁴⁵ the total particles number of synthesized BSA-QDs increased from $1.9 \times 10^{18}\ \text{L}^{-1}$ to $3.2 \times 10^{18}\ \text{L}^{-1}$ when the reaction temperature was elevated from 50 to 100°C (Figure 3.1c). This result agrees well with the simulation data.

Figure 3.1 Effect of reaction temperature on the microfluidic (MF) synthesis of BSA-CdTe QDs.²⁵⁰ (a) Simulation model to predict the concentration distribution of BSA-QDs in the microfluidic channels at different reaction temperatures. (b) Simulation results showing the concentration of BSA-CdTe QDs as a function of time under the reaction temperatures of 50, 65, 85 and 100°C. (c) Experimental results showing the concentration of BSA-CdTe QDs prepared via MF synthesis under different reaction temperatures at the reaction time of 5 min.

Figure 3.2 Effect of channel width and channel length on the fluid velocity and QDs concentration. (a) Simulation models for calculating the velocity and concentration of the QD fluids within MF channels of different widths. The channel height and length was fixed at 200 μm and 50 mm. (b) Experimental results showing the concentration of BSA-QDs synthesized within the MF channels of different widths (200 μm, 400 μm and 600 μm) or lengths (800 mm and 2000 mm) under 65 °C. All the samples were collected at the reaction time of 5 mins. The flow rate were set to be 500 μl min⁻¹ in simulation and experiments.

Besides from the reaction temperature, the dimensions of the MF channels can make a pronounced effect in the properties of the produced nanocrystals.^{223, 251} Simulation study was performed to understand the effect of channel width on the fluid velocity profiles and the concentration distribution profiles of the QDs by using the Transport of Diluted Species (*chds*) and Laminar Flow (*spf*) modules. **Figure 3.2a** shows the theoretically predicted distribution profiles of the velocity of the QDs the MF channels with widths of 200 μm, 400 μm and 600 μm. The channel length is fixed at 50 mm to shorten the computation time and save the storage space. In the straight section of the MF channels, the velocity of QDs fluid increases with decreasing the channel width. This can be explained by Equation 3.7 which presents an

inversely proportional relationship between the fluid velocity and the channel width when the channel height and flow rate are fixed²⁵².

$$v = \frac{Q}{A} = \frac{Q}{WH} \quad (3.7)$$

where v is the fluid velocity, Q is the flow rate, A is the cross-sectional area of the MF channel, W is the channel width and H is the channel height. In straight sections of the MF channel, the narrowed channel width resulted in an increased velocity by fixing the flow rate. From the simulation results (Figure 3.2a), one can see that the fluid at the bend of MF channels also shows an increased velocity upon narrowing the channel width. Afterwards, experimental attempts were made to understand the effect of channel widths by fabricating the BSA-CdTe QDs in MF chips with channel widths of 200 μm , 400 μm and 600 μm . The channel length was fixed at 800 mm and the reaction temperature was set at 65°C for all of these attempts. As shown in Figure 3.2b, the particle numbers of BSA-QDs increased from $1.6 \times 10^{18} \text{ L}^{-1}$ to $2.8 \times 10^{18} \text{ L}^{-1}$ when decreasing the channel width from 600 μm to 200 μm . The narrower MF channels resulted in an increased yield of the QDs at the outlet, which may result from the less deposition of QD by-products on the channel wall at a higher flow velocity. Another advantage reported for using the narrower MF channels is that they produced nanoparticles with improved monodispersity owing to a more homogeneous reaction.^{236, 241} Besides, the effect of MF channel length on the concentration of the synthesized QDs was investigated by simulation study. At a fixed channel width, the concentration of QDs was estimated to increase from 5.06 mM to 6.95 mM when the channel length was increased by 2.5 times (top left inset, Figure 3.2a). This was verified by the experimental result that showed a slight increase of the particle number when using a MF chip with a lengthened channel (Figure 3.2b). The generation of larger population of QDs was attributed to the prolonged residence time of the QDs in the longer MF channel.^{238, 241} In

summary, the microfluidic channel with larger length and smaller width (length : 2000 mm, width : 200 μm) obtained good quality of CdTe QDs with high concentration.

Figure 3.3 Electrophoretic mobility of BSA-CdTe QDs synthesized in MF channels under reaction temperatures of 65°C, 85°C and 100°C.

The attachment of BSA molecules on the QDs surface was confirmed by using gel electrophoresis and FT-IR spectroscopy. Gel electrophoresis experiments were performed on the CdTe QDs synthesized via MF method at different reaction temperatures. Under external electric field, the CdTe QDs resulted in two separate bands representing QDs with different electrophoretic mobility (**Figure 3.3**). The upper band was attributed to the MPA-CdTe QDs with higher mobility while the lower band was attributed to the BSA-CdTe QDs with retarded mobility because of their relatively large size. At the lower band, the QDs synthesized at 65°C exhibited a stronger fluorescence compared to the QDs synthesized at 85°C and 100°C. At the upper band, almost no fluorescence was observed for the BSA-CdTe QDs synthesized at 65°C while considerable fluorescence was emitted from the ones synthesized at 85°C and 100°C. This indicated that the majority of QDs synthesized at 65°C were functionalized with BSA molecules while higher reaction temperature caused the decrease in the functionalization efficiency of BSA. This was attributed to the denature of BSA molecules under high temperatures. To further verify the conjugation of BSA molecules on QDs surface, surface compositions of the QDs synthesized at 65°C were characterized by

Figure 3.4 FT-IR spectra of the free BSA, BSA-(MPA) Cd²⁺ complex and BSA-(MPA) CdTe QDs synthesized in MF chips under 65°C.

FT-IR spectroscopy (**Figure 3.4**). The two peaks at 1653 cm⁻¹ and 1454 cm⁻¹ in the spectra of free BSA were respectively attributed to the C=O bond of the carboxyl group and C-N bond of the imidazole group in the amino acid residues. These two peaks became less pronounced or negligible in the spectra of BSA-(MPA) Cd²⁺ ion complex and BSA-(MPA) CdTe QDs, indicating the interaction of the carboxyl group and histidine residue with Cd²⁺ and CdTe QDs. The peaks at 1402 cm⁻¹ in the spectra of BSA-(MPA) Cd²⁺ and BSA-(MPA) CdTe QDs were attributed to the COO⁻ group of MPA, which originated from the coordination of MPA with Cd²⁺ and CdTe QDs. These results suggested that the MSA and BSA molecules can chelate with Cd²⁺ ions to form complex and anchor on the surface of CdTe QDs^{253, 254} in the MF synthesis process. Based on above characterizations, the optimum reaction temperature was chosen to be 65°C to increase the functionalization efficiency of BSA-CdTe QDs for biomedical applications.

3.3.2 Comparative Study on MF and BT Synthesis Method

The PL spectra of the prepared BSA-CdTe QDs via BT and MF synthesis method were compared and showed in Figure 3.5. Prior to the spectra collection, the QD solutions were diluted to the same absorbance at the excitation wavelength. Two PL spectra are similar in shape and peak position. The QDs synthesized via MF method exhibited a slight higher PL intensity than the QDs obtained by the conventional BT method. It should be noted that the QDs sample synthesized via MF synthesis were obtained at 5 min while the QDs synthesized via BT method were collected from flask at 4 h. The spectra comparison indicates a higher PL efficiency of the QDs by using MF method compared to the conventional BT method. The quantum yield (QY) of the BSA-QDs synthesized via microfluidic (MF) method is determined to be 16.2% by using Rhodamine 123 (QY=96% in ethanol) as the reference.

Figure 3.5 Photoluminescence spectra of the BSA-CdTe QDs synthesized via MF method in 5 min (red line) and BT method in 4 h (black line). The QD solutions were diluted to the same absorbance at the excitation wavelength.

The protein functionalization efficiency of the QDs synthesized via MF and BT method were investigated by examining the electrophoretic mobility of the QDs. **Figure 3.6** shows

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gel electrophoresis results of the BSA-CdTe QDs obtained using either MF or BT method. The QDs synthesized by BT method with MPA but without BSA resulted in an upper band in the gel (first left lane). By using BSA at the same concentration of 0.5 mg ml^{-1} , the QDs synthesized via MF method showed a weaker fluorescence in the upper band and a stronger fluorescence in the lower band compared to the ones produced by BT method. The results indicated that the synthesized QDs were partially functionalized with BSA by using the 0.5 mg ml^{-1} BSA solution in synthesis process. Comparatively, the MF method achieved a higher functionalization efficiency. The possible causes could be: 1) The denaturation degree of the BSA is reduced in the microfluidic synthesis because of the shortened reaction time; 2) The rapid heat transfer and homogeneous precursor mixing in microfluidic synthesis may help to improve the protein functionalization efficiency. For QDs synthesized with 2.0 mg ml^{-1} BSA solution, no fluorescence was observed from the upper band, suggestion a complete BSA functionalization of the QDs under this reaction condition. This study demonstrates that the MF synthesis method resulted in a higher protein functionalization efficiency of QDs when compared to BT method, and the appropriate BSA concentration in synthesis of BSA-CdTe QDs is 2.0 mg ml^{-1} .

Figure 3.6 Electrophoretic mobility of BT-MPA-QDs, BT-BSA-QDs and MF-BSA-QDs synthesized with 0.5 mg ml^{-1} and 2 mg ml^{-1} BSA at 65°C .

The colloidal stability and photostability are two important factors to be considered for QDs used in biophotonics applications.²⁵⁵ The colloidal stability of the prepared BSA-CdTe QDs was evaluated by monitoring the hydrodynamic sizes of the QDs dispersed in solutions with pH ranging from 5 to 12 at different time periods. The time evolution of the hydrodynamic sizes of the QDs synthesized by MF and BT method are shown in **Figure 3.7a** and **b**. In solutions with pH=12, the QDs prepared by MF and BT methods showed almost no changes in their hydrodynamic sizes during 60 h. The good stability of the QDs in alkaline solutions is attributed to the increased repulsive force between the QDs in the presence of excess OH⁻ ions, which can facilitate the dissociation of the anionic groups on the BSA and MPA molecules.²⁵⁶ In solutions at pH 5, 7 and 9, the hydrodynamic sizes of the QDs increased during 8-24 h and the QDs synthesized via MF method exhibited a slower size increase compared to those obtained via BT method. Overall, the BSA-CdTe QDs showed a pH-dependent colloidal stability, and the QDs synthesized by MF method showed comparable or higher colloidal stability compared to the ones prepared by BT method. Besides from the colloidal stability, the photostability of the BSA-CdTe QDs was evaluated and compared. **Figure 3.8** showed the time evolution of the QDs PL under continuous UV radiation. With increasing the irradiation time, the QDs synthesized via MF method exhibited a slower photobleaching rate than the QDs obtained via BT method. After 7 h UV radiation, the PL intensity dropped 74% for the QDs synthesized by MF method, and 90% for the QDs synthesized by BT method. This indicates the QDs synthesized by MF method have an improved photostability compared those obtained via BT method. The improved photostability may result from the higher functionalization efficiency and the better crystallinity of the microfluidic synthesized QDs.

Figure 3.7 Comparison of colloidal stability between BSA-QDs synthesized using MF method in 5 min and BSA-QDs synthesized using BT method in 4 h. The QDs were dissolved in solutions with different pH values (5, 7, 9 and 12) for 60 h.

Figure 3.8 Comparison of photostability between BSA-QDs synthesized using MF method in 5 min and BSA-QDs synthesized using BT method in 4 h. The intensity of the PL maximum were recorded at different UV irradiation time.

3.3.3 Cell Imaging Using CdTe QDs Synthesized via MF Method

To examine the potential of the prepared QDs for biophotonics applications, the BSA-CdTe QDs were applied for live cell imaging by using the RAW264.7 macrophage cell line. As

shown in **Figure 3.9a**, strong fluorescence signals were observed from the cells incubated with the BSA-CdTe QDs. The result indicates that the BSA-CdTe QDs prepared via MF method are promising optical probes for bioimaging. In this study, BSA serves as a model for protein molecules. Other biocompatible molecules with functional groups that can coordinate with Cd^{2+} are also promising coordination and surface ligands. The type, density, and ratio of the functional groups on the ligand molecules can affect the growth kinetics and the

Figure 3.9 Microscopic images of (a) RAW264.7 cells treated with BSA-CdTe QDs, (b) Panc-1 cells treated with MPA-CdTe QDs and (c) Panc-1 cells treated with FA-CdTe QDs. All the QDs used were prepared by MF method.

stability of QDs. For targeted cell imaging, folic acid (FA)-functionalized CdTe QDs were prepared by using the same MF synthesis method. FA is a commonly used targeting molecule for cancer cells that overexpressed folate receptors (FR). Figure 3.9b and c respectively show the fluorescence microscope images of Panc-1 cells treated with MPA- and FA-functionalized QDs. The Panc-1 cells treated with MPA-QDs exhibited weak fluorescence signals while those stained with FA-QDs showed clear fluorescence mostly from the vesicles within the cells. This indicates that the MF synthesis of QDs using FA as ligands can produce QDs with active FA on the surface for facilitating the uptake of QDs in FR-overexpressed cancer cells. In the near future, we envision that the one-step MF synthesis method can be applied to speed up the procedure of preparing the peptide- or antibody-functionalized QDs for *in vitro* and *in vivo* imaging.

3.4 Conclusions

In summary, a detailed study was performed on microfluidic (MF) synthesis of biofunctionalized QDs. Compared to the conventional bench-top (BT) synthesis method, the MF synthesis method provides a more convenient and time-saving route for preparing the aqueous QDs. The simulation study and experimental study were combined to investigate the effect of reaction temperature and dimensions of MF channels on the growth of BSA-CdTe QDs. The results agree well on that the reaction temperature and the dimensions of MF channels play an important role in determining the production yield of QDs. The successful functionalization of the BSA on the QDs surface was confirmed by gel electrophoresis and FT-IR spectroscopy. The same MF synthesis method can be applied to synthesize the folic acid-functionalized QDs. By using the biofunctionalized QDs obtained via microfluidic synthesis method, we carried out the cell imaging study and demonstrated the potential of applying this method to prepare the functionalized QDs for specific biophotonics applications. This facile synthesis method is expected to have a positive impact on developing the

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integrated MF system for producing the bioconjugated QDs, which are capable to be directly used in bioimaging without going through tedious procedures of phase transfer, bioconjugation and multiple cycles of purification.

Chapter 4 The Effects of Surface Ligands on the Physicochemical Properties of QDs for Biophotonics Applications

In Chapter 3, the water-dispersible QDs were synthesized in an aqueous reaction system containing hydrophilic coordination ligands. In this chapter, the QDs were obtained in organic solvents and achieved water-dispersibility by organic-aqueous phase transfer. Versatile ligands used in phase transfer process endow the QDs with different functional groups for future bioconjugation. Because the surface ligand layer directly interacts with the biological components, the type of surface ligands will greatly affect the response from the cells. In this chapter, seven types of hydrophilic ligands with different chain lengths and functional groups were applied to render the organically synthesized CdSe/CdS/ZnS QDs water-dispersible via ligand exchange method. After phase transfer, a systematic study was performed to investigate the effect of surface ligands on the colloidal stability, photostability, cellular uptake and in vitro cytotoxicity of the surface-modified QDs. In general, each set of the prepared QDs with specific type of surface ligands displays certain strengths and limitations in different aspects of their physicochemical properties. Therefore, one should carefully choose the surface ligands to prepare the water-dispersible QDs for minimizing the impacts arising from their limitations.

4.1 Introduction

For QDs applied for biophotonics purpose, a significant challenge remains in preserving the optical properties of the water-dispersible QDs in physiological conditions. Currently, many of QDs are known to have short shelf lives from a few hours to a few days in aqueous solution or biological buffers.^{120, 257-259} As time progresses, the QDs will precipitate or aggregate, thereby adversely impacting their

optical properties. Such phenomena may lead to the skewing of data when different quality of QDs is used in biophotonics applications. Thus, there is an urgent need to investigate and understand the root cause of the degradation of the QDs optical properties in biological media. The majority of QDs are prepared in organic solvents followed by a phase transfer process to achieve water dispersibility.²⁶⁰⁻²⁶² The phase transfer process not only makes the QDs dispersible in water, but also allows one to introduce additional functionalities on the QDs surface, such as conjugation with antibodies for targeted imaging or carrying siRNA for gene delivery. Thus, it is critical to carefully design and optimize the phase transfer methods for preparing QDs with long shelf life and comparable optical properties as they are dispersed in organic phase.

As introduced in Chapter 2, two general approaches, encapsulation method and ligand exchange method, are developed for phase transfer of QDs. The encapsulation method protect the QD core from degradation but may cause the overall QDs size to increase and impact the mobility of the QDs in restricted region.^{120, 263} The ligand exchange method is simpler and could retain the small size of the QDs by using heterobifunctional ligands containing functional groups attaching on QDs surface, and other functional groups extending out to achieve water dispersibility.^{88, 264, 265} Mercaptopropionic acid, cysteine, and thiolated PEG molecules are several examples of commonly used ligands for ligand exchange of hydrophobic QDs. The different types of ligands used for surface modification of the QDs will greatly influence their physical, colloidal and optical properties. For instance, the hydrodynamic size of QDs is governed by the length and structure of the surface ligands.²⁶⁶ The surface charge of QDs is determined by

the existence of cationic/neutral/anionic functional groups on the surface ligands and their grafting density on QDs surface.²⁶⁷ The solubility of the QDs is affected by intermolecular forces and the polarity of the surface ligands and the solvents. The interactions between the solvent and the surfactants, as well as the attachment/detachment of surfactants onto the QDs surface, determine the colloidal stability of QDs. This is why QDs functionalized with less polar ligands like mercaptohexadecanoic acid do not disperse well in water but they do disperse to a good extent in most organic solvents.²⁶⁸ Also, it is reported that the precipitation and degradation of QDs are governed by the types of surface ligands functionalized on their surface.²⁶⁹

In this chapter, a comprehensive study was performed to investigate the effects of surface ligands on the physicochemical properties of QDs. The systematic results can be utilized to evaluate the suitability of each type of ligand-functionalized QDs for intended biological applications. Specifically, seven types of surface ligands with different chain lengths and functional groups (mercaptopropionic acid, mercaptosuccinic acid, cysteine, cysteamine, methoxy-PEG-thiol, carboxymethyl-PEG-thiol and amine-PEG-thiol) were chosen to prepare the water-dispersible CdSe/CdS/ZnS QDs via ligand exchange method. Afterwards, we performed a comparative study on the colloidal stability, photostability, cellular uptake and cytotoxicity of the seven QD formulations. Some important guidelines are highlighted to prepare good quality of QDs with enhanced stability and desirable cellular response. This study provides useful perspective on understanding the effects of surface ligands in designing specific type of QDs for biomedical applications.

4.2 Experimental Methods

4.2.1 Materials

3-Mercaptopropionic acid (MPA), DL-Cysteine (Cys), mercaptosuccinic acid (MSA), 2-aminoethanethiol hydrochloride (cysteamine hydrochloride, AET•HCl), methanol, chloroform, and ammonium hydroxide solution (28.0%-30.0% NH₃ basis) were purchased from Sigma-Aldrich. n-Hexane (95%) was purchased from Tedia. Carboxymethyl-PEG-Thiol (CM-PEG-SH), methoxy-PEG-Thiol (mPEG-SH) and amine-PEG-Thiol (NH₂-PEG-SH) with average molecular weight of 5000 g mol⁻¹ were obtained from Laysan Bio Inc. CertiPUR Buffer Solutions (pH = 4.00, 7.00, 10.00) were purchased from Merck. Phosphate buffered saline buffers (PBS, pH = 7.2) were products of Gibco. 18.2 MΩ•cm deionized water (DI water) was used through the experiment. All chemicals were used as received.

4.2.2 Preparing Water-Dispersible QDs Using Ligand Exchange Method

CdSe/CdS/ZnS QDs capped with TOP/TOPO were prepared using hot colloidal synthesis method based on previous report.²⁵⁵ The obtained hydrophobic QDs were dissolved in chloroform and washed with ethanol before use. Seven types of mercapto ligands, including the short-chain mercapto ligands (MPA, MSA, Cys and AET) and thiolated PEG derivatives (mPEG-SH, CM-PEG-SH and NH₂-PEG-SH), are used to replace the original TOP/TOPO surfactants on QDs surface. In this study, we have optimized the reaction parameters in the phase transfer process for obtaining water-dispersible QDs with enhanced PL intensity as well as good biocompatibility. For preparing QDs capped with MPA, MSA or Cys, 4 µl of MPA in chloroform, or 33 mg of MSA in chloroform, or 4 mg of Cys in methanol was mixed with 1 mg of

TOP/TOPO-capped CdSe/CdS/ZnS QDs dispersed in 5 ml of chloroform and stirred for 5 min. Subsequently, 1 ml of the mixture of 28%-30% $\text{NH}_3 \cdot \text{H}_2\text{O}$ and H_2O with optimal ratio (1:9 for MPA and MSA, 3:7 for Cys) was added. The biphasic solution system was stirred for 5 h or until the majority of QDs were transferred to the aqueous phase. The aqueous phase were then collected and washed by centrifugation with ethanol at 10000 rpm. The precipitation was redispersed in 1 ml of DI water. For preparing QDs capped with AET, 100 μl methanol solution of AET $\cdot\text{HCl}$ (5 mg ml^{-1}) was added drop wise into 1 mg of CdSe/CdS/ZnS QDs dispersed in 5 ml of chloroform, followed by vortex shaking. After flocculation of QDs, the DI water was added to dissolve the QDs by gently shaking. The water phase was collected, centrifuged to remove the agglomerates, followed by filtering to remove the excessive AET and other impurities. To prepare the QDs capped with thiolated PEG derivatives, 8 mg of mPEG-SH, or 5 mg of CM-PEG-SH, or 10 mg of NH_2 -PEG-SH was mixed with 1 mg of QDs dispersed in 2 ml of chloroform solution and stirred overnight. The QDs were then precipitated by adding the hexane. The precipitation was washed with the mixture of ethanol and hexane (v/v ratio 3:5) and redispersed in 1 ml of DI water.

4.2.3 QDs Characterization

Transmission electron microscopy (TEM) images were obtained using a JEOL JEM-2010 microscope. Fourier transform infrared (FT-IR) spectroscopy was performed using a Shimadzu FT-IR spectrometer. The absorption/photoluminescence spectra of QDs dispersion were respectively collected using a Shimadzu UV-2450 spectrometer and a Jobin Yvon Fluorolog-3 Fluorometer (HORIBA, Edison, NJ USA). Hydrodynamic size and zeta potential of QDs were measured by a 90Plus particle size analyzer and ZetaPALS zeta potential analyzer (Brookhaven Instruments, NY USA).

To evaluate the colloidal stability of the prepared QD formulations, the prepared QDs were dispersed in DI water, pH = 4.00, 7.00 and 10.00 buffer solutions, or DMEM medium (Gibco) and their hydrodynamic diameters were monitored at different time points. The photostability of QDs was monitored by using G10T8 lamp for UV irradiation.

4.2.4 Cell Uptake of QDs

RAW264.7 macrophages were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 $\mu\text{g ml}^{-1}$ penicillin (Gibco) and 100 $\mu\text{g ml}^{-1}$ streptomycin (Gibco) at 37 °C with 5% CO₂. The seven QD formulations were diluted to the same PL intensity and added into the wells. After 4 h of incubation, the cells were washed with PBS buffer and fixed using formaldehyde (4%) solution. Afterwards the nuclei were stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma). The cellular uptake was analyzed by using a Nikon Eclipse Ti inverted Microscope for taking fluorescent images and a FACSCalibur flow cytometer system (Becton Dickinson, CA) for obtaining flow cytometry results.

4.2.5 Cell Viability Study

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay was used for evaluating the cell viability of the RAW264.7 macrophages treated with the seven QD formulations. Cells seeded in 96-well plates were incubated with different concentrations of seven QD dispersions for 24 h. The concentrations of the QD dispersions were determined according to the mass of the original TOP/TOPO-capped QDs. The cells treated with PBS solutions were used as control. After incubation with QD dispersions, 18 μl of MTT (5 mg ml^{-1}) was added to each

well and the cells were incubated for another 4 h. Then the solution in the well was carefully removed and the purple precipitate was dissolved in 150 μ l of dimethyl sulfoxide (DMSO, Sigma). The cell viability was calculated by normalizing the absorbance of the sample well against that from the control well.

4.2.6 siRNA Delivery Using AET-Capped QDs

siRNA^{FAM} was mixed with different concentrations of AET-QD dispersions and incubated for 20 min at room temperature. Afterwards, the mixtures were loaded on a 1.2% agarose gel and electrophoresed at 100 V for 15 min. To visualize the siRNA delivery, RAW264.7 macrophages were incubated with the QD-siRNA^{FAM} complexes for cell imaging.

4.3 Results and Discussion

4.3.1 Characterization of QDs

To prepare the hydrophilic QDs, seven types of mercapto ligands, including 3-Mercaptopropionic acid (MPA), mercaptosuccinic acid (MSA), DL-Cysteine (Cys), 2-aminoethanethiol hydrochloride (AET, cysteamine), Carboxymethyl-PEG-Thiol (CM-PEG-SH), Methoxy-PEG-Thiol (mPEG-SH) and Amine-PEG-Thiol (NH₂-PEG-SH), were used for preparing water-dispersible QDs via ligand exchange process. To examine the exchange of the surface ligands, FT-IR spectroscopy was performed to analyze the functional groups on QDs surface. The FT-IR spectra of MSA-QDs, mPEG-QDs were collected and compared with the FT-IR spectra of their corresponding ligand molecules and the TOP/TOPO-QDs (**Figure 4.1**). The broad bands at around 3000-3600 cm⁻¹ in the spectra of all the QD samples may arise from

Figure 4.1 FT-IR spectra of (a) MSA molecules, MSA-QDs, (b) mPEG molecules, mPEG-QDs and TOP/TOPO-QDs.

the hydroxyl groups and H₂O molecules bound on the QDs surface.^{270, 271} For ligand exchange with short chain ligands, the characteristic band of S-H stretch at 2565 cm⁻¹ in MSA spectra was absent in the spectra of MSA-QDs, indicating the attachment of the sulfur atom on the QD surface.^{272, 273} Another evidence of the successful ligand exchange is that the P=O bands around 1074 cm⁻¹^{274, 275} in the spectra of TOP/TOPO-QDs disappeared in the spectra of MSA-QDs. In the FT-IR spectra of the mPEG-QDs, the main characteristic bands of mPEG molecules such as the C-O-C peak around 1114 cm⁻¹ and -CH stretch around 2886 cm⁻¹,²⁷⁶ suggesting the capping of mPEG on QD surface. However, as these bands overlap with the -CH vibrations and P=O stretch, it is difficult to determine whether the TOP/TOPO are completely removed. **Figure 4.2a** shows the absorption and photoluminescence (PL) spectra of the hydrophobic TOP/TOPO-QDs and hydrophilic ligand-modified QD formulations. Upon comparing to the chloroform dispersion of TOP/TOPO- QDs, no significant

changes were observed for the absorption and PL spectra of the aqueous QD formulations. Figure 4.2b and c shows the TEM image of the QDs capped with TOP/TOPO surfactants and modified with AET molecules. The high resolution image shows that the QDs are crystalline particles with average diameter of 6 nm. After ligand exchange process, the hydrodynamic diameters of the QDs were measured using DLS technique. The result shows that AET-QDs (~25 nm) display a larger

Figure 4.2 Characterization of QDs before and after ligand exchange. (a) Absorption (solid line) and PL spectra (dashed line) of TOP/TOPO-QDs in chloroform and MPA-QDs, MSA-QDs, Cys-QDs, AET-QDs, mPEG-QDs, CM-PEG-QDs and NH2-PEG-QDs in water. (b, c) TEM images of TOP/TOPO-QDs and AET-QDs and (inset) the corresponding high resolution TEM images.

hydrodynamic diameter than that of MPA-, MSA- and Cys-QDs (7-10 nm). From the TEM images, we can also observe some agglomerates of QDs after ligand exchange with AET (Figure 4.2c). This is probably because different QD formulations form different extents of ionic combinations. Similar observations have been reported by Hoshino et al.²⁶⁹

Zeta potential values were measured to assess the surface charge of all the prepared surface modified QDs (**Figure 4.3a**). QDs modified with ligands containing carboxyl group show negatively charged surface and the QDs functionalized with ligands containing amino group are determined to be positively charged. For Cys-QDs, the negative zeta potential value could be attributed to the domination of the carboxyl group dissociation rather than the amino group protonation under the pH of DI water (isoelectric point of cysteine is pH = 5.02). For QDs modified with ligands only containing amino or carboxyl group, the ones capped with short chain ligands (e.g. MPA, MSA, AET) exhibit higher absolute zeta potential values upon comparing them to those capped with long-chain ligands (CM-PEG, NH₂-PEG). This could result from the higher ligand packing density or the smaller dimensions of the ligands capped on the QDs surface.²⁶³ The different ligand dimensions may cause a wide diversity in ligand packing density. Herein, we employed a geometric model to estimate the ligand packing density by assuming the spherical nanocrystals and cone-shaped ligands (Figure 4.3b).^{277, 278} Under this assumption, the maximum ligands capped on one QD particle can be computed as,

$$\text{Maximum ligands per QD} = \frac{4\pi r^2}{\text{footprint}} = \frac{4\pi(r + L)^2}{\pi R^2} \quad (4.1)$$

$$\text{footprint} = \pi \left(\frac{D}{2}\right)^2 \quad (4.2)$$

r is the nanocrystal radius, L is the ligand shell thickness, D is the distance between two ligand chains at the particle surface, and R is the radius of ligand cone. For short chain thiol ligands capped nanocrystals with a diameter of ~ 6 nm, the ligands shell thickness is much smaller than the nanocrystals core ($L \ll r$). In this condition, short-chain thiol area (0.025nm^2)²⁷⁹ could be used as approximate footprint of the short ligands. The calculated theoretical capacity is ~ 4524 ligands per QD. This value was even higher than the density of the available anchoring sites on the QD surface. For PEG chain ligands, their ligand shell thickness is comparable to the particle size. Therefore, the varied size of particles could make a difference on the footprint of PEG ligands. According to the ligand cone models, a smaller footprint (i.e. higher percentage of surface coverage) could be achieved on smaller

Figure 4.3 (a) Zeta potential values of the prepared MPA-QDs, MSA-QDs, Cys- QDs, AET- QDs, mPEG- QDs, CM-PEG- QDs and NH2-PEG- QDs. (b) Shematic view of geometric model for estimating the ligand packing density.

particles.²⁷⁸ Therefore, the previous experimental data were classified into two categories depending on whether the particle size is smaller or larger than that of our QDs. For particles with a slightly smaller diameter (2.8 nm),²⁸⁰ we applied their PEG footprint (0.35nm^2) as the upper limit for the footprint of our particles and get a maximum capacity of ~ 323 ligands per

QD. For particles with larger diameter, the results from the previous experimental measurements provide a reference for the dimension of PEG ligand cone. From the given r (4.25 nm), L (11 nm) and D (0.83 nm)²⁸¹, the radius R of the ligand cone could be calculated using Equation 4.1-4.2. Based on the given L , calculated R and measured radius of our QDs, the estimated maximum capacity is ~355 PEG ligands per QD in our condition. These two results (~323 ligands/QD and ~355 ligands/QD) commonly provide a reasonable maximum grafting density for PEG ligands on our QDs. Considering the details such as the incomplete exchange of TOP/TOPO²⁸² and the repulsive force between molecules, the real situation could be more complicated. Nevertheless, this estimation still reveals the significant difference of the packing density between the short chain thiol ligands and PEG long chain ligands.

4.3.2 Colloidal Stability and Photostability of QDs

The colloidal stability of the prepared QD formulations was monitored by measuring their hydrodynamic size in DI water, acidic/neutral/alkaline buffer solutions, and the cell culture medium. In DI water, all the prepared QD formulations have different colloidal stability and shelf life ranging from period of days to weeks (**Figure 4.4**). The MPA- and CM-PEG- QDs are most stable as their hydrodynamic diameters kept almost unchanged over the course of more than 10 days. The MSA- and mPEG-QDs had a slight increase in the hydrodynamic diameter after aging the samples for one week while Cys- and NH₂-PEG-QDs showed an obvious size increase due to the aggregation of the nanoparticles. The Cys-QDs were discovered to be the formulation most prone to aggregation and this may be attributed to the coexistence of carboxyl and amino groups on the QDs surface. In general, QDs terminated with carboxyl groups on their surface displayed a better colloidal stability than those terminated with amino groups on the surface. Some reports have demonstrated that the

aggregation is caused by desorption of the ligands from the QDs surface. This phenomenon is due to the instability of dynamic nature of thiol–Zn interactions on the QDs surface and the ease of oxidation of the ligands in aqueous dispersions.^{120, 264} To examine the colloidal stability of QDs against pH and salt changes, their hydrodynamic sizes in different pH buffer solutions (pH = 4, 7, 10) were monitored using DLS technique (**Figure 4.5**). The colloidal stability of QDs functionalized with short-chain ligands is susceptible to change in different pH environment. Figure 4.5a shows the relatively stable conditions of the QDs capped with short chain ligands (MPA, Cys, MSA, and AET) under different pH conditions. Other formulations not included into the plots exhibited very poor colloidal stability and formed large aggregates during the first two to six hours of stability test. The MPA- and MSA-QDs are relatively stable in alkaline environment due to the ionization of the carboxyl groups. The aggregation and precipitation of other QD formulations might have resulted from the

Figure 4.4 Time course of hydrodynamic size of MPA-QDs, MSA-QDs, Cys-QDs, AET-QDs, mPEG-QDs, CM-PEG-QDs and NH2-PEG-QDs or their aggregates in DI water.

Figure 4.5 The hydrodynamic size of the prepared hydrophilic QDs or their aggregates in pH 4, 7, 10 buffer solutions against time. The size changes of (a) MPA-QDs, MSA-QDs, Cys-QDs, AET-QDs (only relatively stable conditions are displayed), (b) mPEG-QDs, (c) CM-PEG-QDs and (d) NH₂-PEG-QDs were displayed.

weakening of Coulombic interactions between particles when they are dispersed in electrolyte solution.¹²² The Coulombic interaction of the nanoparticles arises from the repulsive electrostatic force between the nanoparticles sharing similar surface charge potentials. On the other hand, the QDs capped with PEG chain molecules had better colloidal stability in buffer solutions. This is due to the steric stabilization from the long PEG chains and the hydrogen bonding of PEG molecules in aqueous solvents.²⁶² Figure 4.5b, c and d suggested that the functional groups on the PEG molecules play an important role in determining the colloidal stability life span of the QDs in solutions of varying pH. The CM-PEG-QDs are much more stable in pH 10 buffer solution than in pH 4 solution while a different trend was observed for NH₂-PEG-QDs.

Figure 4.5d shows that aggregation of particles was observed for NH₂-PEG-QDs dispersed in both pH 4 and 10 buffer solutions. This is not surprising since the ionization of the carboxyl/amino groups in acidic/alkaline environments might have manipulated particle surface charge whereby enhancing or weakening the electrostatic repulsion forces between nanoparticles. Buffer solution at low pH has numerous hydrogen ions (H⁺) and facilitates the protonation of amino groups while suppress the dissociation of carboxyl groups. Correspondingly, high pH solution is rich in hydroxide ions (OH⁻), which eases the dissociation of carboxyl groups but makes it difficult for amino groups to acquire hydrogen to be ionized. Interestingly, we have observed that the PEG modified QDs do not favorably interact with water when they are placed in neutral pH buffer. All PEG modified QDs started to aggregate in pH 7 buffer solution during 25 to 100 h of stability test. The hydrogen bonds forming between the PEG chains and the H₂O molecules are the main factor that supports the colloidal stability of the QDs in electrolyte solution. However, the presence of various salt ions in the neutral buffer solution may have disrupted the formation of hydrogen bonding and thus leading to the aggregation of PEG-modified QDs.²⁸³ For *in vitro* imaging, the synthesized QDs are required to be mixed with the cell culture medium for interaction with the cells. The cell culture medium contains amino acids, salts, glucose and vitamins that are essential for the cells growth and these ingredients may have an important impact the colloidal stability of QDs.²⁸⁴ **Figure 4.6** shows the variation of hydrodynamic diameters of different QDs in DMEM against time. From Figure 4.6, there is clear surfactant dependence for the colloidal stability life span of DMEM dispersion of QDs. For the short-chain ligand modified QDs, they aggregated in DMEM medium in a day. The size distribution of the QD aggregates was observed

to vary from one hundred to a few hundreds of nanometers. The accuracy of these data may be compromised due to the limitation of the DLS technique for particles with broad size distributions, still the result reflects the aggregation state of the particles. For the case of DMEM dispersion of PEG-modified QDs, they are relatively more stable than that of short-ligand capped QDs since their hydrodynamic diameters increases less comparing to their initial size. This may be due to the PEG modified QDs have better resistance to adsorption of surrounding biomolecules.²⁸⁵

Figure 4.6 Time course of the hydrodynamic size of MPA-QDs, MSA-QDs, Cys-QDs, AET-QDs, mPEG-QDs, CM-PEG-QDs and NH2-PEG-QDs or their aggregates in DMEM cell culture medium.

Photostability is an important parameter to be considered for selecting the appropriate fluorescent probes for biophotonics applications. QDs with superior photostability are promising candidates for applications with long-term excitation or requiring accurate quantitative analysis, such as real-time molecule tracking and high-resolution 3D reconstruction.⁸⁶ Generally, the photostability of QDs are affected by solvents,²⁸⁶ oxygen exposure level,²⁸⁷ reducing agents in solvents,²⁸⁸ the core/shell structure of QDs,²⁸⁹⁻²⁹¹ and surface ligands.^{287, 292-294} Monitoring the time evolution of

the PL spectra of QDs under irradiation is a common way to assess their photostability. In some cases, UV irradiation may enhance the PL intensity due to photoinduced smoothing and passivation of QD surface.²⁸⁶ Different mechanisms were proposed to explain this smoothing or passivation process of the QDs surface. For the QDs capped with thiol ligands (e.g. MPA), several studies reported that the surface defects of QDs were passivated by the adsorbed water molecules, surfactant molecules and new-formed oxide layer.²⁹⁵ However, the thioglycolic acid (TGA) stabilized QDs were found to form an additional CdS shell on the surface by using the sulfur source from TGA ligand.²⁹⁶ On the other hand, the UV irradiation may also induce corrosion of the QD core²⁸⁶ or aggregation of QDs,²⁹⁷ which in turn result in PL decrease. In addition, the size of QDs may decrease due to photo-corrosion or increase due to coagulation/ripening, consequently causing a blue- or red-shift in the PL spectra.²⁹² In this study, the PL spectra of all the prepared QDs were monitored under continuous UV irradiation. **Figure 4.7a** shows a representative example of PL spectra changes of QDs under UV radiation. To compare the photostability of the prepared QDs, the peak intensity of the PL spectra for each QDs was plotted against irradiation time (Figure 4.7b). Except for the AET-QDs, an enhancement in the PL intensity was observed for all the QDs in the first few hours of UV irradiation. The changes in PL intensity is attributed to the photoadsorption and photooxidation reactions occurred on the QD-thiol interface under UV irradiation. The photoadsorption reaction will assist to remove the surface defects on the QDs surface through the coordination of thiols or water molecules with QD surface atoms, whereby increasing the PL intensity of QDs.^{286, 298} In the photooxidation process, a thin oxide layer is first formed and passivated the QD surface, thereby increasing the PL intensity. However, further

oxidation leads to the photo-corrosion and aggregation of QDs, both are responsible for the PL decrease.^{295, 297} Figure 4.7b also showed that MPA-, MSA- and Cys-QDs had a more significant PL enhancement that lasted for a longer time period as compared to that of the mPEG-, CM-PEG- and NH₂-PEG-QDs. We speculate that this may arise from the difference between the grafting density of short-chain ligands and PEG ligands on QDs. According to the geometric model depicted in Figure 4.3b and the calculation results, the PEG ligands with large size might have a lower grafting density on QD surface. As a result, the PEG ligands may be more rapidly consumed by photooxidation during the UV irradiation. The naked QDs are susceptible to be corroded and form surface defects, which causes a faster PL decrease. As for the AET-QD formulation, it showed a relatively quick decrease in the PL intensity without PL enhancement process. This might result from the instability of AET ligand, which is easily oxidized to its disulphide state in aqueous solvent.²⁹⁹

Figure 4.7 (a) Time evolution of PL spectra of MPA-QDs under UV irradiation. (b) The PL maximum intensity of surface-modified QDs against UV irradiation time.

4.3.3 Cell Studies on Surface-Modified QDs

Nonspecific cell uptake of QDs determines the contrast of fluorescent images, the delivery efficiency and fate of the QDs. Therefore, it is a common parameter to evaluate the feasibility of using the prepared QDs for bioimaging applications. The cellular uptake of QDs is greatly affected by several factors derived from the surface coatings of the QDs, such as the size, surface charge and functional groups.^{267, 300-302} The influence of these properties on the nonspecific uptake rate of QDs is also cell-type specific and dose dependent.^{303, 304 302, 305} For example, the internalization of nanoparticles by nonphagocytic cells (e.g. Hela, NIH3T3, HepG2) were found to be most efficient for particles with size between 25-50 nm³⁰⁶ whereas phagocytic cells preferentially ingest particles within 2-3 μm size range.³⁰⁵ Positively charged QDs exhibited a higher uptake rate in nonphagocytic compared to the negatively charged QDs, whereas minimal internalization was observed for neutral QDs.^{302, 307} In contrast, most phagocytic cells show stronger interaction with negatively charged nanoparticles.³⁰⁵ To reduce the nonspecific cellular uptake, various strategies were proposed, such as using zwitterionic QDs (e.g. BSA,³⁰⁴), lysine cross-linked QDs²⁶⁸ and hydroxyl-terminated QDs.³⁰⁸ Among these strategies, surface modification of QDs by using PEG molecules is the most commonly used method. The reduction in the cellular uptake rate was attributed to minimal ionizable groups on PEG chains²⁶⁷ and the steric repulsive barrier between PEG-modified QDs and cells.³⁰⁹⁻³¹¹ The length and density of PEG chains also account for different cellular uptake rates.^{267, 303, 312} In this work, the cell uptake of QDs by RAW264.7 macrophages was carried out for all the prepared QD formulations. **Figure 4.8** shows the differential interference contrast (DIC) and fluorescent images of RAW264.7 macrophages cells treated with MPA-,

Cys-, AET-, mPEG-, CM-PEG- and NH₂-PEG-QDs. Upon comparing to short-chain ligands modified QDs, PEG-functionalized QDs showed significantly reduced cell uptake, which is consistent with the previous reports.^{267, 302, 313} A strong uptake was observed for MPA- and Cys-QDs after 4 h of incubation with cells. MSA-QDs formulation was found to exhibit a relatively lower uptake even though their zeta potential value and hydrodynamic diameter were almost the same with MPA- and Cys-QDs. The preference of QDs uptake may be affected by the different aggregation states of the nanoparticles. Commonly, most nonphagocytic cells take up cationic nanoparticles to a higher extent than anionic nanoparticles³⁰² whereas phagocytic cells preferentially internalize anionic ones.³⁰⁵ In our case, we observed that anionic QDs such as MPA-, Cys-, MSA-, mPEG-, CM-PEG-QDs displayed a higher cell uptake by RAW264.7 macrophages in comparison to the cationic QDs such as AET- and NH₂-PEG- QDs. Sakai et al. showed that, the carboxyl-terminated QDs were taken up more effectively by PC12 cells than the amino-terminated QDs.³¹⁴ Clift et al. reported that the carboxyl-group modified QDs were rapidly taken up by the J774.A1 macrophages.³¹³ Our result is consistent with their observation where similarly we have determined that carboxyl-terminated QDs formulation is having a higher uptake than that of the amino-terminated QDs. In addition, the cell uptake rate was quantified by flow cytometry analysis. The representative plots of the fluorescence intensity in RAW264.7 macrophages treated with seven QD formulations or PBS were given in **Figure 4.9**. The results are consistent with the fluorescent imaging. The MPA-QDs

Figure 4.8 Fluorescent imaging of RAW264.7 cells treated with (a) MPA-QDs, (b) Cys-QDs, (c) AET-QDs, (d) mPEG-QDs, (e) CM-PEG-QDs and (f) NH2-PEG-QDs. Cell nuclei were stained with DAPI (rendered in blue) and signals from QDs were rendered in orange.

Figure 4.9 Flow cytometry analysis of the fluorescence intensity in cells treated with different QD formulations. (a) Representative fluorescence intensity distributions, where cells were treated with (i) PBS, (ii) MPA-QDs, (iii) MSA-QDs, (iv) Cys-QDs, (v) AET-QDs (vi) mPEG-QDs, (vii) CM-PEG-QDs and (viii) NH2-PEG-QDs. (b) Average fluorescence intensity from the flow cytometry, $n=3$.

and Cys-QDs had most effective cell uptake in the applied macrophage cell line. In general, a relatively high cell uptake was observed for short-chain ligands modified QDs upon comparing to PEG modified QDs.

To demonstrate the potential of the prepared QDs for biomedical applications, as an example, the AET-QDs were conjugated with small interfering RNA molecules (siRNA) for gene delivery. siRNAs are short interfering RNA molecules containing 20-25 base pairs. For disease therapy, the sequence-specific siRNAs delivered in the cells will initiate the RNA interference process and regulate the expression of the targeting gene.³¹⁵⁻³¹⁷ However, free siRNAs could not penetrate the cell membrane by themselves and are susceptible to degradation in biological fluids.^{318, 319} QDs could provide a platform for facilitating the delivery of siRNA and simultaneously serve as the fluorescent labels. In our study, the negatively charged FAM labelled siRNAs were attempted to be attached on the positively charged AET-QDs through electrostatic interaction. Gel electrophoresis was firstly performed to verify the linking between AET-QDs and siRNA (**Figure 4.10**). All the samples, including mixtures of siRNA^{FAM} and AET-QDs at different weight ratio, mixtures of siRNA^{FAM} and MPA-QDs, neat siRNA^{FAM}, were loaded in the well. Under the external electric field, free siRNA^{FAM} with negative charge migrated downwards and resulted in bright bands at bottom of Figure 4.10. On the other hand, the siRNA^{FAM} conjugated on QDs surface retained in the well due to their limited mobility. Compared to the lane of neat siRNA^{FAM}, the lanes of AET-QDs and siRNA^{FAM} mixtures showed lower intensity at the siRNA band. The fluorescence intensity at this band gradually decrease upon increasing the AET-QDs:siRNA^{FAM} ratio. Correspondingly, the fluorescence intensity retained at well (upper part) became stronger by increasing the AET-QDs:siRNA^{FAM}

Figure 4.10 Gel electrophoresis study of (1-5) the mixture of siRNA and AET-QDs at the weight ratios of 1:6, 1:3, 1:1.5, 1:0.75, 1:0.38, (6) siRNA/MPA-QDs mixture at a weight ratio of 1:3 and (7) pure siRNA.

Figure 4.11 Fluorescent imaging of RAW264.7 cells incubated with (a, e) blank, (b, f) siRNA^{FAM} and (c, d, g, h) AET-QDs-siRNA^{FAM}. (a, b, c) Overlays of bright field and fluorescent image, (d) fluorescent signal overlay of QD and FAM channels, (e, f, g) FAM channel excited with blue light, (h) QD channel excited with green light. Channels of siRNA^{FAM} and QD are rendered in green and red respectively.

ratio. These results indicate the successful conjugation between AET-QDs and siRNA^{FAM}. As a comparison, the mixture of siRNA^{FAM} and negatively charged MPA-QDs showed no fluorescence signal at the well and a bright band at bottom, arising from the migration of the unbound siRNA^{FAM}. Therefore, QD-siRNA complexes are inferred to be formed via electrostatic interactions. The gene delivery efficiency of the QD-siRNA complexes was examined by fluorescent imaging in macrophages. Compared to the neat siRNA (**Figure 4.11f**), the QD-siRNA complexes showed enhanced fluorescence signal from FAM labels (**Figure 4.11g**). This result, combined with the overlap of FAM and QD signals (**Figure 4.11d**), demonstrated the potential of AET-QDs for facilitating the siRNA transfection and serving as fluorescent labels.

The toxicity of QDs remains a major concern for biomedical researchers and has been extensively studied for the last decade. Early studies have revealed that the QDs capped with short thiol ligands could release heavy metal ions (e.g. Cd²⁺, Pb²⁺) due to the degradation of QDs.^{320, 321} Applying cross-linked molecules (e.g. BSA with carbodiimide compound³²⁰) or multidentate polymers on the QDs surface (e.g. DHLA-PEG) may help to minimize the release of the heavy metal components, whereas using heavy-metal-free QDs (e.g. silicon QDs³²²) will lower the QDs toxicity concern. Recently, Zheng et al. have examined the toxicity of QDs capped with a series of thiol ligand-capped ligands including cysteamine (AET), glutathione (GSH), dihydrolipoic acid, polyethylenimine (PEI) and several other antioxidant thiol ligands.³²³ The negatively charged GSH capped QDs have the minimal toxicity while the positively charged PEI capped QDs are the most cytotoxic to HaCaT cells among all the formulations tested. In addition, incomplete purification³²⁴ of QDs may leave

some unwashed impurities in the dispersion and induce the toxicological response.³²⁵ Besides from these factors, toxicity could also arise from the intrinsic property of QDs, such as their size³²⁶ or reactive oxygen species (ROS) generated on the QDs surface under excitation light.³²⁷ It is worth noting that QDs in the physiological environment may also change their size, surface charge, and aggregation states, and these changes will have a significant impact on the toxicological responses from the cells.³²⁸ Also, after QDs are introduced into the cell culture system or in the body, proteins will be absorbed onto the QD surface and form protein corona.³²⁹ This may result in changes of the protein activities whereby leading to dysfunctions of the biological parts *in vitro* and *in vivo*. All these impacts to the biological systems are directly related to the surface modification of the QDs. Thus, continuation studies are needed to further investigate and understand the underlying mechanisms of QDs toxicity. In this study, the cytotoxicity of all the QD formulations was evaluated by MTT assays. **Figure 4.12** shows the cell viability of RAW264.7 macrophages cells after treating them with prepared QDs for 24 h. The cells treated with the MSA-QDs display the highest viability, followed by the NH₂-PEG- and MPA-QD formulations. All the QD formulations maintained higher than 80% cell viability up to concentrations of 70 $\mu\text{g ml}^{-1}$. It is worth to be noted that the concentrations used for fluorescent cell imaging is much lower than the value of 70 $\mu\text{g ml}^{-1}$ used in the MTT assays. This suggests that the prepared QD formulations have negligible toxicity for cell imaging thus could find wide applications for *in vitro* studies.

Figure 4.12 Relative cell viability of RAW264.7 macrophages treated with different concentrations of MPA-QDs, MSA-QDs, Cys-QDs, AET-QDs, mPEG-QDs, CM-PEG-QDs and NH2-PEG-QDs for 24 h, n=5.

4.4 Conclusions

In summary, a detailed study was performed for understanding the effects of surface ligands on the physical property, optical and colloidal stability, cellular uptake and cytotoxicity of QDs. Our result indicates that PEG ligand modified QDs have a higher colloidal stability and a lower cell uptake rate upon comparing to short-chain ligand modified QDs. However, PEG ligand modified QDs displayed a large hydrodynamic diameter and they have relatively poor photostability. MSA-QDs were revealed to be most stable in neutral and alkaline buffer solutions and also possess best photostability. The preference of QDs cell uptake was found to be strongly correlated with the surface charge of QDs. The anionic QDs were having much higher cell uptake upon comparing to the cationic QDs for RAW264.7 macrophage cells. Overall, the physicochemical property of QDs is sensitively influenced by the type of surface ligands used to modify the QDs surface. Thus, it is important to consider the chain length and functional groups of the surface ligands in designing the desirable QDs

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formulation for specific biological applications. It is worth noting that there are diverse protocols for surface modification of QDs in the literature and slight changes in the preparation parameters will result in different quality of QD aqueous dispersion. As such, it is imperative to follow the same set of protocol when preparing the QDs with specific type of surface ligands. In general, each set of the prepared QDs with specific type of surface ligands displayed some limitations in certain aspects of their physicochemical property. Therefore, we should carefully choose the appropriate type of QDs for intended applications to minimize their impacts arising from their limitation factors.

Chapter 5 The Composition Effect on the Optical Properties of Aqueous Synthesized Cu-In-S and Zn-Cu-In-S QDs

Chapter 3 and Chapter 4 focus on the synthesis and surface modification of Cd-based QDs, which contain the highly toxic Cd component. In recent years, Cd-free QDs have recently gained much attention in biomedical research field owing to the low toxicity of their components. From this chapter to Chapter 7, we will present the studies on the preparation and application of several Cd-free QDs, including the multinary QDs and the oxide QDs. Among them, the multinary QDs, because of the large degree of freedom in their structure and composition, have a wide tunability in their band gap but also exhibit the increased complexity and uncertainty in their optical properties. In this chapter, the ternary Cu-In-S (CIS) and quaternary Zn-Cu-In-S (ZCIS) QDs with varied composition ratios were synthesized via a facile aqueous route. The CIS QDs exhibit multi-peak PL with their peak intensity dependent on the Cu:In ratio, which was illustrated using a donor-acceptor recombination process. Upon incorporation Zn into the CIS QDs, the acquired ZCIS QDs showed blue-shifted PL spectra with enhanced emission intensity and narrowed spectral width. A comparative study reveals that, adjusting the Cu:In ratio and varying the Zn content are both feasible strategies for band gap engineering, although the influences on the QDs optical properties were different. Compared to the Cu:In ratio variation, incorporation of Zn is a more effective strategy to achieve a homogeneous absorption band and a wide range of emission wavelength tunability. The deposition of ZnS shell resulted in a further enhancement in PL intensity and a lengthening of the fluorescence lifetime. Finally, the as-prepared ZCIS/ZnS QDs were applied for cell imaging and exhibited a good biocompatibility to macrophage cells.

5.1 Introduction

Most of the high-quality QDs developed so far are composed of heavy metal elements including Cd, Pb, As, and Hg. These heavy metal elements raise tremendous concerns to the biomedical community about the toxicity of these QDs,^{330, 331} especially if the heavy metal ions are released from the degraded QDs during in vivo or clinical applications.²⁰⁸ Development of QDs composed of less toxic compositions, such as InP, CuInS₂, Ag₂S, Si and C-based QDs, is a potential strategy to deal with the heavy metal-related toxic effects.^{204, 332-335} Unfortunately, preparation of Cd-free QDs having comparable optical properties with the conventional QDs remains a challenge to date. For example, the size-tunable emission covering the whole visible range is typically no longer a characteristic property of some Cd-free QDs.^{111, 336} For this reason, other strategies are required to be developed to adjust the band gap or tune the emission wavelength of the Cd-free nanocrystals. Additionally, in comparison to the low-cost and greener aqueous method for Cd QDs preparation,³³⁷ most of the synthesis procedures of Cd-free QDs are much trivial, due to the need for high reaction temperature, volatile and hazardous ligands or pyrophoric precursors.⁹⁶

To make the band gap and emission wavelength of the QDs more adjustable, multinary semiconductor materials have been studied due to their large degree of freedom in composition and lattice structure.³³⁸ For example, by substituting the Zn(II) atoms in ZnS with Cu(I) and In(III) atoms, the ternary Cu-In-S (CIS) preserve the semiconductor properties but narrowing the band gap from 3.68 to 1.55 eV.³³⁹ For Zn-Cu-In-S (ZCIS) preparation, substitution of the Cu and In atoms partly by Zn atoms could result in band gap energy that lies between CIS and ZnS (2.34 eV for CuInZn₂S₄³³⁸).³⁴⁰ Owing to their large tolerance of element off-stoichiometry, the band

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gap of CIS and ZCIS could be further adjusted by tuning composition ratio.^{341, 342} Based on these band gap adjustment strategies, researchers have already developed CIS and ZCIS QDs with emissions tunable from visible to near-infrared region.^{341, 343} However, as the structural and composition freedom increases, more configurations and impurity phases tend to coexist in the multinary materials. Based on the calculational results, small deviation of the element stoichiometry will generate secondary phases (e.g., CuS, Cu₂S, ZnS) and other impurity phases (e.g., vacancies, institutes) in quaternary materials.^{344, 345} Correspondingly, multi-peak or broad PL profiles for the CIS and ZCIS QDs have been observed in many reported experimental works,^{53, 104} which suggest the coexistence of the multi-phases or the inhomogeneity of composition. Moreover, the poor comparability of the optical properties and crystal structures of CIS or ZCIS QDs reported in different studies also imply the complexity and uncertainty of the making of multinary nanocrystals. Therefore, when developing different synthesis methods for preparing these multinary nanocrystals, a systematic investigation is needed to understand the effect of the composition ratio on their optical and structural properties.

A hot colloidal synthesis method has been successfully developed to prepare CIS and ZCIS QDs in the organic phase at temperatures of 200-250 °C.^{341, 346} On the contrary, only a handful of studies reported the preparation of CIS QDs in an aqueous solvent at relatively low temperatures of 95-150 °C.^{54, 347, 348} The aqueous synthesis method usually requires inexpensive precursors and a simpler synthesis setup. More importantly, using water as the reaction medium minimizes the toxicity issue associated with organic solvent evaporation. Furthermore, the as-synthesized QDs are water-dispersible and can be directly used for biological applications after careful washing. Comparatively, the CIS and ZCIS QDs prepared in organic phase need to be

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transferred into aqueous phase. Since these CIS and ZCIS QDs are capped with oleic acid and organothiols, it is difficult to make them water-dispersible by ligand exchange. On the other hand, phase transfer via encapsulation method usually result in nanoparticle agglomeration and significant size increase.^{35, 51, 349} Nowadays, the aqueous synthesis method for Cd-based QDs has been well developed, and optical properties of the QDs are comparable with the ones synthesized in organic solvent.^{250, 350, 351} On the contrary, synthesis of aqueous CIS QDs is still in their infancy stage. Some of the QDs exhibit no PL or low PL efficiency with untunable emission wavelength,³⁴⁷ while improvements have been made and reported in recent works.^{54, 348} Zn alloying is expected to strengthen the crystal structures and eliminate the defects of the CIS QDs. Although controlling of the band gap and optical properties by varying Zn concentration has been demonstrated in the organic method,^{352, 353} As far as we know, only one manuscript has reported the aqueous synthesis of ZCIS QDs. In the work, bright Cu doped In-Zn-S QDs were prepared using autoclave at 150°C for 23 h.³⁵⁴ To our best knowledge, no work has been reported on ZCIS QDs preparation via a low-temperature water bath route. Compared to hot colloidal synthesis method requiring high temperature treatment in the presence of argon/nitrogen or autoclave method requiring high temperature and pressure treatment, the set-up of water bath route is simpler, and the process is greener.

In this work, we employed a quick and facile aqueous phase synthesis method to prepare the ternary CIS and quaternary ZCIS QDs. Compared to hot colloidal synthesis method requiring high temperature treatment in the presence of argon/nitrogen or autoclave method requiring high temperature and pressure treatment, the water bath route employs a simpler set-up, greener synthesis process and could fabricate QDs of different composition ratios within one batch. The CIS QDs show

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multi-peak PL with their peak intensity dependent on the Cu:In ratio. Compared to these CIS QDs, the ZCIS QDs synthesized under similar condition exhibit enhanced PL and narrower spectral widths within certain range of Zn content. The evolution of absorption and emission spectra of the ZCIS QDs when changing the Zn contents were analyzed and correlated with their structural and compositional properties. Based on our results, the influence of Cu:In ratio and Zn content on the optical properties of the QDs was compared and discussed. Next, the deposition of ZnS shell on ZCIS QDs resulted in further enhanced PL intensity together with lengthened fluorescence lifetime of the QDs. Finally, the resulted ZCIS/ZnS QDs were applied for cell imaging and these nanocrystals showed low toxicity to macrophage cells.

5.2 Experimental Methods

5.2.1 Materials

Copper(II) chloride (CuCl_2 , 99%), indium(III) chloride tetrahydrate ($\text{InCl}_3 \cdot 4\text{H}_2\text{O}$, 97%), zinc acetate ($\text{Zn}(\text{OAc})_2$, 99.99%), 3-Mercaptopropionic acid (MPA, $\geq 99\%$) and ammonium hydroxide solution (NH_4OH , 28.0%-30.0%) were purchased from Sigma-Aldrich. Sodium sulfide hydrate ($\text{Na}_2\text{S} \cdot x\text{H}_2\text{O}$, 60-62%) was obtained from ACROS Organics. All chemicals were used as received. Deionized water (DI water, $18.2 \text{ M}\Omega \cdot \text{cm}$) was used throughout the experiment.

5.2.2 Synthesis of Cu-In-S (CIS) QDs

In a typical procedure for synthesizing Cu-In-S(CIS) QDs with a Cu:In ratio of 0.6, aqueous solution of InCl_3 (0.025 mmol) was mixed with MPA (0.2 mmol) in 20 mL of DI water and the pH was adjusted to 9 using NaOH. Afterwards, 0.5 mL of ammonium hydroxide solution of CuCl_2 (0.015 mmol) with MPA (0.3 mmol) were added to the solutions. Subsequently the Na_2S solution (0.04 mmol) was injected into

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the mixtures at room temperature. The reaction mixtures were heated to 100°C and kept at this temperature for 45 min. Then, 0.25 mL of 0.04 M $\text{Zn}(\text{OAc})_2$ and 0.25 mL of 0.04 M Na_2S solutions were injected into the reaction mixtures for four times to obtain CIS/ZnS QDs. The QDs were washed with ethanol at a volume ratio of 1:2 before use. To synthesize the CIS /ZnS QDs with different Cu:In ratios, the feed ratio of Cu:In was adjusted from 0.07 to 1.33 while the amount of MPA was set at the moles of $20 \text{ Cu} + 8 \text{ In}$.

5.2.3 Synthesis of Zn-Cu-In-S (ZCIS) QDs

In a typical synthetic reaction of Zn-Cu-In-S(ZCIS) QDs with Zn content of 30% and Cu:In ratio of 0.6, $\text{Zn}(\text{OAc})_2$ (0.012 mmol) and InCl_3 (0.0175 mmol) were mixed with MPA (0.164 mmol) in 20 ml of DI water. The pH value of the mixture was adjusted to the range of 9-10 by adding NaOH. Then, 0.03 M ammonia solution of CuCl_2 (0.0105 mmol) and MPA (0.21 mmol) were injected in the mixtures. Afterwards, Na_2S solution (0.04 mmol) was added in the solutions and the reaction solution was heated to 100°C. Following the growth of the QDs at 100°C for 40 min, the ZnS shell was coated on the ZCIS QDs. Before use, the QDs were washed with ethanol. The ZCIS/ZnS QDs with different Zn content could be obtained by varying the feed ratio of Zn: (Cu+In) while keeping the gross amount of the metal precursors at 0.04 mmol. The amount of MPA was set at the moles of $20 \text{ Cu} + 8 \text{ In} + 2 \text{ Zn}$.

5.2.4 Characterization of QDs

Transmission electron microscopy (TEM) images were obtained using a FEI TecnaiTM microscope operating at 200 kV. The absorption and photoluminescence (PL) spectra of the synthesized QDs were collected using a UV-2450 spectrometer (Shimadzu) and a Fluorolog-3 Fluorometer (HORIBA Jobin Yvon, Edison). To determine the quantum

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yield (QY) of the samples, CdSe QDs in chloroform was firstly measured as a reference sample. QY of CdSe QDs was calculated by comparing the integrated emission with that of the Rhodamine 6G in ethanol under 480 nm excitation. The QY of the synthesized QDs were then determined by comparing with the CdSe QDs at same excitation wavelengths. Energy dispersive X-ray spectroscopy (EDX) was performed using a JSM-5600LV scanning electron microscope equipped with EDX detector. The powder XRD patterns of the QDs were recorded on a Siemens D5005 X-ray diffractometer. The luminescence decay curve was obtained using a DCS-120 Confocal Scanning Fluorescence Lifetime Imaging System (Excitation wavelength: 375 nm, Repetition rates: 20 MHz). Hydrodynamic diameter of the QDs was measured using 90Plus particle size analyzer (Brookhaven Instruments) using DLS technique.

5.2.5 Cell Imaging and Cell Viability Studies

RAW264.7 macrophages were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone), penicillin ($100 \mu\text{g ml}^{-1}$, Gibco) and streptomycin ($100 \mu\text{g ml}^{-1}$, Gibco) at 37°C with 5% CO_2 . For cell imaging study, the cells seeded on to a 6-well plate were incubated with 30% ZCIS/ZnS QDs for 4 h. Afterwards, the treated cells were washed with PBS buffer for three times and fixed using the 4% formaldehyde solution. The nuclei of the cells were stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma). The cell viability of RAW264.7 macrophages treated with QDs was performed using MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Life Technologies) assay. Cells seeded in 96-well plates were incubated with 30% ZCIS/ZnS solutions with different concentrations for 24 h. Afterwards, $20 \mu\text{l}$ of MTT solution (5 mg ml^{-1}) was added to each well. After another 4 h of incubation, the solution in the wells was

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removed and the purple precipitate was dispersed in 150 μ l of dimethyl sulfoxide (DMSO, Sigma). The absorbance of the solution in the wells was measured with a Bio-Rad microplate reader at a wavelength of 490 nm. The cell viability was calculated by normalizing the absorbance of the sample wells to that of the control wells.

5.3 Results and Discussion

5.3.1 Effect of Cu:In Ratio on the Optical Properties of CIS QDs

CIS QDs with less structural complexity were firstly synthesized to provide guidance for the study of ZCIS QDs. Because ternary CIS structures could accommodate large off-stoichiometry³⁴¹ by forming the antisites (In_{Cu} , Cu_{In}), interstitial sites (Cu_{i} , In_{i}) or vacancies (V_{Cu} , V_{In} , V_{S}), the diversity of the CIS lattice structures facilitate continuous tuning of the optoelectronic properties, which, on the other hand, also raise difficulty in generating CIS products with a pure phase. In comparison with their bulk counterpart, this challenge is more pronounced for CIS QDs materials, because the QDs are so small that even fewer defects or impurity phases could introduce great difference in their properties.³⁵⁵ For this reason, varied optical properties and crystal structures have been reported when the synthesis conditions were slightly changed. In this work, a facile synthesis method was optimized in aqueous solution at a growth temperature of 100°C. The Cu:In feed ratio was studied in a range of 0.07-1.3, and the amount of the stabilizing ligands (MPA) was optimized to achieve high PL efficiency and colloidal stability. EDX measurements determined the Cu:In ratio in the final QD products, which shows an increasing trend when elevating the Cu:In feed ratio (Figure 5.1e). TEM images (Figure 5.1a) reveal that the as-prepared CIS QDs are crystalline nanoparticles and their size is estimated to be 4.5 ± 0.7 nm (Figure 5.1a, upper inset). The interplanar spacings measured from its FFT pattern are 0.32 nm and 0.20 nm, consistent with

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the (112) and (204) faces of the tetragonal CIS phase (JCPDS 27-0159) determined by the XRD pattern (given and discussed later in Figure 5.3). As can be observed in Figure 5.1b, absorption spectra of the QDs shifted to shorter wavelengths in the range of 400-700 nm

Figure 5.1 Characterization of CIS QDs. (a) Representative TEM image, size distribution (upper inset) and FFT pattern (lower inset) of CIS QDs with a Cu:In ratio of 0.6. (b) Absorption and (c) photoluminescence spectra (450 nm excitation) of CIS QDs with respect to Cu: In feed ratio of 0.07-1.33, as given in the legend. (d) PL spectra of the same CIS/ZnS QDs. (e) EDX determined Cu:In ratio in the synthesized CIS QDs as a function of their corresponding feed ratio (dash line). (f) and (g) QY of the CIS and CIS and CIS/ZnS QDs within the range of 470-600 nm and 600-880 nm.

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as the Cu:In ratio decreases, indicating widening of the band gap under a Cu-poor condition.^{104, 356} On the other hand, the absence of distinct exciton features in absorption spectra can be attributed to joint effect of broad size distribution, irregular composition distribution and existence of various intra-band-gap states³⁵⁷. As shown in photoluminescence spectra (Figure 5.1c), the prepared QDs exhibit a broad emission centered at ~700 nm accompanied with a narrow emission at 532 nm. As the Cu:In feed ratio increases from 0.07 to 1.33, the emission at 700 nm was firstly enhanced and then reached a maximum value at Cu:In=0.6, while further increasing the Cu component will lead to a PL QY decrease (Figure 5.1g). On the other hand, elevating the Cu:In feed ratio caused a decrease of the 532 nm emission intensity (Figure 5.1f). Previous reports on CIS QDs synthesis have shown wide variations of their PL properties. Some research groups have obtained deep red-emitting CIS QDs (650-700 nm)^{104, 347, 357} while others observed the emissions can be extended to the near-infrared range of 700-800 nm.^{39, 356} Moreover, in a recent work, aqueous CIS QDs have also been prepared with yellow-green emission peaks at 530 nm.⁵⁴ Typically, the red-NIR emission was attributed to the donor-acceptor pair (DAP) transition, where $\text{In}_{\text{Cu}}/\text{V}_{\text{S}}$ and V_{Cu} served as donor and acceptor, respectively. Considering the energy level of the defect states, the deep red emission was most probably caused by the transition from the conduction band or V_{S} to V_{Cu} , while the NIR emission may originate from the $\text{In}_{\text{Cu}}-\text{V}_{\text{Cu}}$ transition.^{104, 357} According to Chen et al., optimized PL emission of CIS QDs was achieved at Cu:In=0.7, which was thought to be an optimal ratio for accommodating maximum $\text{In}_{\text{Cu}}-\text{V}_{\text{Cu}}$ pairs.³⁴¹ A similar result was obtained in our work, as the maximum intensity of 700 nm emission was obtained at a Cu:In feed ratio of 0.6. Based on the above discussion, the 700 nm emission here should be mainly attributed to the DAP transition of the $\text{In}_{\text{Cu}}-\text{V}_{\text{Cu}}$ pairs. The mechanism for the green emission at 532 nm is not clear. Because the green emission becomes prominent at a Cu-poor and In-rich condition, it may originate from the band-edge transition or defect

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related emission of In_xS_y species. A similar property has been observed in In_2S_3 nanospheres or thin films.³⁵⁸⁻³⁶⁰

After deposition of an additional ZnS shell layer coating over the CIS cores, the QY of the red emission of the obtained CIS/ZnS QDs was 6-8 fold enhanced and the peak wavelength was blue-shifted by 8-45 nm (Figure 5.1 d). The significant PL enhancement can be attributed to passivation of the surface defects by the ZnS shell. As for the pronounced blue shift, several mechanisms were proposed including the interdiffusion of Zn and surface reconstruction. Interdiffusion of Zn, which often proceeds via cation exchange, in presence of free Zn precursors and at a high temperature.^{51, 361} Considering the relatively low temperature (100 °C) applied in our method, the interdiffusion was unlikely to take place.^{204, 362} Surface reconstruction involves partially cation exchange and crystal deformations at the core-shell interface. Although Zn is not expected to diffuse into the core, it may replace Cu and In on the core surface. Since a large proportion (~30%) of atoms are located on the surface for a CIS QD of 5 nm diameter,³⁶³ this partial cation exchange process may have an impact on the emission wavelength. Crystal deformation is suggested as another possible cause for the PL blue shift. Because of the difference in lattice parameters between CIS ($a=5.523 \text{ \AA}$) and ZnS ($a=5.406 \text{ \AA}$), compressive strain can appear on the CIS core after shell coating, which will lead to a widening of the band gap.^{364, 365} The V_S to V_{Cu} or V_S to valence band transition were considered to make a contribution to the PL of CIS core QDs while this recombination pathway should be suppressed in CIS/ZnS QDs. In a recent report, Kraatz et al. investigated the PL mechanism of hydrophobic CIS/ZnS QDs and attributed the PL donor as In_{Cu} rather than the V_S or conduction band.³⁶⁶ The V_S to V_{Cu} transition were considered to make a contribution to the PL of CIS core QDs while this recombination pathway should be suppressed in CIS/ZnS QDs.

5.3.2 Effect of Zn Content on the Optical Properties of ZCIS QDs

As for the proposed ZCIS QDs preparation, different amounts of the Zn precursor were added in reaction solutions while the Cu:In feed ratio was fixed at 0.6. From the TEM image (**Figure 5.2a**), the synthesized ZCIS QDs are crystalline and monodisperse nanocrystals with an average size of 4.9 ± 0.6 nm (Figure 5.2a, upper inset). The absorption and emission spectra of the synthesized ZCIS QDs were analysed and correlated with compositional and structural characterization. As shown

Figure 5.2 (a) TEM image, size distribution (upper inset) and HRTEM image (lower inset) of 30% ZCIS QDs. (b) Absorption and (c) PL spectra of ZCIS QDs with 0-70% Zn content.

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in Figure 5.2b, absorption spectra of the ZCIS QDs gradually shifted to shorter wavelengths with increasing Zn content. The blue-shift of the absorbance profile indicates widening of the band gap as a result of the Zn incorporation.¹⁰⁴ In a recent report by Pan et al., the band gap of ZCIS nanocrystals with varying Zn content has been described and predicted as a function of the band gaps of the CIS and ZnS compounds.³³⁹ In this work, an alloyed structure of our QDs was investigated using XRD spectroscopy. Figure 5.3 shows the XRD spectra of the synthesized CIS and ZCIS QDs. The XRD peaks of the CIS QD samples are compatible with the typical diffraction peaks of the tetragonal CuInS₂ pattern (JCPDS 27-0159). When Zn was introduced at a content ratio of 10%, the diffraction peaks of the nanocrystals shifted to larger angles and lied between those patterns of CuInS₂ and ZnS (JCPDS 05-0566). A more significant right-shift can be observed when the Zn content increased to 50%. On the contrary, shift of the spectra was not that apparent in CIS/ZnS QDs, although the Zn feed content was same as that of the ZCIS QDs (Zn=50%). The continuous shift of XRD spectra in ZCIS QDs was attributed to the incorporation of Zn in the CIS crystal lattice.^{340, 367, 368} As suggested by EDX measurements, the Cu:In ratio reduced as a result of the Zn incorporation, which may also have contributed to the blue-shift of the absorbance spectra. According to EDX results (representatively shown in **Figure 5.4a**) summarized in Table 5.1, the proportion of Zn in the QDs are approximated to the Zn precursors feeding ratio (Figure 5.4b, red line), while the Cu:In ratio showed a decreasing trend as the Zn content increased (Figure 5.4b, black line). This phenomenon reveals that, when forming the ZCIS alloyed structure, the Cu sites are more preferable than the In sites to be occupied by Zn atoms. The asymmetric substitution is a result of energetically favorable formation of Zn_{Cu} substitutes and the weaker strength of the Cu–S bond as compared to the In–S bond.^{104, 369, 370}

Figure 5.3 X-ray diffraction patterns of CIS QDs, 10% ZCIS QDs, 50% ZCIS QDs and CIS/ZnS QDs.

Figure 5.4 EDX analysis of the synthesized ZCIS QDs. a) A typical EDX spectrum of 30% ZCIS QDs. b) EDX showing the Cu:In ratio and Zn proportion (solid line) in the synthesized ZCIS QDs as a function of their corresponding feed ratio (dash line).

Table 5.1 The feed ratio of Zn, Cu, In, S in reaction mixtures and the EDX determined composition ratio in prepared ZCIS QDs.

QDs	Feed ratio (MPA excluded)				EDX determined ratio			
	Zn(%)	Cu(%)	In(%)	S(%)	Zn(%)	Cu(%)	In(%)	S(%)
0% ZCIS	0	18.75	31.25	50	0.00±0.00	18.62±2.44	32.83±2.50	48.55±3.65
10% ZCIS	5	16.875	28.125	50	5.07±0.26	15.74±0.33	25.20±5.84	53.99±6.29
30% ZCIS	15	13.125	21.875	50	14.25±5.04	10.36±2.38	19.97±0.78	55.43±6.50
50% ZCIS	25	9.375	15.625	50	18.83±3.09	6.08±0.59	14.32±1.34	60.77±4.08
70% ZCIS	35	5.625	9.375	50	25.49±0.45	2.83±0.68	9.17±0.82	62.51±1.19

As for PL emission spectra, a significant enhancement of PL intensity was observed when the QD was incorporated with 10% and 30% of Zn, while further increasing the Zn content led to PL decrease (Figure 5.2c). Similarly, dependence of the PL intensity on Zn composition ratio has also been reported for ZCIS QDs forming in organic phase. For example, in a work reported by Nakamura et al., the PL emission of ZCIS QDs reached maximum value when the Zn content was around 20-33% (Cu:Zn=1~2).³⁶⁹ A possible explanation for this phenomenon is that the incorporation of Zn reduced the structural defects, stabilized the crystal structure and inhibited the nonradiative recombination.^{353, 368} However, when a high level Zn substitution was taking place, the impurity phases, which induce radiative recombination, may also be eliminated. As a result, further increasing the Zn content resulted in a lower PL intensity from the QDs. In addition to the changes in PL intensity, the full-width-at-half-maximum (FWHM) of the emission spectra decreased from 158 to 105 nm as the Zn content increased from 0% to 50% (**Figure 5.5**, red line), which means that a more homogeneous irradiation was achieved upon incorporating Zn into the crystal nanostructure. Meanwhile, we observed that the PL peak shifted from 709 nm to 594 nm (Figure 5.5, black line).

Figure 5.5 Evolution of the PL peak position (triangle) and FWHM (circle) with increasing Zn content in ZCIS QDs.

5.3.3 Composition Effect on the Energy Band and Optical Properties of CIS and ZCIS QDs

Figure 5.6 Evolution of the optical band gap of (a) CIS QDs with reducing Cu:In ratio and (b) ZCIS QDs with increasing Zn content.

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Based on our results, reducing the Cu:In ratio in the CIS QDs and incorporating Zn to form the alloyed ZCIS QD are both feasible strategies for band gap engineering. The evolution of optical band gaps were illustrated by plotting the $(ah\nu)^2$ vs $h\nu$ derived from absorption spectra (the molar concentration of the samples is the same) and extrapolating the linear portion to intercept the x abscissa (**Figure 5.6**).^{371, 372} The widening of the band gap when reducing Cu:In ratio or increasing Zn content could be qualitatively confirmed from the continuous right shift of these derived curves. From curves with good linearity, the band gaps of CIS QDs with a Cu:In ratio of 0.14 and 0.07 were estimated to be 2.73 eV and 2.78 eV (Figure 5.6a, dash line) and those of the ZCIS QDs with a Zn content of 50% and 70% were calculated to be 2.75 eV and 2.94 eV (Figure 5.6b, dash line). Although similar band gap widening effect was observed, the influences of adjusting Cu:In ratio or Zn content on the QDs optical properties were different. First, in comparison with tuning the Cu:In ratio, the incorporation of Zn resulted in a more pronounced blue shift in the absorption onset and in the UV region (Figures 5.1b and 5.2b). In other words, the ZCIS QDs exhibit a larger absorption coefficient near the band gap energy while the CIS QDs show a broader distribution of the absorption energy levels. This indicated the formation of a more homogeneous energy band in the ZCIS QDs. Second, varying Zn content ratio resulted in a continuous blue-shift of the PL spectra, while similar results cannot be obtained by altering Cu:In ratio in CIS QDs (Figures 5.1c and 5.2c). This indicates the gap between the donor and acceptor energy band of the ZCIS QDs were widened as the widening of the band gap while same trend was not observed for the CIS QDs. Based on the discussion above, incorporation of Zn into CIS QDs might be an effective strategy to achieve more homogeneous energy band, enhanced PL intensity, narrowed emission spectra and a wide range of emission wavelength tunability. The diameters of 0%, 30% and 70% ZCIS QDs determined from TEM results are in the range of 4-7 nm and close to twice of their Bohr exciton radius (5.0 nm for

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ZnS and 8.2 nm for CuInS₂), which further confirms the composition control is the main cause for the tunable band gap and optical properties.³³⁹ Additionally, tuning the composition ratio is a more controllable strategy for adjusting band gap and optical properties in these ZCIS QDs compared to the size control, for wavelength of their absorption/emission profiles was almost unchanged over reaction time.

5.3.4 Effect of ZnS Shell Growth on the Optical Properties of CIS and ZCIS QDs

Figure 5.7 Synthesis of ZCIS/ZnS QDs. (a) Hydrodynamic particle size distributions of 30% ZCIS QDs (top) and 30% ZCIS/ZnS QDs (bottom). (b) Water dispersion of ZCIS/ZnS QDs (left to right, 50%, 30%, 10%, 0% Zn content) under natural light (top) and UV light (bottom). (c) FFT pattern and HRTEM images of 30% ZCIS/ZnS QDs.

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An additional ZnS shell was deposited on the ZCIS QDs surface for improving their PL efficiency and enhancing their optical stability. Because of small lattice mismatch with CuInS_2 ,^{54, 352} ZnS is expected to have similar lattice parameter with ZCIS. Also, the band alignment of ZCIS and ZnS ³⁴⁰ allows type-I structure formation for effective surface passivation. For 30% ZCIS QDs and their core/shell structures, the hydrodynamic diameters were estimated to be 4.8 ± 0.9 nm and 6.7 ± 0.9 nm, respectively (**Figure 5.7a**). The interplanar spacings measured from the FFT pattern of the HRTEM are 0.19 nm, 0.32 nm, 0.30 nm and 0.29 nm (**Figure 5.7c**), in agreement with zinc blende ZnS phase, tetragonal CIS phase and their alloyed structure. As expected, the resulted ZCIS/ZnS QDs showed enhanced PL intensity. The QY of 30% ZCIS QDs increased from 1.4% to 4.7% after ZnS coating (**Figure 5.8b**) and the QDs showed considerable fluorescence intensity under UV light (**Figure 5.7b**). As an example, **Figure 5.8a** shows the PL spectra of 30% ZCIS QDs before and after shell deposition. Compared to the ZCIS core QDs, the ZnS coated QDs exhibited enhanced emission intensity and blue-shifted PL peak. However, this blue shift was much less pronounced as compared to that observed in CIS/ZnS QDs. As observed in **Figure 5.8b**, the blue shifts for CIS QDs and 10%, 30% and 50% ZCIS QDs are 42 nm, 10 nm, 4 nm, 1 nm, respectively. The inhibition of the blue shift upon shell coating in ZCIS QDs could be attributed to the weakened lattice strain or the reduced cation exchange due to the existed Zn on core surface.³⁶⁸

Time-resolved PL measurement was carried out to investigate the recombination process in ZCIS and ZCIS/ZnS QDs, the time-resolved PL decay was fitted to bi-exponential curves,

$$I(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \quad (5.1)$$

The fitting parameters are summarized in **Table 5.2**. τ_1 , τ_2 are two lifetime

Figure 5.8 (a) PL spectra of 30% ZCIS QDs before and after the ZnS shell growth. (b) PL QY and PL peak emission wavelength of ZCIS and ZCIS/ZnS QDs. The Zn content from left to right is 50%, 30%, 10% and 0%, respectively.

components of the decay curves, and A_1 and A_2 are the relative weights of the lifetime components. Because the excitation pulsed laser has a high frequency of 20 MHz, the fitting parameters were corrected as A_{01} , τ_{01} , A_{02} , and τ_{02} using an incomplete decay model,³⁷³ which will be discussed later in Chapter 6. The transient kinetics can be decomposed into a fast and slow decay component, of which the dynamics are in the time scales of several nanoseconds and more than 100 ns, respectively. The fast decay component is usually correlated to the nonradiative recombination and the slow decay component is consistent with the donor-acceptor recombination related to internal defects.^{104, 364, 374} Average fluorescence lifetime, τ_{avg} , and the time that the fluorescence intensity drops to 1/e of its peak intensity, $\tau_{1/e}$, are calculated to reflect the decay rate. After coating with ZnS shell, the decay rate was decreased and both the average fluorescence lifetime and the $\tau_{1/e}$ of the 30% ZCIS QDs were prolonged (**Figure 5.9** and Table 5.2). The significant reduction of the fast decay component after ZnS shell growth implies that the nonradiative recombination pathways can

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be effectively suppressed upon surface trap passivation. Additionally, growth of the ZnS shell may also lead to delocalization of the electron wave function into the shell region and reduce the electron/hole wave function overlap.³⁷⁵ This change will lower the chance of the electron–hole recombination and prolong both the nonradiative and radiative processes, slightly. Additionally, the fluorescence lifetime of the CIS/ZnS QDs is longer than those of the ZCIS/ZnS QDs, suggesting the alloying of Zn into QD cores.

Figure 5.9 Time-resolved PL decay and fitted curves (solid line) of the 30% ZCIS QDs and 30% ZCIS/ZnS QDs.

Table 5.2 The decay constants of the fitted time-resolved PL decay curves.

QDs	A_1 / A_{01}	τ_1 / τ_{01} [ns]	A_2 / A_{02}	τ_2 / τ_{02} [ns]	$\tau_{1/e}$ [ns]	τ_{avg} [ns]
CIS/ZnS	0.145/0.145	5.090	0.855/0.197	191.067	85.379	112.05
10% ZCIS/ZnS	0.212/0.212	4.835	0.788/0.231	144.376	50.378	77.601
30% ZCIS	0.450/0.450	2.327	0.550/0.180	125.723	4.7607	37.654
30% ZCIS/ZnS	0.145/0.145	4.382	0.855/0.236	154.453	80.582	97.404
50% ZCIS/ZnS	0.274/0.274	3.504	0.726/0.230	131.016	28.309	61.700

5.3.5 Cell Imaging and Cell Viability Study of ZCIS/ZnS QDs

To investigate the potential of the synthesized QDs for biophotonics applications, 30% ZCIS/ZnS QDs were used for optical imaging of RAW264.7 macrophages (**Figure 5.10**). The cells stained with DAPI and ZCIS/ZnS QDs exhibited delocalized emissions from cell nucleus and cytoplasm, while the cells stained with ZCIS/ZnS QDs and Cu:ZnS QDs (synthesized using same water-bath method) show colocalized emissions from cytoplasm. These results demonstrate the potential of using the aqueous synthesized ZCIS/ZnS QDs for cell imaging. To evaluate their cytotoxicity, MTT assay was performed on the 30% ZCIS/ZnS QDs. These ZCIS/ZnS QDs are almost nontoxic to macrophage cells (cell viability >90%) at a concentration of $200\ \mu\text{g ml}^{-1}$ (**Figure 5.11**). It should be noted that the concentration we used for the in vitro imaging in Figure 5.10 is $20\ \mu\text{g ml}^{-1}$, which is nontoxic to RAW264.7 macrophages, according to our MTT results. To investigate the toxicity of

Figure 5.10 Fluorescent imaging of RAW264.7 macrophage cells stained with 30% ZCIS/ZnS QDs and DAPI (upper), and incubated with 30% ZCIS/ZnS QDs and Cu:ZnS QDs (lower).

Figure 5.11 Cell viability of RAW264.7 macrophages treated with 30% ZCIS/ZnS QDs and CIS/ZnS QDs for 24 h (n=6).

the QDs with different Zn content, we also evaluated the cell viability of CIS/ZnS (Zn content: 0%) QDs and observed a higher cytotoxicity of these QDs compared to that of the 30% ZCIS/ZnS QDs. The lowered toxicity of the 30% ZCIS/ZnS QDs may come from its enhanced stability due to the incorporation of Zn. During storage of the QDs, we also observed that the ZCIS/ZnS QDs with a higher Zn content usually have a prolonged shelf life compared to those with lower Zn content. A quicker degradation of the CIS/ZnS QDs may cause toxicity to the treated cells. The prolong exposure to the 30% ZCIS/ZnS QDs may possibly induce the cellular toxicity due to the gradual degradation of the QDs, but may still cause no or minor toxicity due to the low degradation rate.

5.4 Conclusions

This chapter reported the synthesis of the Zn-Cu-In-S (ZCIS) QDs by using a facile aqueous method at a low temperature (100 °C). Before preparing the ZCIS QDs, Cu-In-S (CIS) QDs were first synthesized and showed a multi-peak photoluminescence with the intensity

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dependent on Cu:In ratio. Upon incorporating Zn into the CIS QDs, the PL intensity was significantly enhanced at the Zn content ratio of 10% and 30%, while further increasing the Zn content led to a decrease in the PL. Simultaneously, the FWHM was observed to narrow down, reflecting a more homogeneous irradiation in ZCIS QDs. The incorporation of Zn also resulted in a blue shift for the absorption and PL spectra, which indicated the widening of the QDs band gap. The XRD and EDX spectra revealed that this widening of the band gap was correlated to the alloyed ZCIS nanostructures and the preferentially substitution of Cu by Zn. The influence of Cu:In ratio and Zn content on their band gap and optical properties are compared and discussed. Overall, the incorporation of Zn into CIS QDs is an effective strategy to achieve a more homogeneous absorption band, enhance the PL intensity, narrow down the emission spectral width and increase the ability to tune the emission wavelength of the nanoparticles. Then, a ZnS shell was deposited on the ZCIS QDs and the resulting ZCIS/ZnS QDs showed an enhanced PL intensity and inhibited the pronounced blue shift when compared to CIS/ZnS QDs. Compared to the CIS QDs, the blue shift in PL upon the shell growth was not pronounced for ZCIS QDs, for which a surface reconstruction mechanism was proposed. ZnS shell growth also led to the lengthening of the fluorescence lifetime of the ZCIS QDs, which was attributed to the passivation of surface defects. Using the synthesized ZCIS/ZnS QDs, we examined the feasibility of employing them for imaging of macrophage cells. In macrophages cells, QDs and DAPI exhibited delocalized fluorescence signals from cytoplasm and cell nucleus and indicating the nonspecific in vitro uptake of the QDs. The biocompatibility of these ZCIS/ZnS QDs further demonstrated their potential for future biophotonics applications.

Chapter 6 Engineering of Zn-Ag-In-S QDs for Multiplexed Imaging of Cells

In Chapter 5, the optical properties of multinary ZCIS QDs were found to be highly dependent on the Cu:In ratio and Zn content. In this chapter, this composition control strategy is extended to prepare a similar type of Cd-free QDs, the Zn-Ag-In-S (ZAIS) QDs, using a hot colloidal synthesis method. By varying the Ag:In ratio and the Zn content, we obtained highly luminescent ZAIS QDs with tunable emissions from green to near-infrared (NIR) region. After organic-aqueous phase transfer process, we explored the potential of the water-dispersible ZAIS QDs for spectrally and temporally multiplexed cell imaging. Although their relatively broad emission spectra make them less ideal in spectrally multiplexed imaging, these ZAIS QDs offer new opportunities for temporally multiplexed imaging due to their long fluorescence lifetime. We examined the feasibility of temporally multiplexed cell imaging by using an organic dye, CdSe/ZnS QDs, and ZAIS QDs as model probes. This proof-of-concept study demonstrates the potential of using new types of QDs with distinct fluorescence lifetimes for temporally multiplexed imaging. Upon combining the temporal multiplexing technique with the conventional spectral multiplexing method, more resolvable channels are expected to be achieved in multiplexed imaging and sensing applications.

6.1 Introduction

Multiplexed imaging provides a useful technique for simultaneously identifying multiple biological targets and comprehensively analyzing physiological processes at both the cellular and tissue level.^{376, 377} Among a variety of contrast agents, QDs are particularly suited for multiplexed imaging because of their broad excitation profiles, superior photostability, and

versatile surface chemistry functionalization capability. Up to now, researchers have applied QDs with different emission colors for monitoring the progression of cancer,^{165, 378} identifying the cell types,³⁷⁹ and assessing the genetic expression³⁸⁰. All of these studies are based on spectral multiplexing technique which uses spectrally resolvable QDs and matched optical filters. For such purpose, the conventional Cd-based QDs (e.g., CdSe, CdTe QDs) are commonly used owing to their narrow and widely tunable PL profiles. Comparatively, many new types of QDs (CuInS₂, AgInS₂, Ag₂S, doped ZnS and ZnSe QDs) with relatively broad emission spectra are less ideal for spectrally multiplexed imaging.

Despite their limitations in spectrally multiplexed imaging, these new types of QDs may offer new opportunities for temporally multiplexed imaging due to their distinct temporal characteristics. Unlike the typical cadmium-based QDs with band-edge PL, many new types of QDs exhibit defect- or dopant-related PL. A remarkable feature of these emissions is the long fluorescence lifetimes because of the slow recombination processes via intra-gap levels. For example, the CuInS₂ and AgInS₂ QDs have fluorescence lifetimes typically ranging from a hundred to a few hundred nanoseconds,^{364, 381, 382} while the Cu-doped ZnS and Mn-doped ZnSe QDs show lifetimes up to microsecond and even millisecond scale.³⁸³ These distinct temporal characteristics can be utilized to differentiate these QDs from the cadmium-based QDs with fluorescence lifetimes of several to several tens of nanoseconds, and organic dyes with typical fluorescence lifetimes of a few nanoseconds.^{16, 384} Therefore, by combining these new types of QDs with the conventional optical probes, one can theoretically expect to acquire fluorescent images with temporally resolvable channels. Currently, only a handful of studies have showed the fluorescence lifetime imaging (FLIM) of living cells using a single type of QDs.^{178, 385} To the best of our knowledge, no previous work have examined the concept of temporally multiplexed imaging by using Cd-based and Cd-free QDs with different lifetimes. It is meaningful to assess the feasibility of this concept because it would

be a useful complementary method to spectrally multiplexed imaging of multiple desired biomolecules being delivered to the cells. The combination of temporal and spectral multiplexing may increase or even multiply the number of the channels that can be resolved. Also, the temporally multiplexed imaging itself has some specific advantages. First, the cell autofluorescence in visible region is difficult to be suppressed in spectrally multiplexed imaging, but it can be easily eliminated in temporally multiplexed imaging by setting appropriate time-gates.^{26, 386} Another advantage is that the time gates for separating different channels can be highly customized to meet the specific characteristics of each fluorescent probes. In spectrally multiplexed imaging, the channel splitting is achieved by using a series of spectrally matched optical filters, while in practical conditions the transmission characteristics of filters are not typically customizable. In temporally multiplexed imaging, the channel splitting can be carried out by using the programmable scripts or software, thus can be easily customized according to the specific temporal characteristics of the fluorescent probes.

However, technical challenges must be addressed to realize the potential of temporally multiplexed imaging. Herein, we explore the feasibility of temporally multiplexed imaging by selecting suitable fluorescent probes, optical set-ups and data analysis methods. Because the fluorescence lifetimes are sensitive to local environments,³⁸⁵ the selected fluorescent probes should have widely separated fluorescence lifetimes so that they can be distinguished even if their fluorescence lifetimes vary to some extent in cells. In this study, we selected a 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) dye, CdSe/ZnS (CdSe) QDs, and Zn-Ag-In-S (ZAIS) QDs, which have typical fluorescence lifetimes of a few nanoseconds, tens of nanoseconds, and hundreds of nanoseconds, respectively. The ZAIS QDs with long fluorescence lifetime were synthesized using hot colloidal method and encapsulated in polymer micelles or silica nanoparticles to achieve water dispersibility. The cells separately

treated with DAPI, CdSe QDs, and ZAIS QDs were imaged using a commercialized fluorescence lifetime imaging (FLIM) system. Afterwards, the decay curves in each pixel were exponentially fitted by using an incomplete decay model and the calculated lifetimes were correlated with the hue component of the cell image, showing a successful recognition and distinction of DAPI and two different QDs. Finally, the extracted lifetime features were used in temporally multiplexed imaging for generating separate channels for DAPI and two different QDs. For the first time, our proof-of-concept study demonstrates the feasibility of temporally multiplexed imaging by using Cd-based and Cd-free QDs with differing fluorescence lifetimes.

6.2 Experimental Methods

6.2.1 Engineering of CdSe/ZnS and Zn-Ag-In-S (ZAIS) QDs

Organically capped CdSe/ZnS QDs (simplified as CdSe QDs) were obtained from NN-Labs. Absolute ethanol (Merck Millipore) and 18.2 M Ω •cm deionized (DI) water were used through the experiment. Pluronic F127 is a gift from BASF (NJ, USA). All other chemicals and reagents for synthesis and surface modification of QDs were purchased from Sigma-Aldrich, Alfa Aesar, or Tokyo Chemical Industry.

The organically capped CdSe QDs were transferred into the aqueous phase by a ligand exchange method described in Chapter 4.²⁵⁶ Briefly, 1 mg of CdSe QDs were washed with ethanol and mixed with 30 mg of mercaptosuccinic acid (MSA) in 5 ml of chloroform. After stirring for 5 min, 100 μ l of 30% NH₄OH solution and 900 μ l of DI water were added into the reaction mixture, and the biphasic system solution was stirred for another 5 h. Afterwards, the QDs in aqueous phase were washed by centrifugation with ethanol and dispersed in 1 ml of DI water.

The synthesis of Zn-Ag-In-S (ZAIS) QDs was modified from previous reports.^{19, 387} In a typical synthesis of orange-emitting ZAIS QDs, 0.25 mmol of silver nitrate (AgNO_3), 1 mmol of indium(III) acetate ($\text{In}(\text{OAc})_3$) and 3 mmol of stearic acid (SA) were mixed with 10 ml of 1-octadecene (ODE) and 2 ml of oleic acid (OA) in a flask. The reaction mixture was heated to 100 °C within 30 mins under stirring and nitrogen flow. After cooled down to 90 °C, 2 ml of dodecanethiol (DT) was added and the mixture was stirred for 3 min before the rapid injection of 1.5 mmol of sulfur (S) pre-heated in 3 mL of oleylamine. The Ag-In-S (AIS) QDs were allowed to grow for 5 min. Afterwards, 0.5 mmol of zinc stearate (ZnSt_2) and S dispersed in ODE and DT were injected into the reaction mixture and the temperature was raised to 150°C. After 10 min, the ZAIS QDs were precipitated by centrifugation with ethanol and stored in chloroform. For preparing near-infrared ZAIS QDs, the Ag:In ratio was set at 1:1 and the amount of Zn precursors was reduced to 0.25 mmol. For preparing green-emitting ZAIS QDs, the Ag, In, Zn precursors were adjusted to 0.1 mmol, 0.4 mmol and 0.9 mmol. It should be mentioned that the Zn and S precursors for preparing green-emitting QDs were injected for three times at 1 h interval and the temperature of the reaction mixture was respectively raised to 180°C and 220°C after the second and third injection (**Table 6.1**). The yellow-emitting QDs were obtained before the third injection.

Surface modification of the organically capped ZAIS QDs was adapted from previous methods.³⁸⁸⁻³⁹¹ For preparing F127-encapsulated ZAIS QDs, 1 mg of ZAIS QDs were mixed with 15 mg of F127 in 500 μl chloroform and the mixture was stirred for 30 min. Subsequently, 4 ml DI was added and the mixture was stirred for 24 h in a vial open to the laboratory atmosphere. The resulting solution was filtered through a 0.22 μm syringe filter to remove the large aggregates. For preparing the silica-coated ZAIS QDs, 1 mg of ZAIS QDs dispersed in 100 μl of chloroform were injected into 1 ml aqueous solution of 46.5 mg cetyltrimethylammonium bromide (CTAB). After stirring at 70 °C to evaporate the

chloroform, the resulting aqueous solution was added to the mixture of 4.5 ml of DI water and 12 μl of 0.2 M NaOH. Subsequently, 125 μl of tetraethyl orthosilicate (TEOS) was injected into the solution mixture, and the mixture was heated at 70 °C for 10 h under stirring. Afterwards, the silica modified ZAIS QDs were collected by centrifugation at 9000 rpm for 10 min. The QDs were washed with ethanol and water for five times to remove the impurities and dissolved in DI water with the assistance of sonication.

Transmission electron microscopy (TEM) images were taken with a FEI TecnaiTM microscope at an accelerating voltage of 200 kV. UV-vis absorption spectra of the QDs were measured using a UV-2450 spectrometer (Shimadzu, Japan) and PL spectra of QDs were collected by a Fluorolog-3 Fluorometer (HORIBA Jobin Yvon, USA).

6.2.2 Incubation of QDs with Cells

RAW264.7 macrophage cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 100 $\mu\text{g ml}^{-1}$ penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin and 10% fetal bovine serum (FBS) at 37°C with 5% CO₂. Before treating cells with QDs, cells were seeded onto the glass-bottom microwell dishes. The water-dispersible CdSe and ZAIS QDs were added into the culture media and the cells were incubated for another 4 h. After 4 h, the treated cells were washed with PBS for three times and fixed with 4% formaldehyde. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) was used for nuclei staining. For cross-cell experiment, RAW264.7 cells separately treated with CdSe and ZAIS QDs in two dishes were washed, trypsinized and seeded onto one dish. After incubation for another 4 h, the cells were fixed with 4% formaldehyde.

6.2.3 Fluorescence Decay Measurements and Data Analysis

The time-resolved PL decays of aqueous CdSe and ZAIS QDs were obtained by a DCS-120 Confocal Scanning Fluorescence Lifetime Imaging System (Becker & Hickl,

Germany) operated at a single curve mode. The cell images with 256 x 256 pixels and 256 time channels were taken at a film mode with 100x oil lens. A 375 nm picosecond laser with 20 MHz repetition rate was used as the excitation source. HPM-100-40 high-speed hybrid detector was used to record the fluorescence signals by the control of DCC-100 detector controller.

The fluorescence decay curves of DAPI, CdSe and ZAIS QDs were fitted using an incomplete decay model with the correction method proposed in a previous report.³⁷³ In this model, the measured incomplete fluorescence decay is a summation of the fluorescence decays under excitation of current and previous pulses:

$$I(t) = \lim_{n \rightarrow \infty} [I_0(t) + I_0(t + T) + \dots + I_0(t + nT)] \quad (6.1)$$

where $I(t)$ is the measured fluorescence decay, $I_0(t)$ is original fluorescence decay, and T (50 ns in this study) is the repetition period of the laser pulses.

Given the original fluorescence decay described as,

$$I_0(t) = \sum_{i=1}^N A_{0i} e^{-t/\tau_{0i}} \quad (6.2)$$

and the measured fluorescence decay fitted as,

$$I(t) = \sum_{i=1}^N A_i e^{-t/\tau_i} \quad (6.3)$$

where τ_{0i} , τ_i is the i th lifetime components of the original and measured decay curves, and A_{0i} and A_i is the amplitude of the corresponding lifetime components. By substituting equation (6.2) and (6.3) into equation (6.1) and considering $n \rightarrow \infty$, the parameters of original decays can be obtained by correction of the parameters fitted from the measured decays,

$$A_{0i} = (1 - e^{-T/\tau_i}) A_i \quad (6.4)$$

$$\tau_{0i} = \tau_i \quad (6.5)$$

The corrected lifetime components and amplitudes were used for calculating the lifetime. The average lifetime τ_{avg} of the bi-exponential function can be computed as,^{392, 393}

$$\tau_{avg} = (A_{01}\tau_{01}^2 + A_{02}\tau_{02}^2)/(A_{01}\tau_{01} + A_{02}\tau_{02}) \quad (6.6)$$

Based on this model, the fluorescence lifetime images were analyzed using custom Matlab scripts. First, raw data was imported as a matrix A with 256 rows (time channels) and 256 x 256 columns (pixels). The integrated intensity of each pixel was calculated by summation of the photon counts in the 256 time channels and normalized as a matrix B. After subtraction of the estimated background signal, the fluorescence decay in each pixel was fitted by the standard mono-exponential ('exp1') or bi-exponential ('exp2') functions using a trust-region algorithm. For mono-exponential fitting, the calculated lifetimes were normalized and capped to [0,1], linearly projected to the hue space ([0-1] to [240-0]) and recorded in a matrix C. The values in matrices C and B were taken as the hue and value components in an HSV image and the saturation component was set as 1. Then the HSV images were converted to RGB images and plotted as a 256 x 256 pixel image. By using a similar procedure, the average lifetimes calculated from the corrected bi-exponential models were correlated with the hue component of the HSV image.

6.3 Results and Discussion

6.3.1 Synthesis, Surface Modification, and Characterization of ZAIS QDs

In Chapter 5, the ZCIS QDs exhibit blue-shifted PL spectra upon decreasing the Cu:In ratio or increasing the Zn content. This composition control strategy is extended to synthesize the ZAIS QDs. The composition of ZAIS QDs was adjusted by varying the Ag:In feed ratio in nucleation stage, or by varying the amount of injected Zn and S precursors after formation of

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Table 6.1 The synthetic conditions and procedures for preparing NIR-, orange-, yellow-, green- emitting ZAIS QDs and the representative PL results.

Step	1) Ag-In-S Nucleation			2) + Zn		3) + Zn and S		4) + Zn and S		Representative PL Results	
	Ag ^a	In	S	Zn	Temp [°C]	Zn / S	Temp [°C]	Zn / S	Temp [°C]	Peak [nm]	QY [%]
NIR	1	1	2	0.25	120					724	11.2
Orange	0.25	1	1.5	0.5	150					600	33.8
Yellow	0.1	0.4	0.6	0.1	150	0.4	180			545	52.6
Green	0.1	0.4	0.6	0.1	150	0.4	180	0.4	220	529	66.7

^aThe unit for the amount of precursors is millimole (mmol).

Ag-In-S (AIS) cores. Table 6.1 gives the synthetic conditions for preparation of green-, yellow-, orange-, and NIR-emitting ZAIS QDs (PL peak: 529-724 nm). These organically synthesized ZAIS QDs showed bright emissions under UV excitation (**Figure 6.1a,b**) and the QY reached 33.8-66.7% for orange-, yellow- and green- emitting QDs. The NIR-emitting ZAIS QDs have a considerable QY of 11.2%, which is a promising value as NIR-I (650-950 nm) emitting probes. The EDX results confirmed the compositional variation in the QDs synthesized with different precursor ratios (Figure 6.1e, f). By decreasing the Ag:In ratio or increasing the Zn content, the absorption and emission spectra of the ZAIS QDs showed a pronounced blue shift (Figure 6.1c, d), which is consistent with the result obtained for ZCIS QDs (Chapter 5). Such a large blue shift implied that, rather than forming core/shell structures, Zn is more likely to diffuse into the AIS crystal lattice, and this blue shift was attributed to the band gap widening upon reducing the Ag:In ratio and increasing the Zn content.^{382, 394} It is worth mentioning that the method for incorporating Zn into AIS cores is by alloying Zn into the formed AIS QD cores at an elevated temperature. This is different from what we have done in the case of ZCIS QDs, where the Zn precursors were added as a nucleation agent. We also attempted to incorporate Zn into the AIS cores by co-nucleation of Zn with Ag, In, and S, but the resulting ZAIS QDs is less luminescent than the ones obtained by the alloying method although they showed similar elemental composition.

Figure 6.1 Composition effect on the steady-state optical properties of ZAIS QDs. (a, b) Digital photograph of ZAIS QDs with different Ag:In ratio and Zn content under natural light and UV light. (c, d) UV-vis absorption and PL spectra of ZAIS QDs with different composition ratio. (e, f) Representative EDX spectrum and the composition ratio of ZAIS QDs determined by EDX.

Similar with the ZCIS QDs, increasing the Zn content of ZAIS QDs also resulted in a narrowing of the PL spectral width. From Figure 6.1d, the green-emitting QDs showed reduced full-width at half-maximum (FWHM) compared to the orange- and NIR-emitting QDs. According to TEM results in **Figure 6.2**, the orange- and NIR-emitting QDs had a more homogeneous size and morphology distribution than the green-emitting QDs. This indicates that the reduced FWHM is not because of the increased homogeneity in size or shape. The size of green-, orange- and NIR-emitting QDs estimated from TEM results are 5.5 ± 1.2 , 3.2 ± 0.6 , and 4.7 ± 0.6 nm, respectively, and these QDs show crystalline domains in HRTEM images. The size broadening observed in green-emitting ZAIS QDs may result from non-homogeneous alloying of Zn or Ostwald ripening during the prolonged reaction time.³⁹⁵ As the size and shape factors are excluded, the spectral narrowing upon increasing Zn content may have other origins such as more homogeneous composition distribution, crystal phase distribution, or energy level distribution among QDs.

Figure 6.2 TEM and (insets) HRTEM of (a) green-emitting, (b) orange-emitting and (c) NIR-emitting ZAIS QDs.

The composition ratio of ZAIS QDs not only influenced their steady-state optical properties but also affected their transient optical properties. A shortening of the fluorescence lifetime was observed in ZAIS QDs when decreasing the Ag:In ratio or increasing the Zn

content (**Figure 6.3**). By fitting the time-resolved PL decays to bi-exponential curves, this lifetime shortening was ascribed to two effects, the shortening of the long lifetime component and the reduced weight of the long lifetime component. In Chapter 5, the study in transient optical properties of ZCIS QDs has revealed that the deposition of ZnS shell will cause the lengthening of the fluorescence lifetime. Herein, the addition of Zn and S led to an opposed result. This result provides an additional evidence that the Zn was alloyed into the AIS cores rather than only deposited on the surface as a shell.

Figure 6.3 Composition effect on time-resolved PL decays of green-, yellow-, orange-, and NIR- emitting ZAIS QDs. The PL decays were fitted to bi-exponential curves (solid black line).

The organically-capped ZAIS QDs were subsequently transferred into the aqueous solution for biophotonics applications. According to our experience, the ligand exchange method used in Chapter 4 does not work for these QDs capped with dodecanethiol (DT) ligands because the organothiol ligands can be firmly anchored on the surface of QDs. To render the surface of ZAIS QDs water-dispersible, two encapsulation methods were applied, one is micelle encapsulation using pluronic F127 and the other is CTAB micelle

encapsulation followed by templated silica growth. For both methods, the resulting water-dispersible ZAIS QDs retained a high PL efficiency. From TEM results displayed in **Figure 6.4**, ZAIS QDs showed almost unchanged size and morphology in F127 micelles or silica matrix. In cell studies, the F127-encapsulated QDs showed weak fluorescence from R264.7 cells while the silica-coated QDs can be efficiently taken up by these macrophage cells.

Figure 6.4 TEM image and (insets) HRTEM image of (a) F127-encapsulated NIR-emitting ZAIS QDs and (b) silica-modified orange-emitting ZAIS QDs.

6.3.2 Multiplexed Cell Imaging Using ZAIS QDs

First, we examine the potential of the prepared ZAIS QDs for spectrally multiplexed imaging. Theoretically, the broad emissions of ZAIS QDs will make them less distinguishable in spectrally multiplexed imaging compared to the conventional Cd-based QDs. In practical conditions, the spectral characteristics of the optical filters should be carefully selected to resolve the QD emissions. Herein, the 540/50 nm and 605/35 nm bandpass filters were applied for the cells incubated with the ZAIS QDs. The results in **Figure 6.5** showed that the green- and orange- emitting QDs showed the majority of fluorescence from green (540/50 nm) and red (605/35 nm) channel, respectively, which demonstrated that the emissions from two

QDs can be basically distinguished in spectrally multiplexed imaging. However, the green-emitting QDs also displayed weak fluorescence from the red channel image and the orange-emitting QDs showed moderate fluorescence from the green channel image. This was caused by the broad emission spectra of the QDs that cover the transmission range of both channel filters. Because of this, the number of the resolvable QDs will be limited in spectrally multiplexed imaging.

Figure 6.5 Fluorescent cell imaging using green- and orange-emitting ZAIS QDs with 540/50 nm and 605/35 nm bandpass emission filter.

Although these QDs are less ideal in spectrally multiplexed imaging, their long fluorescence lifetimes make them promising as contrast agents for temporally multiplexed imaging. Herein, we explore the feasibility of using ZAIS QDs for spectrally multiplexed imaging by combining them with DAPI dye and CdSe QDs. The selection criteria for the optical probes is that they have widely separated fluorescence lifetime so that they can be more easily distinguished in cells. Before cell studies, we investigated the steady-state and transient optical properties of the selected fluorescent probes. The PL spectra of the CdSe and ZAIS QDs are severely overlapped (**Figure 6.6a**), making it difficult to distinguish their

emissions by optical filters or visible eyes (Figure 6.6a inset). Despite the spectral similarity between the CdSe and ZAIS QDs, they demonstrate a significant difference in their time-resolved PL decay profiles (Figure 6.6b). Moreover, both of their PL decay profiles show a much lower decay rate compared to that of DAPI. It should be noted that, due to the

Figure 6.6 Spectral and temporal optical properties of CdSe and ZAIS QDs. (a) Absorption (dash line) and PL (solid line) spectra of CdSe and ZAIS QDs dispersed in water. Inset is a digital photograph showing the aqueous dispersions of CdSe (left) and ZAIS (right) QDs under UV light. (b) Time-resolved PL decays of DAPI in cell nuclei, CdSe and ZAIS QDs dispersed in water. The PL decays were fitted to mono-exponential (dash line) and bi-exponential (solid line) curves.

high frequency (20 MHz) of the applied laser, the recorded decays are incomplete fluorescence decays, containing the fluorescence decay upon excitation of current pulse and the decay tails upon excitation of previous pulses. This incomplete effect will skew the decay curves but will nevertheless retain the difference between the fast and slow decays. A lower frequency excitation will allow the acquisition of more complete fluorescence decays, but limit the number of photons that can be collected in a given time. The limited number of

photons increases the difficulty of curve fitting, especially when one needs to record a large number of fluorescence decays (65536 in our study) in a FLIM image. In this study, the applied 20 MHz laser has a repetition period in between the fluorescence lifetimes of CdSe and ZAIS QDs, which increases the photons numbers while retaining the difference of their PL decays. After curve fitting, an incomplete decay model with corrections was used to estimate the fluorescence lifetimes.³⁷³ **Table 6.2** gives the lifetime components and amplitudes (before and after correction) of DAPI, CdSe and ZAIS QDs by bi-exponential fitting. For mono-exponential fitting with only one lifetime component, the original lifetime is theoretically equal to the lifetime calculated from the measured lifetime. For bi-exponential fitting, the values of lifetime components are unchanged according to Equation 6.5. However, the amplitudes can be significantly different before and after corrections, especially when the corresponding lifetime component is comparable or longer than the repetition period of laser. The fluorescence lifetimes calculated from the mono-exponential fitting curves are 1.9, 31.9, and 224.3 ns respectively for DAPI (in cell nuclei), CdSe and ZAIS QDs (in water). Although the mono-exponential fitting is not a tight fitting model for these multi-exponential fluorescence decays, it is recommended to be used in cell imaging because it is more robust when there are limited numbers of photons for curve fitting.³⁹⁶ To evaluate the validity of mono-exponential fitting, the calculated parameters were compared with those obtained from bi-exponential fitting. In mono-exponential fitting, the calculated lifetime is equal to the time that the fluorescence intensity drops to $1/e$ of its peak intensity, $\tau_{1/e}$. For decay curves fitted by bi-exponential functions, we also calculated this $\tau_{1/e}$ by solving t from the formula,

$$A_{01}e^{t/\tau_{01}} + A_{02}e^{t/\tau_{02}} = 1/e (A_{01} + A_{02}) \quad (6.7)$$

From **Table 6.3**, the lifetime τ (*i.e.*, $\tau_{1/e}$) obtained from mono-exponential fitting has no significant difference compared to the $\tau_{1/e}$ calculated from the corrected

bi-exponential models, which proves that the fluorescence lifetimes calculated by using mono-exponential model are reasonable. The average lifetimes τ_{avg} obtained from bi-exponential models are close to the long fluorescence component when there is a large difference between two lifetime components.

Table 6.2 Lifetime components and amplitudes before and after corrections by bi-exponential fitting.

	DAPI	CdSe QDs	ZAIS QDs
A_1 / A_{01}	0.32 / 0.32	0.27 / 0.27	0.08 / 0.08
τ_1 / τ_{01} [ns]	0.75	5.03	0.98
A_2 / A_{02}	0.68 / 0.68	0.73 / 0.44	0.92 / 0.10
τ_2 / τ_{02} [ns]	2.61	55.2	425

Table 6.3 Fluorescence lifetimes calculated using the mono-exponential and bi-exponential fitting models using corrected parameters.

		DAPI	CdSe QDs	ZAIS QDs
Mono-exponential	τ [ns]	1.93	32.4	188
Bi-exponential	$\tau_{1/e}$ [ns]	1.82	28.8	179
	τ_{avg} [ns]	2.39	52.5	424

Although the CdSe and ZAIS QDs dispersed in water can be easily distinguished by distinct fluorescence lifetimes, it is still unknown whether they are resolvable when taken up by cells. To examine this problem, the cells separately treated with DAPI, CdSe and ZAIS QDs were imaged using a commercialized fluorescence lifetime imaging (FLIM) system. The fluorescence decays in each pixel were fitted to mono-exponential curves and the calculated lifetimes were correlated with the hue component of an HSV image. The resulting false-color images show clear distinction between DAPI, CdSe and ZAIS QDs (**Figure 6.7d, e, f**) and

Figure 6.7 Fluorescence lifetime imaging of RAW264.7 cells separately treated with (a,d) DAPI, (b,e) CdSe QDs and (c,f) ZAIS QDs. (a,b,c) Intensity images of cells. (d,e,f) Fluorescence lifetime images of cells. The fluorescence lifetimes were obtained by mono-exponential fitting and mapped to a false-color scale from 0 ns (blue) to 60 ns (red). (g) Histograms of fluorescence lifetimes of DAPI, CdSe and ZAIS QDs were extracted from the corresponding cell images.

Figure 6.8 Fluorescence lifetime imaging of RAW264.7 cells separately treated with (a) DAPI, (b) CdSe QDs and (c) ZAIS QDs. The average fluorescence lifetimes were calculated by bi-exponential fitting and mapped to a false-color scale from 0 ns (blue) to 90 ns (red). (d) Histograms of the average fluorescence lifetimes for DAPI, CdSe and ZAIS QDs were extracted from the corresponding cell images.

the distributions of their fluorescence lifetimes are separated in the extracted histogram (Figure 6.7g). The broad distribution of the fluorescence lifetimes is considered to be a combined result of the lifetime variation of dye and QDs in cells and error in data collection and analysis.^{385, 397, 398} Carlini and Nadeau applied CdSe/ZnS QDs for FLIM imaging and found their fluorescence lifetimes varied in different cellular regions, which are affected by the pH level and redox conditions of local environment.³⁸⁵ Similarly, Orte et al. have observed that the fluorescence lifetimes of CdSe/ZnS QDs varied from 8.7 ns to 15.4 ns when the solution pH was increased from 5 to 9, and they even utilized this property for sensing pH

in live cells.³⁹⁷ However, despite the lifetime variation, the emission of DAPI, CdSe and ZAIS QDs can be well distinguished by their fluorescence lifetimes, which demonstrates the potential of using these probes for temporally multiplexed cell imaging. Upon comparing to mono-exponential fitting, the bi-exponential fitting generated less resolvable FLIM images of the applied dye and QDs (**Figure 6.8**). This may result from a higher instability of bi-exponential fitting under the condition of limited photon numbers and the over-weighting of long lifetime component when calculating the average lifetime.^{385, 396, 399} Therefore, for our FLIM image with limited photons numbers ($<10^4$ photons in each pixel), mono-exponential model is recommended for curve fitting and lifetime calculations.

The obtained fluorescence lifetime distributions (Figure 6.7g) were used to determine the time gates for channel splitting in temporally multiplexed imaging. Accordingly, 10 ns was set as the time gate to separate the signals from DAPI and CdSe QDs and 40 ns was selected to separate signals from CdSe and ZAIS QDs. By using the software-based time gates, the FLIM images containing a mixture of fluorescence signals were split into three channel images (**Figure 6.9**). In original FLIM images, the cells co-stained with DAPI and QDs exhibit a false-color pattern of round nuclei encircled by peripheral cell regions. The cell nuclei were displayed in 0-10 ns channel image, while the peripheral regions were mostly present in 10-40 ns channel image for cells treated with CdSe QDs and in >40 ns channel image for cells treated with ZAIS QDs. This result indicates that in cell imaging, the emissions from QDs and dyes with distinct lifetimes can be temporally resolved. Also, we noticed that a perinuclear, ring-like pattern showing lifetimes in between those of the DAPI and QDs, which was displayed in 0-10 ns channel image for cells treated with CdSe QDs and DAPI, and in 10-40 ns image channel for cells treated with ZAIS QDs and DAPI. According to results in Figure 6.7, DAPI only or QDs only cannot generate such a pattern with these intermediate fluorescence lifetimes. Therefore, this pattern is likely to arise from the

Figure 6.9 Temporally multiplexed imaging of the RAW264.7 cells co-stained with DAPI and CdSe QDs (top row), DAPI and ZAIS QDs (medium row), and the cells separately treated with CdSe and ZAIS QDs, seeded and cultured in a same dish (bottom row). The original FLIM images (left column) were split into 0-10 ns, 10-40 ns, and > 40 ns channel images as indicated.

co-existence of DAPI and QD signals with comparative intensity in the same pixel. To examine the resolvability of two QDs in cell imaging, we performed a cross-cell experiment by co-incubating two group of cells, either pretreated with CdSe or ZAIS QDs, in a same dish for FLIM imaging.⁴⁰⁰ The resulting FLIM images show a spatially separated pattern of cells with different false colors, which is expected when a minor proportion of QDs were

transferred between neighboring cells. After channel splitting, the blue-green pixels in 10-40 ns channel image and red pixels in >40 ns channel image are respectively attributed to emissions from CdSe and ZAIS QDs. This result conceptually proved that the emissions of CdSe and ZAIS QDs can be resolved in temporally multiplexed cell imaging. The same temporal multiplexing technique can improve the reliability of distinguishing the fluorescent probes with similar emission wavelengths but distinct fluorescence lifetimes.

6.4 Conclusions

In this work, highly luminescent ZAIS QDs with different composition ratio were prepared using the hot colloidal synthesis method. The composition effect on the optical properties of ZAIS QDs were investigated, and the results were compared with previous ones obtained from ZCIS QDs. After organic-aqueous phase transfer process, we examined the potential of the surface modified ZAIS QDs for multiplexed imaging. Although the broad emission profiles make them less ideal in spectral multiplexed imaging applications, their long fluorescence lifetime allow new opportunities in temporally multiplexed imaging. For the first time, our proof-of-concept study demonstrates the feasibility of temporally multiplexed imaging of cells by using Cd-based and Cd-free QDs with widely differing fluorescence lifetimes. Future work is needed to extend this technique to a variety of QDs showing magnitude difference of fluorescence lifetimes. Combining a pool of spectrally and temporally distinct fluorescent probes, one can expect to increase the number of resolvable channels for multiplexed imaging and sensing applications. Such technique will serve as a valuable tool for future nanomedicine and biophotonics research.

Chapter 7 Photoluminescence and Plasmonic Properties of ZnO QDs

From Chapter 3 to Chapter 6, all the prepared QDs are made from metal chalcogenide semiconductors, and they rely on the photoluminescence (PL) properties to generate the optical signals for biophotonics applications. In this chapter, we study a type of wide-band-gap metal-oxide QDs, the zinc oxide (ZnO) QDs. In addition to their PL properties, the ZnO QDs can exhibit extraordinary plasmonic properties when they are appropriately doped by the chemical or photochemical methods. In this work, ZnO QDs with different size and dopants were synthesized using a sol-gel method. Their PL and plasmonic properties were characterized and correlated with their size and composition. After photodoping, the plasmonic absorption spectra emerged and are sensitive to oxygen exposure. The effect of size and impurities on the generated plasmonic absorption spectra was studied and discussed. This study provides a guideline for preparing ZnO QDs with specific optical properties for bioimaging or biosensing applications.

7.1 Introduction

As discussed in Chapter 1, Zn is less toxic compared to Cd at the same concentration, and it is an essential element for various biological activities. ZnO QDs with UV to visible emissions are therefore regarded as promising optical probes for biophotonics applications. Another advantage of the ZnO QDs is the flexibility of doping various ions in ZnO semiconductor materials. For example, ZnO materials doped with the transition metal ions (e.g., Mn^{2+} and Co^{2+}) exhibited ferromagnetic behaviors,⁴⁰¹ and ZnO materials doped with some of the trivalent metal ions (e.g. Al^{3+} , In^{3+} , Ga^{3+}) showed plasmonic properties.⁴⁰² For ZnO QDs in the quantum confinement regime, these properties need to be reconsidered due

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to the variation of their electronic structures.⁴⁰³ Also, the small size of ZnO QDs increases the difficulty of controlling the doping process in the formation of the ZnO crystal lattice.

In addition to the chemical doping method, photodoping is another method for modifying the electronic and optical properties of ZnO nanoparticles. Upon continuous excitation by above-band-gap energy photons, ZnO QDs exhibit a significant change in their PL intensity accompanied by the appearance of plasmonic absorption spectra in the near-infrared to mid-infrared region. In recent years, the photodoping mechanism was extensively studied and a common conclusion was drawn that the photoexcited electrons accumulated in the conduction band of the ZnO QDs. For this reason, the process is also called “photocharging”. However, there is still some dispute about the mechanism for the alternation of the optical properties. For example, the researchers previously attributed the photodoping-induced infrared absorption spectra to intraband single-carrier transition.⁴⁰⁴ Until recently, this infrared absorption was thought to be the plasmonic absorption.⁴⁰⁵ Further efforts need to be devoted to investigating the detailed mechanism of this phenomenon and its possible applications.

In this work, the ZnO QDs were synthesized using a sol-gel method. The pH, impurity types, and impurity concentrations were varied to fine-tune their optical properties. The size, morphology, crystal structure, and optical properties of the prepared QDs were characterized to reveal the effect of different synthesis conditions. Finally, a photodoping study was performed on the synthesized ZnO QDs. Varying the size and chemical doping of the QDs was found to influence the photoinduced plasmonic absorption spectra.

7.2 Experimental Methods

7.2.1 Synthesis of ZnO QDs Using Sol-Gel Method

All the chemicals were purchased from Sigma-Aldrich or Tokyo Chemical Industry. The sol-gel method for synthesizing ZnO QDs is modified from previous protocols.^{406, 407} Typically, the Zn(II) precursors were prepared by refluxing 1 mmol $\text{Zn}(\text{oAc})_2 \cdot 2\text{H}_2\text{O}$ in 10 ml of boiling ethanol (99.5%) for 30 min. Afterwards, the flasks were cooled in an ice bath with dry air flow to avoid moisture and 300 μl of KH-560 silane precursors was subsequently added. Meanwhile, 1.4 mmol $\text{LiOH} \cdot \text{H}_2\text{O}$ was dissolved in 10 ml of ethanol under sonication. After cooling in an ice bath, the $\text{LiOH} \cdot \text{H}_2\text{O}$ solvent was added drop by drop to the flask containing Zn(II) precursors while it was stirred vigorously. The stirring was continued for 1 h and the ZnO QDs in ethanol were collected by repeatedly adding hexane followed by centrifugation at 3,700 rpm. The precipitates were redissolved in ethanol for further use. To adjust the pH values, the $\text{OH}^-:\text{Zn}$ ratio was varied from 0.9 to 1.4 to 1.8. At an $\text{OH}^-:\text{Zn}$ ratio of 0.9, NaOH was used in place of $\text{LiOH} \cdot \text{H}_2\text{O}$ and the reaction temperature was elevated to 50°C. To incorporate impurities, a certain amount (5%, 10%, 30% precursor ratio) of $\text{La}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$, $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ or InCl_3 was heated with Zn precursors in the boiling ethanol.

7.2.2 Characterization and Photodoping of ZnO QDs

Transmission electron microscope (TEM) images were taken by a JEOL JEM-2000EX II model operating at 200 kV. Before taking TEM images, concentrated samples were sonicated in acetone, and the solution from the upper layer after placement was dropped and dried on a copper grid. X-ray diffraction (XRD) spectra of the dried samples on cover glasses were acquired on a Rigaku SmartLab diffractometer. UV-vis-NIR absorption spectra were collected using a JASCO V-670 spectrophotometer, and photoluminescence (PL) spectra

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were obtained using a JASCO FP-6500 fluorometer. Before photodoping, the deaerated ethanol solution of ZnO QDs were transferred to the quartz cuvette in an argon glove box. Microcuvettes with an optical length of 1 mm were used for absorption measurement, and a 3 x 3 mm four-window transparent cuvette was used for PL measurement. A Xenon lamp with a 300–330 nm filter was used as the excitation light source for photodoping of the ZnO QDs.

7.3 Results and Discussion

7.3.1 Size Tuning of ZnO QDs

Figure 7.1 (a) Ethanol dispersion of ZnO QDs irradiated by a Xenon lamp with a 300–330 nm filter. (b) Absorption and (c) PL spectra of ZnO QDs synthesized at a OH⁻:Zn ratios of 1.8, 1.4, and 0.9.

ZnO QDs were synthesized via a conventional sol-gel method. Zn(II) precursors were hydrolysed to form ZnO QDs when OH⁻ solutions was injected in the reaction mixture. By varying the ratio of OH⁻:Zn from 1.8 to 0.9, we acquired ZnO QDs with blue to yellow emissions (**Figure 7.1a**). Absorption peak of the ZnO QDs fell in the range of 335–302 nm (Figure 7.1b), which corresponds to the band gap of 3.7–4.1 eV, higher than the band gap of bulk ZnO (3.4 eV⁴⁰⁸). This band gap widening is attributed to the quantum confinement and provides a proof for the formation of ZnO QDs. PL spectra of the ZnO QDs exhibited a weak and narrow emission in the UV region and a bright, broad emission in the visible region. The

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UV emission originated from the band-edge recombination, and the visible emission was usually correlated with oxygen vacancies.^{409, 410}

With an increase in the $\text{OH}^-:\text{Zn}$ ratio, both the absorption and emission spectra exhibited a blue shift, hinting that QDs of different size were formed at varied $\text{OH}^-:\text{Zn}$ ratios. The size variance among QDs was further confirmed using TEM characterization. From the TEM images, the diameters of ZnO QDs synthesized at a $\text{Zn}:\text{OH}^-$ ratio of 1.8, 1.4, and 0.9 were estimated to be 2.85 ± 0.37 nm, 3.42 ± 0.59 nm, and 4.36 ± 0.68 nm, respectively (**Figure 7.2a, b, c**). At a scale comparable to the exciton Bohr radius of ZnO (2.34 nm⁴¹¹), an enhanced confinement due to the size decrease causes a broadening of band gap and results in a blue shift on their absorption and emission spectra. As expected, increasing or decreasing the $\text{OH}^-:\text{Zn}$ ratio affected the size of synthesized QDs, which may result from the varied conditions in sol-gel reactions,⁴¹²

Varying the $\text{OH}^-:\text{Zn}$ ratios may affect the formation of Zn(OH)_2 and its hydrolysis rate, thereby influencing the nucleation and growth kinetics of ZnO QDs. In addition, the high-resolution TEM (HRTEM) images (Figure 7.2d) show that the lattice spacing measures 0.29 and 0.25 nm, consistent with the characteristic of wurtzite ZnO nanocrystals.⁴¹³ This crystal structure was further confirmed by XRD measurements, which will be discussed later.

Figure 7.2 (a, b, c) TEM images of ZnO QDs synthesized at a Zn:OH⁻ ratio of 1.8, 1.4, and 0.9. (d) HRTEM images of ZnO QDs synthesized at a Zn:OH⁻ ratio of 1.4.

7.3.2 Impurity Doping of ZnO QDs

We attempted to incorporate the trivalent ions in the ZnO QDs by preparing Zn(II) precursors in the presence of La, Al, and In precursors. ZnO QDs synthesized with 5%, 10%, and 30% La precursors (simplified as La:ZnO QDs) exhibited similar absorption spectra compared to the pure ZnO QDs except for a slight blue shift at the absorption peak (**Figure 7.3a**). The intensity of visible emissions was greatly enhanced upon the introduction of La precursors in reaction mixtures. The XRD spectra showed similar peak positions of all the ZnO and La:ZnO QDs (**Figure 7.4**), corresponding to the

Figure 7.3 (a) UV-vis absorption spectra and (b) photoluminescence spectra of ZnO QDs and 5%, 10% and 30% La doped ZnO QDs. (c) TEM and (d) HRTEM images of 10% La doped ZnO QDs. Herein, the 5%, 10%, 30% represent the feed ratio of La/(La + Zn).

wurtzite ZnO structure (JCPDS 36-1451). In a previous report, the enhancement of visible PL was attributed to the formation of more radiative defect centers, which may be a result of the increased surface defects due to size decrease or crystal deformations upon La^{3+} incorporation.⁴⁰⁶ In this work, the results were more supportive of the latter explanation. First, the 5% and 10% La:ZnO QDs showed their absorption peak at almost the same position, indicating that nanocrystals of similar size are obtained under these two conditions.

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However, 10% La:ZnO QDs demonstrated a much stronger PL intensity in the visible region than the 5% La:ZnO QDs. The size of 10% La:ZnO QDs obtained from TEM images (Figure 7.3c) are 3.40 ± 0.48 nm, and their morphology is similar with the pure ZnO QDs. Also, the lattice spacing measurements from the FFT derived from HRTEM are 0.29 and 0.25 nm, which is the same as the ZnO QDs. These results illustrate that the size effect is not the main cause for the increased intensity of the visible emission. Second, upon increasing the La impurities from 0% to 30%, the emission intensity in the UV region was decreased (Figure 7.3b), and the XRD peaks were broadened and weakened (Figure 7.4), indicating poor crystallinity and increased structural defects in La:ZnO QDs. Third, the EDX spectra of the carefully washed ZnO and La:ZnO QDs directly confirmed the existence of La in the nanocrystals (**Figure 7.5**). The La content of 10% La:ZnO QDs was measured to be 14.9%. All these results suggested that the enhanced intensity of the visible PL of La:ZnO QDs is a result of the increased structural defects.

Figure 7.4 XRD spectra of ZnO QDs and 5%-30% La doped ZnO QDs.

Figure 7.5 Representative EDX spectra of ZnO and 10% La:ZnO QDs.

Figure 7.6 Characterization of ZnO QDs synthesized with 10% Al precursors. (a) Absorption and PL spectra of 10% Al:ZnO QDs. (b) XRD and (c) representative EDX spectrum of 10% Al:ZnO QDs.

Chapter 7 Photoluminescence and Plasmonic Properties of ZnO QDs

However, the introduction of Al and In impurities were observed to have different effects on the properties of ZnO QDs. From **Figure 7.6**, 10% Al:ZnO QDs showed broadened absorption peak, slightly enhanced visible PL and similar XRD spectra compared to ZnO QDs. The EDX-determined Al content was only 1.3% in 10% Al:ZnO QDs, indicating a minor doping of Al in ZnO QDs. On other hand, the ZnO QDs synthesized in presence of InCl_3 formed aggregates during synthesis. The different effects resulting from doping with La^{3+} , Al^{3+} , and In^{3+} ions may stem from different cation radius and different types of anions.

7.3.3 Photodoping of ZnO QDs

It should be noted that, after doping trivalent ions, we did not observe the generation of absorption spectra in the infrared region in the doped ZnO QDs. However, plasmonic absorption spectra were reported in the trivalent ions doped ZnO nanocrystals synthesized at high temperature in nonpolar solvents.⁴⁰² This infrared absorption was thought to arise from the surface plasmon resonance of the free electrons donated by the trivalent dopants. In this work, the EDX result of La:ZnO QDs confirmed a large amount of incorporation of trivalent La in ZnO nanocrystals. A possible explanation for the inhibition of plasmonic absorption is attributed to the crystal sites of the incorporated trivalent ions. In low temperatures and in ethanol solutions, it may be difficult for the trivalent ions to occupy the substitution sites of ZnO nanocrystals. Another possible reason is that because of the small size of ZnO QDs, even a if few free electrons were produced, they may have been strongly confined to a dimension that is too small to generate a plasmon resonance.

Photodoping has recently been considered as a feasible way to generate plasmonic absorption in ZnO nanocrystals. In this work, the ethanol disperision of ZnO QDs were

Figure 7.7 Evolution of optical properties upon photodoping. (a) UV-vis absorption; (b) infrared absorption; (c) UV emission; and (d) visible emission before and after photodoping were shown.

irradiated by a UV light at 300 to 330 nm under anaerobic conditions. During this process, the electrons were photoexcited to the conduction band, and the holes left in the valence band were rapidly scavenged by the ethanol molecules. Without electron quenchers (e.g., oxygen), the electrons will accumulate in the conduction band and serve as free carriers in ZnO nanocrystals.⁴¹⁴ In this work, the UV-NIR absorption and PL spectra of ZnO QD dispersions were collected before and after the photodoping process for a comparative study. The excitonic absorption was bleached and the infrared absorption spectra emerged upon photodoping (**Figure 7.7a, b**). At the same time, the band-edge emission intensity was enhanced and defect-related emission in the visible range disappeared (**Figure 7.7c, d**). The exact mechanism of these phenomena is still under debate. The emergence of infrared absorption was previously considered to be a result of the intraband transition, but more

recently it has been regarded as plasmonic absorption.^{405, 415} The bleaching of the excitonic absorption was attributed to the screening of the Coulombic interaction between the excitonic states or the filling of the $1S_e$ state in conduction band.^{405, 416} The degradation of the visible PL is usually considered to be a result of the competitive Auger recombination process.⁴¹⁷ In this process, when a photoexcited electron recombines with a hole, the energy is released by elevating another electron to a higher energy level rather than generating a photon. However, if only the Auger recombination occurs, the band-edge emission should also be decreased rather than enhanced. The enhancement of band-edge emission was inferred as a consequence of the stabilization of holes in traps, or contraction of holes into ZnO nanocrystal core.^{418, 419} It should be noted that the samples were prepared in the argon glove box. Once the ZnO dispersions were exposed to oxygen, the absorption and PL properties recovered to their original states. The evolution of the plasmonic absorption during the UV irradiation and upon exposure to oxygen is shown in **Figure 7.8**.

Figure 7.8 Evolution of photodoping-induced plasmonic absorption spectra at different UV irradiation time and after exposure to oxygen.

Figure 7.9 Effect of (a) size and (b) impurity doping on the photodoping-induced plasmonic absorption spectra of ZnO QDs.

Later, the photodoping-induced absorption spectra of ZnO QDs with different sizes or with or without impurity doping were compared. Interestingly, the infrared absorption exhibited a red shift upon increasing the size (i.e.m decreasing OH:Zn ratio) of the QDs (**Figure 7.9a**). This was suggested as a result of the quantum confinement effect on plasmons.^{415, 420} On the other hand, the chemical incorporation of impurities (La^{3+} , Al^{3+}) caused a decreased optical density of the maximum infrared absorption (Figure 7.9b). The size effect was excluded because the size of 10% Al and 10% La:ZnO QDs were in between that of the small and medium ZnO QDs. It was speculated that the doped ZnO QDs have localized charges that may confine the photogenerated electrons in a limited region or perturb the carrier wave function,⁴¹⁵ which causes a weakening of their resonance characteristics.

7.4 Conclusions

In this work, ZnO QDs were synthesized using a facile sol-gel method, and they exhibited blue to yellow fluorescence. With an increase in the OH:Zn ratio, the synthesized ZnO QDs exhibited a blue shift in absorption/emission wavelength and a decrease in nanocrystal size.

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Introducing the trivalent ions (La^{3+}) in precursors caused an enhancement of visible PL and a decrease of UV PL, which was attributed to the decreased crystallinity and increased structural defects. The infrared plasmonic absorption was not observed upon doping the trivalent ions, but it emerged upon photodoping. This process was accompanied by the bleaching of excitonic absorption peak, disappearance of the visible PL, and enhancement of the UV PL. A size increase in the quantum confinement regime led to a red shift in the infrared absorption spectra, and impurity doping resulted in a decreased intensity of the infrared absorption spectra. Further studies of the mechanism of the photodoping-induced plasmonic properties are ongoing with the aim of increasing theoretical understanding and developing biosensing applications.

Chapter 8 Conclusions, Ongoing, and Future Work

8.1 Conclusions

This thesis investigates the synthesis and surface modification methods used to prepare the biocompatible cadmium-based (Cd-based) and cadmium-free (Cd-free) colloidal QDs and examines their potential for use in biophotonics applications. The aqueous dispersion of Cd-based and Cd-free QDs are prepared either via aqueous synthesis or synthesized via the hot colloidal method followed by an organic-aqueous phase transfer process.

Nowadays, the well-documented crystal growth mechanisms of the Cd-based compounds allow one to consistently prepare Cd-based QDs using standard synthesis protocols. Nevertheless, methods for simplifying the synthesis of biofunctionalized Cd-based QDs and for enriching the property profiles of various QDs are still needed. Herein, we have developed preparation methods for water-dispersible Cd-based QDs that address these challenges. First, this thesis introduces a facile synthesis approach to obtain biofunctionalized Cd-based QDs based on microfluidic technique. After parameter optimization through simulation, the biofunctionalized Cd-based QDs were synthesized in a microfluidic chip by using bovine serum albumin (BSA) as surfactants and coordination ligands. The same microfluidic method was extended to prepare folic acid- (FA-) functionalized QDs, and they were successfully applied in receptor-specific cell imaging. This synthesis method avoids the laborious process for preparing biofunctionalized QDs. Compared to bench-top synthesis, the microfluidic synthesis obtained QDs with improved functionalization efficiency and enhanced stability in a shortened reaction time. In summary, this study proposed a simple synthesis method to prepare specifically functionalized QDs for diagnostic and

therapeutic applications. Second, we performed a systematic study to investigate the effect of surface ligands on the physicochemical properties of the water-dispersible Cd-based QDs. Seven representative ligand molecules with different chain lengths and functional groups were employed to modify the surface of Cd-based QDs and render them water-dispersible. The synthesis and purification protocols for preparing the aqueous dispersions of QDs were carefully optimized to prepare the stable and luminescent QDs. A systematic characterization and stability study have been performed to evaluate the potential of these QDs for biophotonics applications. Results showed that the physicochemical properties of QDs are significantly affected by the type of surface ligands. Compared to QDs passivated with long PEG chains, QDs modified with short surface ligands are relatively less colloiddally stable in complex physiological fluids, but they exhibit a better photostability. On the other hand, different end-functional groups and surface charge of QDs greatly affect their colloidal stability and cellular uptake. In short, each type of surface modified QDs has advantages and limitations in certain aspects of their physicochemical properties, suggesting that one should carefully choose the appropriate type of surface ligands in order to obtain QDs with desirable properties for the intended application.

In recent years, Cd-free QDs have received attention owing to the low cytotoxicity of their components and many novel optical properties. In this thesis, three types of Cd-free QDs, Zn-Cu-In-S (ZCIS), Zn-Ag-In-S (ZAIS), and ZnO QDs, were respectively prepared using aqueous synthesis, hot colloidal synthesis, and sol-gel synthesis methods. Owing to the large tolerance of element off-stoichiometry in multinary ZCIS and ZAIS compounds, corresponding QDs with different composition ratios were successfully synthesized. Adjusting the Cu:In or Ag:In feed ratio and varying the Zn contents are two strategies that can be used to adjust the band gap and

tune the emission wavelength. Zn incorporation is proved to be an effective strategy for improving the PL quantum yield (QY), tuning the emission wavelength, and narrowing the spectral width. For aqueous synthesized ZCIS QDs, Zn was incorporated into QDs by adding Zn salts in the nucleation stage. For organic synthesized ZAIS QDs, the Zn elements were introduced by alloying Zn in the formed Ag-In-S QDs at an elevated temperature. By comparing these two studies, the aqueous synthesis is greener, requires a lower temperature, costs less, and yields QDs that are readily dispersed in water. However, aqueous synthesized QDs exhibit moderate QY and short shelf life. QDs synthesized via the hot colloidal method have high QY and can remain stable for months or even years. However, the phase transfer of the organically dispersible Cd-free QDs may greatly increase their size and compromise their optical properties. Because the ligand exchange strategy used to modify TOP/TOPO-capped QDs cannot work for organothioliol-capped QDs, we employed the micelle encapsulation approach to obtain the aqueous dispersion of ZAIS QDs. Both of the water-dispersible ZCIS and ZAIS QDs with tunable emissions can be applied in spectrally multiplexed cell imaging, but their relatively broad spectral width can make their emissions less resolvable compared to Cd-based QDs. Nonetheless, the long fluorescence lifetimes of these Cd-free QDs make them promising candidates for temporally multiplexed imaging. By using the water-dispersible ZAIS QDs, for the first time, we demonstrate the feasibility of temporally multiplexed imaging of cells based on Cd-based and Cd-free QDs with different lifetimes. This temporal multiplexing technique will be a good complement to the conventional spectral multiplexing technique by increasing or even multiplying the number of resolvable channels in multiplexed imaging or sensing.

Finally, we studied the PL and plasmonic properties ZnO QDs, a type of QD made from wide-band-gap oxide materials. The pH, impurity type, and impurity concentration in synthesis process were found to greatly affect the emission wavelength, PL intensity, and photoinduced plasmonic properties of the resulting QDs. Upon photodoping, there is an inverse relationship between PL emission intensity and plasmonic absorption density: one falls as the other rises. Ongoing efforts are focused on mechanistic investigations and biosensing applications of these properties.

8.2 Ongoing and Future Work

8.2.1 Bioconjugation of ZAIS QDs for Two-Photon Imaging and Targeted Imaging

In Chapter 6, ZAIS QDs were synthesized using the hot colloidal synthesis method and exhibited one-photon photoluminescence (PL) under UV-visible excitation. Under the excitation by an 800 nm femtosecond laser, the same ZAIS QDs were found to exhibit up-conversion PL spectra with a similar shape compared to one-photon PL spectra (**Figure 8.1a**). The PL intensity showed a quadratic dependence of 2.13 on the incident power intensity (Figure 8.1b), indicating that up-conversion PL arises from a two-photon excitation process.^{421, 422} This preliminary result demonstrates the potential of using the ZAIS QDs for two-photon imaging, which is an effective technique for increasing imaging depth and reducing overall photodamage to biological tissues.

The silica-modified ZAIS QDs obtained in Chapter 6 were applied in non-specific cell imaging studies. To perform targeted cell-imaging studies, the silica-modified QDs (QD-SiO₂) need to be functionalized with specific biomolecules. In our previous study, we successfully conjugated the dye-encapsulated silica nanoparticles with folic acid (FA) via carbodiimide chemistry and used them for targeted imaging of cancer cells.⁴²³ The same conjugation strategy can be applied to the silica coated QDs because of their similar surface conditions.

Figure 8.1 Two-photon photoluminescence (PL) of ZAIS QDs. (a) PL spectra of ZAIS QDs under excitation of 400 nm laser with 10 mW power density (dash black line) and 800 nm laser with different power intensities (solid grey line). (b) PL intensity dependence on power intensity of 800 nm laser excitation in a log-scale plot.

The QD-SiO₂ can be modified with amino groups, carboxyl groups, or other functional groups by adding silane with different end groups. Using the same carbodiimide chemistry, the amino-modified QD-SiO₂ can be conjugated to FA for specifically imaging folate receptor- (FR-) overexpressed cells. To obtain the QD-SiO₂ nanoparticles modified with other functionalities, the bioconjugation strategies introduced in section 2.3.2 can provide a guideline. The bioconjugated ZAIS QDs will be applied in targeted two-photon imaging or multiplexed imaging.

8.2.2 Surface Modification of ZnO QDs for Biosensing Applications

In Chapter 7, ZnO QDs with photoinduced plasmonic absorption spectra were obtained. Intensities of these infrared absorption spectra and the PL emission spectra are highly associated with the level of reducing and oxidizing agents in solvents. This property can be used for sensing redox changes in biological reactions. For such applications, the ZnO QDs in ethanol are required to attain water dispersibility through surface modification. Because

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the photoinduced properties of ZnO QDs result from their interaction with adsorbed solvent molecules, the encapsulation method is not recommended because it may affect the interaction of QDs with the external environment. By following a previous report,⁴²⁴ we successfully transferred the ZnO QDs from ethanol to nonpolar solvents using the ligand exchange method. The photoinduced properties were preserved in these dodecylamine- or trioctylphosphine oxide- (TOPO-) capped ZnO QDs. Ongoing and future work will focus on transferring these QDs into the aqueous phase and examining their photoinduced plasmonic properties in the presence of different molecules.

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Publication List

1. B. Zhang, C. Yang, Y. Gao, Y. Wang, C. Bu, S. Hu, H. Demir, L. Liu, J. Qu and K.-T. Yong, "Engineering quantum dots with specific fluorescence lifetimes for temporally multiplexed imaging of cells", *Under review*.
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