Synthesis and Functionalization of Copper-Doped Indium Phosphate Quantum Dots with Ratiometric Fluorescence

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The doped quantum dots (QDs) that feature dual emission in one nanocrystal and long fluorescence lifetime have gained great interest in biosensing and bioimaging applications. In this work, we report the synthesis and functionalization of copper ion-doped indium phosphate (Cu:InP) QDs that exhibit simultaneous green InP emission and red Cu²⁺ emission. The oil-soluble QDs were synthesized by adsorbing Cu²⁺ onto InP core, followed by growing zinc selenide (ZnSe) shell via the successive ion layer adsorption reaction. During the synthesis, fluorescence ratiometry (and resultant multiple fluorescence colors) can be generated by changing either the dopant amount or the shell-growth time, but they act in a different manner: increasing the Cu²⁺ amount results in quenched InP emission and oppositely improved Cu²⁺ emission; the increase of shell-growth time leads to continuously improved Cu²⁺ emission relative to constant InP emission. Further, functionalization of the oil-soluble Cu:InP QDs with dihydrolipoic acid-polyethylene glycol (DHLLA-PEG) via ligand exchange produces the water-soluble and biocompatible dual-emission QDs. The PEGylated Cu:InP QDs present desirable charge neutrality and excellent thermal stability and photostability, thereby holding high potential in a diversity of biomedical applications.

Keywords: Quantum dot; indium phosphate; copper doping; ratiometric fluorescence; PEGylation.

1. Introduction

Fluorescent nanocrystals, also called quantum dots (QDs), are widely used in biosensing,¹ biolabeling² and bioimaging.³ Fluorescent nanocrystals have been intensely studied as tunable emitters due to their well-known quantum size effect.^{4,5} Compared with traditional organic fluorescent dyes,

fluorescent nanocrystals own unique advantages, such as broad and strong absorption, tunable emission, reasonable stability and solution processability.⁶ However, some shortcomings of II-VI-group QDs such as CdSe and CdTe are unacceptable to the toxicity of cadmium, which limits their practical applications in biomedical field.⁷ In this case, III-V-group QDs such as InP QDs with the

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advantages of minimal biological toxicity and uncomprised fluorescence have emerged as the excellent substitute for II-VI-group QDs.⁸ As an updated class of emitters, the fluorescent spectrum of InP QDs can cover from blue to near-infrared wavelength.⁹ Moreover, the InP QDs, acting as a nanoscaffold, are facily functionalized with biomolecules or organic receptors by a range of chemical methods.¹⁰ In the past years, the functionalized InP QDs have been successfully used for developing fluorescent probes for selective and sensitive detection of heavy metal ions,¹ small molecules⁷ and nucleic acids.¹¹

Ratiometric fluorescence technique has attracted increasing attention in recent years, owing to its advantages by providing built-in correction for environmental conditions.¹² Compared with single-emission fluorescence, ratiometric fluorescence allows the measurement of changes in the ratio of the fluorescence intensities at two well-resolved wavelengths. In addition to improved sensitivity and accuracy, the dual emission-based ratiometric fluorescence provides enhanced capability of visualization from inconspicuous changes in brightness to significant changes in color. The ratiometric fluorescence is commonly resulted from a combination of two individual QDs. For example, Wu *et al.*¹³

constructed a ratiometric fluorescent probe by directly mixing dual emission QDs and realized the highly sensitive detection of zinc ion. Liu *et al.*¹⁴ designed a ratiometric fluorescent probe by integrating dual emission QDs into one nanohybrid and achieved highly selective detection of nucleic acids. However, encapsulation of at least one is required before the mixing or hybridization of two QDs, to avoid the fluorescence self-quenching between each other.¹³ This increases the difficulty and cost of probe design. In recent years, doped QDs have emerged as a new class of fluorescent nanocrystals that intrinsically offer dual-emission-based ratiometric fluorescence.¹⁵ The dual emission of doped QDs not only retains almost all the characteristics of traditional single-emission QDs, but also can avoid the self-quenching problem due to their substantial ensemble Stokes shift. By introducing trace impurity ions, the electron levels of the parent can be perturbed to generate new fluorescent properties without changing the crystal structure. In addition, new electron levels can generate to form a new electron-hole center, which may lead to the generation of a new fluorescence emission.^{16,17} In this way, dual emission occurs simultaneously from single fluorescent nanocrystals by a single-wavelength

Fig. 1. (a) The synthesis scheme and (b) emission mechanism of Cu:InP QDs and (c) fluorescence decay profiles of InP emission and Cu^{2+} emission.

excitation. With benefits of such optical properties, the so-called dual-emission-in-one QDs are advantageous to construct ratiometric fluorescent probes.

Water solubility and biocompatibility are highly desirable for practical applications of fluorescent nanocrystal in advanced biosensing, biolabeling and bioimaging.¹¹ To enable the water solubility, a most commonly used method is encapsulation of the QDs with liposomes, silicon and polymers.^{18,19} Another representative, also the simplest method, is ligand exchange.²⁰ Xie *et al.*⁹ reported that InP/ZnS core/shell nanocrystals could be readily transferred into water without largely losing its brightness by the ligand exchange with hydrophilic thiol ligands. Followed by their initial work, herein, we reported the synthesis and functionalization of Cu:InP QDs aiming at dual emission-based ratiometric fluorescence. The synthesis scheme of Cu:InP QDs was shown in Fig. 1(a). Copper ion was chosen as the dopant to make the new doping peak appear in the red emission region. The successive ion layer adsorption reaction was employed to grow ZnSe shell onto Cu:InP core. The ZnSe shell functions as a protective layer to prevent desorption of Cu ions and a passivation layer to improve the fluorescence intensity.⁹ The fluorescence ratiometry of Cu:InP QDs could be generated by changing the dopant amount and shell-formation time. Moreover, PEGylation via the ligand exchange was used to realize water solubility as well as biocompatibility of the QDs. The functionalized dual emission-in-one nanocrystals synthesized in this study show great potential for the practical use in the field of biomedical research.

2. Experimental Section

2.1. Materials and reagents

Octadecene (ODE), indium acetate ($\text{In}(\text{Ac})_3$, 99.99%), tri-*n*-octylphosphine (TOP, 97%), myristic acid (MA, 90%), zinc stearate ($\text{Zn}(\text{SA})_2$, 99.99%) were purchased from Sigma-Aldrich. Copper stearate (98%) was purchased from Ark Pharm. Tris(trimethylsilyl) phosphine ($\text{P}(\text{TMS})_3$, 98%) was purchased from J&K. 1-Octylamine (OA, 99%), dihydrolipoic acid (DHLA) and dihydrolipoic acid-polyethylene glycol (DHLA-PEG₂₀₀₀) were purchased from Alfa Aesar. All the chemicals were used without further purification.

2.2. Synthesis of Cu:InP nanocrystals

The injection solution of P precursor was prepared by mixing 0.1 mmol of $\text{P}(\text{TMS})_3$ and 2.4 mmol of ODE (1.5 mL in total) in a glovebox. For a typical synthesis, indium acetate (0.2 mM), myristic acid (0.65 mM) and 4 g of ODE were loaded into a three-neck flask. The mixture was heated to 188°C under argon flow, and then the P precursor solution was injected into the hot reaction mixture, which lowered the reaction temperature to 178°C for 10 min to form the monodispersed InP core. The reaction solution was further cooled to 130°C, and the copper precursor solution (0.006 mmol of copper stearate in ODE) was added into the reaction solution. The reaction mixture was stirred for 10 min to make the copper ions absorbed on the surface of InP core. The reaction mixture was further heated to 210°C for the doping of InP core with Cu ions with a heating rate of $\sim 2^\circ\text{C}/\text{min}$. For the epitaxial growth of ZnSe shell, the reaction solution mentioned above was cooled down to 150°C. Zinc stearate (0.1 M in ODE) and selenium (0.1 M in TOP) precursor solutions (0.8 mL each) were separately added into the reaction flask, with a 10 min-time interval between the two injections at 150°C. After that, the temperature was increased to 220°C to allow the growth of the ZnSe shell and incorporation of Cu^{2+} for 30 min. Samples were taken with the time course of every 5 min from the reaction mixture to get different fluorescent encoded QDs.

For purification, 10 mL of hexane was added to the reaction solution, and the unreacted starting materials and byproducts were removed by successive methanol extractions until the methanol phase was clear. The solid form of the Cu:InP QDs was isolated by the addition of acetone into the purified hexanes/ODE solution, followed by centrifugation, decantation and drying under nitrogen flow.

2.3. Ligand exchange of Cu:InP nanocrystals

The ligand exchanges of Cu:InP QDs were followed with a modified protocol.²¹ First, 5 mg of the Cu:InP QDs was dissolved in ethanol (1 mL) under nitrogen flow. Afterwards, 20 μL of DHLA or DHLA-PEG was added into the solution, followed by heating at 110°C for 10 h. After the reaction, the clarified solution was added with equal volume of *n*-hexane and a small amount of ethanol, followed

by centrifugation at 8000 rpm for 5 min. Finally, the precipitates were ultrafiltrated and washed three times with 1 mL of deionized water.

2.4. Thermal stability and photostability characterization

To access the thermal stability of oil-soluble QDs, both the pure InP QDs and Cu:InP QDs were, respectively, dissolved in chloroform and heated at 110°C in an oil bath. To avoid the influence of air, the whole process was carried out in nitrogen. The fluorescence intensity of QDs was measured every 2 h for time comparison. To access the fluorescence stability of doped QDs, the PEGylated Cu:InP QDs were fluoresced with the time course. Continuous fluorescence monitoring for 7 days was carried out to access the storage fluorescence stability. Also, fluorescence detection under continuous illumination with a laser (0.35 W of excitation intensity and 405 nm of excitation wavelength) was done to investigate the photobleaching resistance of the Cu:InP nanocrystals. The photobleaching resistances of typical fluorescent dyes, 5-carboxy-fluorescein (FAM) and GelRed were also conducted for comparison.

2.5. Instrument

Fluorescence spectra were collected with an F-7000 fluorescence spectrophotometer (Hitachi, Japan), and UV-Vis absorption spectra were obtained by a Hitachi U-4100 spectrophotometer. Fluorescence lifetime measurements were performed on a Horiba Jobin Yvon time-resolved fluorescence spectrophotometer. Zeta potentials were measured on a Malvern Zetasizer Nano-ZS particle analyzer. The morphology was photographed on a JEOL JEM-2100 transmission electron microscope (TEM).

3. Results and Discussion

3.1. Dual emission from one Cu:InP QD

As illustrated in Fig. 1(a), the oil-soluble Cu:InP QDs were synthesized in three steps: the formation of InP core, the adsorption of Cu²⁺ and the growth of ZnSe shell. For comparison, the InP/ZnSe QDs without Cu²⁺ doping were also prepared. The InP/ZnSe QDs showed intense green emission with the peak at ~541 nm. In contrast, the Cu:InP QDs

showed dual emission characteristics: the green emission with the peak at ~548 nm and the red emission with the peak at ~650 nm [Fig. 1(b)]. Clearly, for the doped QDs, the green emission and red emission were assigned to the host (InP) emission and the dopant (Cu²⁺) emission, respectively. The emission mechanism of Cu:InP QDs was shown in the inset of Fig. 1(b). Without Cu²⁺ doping, when the excitation level exceeds the band gap, the QD (InP) absorbs photons and causes electrons to jump from the valence band (VB) to the conduction band (CB). Electrons in the CB then jump back to the VB and emit photons radiatively.²² For the Cu:InP QD, copper ions, attached to the core surface and encased in the ZnSe shell, generate new electron levels which are called doping levels. The doping levels could form new electron-hole centers with great attraction toward electrons in the CB. Upon excitation, part of electrons in the CB of InP core jumps back to the VB and emits green emission, and part of electrons would be trapped by the doping levels and leads to red emission.²³

Next, fluorescence lifetime was investigated for the dual emission-in-one QDs. The fluorescence lifetime can directly reflect the mean time that the QDs exist in an excited state. The green-emission InP/ZnSe QDs have a mean fluorescence lifetime of ~4 ns, which is distinct from the organic fluorescent dyes.²⁴ In contrast, for the Cu:InP QD, the mean lifetime of InP emission was decreased to ~7 ns [Fig. 1(c)]. This in turn indicated the electron trapping by the doping levels. The Cu²⁺ emission showed a long excited-state lifetime of ~1.3 μs, characteristic of triplex emission.^{25,26} This long fluorescence lifetime makes the Cu:InP QDs advantageous in actual bioimaging by effectively reducing the interference from background fluorescence.^{27,28}

3.2. Fluorescence ratiometry by changing the dopant amount

The dopant (Cu²⁺) amount can significantly affect the emission characteristics of doped nanocrystals.^{29,30} Therefore, Cu:InP QDs with varying amounts of Cu²⁺ (0.1, 0.5, 1, 2, 5 and 10% relative to In element, respectively) were synthesized and characterized. As shown in Fig. 2(a), by doping with a dose of Cu²⁺ (0.1%), the Cu:InP QDs exhibited dual emission characteristics: intense green emission

Fig. 2. The changes in (a) fluorescence spectra and fluorescence color, (b) absorption spectra and (c) morphology and size of Cu:InP QDs as a function of the Cu²⁺ amount.

and weak red emission. Along with the increase of dopant amount, the green emission of Cu:InP QDs decreases continuously, and oppositely the red emission kept rising. When the doping amount of Cu²⁺ reached 5% or 10%, the green emission completely disappeared and the intense red emission was obtained. As a result, the Cu:InP QDs displayed distinct fluorescence colors (from green to red) with the varying dopant (Cu²⁺) amounts. Figure 2(b) shows absorption spectra of the Cu:InP

QDs with different dopant amounts. A slight red-shift for the first-order absorption peak could be observed with the increase of dopant (Cu²⁺) amount. Table 1 summarizes the key parameters for photophysical characteristics of the Cu:InP QDs with the varying dopant (Cu²⁺) amounts. The dual emission-based fluorescence ratiometry (I_g/I_r) was dramatically changed from $\sim 7:1$ for the 0.1% doping to 0.5:1 for the 2% doping and then to 0:1 for the $\geq 5\%$ doping. Correspondingly, the

Table 1. The key parameters of dual emission-based fluorescence ratiometry of Cu:InP QDs with the different doping amounts of Cu²⁺.

No.	Doping amount (%)	EM _g (nm)	EM _r (nm)	I_g/I_r	Abs (nm)	PLQY _g (%)	PLQY _r (%)
1	0.1	526	649	7:1	453	23.63	3.01
2	0.5	526	645	2.5:1	454	20.27	8.39
3	1	524	649	1:1	457	17.25	12.82
4	2	525	649	0.5:1	459	13.03	18.63
5	5	—	649	0:1	461	0	22.57
6	10	—	649	0:1	465	0	27.32

photoluminescence quantum yields (PLQY) of the green emission and the red emission were changed with the increase of dopant amount. The calculated PLQY for the green emission decreased from 23.63% to 0, whereas that for the red emission increased from 3.01% to 27.32%. This is because that, on the one hand, the Cu^{2+} doping produced new energy levels that led to the red emission and, on the other hand, its squeezing into the InP core could also significantly quench the InP emission.^{31,32}

Tunneling electron microscopy was conducted to investigate the morphology and size of Cu:InP QDs with the varying dopant (Cu^{2+}) amounts. As shown in Fig. 2(c), all the synthesized Cu:InP QDs were monodispersed. The increase of dopant (Cu^{2+}) amount from 0.1% to 10% led to the slight increase in particle size from 2.1 ± 0.5 nm to 2.6 ± 0.2 nm, which might account for the slightly red-shifted absorption of the Cu:InP QDs.³³

3.3. Fluorescence ratiometry by changing the shell-growth time

Besides controlling dopant (Cu^{2+}) amount in the second step, controlling the growth of ZnSe shell in the third step also can produce the Cu:InP QDs with ratiometric fluorescence.³⁴ In our experiments, by fixing the dopant (Cu^{2+}) amount of 2%, the Cu:InP QDs were synthesized with varying shell-growth times at 220°C. As shown in Fig. 4(a), the fluorescence ratiometry of Cu:InP QDs changed with the shell-growth time. Along with the extension of growing time, the green emission of Cu:InP

QDs remained unchanged, while the red emission kept rising. Accordingly, the Cu:InP QDs displayed a green-yellow-red change in fluorescence color with the extended shell-growth time. Table 2 lists the key parameters of fluorescence ratiometry regulated by changing the shell-growth time. With 5 min of shell-growth time, the I_g/I_r fluorescence ratiometry was 1:0.5. When the shell-growth time was extended to 15 min, the I_g/I_r ratio became approximately 1:1. Further extending the shell-growth time to 30 min or 35 min, the I_g/I_r fluorescence ratiometry reached 1:2. The calculated PLQY for the red emission increased from 13.01% to 26.32%, whereas that for the green emission remained constant at $\sim 14\%$. Despite the constant emission intensity and PLQY, notably, the wavelength of green emission (EM_g) changed from 534 nm to 550 nm, probably due to that the extended growing time resulted in the increased thickness of ZnSe shell. This is in accordance with the observed redshift from 450 nm to 461 nm for the absorption of Cu:InP QDs with the extended shell-growth times [Fig. 3(b)].

3.4. Surface functionalization of Cu:InP QDs

By changing the dopant amount or the shell-growth time, the fluorescence ratiometry of Cu:InP QDs generated multi-colored probes for biosensing, bio-labeling and bioimaging applications.^{35–37} Before the practical applications, water solubility of the QDs is necessary, which requires further surface functionalization.³⁸ In our experiments, the Cu:InP

Fig. 3. The changes in (a) fluorescence ratiometry and fluorescence color and (b) absorption of Cu:InP QDs as a function of the growth time of ZnSe shell.

Table 2. The key parameters of dual emission-based fluorescence ratiometry of Cu:InP QDs with the different shell-growth times.

No.	Time (min)	EM _g (nm)	EM _r (nm)	I _g /I _r	Abs (nm)	PLQY _g (%)	PLQY _r (%)
1	5	534	649	1:0.5	450	14.15	13.01
2	10	540	649	1:0.8	453	15.23	15.39
3	15	542	649	1:0.9	453	14.25	18.82
4	20	546	649	1:1.4	455	14.03	20.63
5	25	548	649	1:1.6	458	13.79	25.52
6	30	550	649	1:1.9	460	13.62	26.05
7	35	552	649	1:2.1	461	13.02	26.32

QDs with the I_g/I_r ratio of 1:2 (made with 2% of Cu²⁺ doping and 35 min of shell-growth time) were functionalized via ligand exchange. The ligand exchange was generally accomplished at high temperature for relatively long time. Therefore, prior to the surface functionalization, the thermal stability of the oil-soluble Cu:InP QDs was investigated at the simulated conditions (110°C, up to 10 h). For comparison, the thermal stability of the undoped InP/ZnSe QDs was also investigated. As shown in Fig. 4, the dual emission of Cu:InP QDs and the green emission of InP/ZnSe QDs were continuously quenched as the time increased, but they exhibited different fluorescence quenching rates. For 10 h of thermal annealing, the green emission and the red

emission were consistently reduced by ~ 30% for the Cu:InP QDs. In contrast, for the undoped InP/ZnSe QDs, the green emission intensity decreased to be ~ 25% of the original for 10 h thermal annealing. The different fluorescence quenching rates clearly revealed the higher thermal stability of Cu:InP QDs versus undoped InP/ZnSe QDs. This is in agreement with the finding that the doping of metal ions (Mn²⁺ or Cu²⁺) can significantly improve the thermal stability of QDs by Xie *et al.*⁹ and Karan *et al.*³⁹

On the basis of excellent thermal stability, the DHLA or DHLA-PEG ligand was used to replace the original OA ligand of Cu:InP QDs via the ligand exchange. As shown in Fig. 5(a), a ~ 30% reduction was observed for the green emission and

Fig. 4. Evolutions of fluorescence spectra and fluorescence intensities showing thermal stabilities of (a, b) Cu:InP QDs versus (c, d) InP QDs.

Fig. 5. (a) The schematic illustration of PEGylation of Cu:InP QDs, and fluorescent spectra of oil- and water-soluble Cu:InP QDs. The comparison in (b) hydrodynamic diameter and (c) zeta-potential of QD@DHLA versus QD@DHLA-PEG.

the red emission of resultant QD@DHLA and QD@DHLA-PEG. The I_g/I_r fluorescence ratio-metry of the QDs remained unchanged after ligand exchange. The PLQY of Cu^{2+} emission was calculated to be 21.35% for QD@DHLA and 20.88% for QD@DHLA-PEG, respectively, and that for the InP emission was calculated to be 11.72% for QD@DHLA and 11.38% for QD@DHLA-PEG, respectively. With respect to the short DHLA ligand, the PEGylation does not alter the emission characteristics, but endows the Cu:InP QDs with good biocompatibility.^{40,41}

As both DHLA and DHLA-PEG are hydrophilic, the resultant QD@DHLA and QD@DHLA-PEG via the ligand exchange became water soluble.^{42–44} Figure 5(b) shows the hydrodynamic diameters of the QD@DHLA and QD@DHLA-PEG measured by dynamic light scattering. The QD@DHLA had the mean hydrodynamic diameter of 5.5 nm, whereas the QD@DHLA-PEG exhibited the mean hydrodynamic diameter of ~ 10 nm. Both the QD@DHLA and QD@DHLA-PEG meet the size requirements for intracellular bioimaging applications.⁴⁵ Figure 5(c) shows zeta potentials of QD@DHLA and QD@DHLA-PEG. QD@DHLA had the zeta potential of -24.8 mV, and in contrast the QD@DHLA-PEG exhibited the zeta potential of -4.6 mV, nearly neutral in charge. The charge-neutral surface of the PEGylated QDs can exclude a variety of unspecific electrostatic adsorption in

complex biological environments, thereby favoring its use for practical biomedical applications.⁴⁶

3.5. Photostability of PEGylated Cu:InP QDs

As a class of fluorescent probes, the photostability of QDs is essential for its diverse applications. The photostability of the Cu:InP QDs was first assessed by storing QD@DHLA-PEG under ambient conditions for different days. As shown in Fig. 6(a), during the storage for a week, both the green emission and the red emission of QD@DHLA-PEG are kept constant, and therefore the I_g/I_r fluorescence ratio-metry of the QDs remained unchanged. In fact, the emission characteristics could remain unchanged for several months at room temperature. Further, the photostability of Cu:InP QDs was assessed by exposing the QD@DHLA-PEG to a continuous laser irradiation (405 nm, 0.35 W) for different times. For comparison, the photostability of two organic dyes, green-emission FAM and red-emission GelRed, was also investigated. As shown in Fig. 6(b), both the green emission and red emission of the Cu:InP nanocrystals slowly quenched with the time course. After 2 h of irradiation, the dual emission intensities were reduced to 50% of the originals. This level of fluorescence attenuation is acceptable for practical biosensing, biolabeling and

Fig. 6. The photostability of PEGylated Cu:InP QDs during (a) storage and (b) exposure to 405 nm-laser illustration for different times, and the photostability of (c) FAM and (d) GelRed upon the 405 nm-laser illustration for different times.

bioimaging applications. In contrast, both the fluorescence of FAM and GelRed showed rapid photobleaching. The emission intensities for both dyes decreased to $\sim 10\%$ within 1 h [Figs. 6(c) and 6(d)]. The significant contrast indicated clearly the excellent photostability of the Cu:InP QDs.

4. Conclusion

In conclusion, we presented two general strategies to synthesize Cu^{2+} -doped InP/ZnSe QDs that feature dual emission-based ratiometric fluorescence. The variation of the dopant amount results in ratiometric fluorescence composed by oppositely changed InP emission and Cu^{2+} emission. Alternatively, variation of shell-growth time leads to the varied Cu^{2+} emission with respect to the constant InP emission. By these synthetic strategies, multicolored Cu:InP QDs could be controllably prepared. Further, surface functionalization via ligand exchange produces water-soluble and biocompatible Cu:InP QDs. In particular, PEGylated Cu:InP QDs exhibited high brightness, relatively small size and excellent photostability as well as charge neutrality. These superior properties make the functionalized doped QDs become indispensable tools in a diversity of biomedical applications.

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