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Fluorescent Probes

Hybrid Silica–Nanocrystal–Organic Dye Superstructures as Post-Encoding Fluorescent Probes**

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The demand for multiplex bioassays without complex instrumentation and processing has driven the development of encoding fluorescent nanoprobes.[1] Organic dyes and luminescent semiconductor nanocrystals (or quantum dots, QDs) are representative fluorescent encoding elements, which have been incorporated into various nano- or microsized spherical supports, such as silica and polymer latex spheres, for high-capacity spectral encoding.[1,2] Organic-dye-doped nanoparticles give an intense fluorescence signal together with highly improved photostability, which makes them especially suitable for ultrasensitive bioassays. However, the number of available organic dyes with the same excitation wavelength and distinguishable emission spectra is limited, which restricts the number of spectrally distinct codes that can be generated.[3] QDs are bright, highly photostable, and have continuous excitation spectra along with narrow, symmetric, size-tunable fluorescence emission. These unique optical properties make them ideal luminophores for wavelength and intensity encoding.[1a]

Various approaches have been employed in the preparation of encoding fluorescent probes. To the best of our knowledge, three major approaches are typically adopted to incorporate fluorescent encoding elements, such as organic dyes and QDs. In the first, the encoding elements are encapsulated inside nano- or microsized spheres through electrostatic and hydrophobic interactions, covalent linkage, hydrogen bonding, and physical encapsulation.[1a,3,4] The second route is to directly assemble the encoding elements on the external surface of spherical supports.[5] In the third approach, the encoding elements are located in concentric shells surrounding a solid core or alternating with nonfluorescent spacer shells through layer-by-layer techniques.[6] Although these approaches have been successfully used to prepare encoding fluorescent nano- or microsized spheres, the process of preparation is rather laborious and time-consuming when the number of codes required for multiplex analysis is large. As the codes manufactured by these methods are based on the incorporation of different fluorescent encoding elements with distinguishable ratios or concentration, the amounts of different encoding elements must be controlled precisely in the preparation of each code. The complex and diverse encoding elements also influence the process of the preparation of spherical supports. Thus, the quest to prepare encoding fluorescent spheres with a more labor-saving process and higher efficiency is of extreme significance.

Herein, we report the synthesis and characterization of a well-defined hybrid superstructure that comprises a fluorescein isothiocyanate (FITC)-doped silica core coated with a dense monolayer of nanocrystalline CdTe QDs, followed by a silica shell. We define this superstructure as a hybrid silica–nanocrystal–organic dye superstructure (HSNOS). The HSNOS has shown unique, well-resolved, dual fluorescence emission under a single excitation wavelength. To endow HSNOSs with multicolor fluorescence combinations, which extend their use in multicolor imaging applications, they were encoded subsequently by incubation with different concentrations of Cu^{2+} ions as an efficient quencher for CdTe QDs. Such a post-encoding strategy, which is different from existing pre-encoding methods, makes the preparation process of multicolor fluorescent spheres rather more labor saving and highly efficient.

HSNOSs were synthesized by a multistep procedure (shown in Figure 1), which involved the synthesis of FITC-doped silica cores, modification of the silica surface with amino groups, self-assembly of CdTe QDs on the particle

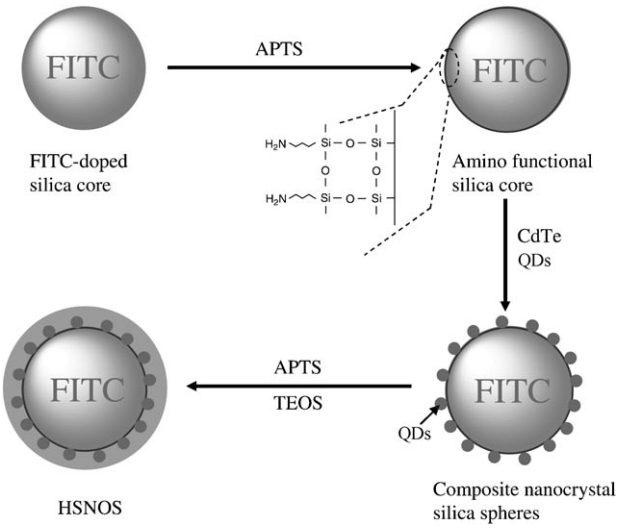

Figure 1. Synthetic procedure to obtain hybrid silica–QD–organic dye superstructures. See text for details.

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surfaces, and final deposition of a silica shell. FITC-doped silica cores were prepared through a typical Stöber-based synthesis method.[3b,7] FITC molecules were first covalently conjugated to an amine-containing silane agent (3-aminopropyltriethoxysilane, APTS), and then APTS and a silica alkoxide precursor (tetraethyl orthosilicate, TEOS) were allowed to hydrolyze and co-condense in a mixture of ethanol and ammonium hydroxide, which resulted in FITC-doped silica nanoparticles. Surface modification of FITC-doped silica cores with amino groups was achieved by direct hydrolysis and co-condensation of APTS with silica cores in the original ethanol/ammonium hydroxide mixture.[7] The surface-modified silica cores were then dispersed in an aqueous solution of thiol-capped CdTe QDs prepared by an aqueous synthesis method with post-photochemical etching.[8] The CdTe QDs self-assembled on the surface of the silica cores as a result of strong binding interactions between the surface amino groups and the QDs.[9] The amounts of CdTe QDs were calculated to be sufficient to provide 100% monolayer coverage of nanoparticles on the silica cores. Finally, the composite nanocrystal–silica spheres were coated with an outer layer of silica, to further improve the colloidal and chemical stability and give the targeted HSNOSs. The outer silica shell improves the stability of the HSNOSs and also offers an ideal anchorage substrate for the covalent binding of specific ligands that are suitable for bioanalysis applications.[9a,10]

High-resolution transmission electron microscopy (HRTEM) was used for the characterization of the prepared composite silica spheres. Figure 2a shows a TEM image of FITC-doped silica cores of diameter 372 ± 15 nm. The particle size can be controlled by changing the amount of ammonium hydroxide in the reaction mixture.[7] Typically, the particle size increases with an increase in the concentration of ammonium hydroxide. A typical HRTEM image of the composite nanocrystal–silica spheres is shown in Figure 2b. It can be observed that many CdTe QDs exist as dispersed single particles on the surface of each silica core (energy-dispersive X-ray analysis is also provided in the Supporting Information). The TEM image of the targeted HSNOSs shown in Figure 2c indicates that HSNOSs in aqueous solution are well-dispersed without aggregation. The thickness of the outer shell can be controlled by the added amount of TEOS.[10]

Figure 3a shows the normalized absorption and spectrofluorometric emission spectra of FITC dye and thioglycolic acid (TGA)-capped CdTe QDs. As a result of the continuous absorption spectra and narrow, symmetric, size-tunable fluorescence emission from CdTe QDs, these two fluorophores share a broad overlapped absorption spectrum but have two distinct maximum emission wavelengths, with that for FITC at 510 nm and that for CdTe QDs at 615 nm. The fluorescence property of FITC and CdTe QDs was monitored in each synthesis step of the HSNOSs. The fluorescence spectra and intensity of FITC–silica cores remain nearly unchanged before and after the surface deposition of CdTe QDs, as well as after final coating with an outer silica shell. The assembly of CdTe QDs on FITC-doped silica cores results in well-resolved dual fluorescence emission (see Figure 3b). In addition, it can be seen that when the composite dual-fluorescent spheres are finally coated with an outer layer of silica, a slight red shift of the emission spectrum of the CdTe QDs occurs. The width of the spectra of the QDs does not change during all the synthesis steps. This finding indicates that the polydispersity of the QDs does not change, which is corroborated by TEM data. As this HSNOS displays well-resolved dual fluorescence emission, multicolor encoding fluorescent spheres can be generated by controlling the relative peak intensity ratio at 510 and 615 nm through a simple post-manufacture (post-encoding) process.

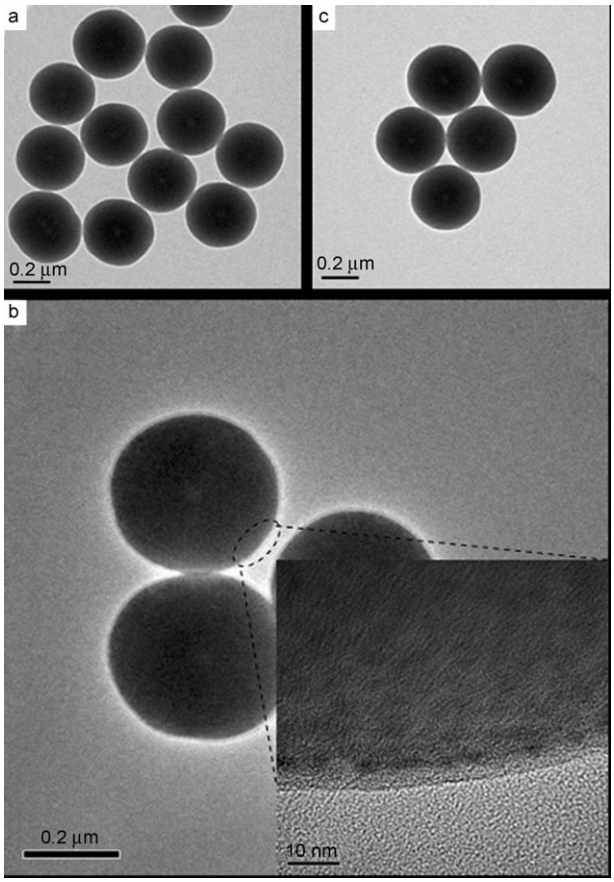

Figure 2. a) TEM image of FITC-doped silica cores. b) HRTEM image of silica cores assembled with CdTe QDs. c) TEM image of the targeted HSNOSs.

The fluorescence properties of QDs are closely related to the nature of their surface.[11] The interaction of small molecules or ions with the surface of Cd chalcogenide QDs has been a subject of wide interest. Some heavy-metal ions, such as Cu^{2+}, Hg^{2+}, and Ag^{+}, show fluorescence quenching of various Cd chalcogenide QDs. Nontoxic and stable Cu^{2+} ions were found to be an effective quencher of the fluorescence intensity of CdTe QDs.[11,12] It was proposed that Cu^{2+} ions bound to the surface of the QDs facilitate nonradiative electron–hole annihilation, thus resulting in quenching of the fluorescence emission. Therefore, to generate multicolor fluorescence combinations from the prepared HSNOSs, Cu^{2+} ions were used to change the fluorescence emission from the embedded CdTe QDs. HSNOSs were incubated with

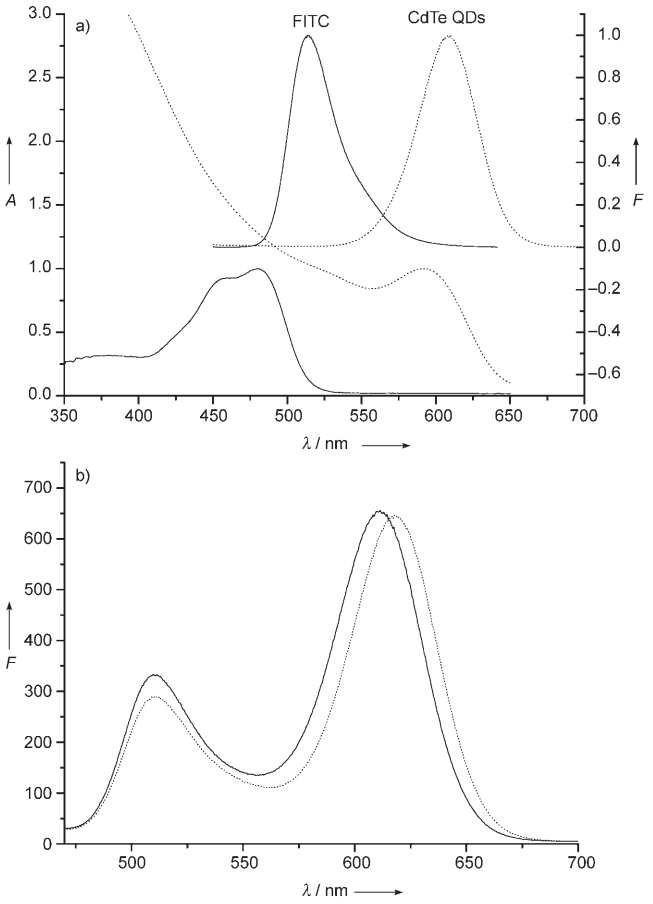

Figure 3. a) Normalized absorption and fluorescence emission spectra of FITC (——) and CdTe QDs (•••••). b) Fluorescence emission spectra of HSNOSs before (——) and after (•••••) coating with an outer silica shell.

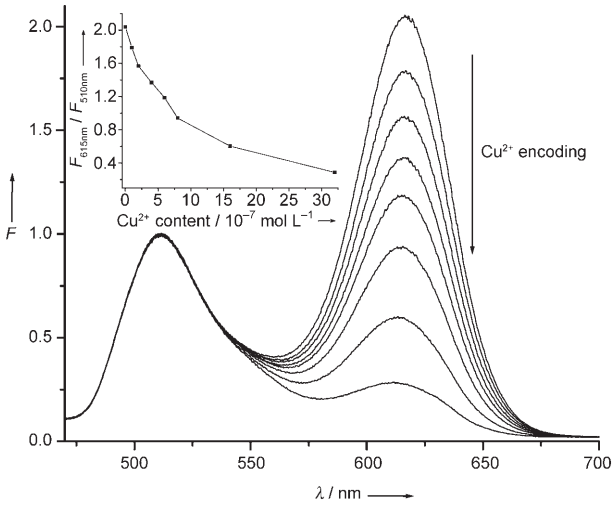

Figure 4. Normalized fluorescence emission spectra of multicolor post-encoding HSNOSs. Inset: correlation between the intensity ratio and the added amounts of Cu^{2+} ions.

different amounts of Cu^{2+} ions for 30 min, which rendered HSNOSs with distinguishable multicolor fluorescence emission. Figure 4 shows the fluorescence spectra taken from the post-encoding HSNOSs with Cu^{2+} ions. The fluorescence intensity of FITC is not influenced by the addition Cu^{2+} ions. However, the fluorescence intensity of the embedded CdTe QDs is reduced successively with increasing Cu^{2+} ion concentration. Furthermore, the fluorescence intensity ratio of the dual emission can be accurately controlled by varying the amounts of the Cu^{2+} ions added. The inset of Figure 4 shows the correlation between the intensity ratio and the relative amounts of added Cu^{2+} ions. To test the reversibility of the fluorescence quenching of QDs by Cu^{2+} ions, the post-encoding HSNOS particles were washed and separated from the supernatant, redispersed in a fresh deionized water solution, and then the fluorescence spectra were recorded. The spectra remained nearly unchanged, which indicates that the quenching of the fluorescence from CdTe QDs by Cu^{2+} ions was nonreversible because of the large association constant between Cu^{2+} ions and the surface of the QDs. The nonreversibility is absolutely necessary for the post-encoding fluorescent probes to be used in bioanalysis. To determine whether the intensity ratios have batch-to-batch reproducibility, the average peak intensity ratios from five parallel post-encoding HSNOS solutions were compared; the standard deviations for these intensity ratios were found to be less than 3%. This high level of reproducibility allows not only more intensity ratios to be distinguished, but also higher precision in multiplex analysis. In addition, compared to fluorescence encoding with absolute intensity, ratiometric encoding is much more reliable because the ratio values do not suffer from simultaneous drifts or fluctuations of individual signals.[13]

Based on the same strategy, a new batch of HSNOSs was prepared from CdTe QDs with a different size (the fluorescence spectra are provided in the Supporting Information). This indicates that even more batches of HSNOSs can be prepared by simply changing the size of the embedded CdTe QDs. In addition, the FITC doped in the silica core can be replaced by other fluorescent dyes. Controlled assembly of various QDs on the surface of silica spheres can also be achieved by several other procedures,[5,9,10a] such as layer-by-layer techniques using oppositely charged polyelectrolytes, methods based on electrostatic and hydrophobic interactions, and techniques based on binding of the nanoparticles on thiol-functionalized silica spheres. These alternative procedures allow the preparation of various HSNOSs with different fluorescence emissions. After the preparation of various HSNOSs, the number of fluorescence codes can be strongly amplified through a simple post-encoding method, thus making high-throughput multiplexed detection more labor saving and convenient.

In conclusion, we have reported the multistep synthesis of HSNOSs that comprise FITC-doped silica cores, embedded CdTe QDs, and an outer silica shell. These HSNOSs display well-resolved dual fluorescence emission, with FITC at 510 nm and CdTe QDs at 615 nm. We used these HSNOSs as encodable precursors to prepare for the first time various HSNOSs with different fluorescence emissions. The encoding strategy is based on the selective quenching by Cu^{2+} ions of the fluorescence emission from CdTe QDs embedded in

HSNOSs. Compared to the existing pre-encoding methods, this post-encoding strategy makes the preparation process of multicolor fluorescent spheres more labor saving and highly efficient. In addition, various HSNOSs with different fluorescence emissions can be prepared by simply changing the embedded QDs or the doped organic fluorescent dye. It is expected that post-encoding will be a new concept to design encoding fluorescent probes, which could find application in multiplex bioassays.

Experimental Section

FITC-doped silica cores: FITC-doped silica cores were prepared by a typical Stöber-based synthesis method.[3b,7] To prepare the silica core, FITC was first covalently linked to the silane coupling agent APTS. APTS (0.20 mL) was added to FITC (0.0404 g) in anhydrous ethanol (5 mL) medium. The reaction proceeded for 20 h in the dark with magnetic stirring and under nitrogen. Then a solution of the prepared APTS–FITC conjugates (0.5 mL) was added to a clean glass reaction vessel containing anhydrous ethanol (20 mL) and ammonium hydroxide (28%, 2.0 mL). TEOS (1.0 mL) was added and the mixture was stirred for 24 h in the dark.

Silica cores modified with amino groups:[7] To modify the silica surface with amino groups, APTS (0.05 mL) was added to the above solution and stirred for another 24 h. After the reaction, the prepared samples were centrifuged at 13000 rpm for 15 min to collect the silica cores. The cores were further washed with ethanol and deionized water by centrifugation and decantation several times to remove the unreacted chemicals. The obtained orange-yellow silica particles were dried in a desiccator before use.

CdTe QDs: TGA-capped CdTe QDs were synthesized according to a previously published procedure.[8] Typically, $CdCl_2{\cdot}6H_2O$ (0.5366 g) was dissolved in deionized water (100 mL) followed by the addition of TGA (0.24 mL). The pH value of the solution was fixed at 11–12 by dropwise addition of NaOH solution (1M). The mixture was then deaerated by bubbling nitrogen for 30 min. Under stirring, a certain volume of a NaHTe solution, prepared by the reaction of tellurium powder with $NaBH_4$, was injected into the reaction mixture. After approximately 20 min, the mixture was refluxed; the particle size was controlled by the reflux time. The prepared CdTe QDs were then illuminated under sunlight to enhance the fluorescence quantum yield. After illumination, highly fluorescent CdTe QDs were precipitated by the addition of pure ethanol. The resultant precipitate was centrifuged, washed with ethanol, and dried in a desiccator before use.

Composite nanocrystal–silica spheres: A dispersion of CdTe QDs (5 mg) in deionized water (2 mL) was mixed with FITC-doped silica cores (10 mg) modified with amino groups in deionized water (2 mL) and phosphate buffer (0.5 mL, pH 7.0). The reaction mixture was stirred for 8 h at room temperature in the dark. The resulting nanocrystal-coated silica composites were isolated by centrifugation and washed with deionized water to remove the excess QDs.

HSNOSs: Silica spheres coated with CdTe QDs were further coated with an outer layer of silica in water/ethanol (1:4, 5 mL) containing APTS (2 μL) and TEOS (10 μL).[10] After the hydrolysis and condensation of the silanes on the surface of the composite spheres (24 h), an outer silica shell was formed. The obtained HSNOSs were isolated by centrifugation and redispersed in deionized water (5 mL).

HSNOSs post-encoded with Cu^{2+} ions: Different amounts of Cu^{2+} ions in aqueous solution were added to a clean, calibrated glass test tube containing the above HSNOS solution (0.2 mL) and deionized water (3 mL) with magnetic stirring. The reaction was complete after approximately 30 min. Then the solution was diluted to the mark with water and mixed thoroughly.

Characterization of particles: The fluorescence spectra were recorded with a Shimadzu RF-5301pc spectrofluorometer. HRTEM was performed on a Tecnai F30 electron microscope. Sample preparation was carried out by placing a drop of the freshly prepared colloidal solution on a carbon-coated copper grid and allowing the solution to evaporate.

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