J Fluoresc (2007) 17:133–138
DOI 10.1007/s10895-007-0157-5

BRIEF COMMUNICATION

A Feasible and Quantitative Encoding Method for Microbeads with Multicolor Quantum Dots

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Received: 20 June 2006 / Accepted: 2 January 2007 / Published online: 8 February 2007
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Abstract Multicolor encoded beads were achieved by incorporating two color core-shell quantum dots (QDs) (CdSe/ZnS) to commercial polystyrene (PS) beads. By controlling the concentration ratios of the two quantum dots (QDs) in doping solutions, a series of codes with different intensity ratios were obtained. Based on the multiple encoded carboxylic modified polystyrene beads, fluorescent dyes labeled antibodies were distinguished successfully on the beads' surface. It suggests that the encoded beads from this method have the practicability in biological applications and chemical analysis.

Keywords Quantum dots · Microbeads · Fluorescence spectra · Antibody conjugation

Introduction

In recent years, the rapid increase of interest in carrying out comprehensive analysis on classes of biologically relevant molecules (e.g., genomics, proteomics, and antigen/antibodies), has been promoting the invention and development of new analytical and detecting technology [1–3]. Compared with the traditional analytical and detecting technologies (such as planar array-based analytical technologies), the microbeads-based analytical technology invented lately, known as suspension array technology (SAT) [1, 4], possesses some special advantages. As a carrier, microbead has a better fluidity in devices and a huge capacity to bind larger numbers of molecules per unit volume, which are likely to promote the development of multiplexed analytical technology. The latter may improve detecting limits by providing a degree of preconcentration also. Besides, due to its potential applied value in multiplexed bioassays, medical diagnostics, high-throughput screening and combinatorial chemical synthesis, the technology has drawn closer attention these years [4]. Nevertheless, the bottleneck of the technology is how to encode the microbeads effectively [5, 6]. The encoding methods appeared in the past years such as optical encoding, electronic encoding, graphical encoding and physical encoding have their own advantages and disadvantages respectively [7], among which microbeads (such as polymeric, silicon or glass microbeads) with embedded optical signatures (such as the fluorescence of quantum dots (QDs)) have been made the focus of attention due to their better stability, repeatability and their ability to accomplish high-through detection easily.

The special properties of quantum dots (such as characteristic narrow and symmetric spectra, size-tunable emission, simultaneous excitation and more photostable) have made it the ideal fluorescence material in optical encoding of microbeads. In this field, multicolor microbeads encoded with QDs are concerned particularly. In 2001, Nie et al. encoded polystyrene beads (with a 1.2-μm diameter and a standard deviation of 2–3% in size) with three different color QDs (484 (blue), 547 (green), and 608 nm (red)) and used them for DNA hybridization studies after integrating it with Cascade Blue (425 nm) [5]. They did a significant try in the multicolor encoding and the application of encoded microbeads in

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bioscience. They predict that a large number of codes could be obtained by encoding microbeads with QDs with different sizes (for example, the use of 10 intensity levels and 6 colors gives one million unique codes). In 2003, Hongxia Xu et al. obtained nine unique codes by encoding microbeads with green (530 nm) and yellow (565 nm) QDs. After being integrated with Streptavidin-PE-Cy5 (670 nm), the beads were used to study multiplexed SNP genotyping [6].

Nevertheless, the multicolor encoding of beads with QDs still has deficiency and needs to be improved further. The emission wavelengths of many fluorescence dyes used in biological field locate between blue and red (such as FITC 520 nm, Rh.6G 555 nm and Cy3.5 TM 596 nm). They are not suitable to be integrated to Nie's beads encoded with blue, green and red QDs for biological applications as the existence of overlapping problem with the fluorescence from the QDs (blue, green or red) in encoded beads. Although Hongxia Xu et al. used two kinds of QDs with closer emission wavelengths (530 and 565 nm) to encode microbeads, the signal intensity of the corresponding codes contained only three qualitative levels (high, medium and low) and the details how to encode beads with QDs was not offered either. In order to extend the application of microbeads encoded with QDs in biological field, in this paper, we hope to choose two or more kinds of suitable QDs with different sizes in accordance with the special requirements of fluorescent dye by biological research to encode microbeads. Then, the special band of wavelength without overlap with QDs emission can be remained and used as a detecting window for the fluorescent dye. Making a comprehensive view of the work of Nie et al., Hongxia Xu et al. and other biological researchers, it is necessary and valuable to develop a method of encoding microbeads with multicolor QDs, which is characterized by its tuning window for detection, the enormous amount of coding library and quantitative control of encoding. To test the performance of the encoded beads in bio-application, antibody conjugation analysis were carried out on multicolor encoded carboxyl-modified beads in experiment.

Experimental part

Materials and apparatus

Tri-n-octylphosphine (TOP), Tri-n-octylphosphine oxide (TOPO), bis(trimethylsilyl)sulfide ($(TMS)_2S$) and hexadecylamine (HDA) were purchased from Sigma-Aldrich Fine Chemicals. Cadmium acetate ($Cd(Ac)_2$), zinc acetate ($Zn(Ac)_2$), selenium, toluene, chloroform and anhydrous methanol were obtained from Acros Organics. Polystyrene microbeads (average size: $\sim$110 μm) were ordered from Aldrich (USA). IgG (Goat anti-human IgG/FITC), NHS and EDC were ordered from Sigma (USA). Rhodamine (TRITC)-Conjugated ImmunoPure Goat Anti-Mouse IgG was obtained from Pierce (USA). All other chemicals were of at least analytical reagent grade.

The UV-visible absorption spectra of QDs were recorded on a UV-visible Spectrophotometer 2550 (Shimadzu, Japan). And the fluorescence spectra of QDs were achieved with a Luminescence Spectrometer 55 (PerkinElmer, USA) at room temperature. The quantum yields of QDs were measured by the optically dilute measurement method using Rhodamin 6G (whose Φ_f is assumed to be 95% in ethanol at an excitation of 475 nm) as the reference [8]. The emission spectra of single bead encoded with QDs were acquired by using an inverted microscope (IX71, Olympus, Japan) equipped with a fiber optic spectrometer (HR2000, Ocean Optics, USA).

Synthesis of ZnS-capped CdSe quantum dots

Core-shell quantum dots (CdSe/ZnS) were synthesized according to literature procedures [9–11]. In a typical recipe, 6 g TOPO and 4 g HAD were dried and degassed at 180°C for 2 h in a 50-ml three-neck flask. Then 0.125 g $Cd(Ac)_2$ was added into the solution. After the mixture was heated to 310°C, the heater was removed. And the stock solution of TOP/Se prepared by dissolving 0.2 g selenium in 5 g of TOP was quickly injected in under vigorous stirring, resulting in nucleation of CdSe nanocrystals. Desired size of QDs was obtained by controlling the nucleation time. The synthesis of shell of CdSe QD is as flows: 6 g TOPO, 2 g HDA and 0.2 g ($Zn(Ac)_2$) were mixed in three-necked flask. Then the previously synthesized CdSe nanocrystals in chloroform was added into the mixture and the temperature was increased to the desired temperature quickly. The desired stock solution of TOP/S by adding 0.1 ml $(TMS)_2S$ to 3 ml TOP was dropped with the rate 0.5 ml per 30 s. In the following 2 h, the temperature maintained 90°C to keep the inorganic epitaxial growth of the shell proceed on the surface of the core. By controlling the temperature and the molar ratio of reactant in the reaction, different size of quantum dots were synthesized. The resulting quantum dots were coated with a layer of tri-n-octylphosphine xoide (TOPO).

Quantitative doping of beads with multicolor quantum dots

The solvent of doping solution was mixed by propanol (40%) and chloroform (60%) (vol/vol) [12]. Certain amount of two doping QDs with a certain molar ratio was added into the solvent to make up of the doping solution. In this experiment, 540 and 652 nm quantum dots were chosen to investigate the multicolor encoding of beads. Firstly, by changing the molar ratio ($C_{540\ nm}/C_{652\ nm}$) of the two QDs in doping solution, the resulting beads with fluorescence intensity ratio ($I_{540\ nm}/I_{652\ nm}$) about 1: 1 were obtained. Then the concentration of one QD was maintained and the other

concentration of QD was changed to obtain different doping solutions with different molar ratios. After polystyrene microbeads were added into every doping solution, the mixtures were stirred for 24 h until the QDs penetrated adequately into the beads. Finally, the solvents were got rid of, and the resulting beads were washed with ethanol three times respectively. In this way, a series of encoded beads with different codes were accomplished. The emission spectra of single encoded bead were obtained by using an inverted microscope (IX71, Olympus, Japan) equipped with fiber optic spectrometer (HR2000, Ocean Optics, USA).

Antibody conjugation on encoded carboxylic modified beads

Carboxyl modified (PS-COOH) encoded beads were obtained by two steps: Firstly, the beads were modified with carboxyl according to previous protocols [13, 14]; then the beads were encoded with the method described above. Concretely, sample a and sample b, encoded PS-COOH beads, were achieved respectively by doping the carboxylic beads with 590, 652 nm QDs and 521, 652 nm QDs. Antibody conjugation between human IgG and organic dye fluorescein isothiocyanate (FITC)($\sim$ 520 nm) tagged goat anti-human IgG was carried out on beads from sample a. And antibody conjugation between mouse IgG and organic dye Rhodamine (TRITC)-Conjugated ImmunoPure Goat Anti-Mouse IgG was carried out on beads from sample b. After being washed with PBS (0.01 M, pH = 7.4) solution, encoded PS-COOH beads were dispersed in 400 μl PBS solution (0.01 M, pH = 7.4) in centrifugal tube, then 20 μl EDC (200 mg/ml) was added in and stirred for 15 min, after that, 100 μl NHS (0.15 mg/ml) was added in and stirred for another 30 min. Then 100 μl mouse IgG (2 mg/ml) was added in, incubated for 3 h with stirring, and then 50 μl Tris was added and stirred for 30 min. Washed with buffer after all the solvent was removed, then the beads were dispersed in 500 μl solution containing 400 μl PBS (0.01 M) and 100 μl FITC($\sim$ 520 nm) tagged goat anti-human IgG (2 mg/ml), and incubated for 1 h. Finally, the beads were washed with PBS buffer several times. Similar protocols were used for the Rhodamine (TRITC)-Conjugated ImmunoPure Goat Anti-Mouse IgG antibody conjugation.

Results and discussion

Figure 1 shows the UV-visible absorption and the fluorescence spectra of the two QDs used in quantitative multicolor encoding investigation. The photoluminescence quantum yields (QY) of the green (540 nm) and red (652 nm) QDs are 8.6 and 14% respectively, which is effectual for encoding of microbeads.

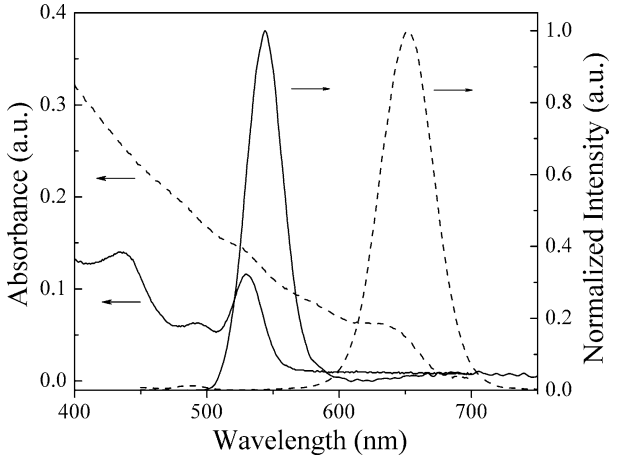

Fig. 1 Absorbance and fluorescence spectra of QDs. Solid: absorbance and fluorescence spectra of the green QDs (540 nm); Dash: absorbance and fluorescence spectra of the red QDs (652 nm)

In this experiment, green (540 nm) and red (652 nm) QDs synthesized in our lab were used to quantitatively investigate the multicolor encoding of microbeads. In order to find out the proportion, by which the two QDs in doping solution was adsorbed into microbeads, the molar ratios of the green and red QDs in doping solution were changed. And a series of doping solution with different molar concentration ratios were obtained. As shown in Fig. 2, the spectra (A $\sim$ M) of single bead of every sample were obtained from the average of twenty random beads. By controlling the molar ratio of the two QDs in doping solution, various intensity ratios ($I_{540\ nm}$: $I_{652\ nm}$) of fluorescence of encoded beads were obtained (Fig. 2).

According to the previous reports, the emission wavelength of CdSe/ZnS quantum dots can locate at any

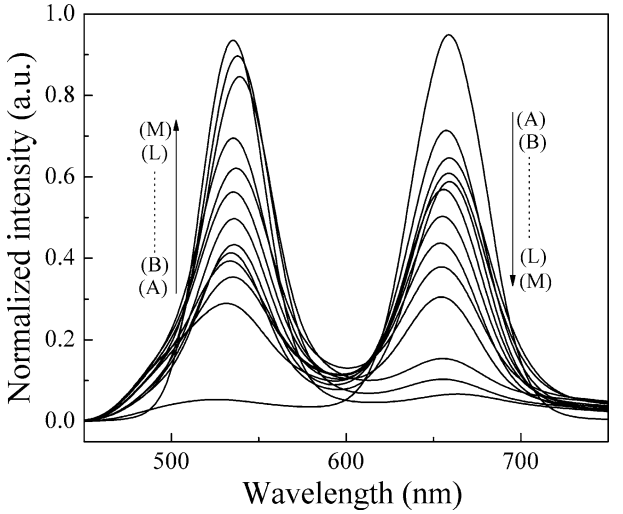

Fig. 2 Emission spectra of single bead encoded with 540 and 652 nm QDs. Fluorescence intensity ratio of green and red QDs ($I_{540\ nm}/I_{652\ nm}$) ranges from 14.6: 1 to 1: 18.9

wavelength between 500 and 700 nm by tuning their size. The full width at the half-maximum (fwhm) of the fluorescent spectrum of CdSe/ZnS QDs is ~30 nm. Then, generally a 30 nm interspace between the two emission wavelengths of two QDs is required for spectrophotometer to effectively distinguish the two quantum dots. Otherwise, overlap between the two emission peaks would occur, which brings difficulty in distinguishing the two fluorescence spectra of the two QDs. So there would be 7 ((700–500)/30 nm ≈ 7) kinds of QDs with different emission wavelengths can be simultaneously chosen for multicolor encoding of beads. However, in practical application, an organic dye is usually used as tag of a specifically bio-molecular, which is going to be screened. Then a specifically wavelength band must be remained as the detecting window for the specifically dye. So, commonly, 6 different kinds of QDs can be chosen simultaneously for encoding of beads in practical applications. Based on the above analysis, with six CdSe/ZnS quantum dots with different emissions, the total number of discriminable codes could be calculated by the following equation:

$$\mathrm{Num}\,(n) = C_6^1 + C_6^2 \times n^2 + C_6^3 \times n^3 + C_6^4 \times n^4 + C_6^5 \times n^5 + C_6^6 \times n,$$

here *Num* is the total number of discriminable codes could be obtained based on CdSe/ZnS QDs, and n is the number of fluorescence intensity levels of every QD. Such as, $n = 3$, which means that there are three (high, medium and low) kinds of fluorescence intensity levels could be chosen for every kind of QD in encoding. *Num*(3) can be calculated to be 4083, that is to say, 4083 different discriminable codes could obtained. In fact, n, the number of discriminable intensity levels, usually exceeds 3, even reachs 10. Figure 3 shows that the fluorescence intensity ratio ($I_{540\ \mathrm{nm}}/I_{652\ \mathrm{nm}}$) of encoded bead increases with the rise of the molar concentration ratio ($C_{540\ \mathrm{nm}}/C_{652\ \mathrm{nm}}$). While the concentration ratio $C_{540\ \mathrm{nm}}/C_{652\ \mathrm{nm}}$ is under 1700, the I_G/I_R increase linearly with $C_{540\ \mathrm{nm}}/C_{652\ \mathrm{nm}}$ ($I_{540\ \mathrm{nm}}/I_{652\ \mathrm{nm}} = 1.002 + 0.00815^{*}C_{540\ \mathrm{nm}}/C_{652\ \mathrm{nm}}$, $R = 0.9924$). It also can be seen from Fig. 4 that, the $I_{652\ \mathrm{nm}}/I_{540\ \mathrm{nm}}$ increase linearly with the rise of $C_{652\ \mathrm{nm}}/C_{540\ \mathrm{nm}}$ before $C_{652\ \mathrm{nm}}/C_{540\ \mathrm{nm}}$ reaching ~1.9 ($I_{652\ \mathrm{nm}}/I_{540\ \mathrm{nm}} = 0.32215 + 7.18665^{*}C_{652\ \mathrm{nm}}/C_{540\ \mathrm{nm}}$, $R = 0.99728$). Furthermore, it indicates in Fig. 3 that while maintaining the concentration of 652 nm QDs and increasing the concentration of 540 nm QDs, 7 different discriminable codes of beads are accomplished. And 6 different discriminable codes were obtained when maintaining the concentration of 540 nm QDs and increasing the concentration of 652 nm QDs shown in Fig. 4. It is can be supposed that, if every kind of QDs for encoding could provide with 6 different discriminable coding intensity levels (Here, it is needed

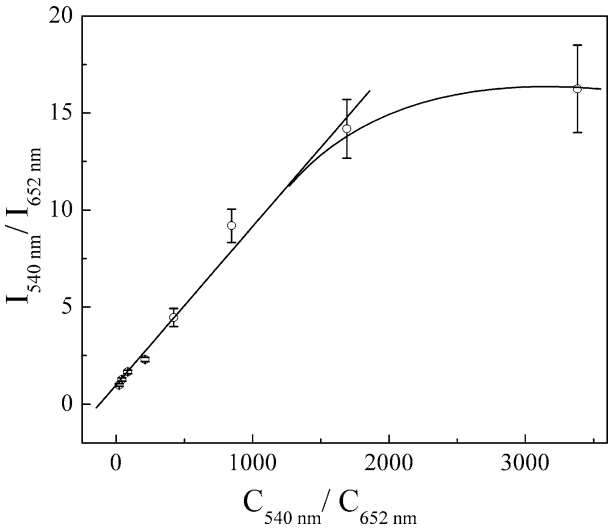

Fig. 3 Change of fluorescence intensity ratio ($I_{540\ \mathrm{nm}}/I_{652\ \mathrm{nm}}$) of single bead with the increase of green QDs' concentration ($C_{540\ \mathrm{nm}}$) while the red QDs' concentration ($C_{652\ \mathrm{nm}}$) is changeless

to point out that the 6 (or 7) discriminable coding intensities were obtained with doping solutions, which were confected random with interspaced molar ratios in a wide range. If the molar ratios of doping solution is confected and controlled more smartly, even continuously, more discriminable codes could be accomplished), then according to the previously described equation, 117618 different codes could be accomplished with 6 kinds of QDs in experiment.

In order to investigate the applicability of QDs encoded beads in biological applications, antibody conjugations were carried out on carboxyl-modified beads, which were encoded with multicolor QDs. According to the emission wavelength of the dye labeled on antibodies, different combinations of

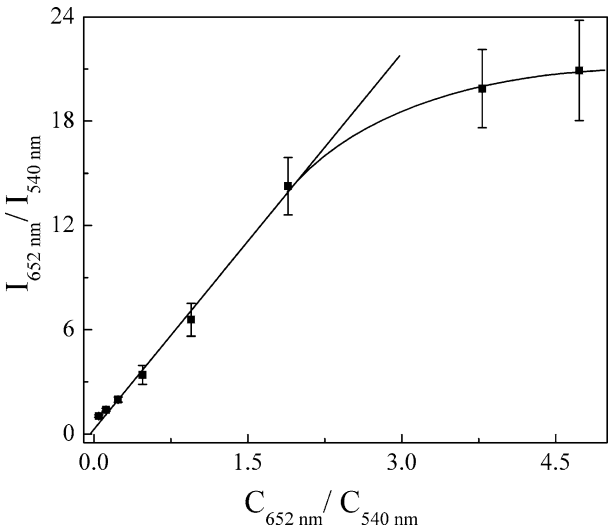

Fig. 4 Change of fluorescence intensity ratio ($I_{652\ \mathrm{nm}}/I_{540\ \mathrm{nm}}$) of single bead with the increase of red QDs' concentration ($C_{652\ \mathrm{nm}}$) while the green QDs' concentration ($C_{540\ \mathrm{nm}}$) is changeless

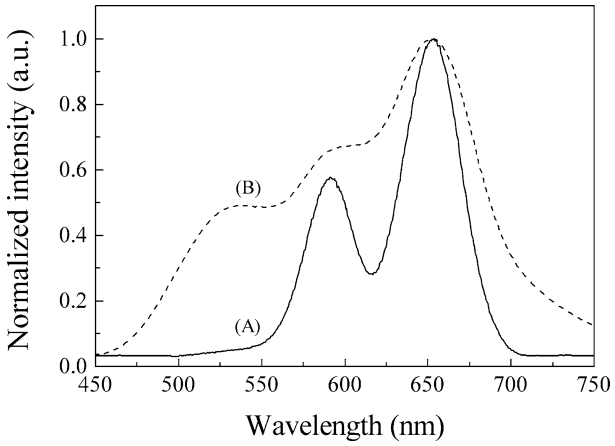

Fig. 5 Fluorescence spectra of single bead encoded with 590 and 652 nm QDs before (A) and after (B) antibody conjugation between human IgG and Goat anti-human IgG/FITC on its surface

QDs with appropriate emission wavelength were chosen to code the beads. In this experiment, 590 and 652 nm QDs were used to encode the beads, on which FITC (520 nm) labeled antibody conjugation was carried out. And 525 and 652 nm QDs were chosen to dope the beads, on which Rhodamine (580 nm) labeled antibody conjugation was carried out.

The fluorescence spectra of single bead before and after antibody conjugation were shown in Figs. 5 and 6. Spectrum A is captured from encoded carboxylic modified beads, which is treated in the same process as the beads in antibody conjugation process without adding Goat anti-human IgG/FITC. Line B is the fluorescence spectrum of encoded bead after antibody conjugation on it surface. As shown in Fig. 5, a new fluorescence peak ($\sim$ 520 nm) appears in spectrum B, which is considered to be the emission of organic dye FITC labeled on goat anti-human IgG. It suggests that the antibody conjugation has occurred on the beads surface.

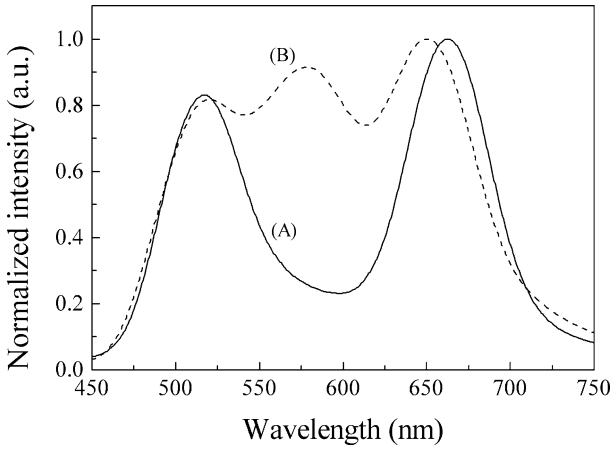

Fig. 6 Fluorescence spectra of single bead encoded with 525 and 652 nm QDs before (A) and after (B) antibody conjugation between mouse IgG and Rhodamine-Conjugated ImmunoPure Goat Anti-Mouse IgG on its surface

That is to say, the FITC labeled goat anti-human IgG has successfully coupled to the human IgG probed on beads. The code ($I_{590\ nm}$: $I_{652\ nm}$) of bead changes from 0.57: 1 to 0.66: 1 after antibody conjugation. The increase of fluorescence intensity at $\sim$590 nm should be attributed to the fluorescence overlapping between FITC and 590 nm QDs. In Fig. 6, it can be seen that the third fluorescence peak ($\sim$ 580 nm) appears in spectrum B compared with the two peaks of QDs in spectrum A. The third peak is considered to be the emission of Rhodamine (TRITC) labeled on Goat Anti-Mouse IgG. It indicates that the Rhodamine (TRITC)-Conjugated ImmunoPure Goat Anti-Mouse IgG has successfully coupled to the mouse IgG probed on beads. Here, the code ($I_{525\ nm}$: $I_{652\ nm}$) of bead after antibody conjugation did not change compared with its original value. Furthermore, 3 nm-red shift of fluorescence peak of 521 nm QDs and 10 nm-blue shift of fluorescence peak of 652 nm QDs were observed after antibody conjugation, which is estimated to the fluorescence overlap between Rhodamine (TRITC) and the two QDs.

Besides, we used cheap and available commercial polystyrene microbeads in the experiment. Its diameter ranges from 75 to 150 microns, and its average diameter is 110 microns. According to the previous report [15], the encoded porous beads are $\sim$1000 times brighter in fluorescence intensities and 5 times more uniform than those nonporous beads. So it can be supposed that a much better result is likely to be achieved if a kind of higher quality bead is used in the method.

Conclusions

By controlling the molar ratio of the QDs in doping solution, various encoded beads with different discriminable codes could be obtained. For two certain QDs, the relation curve about the intensity ratio of single encoded bead and the molar ratio of the two QDs in doping solution could be achieved firstly. Then the curve could be used to estimate the capacity of encoding based on these two QDs, and guide the quantitative encoding in the coming experiment. For different dyes with special emitting wavelengths, we always could choose proper QDs for encoding to effectively avoid overlapping problems. The results of antibody conjugation suggest that the encoded beads obtained in this method could be effectively used in biological applications.

Acknowledgements The work was supported by the National High Technology Research and Development Program of China (863 Program: 2003AA234010), the National Natural Science Foundation of China (Grant No. 30670553, 30370387), the Trans-Century Training Program Foundation for Talents by the Ministry of Education, the Program for Distinguish Young Scientist of Hubei Province (2006ABB020).

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