

Quantum dot optical encoded polystyrene beads for DNA detection

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Abstract. A novel multiplex analysis technology based on quantum dot (QD) optical encoded beads was studied. Carboxyl functionalized polystyrene beads, about 100 μm in size, were precisely encoded by the various ratios of two types of QDs whose emission wavelengths are 576 and 628 nm, respectively. Then the different encoded beads were covalently immobilized with different probes in the existing of sulfo-NHS and 1-[3-(Dimethylamino) propyl]-3-ethylcarbodiimide methiodide, and the probe density could reach to 3.1 mmol/g. These probe-linked encoded beads were used to detect the target DNA sequences in complex DNA solution by hybridization. Hybridization was visualized using fluorescein isothiocyanate-labeled DNA sequences. The results show that the QDs and target signals can be obviously identified from a single-bead-level spectrum. This technology can detect DNA targets effectively with a detection limit of 0.2 $\mu\text{g}/\text{mL}$ in complex solution. © 2006 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2358957]

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1 Introduction

In the recent decade, with the development of many subjects such as life science, chemistry, and so on, a number of compounds and biology molecules have come forth for screening or identifying. How to screen out the target compounds or molecules quickly and with high efficiency from the complex system, which contains a number of candidates, is one of the big problems for the biologist and chemistry analyst.^{1–3} The analysis technologies, such as electrophoresis and lab-on-chips, make big contributions to the development in the abovementioned fields,^{4,5} but they are complex to manipulate or have high costs or long time periods and have met the challenges of miniaturization, speediness, and veracity.^{6,7}

The multiplex and high-throughput analysis based on the decoded beads is a new analysis strategy and has been developed to solve these challenges.^{8–10} This strategy's basic principle is that the beads are predecoded by chemical,¹¹ spectral,^{12,13} electronic, or physical technologies¹⁴ and each coding represents a kind of given information for recognition, at the same time, each of the beads also offers the sites to react with the probes. Before the detection, the different coded beads are linked with different probes such as oligonucleotides or proteins (antibodies), and so each bead has a different probe to capture different targets. The immobilized probes capture the fluorescein-labeled targets in the solution through specific recognitions, and so there appears to be an extra target fluorescent signal in the spectrum of the beads. The

beads are excited when passing through the detection channels by microfluids technology, and the signals (including the coding and target signals) of the bead are collected for analysis and decoding, and the results are given.^{12,15}

In the coding aspect, the optically distinct polystyrene (PS) beads, which are encoded by varying the ratios of red and infrared dyes,^{16,17} have been reported; each optically encoded bead has a unique spectrum that contains of two emission wavelengths and intensities, which effectually can identify the beads' encoding type. However, because of the unfavorable properties of the dyes, such as broad emission profiles, spectrum overlapping, and the limited kinds of dyes, the amounts of the beads that can be coded are so far dissatisfactory for the needs. This coding method of 10 intensity levels of two dyes could only yield an array of about 100 beads at the present time.^{18,19} Compared to the organic dyes, quantum dots (QDs) as coding fluorophores have many unique properties. The intrinsic QDs' spectral width is about one-third as wide as that of organic fluorescence dyes: the fluorescence intensity of a single CdSe QD is about 20 times that of a dye molecule.²⁰ Furthermore, the emission wavelength can be tuned continuously by changing the size of the QD particles. Use of these emission-wavelength-different QDs can yield an enormous number of encoded beads, for example, 6 kinds of QDs with 10 intensity levels theoretically can code 1 000 000 beads. The most important is that the emission-wavelength-different QD particles can be excited simultaneously by one excitation wavelength due to their the broad-excitation wavelength. That is to say, we can choose the excitation wavelength specific to fluorescent molecules labeling to the target to excite the cod-

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ing and target signals simultaneously; but for the organic dye-encoded beads, at least two kinds of exciting laser are needed to excite the coding and target signals, respectively.^{16,17} All of these unique properties are required advantages for optical coding, and therefore, QDs become a perfect luminescent material for optically encoded beads, which have attracted much attention recently.^{21–23} Recently Nie et al. demonstrated the feasibility of DNA detection using the QD-encoded beads,²² and here we attempted to carry out some trials to put this strategy to practice in a complex system and studied other aspects such as detection range, limitation, sensitivity, and so on.

We even doped the QDs into the porous PS beads by adsorption in solution, thereby encoding the beads, and coated the beads with a thin layer of silica to get precise encodings. In this paper, we immobilized DNA probes to the abovementioned beads and used these beads to detect the fluorescein isothiocyanate (FITC)-labeled target DNA sequences in solution and successfully read out the coding and target signals. This work was a successful attempt to study the multiplex and high-throughput analysis and practice based on QD-encoded beads. This technology also provides an alternative approach to detect and analyze specific DNA sequences, and it will be a powerful tool for advanced drug discoveries, disease diagnostics, and biological assays.

2 Materials and Methods

2.1 Chemicals and Reagents

Tri-*n*-octylphosphine oxide (TOPO, 90%), CdO (99.99%), Triton X-100, selenium powder (99.99%), 1-[3-(Dimethylamino) propyl]-3-ethylcarbodiimide methiodide (EDC), sulfo-NHS, and morpholineethanesulfonic acid (MES) were ordered from Aldrich. Chlorosulfonic acid (80 wt%, 1.75 g/mL), cyclohexane (99.9%), 1-octanol (99%), 6-amino-caproic acid (98.5%), dodecyl sulfonic acid sodium salt (SDS, 99%), and sodium citrate were purchased from China National Pharmaceutical Group (Shanghai, China). The 5× Denhardt solution was ordered from Fluka. The terminal modified 30mer oligonucleotides (dT₃₀, seen in Table 1) were purchased from AuGCT Biotechnology Ltd. (Beijing, China). Calf thymus DNA was purchased from Sigma. PS beads (75 to 150 μm, 1% cross-linked) were obtained from Aldrich; the beads are 97.7% between 75 and 150 μm, and 107.3 μm is the median. All other reagents required for use in the coupling, washing, or hybridization buffer were of analytical grade.

2.2 Preparation of Carboxyl Functionalized Beads

Carboxyl functionalized beads (PS-COOH) were prepared by chlorosulfonation reaction on the surface of beads.²⁴ The 0.5-g PS beads were swollen in 20 mL dichloromethane in a flask and stirred for 1 h, and then 2 mL chlorosulfonic acid was dropped into the flask and stirring continued for 0.5 h. Then, only the suspended particles were removed, 10 mL 6-amino-caproic acid solution (25% concentration, excessive) were added to the flask, stirred for 24 h, and filtered. Then the beads were washed with 1% HCl, the excess water was removed, and then the beads were dried in a vacuum. This process can be seen in Fig. 1.

Table 1 Oligonucleotide sequences used in this paper.

| Oligonucleotide | Sequence |
|-----------------|--|
| Oligo-DNA0 | 5'-FITC-CAC CCG ACG GGG CGG AGT TCG ATC GCG GTC-NH ₂ -3' |
| Oligo-DNA1 | 5'-ACG CAC GGG CCG CGG GCG GTC AGT TCG ATC-NH ₂ -3' |
| Oligo-DNA2 | 5'-CAG GAT GCG CTC GCC CCG CCC CGA TCG AAT-NH ₂ -3 |
| Oligo-DNA2' | 5'-FITC-ATT CGA TCG GGG CGG GGC GAG CGC ATC CTG-3' |
| Oligo-DNA3 | 5'-GAT CGA ACT GAC CGC CCG CGG CCC GTA AGC-NH ₂ -3' |
| Oligo-DNA4 | 5'-ATT CGA TCG GGG CGG GGC CGA CCG CGG CCC-3' |

2.3 Preparation of ZnS-Capped CdSe QDs and QD-Encoded PS Beads

Core-shell QDs (ZnS-capped CdSe) were synthesized according to Refs. 25 and 26. The synthesized QDs were coated with a layer of TOPO, which was used as a high-temperature coordinating solvent.

Two-color encoded beads were accomplished by doping a controlled amount of QDs into porous beads.^{27–29} This was completed in 5 mL of the mixture solution of 5% butanol and 95% chloroform (v/v), which had been added to 0.25 g of grafted PS beads (PS-COOH) and 2 mL of 15-nM QD solution. The mixture was stirred until no QDs were left in the solution. The doping process was completed within 2 h at room temperature. After the doping, the encoded beads were isolated by a filter and washed several times with ethanol.

2.4 Preparation of Oligo-DNA Conjugated Beads

The 0.25-g PS-COOH beads, sulfo-NHS (excessive), and EDC (500 mg) were directly added to 25 ml of 0.1-M MES buffer (pH 4.5), then added 3 μg of oligo-DNA. The mixtures were shaken and incubated at 25°C for 2 h. Then the mixtures were filtrated to get the beads, washed with phosphate buffer saline [(PBS), pH 7.2] until there was no fluorescence in the washed PBS solution, and dried in a vacuum. These DNA probe-linked beads were kept in an airtight container at 4°C until use.

2.5 Hybridization Studies of Probe-Immobilized Beads

Hybridization assay based on the QD-encoded beads was carried out in hybridization solution containing 6×SSC (1×SSC:0.015 M sodium citrate, 0.15 M sodium chloride, pH 7.0) and 0.05% SDS at 45°C. Denatured calf thymus DNA was dissolved in 2 ml 6×SSC and the final OD₂₆₀ was 0.588 (about 23.5 μg/ml). Oligo-DNA2' was dissolved in 6×SSC, at graduated concentrations of 0, 2, 4, 8, 10, 16, 24, 30, 35, and 40 μM. The 0.01-g probe-linked beads were added to the hybridization solution and incubated for 1 h at

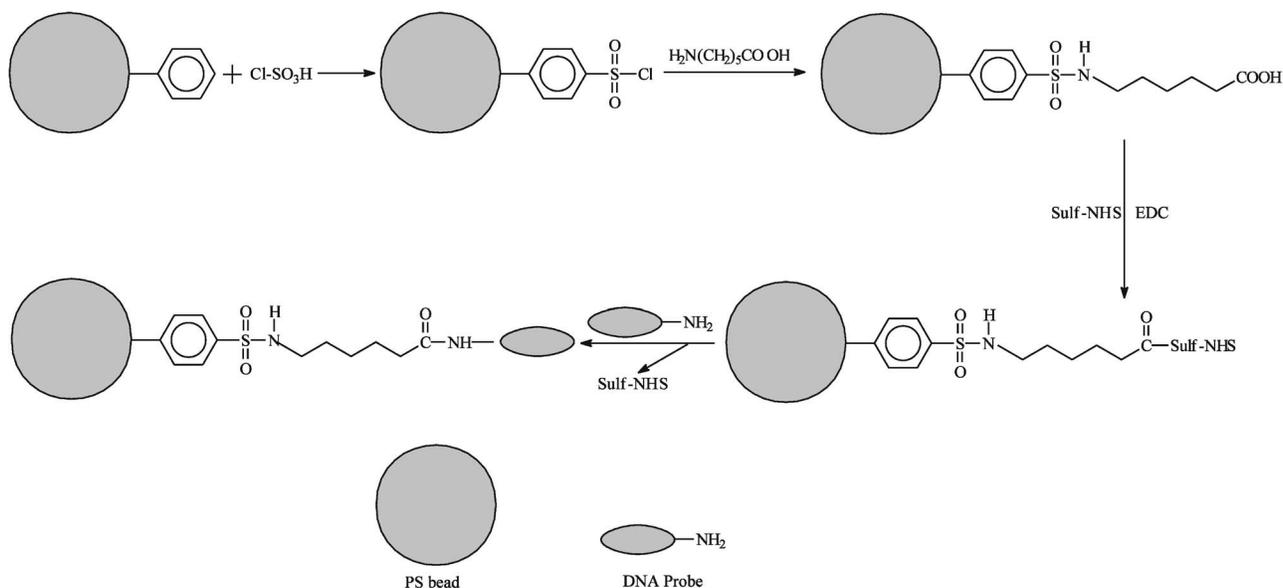


Fig. 1 The sketch of preparation carboxyl functionalized PS beads and covalent of DNA onto the PS beads.

45°C. After hybridization, the beads were washed sequentially with buffers of $1\times\text{SSC}+0.03\%$ SDS, $0.2\times\text{SSC}$, $0.05\times\text{SSC}$, and with deionized water at 30°C to remove the nonspecifically bound oligo-DNAs. Then they were dried in a vacuum before the fluorescence measurements were taken.

2.6 Characterization

The OD_{260} of calf thymus DNA was determined by uv-visible (UV/VIS) spectrophotometer (UV-2550, Shimadzu, Japan). The fluorescence spectrum and intensity of the beads were taken from the beads using an UV/VIS fiber optic spectrometer (HR2000, Ocean Optics, USA).

The terminal $-\text{COOH}$ content on the beads was determined by titrating with KOH. Thus, the 0.25-g beads were left in contact with a methanolic solution (15 mL) of 1.0-g KOH for 24 h at room temperature, filtered, and then 5 mL of the filtrate was diluted to 20 mL with distilled water. Then, the solution was titrated with 0.1-M HCl.

3 Results and Discussion

The encoding signals of the beads are determined by two main factors: fluorophore emission wavelength and fluorescence intensity if the self-absorption of QDs is negligible. Emission wavelength is the inherent characteristic of the fluorophore and it has no relation with the quantity of the fluorophores that the bead loaded; but fluorescence intensity is in linear ratio with the quantity of fluorophores that loaded on the bead. Compared with organic dyes, QDs as fluorophore have many unique properties, which are suitable for optical coding. First, because of the broad-excitation wavelength, different emission-wavelength QDs can be excited simultaneously by the same excitation wavelength, that is to say, the excitation wavelength specific to the target-labeled fluorescent molecules could be chosen to excite the coding and target signals simultaneously. In this paper, FITC-labeled target sequences can be seen as study objects, and the FITC excitation wavelength is 480 nm, which can also excite the QDs. So, in

the following experiments, 480 nm was chosen as excitation wavelength to detect the coding ($\lambda_{\text{QD1 em}}=576$ nm, $\lambda_{\text{QD2 em}}=628$ nm) and target ($\lambda_{\text{FITC em}}=520$ nm) signals at the same time. Because the full width at half-maximum of QDs is very narrow (usually about 20 to 35 nm), more coding signals can be contained in the detecting range; this is the second advantage. Third, the QDs have high intensity and stability of fluorescence; the fluorescence intensity of a single CdSe QD is about 20 times that of the dye molecule.²⁰ Several types of beads were coded in this paper by two different emission wavelength QDs, and the coding spectrums of these beads can be seen in Fig. 2, for the PS bead *a*, the normalized intensity ratio (intensity of 576 nm/intensity of 628 nm, the same in the following) is 1:3, for *b* is 1:1, and for *c* is 3:1. At the same time, FITC-labeled DNA sequences were chosen as detection targets and the detection principle is showed in Fig. 3.

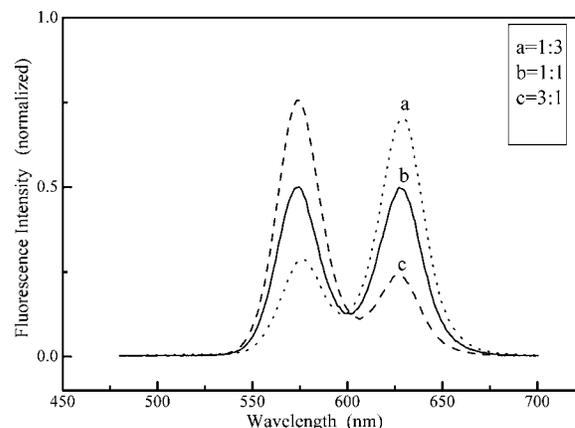


Fig. 2 Three spectra of QD-encoded beads. The coding QDs' emission wavelengths are 576 and 628 nm, respectively. The encoding signals are composed of wavelength and intensity. For the PS bead *a*, the normalized intensity ratio (intensity of 576 nm/intensity of 628 nm) is 1:3, for *b* is 1:1, and for *c* is 3:1.

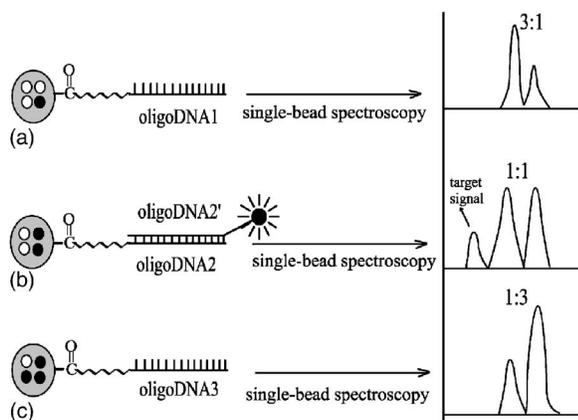


Fig. 3 Bead-based array analysis strategy. The beads were encoded by two-color QDs and covalently linked with DNA probes for hybridization array.

As a carrier of bioassays, PS beads have various applications.¹ Considering the encoding reproducibility and feasibility to establish the strategy of detecting target DNA in a complex system, 100- μm -sized beads were chosen to use as platforms. At 100 μm in diameter, the beads present a much larger surface area and higher surface reaction activity than the flat surface.^{29,30} In order to decrease the steric hindrance and increase the space freedom for DNA probes in hybridization, a “spacer arm” (chlorosulfonic acid and 6-amino-caproic acid) was introduced between the bead and DNA probe, and furthermore, carboxyl functionalized beads can be easily covalently conjugated with biomolecules or probes, which much interest is focused on.^{31,32} In our former work, the sulfonation group was even introduced to the surfaces of the PS beads by sulfonation reaction, and then grafted with 6-amino-caproic acid as the spacer arm to get -COOH functionalized PS beads (PS-COOH); further acid-alkali titration³² results indicated a -COOH density of 3.88 mmol/g.

At the terminus of the spacer arm, the -COOH has more space freedom to chemical reactions, and it can be considered that no influence is caused by the beads.³³ Generally, the immobilization of DNA probes is accomplished by abbreviating the probe's -NH₂ and the PS-COOH bead's -COOH to form stable amide linkage in the existing of EDC and sulfo-NHS.³⁴ In this paper, this method was used to covalently immobilize the DNA probes to the carboxylic beads to detect the target sequences in solution, and the steps can be seen in Fig. 1. Comparing the spectra of the beads before [Fig. 4(a)] and after [Fig. 4(b)] the immobilization of FITC-labeled oligo-DNA0, a clear FITC signal appeared at 520 nm. The FITC signal indicates the presence of probes and also proves that the -NH₂ modified DNA can be well covalently immobilized to the carboxyl beads. There are two intentions to introduce the spacer arm molecular 6-amino-caproic acid. First, conjugation efficiency of probes with the spacer arm grafted beads is higher than that with the beads, which have no spacer arm molecules. It was found that the time of reaction with the -COOH only needs 10 min, while it needs 40 to 60 min when directly reacted with the sulfonation group on the surface of the beads, therefore, the reaction time is sharply shortened. Furthermore, by comparing the probe concentration be-

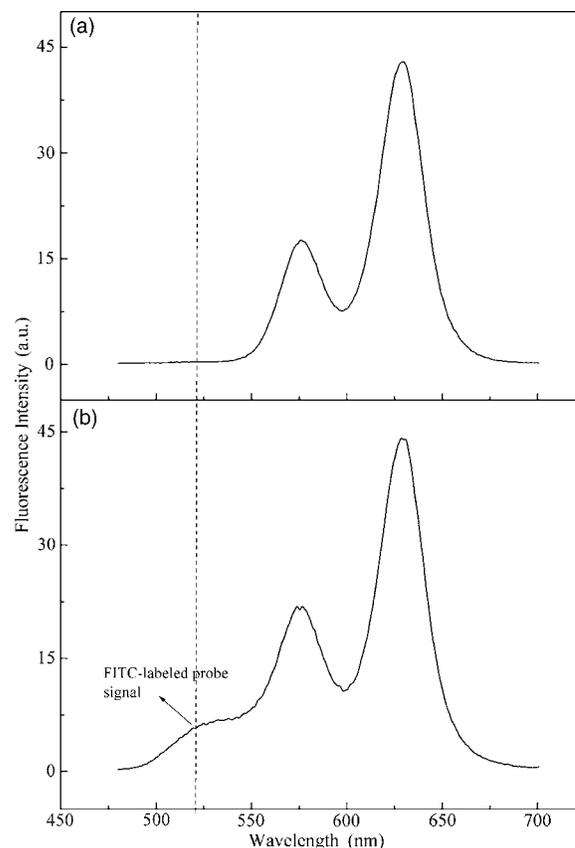


Fig. 4 Spectra of the carboxyl beads before (a) and after (b) immobilized with FITC-labeled DNA0. The spectra were examined from the beads with the integral time of 40 ms. The exciting wavelength is 480 nm. Therefore, 576 and 628 nm are the encoding signals, and 520 nm is the FITC signal.

fore and after the immobilization in the solution, it can be concluded that the DNA probe density on the -COOH spacer arm beads is 3.1 mmol/g. Considering the original carboxyl density of 3.88 mmol/g, the covalent efficiency on the spacer arm beads can reach 79.9%, while it can only reach about 40% when the probes were directly immobilized on the beads' surfaces, which shows that the efficiency is greatly improved. On the other hand, the spacer arm molecules enabled the immobilized probes more space freedom, which is favorable for the next probe-target hybridization.

To study the possibility for the detection of target DNA sequences based on the QD-encoded PS beads, three encoding types of PS beads that immobilized with different probes, respectively, were used in the experiments, named PS-oligo-DNA1 (a), PS-oligo-DNA2 (b), PS-oligo-DNA3 (c). These three types of beads were put into the same hybridization solution that only contains the FITC-labeled oligo-DNA2' sequences (complements of the oligo-DNA2, the probes on b beads), and then the spectra of the three types of beads were detected. Because the target DNA (see Table 1, oligo-DNA2'), on both its terminals, has no active groups such as -NH₃ to react with the activated PS-COOH, so it was hypothesized that few target DNA could be immobilized in the process of hybridization. After the hybridization, the beads were thoroughly washed with buffers of SSC to ensure that

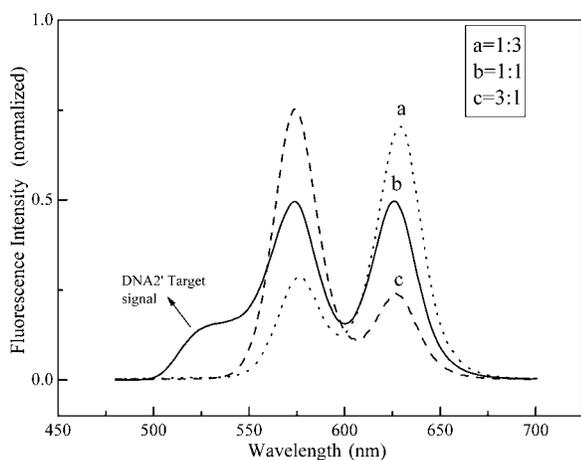


Fig. 5 Spectra of the beads (*a*, *b*, *c*) that hybridized in the solution containing FITC-labeled oligo-DNA2'. Oligo-DNA2' is the complement sequence of oligo-DNA2, which immobilized as probe on the bead *b*'; the sequences are shown in Table 1. The experiment parameters were the same as in Fig. 4.

there was no adsorption and nonspecifically bound target DNA on the beads' surfaces. The results are shown in Fig. 5. It can be seen that the FITC fluorescence signal did not appear at 520 nm in the spectra of beads *a* and *c*, while a strong FITC fluorescence signal appeared in the spectra of *b*. The FITC signal indicates the hybridization of probes with target sequences on the *b* beads. These results also indicate there was no reaction between the noncomplementary probes and target sequences, so the FITC signal can be detected only on *b* beads. By varying the target DNA concentration, it could be found that in the range of 0.2 $\mu\text{g}/\text{mL}$ to 30 $\mu\text{g}/\text{mL}$, the FITC signal intensity is in linear relation with the corresponding target concentration under the same conditions (relative standard deviation $<2\%$), as shown in the inset of Fig. 6.

When the hybridization solution contains oligo-DNA2' and oligo-DNA4, which has the same sequence length but different base pairs, it could be found the beads of type *b* still

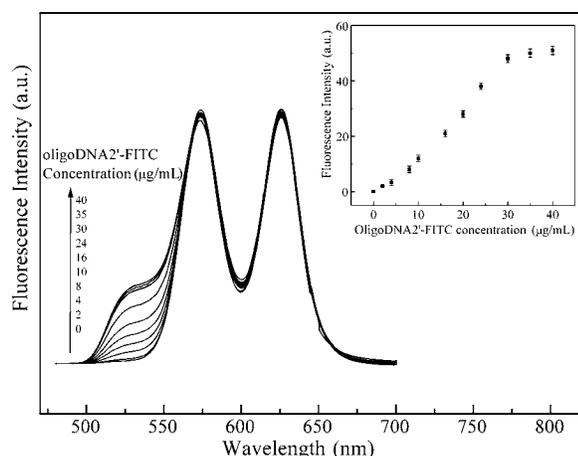


Fig. 6 Spectra of the bead *b* hybridized with FITC-labeled oligo-DNA2' in different concentrations. Inset shows the relation of FITC signal intensity to the concentration of target oligo-DNA2'. The experiment parameters were the same as in Fig. 4.

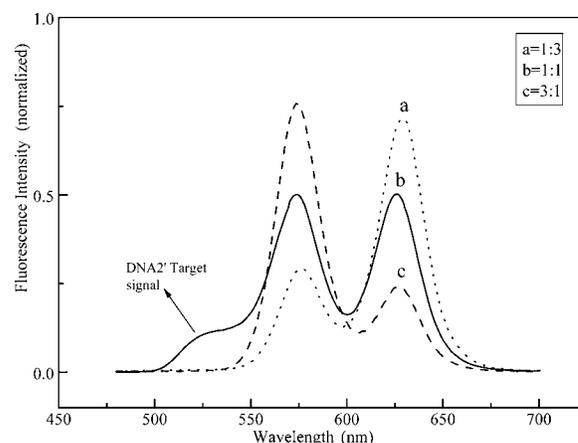


Fig. 7 Spectra of beads hybridized in the solution that contains FITC-labeled oligo-DNA2' and oligo-DNA4. Oligo-DNA2' and oligo-DNA4 is the same sequence length but the base pairs are different. The experiment parameters were the same as in Fig. 4.

captured the FITC-labeled target DNA (oligo-DNA2') successfully, which was proved by the obvious FITC signal in the spectrum of curve *b* in Fig. 7. Compared with the spectrum of curve *b* in Fig. 5, the hybridization in the solution that only contains target sequences, the FITC fluorescence signal intensity decreased about 11%. This indicates that the complementary hybridization efficiency decreased. The oligo-DNA2' and the oligo-DNA4 have the same 10-base-pair sequence (5'-CGA TCG GGG CGG GGC) and the oligo-DNA4 possibly partly hybridized with the oligo-DNA2 probe, which disturbed the normal complete hybridization between oligo-DNA2' and oligo-DNA2.

To validate the feasibility of the target DNA detection in a complex solution based on the QD-encoded PS beads, the hybridization properties of the probe-immobilized beads in the denatured calf thymus solutions were studied. The denatured calf thymus DNA solution contains a large number of ssDNA sequences with different base sequence and length and is a perfect complex solution for target DNA detection. The FITC-labeled oligo-DNA2' in the denatured calf thymus DNA solution, complement to the conjugated probes, can be seen as target DNA; the results are shown in Fig. 8. The FITC signal appeared only in the spectrum of *b* that immobilized complementary probes and could be well identified, while the target signal of other types of beads was not detected. However, comparing it with curve of *b* Fig. 5, hybridization in a single-component solution, the FITC signal intensity decreased 30%, indicating the further hybridization efficiency decreasing of the complementary sequences. This may have been caused by the partial base-pairs hybridization between the probe and short ssDNA that occupied a few bases on the probe sequence and thereby blocked the complete hybridization between target DNA and probe. The partial hybridization of probe and short ssDNA is not stable and dissociation takes place. This case may be overcome by changing certain conditions, such as prolonging the reaction time and washing with the high concentration of salts solution. From Fig. 8, it can be also concluded that, although the target DNA signal can be disturbed by the noncomplementary sequences, this disturbance is not enough to affect the detection. Series ex-

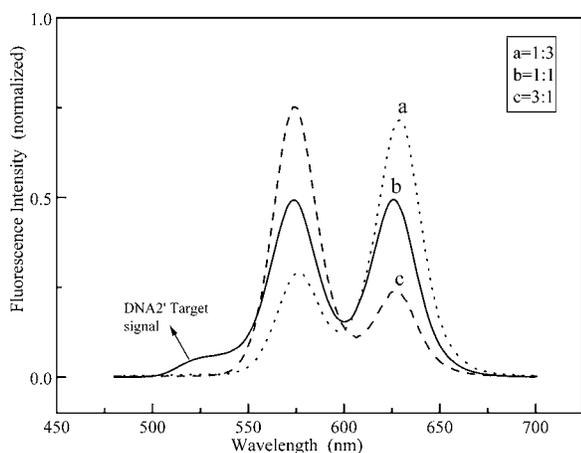


Fig. 8 Spectra of the beads that hybridized in the solution contains denatured calf thymus DNA and FITC-labeled oligo-DNA2'. The experiment parameters were the same as in Fig. 4.

periments validate that the DNA targets can be detected effectively with a detection limit of $0.2 \mu\text{g/mL}$ in a complex solution.

4 Conclusions

In this paper, a multiplex analysis technology based on the novel fluorophores QD-encoded beads was systematically studied, and the principles of QD encoding and DNA hybridization analysis were also expatiated. Due to the good optical encoding performances, carboxyl functionalized beads were precisely encoded with two different emission-wavelength QDs in various ratios. Then the differently encoded beads were covalently immobilized with different probes. The target DNA sequences can be successfully detected by using the probe-linked encoded beads in a complex solution. This analysis technology based on QD-encoded beads has advantages in the aspects of sensitivity and portability, and by further improving the detection performances, it will provide a powerful tool in the fields of bioassays, drug discoveries, disease diagnoses, and so on.

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