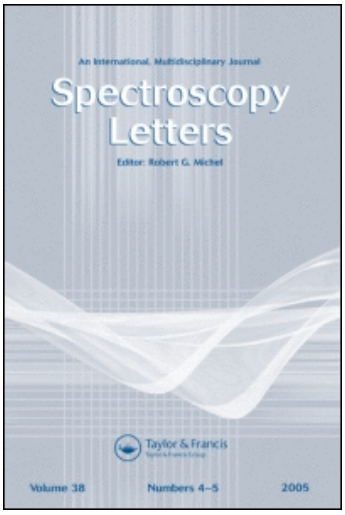

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Abstract: The use of polystyrene fluorescent microspheres conjugated to antigens and CdTe quantum dots (QDs) conjugated to antibodies as sensitive fluorescent labels in fluoroimmunoassay is described. CdTe QDs were attached to antibodies via electrostatic/hydrophilic self-assembly. Both CdTe QD–labeled antibodies (anti-IgGQD) and microsphere-labeled antigens (IgGFM) were used to form a typical immunoassay on the surface of fluorescent microspheres. The immunoreaction system with different microsphere emissions and QDs can be detected effectively at a single excitation wavelength, because fluorescent QDs have broad excitation spectra and narrow emission bandwidths. This approach allowed detection of goat-anti-rabbit IgG in the range of 0.5 to 10 μg/mL. Furthermore, the immunocomplexes of anti-IgGQD–IgGFM were confirmed by coincidence detection of the fluorescence signals of CdTe QDs and microspheres on a microfluidic on-chip device.

Keywords: Fluorescent polystyrene microspheres, fluoroimmunoassay, quantum dots

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INTRODUCTION

Fluorescent labeling of biological materials is widely employed in the life sciences and the fluoroimmunoassays. Fluoroimmunoassays using conventional fluorophores can be multiplexed to screen for multiple analytes simultaneously. However, when conventional organic dyes are used, the situation is often complicated by requirements for elaborate excitation and detection schemes and challenging data collection and analysis.[1]

Luminescent colloidal semiconductor nanocrystals (known as quantum dots; QDs) are inorganic fluorophores that have the potential to circumvent some of the functional limitations encountered by organic dyes in biotechnological applications by combining the advantages of readily tunable spectral properties, high photobleaching threshold, and good chemical stability.[2] QDs exhibit size-dependent tunable photoluminescence with narrow emission bandwidths (full width at half height of the maximum [fwhm] of $\sim$30–45 nm) that span the visible spectrum and broad absorption spectra. These allow simultaneous excitation of several particle sizes at a single wavelength with emission at multiple wavelengths far from the single excitation source.[3] Moreover, unlike organic fluorophores, the inorganic nature of QDs makes them resistant to metabolic degradation: QDs have been shown to remain fluorescent and to be retained in live cells and organisms for several weeks to months, with no detectable toxicity.[4,5] The QDs can be conjugated to the linker (e.g., avidin, protein A or protein G, or a secondary antibody) either by covalent binding[6–8] or by self-assembly based on electrostatic interactions.[4,9] QDs show exceptional photostability: when illuminated constantly with a 50-mW light, QDs do not photobleach even after 14 hr.[4] These features make them desirable fluorescent tags for cell and developmental biological applications that require long-term, multitarget, and highly sensitive imaging.[10–12] The potential of QDs to address multiplex immunoassays has been realized recently, including multiplexed toxin analysis,[1] multianalyte electrochemical biosensor,[13] and microbead QD encoded bar codes for use in DNA hybridization.[14,15] However the applications of QD–antibody conjugates combined with fluorescent microspheres in fluoroimmunoassays are few.

Immunoassays require high sensitivity and direct detection, while small, cell-sized polystyrene/latex microspheres ($\sim$3–5 μm in diameter) have proved to be ideal.[16–18] Latex particles with bound proteins have been used in many medical diagnostic tests, usually as agents magnifying effects of antigen–antibody interactions.[19] The surface of microspheres should be equipped with chemical groups suitable for covalent binding of proteins (antibodies or antigens).[20] The 5.5-μm spheres provide a large surface area that can accommodate up to 100,000 capture antibodies per bead.[21] The high density of capture antibodies ensures maximum antigen binding, thereby enhancing assay sensitivity. Other capture agents, including enzyme substrates, oligonucleotides, peptides, and biotin-conjugated molecules, can

be coupled to the microspheres; thus, assay capabilities are greatly expanded. Because microspheres are freely suspended in solution, antigens experience a short diffusion path to antibody binding sites on the spheres; thus, rapid reactions are kinetically favored.[21] Additionally, sensitive immunoassays have been developed using fluorescent-label microspheres. The use of fluorescent microspheres affords greater sensitivity than can be achieved using soluble fluorescent labels.[22]

Although the fluorescent efficiency of the QDs produced in high-temperature coordinating solvents (organic solvents) is usually high, current phase transfer schemes still require many experimental steps (phase transfer or coating with hydrophilic groups, are essential for this kind of QD to be used as a biological reporter), and ligand exchange is commonly associated with decreased fluorescence efficiency and a propensity to aggregate and precipitate in a biological buffer.

In the current study, the CdTe QDs used were prepared in aqueous solution. A fluoroimmunoassay method based on bioconjugation of antigens and antibodies is described in this paper. The CdTe QDs were conjugated to antibodies (goat-anti-rabbit IgG) by electrostatic interaction. The antibody-specific captured antigens (rabbit IgG) were immobilized on the surface of polystyrene microspheres. Both CdTe QD–labeled antibodies and microsphere-labeled antigens were used to form a typical immunoreaction system for the detection of goat-anti-rabbit IgG. Also, a typical competitive immunoassay format was realized through the inhibition of goat-anti-rabbit IgG-labeled QDs binding to rabbit IgG, which were coupled to fluorescent microspheres by free rabbit IgG. In addition, a novel microfluidic chip device with a laser-induced fluorescence (LIF) system was established and used for the determination of the immunocomplexes of biofunctional QD-labeled microspheres.

MATERIALS AND METHODS

Instrumentation

Fluorescence experiments were performed on a Shimadzu RF-5301 PC spectrofluorophotometer (Shimadzu Co., Kyoto, Japan). A 1-cm-pathlength quartz cuvette was used to measure the fluorescence spectrum. The fluorescence intensity was measured with a delay time of 50 ms and an integration time of 1 ms with 5 nm and 5 nm slit widths for excitation and emission.

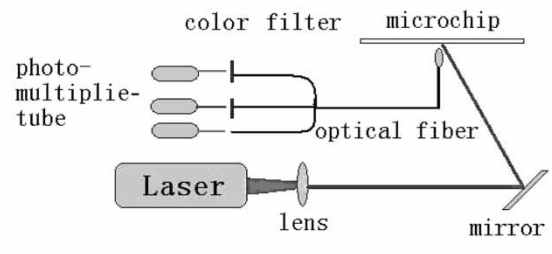
Fluorescence microscopy (at 40 ×) was done using an inverted optical microscope (Leica DM IRB, Wetzler, Germany) equipped with a charge-coupled device camera (TOTA 500II, Kyoto, Japan) and a 100-W Hg excitation lamp. Samples were spread on glass slides and dried under air. All optical measurements were done at room temperature under ambient conditions. A bath ultrasonic cleaner (Autoscience AS 3120, Tianjin, China)

was used to disperse the spheres. All pH measurements were made with a PHS-3C pH meter (Tuopu Co., Hangzhou, China).

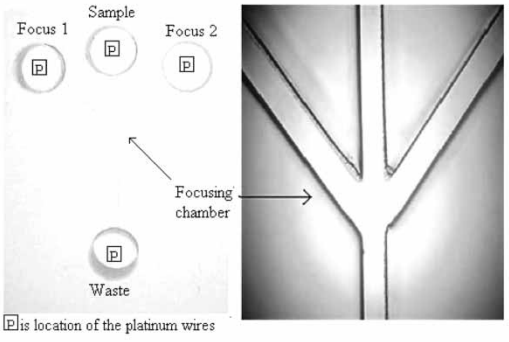
In this experiment, mean fluorescence values are reported in arbitrary units that are internally consistent within a given experiment. However, these units are not absolutely comparable between experiments. All samples were prepared in triplicate, and average values at each concentration were calculated.

A photo of the microfluidic chip device is depicted in Fig. 1. Laser-induced fluorescence (LIF) system was established by a laser excitation source (488 nm, 10 mW) and three photomultiplier tubes. For coincidence measurements, light-scattering and LIF signals were acquired simultaneously from the chip using an optical fiber, filtered with 520-nm and 600-nm filters to receive the corresponding single fluorescent signals. The signals were amplified and digitized with multifunction I/O cards. The microfluidic chip device was provided by the New Tech Co. (Changchun, China).

The polydimethyl siloxane (PDMS) chips were obtained from the Dalian Institute of Chemical Physics, Chinese Academy of Sciences. The channels on



(a)



(b)

Figure 1. (a) Detection setup used for coincident laser light scattering and fluorescence measurements. (b) Schematic of the microchip used for detection with eletrokinetic focusing.

the microchip were 100-μm wide and 30-μm deep (see Fig. 1b). The probe region was designed to be 50 μm downstream of the focusing chamber. Electrical contact with the solutions was achieved by placing platinum wires into each of the reservoirs, and a computer was used for the voltage control. The fluid motion was driven by varying the potential applied to the reservoirs. Under optimal focusing conditions, 400 and 500 V were applied to the sample and two focus reservoirs, respectively.[23,24] The waste reservoir was grounded. When the sample was continuously infused into the focusing chamber, hydroxypropyl methyl cellulose (HPMC: Sinopharm Chemical Reagent Co., Shengyang, China) solution was added from the focus reservoirs for reducing the microspheres' adhesion. The average flow rate when the scatter/fluorescence tests were done was 5 μL/min.

Reagents and Materials

All chemicals used were of analytical reagent grade and were used without further purification. 3-Mercaptopropyl acid (MPA)(>99%), tellurium powder (~200 mesh, 99.8%), $CdCl_2$ (>99%), and $NaBH_4$ (99%) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Carboxylated fluorescent microspheres (1% w/v) were obtained from Spherotech Co. (Libertyville, IL, USA). The microsphere average diameter was about 0.4 μm. The emission maximum is at about 510 nm (green emitting) and the excitation maximum is at 465 nm. The suspension of fluorescent microspheres was ultrasonically vibrated prior to immediate use. The compound 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was obtained from JK Chemical Co. (Beijing, China). Rabbit immunoglobulin G (rabbit IgG, 10 mg, Dingguo Biotechnology Ltd., Beijing, China) and goat-anti-rabbit IgG (10 mg/mL, Dingguo Biotechnology Ltd.) were used in this study. The antigen powder was dissolved in a 2 mmol/L phosphate-buffered saline solution (PBS; pH 7.4, 13.8 mmol/L NaCl, 0.27 mmol/L KCl) to obtain 1 mg/mL solution and stored at 0–4°C. All antigens, antibodies, and other reagents used in the fluoroimmunoassay were diluted with PBS to the concentrations used prior to immediate use. The water used in all experiments had a resistivity higher than 18 MΩ · cm.

Synthesis of Aqueous-Compatible CdTe Quantum Dots

The stable water-compatible CdTe quantum dots used in our studies were synthesized and characterized as previously described.[25] Sodium hydrogen telluride (NaHTe) was produced in an aqueous solution by reaction of sodium borohydride ($NaBH_4$) with tellurium powder at first. Later, NaHTe solution was mixed with nitrogen-saturated 1.25×10^{-3} mol/L $CdCl_2$ aqueous solution with MPA as a stabilizing agent. The molar ratio of Cd^{2+}:

MPA:NaHTe$^-$ was fixed at 1 : 2.4 : 0.5. The resulting mixture was then subjected to refluxing to control the size of the CdTe nanocrystals. A luminescence quantum yield of 15 $\sim$ 25% was measured for the CdTe nanocrystals at room temperature by comparing with the fluorescence emission of Rhodamine 6G.[26] The CdTe QDs synthesized in this study can be stable at 4°C for 1 year. Stable water-compatible MPA-capped CdTe QDs with emission maximum at about 606 nm (red emitting, 3.9 nm) were used in the current experiments.

Covalent Coupling of Antigens to Fluorescent Microspheres

The size of microsphere (0.4 μm) used here can capture mass IgG biomolecules on the surface. This work chose a saturated quantity of IgG for the high-density capture surface. In order to covalently attach the carboxylic acid groups on the microspheres' surface to rabbit IgG molecules, 25 μL EDC (50 mg/mL) solution was added to 500 μL carboxylated microspheres in PBS buffer, followed by addition of 500 μL rabbit IgG (125 mg/L). After 5 min ultrasonic vibration, the reaction mixture was vortexed and incubated at room temperature in the dark for at least 2 hr. The nonspecific binding sites on the activated microspheres were blocked with 1-aminoethanol for the purpose of eliminating covalent binding of proteins. Dialysis can be used to remove small molecules from protein, but dialysis was also found to be problematic, with adsorption of microspheres on the dialysis films and unsuitabile diameter. Thus, centrifugation for microspheres has been commonly employed in the applications reported in the literature.[27] In this study, the unbound antigens were removed by centrifugation (10 min, 1200 × *g*). After that, the rabbit IgG–labeled fluorescent microspheres (IgGFM) were resuspended in PBS and stored in the dark at 2–8°C for further studies.

Formation of QD–Antibody Conjugates

The water-stable CdTe QDs with carboxylic acid terminal groups can link with biomolecules to form QD–bioconjugate, where conjugation is driven by electrostatic self-assembly. QD–goat-anti-rabbit IgG bioconjugates (anti-IgGQD) were formed by incubating 1 mL 2.5 mmol/L CdTe QDs in the PBS with 1 mL 30 μg/mL goat-anti-rabbit IgG for approximately 1 hr at room temperature. In this study, excess CdTe QDs was added to ensure that all goat-anti-rabbit IgG were labeled. After incubation, the mixture was centrifuged for 30 min at 5600 × *g* and the supernatant was removed to remove excess free CdTe QDs. Then the CdTe QD–labeled goat-anti-rabbit IgG was resuspended in 1 mL PBS and stored in the dark at 4°C for further studies.

The binding ratio of CdTe QDs with anti-IgG can be determined by the related UV-Vis absorption signals of the CdTe QDs and anti-IgG. At

this time, we are unable to determine the exact stoichiometry of the CdTe QD–goat-anti-rabbit IgG conjugate formed. A rough calculation of the average number of anti-IgG molecules conjugated to a single CdTe QD was estimated to be about 1–2.

Formation of Anti-IgGQD–IgGFM Immunocomplexes

Anti-IgGQD–IgGFM immunocomplexes were formed by incubating various concentrations (0.5 mg/L to 15.0 mg/L) of anti-IgGQD with 1.0 mg/L IgGFM in PBS for approximately 1 hr at room temperature. Then the reaction mixtures were centrifuged (10 min, 1200 × *g*), supernatant was discarded, and the immunocomplexes were washed with 1 mL PBS (2 mmol/L) followed by centrifugation and ultrasonic vibration before detection.

The anti-IgGQD, IgGFM, and the anti-IgGQD–IgGFM immunocomplexes can be stable at 4°C for 4 months.

Competitive Immunoassay

Competitive assay for the detection of no-labeled rabbit IgG through the inhibition of 1 mL 10.0 mg/L anti-IgGQD binding to 1 mL 1.0 mg/L IgGFM at various concentrations (5 mg/L to 200 mg/L) free rabbit IgG was adopted. For this purpose, the no-labeled rabbit IgG and anti-IgGQD were mixed for the primary capture step. After incubation for 1 hr with a gentle vortex, IgGFM was added and incubated for 1 hr with vortexing for the second competition step to form the competitive immunoassay complexes. Then the competition reaction mixture was centrifuged (10 min, 1200 × *g*). The supernatant was discarded and PBS was added as described above, and the reaction mixtures were ultrasonically vibrated before detection. A schematic diagram of the principles of the fluoroimmunoassays for detection of goat-anti-rabbit IgG and competitive assay for free rabbit IgG is shown in Fig. 2.

RESULTS AND DISCUSSION

Immunoreactions Between IgGFM and Anti-IgGQD

There are two luminescence peaks that can be observed (510 nm and 606 nm) after the immunoreaction between IgGFM (green emitting) and anti-IgGQD (red emitting), as shown in Fig. 3. The immunocomplex solutions contain the spectroscopic signature of both red CdTe QDs and green fluorescent microspheres, which confirms the presence of both the goat-anti-rabbit IgG and rabbit IgG. This result clearly shows that the anti-IgGQD can bind with IgGFM through immunoreaction.

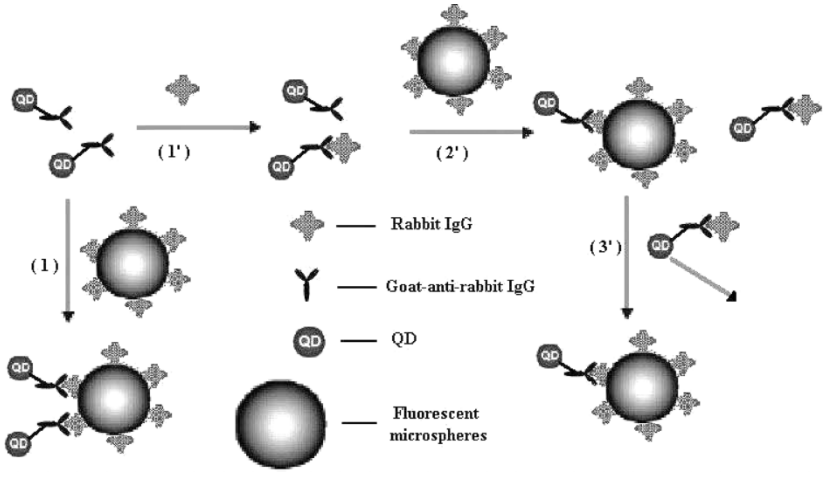


Figure 2. The scheme of the principles of the fluoroimmunoassays for detection of goat-anti-rabbit IgG and competitive assay for free rabbit IgG. As a first step, QD-labeled antibodies as analyte were bound to fluorescent microspheres coated with a layer of antigens (1). In the competitive assay, QD-labeled antibodies were mixed with free antigens in the solution (1′). Subsequently, the rabbit IgG–labeled fluorescent microspheres were added for the competition (2′). Finally, anti-IgGQD immobilized on the microspheres were separated and detected (3′). The ratios and the sizes of microspheres (0.4 μm) and QDs (5 nm) with biomolecules are not proportional in the illustration.

In this study, the effect of reaction time on the fluorescence emission spectra of the anti-IgGQD–IgGFM immunoreaction system was studied. For this purpose, the reaction time of anti-IgGQD and IgGFM was changed from 10 min to 10 hr. The results are shown in Fig. 3, in which the inset shows a graph of the reaction time versus fluorescence intensity at 606 nm. From Fig. 3, it can be seen that the fluorescence intensity at 606 nm increases rapidly in the first hour, while the fluorescence intensity at 510 nm keeps constant; the fluorescence intensity at 606 nm reaches a plateau when the reaction time exceeds 2 hr. In this study, a 2-hr reaction time was adopted.

Detection of Goat-Anti-Rabbit IgG

The change of fluorescence spectra of immunocomplex solutions with gradually increasing quantity of anti-IgGQD using a fixed amount of rabbit IgGFM was studied (see Fig. 4a). The fluorescence intensity at 606 nm increases with the increasing concentration of anti-IgGQD, while the fluorescence intensity at 510 nm keeps constant. Figure 4b displays the relationship between the fluorescence intensity at 606 nm and the concentration of anti-IgGQD. It can be seen that it was linear in the range from 0.5 to 10 mg/L,

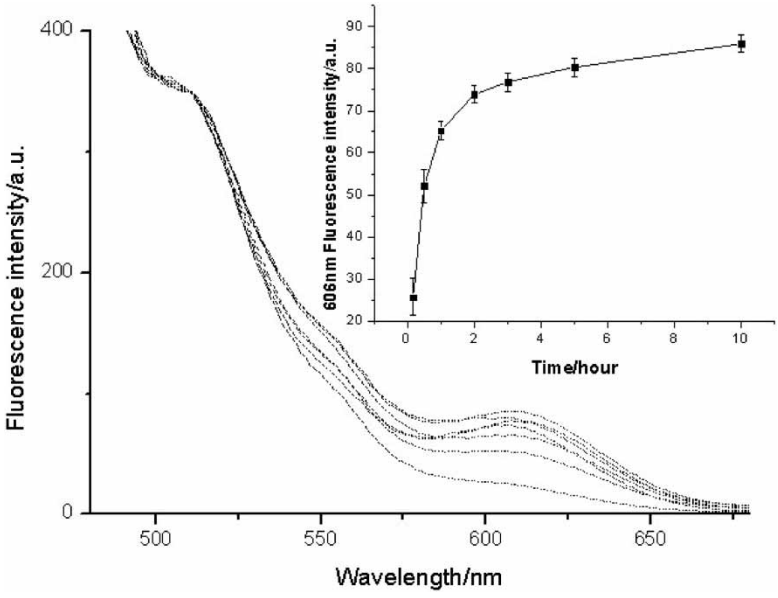


Figure 3. The effect of the different reaction time on the fluorescence emission spectra of the IgGFM–anti-IgGQD immunoreaction system: (a) 10 min; (b) 30 min; (c) 1 hr; (d) 2 hr; (e) 3 hr; (f) 5 hr; (g) 10 hr. The inset shows the relation curve between the reaction time and fluorescence intensity at 606 nm.

and the correlation coefficient reaches 0.999. After that, even higher concentrations of anti-IgGQD were added, and the fluorescence intensity at 606 nm increased slowly. The results suggest that the reaction between anti-IgGQD and IgGFM was saturated at a high concentration of antibodies for the 1.0 mg/mL IgGFM capture surface. Thus, the ratio of anti-IgG to IgGFM was estimated to be 10:1.

Compared with ELISA, the approach displayed a low sensitivity but a significantly shorter assay time, and the procedure is simple. Further optimizations, such as the use of systems with lower background signal or higher affinity, should allow the detection of antibodies with higher sensitivity. Importantly, the fluorescent microsphere combined with the conjugation of QDs with an antibody protein in microfluidic devices can be used for bioanalytical applications.

Fluorescence Micrograph of the Immunocomplexes of Anti-IgGQD–IgGFM

The immunocomplexes of 1.0 mg/L IgGFM with 1.5 mg/L anti IgGQD were flowed into the microfluidic chip. Figure 5 shows the scatter/fluorescence

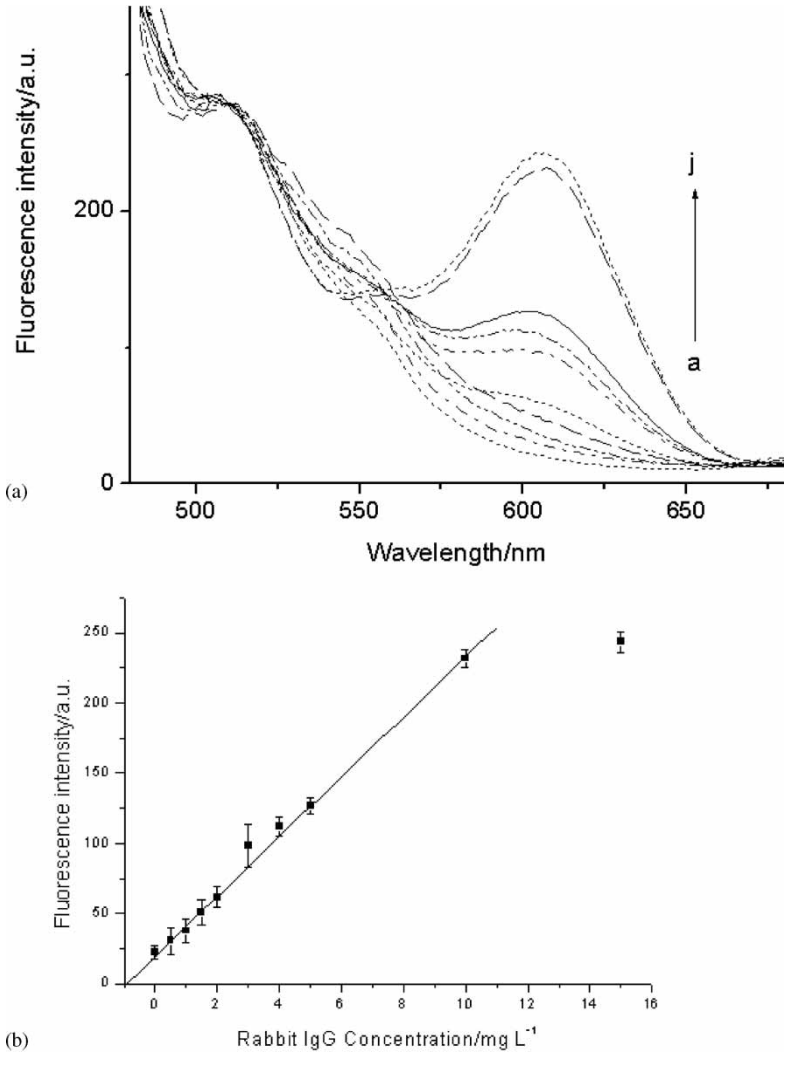


Figure 4. (a) Fluorescence emission spectra of 1.0 mg/L IgGFM solutions containing (a) 0 mg/L; (b) 0.5 mg/L; (c)1.0 mg/L; (d) 1.5 mg/L; (e) 2.0 mg/L; (f) 3.0 mg/L; (g) 4.0 mg/L; (h) 5.0 mg/L; (i) 10.0 mg/L; (j) 15.0 mg/L goat-anti-rabbit IgG labeled with CdTe QDs. An excitation wavelength of 465 nm was used for all samples. (b) The relation curve between the fluorescence intensity at 606 nm and the concentration of anti-IgGQD. An excitation wavelength of 465 nm was used for all samples.

coincidence data obtained from the immunocomplexes flowed through the channel of the microchip. Each peak in the scatter channel represents a microphere passing through the interrogation region. It can be seen that the fluorescent signals of IgGFM and anti-IgGQD can be detected in the 520-nm

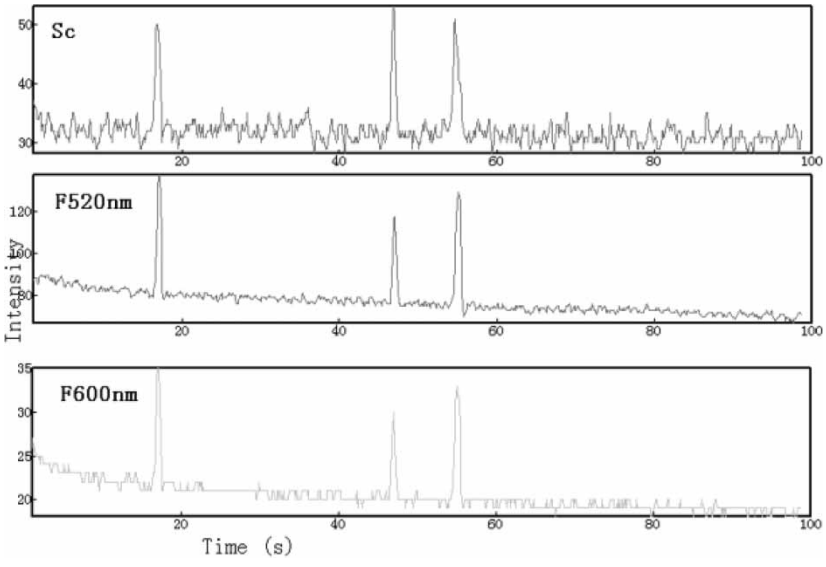


Figure 5. Light scattering (Sc) and fluorescence signals (520 nm and 600 nm) for 1.0 mg/L IgGFM conjugated with 1.5 mg/L anti-IgGQD on the microchip.

and 600-nm channels synchronously, which indicates that the conjugation of anti-IgGQD–IgGFM was formed via the biospecific binding between antigens and antibodies, and the conjugations of IgGFM and anti-IgGQD were also confirmed when the signals of QDs and fluorescent microsphere were detected synchronously. Each peak in Fig. 5 stands for one immuno-complex. The lowest and highest concentrations required for obtaining optimal separated individual fluorescence signal are 1.0 mg/L IgGFM bound with 1.5 mg/L anti-IgGQD and 1.0 mg/L IgGFM bound with 10 mg/L, respectively.

Nonspecific Binding of Fluorescent Microspheres–Rabbit IgG

In order to study the nonspecific binding of IgGFM, QD–goat-anti-mouse IgG conjugates were formed using the same method as QD–goat-anti-rabbit IgG (anti IgGQD). Then QD–goat-anti-mouse IgG and IgGFM were mixed according to the procedure for goat-anti-rabbit IgG–QD–IgGFM conjugates. Fluorescence spectra of the mixed solution, the supernatant, and resuspended deposit after centrifugation are shown, respectively, in Fig. 6. From Fig. 6, it can be seen that the luminescence peaks at 606 nm can be observed in the mixed solution and the supernatant after centrifugation, but the luminescence peak at 606 nm does not appear in the fluorescence spectrum of the resuspended deposit. For the positive sample (IgGFM conjugated with goat-anti rabbit IgG–QDs), the luminescence peak at 606 nm appears in both the fluorescence

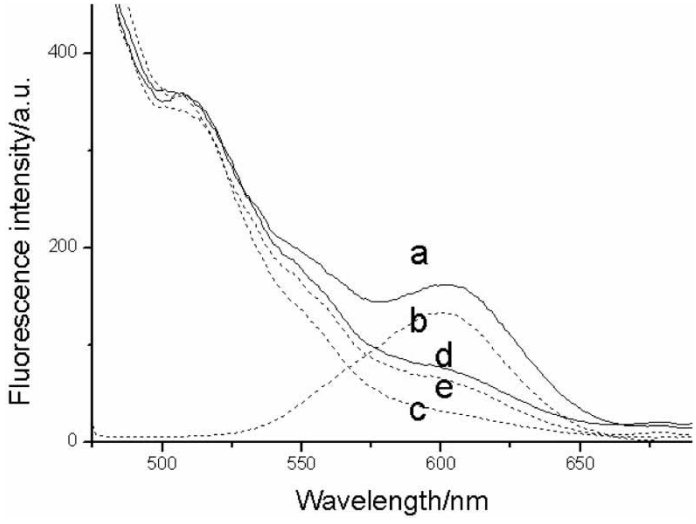


Figure 6. Fluorescence emission spectra obtained from solutions of 5.0 mg/L goat-anti-mouse IgG–labeled QDs mixed with 1.0 mg/L IgGFM before centrifugation (a), the supernatant after centrifugation (b), the resuspended deposit after centrifugation (c), the fluorescence emission spectra obtained from solutions containing 2.0 mg/L goat-anti-rabbit IgG–labeled QDs mixed with 1.0 mg/L IgGFM before centrifugation (d), and the resuspended deposit after centrifugation (e). Excitation wavelength is 465 nm.

spectra of the mixed solution and the resuspended deposit. This indicates that there is no immunoreaction between goat-anti-mouse IgG and rabbit IgG and the nonspecific binding is probably negligible in this study. On the other hand, it also confirmed the interaction between IgGFM and anti IgGQD.

Competitive Assay for the Detection of Rabbit IgG

A competitive assay was investigated through the inhibition of goat-anti-rabbit IgG–labeled QDs binding to IgGFM by free rabbit IgG. For this purpose, goat-anti-rabbit IgG–QD–IgGFM immunocomplexes were employed. The fluorescence intensity was measured with various concentrations of free rabbit IgG added to the anti-IgGQD–IgGFM conjugates. Figure 7 shows the results of this competitive assay. It can be seen that free rabbit IgG competed effectively with IgGFM for binding with goat-anti-rabbit IgG–labeled QDs, as indicated by the decrease of the fluorescence intensity at 606 nm with the increasing concentration of unlabeled rabbit IgG. The fluorescent signal almost reached background values at the higher unlabeled rabbit IgG concentration (i.e., $\sim$200 mg/L). This result indicates that the immunoreaction between IgGFM and goat-anti-rabbit IgG/QD occurred on the surface of fluorescent polystyrene microspheres.

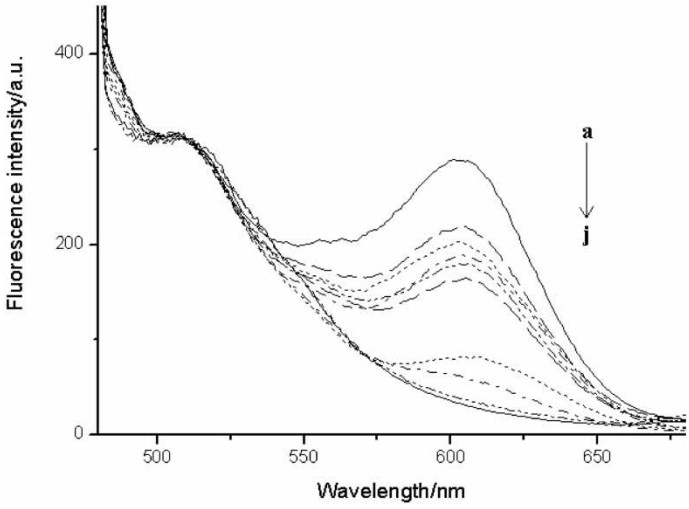


Figure 7. The fluorescence emission spectra of the IgGFM–anti-IgGQD immunoreaction system with a series of different concentrations of unlabeled rabbit IgG added: (a) 0 mg/L; (b) 5 mg/L; (c) 10 mg/L; (d) 15 mg/L; (e) 20 mg/L; (f) 30 mg/L; (g) 50 mg/L; (h) 100 mg/L; (i) 150 mg/L; (j) 200 mg/L.

CONCLUSIONS

In the current study, we have reported a novel strategy for fluoroimmunoassay with CdTe QDs, which were prepared in aqueous solution to label biological molecules without any postpreparative treatment. In comparison with other fluorescent microsphere–based immunoassays, the use of QDs provides a new approach to make probes that fluoresce at different wavelengths with a single excitation wavelength. Using this nature, a simple and convenient microfluidic device was used to monitor the anti-IgGQD–IgGFM immunocomplexes in this study. Based on some of the unique properties of nanocrystals and fluorescent microspheres, both CdTe QD–labeled antibodies and microsphere-labeled antigens were used to form a typical immunoreaction system for the detection of goat-anti-rabbit IgG effectively. It was shown that by use of a microfluidic chip device and fluorescence micrographs, the change in the fluorescence spectra was the result of the formation of immunocomplexes between anti-IgGQD and IgGFM. We consider that this approach will be particularly useful in the development of sphere-based fluoroimmunoassays using microfluidic devices.

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