**Polyelectrolyte-protected Dual-color-quantum-dot Assembled Silica Nanoparticles and Their Application in Simultaneous Fluorescence Determination of e Antigen and Surface Antigen of Hepatitis B**

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Cationic poly-diallyldimethylammonium (PDADMAC), green CdTe quantum dots (QDs) or red CdS coated CdTe QDs, and anionic polyacrylic acid (PAA) were respectively assembled on the nano-carrier SiO₂ to prepare green fluorescence composite nanoparticles (GF-QDs) and red ones (RF-QDs) with the structure SiO₂/PDADMAC/QD/PDADMAC/PAA. The sandwich structure “PDADMAC/QD/PDADMAC” on the nano-carrier not only realized the protection to fluorescence of QDs but also avoided the fluorescence shielding of silica shell for the assembled QDs. In 7 days, the diluent solutions of GF-QD and RF-QD all have a very stable fluorescence. On the contrary, the fluorescence of diluent solutions of red and green QDs reduced by 75.99 and 94.35%, respectively. Indeed, they have not fluorescent shielding and have a very slight fluorescent enhancement. Based on GF-QD and RF-QD, the simultaneous determination of Hepatitis B e antigen and surface antigen has been established. Their determination in buffer and plasma all showed good precision and accuracy.

**Keywords** Quantum dot, silica nanoparticle, self-assembly, fluorescent stability, fluorescence shielding, immuno-determination

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**Introduction**

It is necessary to detect several biomarkers for clinical diagnosis. Immuno-reaction-based detection for multiple biomarkers, especially simultaneous detection of dual-markers, has received extensive attention.1-6 However, dual-tag-based simultaneous detection usually has one main problem, that is, how to get two tags without mutual signal interference.2-5 Because quantum dots (QDs) have a narrower fluorescent spectrum with an adjustable emission wavelength, it could be used as a dual-tag.2-12 There are usually two kinds of QDs, that is, oil-soluble3,4 and water-soluble ones. For water-soluble QD, its surface ligands, such as mercaptopropionic acid, could be directly used to label antibodies.5 However, because of their weak coordinate bonds, these surface ligands are dropped easily down from QDs after bio-labeling, and then the labeling QDs are usually aggregated and their fluorescence is reduced.5,13

The indirect labeling of water-soluble QD has been a focus.5,14-21 SiO₂ nanoparticles are usually used as carriers of QDs due to good hydrophilicity and easy surface functionalization. Meanwhile, functionalized activity groups could also be used to label bio-molecules.5,14,15,20,21 However, after QDs were embedded into SiO₂ nanoparticles, their fluorescence would be shielded by the outer silica shell.16-19 To solve this problem, QDs were assembled on the surface of each silica nanoparticle by chemical bonds between the functionalized activity group of the silica nanoparticle and the surface ligand of QD.5,14,16,20,21 In fact, a fluorescent reduction of QD is also produced because their cross-linking assembly has been based on the chemical reaction of the surface ligand, which also results in surface ligand dropping.16,17,22 This has indicated that the surface ligands of water-soluble QD could not be used to chemically connect nano-carrier or (and) bio-molecule for sensitive bioanalysis.

In the present work, SiO₂ nanoparticles were also used as nano-carriers. Cationic polyelectrolyte PDADMAC (poly-diallyldimethylammonium chloride), water-soluble QDs with negative charge (green CdTe QDs or red CdS coated CdTe QDs (CdTe@CdS QDs)), and anionic polyelectrolyte PAA (polyacrylic acid) were assembled layer-by-layer on the surface of each silica nanoparticle by electrostatic interaction, to prepare composite nanoparticles having green fluorescence (GF-QDs) and also red (RF-QDs). They all have the same assembled structure, SiO₂/PDADMAC/QD/PDADMAC/PAA. Since an electrostatic assembly occurred between the nanoparticle and the polymer, or one polymer and another polymer, their electrostatic interaction was very strong, and thus the as-prepared fluorescent nanoparticles have a stable structure.
Meanwhile, the surface ligands of QDs were also protected by a sandwich structure, "PDADMAC/QD/PDADMAC", on the surface of the SiO2 nanoparticle. Certainly, there was also no fluorescent shielding for the assembled QDs. Based on the surface carboxyl of the assembled fluorescent nano-particle, antibody labeling was easily carried out. Using the green GF-QDs and red RF-QDs as a dual-tag for two kinds of assaying-antibodies, we established the simultaneous fluorescence determination of Hepatitis B e antigen (HBeAg) and surface antigen (HBsAg) with magnetic nanoparticles as the carrier of capturing-antibodies (Fig. 1), and also satisfactory selectivity, precision and accuracy of this simultaneous fluorescence determination were verified.

**Experimental**

**Materials and instrument**

Poly-diallyldimethylammonium chloride ($M_w = 10000$, 35.0 wt% in water) and polyacrylic acid ($M_w = 10000$, 35.0 wt% in water) were purchased from Aldrich. HBeAg, HBeAb, mouse anti-HBeAg antibody (M-HBeAb) and mouse anti-HBsAg antibody (M-HBsAb) were all purchased from Shanghai GenoMintel Bioscience & Technology Development Co., Ltd. Rabbit anti-HBeAg antibody (R-HBeAb) and rabbit anti-HBsAg antibody (R-HBsAb) were all purchased from Suzhou Pulong Bio. Co., Ltd. Other chemicals were all of analytical grade or better. Milli-Q water at 18.2 MU cm was used throughout. Plasma with negative hepatitis B was obtained from Suzhou Blood Center, China.

LS55 fluorescence spectrophotometer (Perkin Elmer, USA) was used to measure the fluorescence spectrum. A TecnaiG220 transmission electron microscope (TEM) (FEI, USA) was used to characterize the morphology of nanoparticles. A NICOMPTM 380ZLS Zeta analyzer (PSS, America) was used to determine the surface potential of nanoparticles.

**Preparation of fluorescent composite nanoparticles**

Firstly, SiO2 nanoparticles were prepared using the Stöber approach.[16] Green CdTe QDs were prepared using the water-phase approach previously reported.[16] On the basis of the as-prepared green CdTe QDs, red CdS-coated CdTe QDs (CdTe@CdS QDs) were prepared through the coating of CdS. After a 1.0 mL SiO2 nanoparticle solution (18.0 mg mL$^{-1}$) and 1.6 mL PDADMAC solution (10.0 mg mL$^{-1}$) were mixed in an ampoule and stirred for 30.0 min, excess PDADMAC was removed by high-speed centrifugation at 12000 rpm for 20 min. The precipitation was re-dispersed into 1.0 mL of water. Then, 24.5 mL of CdTe QD or 7.0 mL CdTe@CdS QD solution was added into the above-mentioned nanoparticle solution. After being stirred for 30.0 min, excess QDs were removed by high-speed centrifugation. The precipitation was re-dispersed in 1.0 mL of water. We then successively repeated the above steps with the 1.0 mL PDADMAC (10.0 mg mL$^{-1}$) and 500.0 μL PAA (10.0 mg mL$^{-1}$), respectively. Green fluorescent composite nanoparticles GF-QDs and the red RF-QDs with the structure SiO2/PDADMAC/QD/PDADMAC/PAA were prepared.

**Labeling of assaying-antibodies and immobilizing of capturing-antibodies**

For the immune sandwich analysis of HBeAg and HBsAg, the prepared green and red nanoparticles were used to label the assaying-antibodies for the simultaneous determination of HBeAg and HBsAg, respectively. Then a 100.0-μL of the GF-QD solution (3.0 mg mL$^{-1}$) was activated by 100.0 μL EDC (1.0 mg mL$^{-1}$) and 100.0 μL NHS (0.6 mg mL$^{-1}$) in a 1.5-mL EP tube at 37°C for 60 min. Excess EDC and NHS were removed by high-speed centrifugation at 12000 rpm for 20 min, and the precipitation was washed 3 times with water. Subsequently, a 100.0-μL R-HBeAb solution (20.0 μg mL$^{-1}$) was added and incubated at 37°C for 60 min. Then a 100.0-μL 1% BSA was added to block any remaining active sites for 60 min. The supernatant was removed by high speed centrifugation and re-dispersed the precipitation into 100.0 μL of Tris-HCl buffer (pH 7.0). The assaying-antibody, R-HBeAb, labeled by GF-QD (R-HBeAb-GF-QD) was obtained. Another assaying-antibody R-HBsAb was labeled by the red RF-QD according to the aforementioned similar approach, and then the labeled assaying-antibody R-HBeAb-GF-QD was also obtained.

To immobilize two capturing-antibodies, the carboxyl-modified Fe3O4 magnetic nanoparticle[19] was used to assemble PDADMAC and PAA sequentially, that is, to prepare a new carboxyl-modified Fe3O4 nanoparticle with the structure Fe3O4/PDADMAC/PAA. This nanoparticle has an average size of about 20 nm and a Zeta potential of ~26.7 (Figs. S1 and S2 in Supporting Information). After that, the capturing-antibodies M-HBeAb and M-HBsAb were chemically connected to obtain the immobilized capturing-antibodies Fe3O4-M-HBeAb and Fe3O4-M-HBsAb, respectively.

**Determinations of HBeAg and HBsAg**

First, 100.0 μL capturing-antibody Fe3O4-M-HBeAb and...
characterization of fluorescent composite nanoparticles

Results and Discussion

Characterization of fluorescent composite nanoparticles

Characterization of the used QDs can be seen in Figs. S3 – S5 in Supporting Information. It could be found that these QDs had a diameter of about 4 nm with a uniform distribution, and no aggregation; further, they had a typical fluorescence emission spectrum of a quantum dot at 540.0 and 625.0 nm for the green and red QDs, respectively. Images of the transmission electron microscopy (TEM) of fluorescent composite nanoparticles and their correlative ones are given in Fig. 2. It could be found that a large number of the QDs were assembled on the surface of the SiO₂ nanoparticles, and that they looked like granular spots with a uniform distribution, and no aggregation. Meanwhile, the shapes of these fluorescent composite nanoparticles were still spherical, which was the same as their carrier SiO₂ nanoparticles.

To characterize the layer-by-layer assembly of the target nanoparticles, the Zeta potential of all nanoparticles in each step of the preparing process was also measured. After PDADMAC, QD, PDADMAC and PAA were sequentially assembled on the SiO₂ carrier by electrostatic interaction, the alternate positive and negative surface charge of the nanoparticles were determined, that is, from –21.98 of SiO₂ carrier to +20.4, –19.57, +27.14, and –33.54 for RF-QD, and to +20.4, –21.4, +18.2 and –21.65 for GF-QD, which was consistent with the potential of each layer, itself. It was shown that the as-prepared fluorescent composite nanoparticles GF-QD and RF-QD all have a same assembling structure SiO₂/PDADMAC/QD/PDADMAC/PAA.

Besides, the absolute potential of the nanoparticles in each step was all over 20 mV, indicating that these nanoparticles had good dispersibility in the aqueous medium. In fact, the good dispersibility can be observed in Fig. 2.

Fluorescent spectra and stability

Since the quantum dots were assembled on the surface of the carrier SiO₂ nanoparticle, there was not fluorescent shielding from the outer shell of the carrier. Indeed, the assembled QDs had a very slight fluorescent enhancement (Fig. 3). At the same time, we also found spectral blue shifts for two kinds of the fluorescent composite nanoparticles, GF-QD (2 nm) and RF-QD (4.5 nm), after the quantum dots were assembled. This was coincident with the spectral blue shift of the sandwich structure in the solution without any nano-carrier.22 This indicated that the quantum dots in the prepared composite nanoparticles had been protected by a sandwich structure “PDADMAC/QD/PDADMAC” on the surface of the SiO₂ nanoparticle. In fact, when the preparation solution of QD was diluted, or the surface ligands of the prepared QDs were used to label biomolecule, the surface ligands of QDs easily dropped, and then their fluorescence obviously weakened with their storage time.22 However, this protective structure could not only prevent those ligands dropping, but also stopped those ligands away from the surface of QD. This was because of the former reason that the fluorescent stability of the composite nanoparticles could be strengthened. Certainly, owing to the latter reason, the coordination action of those ligands may also be slightly strengthened, and then this would result in a slight fluorescent enhancement. To illustrate its protection, we investigated the fluorescent stability of the composite nanoparticles with their storage time (Fig. 4). In 7 days, the diluent solution of the GF-QD or RF-QD all has a very stable fluorescence, seen in lines
A and C in Fig. 4. However, the diluent solutions of their corresponding quantum dots have very poor stability (lines B and D in Fig. 4). The fluorescent intensity of the red QD/CdS was reduced by 75.99%, and the green QD was up to 94.35%. For such weak fluorescence, they could almost not be used to label bio-molecule after 7 days. In summary, the a-prepared fluorescent composite nanoparticles have a stable structure and keep the strong fluorescence of quantum dots without fluorescent shielding. Certainly, the two kinds of nanoparticles had still the character of the fluorescence emission spectra of a typical quantum dots, that is, the narrow spectral band, and rich carboxyl on the surface was also beneficial to label biomolecules.

Cross immuno-reaction of simultaneous determination

In the simultaneous detection of double antigens HBeAg and HBsAg, there are not only the labeled assaying-antibodies, R-HBeAb-GF-QD and R-HBsAb-RF-QD, but also immobilized capturing-antibodies, Fe₃O₄-M-HBeAb and Fe₃O₄-M-HBsAb. The possible cross immunoreaction among these antibodies would be a major factor that would interfere with the precision and reliability of simultaneous determination, so it is necessary to consider the potential cross immuno-reaction of these antibodies here.

The fluorescent intensities of the two labeled assaying-antibodies, R-HBeAb-GF-QD and R-HBsAb-RF-QD at the peak wavelength 540 nm and R-HBsAb-RF-QD at 625 nm, was used as the control of cross reaction (line a in Fig. 5). When only the capturing-antibody Fe₃O₄-M-HBeAb was mixed and incubated with the two labeled assaying-antibodies, after magnetic separation, the remaining fluorescence of the assaying-antibodies R-HBeAb-GF-QD (at 540 nm of line b in Fig. 5) and R-HBsAb-RF-QD (at 625 nm of line b in Fig. 5) did not decrease significantly. This suggested that there was no cross immuno-reaction between the capturing-antibody M-HBeAb and the assaying-antibody, R-HBeAb or R-HBsAb. For another capturing-antibody Fe₃O₄-M-HBsAb, the same conclusion was also obtained (line c in Fig. 5).

When the two capturing-antibodies, Fe₃O₄-M-HBeAb and Fe₃O₄-M-HBsAb, were simultaneously added and incubated with the two labeled assaying-antibodies, the potential cooperative cross immune-reaction was studied (line d in Fig. 5). There was no significant decrease of their remaining fluorescence yet, no matter that of the labeled assaying-antibody, R-HBeAb-GF-QD or R-HBsAb-RF-QD. Therefore, there was no cooperative cross immuno-reaction in the simultaneous determination.

Selectivity of simultaneous determination

Though the above investigation has shown that the used four capturing-antibodies and assaying-antibodies have not cross immuno-reaction, the determining selectivity of one antigen may still be influenced by another one in the simultaneous determination system. When the selectivity was poor, the accuracy of the analytical result would be decreased. Therefore, the selectivity of the determination system in this paper has been studied (Fig. 6, and Figs. S6 and S7 in Supporting Information).

At first, the selectivity for the determining system of a single antigen was investigated (Figs. S6 and S7). It could be found that the determining system of one antigen has not been influenced by another determined antigen. After that, the simultaneously determining system of double antigens was also
investigated (Fig. 6). The system of simultaneous determination without any determined antigen, i.e., the mixture solution of the two capturing-antibodies and two assaying-antibodies, was used as a blank control (line a in Fig. 6). When the antigen HBeAg was added, it could be found according to the spectrum of the supernatant after incubation and magnetic separation that the green fluorescence of HBeAg’s corresponding assaying-antibody R-HBeAb-GF-QD decreased significantly, and the red fluorescence of another assaying-antibody R-HBsAb-RF-QD had no significant change (line b in Fig. 6). It was shown that the immune complex of the determined antigen HBeAg, i.e., Fe3O4-M-HBeAb···HBeAg···R-HBeAb-GF-QD, had been correctly formed, and the assaying-antibody and capturing-antibody of another determined antigen HBsAg all had no interference. For the determined antigen HBsAg, a similar result could also be found from line c in Fig. 6; that is, the assaying-antibody and capturing-antibody of another determined antigen HBsAg have no interference for the determination of HBsAg. When the two antigens HBeAg and HBsAg were all added into this simultaneous determining system, the two immune complexes Fe3O4-M-HBeAb···HBeAg···R-HBeAb-GF-QD and Fe3O4-M-HBsAb···HBsAg···R-HBsAb-RF-QD could all be formed correctly (line d in Fig. 6).

Simultaneous determination of HBeAg and HBsAg and their linear ranges

Since there was a small overlap of the fluorescense peak between R-HBeAb-GF-QD and R-HBsAb-RF-QD, Fig. 6, their semi-peak areas were used to characterize the fluorescence intensities. The left semi-peak area (Sg1/2) of the green R-HBeAb-GF-QD and the right semi-peak area (Sr1/2) of the red R-HBsAb-RF-QD were selected for the determination of HBeAg and HBsAg, respectively.

According to the above method, the standard samples of the analyzed HBeAg with concentrations of 20.0, 40.0, 60.0, 80.0 and 100.0 ng mL⁻¹ were prepared. After incubation and magnetic separation, the fluorescence spectra of their corresponding supernatants were measured (Fig. 7A). The fluorescence spectrum of the blank control without the determined antigen HBeAg was also obtained (Fig. 7A). Compared with the semi-peak area of blank control, the decreased semi-peak area of the analyzed samples were positively related to the concentration of the determined antigen HBeAg, that is, ΔSg1/2 = 391.11 + 104.08CBe. Here CBe denotes the concentration of the determined antigen HBeAg in the analyzed sample, and ΔSg1/2 = Sg1/2(0) – Sg1/2(CBe), where Sg1/2(0) and Sg1/2(CBe) denotes the semi-peak areas of the green fluorescence spectra for the blank control and the analyzed sample.

From the correlation coefficient (r = 0.9977) and the regression curve, it could be found that the determination of HBeAg had good linearity in the concentration range of 20.0 - 100.0 ng mL⁻¹.

For another component HBsAg of the simultaneous determination, its linear range has also been investigated with a similar method to that above (Fig. 7B). The similar result was obtained; that is, the linear equation ΔSr1/2 = 151.73 + 126.92Cb, where Cb denotes the concentration of HBsAg in the analyzed sample. ΔSr1/2 = Sr1/2(0) – Sr1/2(Cb), where Sr1/2(0) and Sr1/2(Cb) denotes the semi-peak areas of the red fluorescence spectra for the blank control and analyzed sample, respectively. Similarly, the determination of HBsAg had also a good linearity in the concentration range of 20.0 - 100.0 ng mL⁻¹, and its correlation coefficient was 0.9954.

Methodological accuracy and determining interference

Because of the infectivity of hepatitis B, its real samples with positive hepatitis B could not be obtained and detected in a usual laboratory, the methodological accuracy and determining interference of the established approach were studied by the spiked samples in the buffer and plasmas, respectively.

At first, the accuracy of this established approach was evaluated with the spiked recoveries of three groups of the analyzed samples with high, middle and low concentration of the determined antigens. According to the above method, three groups of measuring samples were prepared in Tris–HCl buffer (pH 7.0) by using the analyzed antigen with concentrations of 30.0, 60.0 and 90.0 ng mL⁻¹, and their fluorescence spectra were measured. The concentrations of the determined antigens were calculated according to the aforementioned regression equations, and the average recovery of HBeAg spiked in Tris-HCl buffer (pH 7.0) was in the range of 98.1 - 105.9% with a range 0.9 - 2.8% of the relative standard deviation (RSD), and HBsAg in the range of 104.0 - 106.7% with the RSDs 1.6 - 2.2%. Certainly, such recoveries were already satisfactory for an approach of biological analysis, especially for simultaneous determination. Thus, the above results indicated that this established approach has good accuracy for the simultaneous determination of HBeAg and HBsAg.
Since the analyzed HBeAg and HBsAg were usually from plasma, those complex components in plasma may disturb their determination. In this paper, the effect of plasma on this simultaneous determination system was studied by the spiked samples in plasma. The antigens HBeAg and HBsAg were added into the blank plasma, which was a mixture of blank plasma with negative hepatitis B from different people, to prepare the measuring samples. The blank plasma was used as the control sample. Two groups of the measuring samples, i.e. the middle concentration group (all 60 ng mL⁻¹ for HBeAg and HBsAg) and another high-low concentration group (the low concentration 30 ng mL⁻¹ for HBeAg and the high concentration 90 ng mL⁻¹ for HBsAg). Each group still had three parallel samples. For the middle concentration group, the average recovery was 99.0 and 94.0% with RSD 5.7 and 7.3% for HBeAg and HBsAg, respectively. For the high-low concentration group, the average recovery of the low concentration HBeAg was 100.7% with the RSD 4.9%, and the high concentration HBsAg was 104.8% with the RSD 0.6%. It could be found that no matter whether in high, middle or low concentrations for the two determined antigens, their simultaneous determination in plasma both had satisfactory precise and accuracy. This suggested that the rapid and simultaneous determination of HBeAg and HBsAg could be carried out in a plasma sample.

Conclusions

Based on electrostatic layer-by-layer self-assembly of SiO₂ nanoparticles, PDADMAC, green CdTe QDs or red CdTe@CdS QDs, PDADMAC and PAA, the fluorescent composite nanoparticles GF-QD and RF-QD with the assembling structure SiO₂/PDADMAC/QD/PDADMAC/PAA have been prepared. Since the assembled QDs have a sandwich structure, “PDADMAC/QD/PDADMAC”, on the surface of the SiO₂ nano-carrier, the as-prepared composite nanoparticles not only realized protection to the fluorescence of QDs, but also avoided the fluorescence shielding of silica shell for the assembled QDs. In 7 days, the diluent solution of GF-QD and RF-QD all have a very stable fluorescence. On the contrary, the diluent solutions of their corresponding quantum dots were poor, and their fluorescent intensity of the red QD/CdS was reduced by 75.99%, and the green QD was up to 94.35%. Meanwhile, for the assembled QDs, there was no fluorescent shielding from the nano-carrier, and indeed they have a very slight fluorescent enhancement. Based on the green GF-QD and red RF-QD as two tags of the determination of HBeAg and HBsAg, respectively, their simultaneous-determination approach has been established. The simultaneous determination of the two antigens in Tris-HCl buffer and plasma all showed good precision and accuracy.

Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

References