Enhancement of sensitivity and specificity of the fluoroimmunoassay of Hepatitis B virus surface antigen through “flexible” coupling between quantum dots and antibody

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A B S T R A C T

Quantum dots (QDs) are widely used in the immune detection. Yet, the sensitivity and specificity of the immune detection are not satisfactory because the binding sites of QDs onto antibody (Ab) are often arbitrary and the influence of the large surface electronic potential energy of QDs on the directly conjugated Ab is nonnegligible. In this work, we provide a “flexible” coupling method, in which protein G (PG) is selected as the flexible bridge between the QDs and the Hepatitis B virus surface antibody (HBsAb), to improve the sensitivity and specificity of the fluoroimmunoassay compared to the directly covalent conjugation. Successful coupling of the HBsAb to our highly luminescent CdTe/CdS core/shell QDs is proven with Gel electrophoresis and atomic force microscopy (AFM). The assay results, based on the microelisa well plate as matrix to immobilize the sandwich structure, show that both sensitivity and specificity can be improved greatly through the flexible coupled QDs–PG–Ab conjugates.

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1. Introduction

Quantum dots (QDs) hold immense promise for optoelectronical and biological/biomedical applications due to their unique optical properties, such as size-tunable photoluminescence (PL), high PL quantum yields (QY), narrow emission line width, broad absorption profile, superior photostability, and flexible solution-processing [1–3]. Compared to organic fluorophores, the QDs also have high resistance to chemical degradation and photodegradation [4]. The number of publications on applications of QDs in bio-labeling and bio-detection has increased enormously during the recent years [3,5–12]. Attempt of in vivo applications, e.g., cell-imaging with the glutathione capped CdTe QDs has been reported [7]. In the mean time, focus has been given on in vitro applications based on the toxicity concern of the QDs. Mattoussi’s group reported the detection of pathogens by using the conjugates of dihydrolipoic acid capped CdSe/ZnS QDs and the recombinant maltose binding protein [8,9]. Peng et al. improved the sensitivity of pathogens detection to be as low as 5 ng/ml via CdSe/ZnS dendron QDs and a porous membrane immunofilter [10]. In spite of these efforts, the large surface electronic potential energy and the resulting influence of the QDs on the antibody (Ab) are still large, which will ultimately reduce the immunoreactivity and increase the nonspecific binding of the labeled antibody. This disadvantage will be reduced by applying “flexible” coupling where a proper soft bridge is built up providing a single coupled site to the Ab and the single site linkage can minimize the orientation alteration of the Ab, thus maintaining its native immunoreactivity.

The biotin–avidin system (BAS) has been widely applied in the bio-conjugation of QDs and proteins as a “bridge” [4,8]. However, this requires the antibody (or antigen) to be conjugated to biotin molecules, the non-epitaxial conjugation will finally sacrifice the sensitivity and specificity of the bio-detection. It is well known that the Fab fragments of the antibody contain the antigen binding regions. And protein G (PG) is an immunoglobulin-binding protein expressed in group C and G streptococcal bacteria. It is a cell surface protein that has found application in purifying or coupling antibodies because the β2 domain of PG can bind strongly with the Fc fragment of the antibody [9]. The fluorescent labels can thus be connected to the antibodies through protein G – a “flexible bridge” – without occupying the Fab domain and influencing the bio-specificity of the Ab. Early in 2002, Mattoussi et al. reported the detection of explosive 2,4,6-trinitrotoluene with conjugates of...
QDs and a specific antibody using PG as a bridge [13], however, they did not give a systematical discussion and manifestation on the high-specificity of the fluorimunosassay. In this work, flexible conjugation is realized by covalently coupling carboxylic highly luminescent aqueous CdTe/CdS core/shell QDs with PG to form a QDs–PG complex which is then bound to the antibodies. The effect on the specificity and sensitivity of detection based on the sandwich structure is studied with choosing microelisa well plate as the matrix for antibody immobilization. As far as virus is concerned we have chosen Hepatitis B virus (HBV)—known to be one of the leading causes of death worldwide [14]. In our experiments, Hepatitis B surface antigen (HBsAg) are detected with much lower detect limitation and non-specificity when the flexible coupling is realized of the HBsAb with highly luminescent CdTe/CdS QDs.

2. Experimental

2.1. Materials

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (99%), sulfo-N-hydroxysuccinimide (sulfo-NHS) (98.5%), aminopropyltriethoxysilane (APTES) (98%) and glutaraldehyde (Glu) were purchased from Aldrich. Protein G, HBsAb, HBsAg, Hepatitis B surface antigen (HBsAg) were purchased from Beijing DingGuo Biotechnology co. Ltd. All the reagents were used without further purification. Deionized water was purified through Milli-Q water purification system and the resistivity was 18.2 MΩ cm.

2.2. Apparatus

Ultraviolet–visible (UV–vis) absorption and fluorescence spectra were measured at room temperature (RT) by a UV-3101 spectrophotometer and a Hitachi F-4500 fluorescence spectrofluorimeter, respectively. Unless claimed otherwise, the excitation wavelength was set to 350 nm and the emission and excitation splits are 5 nm. The QY of nanocrystal solutions was calculated based on the wavelength was set to 350 nm and the excitation and emission spectra were measured at room temperature (RT) by a UV-3101 spectrophotometer and a Hitachi F-4500 fluorescence spectrofluorimeter. The samples were prepared by the 1:200 dilution of a stock solution.

2.3. Synthesis of highly luminescent CdTe/CdS core/shell QDs

CdTe/CdS core/shell QDs were prepared according to our previous publication [16]. The CdTe QDs were capped with 2 monolayers (ML) of CdS shell with a 530 nm emission and QY of 40%. The average size of the aqueous core–shell QDs was calculated to be only about 3 nm, which successfully discards the drawback of the large size (12–20 nm) water-soluble QDs after the multilayer coating, e.g., phospholipids [17], silica [2,18], or polymer [19,20], etc. This is because that the small dimension would dramatically improve the biological labeling and detection efficiency.

2.4. Conjugation of antibody onto CdTe/CdS QDs

The HBsAb was conjugated to the CdTe/CdS QDs classically by using EDC and sulfo-NHS (S-NHS) as crossing-linking reagent [10]. The schematic route of the conjugation is shown in Scheme 1. Firstly, 0.1 ml of QDs (60 μM) was mixed with 10 μl of EDC and sulfo-NHS in phosphate buffer saline (PBS, pH 7.4) with the molar ratio of 1:100:200. After 30 min of magnetic stirring, 3 nmol HBsAb was added into the reacted bottle and stirred at room temperature for another 2 h. In this way, the amide linkage can be formed through the amino of the Ab and the active carboxyl of QDs. In order to remove the excess small molecules, e.g., EDC and sulfo-NHS, the resulting samples were centrifuged in Microcon Centrifugal Filter Devices (50,000 Nominal Molecule Weight Limit). Afterwards, the product was purified by sephadex-150 filtration column chromatography. The conjugation of Ab and QDs was confirmed by the agarose gel (0.8%) electrophoresis technique.

2.5. Flexible conjugation of antibodies onto CdTe/CdS QDs

The HBsAb was conjugated with QDs flexibly by using protein G as a bridge. Firstly, the PG was covalently linked with QDs by an EDC-mediated coupling reaction as described above. Then the complex was purified by centrifugation in Microcon Centrifugal Filter Devices (50,000 Nominal Molecule Weight Limit). Afterwards, the HBsAb was added into the QDs–PG PBS solutions with equal molar ratio under stirring for 2 h. Because the β2 domain of protein G can bind with the Fc domain of the Ab specially, the Ab can be oriented as shown in Scheme 2. The product was then purified by sephadex-150 filtration column chromatography to separate the by-products and the excessive QDs. The conjugation of Ab and QDs was also confirmed by the agarose gel (0.8%) electrophoresis technique.

2.6. Fluorescent immune detection

As depicted in Scheme 3, firstly, 200 μl designed amount of monoclonal HBsAb was planted into the transparent microelisa well plate and incubated at 4 °C for about 16 h. Then, the solution was discarded away and 200 μl 5% (w/v) nonfat powdered milk in PBS was added into the well to block the plates. After 2 h of blocking time at RT, the nonfat dried milk solutions were also discarded and the wells were rinsed with PBS for 3 times. Afterwards, 200 μl series of
dilutions of HBsAg solutions were dropped into the well for about two hours' specific adsorption reaction and then the solutions were removed again and rinsed with PBS for 3 times. Control well was always included in which no HBsAg was added to capture the Ab adsorbed on the walls of the wells. At last, the designed concentration of QDs labeled HBsAb was added into the well and reacted specifically with HBsAg for 2 h. The solutions were taken out for additional PL measurement to obtain the detection curves.

2.7. AFM measurement

The atomic force microscopy (AFM) (Veeco Instrument, DI-3100) was measured via immobilizing the QDs labeled Ab covalently onto the silicon slice to evaluate the flexible coupling. Tapping mode silicon probe with spring constants of $\sim 180$ N/m and resonance frequencies of $\sim 1$ kHz was used. Prior to silanization, the silicon slice surface was pre-cleaned by treatment in HCl:CH$_3$OH = 1:1 solution, followed by strong vitriol for 30 min and being boiled for 10 min, then extensive rinsing with deionized water, and were stored in 25% ethanol. Chemically cleaned silicon slice surface was reacted with an acetone solution of APTES for 90 min at RT, followed by rinsing several times with acetone, and dried with nitrogen flowing and baked for 2 h at 120 °C in a vacuum oven. Finally, the APTES-treated silicon slice was immersed in the 10% solution of glutaraldehyde in PBS (pH 7.4) for 2 h, followed by rinsing with PBS (pH 7.4). QDs labeled Ab was immobilized on the APTES-Glu surface by incubation at 4 °C with an aqueous solution overnight. The QDs labeled Ab was covalently immobilized on this surface by an imine linkage between the aldehyde group on surface and primary amine of the Ab. The images were obtained in air after drying the samples in a gentle stream of nitrogen.

3. Results and discussion

The absorption and emission spectra of bare CdTe and CdTe/CdS core/shell QDs are shown in Fig. 1a, the calculated QY of the CdTe QDs increases from 15 to 40% after growing 2 monolayers CdS shell. The photo-irradiation experiments of the diluted samples (0.3 μM) were performed under ambient condition and irradiation from a 365-nm ultraviolet lamp at certain time intervals. It is reported that the PL peak will shift to blue side with time under ultraviolet light irradiation due to photo-oxidation reaction of the diluted samples [21]. In our experiments, a blue-shift of 39.6 nm of the PL spectrum of the CdTe bare core is observed after four hours' photo-irradiation, however, the blue-shift of the CdTe/CdS core/shell QDs is only 2.8 nm under the same conditions (cf Fig. 1b). In fact, these aqueous core/shell QDs are found to be stable and the optical properties of them in a sealed vial remain invariable over one year's storage under ambient condition. Therefore the core/shell QDs are capable to act as the fluorescent marker in the immunoassay.

In order to purify the QDs–Ab complexes and investigate the combination of proteins and CdTe/CdS QDs, the sephadex G-150
strate the success in flexible conjugation of the QDs to Ab based on the fact that the height of the flexible coupling should be higher compared to that of the normal covalent coupling. In order to keep the proteins stable on the silicon surface, covalent immobilization is a preferred method. APTES surfaces are used to immobilize the proteins covalently, because the amido groups on their surfaces can react with glutaraldehyde, yielding aldehyde groups that could form an imine linkage with the primary amine groups on the surface of the proteins. The size of a protein measured from AFM is directly correlated to its molecule weight [26]. Hence, AFM can be used to study the binding behavior of Ab to QDs. Fig. 3 is the AFM image of QDs–Ab and QDs–PG–Ab bio-conjugates. Because the binding sites of QDs onto Ab are arbitrary, the conformational dimension of QDs–Ab complex must be tanglesome. On the contrary, the conformational dimension of QDs–PG–Ab complex should be uniform. In our image, the QDs–Ab exhibits a heterogeneous and dissymmetrical shape with the AFM height of approximately 6–8 nm, and reversely, the QDs–PG–Ab exhibits a homogeneous and symmetrical shape with the AFM height of approximately 10–12 nm. Considering that the sizes of QDs, Ab and PG are 3, 7 and 3 nm, respectively, the result from AFM match closely with the heights of QDs–Ab and QDs–PG–Ab with only a few nanometers less, and this shortening can be ascribed to the fact that Ab protein molecules on QDs surface can be compressed by the imaging process due to forces applied to the samples [27,28]. In line with this idea, the average AFM heights of QDs–Ab and QDs–PG–Ab are a little smaller than the estimated ones. In any case, the height of QDs–PG–Ab is obviously higher than that of QDs–Ab, which can be used to efficiently testify the flexible coupling between QDs and Ab via PG bridges.

The fluorescent immune detection is based on the sandwich method with bright and robust CdTe/CdS QDs as the indicator. In order to enhance the efficiency of the immunoreaction between Ab and Ag, shorten the detection time, and reduce the detection limit, the flexible coupling based immunoadsay is employed with microelisa well plate as the matrix. The microelisa well plate can provide a large surface area for protein immobilization and properly sized pores in the plate to force the immunoreaction between Ab and Ag in a limited space that is small but sufficiently large for the volume of the sample solution. In order to immobilize the Ab sufficiently, the adsorption time of the Ab on the pores should be enough (not less than 12 h). The detecting signal of the HBSAg is based on measuring the subtraction between the PL intensity of the control value of the QDs–Ab without any Ag injection and the value of the excessive unreacted QDs–Ab on the sandwich immunoplexes of the wells. The schematic bio-detection of HBSAg by using the microelisa well plate is given in Scheme 3, whereas Fig. 4 illustrate the PL spectra of the normal coupling (QDs–Ab) and flexible coupling (QDs–PG–Ab) with different concentrations of the HBSAg. Fig. 4C and D show the experimental results where

**Fig. 2.** The digital photos of the QDs-based AGE gel electrophoresis for probing the proteins under the excitation of a 365-nm ultraviolet lamp. (A) Photo shows AGE electrophoresis of the QDs and QDs–Ab. (B) photo shows AGE electrophoresis of the QDs and QDs–PG. (C) photo shows AGE electrophoresis of the QDs and QDs–PG–Ab.

**Fig. 3.** AFM images of the complexes of QDs–HBsAb (A) and QDs–PG–HBsAb (B).
the PL intensity from bound HBsAb conjugate is measured over a range of HBsAg concentration till 2000 ng/ml. It should be noted that the signal increases monotonically with the increase of the concentration of HBsAg until saturation is reached at ∼1000 ng/ml. The detection limit can be defined as the lowest concentration of detected sample that give positive value of the PL intensity difference between control (without antigen) and detected sample (with antigen) [4,13]. It can be clearly seen from Fig. 4 that the subtracted PL intensity is still discernable to be positive even when the concentration of HBsAg is as low as 62.5 ng/ml for normal conjugation and 15.6 ng/ml for flexible conjugation, which indicates that the sensitivity of detection is improved due to flexible coupling. The lower detection limit and broad working range in the flexible situation result from high capturing efficiency of QDs with the microelisa well plate. The higher capturing efficiency is due to the flexible coupling of QDs and Ab (as shown in Scheme 2) which enables sufficiently mutual affinity between QDs labeled HBsAb and the HBsAg immobilized onto the pores of the well plates. On the contrary, the normal coupling are commonly accompanied with certain non-efficient conjugation by occupying the specific site or influencing the bioactivity of the Ab (as shown in Scheme 1), which will reduce the ability of the recognition between the QDs labeled Ab and the detected Ag consequently and result in a lower detection sensitivity.

The specificity of an immunoassay system mainly depends on the immunoreactivity of the labeled monoclonal Ab. However, due to the larger surface electronic potential energy of the nanocrystals, the property of the Ab can be easily manipulated by the nature of the fluorescence labels. PG acts as a “flexible bridge” for connecting the QDs with Ab to avoid the non-specific and null combination, and thus to increase the specificity of the immunoassay. As is shown in Fig. 4C, detect signal shows negative value at certain low concentrations, which is not observed in Fig. 4D. The negative value is mainly a consequence of the non-specific adsorption between QDs–Ab and the blocking molecules immobilized on the wall of the well. To further validate the specificity enhancement based on the flexible coupling, a control experiment was designed by using a 1% BSA PBS solution as the detected sample. The results are given in Fig. 5, in which the PL intensity is normalized to the value of HBsAg detection. When the QDs–Ab is used to monitor the nonspecific binding of the BSA molecules, a low PL intensity can be obtained, which indicates that there is 31.5% non-specific adsorption of BSA molecules during the detection process. Comparably, when the QDs–PG–Ab is used to monitor the nonspecific binding of the BSA molecules, only a small PL signal is observed under the same detection condition, which indicates that only 2.4% non-specificity with BSA exists. Clearly the immunoassay using the QDs–PG–HBsAb conjugate is much
more specific for HBsAg than that using the QDs–HBsAb conjugate.

4. Conclusions

Highly luminescent aqueous CdTe/CdS core/shell QDs are covalently conjugated with HBsAb and PG by using EDC and S-NHS as cross-linking reagents. The flexible coupling using protein G as a bridge between the QDs and HBsAb is characterized by the gel electrophoresis technique and AFM measurement. The fluoroimmunoassay based on the microlisa strip plates via the conjugates of QDs and HBsAb is applied to detect the HBsAg. The detection limit can reach about 15.6 ng/ml and 62.5 ng/ml by using the complexes of QDs–PG–Ab and QDs–Ab, respectively. In addition, the immune detection using QDs–PG–Ab conjugate shows higher specificity, accompanied with the higher sensitivity. Therefore, the flexible coupling by using PG as a bridge is feasible and the fluorescent immune detection via microelisa well plate based on the coupling of the highly luminescent aqueous CdTe/CdS QDs and biomolecules is also acceptable and can be applied into the future biological diagnosis.

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