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RESEARCH PAPER

Improving colloidal properties of quantum dots with combined silica and polymer coatings for in vitro immuofluorenscence assay

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Abstract Semiconductor quantum dots (QDs) are promising fluorescence probes for immuofluorescence assay in the biological applications. However, water solubilization and non-specific binding are two critical issues to be addressed for the practical uses. Here, we reported a new type of QDs with combined silica and polymer coating. QDs with excellent colloidal properties were prepared via carboxylation of the amino groups on the surface of silica-coated QDs by reacting with multi-carboxyl poly (acrylic acid) (PAA). Hydrodynamic size of PAA-functionalized silica-coated QDs was around 40 nm. They were highly fluorescent (about 47.8% quantum yield). No precipitate of QDs was observed after 3 month storage at 4 °C. When cancer cells (HeLa) were used, the functionalized QDs exhibited little or no non-specific cellular binding. The results from in vitro experiments indicated that PAA-functionalized silica-coated QDs-antibody bioconjugates had excellent antigen-capture ability and exhibited little or no non-specific binding to polystyrene spheres which were used to immobilize the antigen for immuoflurescence assay. The PAA-functionalized silica-coated QDs with improved colloidal properties could serve as excellent alternative fluorescent probes for biodetection.

Keywords Quantum dots · Colloidal properties · Poly (acrylic acid) · Silica · Immunofluorescence assay · Nanomedicine

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Introduction

Quantum dots (QDs) are used as a new class of fluorescent probes in biological fields such as bioimaging, biolabeling, and biodetection (Biju et al. 2010; Edgar et al. 2006; Fu et al. 2007). In those fields, QDs have many unique advantages due to their inherent properties, including tunable fluorescence wavelength, broad excitation spectra, sharp and symmetrical fluorescent peak, strong and stable emission, and high quantum yield (Kirby et al. 1994; Mews et al. 1996; Yang and Li 2006). However, practical uses of QDs in the biological fields require us

to solve the issues related to their colloidal properties (e.g., water solubility, redispersion capability, chemical stability, and non-specific binding property). To improve colloidal properties of QDs, a number of surface encapsulation methods have been used, including ligand exchange (Mitchell et al. 1999; Potapova et al. 2003; Uyeda et al. 2005), polymer coating (Gao et al., 2004; Luccardini et al. 2006; Pellegrino et al. 2004), and silica coating (Darbandi et al. 2005; Yang and Gao 2005; Zhelev et al. 2006). Polymeric coatings such as polyethylene glycol (PEG) and poly (acrylic acid) derivatives keep QDs stable for months, but the photostability of the QDs is drastically reduced in the aqueous solution carrying oxygen. Oxygen in water catalyzes photoanodic dissolution of chalcogenide semiconductor particles in water (Nann and Mulvaney 2004). While silica coating is a classic method for surface modification that can effectively enhance the colloidal properties of QDs by providing the chemical functionality, and protecting QDs from oxidation and agglomeration (Jovanovic et al. 2006). Although pure silica-coated QDs are well dispersed in water, the charge from chemical groups (e.g., amino groups) functionalized on the surface of silica-coated QDs will cause the particles to aggregate (Yang et al. 2006).

Willem J. M. Mulder group reported a strategy to coat silica particles with a dense monolayer of paramagnetic and PEGylated lipids for multi-modal imaging (Koole et al. 2008). And other articles reported (Cavalieri et al. 2009; Xia et al. 2009) drug/gene delivery by using lipid or polymer-coated colloidal silica particles without quantum dots. However, the functional lipid or polymer used in the above reports aimed for the imaging or drug/gene delivery. The authors did not discuss the colloidal properties of nanoparticles. In this study, silica-coating and polymer-coating methods were combined to improve the colloidal properties of QDs. The silica coating was applied to incorporate hydrophobic QDs into the silica matrix via the reverse microemulsion approach. Polymer coating was then employed to modify the silica-coated QDs to enhance the water solubility and redispersion capability of the silica-coated QDs. Amino groups were first introduced on the surface of the silica-coated QDs with 3-aminopropyltriethoxysilane (APTES). PAA was then grafted on the silica-coated QDs via the carboxylation of the amino groups. Due to its abundant carboxyl groups, PAA could not only improve the water solubilization of QDs but also provide a lot of anchors to bioconjugate biological molecules. Furthermore, compared to APTES, PAA molecules have much longer and more flexible chains, which can reduce the steric hindrance for conjugating biomolecules (Maxwell et al. 2002). Moreover, in this study, PAA modified silica-coated QDs showed little or no non-specific binding to human cervical cancer cells. In vitro immunofluorencence assays were used to test the specificity of bioconjugates formed by PAA-functionalized silica-coated QDs and anti-human IgG.

Experimental section

Materials

The materials used in this study included selenium powder (100 mesh, 99.99%, Sigma-Aldrich), cadmium oxide (CdO, 99.5%, Sigma-Aldrich), zinc oxide (ZnO, 99.9%, Sigma-Aldrich), sulfur (99.98%, Sigma-Aldrich), tri-*n*-butylphosphine (TBP, 90%, TCI), tri-*n*-octylphosphine oxide (TOPO, 90%, Sigma-Aldrich), octadecylamine (ODA, 90%, ACROS), 1-octadecene (ODE, 90%, ACROS), oleic acid (OA, 90%, Sigma-Aldrich), carboxyl-polystyrene (PS) microspheres, and purified total IgG from normal human serum in which the heavy chain is 50000 Da and the light chain is 25000 Da, goat anti-human IgG, BSA, 1-ethyl-3-(3-dimethyllaminopropyl)carbodimide hydrochloride (EDC), 3-aminopropyltriethoxysilane (APTES, Sigma-Aldrich), poly (acrylic acid) (PAA, Sigma-Aldrich, M_w 2,000), NP-40 (Fluka), TEOS (99%, Aldrich), aqueous ammonia solution (25 wt%), cyclohexane, acetone, butanol, chloroform, ethanol, dichloromethane, and Argon (oxygen free). All chemicals were used without further purification.

Methods

Synthesis of shell/core QDs

TOPO-capped QDs were synthesized according to a previously reported protocol with some minor modifications (Li et al. 2003; Peng and Peng 2001). For synthesis of CdSe-core QD, 0.3 mmol of CdO,

0.4 mL of OA, and 4.0 mL of ODE were loaded into a 50 mL flask. The mixture was heated to 300 °C under an Ar flow, and CdO was dissolved in the mixture. After a colorless homogeneous solution was formed, the solution was cooled to room temperature (RT). 2.50 g ODA and 0.50 g TOPO were added to the flask, which was heated again to 280 °C under an Ar flow. Next, a selenium solution (1.8 mmol of Se powder dissolved in 2 mL of TBP) was injected quickly into the flask. Following the injection of selenium, nanocrystals were grown at 260 °C over different durations to obtain the desired sizes. The solution underwent color changes from first colorless, to green, yellow, and finally to red, which were typical indications of QD formation. Upon formation, the QD solution was injected into chloroform. The TOPO-capped QDs were precipitated by adding dry ethanol, collected by centrifugation, washed with methanol several times, and vacuum dried for use.

For shell/core QDs synthesis, CdSe nanocrystals were dispersed in 10 mL of hexane. The hexane solution was then mixed with 1.5 g of ODA and 5.0 g of ODE in a 25 mL three-neck flask. The flask was switched to Ar flow to replace the air for 30 min, and then heated to 100 °C for another 5–10 min to remove hexane from the system. The reacted mixture was further heated to 240 °C for the injections. The procedures for injections of Cd, Zn, and S resources can be found in literature (Li et al. 2003). The final product was diluted by hexane, followed by a methanol extraction, or acetone precipitation of the nanocrystals. Excess amines were further removed by dissolving the nanocrystals in chloroform and precipitating them with acetone. CdSe/CdS/CdS/$Cd_{0.75}Zn_{0.25}S$/$Cd_{0.5}Zn_{0.5}S$/$Cd_{0.25}Zn_{0.75}S$/ZnS/ZnS (7 layers) with quantum yield (QY) of 47.8% were prepared for incorporating into silica.

Synthesis of silica-coated QDs and PAA-functionalized silica-coated QDs

Silica-coated QDs were synthesized according to previously published procedures (Zhang et al. 2008b). Typically, 10 mL of cyclohexane, 1.3 mL of NP-40, and 200 μL of QD (3.3×10^{-6} M) stock solution in chloroform, and 120 μL of TEOS were added into a flask under vigorous stirring. Thirty minutes after the microemulsion system was formed, 100 μL of ammonia aqueous solution (25 wt%) was introduced to initiate the polymerization process. The silica growth was completed after 24 h of stirring at RT. Chemically activated silica-coated QDs were prepared by adding 20 μL APTES for the introduction of amino groups into above reaction system after 24 h polymerization. The PAA-functionalized silica-coated QDs were obtained by treating the amino-functionalized silica-coated QDs with PAA. Briefly, 0.08 g PAA and 0.23 g EDC were added into the amino-functionalized silica-coated QDs. Upon completion of the 24-h carboxylation process, the resulting QDs were isolated from the microemulsion using acetone and ethanol to remove any surfactant and unreacted molecules. Succinic anhydride modified silica-coated QDs were obtained by treating the amino-functionalized silica-coated QDs with succinic anhydride in DMF for 8 h (Kumar et al. 2008).

Gel electrophoresis analysis

PAA-functionalized silica-coated QDs were prepared with different amounts in each well with a 6× loading buffer on a 1% agarose gel at 100 V in TAE buffer (1X) for 30 min (PowerPak Basic, Bio-Rad). The gel was illuminated with an ultraviolet trans-illuminator (Tanon Gel Image System) for fluorescence imaging of the emission bands.

Quantitative analysis of non-specific binding

The 96-well Thermo Microtiter Microplate was blocked first by using 250 μL 0.5% BSA in each well at moisture chamber, at 37 °C for 3 h. After blocking, the plate was washed three times with 10 mM PBS. Next, 100 μL PAA-functionalized silica-coated QD samples with different concentrations were loaded and incubated for 40 min at 37 °C moisture chamber. After washing the plates three times with 10 mM PBS containing 0.02% Tween-20, each was measured in duplicate on a Thermo Fluoroskan Ascent FL plate reader (excitation at 355 nm, emission at 620 nm).

Qualitative analysis of non-specific binding with cancer cells

HeLa cells (ATCC number CCL-2) were cultured in RPMI media with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL) at 37 °C in a

humidified atmosphere containing 5% CO_2. Cells were plated in 6-well plates and cultured for 24 h. The cells were then washed twice with fresh 1× PBS, fixed with 4% formaldehyde for 2 h, permeabilized with 0.1% Triton X for 5 min, and washed again three times with 1× PBS. A 2% bovine serum albumin (BSA) blocking solution was added to the wells and incubated for 20 min. After the solution was aspirated, QDs in blocking solution were added into the wells and incubated with the cells for 20 min. The cells were then washed three times with 1× PBS to remove the QDs, and the cellular nucleus was stained with 4′,6-diamidino-2-phenylindole (DAPI) solution (2 μg/mL). Finally, the cells were washed three times with deionized water, and the slide was mounted and imaged under fluorescence microscopy.

Covalent immobilization of antibody and antigen onto PAA-functionalized silica-coated QDs and carboxylated PS microspheres

The antibody and antigen were covalently immobilized onto the PAA-functionalized silica-coated QDs and carboxylated PS microspheres, respectively. The PAA-functionalized silica-coated QDs reacted with the QDs/goat anti-human antibody/EDC at the molar ratio of 1:8:4000 in PBS (0.01 M, pH 7.2) for 2 h at RT. The final QD-antibody bioconjugates were purified by ultracentrifugation with PBS several times to remove excess IgG antibody and kept at 4 °C in PBS–BSA buffer (0.01 M, pH7.2, 0.5% BSA). Highly carboxylated PS microspheres were coated with human IgG (as positive control) and rabbit IgG (as negative control) via the covalent bonds between human IgG molecules and microspheres. The IgG solutions (10.0 mg), EDC (10.0 mg) and microspheres (10.0 mg) were gently mixed in the 1× PBS buffer for 2 h at RT on an orbital shaker followed by centrifugal purification.

In vitro immunofluorescence assay

The immunofluorescence assay for both the experiments and controls were conducted using the same procedure. The IgG-coated PS microspheres were first blocked for 30 min at RT in the PBS–BSA buffer (0.01 M, pH 7.2, 0.5% BSA), then gently mixed with bioconjugates of IgG-coated PAA-functionalized silica-coated QDs for 45 min at RT, using an orbital shaker. After the antibody–antigen reaction, centrifugations/redispersion procedures were applied to separate the unbound IgG-coated PAA-functionalized silica-coated bioconjugates. The microspheres were examined under an OLYMPUS.BX51 fluorescence microscope equipped with an OLYMPUS MICRO DP 70 camera and a broad band light source (ultraviolet 330–385 nm). The illumination light was from an O-LH100HG 100 W mercury lamp with automatic exposure control.

Characterization of QDs, silica-coated QDs, and PAA-functionalized silica-coated QDs

Ultraviolet–visible (UV–vis) absorption spectra were recorded on a UV-2450 spectrophotometer (Shimadzu). Photoluminescence measurement was performed at RT using an F-4500 spectrophotometer (Hitachi). PAA-functionalized silica-coated QDs were photographed on a transmission electron microscope (TEM, JEOL JEM-100CX II) operating at an acceleration voltage of 100 kV. Fourier transform infrared spectroscopy (FTIR) spectra were recorded on a Bio-Rad FTS 6000 spectrometer at RT. The dispersion property of PAA-functionalized silica-coated QDs in solution was measured using a particle size analyzer (Nano ZS, Malvern). The fluorescence quantum yield of the QDs were measured using Rhodamin 6G as a fluorescence standard.

Results and discussions

The size properties of nanoparticles

The silica-coated QDs were synthesized using the reverse microemulsion approach. The as-prepared amino-functionalized silica-coated QDs were examined by a TEM. As shown in Fig. 1a, the amino-functionalized silica-coated QDs were uniform, with size around 40 nm. There were no significant morphological changes after PAA introduction, and the nanoparticles also exhibited a uniform distribution. The center cores shown in Fig. 1a and b were hydrophobic QDs. The hydrodynamic size of amino-functionalized silica-coated QDs and PAA modified ones was measured using a particle size analyzer. The measured hydrodynamic size was around 37 nm

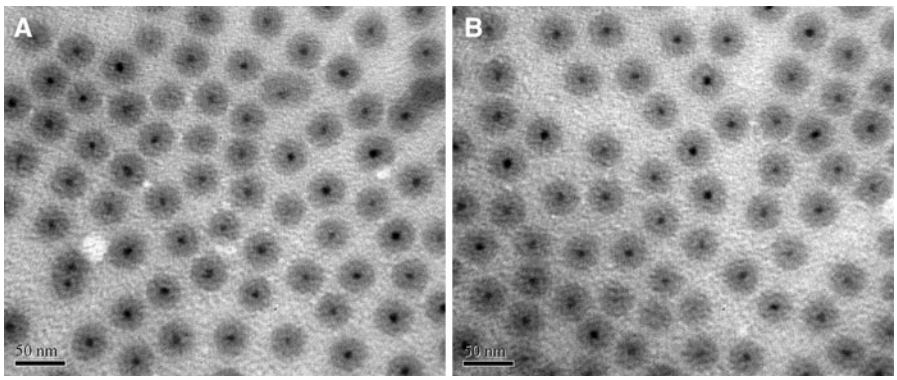

Fig. 1 TEM images of amino-functionalized silica-coated QDs (**a**) and PAA-functionalized silica-coated QDs (**b**)

which was consistent with the TEM observations. It indicated that both amino-functionalized silica-coated QDs and PAA-functionalized ones had no observable aggregates and exhibited excellent dispersions in water.

The surface properties of nanoparticles

The unmodified silica-coated QDs with hydroxyl groups are usually found to be difficult to conjugate with biomolecules. In order to make the conjugation reaction happen, active chemical groups (e.g., amine, thiol, and carboxyl) are grafted on the surface of QDs. In this study, 3-aminopropyltriethoxysilane (APTES), containing primary amine, was added to the reaction system after 24 h of silica-polymerization. FTIR spectra (Fig. 2) of as-prepared silica-coated QDs and modified silica-coated QDs showed vibrations at 1095.5, 945.1, and 801.2 cm^{-1}, confirming the formation of silica. Vibrations at 2931.7 cm^{-1} in the spectra (Fig. 2b, c) were from the methylene groups of APTES. While the characteristic vibrations at 1697.3/1558.4 and 1651 cm^{-1} indicated the amine and carboxyl groups on the surface of silica-coated QDs and PAA modified ones, respectively. Furthermore, the zeta potentials of nanoparticles were monitored before and after the introduction of chemical groups. The negatively charged silica-coated QDs turned positive after amine groups were introduced. Zeta potential changed from −50.31 to 22.09 mV. It became negative after –COOH groups were introduced, with a value of –27.33 mV. These results were consistent with those obtained from the FTIR spectra.

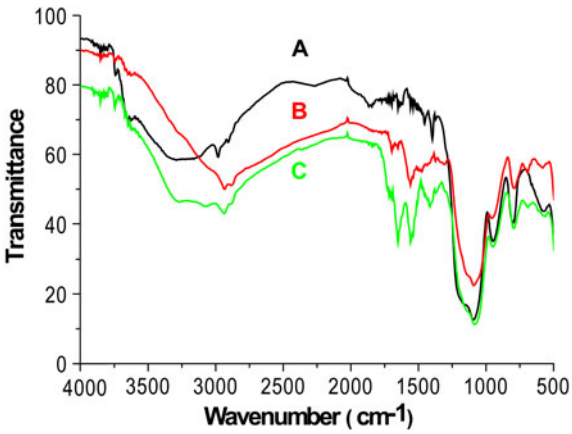

Fig. 2 FTIR Spectra of as-prepared silica-coated QDs (**a**), amino-functionalized silica-coated QDs (**b**), and PAA-functionalized silica-coated QDs (**c**)

The colloidal properties of nanoparticles

Centrifugal separation and redispersion experiments were conducted to test the water solubility of both PAA-functionalized silica-coated QDs and succinic anhydride modified ones. Experimental results showed that the PAA-functionalized silica-coated QDs obtained by centrifugation (14000 rpm ($25800\times g$), 25 min) redispersed quite well after adding DI water. However, the succinic anhydride modified silica-coated QDs did not redisperse under the same procedures (Fig. 3a). Bioconjugates of PAA-functionalized silica-coated QDs purified by centrifugal separation were also easily redispersed by adding DI water, with no evident sediments at the bottom after 3 month storage at 4 °C (Fig. 3b). Although PAA and succinic anhydride both introduced –COOH groups on the surface of nanoparticles, the improved colloidal properties of PAA-functionalized silica-coated QDs may relate to the charge density and steric hindrance of the long flexible chains of PAA polymer. Gel

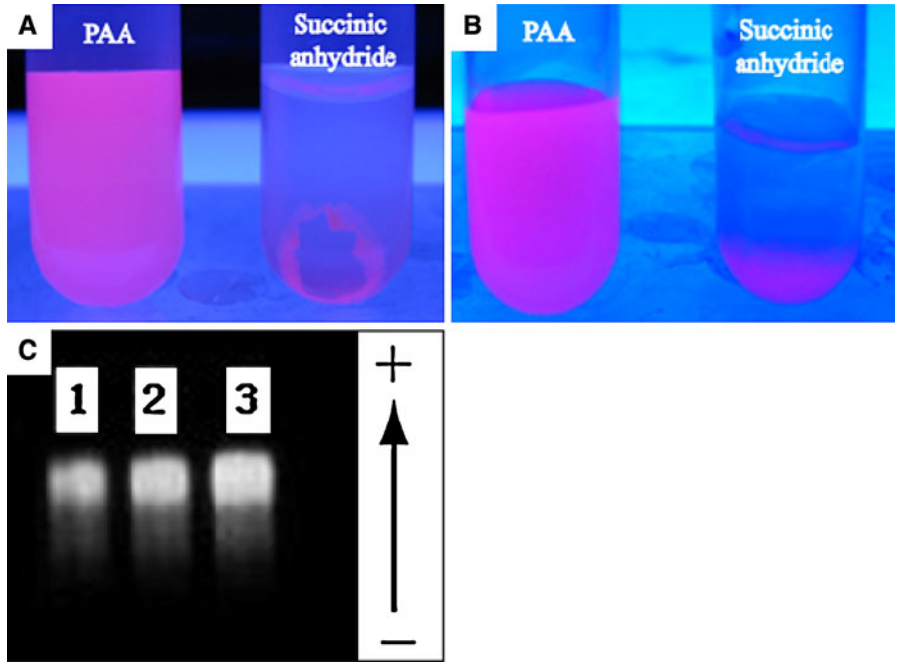

Fig. 3 Colloidal properties: **a** redispersion capability and water solubility—note the difference between the PAA-functionalized silica-coated QDs and succinic anhydride modified silica-coated QDs after centrifugation and redispersion by adding DI Water, **b** storage stability, **c** Gel electrophoresis analysis of PAA-functionalized silica-coated QDs with different concentrations. From *lane 1 to lane 3* concentrations of QDs increased

electrophoresis was used to study the size and surface charge distribution of the PAA-functionalized silica-coated QDs and to check for the presence of nanocrystal aggregates. The gel electrophoresis analysis in Fig. 3c showed that the bands on the gel were remarkably narrow. Since the particle mobility in gels depended on both charge and size, the appearance of any possible aggregates would result in a long migrating tails. The bands (lane 2 and lane 3) from high concentration of PAA-functionalized silica-coated QDs were found to be as narrow as that (lane 1) of the low concentration. In general, the nanoparticles of high concentration were prone to aggregation, resulting in broadened band. However, there were no aggregates even in a solution with high concentrations of PAA-functionalized silica-coated QDs. Although PAA derivatives-coated quantum dots are well water-soluble, their colloidal stability and oxidation resistance are not good, especially in some harsh chemical conditions (Nann and Mulvaney 2004). Some post-treatment should be employed to stabilize the PAA derivatives-coated quantum dots (Wu et al. 2003). The performance of both PAA and silica-coated quantum dots is better than that of PAA derivatives-coated quantum dots, since PAA and silica coating provides both advantages of better colloidal property and oxidation resistance.

Non-specific binding analysis

The low non-specific binding property of PAA-functionalized silica-coated QDs was studied in a cell experiment by co-incubating QDs with HeLa cells (Fig. 4). PAA-functionalized silica-coated QDs showed no detectable non-specific binding, while succinic anhydride modified silica-coated QDs exhibited non-specific binding to the cancer cells at a similar experimental condition. The non-specific binding test was also quantitatively characterized by a plater reader (Thermo Fluoroskan Ascent FL). The fluorescent emission from the PAA-functionalized silica-coated QDs sample wells after washing decreased to the intensity level of the background, while succinic anhydride modified silica-coated QDs still emitted fluorescence with 20% of the original intensity after washing. It indicated that PAA-functionalized silica-coated QDs could be used in fluorescence-based immunoassay.

In vitro immunofluorescence assay

In vitro immunofluorescence assay was used to test the specificity of PAA-functionalized silica-coated QDs-anti-human IgG bioconjugates. Figure 5a and b

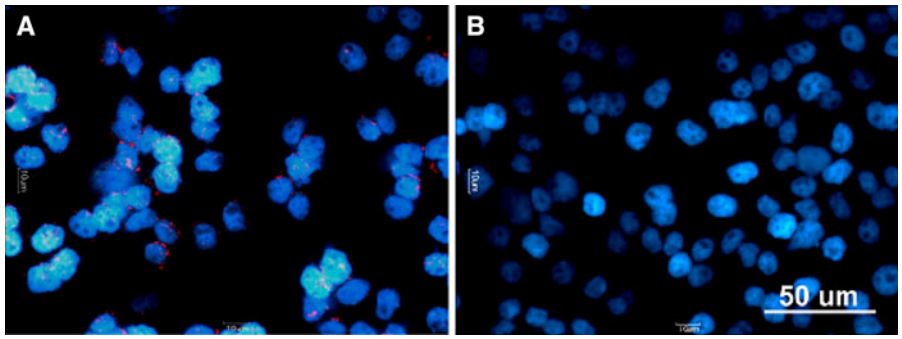

Fig. 4 Fluorescence microscopy images of succinic anhydride modified silica-coated QDs (**a**) and PAA-functionalized silica-coated QDs (**b**) non-specifically bound to fixed human HeLa cells with DAPI counterstaining of cell nuclei

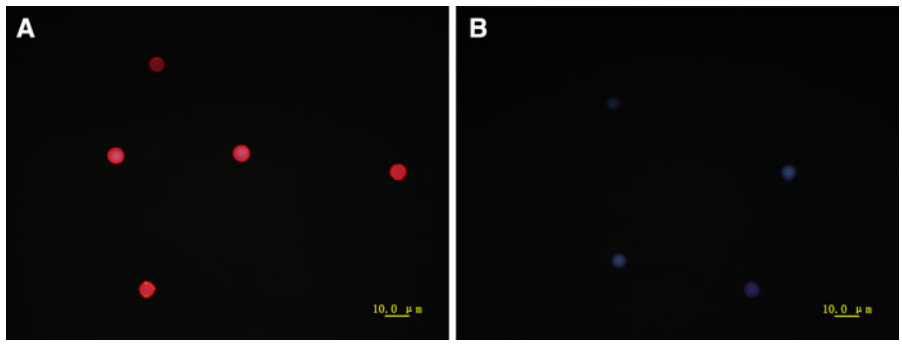

Fig. 5 The immunofluorescence images of human IgG conjugated PS microspheres (**a**) and rabbit IgG conjugated PS microspheres (**b**) treated with PAA-functionalized silica-coated QDs-anti-human IgG bioconjugates

showed the immunofluorescence images of human IgG (positive control) and rabbit IgG (negative control) conjugated PS microspheres treated with PAA-functionalized silica-coated QDs-anti human IgG probes. These images indicated a brighter red fluorescence on the positive control microspheres in Fig. 5a, while nearly no red fluorescent signal existed on the surface of negative control microspheres in Fig. 5b. The bright red fluorescence signal on the positive control microspheres was homogeneous as the PAA-functionalized silica-coated QDs-anti-human IgG probes were evenly dispersed.

The excellent water solubility and redispersion capability are critical for developing the QD-probes for biological applications. As reported previously, QD-bioconjugates are usually purified by gel chromatography (Gao et al. 2004; Zhang et al. 2008a; Lingerfelt et al. 2003). Unfortunately, chromatography is procedurally complicated, time-consuming, and costly. Centrifugal separation is a straight-forward approach. However, once particles settle down, they are very difficult to redisperse. Although there are methods for redispersing these sediments, such as ultrasonic dispersion, warming, and pH adjusting, each has disadvantages. In this study, PAA, a water-soluble, biocompatible, low cost, and commercially available polymer, was used to modify silanized QDs, yielding silica-coated QDs with improved water solubility, redispersion capability, and colloidal stability. No precipitate was observed at the bottom of the tube after 3-month storage at 4 °C. The above characterization results indicated that the PAA-functionalized silica-coated QDs can satisfy the requirements of colloidal properties as fluorencent probes for the biological applications. Furthermore, the density of linked biomolecules can be optimized by PAA-functionalized silica-coated QDs as a result of active chemical groups on their surfaces. The PAA polymer on the QDs, acting as spacers to reduce the steric hindrance, can provide sufficient space for unhindered biomolecular interactions. Excellent solubility and improved dispersion behavior of the PAA-functionalized silica-coated QDs may attribute to multiple carboxyl groups and long flexible polymer chains from PAA polymer, which can provide electrosteric stabilization between nanoparticles.

Conclusions

In conclusion, we developed a new class of QDs with combined silica coating and polymer (PAA) coating. The experimental results indicated PAA-functionalized silica-coated QDs had significantly improved colloidal properties. The improved redispersion properties of the modified QDs could possess advantages in the purification of QD-probes because they could be easily redispersed after centrifugation. Non-specific binding was greatly reduced as a result of modified QD surfaces. And the bio-fluorescent probes based on the prepared PAA-functionalized silica-coated QDs exhibited high specificity to the corresponding substances.

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