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Silica-encapsulated quantum dots for highly efficient and stable fluorescence immunoassay of C-reactive protein

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ABSTRACT

In this study, we presented a new coating regulation of silica by the reverse microemulsion method to obtain biocompatible high quality silica-encapsulated quantum dot (QD). The hydrophobic CdSe/ZnS QDs were coated with silica successfully and demonstrated good photostability and thermal stability and high fluorescent intensity. The corresponding silica-encapsulated QDs-antibody probes were used to detect C-reactive protein (CRP) with quantum dot-labeled immunosorbent assay (QLISA). Only micro dosage of probes was required due to good antibody-antigen combination in optimized buffer environment. The assay results are comparable to commercial assay kits with a wide analytical range of 1-1000 ng/mL, a low limit of detection of 0.76 ng/mL, and good recovery rates of 87.31–105.48%. The convenience and cost effective aspects of this silica-encapsulated QDs immunosorbent assay motivate future development of *in vitro* diagnostic kits.

1. Introduction

Photoluminescent semiconductor nanocrystals quantum dots (QDs), due to outstanding fluorescent properties, were widely used in biosensor and biomedical applications such as visualization of cellular processes, delivery of drugs to specific targets, gene sequencing, photoelectrochemical immunoassays and *in vitro* diagnostics (IVD) [1–7]. As ideal labels in bio-detection, QDs generally have high photoluminescence (PL) efficiency, high quantum yields (QYs), high resistance to photobleaching, excellent biocompatibility, broad excitation band, and narrow band of fluorescence [8–10]. Generally, one excitation band could simultaneously excite many QDs with different emissions, which opens new prospects for simultaneous multiplex assay of many compounds [11]. Therefore, QDs begin to have a broad application prospect for IVD recently [12–15].

With the development of the immune-diagnosis reagents, the detection of proteins plays an important role in IVD, especially the detection of tumors and inflammatory biomarkers [16,17]. Many methods using QDs reported previously were based on the antibody (Ab)-antigen (Ag) specific recognition, and only good for qualitative detection. As an example, Liu's group achieved qualitative detection of hepatitis B surface Ag (HBsAg) with CdSe/ZnS nanocrystals [18]. Based on QD-lateral flow immunoassays (LFIA) system, we also applied $CuInZn_xS_{2+x}$/ZnS core/shell QDs as the fluorescent label to detect C-reactive protein (CRP) qualitatively [19]. Meanwhile, different concentrations of an immune-protein may predict different diseases. For instance, CRP is of clinical importance as an independent marker for coronary heart disease, with stratifications corresponding to low (< 1 μg/mL), moderate (1–3 μg/mL), and high (> 3 μg/mL) levels of cardiovascular risks; and high levels CRP (10–100 μg/mL) correlating with the severity of bacterial infection [20–22]. As a result, quantitative detection is widely needed in some bio-applications, and high sensitive and accurate detection is crucial in IVD [23–25]. Thus, it is very important to make high quality QD-based biolabeling probes, not only with high QYs, but also with high photostability. Since PL intensity is expected to be

Abbreviations: Ab, antibody; Ag, antigen; BS, buffersodium borate buffer; BSA, bovine serum albumin; CB buffer, carbonate-bicarbonate buffer; CRP, C-reactive protein; DLS, dynamic light scattering; ELISA, enzyme-linked immunosorbent assay; EDC, 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide; HBsAg, hepatitis B surface Ag; IVD, *in vitro* diagnostics; LFIA, lateral flow immunoassay; LOD, the limit of detection; LOQ, the limit of quantification; mAb, monoclonal antibody; ODE, 1-octadecene; OTMS, trimethoxy(octadecyl)silane; PMAH, polymaleic acid n-hexadecanol ester; POCT, point-of-care testing; PBS, phosphate buffered saline; PL, photoluminescence; QDs, quantum dots; QLISA, quantum dot-labeled immunosorbent assay; QYs, quantum yield; R, the correlation coefcient value; sulfo-NHS, *N*-hydroxysulfosuccinimide; TEM, transmission electron microscopy; TEOS, tetraethyl orthosilicate.

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stable during the detection process, we are focusing on developing methods to make highly stable fluorescent QDs.

In our previous work, we used amphiphilic oligomers (polymaleic acid n-hexadecanol ester, PMAH) as surfactants to prepare aqueous CdSe/ZnS QDs. By using such oligomer-coated CdSe/ZnS QDs as fluorescence labels, we were able to quantify CRP antigen *in vitro* based on quantum dot-based fluorescence-linked immunosorbent assay (QLISA) successfully [26]. However, we also noticed that the separation and stability of QDs were still difficult to control in some cases [27]. Especially, PL intensity might be inevitably weak or self-quenched in harsh conditions (such as high concentrations of acids), and the aggregation of QDs might happen in certain bio-buffers. It would be ideal to have better surface modifications to protect the QD core, improve colloidal stability, and introduce chemical functionality for bio-detection [28,29].

Strategies used to make QDs more stable in harsh conditions include coating the QDs with a silica layer by a Stöber or reverse microemulsion method [30–32]. The silica coating, as a very good surface modifier, can conjugate with various functional groups with excellent biocompatibility and stability and nontoxicity [33,34]. After silica coating, QDs enable the coupling and label of biotargets with selectivity and specificity [35–39]. Here, we prepared silica-encapsulated QDs *via* the reverse microemulsion method and used new developed QLISA method to detect CRP [38]. Comparing to PMAH-coated QDs, the silica-encapsulated QDs as fluorescent probe, demonstrated the following advantages: First, the aqueous QDs kept excellent fluorescent properties with silica coating, as well as improved pH stability of silica-encapsulated QDs-antibody; Second, only micro dosage of probes was required in suitable buffer environment; Third, a much wider linear detection range was obtained with silica-encapsulated QDs. Thus, this new silica-encapsulated QDs based QLISA meets the needs of high efficiency, stable, sensitive, and high-throughput determination of CRP levels with less materials and can be potentially applied to other *in vitro* diagnostics.

2. Materials and methods

2.1. Materials and instruments

Zinc oxide (ZnO, 99.99%, power), cadmium oxide (CdO, 99.99%), selenium (Se, 99.99%, power), sulfur (S, 99.98%, power), 1-octadecene (ODE, 90%), oleic acid (OA, 90%) and Igepal CO-520 were purchased from Aldrich (Shanghai, China). NaOH, HCl, KCl, NaCl, $NaHCO_3$, Na_2CO_3, Na_2HPO_4, KH_2PO_4, H_3BO_3, $Na_2B_4O_7{\cdot}10H_2O$, Tris, Hepes, and Tween-20 were purchased from Shanghai Sangon Ltd (Shanghai, China). Cyclohexane (analytical grade), 1-hexyl alcohol (analytical grade), methanol (analytical grade) and ethanol (analytical grade) were obtained from Beijing Chemical Reagent Ltd (Beijing, China). Tetraethyl orthosilicate (TEOS) and trimethoxy(octadecyl)silane (OTMS) were purchased from Alfa Aesar. Calf serum and bovine serum albumin (BSA) were purchased from Sigma. N-Hydroxysulfosuccinimide (sulfo-NHS), 1-ethyl-3-(3-(dimethylamino) propyl) carbodiimide (EDC) and the microplates were purchased from Thermo Fisher Scientific (USA). Mouse anti C-reaction protein monoclonal antibody and CRP antigen were obtained from Abcam (USA). The fluorescence spectra were detected using SpectraMaxi3 (Molecular Devices, Sunnyvale, USA). Purified water (18.2 mΩ, Millipore USA) was used in all experiments.

2.2. Synthesis of silica coated PL QDs (silica-encapsulated QDs)

The hydrophobic CdSe/ZnS QDs (PL 625 nm) was prepared according to previous work [40]. For purification, 10 mL of hexanes was added and the unreacted compounds and byproducts were removed by successive methanol extraction (at least three times). Then, QDs were redissolved in cyclohexane before further treatment. The silica-encapsulated QDs were synthesized by reverse microemulsion method according to the following protocol. TritonX-100 (1.77 mL), 1-hexyl alcohol (1.80 mL), cyclohexane (7.50 mL), and hydrophobic CdSe/ZnS QDs (3.3×10^{-6} M) were added in a flask and ultrasonically dispersed to uniformity. Before a certain volume of TEOS was added, ammonia solution (60 μL, 25 wt%) was added into the mixture, then TEOS (100 mL) was added into the flask under vigorous stirring. The silica growth was completed after stirring for 48 h at room temperature. The as-synthesized silica-encapsulated QDs were then purified using ethanol by centrifugation method. Then they were redispersed in ethanol. 5 mL of silica-encapsulated QDs ethanol solution was mixed with 50 μL of ammonia solution (28%). 1 mL of OTMS solution (dispersed in $CHCl_3$) was added dropwise into the silica-encapsulated QDs suspension under vigorous stirring. After 24 h, the particles were collected after centrifuging and washing with ethanol, and dispersed in 1 mL of $CHCl_3$. Amphiphilic oligomer (polymaleic acid n-hexadecanol ester, PMAH) [9] was used to further prepare aqueous silica-encapsulated QDs. First, 1 g of PMAH was dissolved in 5 mL of chloroform. Then, 2 mL of silica-encapsulated QDs chloroform solution was added into PMAH solution and stirred for 24 h in a closed container. The chloroform was slowly evaporated by rotary evaporation, and the remaining QDs were dispersed in ammonia water (pH = 9) with sonication to obtain a clear and colored solution of aqueous QDs. The solution was centrifuged for 30 min at 20,000 rpm to remove excess oligomers.

2.3. Preparation of silica-encapsulated QDs-antibody probe

The silica-encapsulated QDs-antibody conjugates were prepared according to a previously described protocol [26]. Briefly, 150 μL of silica-encapsulated QDs (PL 625) nanocrystals (5 mg/mL) were first dispersed in 750 μL sodium borate buffer (5 mM, pH 7.2, BS buffer). Subsequently, 50 μL of 0.226 M sulfo-NHS and 50 μL of 0.09 M EDC, respectively, were added into the reaction solution and were activated to react for 5 min at 4 °C under ultrasonic. Therewith, the supernatant was discarded to remove unreacted reagents. Later, the mixture was redissolved in 400 μL BS buffer (5 mM, pH 8.0) and 68.8 μg CRP mAb was added into the mixture, then incubated at 4 °C overnight. Afterwards, the composite was blocked with 1% BSA solution and the active sites were stopped with 2.4 μL ethanolamine. Finally, the probes were washed with 5 mM BS buffer (pH 9.0). The silica-encapsulated QDs-antibody were stored in 50 μL BS solution (5 mM, pH 8.0) for assay tests.

2.4. Preparation of antibody coated fluorescence microplate

The coating CRP antibody (1.8 mg/mL) was diluted by carbonate-bicarbonate buffer (0.05 M, pH 9.6, CB buffer) and incubated at 4 °C overnight. Subsequently, the wells were washed with phosphate buffered saline buffer (0.01 M PBS, pH 7.4) containing 0.05% Tween-20 three times to avoid nonspecific binding. For blocking to excess binding sites, 0.5% (w/v) BSA in 0.01 M PBS (pH 7.4) was added and incubation overnight at 4 °C. The microplate was dried in a constant temperature humidity chamber for 24 h, and stored at 4 °C until use.

2.5. The QLISA method for quantitative detection of the CRP standard antigens

As shown in Scheme 1, we designed the silica-encapsulated QDs as fluorescent probe to detect CRP. 100 μL of a series of CRP antigens solutions (0, 1, 5, 10, 50, 100, 200, 500, and 1000 ng/mL in 0.01 M PBS with 10% calf serum (v/v)) were loaded into the 96-well microplate,