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Efficient Incorporation of Quantum Dots into Porous Microspheres through a Solvent-Evaporation Approach

Gang Wang, Pengfei Zhang, Hongjing Dou, Wanwan Li, Kang Sun, Xiaotian He, Junsong Han, Huasheng Xiao, Yao Li

1 The State Key Lab of Metal Matrix Composites, School of Materials Science and Engineering, Shanghai Jiao Tong University, Shanghai, 200240, P. R. China

2 Shanghai Biochip Co., Ltd. & National Engineering Center for Biochip at Shanghai, 151 Libing Road, Zhangjiang Hi-Tech Park, Pudong, Shanghai, 201203, P. R. China.

E-mail addresses: hjdou@sjtu.edu.cn (H. Dou), ksun@sjtu.edu.cn (K. Sun)

* Corresponding author. Tel.: +86-21-34202956; fax: +86-21-34202745

# Pengfei Zhang and Gang Wang contributed equally to this work. The present address of Pengfei Zhang is Zhejiang California International Nanosystems Institute, Zhejiang University, Hangzhou, Zhejiang, 310029, P. R. China
Abstract

Quantum dot (QD) -encoded microspheres play an important role in suspension arrays by acting as supports for various reactions between biomolecules. With regard to QD-encoded microspheres utilized in suspension arrays, three key requirements are controllable size, abundant surface functional groups and especially excellent fluorescence properties. In this paper, narrowly dispersed poly(styrene-co-divinylbenzene-co-methylacrylic acid) (PSDM) microspheres with specific size, surface carboxyl groups, and porous structures were synthesized by seeded copolymerization. In order to improve the incorporation efficiency of QDs within microspheres, we developed a swelling-evaporation approach in which the swelling process was combined with gradual evaporation of the solvent, thus gradual concentration of QDs in the dispersion solution. This approach was demonstrated to be an efficient method for improving the fluorescence intensity of resultant microspheres compared with the use of swelling alone. Moreover, the porous structure was shown to aid the penetration of QDs into the interiors of the microspheres. Through this approach, microspheres encoded with either single or multiple wavelength-emitting QDs were fabricated effectively. The suspension immunoassays were then founded based on the QD-encoded microspheres, by coating mouse anti-human chorionic gonadotropin as the probe for goat anti-mouse IgG detection. The positive results determined by Luminex 100 and the low cytotoxicity of the QD-encoded microspheres demonstrated their great potential in suspension arrays.

Gang Wang, Pengfei Zhang, Hongjing Dou*, Kang Sun * and et al.

For improving the efficiency of QD incorporation into polymeric microspheres, here we developed a swelling-evaporation approach, in which the swelling process was combined with gradual evaporation of the solvent. From this approach, it was demonstrated that appropriate choice of the porous structure could yield microspheres with uniform high brightness.

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1. Introduction

Microspheres are a versatile diagnostic platform for constructing bio-assays to detect antibodies, peptides, oligonucleotides, or other targeting bio-markers.\textsuperscript{1-4} The core technology of microsphere-based bioassays is the identifier-encoded microspheres, and either optical emitters\textsuperscript{2-4} or metal ions\textsuperscript{5, 6} can be utilized as identifiers to encode these microspheres. Among various identifiers, optical emitters, such as organic fluorophores, Raman probes, and especially Quantum Dots (QDs) were shown to be effective for constructing microsphere barcodes, which have applications in existing biological instruments such as flow cytometers and the Luminex xMAP platform.\textsuperscript{2-4, 7} In comparison with microsphere barcodes doped with organic fluorophores, QD-encoded microspheres (QD-microspheres) display excellent optical properties, including narrow and symmetrical emission spectra, enhanced brightness, and higher resistance to photobleaching.\textsuperscript{8} Therefore these systems have received increasing attention since the concept was first raised by Nie et al.\textsuperscript{7}

There are three essential requirements for QD-microspheres to be applicable in microsphere based bioassays. These include controllable size, abundant surface functional groups, and especially excellent fluorescence properties. Researchers have developed various strategies to fabricate QD-microspheres with high fluorescence intensities that are also tunable.\textsuperscript{7, 9-25} According to the order of microsphere synthesis and QD encoding, the strategies can be categorized as the “Microsphere First” strategy in which the microsphere synthesis was followed by QD encoding,\textsuperscript{7, 9-14, 16-19} or the “Microsphere Combined Encoding” strategy in which the QDs were embedded into the microspheres as the microspheres were synthesized.\textsuperscript{20-25} Although the “Microsphere Combined Encoding” strategy improves the efficiency of the preparation by combining microsphere synthesis and QD encoding in a single step, this strategy did not improve the fluorescence properties or the size distribution of the resultant microspheres. Damage to the fluorescence properties of the QDs may have resulted from
exposure to solvents and monomers used for the microsphere synthesis. Meanwhile, diminished size control may have arisen from the phase separation between QDs and the polymers.\textsuperscript{20-25} Chan et al. established the “Microsphere Combined Encoding” strategy by using Poly (styrene-co-maleic anhydride) (PSMA) in a continuous-flow focusing apparatus to obtain monodisperse QD-in-PSMA microspheres.\textsuperscript{26} However, a limitation of this technique is that it requires a specialized continuous-flow focusing apparatus. Therefore, the “Microsphere First” strategy has been applied more frequently to obtain QD-microspheres for applications in bioassays. In this strategy, two approaches, named as the “Swelling Approach” and “Layer-by-Layer (LBL) Assembly”, were developed by embedding QDs within the interiors of microspheres or depositing QDs on the surfaces of microspheres, respectively. An advantage of LBL assembly is that it improves the encoding accuracy by allowing the designer to control the number of QD layers.\textsuperscript{16-19} However, the QDs on the surface are exposed to the environment and might become quenched if the microspheres are not modified. Accordingly, the microsphere barcodes fabricated by the swelling approach usually display stable fluorescence properties owing to the protection provided by the microsphere matrix to the QDs.\textsuperscript{7, 9-14} The advantages of the swelling approach make it an effective approach to fabricate microsphere barcode candidates.

In the swelling approach, the generation of a force driving the infiltration of QDs into the interiors of microspheres is crucial for improving the amount of QDs that may be embedded within microspheres and the homogeneity of the QD distribution within the microspheres. By adding a designated amount of solvent that was selectively poor for QDs to a solution of QDs and porous microspheres, Nie et al. successfully embedded QDs within microsphere interiors.\textsuperscript{7, 9} However, because of the use of poor solvent, the fluorescence properties of the QDs were often diminished during the process, and thus it would be desirable to improve the fluorescence intensity of the resultant microspheres. Herein, we have attempted to develop a swelling-evaporation (SE) approach in which the swelling process was combined with gradual solvent evaporation, thus gradually concentrating the QDs in the dispersion solution. The use of poor solvent was avoided in this approach and the fluorescence intensities of the resultant microspheres were improved. Moreover, considering the importance of the porous structure of these
microspheres, we synthesized narrowly dispersed poly(styrene-co-divinylbenzene-co-methylacrylic acid) (PSDM) microspheres with specific sizes, surface carboxyl groups, and porous structures by an improved seeded copolymerization based on literature examples. In addition, we studied the effect of the porous structure on the microsphere’s fluorescence properties by comparing three classes of microspheres with different porous structures prepared by this approach.

Furthermore, the application of resultant QD-microspheres in suspension assays was also explored in this study. Compared with other conventional bioassay technologies, suspension assays based on fluorescently encoded microspheres offers several advantages, such as high throughput and flexibility. As a typical suspension assay technology, the xMAP platform developed by Luminex Corporation has attracted wide attention owing to its cost-effectiveness and reliable data processing. However, there are still few reports on the application of QD-microspheres in this platform. The reason for this might lie in the requirement of fluorescence wavelengths at 658 and 712 nm for the platform, i.e., the difficulty in both the synthesis of near infrared-emitting QDs and their subsequent incorporation into microspheres. Here, we synthesized near infrared-emitting CdSeTe QDs with an emission wavelength of 685 nm. After embedding these near infrared QDs into PSDM microspheres by the SE approach, we realized successful determination of goat anti-mouse IgG by applying these QD-microspheres in the xMAP platform.

2. Experimental Section

2.1. Materials. Styrene (St, analytical grade), methacrylic acid (MAA, analytical grade), azoisobutyronitrile (AIBN, analytical grade), and benzoyl peroxide (BPO, analytical grade) were purchased from Sinopharm Chemical Reagent Co., Ltd., China and purified before use. Divinylbenzene (DVB, technical grade) was purchased from Aldrich and also purified before use. Polyvinylpyrrolidone (PVP, K-30, $M_n = 36,000$), sodium dodecylsulphonate (SDS, analytical grade), 2-methoxyethanol (analytical grade), dimethylbenzene (analytical grade), and methylene chloride (analytical grade) were purchased from Sinopharm Chemical Reagent Co., Ltd., China. 1-Chlorododecane (CD), Cadmium
oxide (CdO, 99.5%), selenium powder (Se, 99.99%), tellurium powder (Te, 99.999%), oleic acid (90%),
tri-\textit{n}-octylphosphine (TOP, 90%), and Rhodamine 6G were purchased from Aldrich. Liquid Paraffin
(chemical grade) was purchased from Sinopharm Chemical Reagent Co., Ltd., China. This was a
mixture of long chain hydrocarbons (\(\text{CH}_3(\text{CH}_2)_n\text{CH}_3, n = 16–22\)) with boiling points higher than 300 °C.
Methanol (analytical grade), ethanol (analytical grade), and n-Hexane (analytical grade) were also
purchased from Sinopharm Chemical Reagent Co., Ltd. Sulfo-\textit{N}-hydroxy-sulfosuccinimide (sulfo-
\textit{NHS}), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (EDC) and Streptavidin-
Phycoerythrin were purchased from Aldrich. Mouse anti-human chorionic gonadotropin and
biotinylated goat anti-mouse IgG detection were purchased from Shanghai Linc-Bio Science Co., Ltd.
Monodisperse non-porous polystyrene microspheres (PS06N/5623, 5.09 \(\mu\)m) were purchased from
Bangs Laboratories, Inc.

2.2. Preparation of monodisperse porous poly(styrene-\textit{co}-divinylbenzene-\textit{co}-methylacrylic acid)
(PSDM) microspheres. The porous PSDM microspheres were synthesized by seeded
copolymerization.\(^{40}\) Firstly, linear polystyrene (PS) particles were prepared by dispersion
polymerization: a mixture of an initiator (AIBN) and styrene was added dropwise into a continuous
phase solution (as recipes in Table S1). After it was sealed in a nitrogen atmosphere, the solution was
stirred with a rotation speed of 120 rpm at 70 °C for 12 hours. The resultant linear PS particles were
washed with ethanol and collected via centrifugation. The concentrations of the monomer, the
composition of the continuous phase solution, and the initiator in the polymerization system were
regulated to control the molecular weight of the linear PS particles (as shown in Table S1). Secondly,
seed particles of linear PS were redispersed via ultrasonication into 0.25 wt.% SDS aqueous solution.
These seeds were swollen by 0.1 mL of CD in a 0.25 wt.% SDS aqueous solution at 30°C for 12 hours.
After the CD droplets had completely disappeared from solution, the seeds were swollen for another 12
hours by a mixture of St, DVB, MAA and BPO (according to the recipes shown in Table 1), which was
emulsified in 0.25 wt.% SDS aqueous solution in advance. Subsequently, aqueous PVA solution was
added, and the concentration of PVA was fixed at 1 wt.% of the whole solution. Polymerization was
then carried out for 12 hours by heating the solution to 70˚C under a nitrogen atmosphere. The resultant microspheres were washed with ethanol and collected via centrifugation. Finally, these microspheres were extracted with methylene chloride for 48 hours in a Soxhlet apparatus to remove linear PS chains from within the microspheres, which behaved as porogens. Thus, porous PSDM microspheres were obtained.

2.3. Characterization of PS seed particles and PSDM microspheres. Molecular weights of seed particles were measured by gel permeation chromatography (GPC, Agilent 1100). The elution phase was tetrahydrofuran, and a flow rate of 1 mL/min was used. The morphologies of the seed particles and the PSDM microspheres were observed by scanning electron microscopy (SEM, JSM-7601F, JEOL). Fourier transform infrared spectroscopy (FT-IR) spectra of porous PSDM microspheres were recorded using a Paragon 1000 spectrometer, with the samples prepared as KBr discs. Carboxyl groups on the surfaces of the porous PSDM microspheres were measured by acid-base neutralization titration with an automatic titration system (MET mode, 785 DMP Titrino, Metrohm Co., Ltd).

2.4. Synthesis and purification of CdSe QDs and near-infrared CdSeTe QDs. CdSe QDs with emission wavelengths at 460, 520 and 560 nm were synthesized as previously reported.\(^\text{41}\) 2 mmol of CdO was dissolved in a mixture of 4 mmol of oleic acid (OA) and 8 mL of liquid paraffin in a three-necked round-bottom flask at 150 °C to prepare the Cd precursor solution. Following this, 0.4 mmol of Se was dissolved into 20 mL of liquid paraffin at 220 °C with vigorous stirring to prepare the Se precursor solution. Subsequently, 4 mL of solution containing 0.8 mmol of Cd precursor was quickly injected into the Se precursor solution with vigorous stirring, and the growth temperature was kept at 200 °C. The emission wavelengths were varied by controlling the growth time of the CdSe QDs.

Due to the nonlinear characteristics of ternary CdSeTe QDs, it was feasible to synthesize high quality CdSeTe QDs displaying near-infrared fluorescence, which was difficult for binary II-VI QDs. CdSeTe QDs displaying emission at 685 nm were also synthesized as previously reported.\(^\text{42}\) The Cd solution was prepared by dissolving 1.2 mmol of CdO in a mixture of 0.4 mL oleic acid and 20 mL of liquid paraffin at 150°C, and increasing the temperature to the desired reaction temperature. The tellurium
stock solution was prepared by dissolving 1 mmol of tellurium powder into 5 mL of tri-n-octylphosphine (TOP), and the Se stock solution was prepared by dissolving 1 mmol of Se powder into 5 mL of TOP. The premixed Te and Se solutions were prepared from the individual stock solutions with Se:Te molar ratios of 3:1. The initial molar ratio of Cd to the total amount of Se and Te in the reaction solution was kept constant at 2:1 (Cd:Se+Te), and then 3 mL of the premixed solution was rapidly injected into the Cd solution with vigorous stirring, with the solution kept at the desired growth temperature. The reaction was stopped when the desired emission wavelength was reached.

The purification of QDs was carried out by firstly precipitating the QDs by adding a mixture of methanol and chloroform (v/v = 1/1) to liquid paraffin, and then separating via centrifugation. Finally, the required amount of QDs was redispersed into chloroform to form a well-dispersed QD solution for further use.

2.5. Incorporation of QDs into microspheres. In our work, a swelling-evaporation approach (SE approach) was developed to embed the QDs into the microspheres. The detailed procedure is as follows: 0.5 mL of a 10 nM solution of QDs in chloroform was added into a 0.5 mL chloroform solution containing $10^7$ microspheres. Initially, the solution was sealed in a vial and stirred for 2 hours to swell the microspheres. Subsequently, the solution was exposed to the atmosphere for another 2 hours at room temperature to permit complete evaporation of the chloroform. Thereafter, the microspheres were washed with dimethylbenzene and collected via centrifugation. By following the SE approach, the concentration of QDs in solution could be kept at a high level, which would accelerate the diffusion of QDs into the swollen microspheres. To make a comparison, the conventional swelling approach was also performed by following a literature method, the original concentrations of QDs in swelling approach were kept same as that in SE approach.\(^7\)

2.6. Characterization of the fluorescence properties of the QDs and QD-microspheres. The fluorescence spectra of QDs in chloroform solution and of QD-microspheres ($1.5 \times 10^7$ microspheres/mL in water) were recorded using a Shimadzu RF–5301PC spectrofluorophotometer. Fluorescence images of various focalized planes of QD-microspheres in water were recorded using a
laser scanning confocal microscope (LSM 510 META) with a He-Ne laser (λ = 632.8 nm) as the excitation source. True-color fluorescence images of QD-microspheres in water were obtained by fluorescence microscopy (IX-71, Olympus) with a 100-W mercury arc lamp as the excitation source. The uniformity of the fluorescence signals of the QD-microspheres was characterized using a flow cytometer (Beckman coulter FC500). QD-microspheres were suspended in water and then injected into the flow cytometer. Each individual QD-microsphere was excited by a 488 nm laser, and the fluorescence signal of the QD-microsphere was measured by a FL4 detection channel which is able to detect the fluorescence signal at 675 nm.

2.7. Suspension immunoassays for goat anti-mouse IgG. Four kinds of buffer solution were used in the preparation of suspension immunoassays, including a washing buffer, an active buffer, a blocking buffer, and a storage buffer. The QD-microspheres were stored after 10 days before being used as suspension immunoassay to avoid possible decrease of fluorescent intensity in the early stage of storage. The recipes of the four buffers are listed in the Table S2 of the Supporting Information (SI). A 100 µL aqueous suspension of QD-microspheres (1.25 × 10^7 microspheres/mL) was washed three times with the washing buffer. Each sample was centrifuged and resuspended in 100 µL of active buffer (pH = 6.2). A 10 µL solution of sulfo-NHS (10 mM in active buffer) and a 10 µL solution of EDC (4 mM in active buffer) were then added to the sample. After the sample was incubated on a vortex for 20 minutes at room temperature, it was washed three times with a washing buffer and subsequently centrifuged and resuspended in 100 µL of PBS buffer (pH = 7.4). Subsequently, 50 µg of mouse anti-human chorionic gonadotropin was added into the sample as a probe for goat anti-mouse IgG detection. The probe was incubated for 2 hours on a vortex at room temperature, and the sample was subsequently washed three times with the washing buffer and then centrifuged and re-suspended in the blocking buffer (pH = 7.4) for another 20 minutes to block free carboxylates on the surface of the QD-microspheres. Blocked probe-coated QD-microspheres were washed with the washing buffer and stored in a storage buffer at 4 °C.
To prepare a suspension immunoassay, a 100 µL solution of prepared probe-coated QD-microspheres (1.25 x 10^7 microspheres/mL in PBS buffer) was added into a 100 µL solution of biotinylated goat anti-mouse IgG (1 wt.% in PBS buffer) for each sample. After it was incubated for 2 hours in a vortex at room temperature, the sample was washed three times with the washing buffer and centrifuged. Following this step, a 100 µL solution of Streptavidin-Phycoerythrin (1µg/mL in PBS buffer) was then added, and the sample was incubated for 10 minutes on a vortex at room temperature. Finally, the sample was washed three times with the washing buffer and subsequently centrifuged and resuspended in a 100 µL assay buffer (pH = 7.4). The fluorescence signals of the final product were measured on a Luminex 100 Bio-plex System (Luminex Corporation).

2.8. Cytotoxicity tests of the QD-microspheres. MTT assays are based on a cell’s ability to metabolize yellow tetrazolium salt into a highly colored formazan product.

HeLa cells (human carcinoma) were seeded in Dulbecco’s modified Eagle’s medium (DMEM, 200 µL for each well of a 96 well-plate) containing l-glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 units/mL), and 10% heat-inactivated fetal bovine serum (FBS) at 25000 cells/mL. The samples were incubated at 37 °C for 12 hours under 5% CO₂. Subsequently, fresh media containing different concentrations of QD-microspheres (from 1.5 x 10^5 to 1.5 x 10^7 microspheres/mL) were used to replace the original media. After the samples were incubated at 37 °C for 24 hours under 5% CO₂, the media was removed. The cells were then washed twice with PBS buffer (pH = 7.4) and incubated in MTT solution (5 mg/mL, 20 µL for each well) at 37 °C for 4 hours. Thereafter, 100 µL of DMSO was added to dissolve the formazan crystals. The UV absorbance of the solubilized formazan crystals was measured at 492 nm. Cell viability was expressed as the ratio between the amount of formazan determined for cells treated with the QD-microspheres and for non-treated control cells. The results were expressed as an average of three nominally identical measurements.

3. Results and discussion
3.1. Preparation of porous poly(styrene-co-divinylbenzene-co-methylacrylic acid) (PSDM) microspheres. According to the mechanism proposed in previous reports describing seeded copolymerization, the linear polymers generated by seed particles and distributed among the crosslinked polymeric aggregates that are swelled by monomers can behave as porogens during the formation of porous structures. The sizes and size distributions of the polymer aggregates were determined by phase separation between linear polymers of seed particles and polymers formed by seeded copolymerization. They are related to several parameters of the seeded copolymerization, such as the molecular weights of the seed particles and the ratios of crosslinker, monomer and seed particles.

In this study, the effect of the molecular weights of the seed particles on the porous structures of the microspheres was initially investigated. The SEM images of the prepared microspheres are shown in Figure 1. Some crater-like cavities were observed on the surfaces of both PS1-33-15 and PS2-33-15 microspheres, which were respectively prepared from PS1 seed particles (\(M_n = 87,900\)) and PS2 seed particles (\(M_n = 28,400\)). The SEM images of the PS1 and PS2 seed particles as well as their characteristic parameters are shown in Figure S1 and Table S1, respectively, in the SI. However, the crater-like cavities disappeared from the surfaces of PS3-33-15 microspheres prepared from PS3 (\(M_n = 17,500\)) seeds which had lowest molecular weight. Obviously, there were many submicron-sized pores on the surface of PS3-33-15, even before the extraction of linear polystyrene porogen by methylene chloride. As shown in Figure 1d, after the porogen was extracted from the microspheres, no significant difference was observed on the surface of PS3-33-15E (PS3-33-15 microspheres that were subjected to extraction with methylene chloride) except for a few more cavities. This indicates that linear polymers of seed particles with low molecular weights can distribute homogeneously throughout the crosslinked polymeric aggregates, thus yielding porous microspheres with relatively homogenous structures.

The effect of crosslinker concentration was investigated by adjusting the ratio of DVB added to the mixture of monomers during seeded copolymerization, while all other experimental parameters were kept constant. The SEM images of microspheres prepared with various crosslinker concentrations are shown in Figure 2. Results demonstrated that reducing the concentration of the crosslinker caused the
microspheres to solidify and become smoother, and also yielded microspheres with smaller pores. Moreover, after porogen extraction, the porous structures of all three kinds of microspheres became more apparent. According to the magnified SEM images of these microspheres, the diameters of the pores were on the scale of $10^2$ nanometers. Due to the hydrophobic nature of the polystyrene matrix of the microspheres, the pores should provide ideal hosts for embedded QDs.

In seeded copolymerization, the amount of monomer added can affect both the porosity and the size of the resultant microspheres. Therefore, the weight ratio of monomer to seed particles ($r_{ms}$) was investigated as another parameter controlling the size and structure of the microspheres. As shown in Table 1, $r_{ms}$ was adjusted from 15 to 40, and the SEM images of the resultant microspheres both before and after porogen extraction are shown in Figure 3. The results demonstrate that as $r_{ms}$ was increased from 15 to 40, the microsphere diameters increased from 4.79 to 7.15 µm (as displayed in Table 1). Moreover, the structures of the microsphere became more dense with increasing $r_{ms}$, indicating that the pore diameter decreased as $r_{ms}$ increased. The variation of both size and morphology with changes to $r_{ms}$ is reasonable considering the effects as follows. When $r_{ms}$ is increased, the amount of monomer polymerized in every seed particle increases whereas the amounts of the other reagents, especially crosslinker, remain constant, resulting in an increase of microsphere size and density. In addition, as shown by Figures 3a2 to c2, the porous structures of the microspheres became more distinct upon porogen extraction, which was a common characteristic of the microspheres synthesized by this approach.

Our investigations demonstrated not only the validity of seeded copolymerization to prepare porous microspheres, but also the possibility of controlling the size and structure of microspheres by adjusting the parameters of the polymerization. Moreover, a carboxyl group-bearing comonomer was added to the seeded copolymerization to endow carboxyl groups, which can facilitate biomolecule coupling. The presence of carboxyl groups was confirmed by FT-IR, as shown in Figure S2a in the SI. In addition, the number of carboxyl groups per microsphere could be controlled by adjusting the amount of MMA comonomer, as shown in Figure S2b.
3.2. Swelling-Evaporation Approach for QD encoding of porous PSDM Microspheres. The swelling approach has been considered an effective approach to achieve QD encoding of microspheres since it was first developed by the Nie group.\textsuperscript{7,9,10} Researchers have proposed various methods to improve the efficiency of QD incorporation, such as the addition of poor solvent to swelling solvents.\textsuperscript{48,49} However, the usage of poor solvent may decrease the fluorescence intensity of the QDs, and it would be desirable to improve the fluorescence properties of the resultant microspheres. Herein, we propose a swelling-evaporation approach (SE approach) to induce the continuous incorporation of QDs into microspheres. As illustrated in Scheme 1, after completely swelling microspheres together with QDs in a common solvent, an extra solvent evaporation step is introduced to gradually increase the concentrations of both the microspheres and the QDs in the solution. Due to the diffusion equilibrium of QDs inside and outside the microspheres, the gradual concentration of QDs during the evaporation step is anticipated to induce the further entry of QDs from the dispersion solvent into the microspheres, and thus further enhance the fluorescence intensity of the QD-microspheres.

With this in mind, we wanted to study the effectiveness of the SE approach in improving the fluorescence intensity of QD-encoded microspheres. PS3-17-15E microspheres were chosen for comparing the effectiveness of the SE approach with the swelling approach. The concentration of both QDs and microspheres were kept constant to compare the effect of the two approaches. Shown in Figure 4 are the normalized fluorescence spectra of the resultant QD-encoded microspheres, the concentration of the different samples were kept constant. Obviously, increasing the value of $V_{B/C}$ (the volume ratio of butanol/chloroform) from 5/1 to 20/1 in the swelling approach, caused the fluorescence intensity of the microspheres to increase by approximately four-fold. However, although the addition of butanol is effective in increasing the fluorescence intensity of microspheres, the SE approach still exhibits an obvious advantage compared with the swelling approach. As shown in Figure 4a, the fluorescence intensity of the microspheres prepared by the SE approach is 20% higher than that of the microspheres prepared by the swelling approach. For the preparation of QD-encoded microspheres, a key issue lies in the creation of a driving force to induce the QDs to migrate into the interiors of the microspheres.
microspheres. As shown in Figure 4b, the primary driving forces of the SE and swelling approaches arise from the gradual concentration of the QDs and the immiscibility of butanol with QDs, respectively. In the swelling approach, the addition of poor solvent may destroy the crystalline structure on the surfaces of the QDs. This is a possible reason for the diminished effect of the swelling approach in improving the fluorescence intensity, in comparison with the SE approach.

For the incorporation of QDs into microspheres, a porous structure is crucial to determine the encapsulation efficiency of QDs. Therefore, three kinds of microspheres with similar sizes but different porous structures were prepared to study the effect of their porous structure on QD encapsulation. These included PS3-33-15E microspheres bearing larger pores ranged from $10^2$ nanometers to approximately $10^3$ nanometers, PS3-17-15E microspheres bearing smaller homogeneous pores with diameters ~100 nm, and nPS microspheres, which had smooth nonporous structures. As shown by Figure 5a, after they were encoded with the same QDs, the PS3-17-15E microspheres possessed the highest fluorescence intensity among the three classes of microspheres. In addition, the fluorescence emission peak of the QD-microspheres displayed a 5 nm red-shift compared with that of non-encapsulated QDs. This may be due to a mild degree of aggregation among the QDs during their incorporation into the microspheres.

To further explore the QD encapsulation, the distributions of QDs within the microspheres were observed by laser confocal microscope. As shown in Figure 5, microspheres bearing small pores (PS3-17-15E) displayed homogeneous QD encapsulation throughout the entire microsphere, indicating that this kind of structure is ideal for QD encapsulation. Meanwhile, the fluorescence intensities within the microspheres bearing larger pores (PS3-33-15E) declined sharply near the periphery of the microspheres. The diminished fluorescence intensity could be ascribed to the larger pores of these microspheres, which might result in a leakage of QDs to the surroundings. Meanwhile, the nonporous structure was found to be the least suitable structure for QD encapsulation. As confirmed by confocal microscopy images, the QDs had difficulty penetrating into the interiors of the nonporous microspheres (nPS). The study on these three classes of microspheres confirmed the importance of choosing an appropriate porous structure for improving their fluorescence intensities upon QD encapsulation.
In addition, the SE approach was shown to be effective for fabricating fluorescent microspheres with various emission wavelengths. Figure 6 shows fluorescence spectra and fluorescent microscopy images of microspheres encoded with 460, 520, 560, and 685 nm QDs. Still, there was about 5nm red-shift of fluorescence emission peak of QD-microspheres compared with non-encapsulated QDs. These results indicate that encoding microspheres with various QDs can provide a convenient route toward QD-encoded microspheres with different emission wavelengths and high brightness.

Single microspheres endowed with multiple distinguishable emission wavelengths would provide a greater diversity of potential codes and thus enlarge the microsphere barcode library. Theoretically, the number of barcodes would increase exponentially with the combination of QDs encoded within a single microsphere. Herein, we chose 520 and 685 nm emitting QDs as two encoding markers and attempted multiple encoding through the SE approach. Here multiple encoding was defined as encoding one microsphere sample with more than one type of QD. As shown by the fluorescence spectra in Figure 7, the characteristics of the emission spectra could be easily controlled by adjusting the molar ratio of two types of QDs. The emission spectra display distinct characteristics at three different ratios of 685 and 520 nm QDs.

3.3. Suspension immunoassays of QD-encoded microspheres. As a typical suspension assay technology, the xMAP platform developed by Luminex Corporation has attracted wide attention owing to its cost effectiveness, compact design, and reliable data processing. Herein, by designing and synthesizing carboxyl group bearing microspheres (PS3-17-15E) encoded with CdSeTe QDs possessing a 690 nm emission wavelength, we achieved successful determination of goat anti-mouse IgG by using the xMAP platform.

As a platform based on flow cytometry technology, fluorescent microspheres utilized on the xMAP platform should possess uniform size and fluorescence properties. Therefore, the 690 nm QD-microspheres were first tested on a flow cytometer to verify their feasibility in suspension assays. As shown in Figure 8a, the fluorescence signal of QD-microspheres display a narrow peak on the flow cytometric analysis histogram. In the histogram, the x-axis represents the fluorescence intensity of the
QD-microspheres in the FL4 channel (which detects fluorescence signals at 675 ± 15nm), the y-axis is the number of counts of the tested QD-microspheres, which was set as 10,000 microspheres. These results indicate that these QD-microspheres possess a uniform fluorescence signal and are promising candidates for use in suspension immunoassays on the xMAP platform.

To test these microspheres in suspension immunoassays, 690 nm QD-microspheres (PS3-17-15E) were coated with mouse anti-human chorionic gonadotropin as probes for capturing goat anti-mouse IgG. After IgG was captured by the probe-coated QD-microspheres, fluorescent dyes of streptavidin-phycoerythrin were added to couple with IgG as a reporter molecule. The final QD-microspheres bearing sandwich immunoassay structures on their surfaces were measured on a Luminex 100 system. In this apparatus, each single microsphere was excited by two lasers (511 nm for the reporter molecule, i.e. fluorescent dyes of streptavidin-phycoerythrin, and 621 nm for the QD-microsphere). Due to the unique fluorescence properties of the QDs, both lasers can excite the QDs of the microspheres to emit fluorescence signals. Therefore only one laser was needed for the excitation of these QDs-microspheres, which may help to reduce the costs of manufacturing apparatus in future applications. As shown in Figure 8b, highly positive signals were detected from QD-microspheres bearing sandwich immunoassay structures on their surfaces, whereas both the probe-coated QD-microspheres and the mixture of probe-coated QD-microspheres with reporter molecules showed negative fluorescence signals. These results indicated that there was no apparent non-specific adsorption occurring during the coupling process. Goat anti-mouse IgG could be detected effectively by probe-coated QD-microspheres, thus demonstrating the potential application of these QD-microspheres in suspension arrays.

Furthermore, QD-microspheres with low cytotoxicity would be ideal for various biomedical applications. Therefore, a MTT assay test was performed with HeLa cells to evaluate the cytotoxicity of the QD-microspheres. The cell viability was normalized to that of cells incubated without QD-microspheres as a blank control. As shown in Figure 9, the viabilities of the cells incubated with 690 nm QD-microspheres (PS3-17-15E) at three concentrations were above 100% at 1.5 × 10^5/mL, 78% at 1.5 × 10^6/mL and nearly 50% at 1.5 × 10^7/mL. These results indicate that these QD-microspheres have...
low cytotoxicity on HeLa cells at concentrations normally used in suspension arrays (~10^6 microspheres/mL).

4. Conclusions

In summary, porous PSDM microspheres with specific sizes, surface carboxyl groups, and various porous structures were prepared by seeded copolymerization. In order to improve the efficiency of QD incorporation into swollen microspheres, we developed a swelling-evaporation approach, in which the swelling process was combined with gradual evaporation of the solvent. Gradually concentration of the QDs in the dispersion solution was proposed as a strong driving force to accelerate the diffusion of QDs into the interiors of the microspheres. From this approach, it was demonstrated that appropriate choice of the porous structure could yield microspheres with high brightness and uniform fluorescence signals. Both single- and dual-color codes were also obtained by using various QDs to encode the microspheres. In addition, near infrared-emitting 690 nm QD-encoded microspheres were adopted to perform a suspension immunoassay for goat anti-mouse IgG on the xMAP platform. Encouraging results with good detection sensitivity and low cytotoxicity demonstrated the great potential of these QD-microspheres in suspension assays.

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Supporting Information Available. Recipes of preparing PS seed particles by disperse polymerization, SEM images of seed particles, characterization of the surface carboxyl groups of PSDM
microspheres, TEM images of CdSe and CdSeTe QDs, and the recipes of buffers for suspension immunoassays. This information is available free of charge via the Internet at http://pubs.acs.org.

References


Table 1. Recipes and properties of PSDM microspheres prepared by seeded copolymerization.

Figure 1. SEM images of (a) PS1-33-15, (b) PS2-33-15, (c) PS3-33-15, and (d) PS3-33-15E PSDM microspheres. PS1-33-15, PS2-33-15, and PS3-33-15 are PSDM microspheres polymerized from PS1, PS2, and PS3 seed particles, respectively. PS3-33-15E is a PS3-33-15 microsphere that was subjected to porogen extraction. In the synthesis of PS1-33-15, PS2-33-15, and PS3-33-15, the volume ratio of DVB in the monomer and the weight ratio of swelling monomers to the seeds ($r_{ms}$) were kept as 33 and 15, respectively.

Figure 2. Images (a1), (b1) and (c1) are SEM images of PS3-25-15, PS3-17-15, and PS3-10-15 microspheres, respectively, which were synthesized using different crosslinker concentrations. Images (a2), (b2), and (c2) are SEM images of PS3-25-15E, PS3-17-15E and PS3-10-15E microspheres, respectively. PS3-25-15E, PS3-17-15E and PS3-10-15E microspheres were obtained from PS3-25-15, PS3-17-15, and PS3-10-15 microspheres, respectively, via porogen extraction.

Figure 3. Images (a1), (b1) and (c1) are SEM images of PS3-17-22, PS3-17-30, and PS3-17-40 microspheres, which were synthesized using $r_{ms}$ of 22, 30, and 40, respectively. Meanwhile, (a2), (b2), and (c2) are SEM images of PS3-17-22E, PS3-17-30E, and PS3-17-40E microspheres, respectively. PS3-17-22E, PS3-17-30E, and PS3-17-40E microspheres were prepared from PS3-17-22, PS3-17-30, and PS3-17-40 microspheres respectively, via porogen extraction.

Scheme 1. Schematic Process for embedding QDs into porous PSDM Microspheres by the SE approach.

Figure 4. (a) Fluorescence spectra of 525 nm QD-microspheres (PS3-17-15E) prepared by the SE approach and the Swelling approach. $V_{BC}$ is the volume ratio of butanol to chloroform in a swelling solvent prepared by the swelling approach. (b) Schematic illustration of the primary diffusion driving force of the SE approach and the swelling approach.
Figure 5. (a) Fluorescence spectra of 685 nm QDs and 690 nm QD-microspheres with various porous structures, the QD-microspheres were encoded with 685 nm QDs. Images (b1) to (d1) are SEM images of PS3-33-15E, PS3-17-15E and nonporous microspheres (nPS), respectively. Images (b2) to (b4), (c2) to (c4), and (d2) to (d4) are laser confocal fluorescent images recorded at different focalized planes from the top to the bottom of microspheres for PS3-33-15E, PS3-17-15E and nPS, respectively, which were encoded with 685 nm QDs.

Figure 6. Images (a), (b), (c) and (d) are true-color fluorescence images of QD-microspheres (PS3-17-15E) with emission wavelengths at 465, 525, 565 and 690 nm. Image (e) shows corresponding fluorescence spectra of QD-microspheres with various emission wavelengths.

Figure 7. Fluorescence spectra of microsphere (PS3-17-15E) barcodes encoded with both 520 and 685 nm QDs at different molar ratios. The ratios marked in the figure are the molar ratios of 520nm and 685 nm QDs used in the embedding process.

Figure 8. (a) Flow cytometric analysis histogram of 690 nm QD-microspheres (PS3-17-15E), (b) suspension immunoassays of goat anti-mouse IgG on Luminex 100. Product-1 is obtained from probe-coated 690 nm QD-microspheres, Product-2 represents probe-coated 690 nm QD-microspheres that were reacted with reporter molecules, while Product-3 represents 690 nm QD-microspheres bearing a sandwich immunoassay structure. The reporter molecule used in the experiments is a fluorescent dye of streptavidin-phycoerythrin.

Figure 9. Cytotoxicity profiles of HeLa cells that were incubated with three concentrations of 690nm QD-microspheres (PS3-17-15E) as determined by an MTT assay test. Data represent mean ±SD, n = 3.
Table 1. Recipes and properties of PSDM microspheres prepared by seeded copolymerization.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Seeds (g)</th>
<th>St (mL)</th>
<th>DVB (mL)</th>
<th>MAA (mL)</th>
<th>BPO (g)</th>
<th>$D_{av}$ (µm)</th>
<th>CV $^d$</th>
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<td>1.00</td>
<td>0.06</td>
<td>7.15</td>
<td>6.05</td>
</tr>
</tbody>
</table>

$^a$ PS-$\alpha$-$\beta$: $\alpha$ is the volume ratio of DVB in the monomer, and $\beta$ is the weight ratio of swelling monomers to the seeds ($r_{ms}$).

$^b$ Seed particles: PS1 ($M_n = 87,900$), PS2 ($M_n = 28,400$), PS3 ($M_n = 17,500$).

$^c$ $D_{av}$ is the number average diameter, which is calculated from the diameters of 200 microspheres measured from SEM images.

$^d$ CV is the coefficient of standard deviation of microspheres, which is calculated as:

$$CV = \sqrt{\frac{\sum_{i=1}^{n} D_i^2 - D_{av}^2}{N}} / D_{av}$$

$D_i$: Diameter of a single microsphere; $N$: Number of microspheres measured from SEM images.
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