Use of semiconductor nanocrystals to encode microbeads for multiplexed analysis of biological samples

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Abstract. Microbeads encoded with semiconductor quantum dots (QDs) are suitable tools for multiplexed analyses of various biological markers using flow cytometry. We have prepared a panel of microbeads encoded with QDs of different colors emitting with different luminescence intensities using the layer-by-layer deposition technique, which consists in layering of alternately charged polyelectrolytes and negatively charged QDs onto the surface of microbeads. This method allows QDs to be separated with one or several polymer layers in order to prevent Förster resonance energy transfer (FRET) and the resultant quenching of QD fluorescence in multicolor microbeads.

1. Introduction

In recent years, interest in optically encoded microbeads has been growing due to their potential applications in medical diagnostics [1]. Microbeads can be encoded with either organic fluorophores or semiconductor quantum dots (QDs) [2–6]. The use of microbeads encoded with organic fluorophores with different fluorescence maxima requires complex equipment with multiple excitation lasers for decoding, which complicates the analysis and increases the cost of instrumentation. QDs constitute a new class of fluorophores that have a number of advantages over routinely used fluorescent organic dyes, such as a high brightness, resistance to photobleaching, a wide excitation spectrum, and a narrow emission spectrum (Figure 1) [7, 8]. The unique optical characteristics of QDs make it possible to use them in multiplexed analyses, as well as in multicolor imaging [9], because QDs of different colors can be simultaneously excited using a single radiation source, whereas their fluorescence peaks can be effectively separated and detected in different channels of a flow cytometer. Therefore, multiplexed analysis by means of flow cytometry using QD-encoded microbeads is easy to perform and does not require sophisticated equipment.
A number of approaches have been designed for engineering QD-encoded microparticles: embedding of QDs into the microbeads during the manufacturing [3], incorporation of QDs into pores of swollen microbeads, [2, 4], doping of mesoporous microbeads with QDs [5], the concentration-controlled flow-focusing technique [6], and layer-by-layer deposition [10]. By using several colors of QDs and several intensity levels, it is theoretically possible to create thousands or even millions of individual optical codes [2].

In this study, we have prepared a panel of microbeads encoded with QDs of different colors at different luminescence intensity levels using the layer-by-layer deposition technique, which consists in layering of alternately charged polyelectrolytes and negatively charged QDs onto the surface of microbeads. We established the number of QDs that was adsorbed onto the microbeads and investigated some physicochemical aspects of electrostatic adsorption of water-soluble negatively charged QDs onto a positively charged microbead surface. Fluorescence lifetime measurements were used for investigation of FRET between QDs in the solution and within QD-encoded microbeads. The technique of optical encoding developed in this study makes it possible to fabricate various multicolor microbead populations for multiplexed flow-cytometry analysis.

2. Materials and Methods

2.1 Preparation and characterization of water-soluble QDs
CdsSe/ZnS core/shell QDs [7] with fluorescence maxima at 508, 590, and 640 nm and CdsSe/ZnS/CdS/ZnS multishell QDs [9] were synthesized and kindly provided by Dr. Pavel Samokhvalov (Laboratory of Nano-Bioengineering, Moscow Engineering Physics Institute, Moscow, Russia). Originally water-insoluble QDs coated with trioctylphosphine oxide (TOPO) were solubilized by replacing TOPO with D.L-cysteine [11] and subsequently replacing the cysteine with derivatives of polyethylene glycol containing thiol and carboxyl terminal groups (SH-(PEG)_{12}-COOH, Thermo Fisher Scientific, USA) as described earlier [12]. The QD concentrations were determined from their extinction spectra at the positions of the first excitonic maxima [13]. The concentration of multishell QDs was estimated from the experimental values of molar extinction coefficient and molecular weight (100,000 g/(mol⋅cm) and 92,600 g/mol, respectively) (data not shown). The hydrodynamic sizes of water-soluble QDs were determined by dynamic light scattering measurements using a Zetasizer NanoZS analyzer (Malvern, UK).

2.2 Preparation of QD-encoded microbeads
Optical encoding of microbeads with QDs was performed using the adapted procedure of layer-by-layer deposition of alternately charged polymer layers and water-soluble QDs described in our
previous studies (Figure 2) [14, 15]. According to the adapted protocol, $3 \times 10^7$ carboxylated melamine resin microbeads with a diameter of 4.08 μm (Microparticles GmbH, Berlin, Germany) were suspended in 0.5 ml of ultrapure water and mixed with 0.5 ml of 2 mg/ml poly(allylamine hydrochloride) (PAH, Mw = 15,000 Da, Sigma-Aldrich, USA) dissolved in 0.5 M NaCl. The suspension was sonicated and incubated while shaking for 20 min; then, the microbeads were washed from excess polymer by triple centrifugation (2000g for 5 min) and resuspension in ultrapure water. For deposition of the next polymer layer, the microbeads were resuspended in 0.5 ml of ultrapure water and mixed with 0.5 ml of 2 mg/ml poly(sodium 4-styrenesulfonate) (PSS, Mw = 70,000 Da, Sigma-Aldrich, USA) dissolved in 0.5 M NaCl. As with the first layer, the suspension was sonicated and incubated while shaking for 20 min, and the microbeads were washed by triple centrifugation and resuspension in ultrapure water. The procedure of polymer deposition and washing was repeated until five alternately charged polymer layers were formed on the microbead surface, the outer layer consisting of positively charged PAH. Then, the microbeads were resuspended in 100 μl of ultrapure water, and various amounts (from 0.4 to 146 μg) of negatively charged water-soluble QDs were added while stirring the suspension. The mixture was incubated while shaking for 20 min, after which the microbeads were washed by three cycles of centrifugation and resuspension. For preparation of single-color microbeads, the next stage was the deposition of five layers of alternately charged polymers, as described above. In the case of two- or three-color microbeads, the deposition of QDs of different colors was alternated with deposition of positively charged PAH layers, after which five alternately charged polymer layers were deposited. The resultant encoded microbeads were stored at 4°C in the dark.

Figure 2. The method of layer-by-layer deposition [10] was used to obtain a population of microbeads encoded with QDs with different fluorescence peaks and fluorescence intensities: the surface of microbeads was successively coated with layers of differently charged polyelectrolytes and negatively charged QDs.

2.3 Analysis of photoluminescence properties
The fluorescence spectra of all samples were measured using an Infinite 200 PRO multimode reader (TECAN, Switzerland). The fluorescence decay times of QDs and QD-encoded microbeads were measured by means of a specially designed experimental setup. All the samples were excited with the use of a Tsunami pulse laser (Spectra Physics) (wavelength, 395 nm; pulse duration, 300 fs; pulse repetition rate, 8 MHz; pulse energy, 0.5 nJ). The photoluminescent signal from a sample was collected with an optical condenser and supplied to a monochromator, which was adjusted to the photoluminescence maxima of QDs. The signal from the detector (an avalanche photodiode) was analyzed using a PicoHarp time-correlated single photon counting (TCSPC) system (PicoQuant) with a set resolution of 256 ps.

2.4 Flow cytometry
QD-encoded microbeads were analyzed with the use of a FACSCanto flow cytometer (Becton Dickinson, USA). The three-color spectral code of the microbeads was excited with a blue laser (488 nm), and the fluorescence intensity was analyzed in three channels: FITC (530/30 nm), PE (575/25 nm), and PerCP (695/40 nm). The data were processed using the FACSDiva software (Becton Dickinson, USA).

2.5 Fluorescence microscopy
For studying the fluorescence of the QD-encoded microbeads, we used a Carl Zeiss Axio Scope A1 fluorescent microscope (Carl Zeiss, Germany). Before the experiment, QD-encoded microbead samples were mixed with a 20% glycerol solution and fixed on microscope slides. Imaging of the specimens was performed with the use of two optical filters, FITC (excitation wavelength, 495 nm; emission wavelength, 520 nm) and Alexa Fluor 568 (excitation wavelength, 568 nm; emission wavelength, 593 nm), and a Zeiss N-achroplan 40×/0.65 Ph2 lens. Image processing was performed using the ZEN2012 SP1 software (blue edition) (Carl Zeiss, Germany).

3. Results and Discussion
The layer-by-layer deposition technique for optical encoding of microbeads consists in applying layers of alternately charged polyelectrolytes and negatively charged QDs on the surface of microbeads. Polystyrene sulfonate (PSS) and polyallylamine (PAH) contain sulfo and amino groups ensuring the formation of multiple salt bonds between the polymers. For layering the polymers, preliminarily carboxylated microbeads are incubated with PAH to form a positively charged polymer layer on their surface. Then, excess PAH is removed from the solution, and negatively charged PSS is added. The layering is repeated until five polymer layers, the outer layer consisting of PAH, are formed. Then, the microbeads are incubated in the presence of negatively charged QDs carrying carboxyl groups on their surface. The QDs are electrostatically adsorbed onto the microbeads and form their optical code. Finally, five additional polymer layers are deposited onto the microbeads to give them stability.

Populations of monocolor microbeads encoded with QDs with fluorescence maxima at 508, 590, and 640 nm were studied in detail using flow cytometry. The range of fluorescence was analyzed in different channels of the flow cytometer (Figure 3). Fluorescence of microbeads encoded with QDs was excited using a single light source (a laser with a wavelength of 488 nm). Microbeads encoded with QDs had a high level of fluorescence and were detected at a high resolution.

![Figure 3](image-url). Histograms of populations of microbeads encoded with QDs of different colors. The range of fluorescence analyzed in different channels of the flow cytometer.
We estimated the maximum number of adsorbed QDs on the surface of microbeads. For this purpose, we compared the absorbance spectra of QDs in the stock solution and in the supernatant after incubation with microbeads. From the values of molecular weight and emission maximum of QDs, we calculated that number of QDs adsorbed on the surface of a single microbead, 4.08 µm in diameter, is \(1.8 - 2 \times 10^6\).

We performed fluorescence lifetime measurements of QDs in the solution and the same QDs adsorbed on microbeads. The photoluminescence decays of QDs emitting at 508 nm (QDs\(_{508}\)) in the solution and those adsorbed on microbeads are shown in Figure 4. The decay was significantly faster for QDs adsorbed on microbeads than for QDs in the solution. Specifically, adsorption onto microbeads shortened the decay time of QD\(_{508}\) fluorescence from 16.1 to 5.1 ns. This may have been accounted for by FRET between QDs [16] or specific influence of PAH, which interacts with functional groups on the QD shell surface.

In multi-color QD-encoded microbeads, distance-dependent optical effects, such as FRET and photon reabsorption, may occur, affecting the resultant relative levels of the QD fluorescence intensity [6]. A way to reduce these optical effects is to spatially separate QDs of different colors, so that the distance between them is larger than the Förster energy transfer radius (5–8 nm for QDs) [17]. Furthermore, homo-FRET within a monochromatic QD population may occur due to distribution of QD sizes within the population and, hence, broadening of their fluorescence peak [16]. An overlap between the absorbance and emission spectra of the QD population leads to quenching of the blue side and enhancement of the red side of the photoluminescence peak, causing a red shift of the photoluminescence spectrum.

In order to prevent FRET between two QD populations, we spatially separated them with one or several polymer layers during the encoding process. We prepared single-color microbeads encoded with QDs\(_{508}\) (1) and two types of bicolor microbeads encoded with QDs\(_{508}\) and QDs\(_{590}\) that are spatially separated from each other by one (2) or three (3) polymer layers. The decay times of the samples were measured upon excitation at 508 nm (Table 1). All the three samples were characterized by comparable decay times of about 8 ns, which were almost independent of the number of polymer layers between two types of QDs and were considerably shorter than that in the QD\(_{508}\) solution (16 ns). If there were to be FRET, the decay times in cases (2) and (3) would be shorter than in case (1). However, reduction of decay time was not observed in these samples, which confirms the absence of

![Figure 4. Fluorescence lifetime of QDs\(_{508}\) in the solution and those adsorbed on microbeads.](image-url)
FRET. This result has allowed us to use an effective strategy of FRET prevention in preparing multicolor microbeads with a hundred of optical codes.

Table 1. The average decay time ($t_{av}$) values for QDs508 in a solution, single-color microbeads encoded with QDs565, and two types of bicolor microbeads encoded with QDs508 and QDs590 spatially separated by one and three polymer layers, respectively.

| Sample | QDs508 in solution | Single-color QD508-encoded microbeads | Bicolor microbeads encoded with QDs508 and QDs590 separated by: |
|--------|-------------------|-------------------------------------|---------------------------------------------------------------|
|        |                   |                                     | One polymer layer | Three polymer layers |
| $t_{av}$, ns | 16.0              | 7.35                                | 7.86              | 9.5                  |

The populations of QD-encoded microbeads have been studied in detail using fluorescence microscopy. QD-encoded microbead samples were mixed with 1% glycerol and fixed on microscope slides. Imaging of the specimens was performed with the use of two optical filters, FITC (excitation wavelength, 495 nm; emission wavelength, 520 nm) and Alexa Fluor 568 (excitation wavelength, 568 nm; emission wavelength, 593 nm), and a Zeiss N-achroplan 40×/0.65 Ph2 lens. The microbeads had a high brightness and were easily discerned under the microscope (Figure 5).

4. Conclusions
In this study, we have investigated some physicochemical aspects of electrostatic adsorption of negatively charged QDs on a positively charged microbead surface. In particular, we have estimated the maximal number of QDs that may be adsorbed in a single stage on the surface of a single microbead. We have also developed a technique of multistage QD adsorption for preparation of a panel of QD-encoded microbead populations with a wide range of fluorescence intensities. Using fluorescence lifetime measurements, we have found that the layer-by-layer deposition technique prevents FRET between QDs of different colors adsorbed at different stages.

The layer-by-layer technique for optical encoding of polymer microbeads with water-soluble QDs developed in this study makes it possible to obtain microbead populations for multiplexed analysis of biological markers using flow cytometry.
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