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MEDICAL PHYSICS AND BIOPHYSICS

Design of Optically Encoded Microspheres of Different Sizes for Multiplexed Flow Cytometry

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Abstract—The development of innovative fluorescence detection methods based on optically encoded microspheres of different colors and sizes is an important area of multiplexed detection and diagnosis of various diseases. For example, the xMAP technology of the American company Luminex employs polystyrene microspheres labeled with two or three organic fluorophores at different ratios, each with its unique spectral characteristics. Although xMAP can detect as many as 80 proteins or DNA sequences in a single test system, the need to use special equipment, produced only by the same company, for multiplexed analysis, as well as inherent disadvantages of organic fluorophores (a large Stokes shift and photobleaching under laser excitation) limit the applications of this technology. The objectives of this study were to design and fabricate microspheres encoded with semiconductor quantum dots. Carboxylated melamine-formaldehyde microspheres of three sizes were optically encoded with quantum dots of two colors immobilized between layers of oppositely charged polyelectrolytes on the surface of the microspheres. As a result, six populations of microspheres with different sizes and/or unique optical codes were obtained, characterized by a long-term stability and homogeneity in aqueous solutions. Analysis of the microspheres by the dynamic light scattering, epifluorescence microscopy, and flow cytometry methods showed their suitability for multiplexed analysis. At the same time, the use of quantum dots for optical encoding makes it possible to exclude photodegradation of the signal and to excite all quantum dot populations at the same wavelength of radiation, with effective separation of signals from the microspheres into different channels of a standard flow cytometer.

Keywords: microspheres, quantum dots, fluorescence, multiparametric analysis, flow cytometry, diagnostics

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INTRODUCTION

Modern biomedical research urgently needs high-throughput and highly sensitive detection technologies, which become feasible due to the development of multiplexed systems with an extended range of detectable concentrations enabling simultaneous quantitative measurement of several analytes with high speed and accuracy [1, 2]. Molecular diagnostic technologies using microspheres optically encoded with fluorescent dyes (fluorophores) are of particular interest for multiplexed bioanalysis because they are easy to use and optical signal reading equipment (microscopes, readers, and flow cytometers) is widely available in clinical practice [2].

The Luminex and BD Biosciences companies have developed multiplexed systems based on microspheres optically encoded with classical organic dyes [3]. The surface of each type of microspheres contains capture molecules, which specifically interact with the markers (analytes) under study. A Luminex flow cytometer adapted for reading xMAP microsphere signals is used to read the spectral address of a specific microsphere and the signal of the reporter fluorophore, whose intensity depends on the amount of the bound analyte [4, 5]. Although a single test system is capable of

Table 1. The sizes and surface charges of the modified water-soluble quantum dots (QDs)

No.	Sample	Temperature, °C	Hydrodynamic diameter, nm*	Standard deviation	ζ-potential, mV*	Standard deviation
1	QD-534 (SH)-PEG-COOH	25	30.17	0.68	−55.4	1.81
2	QD-593 (SH)-PEG-COOH	25	29.36	0.21	−37.0	1.77

* The average of three independent measurements.

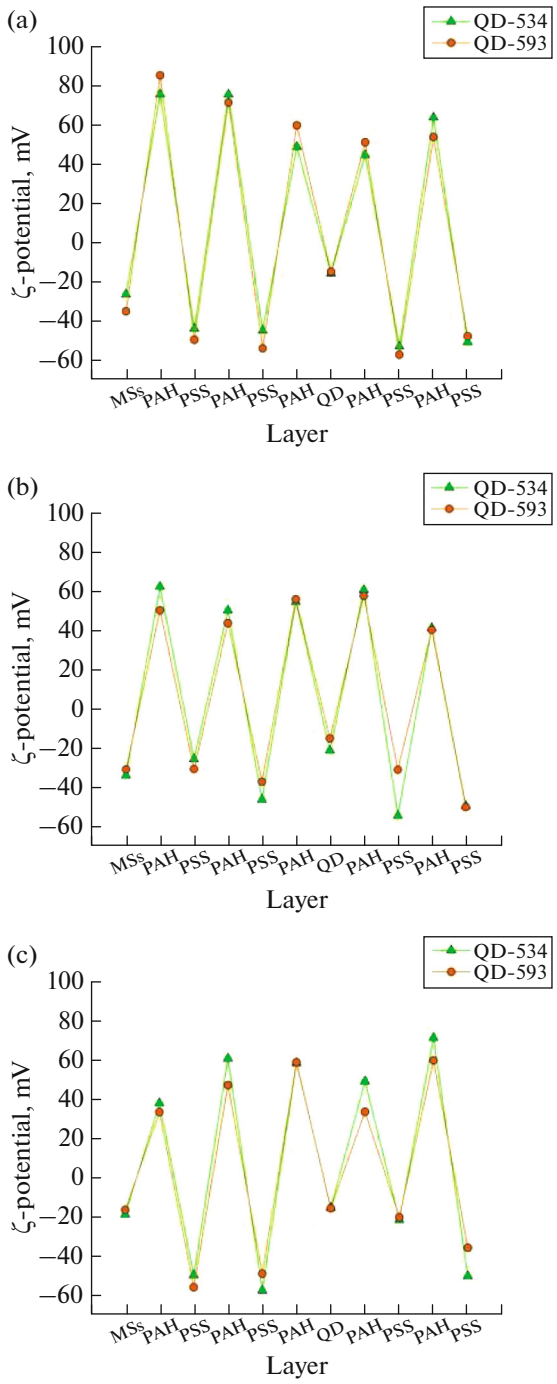

Fig. 1. Changes in the surface charge in the course of layer-by-layer-deposition of polyelectrolytes onto the surface of melamine-formaldehyde microspheres (a) 4.02, (b) 6.10, and (c) 8.24 μm in diameter. Designations: QDs, quantum dots; MSs, melamine-formaldehyde microspheres; PAH, poly(allylamine hydrochloride); PSS, poly(sodium 4-styrene sulfonate).

detecting as many as 80 proteins or DNA sequences, the need to use special equipment for multiplexed analysis, produced only by the same company, and inherent disadvantages of organic fluorophores (a large Stokes shift and photobleaching under laser excitation) limit the applications of this technology. Indeed, the spectral properties of organic dyes require special equipment with several excitation lasers for reading the optical code, which makes the analysis time-consuming and expensive. These problems can be solved by using semiconductor quantum dots (QDs) for optical encoding. They are characterized by a high quantum yield, resistance to photodegradation upon excitation with laser irradiation, and a quasi-continuous absorption spectrum, which makes it possible to excite fluorescence of QDs of different colors at the same radiation wavelength [6–15]. The unique optical properties of QDs make them ideal candidates for multiplexed analysis using standard flow cytometers: QDs can be excited from a single radiation source, and their fluorescence peaks can be effectively separated and detected in different channels of the flow cytometer [7]. Thus, multiplexed flow cytometry analysis using QD-encoded microspheres is easy to perform and does not require special equipment.

Here, we obtained six populations of melamine-formaldehyde microspheres of different sizes encoded with QDs of different colors (with fluorescence maxima at 534 and 593 nm; QD-534 and QD-593, respectively) with different levels of fluorescence intensity. Optically encoded microspheres were obtained via layer-by-layer deposition of alternating layers of oppositely charged polyelectrolytes and negatively charged QDs on the surface of the melamine-formaldehyde microspheres.

METHODS

We used carboxylated melamine-formaldehyde microspheres with diameters of 4.02, 6.1, and 8.24 μm (Microparticles, GmbH). Stable water-soluble QD-534 and QD-593 consisting of a cadmium selenide (CdSe) core coated with a zinc sulfide (ZnS) shell were obtained using a two-step procedure that consisted of the substitution of a trioctane phosphine (TOPO) organic shell with a DL-cysteine shell and subsequent substitution of cysteine with a low-molecular-weight polyethylene glycol derivative containing thiol (–SH) and carboxyl (–COOH) groups [1]. The concentration of the obtained QDs was determined spectrophotometrically using a Cary 60 spectrophotometer (Agilent Technologies). The principle of optical encoding of microspheres was to coat the carboxylated melamine-formaldehyde core with a multilayer polymer shell containing embedded water-soluble QDs. The method of layer-by-layer deposition of oppositely charged polyelectrolytes, poly(allylamine hydrochloride) (PAH) with $M_w \approx 65\,000$ Da and poly(sodium 4-styrene sulfonate) (PSS) with $M_w \approx 70\,000$ Da, according to a previously described procedure [15] was used for the formation of the polymer shell. The size and surface charge of the water-soluble QDs and those of the optically encoded microspheres

Table 2. The sizes of the microspheres (MSs) encoded with the QD-534 and QD-593 quantum dots

No.	Sample (size); luminescence wavelength	Temperature, °C	Polydispersity index	Hydrodynamic diameter, μm
1	MSs (4.02 μm); 534 nm	25	0.268	4.449
2	MSs (4.02 μm); 593 nm	25	0.240	4.453
3	MSs (6.10 μm); 534 nm	25	0.152	6.887
4	MSs (6.10 μm); 593 nm	25	0.298	6.931
5	MSs (8.24 μm); 534 nm	25	0.267	9.128
6	MSs (8.24 μm); 593 nm	25	0.305	8.968

after depositing each polymer layer were measured using an analyzer of dynamic light scattering and ζ-potential of particles (Zetasizer Nano ZS, Malvern Instruments). The optical characteristics of the encoded microspheres with different diameters were studied using an epifluorescence microscope (Nicon Eclipse TE2000-S). The QD-encoded microspheres were analyzed using a flow cytometer (FACSCantoII, Becton Dickinson). The fluorescence of the QDs, which determined the optical codes of the microsphere populations, was excited using an argon laser with an emission wavelength of 488 nm. The fluores-

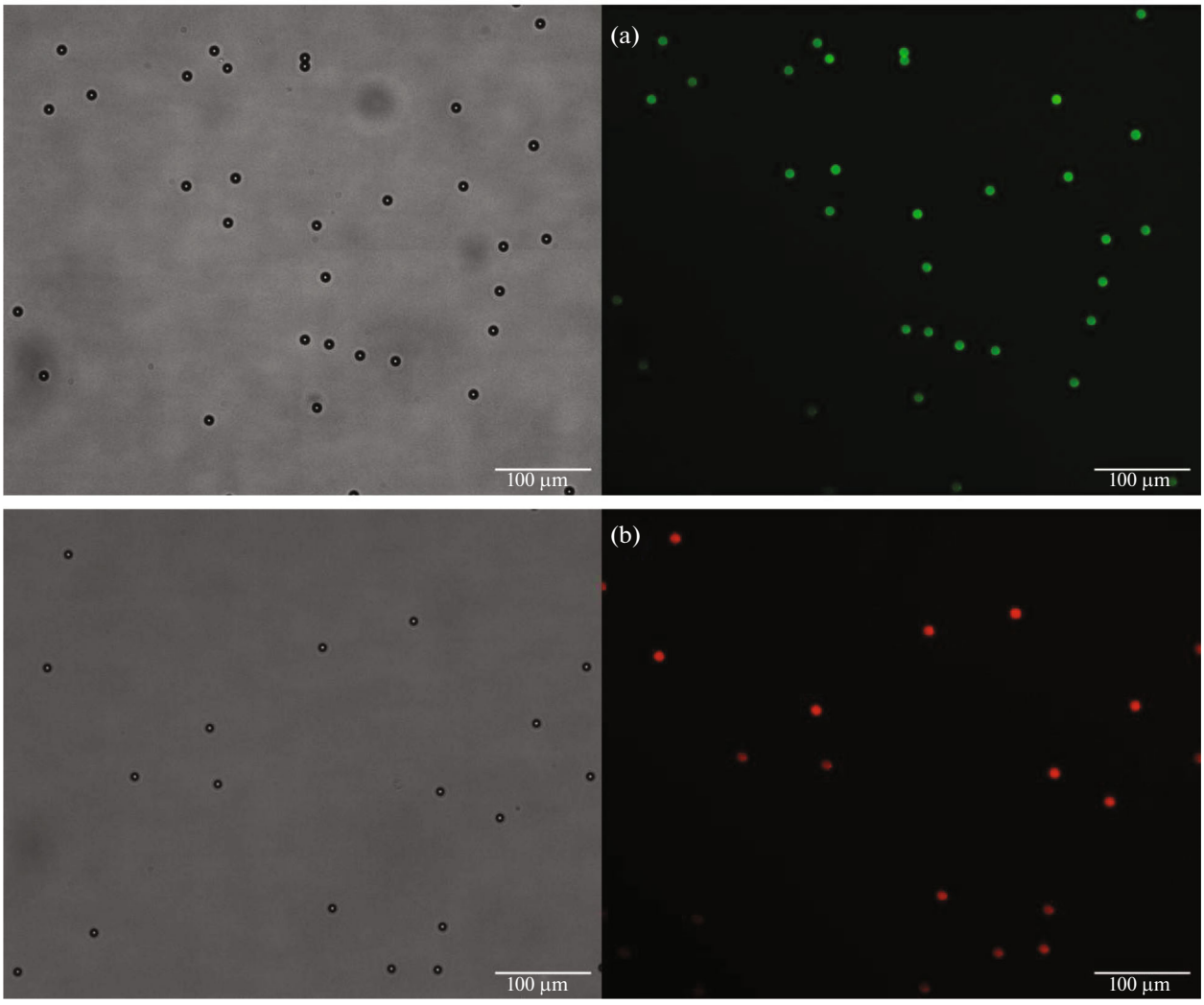

Fig. 2. Microspheres of different sizes encoded with quantum dots (QDs) of different colors. (a) 8.97-μm microspheres encoded with QD-593, (b) 9.12-μm microspheres encoded with QD-534. Left, light microscopy images; right, fluorescence images.

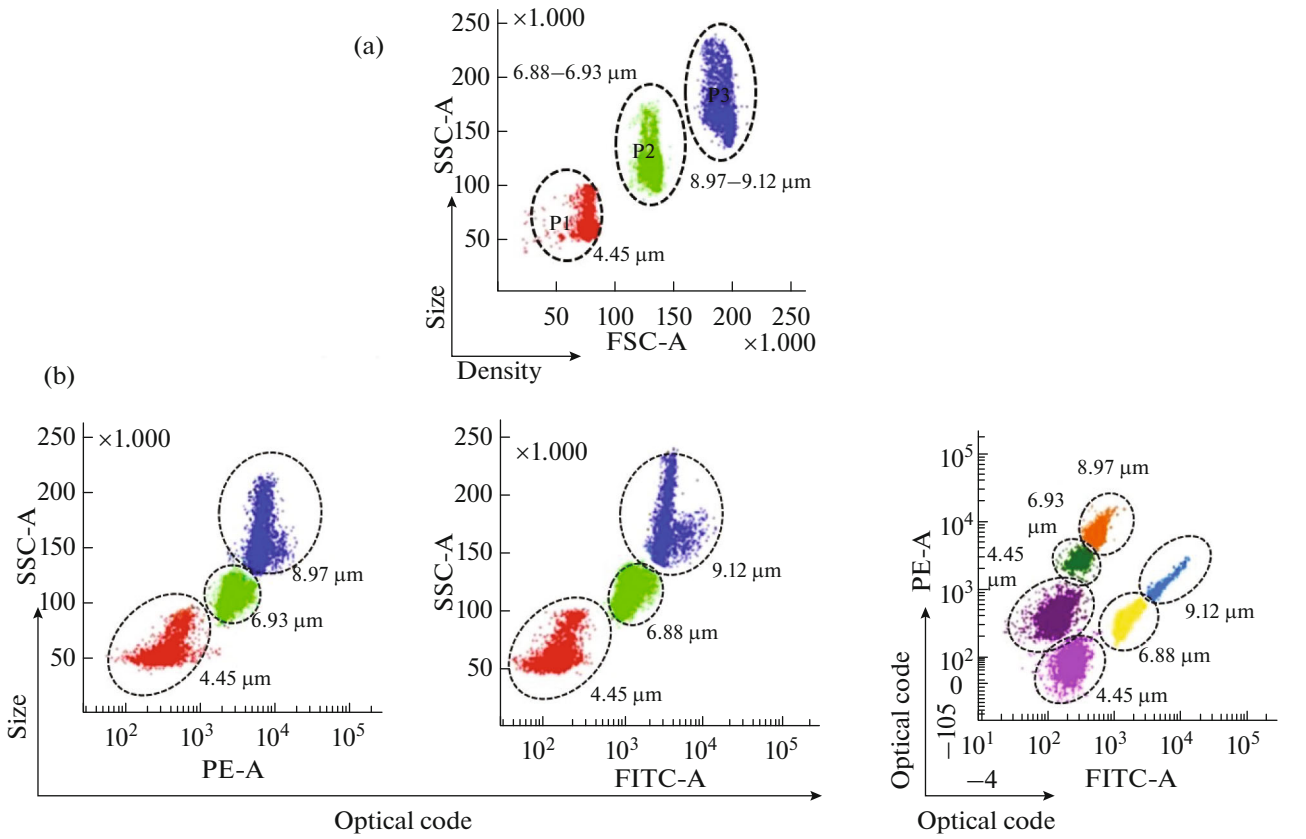

Fig. 3. Results of flow cytometry analysis of fluorescent microspheres encoded with quantum dots. (a) Differentiation of the microspheres by size and density using light scattering analysis, (b) differentiation of the microspheres by optical code. SSC, side scatter; FSC, forward scatter; FITC, fluorescein; PE, phycoerythrin.

cence intensity was determined in two standard channels of the flow cytometer: FITC (530/30 nm) and PE (575/25 nm).

RESULTS AND DISCUSSION

At the first stage, water-soluble QD-534 and QD-593 modified with the (SH)-PEG-COOH polymer (with fluorescence maxima of 534 and 593 nm) were obtained at concentrations of 4.9 and 5.4 mg/mL, respectively. The hydrodynamic diameters and ζ-potentials of these QDs were estimated (Table 1).

Layer-by-layer adsorption of oppositely charged polyelectrolytes was used for optical encoding of the microspheres [1]. The original carboxylated melamine-formaldehyde microspheres of different diameters had a negative surface charge, which promoted efficient adsorption of the positively charged polymer (PAH). This led to a change in the surface charge of the microspheres to the positive one, which, in turn, allowed the adsorption of the negatively charged polymer (PSS), and so on, until a shell of several alternating layers of oppositely charged polymers was formed. The water-soluble QDs modified with (SH)-PEG-COOH had a negative charge, which favored their adsorption onto the positively charged PAH layer. In this way, we used layer-by-layer deposition of polyelectrolytes and QDs to obtain six types of microspheres with the shell compositions shown in Fig. 1.

The sizes of the QD-encoded microspheres (Table 2) were determined by means of an analyzer of dynamic light scattering and ζ-potential.

The optically encoded microspheres were studied by means of epifluorescence microscopy. The images were acquired using two optical filters, FITC and PE(CY3), and a 20 × 0.35 objective lens. The analysis has shown that the resultant QD-encoded microspheres exhibit bright fluorescence and high homogeneity (Fig. 2).

Six types of the microspheres encoded with QDs fluorescing at 534 and 593 nm were analyzed by flow cytometry. The results have shown that the locations of the microsphere populations in the forward and side light scatter diagrams allow the separation of microspheres by size (Fig. 3a). The optical codes of the microsphere populations encoded with QD-534 and QD-593 were detected in the FITC and PE channels, respectively, which made it possible to separate them according to optical codes (Fig. 3b). It is important to

emphasize that the populations of microspheres of different sizes and with different optical codes are distinctly differentiable by means of flow cytometry.

The intensity of fluorescence of the microspheres has been shown to depend on their size. The fluorescence intensity of the 4.02-μm microspheres was approximately 10^2 arb. units, and that of the 6.1 and 8.24 μm microspheres ranged from 10^2 to 10^4 arb. units.

The results of cytometric analysis have shown that microspheres of different diameters encoded with QD-534 and QD-593 are homogeneous and can be identified on the basis of their size and color. The optically encoded microspheres can be distinctly separated in a mixture of fluorescent microspheres, which ensures their accurate differentiation. Thus, the obtained QD-encoded microspheres can serve as a basis for a multiplexed analysis system using flow cytometry.

CONCLUSIONS

Thus, we have proposed a design of microspheres of different sizes optically encoded with QDs fluorescing at 534 and 593 nm and fabricated these microspheres. They are characterized by high homogeneity and stability and a bright fluorescence, which have been confirmed by dynamic light scattering and epifluorescence microscopy analyses. The optically encoded microspheres are efficiently separated into different channels of a flow cytometer upon excitng at the same wavelength of laser irradiation and are resistant to photodegradation. Thus, the method of optical encoding of polymeric microspheres with water-soluble QDs through layer-by-layer adsorption of oppositely charged polyelectrolytes yields populations of fluorescent microspheres with properties superior to those of traditional systems based on organic fluorophores. These microspheres are good candidate components of test systems for multiplexed analysis with the use of flow cytometry.

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CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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