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Multiplexed analysis of serum breast and ovarian cancer markers by means of suspension bead–quantum dot microarrays

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Abstract

Multiplexed analysis of cancer markers is crucial for early tumor diagnosis and screening. We have designed lab-on-a-bead microarray for quantitative detection of three breast cancer markers in human serum. Quantum dots were used as bead-bound fluorescent tags for identifying each marker by means of flow cytometry. Antigen-specific beads reliably detected CA 15-3, CEA, and CA 125 in serum samples, providing clear discrimination between the samples with respect to the antigen levels. The novel microarray is advantageous over the routine single-analyte ones due to the simultaneous detection of various markers. Therefore the developed microarray is a promising tool for serum tumor marker profiling.

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Keywords: quantum dots; cancer markers; lab-on-a-bead; multiplexed analysis; flow cytometry.

1. Introduction

The development of multiplexed fluorescent suspension arrays is currently of particular interest for clinical diagnostics (Nolan et al. (2002); Elshal et al. (2006); Sukhanova et al. (2008)). This technology uses combinations of fluorophores incorporated into microparticles to obtain individual spectral codes (Czarnik (1997)). Fluorophore-encoded beads can be rapidly analyzed using classical flow cytometry. Microparticles can be optically encoded with either conventional organic dyes or novel advanced fluorophores, inorganic semiconductor fluorescent nanocrystals or quantum dots (QDs). Specific characteristics of conventional organic dyes considerably restrict the number of their possible combinations, limiting the number of color sets in a suspension array. QDs have unique advantages over classical organic fluorophores (Resch-Genger et al. (2008)). These are, e.g., high extinction coefficients and, hence, a high brightness; narrow, symmetrical fluorescence peaks; the possibility to excite QDs of different colors with a single light source and a rock-stable photostability (Nabiev et al. (2008)). Due to the unique spectral characteristics, QDs can act as efficient donors for Förster resonance energy transfer (FRET) to a suitable acceptor (Akinfiyeva et al. (2012)). This significantly improves the detection quality and increases the sensitivity of diagnostic assays. The benefits of FRET-based suspension arrays were demonstrated in our earlier study (Sukhanova et al. (2007)). Recently, we have developed an alternative diagnostic suspension bead–QD based array for identification of prostate-specific antigens serving as molecular markers of prostate pathology in serum samples from cancer patients.

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Nomenclature

Abs	antibodies
ELISA	enzyme-linked immunosorbent assay
CA	cancer antigen
CEA	carcinoembryonic antigen
QDs	quantum dots

In this study, we have designed a similar highly sensitive and specific diagnostic system based on QD-encoded microbeads to detect specific markers of female reproductive system tumors. Preparation of optically encoded fluorescent microbeads of different sizes intended for immunodiagnosics is based on layer-by-layer electrostatic deposition of charged polymers onto the charged surface of polystyrene latex beads. Water-soluble CdSe/ZnS QDs emitting in the orange region (585 nm) were deposited between polymer layers to form an individual optical code (Brazhnik et al. (2014); Brazhnik et al. (2015)). This technology makes it possible to obtain an almost unlimited number of individual identification codes for biomolecule tagging by using multiple QD color combinations and different sizes of encoded microparticles.

We prepared sets of QD-encoded microbeads of different sizes and bound capture antibodies (Abs) against different cancer biomarkers (CA 15-3, carcinoembryonic antigen (CEA), and CA 125) to their surfaces. These cancer-specific biomarkers are generally found in serum of women with reproductive system disorders, in particular, breast cancer. The capture monoclonal Abs were chemically linked to the bead polymer shell. These antigen-specific microbeads have been calibrated with the calibrator standards and used to test serum samples from cancer patients in comparison to healthy donors. A set of serum samples from patients with different stages of breast cancer and from healthy donors were collected for quantitative analysis of target biomarkers. The collected data were compared with the results of the “gold standard” enzyme-linked immunosorbent assay (ELISA).

The results obtained pave the way to the development of multiplexed arrays based on QD-encoded beads as an advanced alternative to the conventional techniques of cancer marker detection, especially for early diagnosis.

2. Experimental part

2.1. Preparation of suspension array based on QD-encoded beads

CdSe/ZnS semiconductor fluorescent nanoparticles or QDs emitting at 585 nm were synthesized from organometallic compounds by colloidal chemistry methods (Sukhanova, Even-Desrumaux et al. (2012); Stsiapura et al. (2006)) their surface properties were analyzed using Fourier Transform Infrared Spectrophotometer (FTIR-8400S, Shimadzu); QDs were kindly provided by Dr. Pavel Samokhvalov (Laboratory of Nano-Bioengineering, Moscow Engineering Physics Institute, Moscow, Russia). The QDs were solubilized with derivatives of polyethylene glycol containing both thiol and carboxyl groups (Thermo Fisher Scientific, Moscow, Russia) as described previously (Brazhnik et al. (2015); Sukhanova et al. (2004)).

Carboxylated melamine resin microparticles of different diameters (4.08, 6.1, and 8.24 μm) were purchased from Microparticles GmbH (Berlin, Germany) and used as matrix cores for the preparation of QD-encoded microbeads. Differently charged organic polyelectrolytes, namely, poly(allylamine hydrochloride), poly(sodium 4-styrenesulfonate) and sodium salt of poly(acrylic acid) (Sigma-Aldrich, Moscow, Russia) were used to form multilayer shells on the surface of the carboxylated particles (Susha et al. (2000)). Water-soluble CdSe/ZnS QDs emitting at 585 nm were attached to the pre-surface layers of monodispersed polymer-coated melamine resin microbeads with different diameters using the procedure of layer-by-layer assembly as described previously [Brazhnik et al. (2015)].

Monoclonal capture Abs, detection Abs (different for different antigens), and standard ELISA kits for three markers of female reproductive system tumors (CA 15-3, CEA, CA 125) were purchased from Fujirebio Diagnostics, Inc (Göteborg, Sweden). Capture monoclonal antibodies were conjugated to the surface of QD-encoded microbeads through carbodiimide crosslinkers (Thermo Fisher Scientific, Moscow, Russia). Detection Abs were biotinylated with the Sulfo-NHS-LC-biotin reagent (Thermo Fisher Scientific, Moscow, Russia) according to the manufacturer's protocol. The secondary fluorescent label, streptavidin-Tri-COLOR, was purchased from Sigma-Aldrich (Moscow, Russia). Serum samples from patients with breast cancer and healthy donors were provided by Blokhin Cancer Research Center of the Russian Academy of Medical Sciences (Moscow, Russia).

2.2. Immunodiagnostic analysis with the use of the array for cancer marker profiling based on QD-encoded beads

To perform immunodiagnostic suspension analysis, we employed the “lab-on-a-bead” detection principle (Brazhnik et al. (2015)). According to it, monoclonal capture Abs against each target antigen (CA 15-3, CEA, and CA 125) were chemically linked to the surface of the respective population of QD-encoded beads (Table 1).

Capture Abs were conjugated with the microbead polymer shell using carbodiimide chemistry according to the manufacturer's protocol. The beads were then incubated with blocking solutions (containing bovine serum albumin and casein) to prevent nonspecific binding and false-positive results.

Table 1. Individual QD-encoded bead populations and specific antibodies adapted for simultaneous detection of markers of female reproductive system tumors.

Bead population	Cancer marker	Capture Ab (Fujirebio Diagnostics)	Detection Ab (Fujirebio Diagnostics)
4.08 μm /QD 585 nm	CA 15-3	Ma695	Ma552
6.1 μm /QD 585 nm	CEA	12-140-10	12-140-1
8.24 μm /QD 585 nm	CA 125	Ov197	Ov185

To balance/equilibrate the effective working surface of the antigen-specific populations of beads of different diameters, the total effective amount of 6.1- and 8.24- μm microbeads was calculated as follows:

$$N = \frac{50000 \times s(4.08 \mu\text{m})}{S} \quad (1)$$

Here, N is the amount of 6.1- and 8.24- μm microbeads for each analysis, S is the surface area of the 6.1- and 8.24- μm microbeads, and $s(4.08 \mu\text{m})$ is the surface area of the 4.08- μm microbeads.

The antigen-specific fluorescent microbeads were calibrated using the corresponding commercial standards (Fujirebio Diagnostics). For each analysis, 50 000 beads with the diameter of 4.08 μm and the corresponding calculated amounts of 6.1- and 8.24- μm microbeads were used. For clinical tests, a mixture of the three antigen-specific microbead populations was sequentially incubated with all components of the immunodiagnostic complex. The resultant complete surface immune complexes consisted of capture monoclonal Abs, the target analyte (cancer antigen), biotinylated detection monoclonal Abs, and the streptavidin-linked fluorophore (streptavidin-Tri-COLOR). The microarrays based on QD-encoded microbeads were processed and analyzed using classical flow cytometry (FACSCanto II, Becton Dickinson, USA).

Serum samples from patients with different stages of breast cancer and samples from healthy donors were collected for quantitative analysis of cancer serum antigens. All the samples were analyzed using the "gold standard" ELISA test to determine the exact concentration according to the standard clinical diagnostic requirements. Several cancer-positive serum samples have been analyzed using the designed multiplexed suspension array in comparison with serum of healthy donors serving as a control.

3. Results and discussion

3.1. Characteristics of the suspension array based on QD-encoded beads

The adapted procedure of charged polymer deposition onto the surface of beads and incorporation of water-soluble carboxylated QDs was shown to be suitable for preparing optically encoded microparticles and using them in immunodiagnostic suspension arrays (Sukhanova et al. (2007); Brazhnik et al. (2014); Brazhnik et al. (2015)). The resultant single-color QD-encoded microbeads exhibited intense fluorescence with emission wavelengths that were nearly identical to those of the original QDs (data not shown). Flow cytometry analysis demonstrated that individual microbead populations (4.08 μm /QDs 585 nm, 6.1 μm /QDs 585 nm, and 8.24 μm /QDs 585 nm) were extremely bright and homogenous and were clearly distinguishable in the mixture by size and optical code (see Fig. 1).

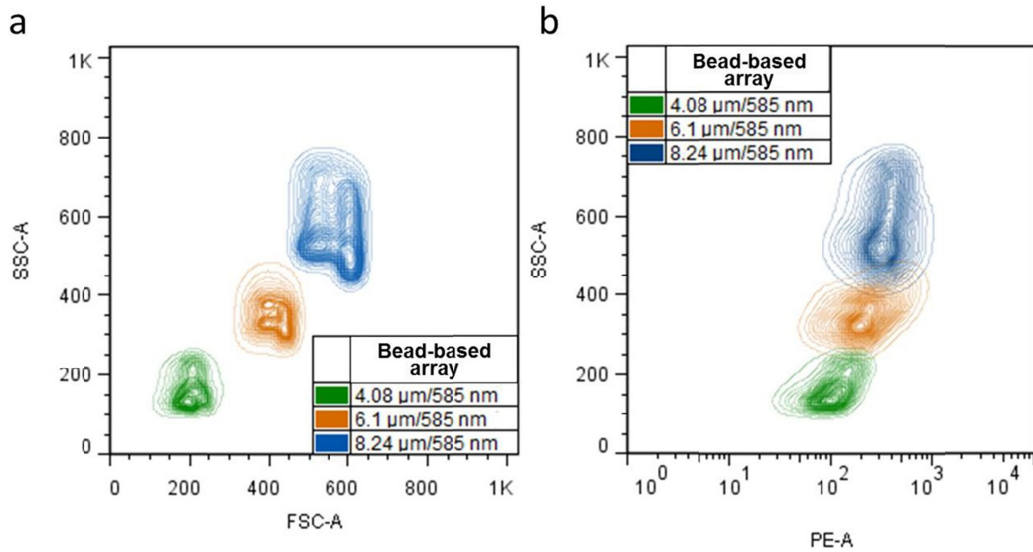


Fig. 1. The results of flow cytometry analysis of the mixture of QD-encoded bead populations (4.08 μm /QDs 585 nm, 6.1 μm /QDs 585 nm, and 8.24 μm /QDs 585 nm) showing the microparticle (a) size and granularity (FSC-A and SSC-A) and (b) fluorescence (PE-A) distributions. FSC-A, size; SSC-A, granularity; PE-A, optical code (QD 585 nm fluorescence).

3.2. Flow cytometry analysis of immunodiagnostic arrays based on QD-encoded beads

Specific capture monoclonal Abs against the cancer markers CA 15-3, CEA, and CA 125 were conjugated with carboxyl groups on the surface of 4.08 μm /QDs 585 nm, 6.1 μm /QDs 585 nm, and 8.24 μm /QDs 585 nm microbeads, respectively.

In order to perform accurate multiplexed analysis for profiling and quantitative detection of the three cancer markers with the use of the designed suspension array, we first analyzed calibrator samples containing precise concentrations of recombinant antigens. For testing each clinical sample, we prepared fresh conjugates of the beads and capture Abs, and the conjugation efficiency was estimated. The results of calibrator sample analysis were used to plot calibration curves (data not shown) for accurate quantification of the amount of the target biomarkers in clinical samples of serum.

Serum samples taken from patients with different stages of breast cancer and serum samples from healthy donors were analyzed using the “gold standard” ELISA test to determine the precise antigen concentrations according to the standard clinical diagnostic requirements and correlate these results with our data (data not shown).

For clinical tests with the designed suspension array, we used several clinical samples from breast cancer patients with elevated levels of CA 15-3, CEA, and CA 125 and compared them with control samples from healthy donors. The data were validated by comparing with the results of the “gold standard” ELISA.

The results of flow cytometry analysis using QD-encoded microbeads corresponded to those of quantitative detection with the use of the standard clinical diagnostic techniques. The fluorescence shift to the red region (Tri-COLOR label, 670 nm) after incubation of the antigen-specific bead mixture with the serum of cancer-positive patients (see Figs. 2a, 2b) as compared with the serum of cancer-negative patients (see Fig. 2b) provided clear discrimination between the samples with respect to the antigen content. The results entirely agreed with those of traditional ELISA detection techniques (data not shown).

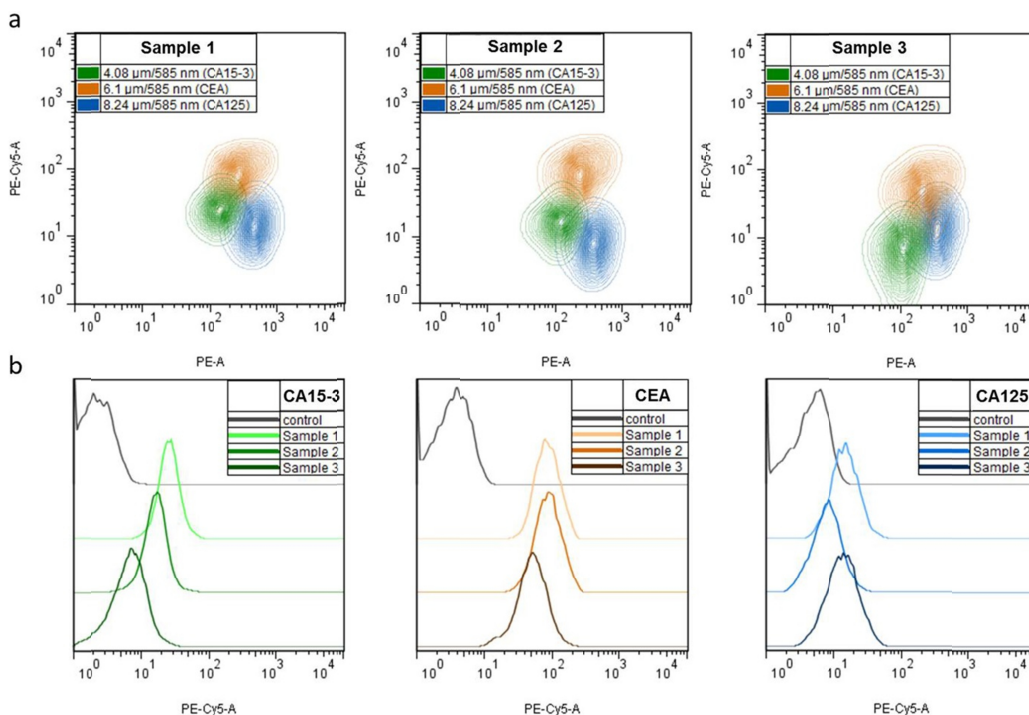


Fig. 2. Flow cytometry analysis of multiplexed QD-bead microarrays used for simultaneous detection of CA 15-3, CEA, and CA 125, markers of female reproductive system tumors, in clinical serum samples. (a) Simultaneous detection of three cancer markers in three individual serum samples from patients with different stages of breast cancer. (b) Comparative histograms indicating different levels of each target marker in three analyzed serum samples in comparison with the control sample. FSC-A, bead size; PE-A, bead optical code (QD 585 nm fluorescence); PE-Cy5-A, the amount of the cancer marker detected (fluorescence of the streptavidin-Tri-COLOR visualization label).

4. Conclusions

Modern clinical diagnostics of cancer rely on the profiling and quantitative detection of known cancer-specific antigens, the main biomarkers of malignant growth, in serum samples. Early detection of specific cancer markers is essential for timely diagnosis and proper therapy of cancer. In most cases, accurate diagnosis requires quantitative profiling of a panel of biomarkers in the same biological sample (Sturgeon et al. (2010); Maruvada et al. (2005); Shemetov et al. (2012)).

Currently, the conventional planar surface arrays remain the most widely used tools for multiplexed biomarker detection and clinical diagnosis. Unfortunately, the number of selected parameters and analyzed characteristics, sensitivity, quality of the results, and quickness of analysis are considerably limited by the properties of a fixed two-dimensional planar matrix (Gonzalez-Gonzalez et al. (2012)).

Liquid-phase microbead-based assays utilizing a wide panel of unique optical and size codes to label different antigens are free from most of these limitations. These assays are easily modifiable to fit the analyzed target profiles and are characterized by a fast binding kinetics and high sensitivity and quality of analysis (Nolan et al. (2002); Elshal et al. (2006)). Multiplexed suspension arrays can be successfully used to detect multiple proteins, viruses, antibodies against allergens and autoantibodies, gene polymorphism, etc. (Sukhanova et al. (2007); Kellar et al. (2003); Gao et al. (2011); Long et al. (2010)).

In this study, we have developed a novel lab-on-a-bead suspension diagnostic assay based on optically encoded microbeads for simultaneous multiplexed detection of several cancer-specific antigens. We used QD-encoded microbead populations of different sizes emitting in the orange range to label three biomarkers of female reproductive system tumors (CA 15-3, CEA, CA 125). We used antigen-specific monoclonal capture Abs linked to the bead surface and biotinylated detection Abs to catch and bind the target analyte. A complete immune complex was visualized with a secondary fluorescent streptavidin-Tri-COLOR label to quantify each target antigen in the sample. The designed QD-encoded beads linked to specific Abs have been calibrated with calibrator samples and used to test cancer-positive serum samples as compared with samples from healthy donors. The multiplexed (triplex) array was analyzed by means of flow cytometry.

The data have been validated by comparing with the results of the “gold standard” ELISA. Thus, we have demonstrated that the designed bead-based suspension assays are applicable to efficient and accurate multiplexed quantification of several markers in clinical serum samples from cancer patients.

Our results offer new prospects for high-throughput screening that could make use of the unique and robust fluorescence properties of QD-encoded beads. The new generation of bead-based assays can ensure efficient simultaneous determination of multiple antigens and enhance the clinical sensitivity and specificity of cancer marker screening in multiplexed diagnostics.

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