Development and potential applications of microarrays based on fluorescent nanocrystal-encoded beads for multiplexed cancer diagnostics

Kristina Brazhnika, Regina Grinevicha, Anton E. Efimovb,c, Igor Nabieva,d, and Alyona Sukhanovaad,e

Laboratory of Nano-Bioengineering, Moscow Engineering Physics Institute, 115409 Moscow, Russian Federation; Laboratory of Bionanotechnology, V.I.Shumakov Federal Research Center of Transplantology and Artificial Organs, 123182, Moscow, Russian Federation; SNOTRA, LLC., 105318 Moscow, Russian Federation; European Technological Platform “Semiconductor Nanocrystals,” Laboratory of Research in Nanosciences - EA4682, Université de Reims Champagne-Ardenne, 51100 Reims, France

ABSTRACT

Advanced multiplexed assays have recently become an indispensable tool for clinical diagnostics. These techniques provide simultaneous quantitative determination of multiple biomolecules in a single sample quickly and accurately. The development of multiplex suspension arrays is currently of particular interest for clinical applications. Optical encoding of microparticles is the most available and easy-to-use technique. This technology uses fluorophores incorporated into microbeads to obtain individual optical codes. Fluorophore-encoded beads can be rapidly analyzed using classical flow cytometry or microfluidic techniques.

We have developed a new generation of highly sensitive and specific diagnostic systems for detection of cancer antigens in human serum samples based on microbeads encoded with fluorescent quantum dots (QDs). The designed suspension microarray system was validated for quantitative detection of (1) free and total prostate specific antigen (PSA) in the serum of patients with prostate cancer and (2) carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA 15-3) in the serum of patients with breast cancer. The serum samples from healthy donors were used as a control. The antigen detection is based on the formation of an immune complex of a specific capture antibody (Ab), a target antigen (Ag), and a detector Ab on the surface of the encoded particles. The capture Ab is bound to the polymer shell of microbeads via an adapter molecule, for example, protein A. Protein A binds a monoclonal Ab in a highly oriented manner due to specific interaction with the Fc-region of the Ab molecule. Each antigen can be recognized and detected due to a specific microbead population carrying the unique fluorescent code.

100 and 231 serum samples from patients with different stages of prostate cancer and breast cancer, respectively, and those from healthy donors were examined using the designed suspension system. The data were validated by comparing with the results of the “gold standard” enzyme-linked immunosorbent assay (ELISA). They have shown that our approach is a good alternative to the diagnostics of cancer markers using conventional assays, especially in early diagnostic applications.

Keywords: fluorescent nanocrystals; quantum dots; microbeads; optical encoding; antigen; antibody; cancer diagnostics; multiplex suspension assays; flow cytometry.
1. INTRODUCTION

Common approaches to multiplex analysis of biological samples employ solid-state two-dimensional planar arrays\(^1,2\) or liquid-state suspension arrays based on encoded microparticles\(^3-5\). Both detection systems have their specific advantages and shortcomings\(^6\). The liquid-state suspension arrays are easily modifiable to fit the analyzed target profiles and are characterized by a fast binding kinetics and high sensitivity and quality of analysis\(^3,7\). The development of multiplex suspension arrays is currently of particular interest for clinical diagnostics. This technology uses combinations of fluorophores incorporated into microbeads to obtain individual spectral codes. Fluorophore-encoded beads can be rapidly analyzed using classical flow cytometry\(^4,8\). Specific characteristics of conventional organic dyes considerably restrict the number of their possible combinations, limiting the number of color sets in a detection array\(^5\). Quantum dots (QDs) have unique advantages over classical organic fluorophores\(^9\). 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\(^10,11\). Due to the unique spectral characteristics, QDs can act as efficient donors for Förster resonance energy transfer (FRET) to a suitable acceptor. This significantly improves the detection quality and increases the sensitivity of diagnostic assays\(^12\). We have recently developed diagnostic suspension arrays for identification of specific autoantibodies in serum samples from systemic sclerosis patients. These benefits of FRET-based suspension arrays have been demonstrated experimentally\(^13\).

In this study, we have designed a new, highly sensitive and specific diagnostic system based on QD-encoded microbeads for the detection of specific markers of prostate cancer and breast cancer. Preparation of optically encoded fluorescent microbeads suitable for immunodiagnostics is based on layer-by-layer electrostatic deposition of charged polymers onto the charged surface of polystyrene latex beads. Different molar ratios of water-soluble CdSe/ZnS QDs emitting in the green (515 nm) and orange (581 nm) regions were used to deposit them between polymer layers. A huge amount of individual spectral codes of microbeads for biomolecule tagging could be obtained with this technology by using multiple color combinations.

We prepared multicolor sets of QD-encoded microbeads and bound capture antibodies (Abs) against different cancer biomarkers (PSA, CEA, and CA15-3) to their surfaces in a highly oriented manner. These biomarkers are generally found in the serum of patients with prostate cancer and breast cancer. The capture Ab is bound to protein A or another adapter molecule chemically conjugated with the polymer shell of the microbead. Protein A binds the monoclonal Ab in a highly oriented manner due to specific interaction with the Fc-region of the Ab molecule. The proposed protocol prevents nonspecific binding and decreases the false positive signal in the case of using the Fc-specific F(ab)\(_2\)-fragment as a reference detection Ab. The system was calibrated by using recombinant antigens (Ags) and used to test serum samples from cancer patients and healthy donors. 100 and 231 serum samples from prostate cancer and breast cancer patients, respectively, and serum samples from healthy donors were taken for quantitative analysis of cancer serum Ags. The data have been validated by comparing with the results of the “gold standard” enzyme-linked immunosorbent assay (ELISA).

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

2. MATERIALS AND METHODS

2.1 Materials

Carboxylated microparticles (melamine resin particles, 4.08 µm) were purchased from Microparticles GmbH (Berlin, Germany) and used as matrix cores for the preparation of QDs-encoded microbeads. Organic polyelectrolytes with different electric charges, namely, poly(allylamine hydrochloride) (PAH), poly(sodium 4-styrenesulfonate) (PSS), and sodium salt of poly(acrylic acid) (PAA) (Sigma-Aldrich, Moscow, Russia) were used to form multilayer shells on the surface of carboxylated particles. CdSe/ZnS semiconductor fluorescent nanoparticles or QDs were synthesized from organometallic compounds by colloidal chemistry methods as described previously\(^14,15\); they were kindly provided by Dr. Pavel Samokhvalov (Laboratory of Nano-Bioengineering, Moscow Engineering Physics Institute, Moscow, Russia).
QDs were solubilized with derivatives of polyethylene glycol (PEG) (Thermo Scientific) containing both thiol and carboxyl groups. Protein A (Thermo Scientific, Moscow, Russia) was conjugated to the surface of QD-encoded microbeads through carbodiimide crosslinkers (Thermo Scientific, Moscow, Russia). Monoclonal Abs against cancer markers and cancer protein Ags were purchased from Fujirebio Diagnostics, AB (Göteborg, Sweden). Recombinant prostate specific antigen (PSA) was purchased from Abcam plc. (Cambridge, England). Secondary antibodies and labeled conjugates were purchased from Sigma-Aldrich (Moscow, Russia). Serum samples from patients with breast cancer and prostate cancer and healthy donors were provided by Blokhin Cancer Research Center of the Russian Academy of Medical Sciences (Moscow, Russia).

2.2 Water solubilization of quantum dots

CdSe/ZnS core/shell QDs coated with trioctyl phosphine oxide (TOPO) that emitted in the green (515 nm) and orange (581 nm) regions were synthesized as described earlier. In order to obtain highly stable water-soluble QDs, we used a two-step procedure of the replacement of the TOPO shell with a cysteine shell followed by the replacement of cysteine with carboxy-PEG12-thiol.

2.3 Optical encoding of microbeads with water-soluble QDs

Preparation of QD-encoded microbeads suitable for immunodiagnostics was carried out using a procedure of layer-by-layer assembly adapted for deposition of alternatively charged polymer layers and a layer of carboxyl-modified QDs. In this study, CdSe/ZnS QDs emitting in the green (515 nm) and orange (581 nm) regions with quantum yields of 55% and 70%, respectively, in aqueous solutions were attached to pre-surface layers of polymer-coated monodispersed melamine resin microbeads with a diameter of 4.08 µm.

2.4 Immunodiagnostic suspension assays with the use of QD-encoded microbeads for quantitative detection of cancer antigens

To perform immunodiagnostic suspension analysis, we employed the “lab-on-a-bead” detection principle. According to it, we bound monoclonal capture Abs against each target Ag (total PSA and free PSA, CEA, and CA15-3) to the surface of each QD-encoded bead population carrying a unique optical code. To ensure the desired orientation of capture Abs and, hence, formation of an effective complete immunodiagnostic complex, protein A adapter molecules were conjugated with the microbead polymer shell using carbodiimide chemistry. The beads conjugated with protein A were sequentially incubated with all elements of the immunodiagnostic complex according to the scheme: QD-encoded beads–protein A : capture monoclonal Abs : sample (Ags) : detection monoclonal Abs : visualization (secondary) Abs–biotin conjugate : streptavidin–fluorophore (streptavidin-Tri-COLOR (TC), or phycoerythrin-cyanine 5 (PE-Cy5)). As a secondary antibody, we used the biotinylated F(ab)2 Ab fragment lacking the Fc-region to minimize nonspecific binding and decrease the false positive signal. To demonstrate the efficiency of the resultant detection system for the profiling and quantitative detection of cancer markers, we calibrated the system with known concentrations of recombinant Ags. Different techniques and protocols and a variety of blocking chemistries (bovine serum albumin (BSA) and casein-containing solutions) were tested to select the appropriate conditions for suspension analysis. The microarrays based on QD-encoded beads were processed and analyzed using classical flow cytometry (BD FACSCanto II).

100 and 231 serum samples from patients with different stages of prostate cancer and breast cancer, respectively, and samples from healthy donors were taken for quantitative analysis of cancer serum Ags. 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 multiplexed detection system developed, with serum of healthy donors serving as a control.

3. RESULTS AND DISCUSSION

3.1 Analysis of QD-encoded microbead structure and fluorescent properties

First, we selected the optimal conditions for the formation of a polymeric shell with incorporated water-soluble single-color QDs on the surface of microbeads with the use of the layer-by-layer technique. 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 for immunodiagnostic suspension assay.
resultant single-color QD-encoded microbead populations exhibited intense fluorescence with emission wavelengths that are nearly identical to those of the original QDs. Fig. 1A and Fig. 1B show single-color fluorescence images of the populations of beads encoded with QDs emitting at 515 nm obtained using fluorescent (Nicon Eclipse TE2000-S) and confocal (A1Rsi Spectral Imaging Confocal System) microscopy. It is important to note that a single light source was used in this experiment. These techniques allowed all the encoded beads to be visualized; there were no bead aggregates in the sample, and the emission colors of different populations were clearly distinguishable (data not shown). Confocal imaging has shown that the QDs are mainly located in the outer 25% of the bead radius, as shown in Fig. 1B. However, this is only an approximate estimate, because the microbeads refract light and cause image distortion. Fig. 1C shows a detailed core/shell structure of a bead and the most likely distribution of fluorescent nanocrystals in the pre-surface area of the encoded microbeads. This image was obtained by means of a “slice-and-view” combination of ultramicrotomy and scanning probe microscopy (scanning probe nanotomography, SPNT) as described previously.

Figure 1. Nanocrystal-encoded microbeads for cancer diagnostics. (A) A fluorescence image of microbeads encoded with QDs emitting at 515 nm. (B) A confocal fluorescence image of a microbead encoded with QDs emitting at 515 nm: approximate estimation of the QD distribution in the outer pre-surface area. (C) Scanning probe nanotomography image of microbead encoded with QDs emitting at 515 nm showing a detailed core/shell structure of the bead.

3.2 Flow cytometry analysis of suspension immunodiagnostic assays based on QD-encoded microbeads

In our study, protein A was conjugated with carboxyl groups on the surface of the microbeads encoded with QDs emitting in green (515 nm) and orange (581 nm). Protein A binds an antibody molecule in a highly oriented manner due to specific interaction with the Fc-region of Ab. The proposed protocol provided specific orientation of all components of the diagnostic system and decreased the false positive signal in the case of using the Fc-specific F(ab)2 fragment as a reference detection agent. The QD-encoded and protein A-conjugated microbeads were finally back-coated with 1-2% casein from bovine milk.

The results of the conventional flow cytometry analysis of suspension immunodiagnostic assays based on QD-encoded microbeads are shown in Fig. 2A. Flow cytometry analysis demonstrated that the designed QD-encoded and protein A-coated microbeads were extremely bright and homogeneous.

In order to confirm the efficiency of conjugation and the presence of protein A on the microbead surface, the microbeads were incubated with full-length FITC-labeled anti-mouse immunoglobulin (IgG). In this control assay, protein A, which was efficiently conjugated with the surface of QD-encoded microbeads, bound the FITC-labeled Fc-region of IgG. Fig. 2B demonstrates distinct shifts of FITC-negative control signal towards green fluorescence depending on labeled...
antibody concentrations. These control experiments clearly demonstrate the presence of protein A adapter molecules on the microbead surface. The negative controls included incubation of conjugated microbeads with the FITC-labeled anti-mouse F(ab)\textsubscript{2} fragment of IgG lacking the Fc-region (Fig. 2C), where no significant fluorescence shift has been detected.

Figure 2. Flow cytometry dot plots of QD-encoded and protein A-conjugated microbeads used for detection of the PSA cancer antigen. (A) Uncoded MF 4.08 µm microparticle control (light scattering measurements, green (FITC-A) versus orange (PE-A) fluorescence profiles versus protein A-coated microbeads encoded with QDs emitting at 581 nm. (B) Protein A conjugation control: QD-encoded, protein A-conjugated microbeads incubated with full-length FITC-labeled anti-mouse IgG antibodies. (C) Negative control: QD-encoded, protein A-conjugated microbeads incubated with FITC-labeled anti-mouse F(ab)\textsubscript{2}-fragment of IgG lacking the Fc-region. Excitation is provided by the 488 nm line of an argon laser.

The principles of experimental procedures and flow cytometry assay for antigens profiling are shown below.

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Cancer antigen profiling with flow cytometry
(bead-based assay)

+ QD-encoded fluorescent beads conjugated with protein A
+ Antigen-specific capture monoclonal antibodies
  + Calibrator or serum sample containing target cancer antigen
  + Antigen-specific detection monoclonal antibodies
    + Secondary biotinylated F(ab)\textsubscript{2}-fragment antibodies
    + Fluorescent conjugate of streptavidin and organic dye (Tri-COLOR)

Cancer antigen profiling with flow cytometry analysis
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The details of the designed “lab-on-a-bead” complete detection system are schematically shown in Fig. 3.

Figure 3. Scheme of a flow cytometry assay for immunodetection of a cancer antigen with QD-encoded, protein A-conjugated microbeads. Microbeads optically encoded with orange-emitting QDs and conjugated with protein A adaptor molecules bind the Fc-region of antigen-specific capture monoclonal antibodies, ensuring effective orientation of the entire immunodiagnostic complex. These beads are incubated with a sample containing the target antigen and antigen-specific detection antibodies and stained with the complex of biotinylated F(ab)2-fragment antibodies and fluorescent-labeled streptavidin (streptavidin Tri-COLOR or PE-Cy5).

In order to perform accurate multiplexed analysis for profiling and quantitative detection of several cancer markers with the use of the suspension system developed, we first analyzed samples containing known concentrations of a recombinant Ag. We used the range of recombinant Ag dilutions from picograms to nanograms per sample. For each clinical sample test, we prepared fresh conjugates of the beads and protein A, and the conjugation efficiency was controlled. Moreover, the system calibration using serial dilutions of the recombinant Ag in a buffer and in the serum from healthy donors was carried out for each experiment. The results of calibrator sample analysis were used to plot calibration curves (Fig. 4) for accurate quantification of the amount of the target biomarker in clinical serum samples.

Figure 4. Standard curves of flow cytometry analysis of clinical serum samples based on the results of recombinant cancer antigen detection using the designed suspension assay. The results of three independent experiments are presented.

100 and 231 serum samples from patients with different stages of prostate cancer and breast cancer, respectively, and serum samples from healthy donors were recently taken for functional clinical tests and quantitative analysis of cancer
serum Ags with the use of the designed diagnostic suspension system. First, all the samples were analyzed using the “gold standard” ELISA test to determine the exact Ag concentrations according to the standard clinical diagnostic requirements and correlate these results with our data (data not shown).

For functional clinical tests with the designed suspension system, we used several clinical samples from prostate cancer patients with confirmed elevated levels of the total and free forms of PSA 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 obtained with flow cytometry assay utilizing QD-encoded microbeads corresponded to those of quantitative detection with the use of the standard clinical diagnostic techniques. The fluorescence shift from the orange to the green region after incubation of free-PSA-specific and total-PSA-specific beads with serum of cancer-positive patients (Fig.5C) as compared with the serum of cancer-negative patients (Fig. 5B) provided clear discrimination between the samples with respect to the Ag content and excellently agreed with the results of traditional ELISA detection techniques (data not shown).

Figure 5. Flow cytometry dot plots of QD-encoded microbeads used for detection of the total PSA form, a marker of prostate cancer, in the clinical serum of healthy donors and cancer-positive patients. PE-A (orange) versus PE-Cy5-A or TC (red) fluorescence profiles. (A) TC-negative control of microbeads encoded with QDs emitting at 581 nm versus TC-background fluorescent signal from 2% casein antigen in the complete detection suspension assay. (B, C) Microbeads encoded with QDs emitting at 581 nm and conjugated with protein A incubated in serum of (B) a healthy donor or (C) a prostate cancer-positive patient and stained with the Tri-COLOR-labeled visualization complex according to the scheme shown in Figure 3.

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 patients’ serum samples. Early detection of the specific oncological markers is essential for prompt diagnosis and proper therapy of cancer. In most cases, accurate diagnosis requires quantitative profiling of a panel of biomarkers in the same biological sample.
Currently, the conventional planar surface arrays still remain the most widely used tool for biomarker detection and clinical diagnostics. 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.

Liquid-phase microbead-based assays utilizing a wide panel of unique optical 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. Multiplex suspension arrays can be successfully used to detect multiple proteins, viruses, antibodies against allergens and autoantibodies, gene polymorphism, etc.

In this study, we have developed a novel suspension diagnostic assay based on optically encoded microbeads for simultaneous multiplexed detection of several cancer-specific antigens. We used QD-encoded microbead populations emitting in green and orange to label two antigen biomarkers (free PSA and total PSA in the case of prostate cancer; CEA and CA15.3 in the case of breast cancer). We ensured highly effective orientation of immunodiagnostic complex on the bead surface with the use of protein A adaptor molecules. A biotinylated F(ab)\textsubscript{2} Ab fragment lacking the Fc-region was used as a secondary antibody in order to minimize nonspecific binding and decrease the false positive signal. This allowed us to improve detection quality, including specificity.

The designed QD-encoded beads linked to specific Abs have been calibrated with recombinant antigens and used to test cancer-positive serum samples as compared with samples from healthy donors. 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 for efficient and accurate multiplexed detection of several Ags in clinical serum samples from cancer patients.

Our results open 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 may enable efficient simultaneous determination of multiple Ags and enhance the clinical sensitivity and specificity of Ag screening in multiplexed diagnostics.

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