Analytica Chimica Acta 665 (2010) 63–68

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Multiplex immunodetection of tumor markers with a suspension array built upon core–shell structured functional fluorescence-encoded microspheres

Yao Long[a], Zhiling Zhang[a], Xiaomei Yan[a,*], Jinchun Xing[b,**], Kaiyan Zhang[b], Jingxiong Huang[b], Jiaxin Zheng[b], Wei Li[b]

[a] Department of Chemical Biology, College of Chemistry and Chemical Engineering, The Key Laboratory for Chemical Biology of Fujian Province, Key Laboratory of Analytical Science, Xiamen University, Xiamen 361005, China
[b] The First Hospital of Xiamen, Xiamen 361005, China

ARTICLE INFO

Article history:
Received 3 February 2010
Received in revised form 1 March 2010
Accepted 7 March 2010
Available online 12 March 2010

Keywords:
Polystyrene beads
Flow cytometry
Suspension array
Multiplex assay
Tumor marker detection

ABSTRACT

A new suspension array built upon laboratory-prepared functional fluorescence-encoded polystyrene beads (FFPBs) was developed for multiplex immunodetection of tumor markers. The FFPBs were synthesized by copolymerizing rhodamine 6G (R6G) and carboxyl function groups on the surface of the seed beads forming a core–shell structure. The fabrication process was facile and the encoding fluorescence intensity of the beads can be precisely controlled by adjusting the quantity of R6G. In present work, we demonstrated that the quantity variation of impregnated R6G had negligible effect on the coupling efficiency of biomolecules onto the surface of the FFPBs. The R6G encoding fluorescence remained good monodispersity upon capture probe coupling and immunocomplex formation. No fluorescence resonance energy transfer was observed between the R6G doped in the bead shell and fluorophore used for antibody labeling. Under the optimal conditions, the proposed suspension array allowed simultaneous detection of α-fetoprotein, carcinoembryonic antigen, and prostate specific antigen in the ranges of 0.07–500 ng mL^{-1}, 1–2000 ng mL^{-1}, and 0.5–500 ng mL^{-1}, respectively, with detection limits of 0.0626 ng mL^{-1}, 0.554 ng mL^{-1}, and 0.250 ng mL^{-1}. Test on clinical serum samples demonstrated that the results obtained with suspension array were in good agreement with those of the reference electrochemiluminescence immunoassay method. We conclude that the laboratory-made FFPBs are sufficient as the microcarrier for the construction of suspension array in clinical diagnosis.

1. Introduction

Early diagnosis and treatment of cancer is the key to improving the survival rate. Studies have shown that the concentrations of tumor markers in easy-to-access biological fluids are associated with the stages of tumor progression [1–4]. However, no single tumor marker is sensitive and specific enough to meet stringent diagnostic criteria. Researches have shown that the measurement of a panel of tumor markers can greatly increase the accuracy of disease diagnosis [2,3,5–10]. Compared with traditional single-analyte test, such as enzyme-linked immunosorbent assay (ELISA), simultaneous multi-analyte detection can not only reduce sample and reagent consumption, but also save time and efforts [11]. Protein microarray is the predominant analytical technique currently used for protein expression profiling [5,12,13], however it suffers from slow diffusion of analyte to the surface and narrower dynamic range [14–17].

As an evolution of the planar microarray, suspension array built upon microbeads offers distinct advantages in greater flexibility in array preparation, faster binding kinetics, less expense, higher reproducibility, and superior detection sensitivity [13,17–21]. As a result, suspension array has become a highly attractive platform for multiplexed analysis of biomarkers, drug screening, food and environmental monitoring, and other applications [8,10,22–31]. Suspension array is usually created by capturing analytes on the surface of microspheres encoded with distinct optical properties in conjunction with flow cytometric analysis. As the microcarriers in suspension arrays, micron-sized microspheres with large encoding capacity and good functionality are critical to assay preparation. Using solvent swelling method, Luminex Corp. (Austin, TX) successfully created the xMAP arrays, which employ 100 different color-coded microsphere sets to enable the simultaneous test of 100 different biomolecules in a single run [32]. Recently, we reported a new and efficient strategy for the facile preparation of fluorescence-encoded polystyrene beads with desirable optical and surface properties for suspension arrays [33].

In this report, we investigate the applicability of the laboratory-synthesized functional fluorescence-encoded polystyrene beads (FFPBs) in suspension array by examining (1) whether the variation

* Corresponding author. Tel.: +86 592 2184519; fax: +86 592 2189959.
** Corresponding author. Tel.: +86 13806089889.
E-mail addresses: xmyan@xmu.edu.cn (X. Yan), xmcua2007@sina.com (J. Xing).

0003-2670/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2010.03.009

of doped fluorophore quantity would affect the capture molecule coupling efficiency using standard carbodiimide conjugation, (2) whether the monodispersity of R6G encoding fluorescence would change upon capture probe immobilization and immunocomplex formation, and (3) the performance of FFPBs in the multiplex immunodetection of tumor markers. Because α-fetoprotein (AFP), carcinoembryonic antigen (CEA), and prostate specific antigen (PSA) play important roles in early screening, clinical diagnosis and prognosis of some malignant diseases including hepatocellular cancer, colorectal cancer, and prostate cancer [4,34,35], these three conventional tumor markers are used as the model analytes. The suspension immunoassay adopts a standard sandwich format where antigen-specific capture antibodies are immobilized on the FFPBs, specimen containing the antigen proteins are then added and allowed to bind to the beads. The bead-captured antigens are subsequently recognized by the biotinylated reporter antibodies which are then labeled by streptavidin–allophycocyanin (streptavidin–APC) conjugate. For clinical serum samples, the multiplex detection results obtained from the suspension array using FFPBs are in good agreement with those of the reference electrochemiluminescence immunoassay method. The laboratory-prepared FFPBs are sufficient as the microcarriers of suspension array for clinical diagnostics.

2. Experimental

2.1. Materials

Human CEA, PSA antigens and their monoclonal antibodies were obtained from Linc-bio Science Co., Ltd. (Shanghai, China). Human AFP antigen and its monoclonal antibodies were purchased from Boson Biotechnology Corp. (Xiamen, China). Goat anti-mouse antibody (GAM) was obtained from Huamei Scientific Corp. (Beijing, China). The antibodies were biotinylated according to the manufacture's instructions (Pierce, IL, USA). N-hydroxy-succinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and sulfosuccinimidyl-6-(biotinamido)-6-hexanamido hexanoate (Sulfo-NHS–LC–LC–Biotin) were obtained from Pierce. Fetal bovine serum (FBS) was obtained from Hyclone Co. (Logan, UT, USA). Streptavidin–APC conjugate was purchased from Molecular Probes (OR, USA). SPHERO™ Rainbow Calibration Particles were purchased from Spherotech, Inc. (Libertyville, IL, USA). Clinical serum samples were collected in the First Hospital of Xiamen, China. Divinyl benzene (DVB, 80%) was obtained from Fluka. Rhodamine 6G (R6G) was purchased from Acros Organics (Geel, Belgium). Styrene was obtained from Xilong Chemical Co., Ltd. (Guangdong, China) and was washed with 1.25 M sodium hydroxide solution to remove potential inhibitors. Analytical grade potassium persulfate (KPS), undecylenic acid, ethanol (99.5%), and dodecyl sulfonic acid sodium (SDS) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other chemicals were obtained from Sigma (St. Louis, MO). Deionized and sterilized water were used throughout the experiments.

2.2. Preparation of functional fluorescence-encoded polystyrene beads

The functional fluorescence-encoded polystyrene beads were fabricated according to the method described in our early report [33]. Briefly, the micron-sized, monodisperse polystyrene seed beads were synthesized by dispersion polymerization. Then dye molecules (R6G) and carboxyl function groups were copolymerized on the surface of the seed beads by forming a core–shell structure (Fig. 1). The fluorescence intensity of the beads can be precisely controlled by adjusting the R6G quantity. For multiplex detection using suspension array, seed beads with the diameter of 3.77 μm (size distribution coefficient of variation of 2.4%) were used to fabricate five sets of FFPBs that can be clearly distinguished on the flow cytometer. The R6G encoded beads were prepared by mixing up 0.05 g of seed beads, 12.5 mg of KPS, 1.25 mg of SDS, 25 μL of undecylenic acid/methanol (1:10, v/v), 10 μL of DVB/methanol (1:100, v/v), R6G, and 2 mL of water. The quantities of R6G were 1.5 μg, 6 μg, 10 μg, 20 μg, and 35 μg, respectively for the five sets of FFPBs. The average diameter of these fluorescent beads was 3.82 μm and the final concentrations of these beads were adjusted to 10^9 mL^{-1}.

2.3. Capture probe immobilization

A two-step coupling procedure optimized in our laboratory was used to covalently attach the anti-tumor marker capture MAbs to the FFPBs. Briefly, 5×10^6 of the FFPBs were pipetted into 80 μL of activation buffer (0.01 M sodium acetate, pH 5.4), to which freshly made solutions of NHS (10 μL, 50 mg mL^{-1}) and EDC (33 μL, 100 mg mL^{-1}) in the activation buffer were added. After incubation at room temperature for 20 min, excess EDC/NHS was removed by centrifugation and resuspension. After washing twice with 400 μL activation buffer, the activated FFPBs were incubated with anti-tumor marker capture MAbs in 400 μL coupling buffer (same composition as the activation buffer) at room temperature for 4 h. The reaction was stopped by washing the MAb-bearing FFPBs twice with 500 μL washing buffer (0.05% Tween 20 in PBS pH 7.4). The FFPBs immobilized with the anti-tumor marker capture MAbs were then stored in 500 μL blocking/storage buffer (1% BSA, 0.05% sodium azide in PBS pH 7.4) at 4 °C for further detection.

2.4. Evaluation of coupling efficiency

The coupling capability of FFPBs was evaluated by incubating each set of MAb-bearing FFPBs (1×10^4) with 50 μL of self-prepared biotinylated goat anti-mouse (GAM) IgG (2 μg mL^{-1}) diluted in incubation buffer (0.2% Tween 20, 1% BSA in PBS pH 7.4) at 37 °C for 30 min. After washing twice with 500 μL of washing buffer, these beads were incubated with 50 μL of streptavidin–APC (4 μg mL^{-1}) diluted in incubation buffer at 37 °C for another 20 min. After washing once with 500 μL of washing buffer, these beads were resuspended in 100 μL of PBS and analyzed on the FACSAria flow cytometer (BD Biosciences, San Jose, CA, USA).

2.5. Collection and storage of blood sera

Blood was drawn using standard phlebotomy procedures and collected without anticoagulant. Sera were separated by centrifugation, immediately aliquoted, frozen, and stored at −80 °C. No more than 2 freeze–thaw cycles was allowed for each sample.

2.6. Multiplex detection of tumor markers

Equal amounts of the MAb-bearing FFPBs for AFP, CEA, and PSA detection were mixed together. Three microliters of the FFPBs mixture (~30 000 FFPBs total) was added into 100 μL of human serum, serum diluent, or tumor marker standard prepared in sample dilution buffer (0.2% Tween 20, 1% BSA, 20% FBS in PBS pH 7.4). After incubation at 37 °C for 45 min, two washes with 500 μL washing buffer were carried out to remove the unbound analytes. Then 50 μL of self-prepared biotinylated anti-tumor marker reporter antibodies (2 μg mL^{-1}) dissolved in incubation buffer was added. After incubation at 37 °C for 30 min, the beads were washed twice with 500 μL of washing buffer. Then 50 μL of streptavidin–APC (1 μg mL^{-1}) in incubation buffer was added. After 20 min of incubation at 37 °C, the beads were washed twice with 500 μL of washing buffer and suspended in 100 μL PBS for analysis on the flow cytometer.