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Microfluidic beads-based immunosensor for sensitive detection of cancer biomarker proteins using multienzyme-nanoparticle amplification and quantum dots labels

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ABSTRACT

This study reports the development of a microfluidic beads-based immunosensor for sensitive detection of cancer biomarker α-fetoprotein (AFP) that uses multienzyme-nanoparticle amplification and quantum dots labels. This method utilizes microbeads functionalized with the capture antibodies (Ab_1) and modified electron rich proteins as sensing platform that was fabricated within a microfluidic channel, and uses gold nanoparticles (AuNPs) functionalized with the horseradish peroxidase (HRP) and the detection antibodies (Ab_2) as label. Greatly enhanced sensitivity for the cancer biomarker is based on a dual signal amplification strategy: first, the large surface area of Au nanoparticle carrier allows several binding events of HRP on each nanosphere. Enhanced sensitivity was achieved by introducing the multi-HRP-antibody functionalized AuNPs onto the surface of microbeads through "sandwich" immunoreactions and subsequently multiple biotin moieties could be deposited onto the surface of beads resulted from the oxidation of biotin–tyramine by hydrogen peroxide. Streptavidin-labeled quantum dots were then allowed to bind to the deposited biotin moieties and displayed the signal. Secondly, enhanced mass transport capability inherent from microfluidics leads to higher capture efficiency of targets because continuous flow within micro-channel delivers fresh analyte solution to the reaction site which maintains a high concentration gradient differential to enhance mass transport. Based on the dual signal amplification strategy, the developed microfluidic bead-based immunosensor could discriminate as low as 0.2 pg mL^{-1} AFP in 10 μL of undiluted calf serum (0.2 fg/chip), and showed a 500-fold increase in detection limit compared to the off-chip test and 50-fold increase in detection limit compared to microfluidic beads-based immunoassay using single label HRP-Ab_2. The immunosensor showed acceptable repeatability and reproducibility. This microfluidic beads-based immunosensor is a promising platform for disease-related biomolecules at the lowest level at their earliest incidence.

1. Introduction

Sensitive detection of protein biomarkers is critical to many areas of biomedical research and diagnostics, systems biology, and proteomics (Smith et al., 2007; Kitano, 2002; Hood, 2003). For example, the clinical measurement of cancer biomarkers shows great promise for early disease detection and highly reliable predictions (Bensalah et al., 2008). Conventional immunoassay methods, including the enzyme-linked immunosorbent assay (ELISA) (Voller et al., 1978), fluorescence immunoassay (Matsuya et al., 2003), chemiluminescence assay (Fu et al., 2008), electrochemical immunoassay (Du et al., 2010), radioimmunoassay (Teppo and Maury, 1987), electrophoretic immunoassay (Schmalzing and Nashabeh, 1997), mass spectrometric immunoassay (Hu et al., 2007), and immune-polymerase chain reaction (PCR) assay (Saito et al., 1999) allow reliable clinical diagnosis and disease monitoring. Nevertheless, along with more growing incidence of lethal diseases (e.g. cancer) caused by pollution and viral means, the increasing demand for early and ultrasensitive screening of cancer biomarkers is pushing the enhancement of detection sensitivity by signal amplification or novel detection technologies.

Enzyme-functionalized nano-structured materials have become one of the most popular and practical solutions for signal amplification due to the increased enzyme loading toward one reaction event, which subsequently enhanced the ensemble signal for target detection (Tang et al., 2010; Mao et al., 2009). For example, Enzyme-functionalized silica nanoparticles have been used as label for sensitive detection of α-fetoprotein based on electrochemical and chemiluminescence measurement (Wu et al., 2009). Multi-wall carbon nanotubes (MWCNTs) functionalized with multiple

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enzyme labels and detection antibodies have achieved the sensitive detection of prostate specific antigen when compared with conventional singly labeled detection antibodies (Yu et al., 2006). Horseradish peroxidase (HRP)-functionalized gold nanoparticles are employed for the detection of human IgG by electrochemical immunosensor based on gold nanoparticles (GNPs)/carbon nanotubes (CNTs) hybrids platform (Cui et al., 2008). An electrochemical immunosensor using multi-enzyme functionalized carbon nanospheres labeled with HRP-secondary antibodies has been developed for sensitive detection of cancer biomarker α-fetoprotein (Du et al., 2010). A novel electrochemical immunosensor based on Fe_3O_4/Au nanoparticles as a carrier using a multienzyme amplification strategy has been developed for ultrasensitive detection of α-fetoprotein (Gan et al., 2011).

Microfluidics technology has attracted increasing attention owing to its advantages over macroscopic instrumentation such as reduced sample and reagent consumption, portability, disposability (Dittrich et al., 2006; Vilkner et al., 2004; Zhang et al., 2006; Tanaka et al., 2007). Recently, the integration of efficient sample handling of microfluidics and high-throughput parallel analysis of microbead based arrays has been demonstrated for multi-target detection, making it an ideal platform for sensitive detection of disease-related biomolecules (Lucas et al., 2007; Gunderson et al., 2005). For example, a microfluidic beads-based immunosorbent assay system has been developed with sample volume reduced to microliter range and antigen–antibody reaction time shortened from 15 h to 10 min (Sato et al., 2000). A one-dimensional microfluidic bead array chip has been fabricated for the detection of low-abundant DNA point mutations with better sensitivity compared to the off-chip assays (Zhang et al., 2008). A novel microfluidic bead-based array has been constructed for sensitive genotyping of Hepatitis B virus using quantum dots as labels. (Zhang et al., 2010). A microbead-based rolling circle amplification method in a microfluidic chip has been developed for sensitive DNA detection (Sato et al., 2010). Based on the hydrodynamic principle, some novel hydrodynamic traps have been designed and fabricated for manipulation and immobilization of one particle to construct the particle array (Bithi and Vanapalli, 2010; Huebner et al., 2009).

Our present work is motivated by the promising results of enzyme-functionalized nanoparticles for signal amplification and microfluidic beads-based array for parallel detection of multiple targets and sensitivity enhancement. Biomolecules-functionalized gold nanoparticles are advantageous over conventional probes in a variety of bio-detection schemes as a result of their unique chemical and physical properties (Mirkin et al., 1996; Rosi and Mirkin, 2005). Quantum dots have been utilized for labeling biomolecules with several attractive features: broad excitation spectrum but narrow and precise emission that is tunable by varying the size of the nanoparticles, negligible photobleaching, fairly high quantum yields, stability, and negligible phototoxicity (Stroh et al., 2005; Gao et al., 2004). In this paper, streptavidin-conjugated quantum dot (CdSe/ZnS, 605 nm), Au nanoparticles functionalized with detection antibody and HRP, and microbeads functionalized with the capture antibodies and modified electron rich proteins are combined for sensitive detection of AFP antigen. AFP is a major plasma protein produced by the yolk sac and the liver. The AFP expression is often associated with hepatoma and teratoma and has been widely used as a diagnostic biomarker for hepatocellular carcinoma (Tamura et al., 2009). The combination of microfluidic beads array with enzyme-functionalized nanoparticles for AFP analysis is 3-folded: (1) high detection sensitivity by multienzyme-nanoparticle amplification and enhanced mass transport capability inherent from microfluidics, (2) efficient liquid handling capability inherent from microfluidics, and (3) rapid binding kinetics offered by homogeneous microbead assays.

2. Materials and methods

2.1. Materials and reagents

PDMS prepolymer and curing agent used for the fabrication of microfluidic chip were purchased from Dow Corning Corporation (Saginaw, MI). Polystyrene microbeads were purchased from Bangs Laboratories and streptavidin-conjugated quantum dot (605 nm) was purchased from Wuhan Jiayuan Quantum Dots Co., Ltd. (Wuhan, Hubei, China). 3-(4-Hydroxyphenyl) propionic acid N-hydroxysuccinimide ester (4-OH-PPA-NHS), tyramine hydrochloride, biotin N-hydroxysuccinimide ester (biotin-NHS), casein were purchased from Sigma–Aldrich Chemical Co. Horseradish peroxidase (HRP)-labeled mouse monoclonal antibody to AFP (HRP-anti-AFP, HRP-Ab_2) and human AFP ELISA KIT were purchased from Abcam, Inc. AFP and biotin-labeled antibody to AFP (biotin-anti-AFP, biotin-Ab_1) were purchased from Shanghai Linc-Bio Co., Ltd.

2.2. Microchip fabrication

Microfluidic structures were prepared by molding PDMS against a printed circuit board (PCB) master (Li et al., 2006). In brief, the procedure involved the design of photomasks, the transfer of patterns on the photoresist of the PCB (Kinsten, Chiefskill, Taiwan) by UV irradiation, the removal of the exposed photoresist, the etching of the unprotected copper layer on the PCB, and the casting of PDMS on the PCB master to form negative replicas. The detailed chip fabrication and the loading of functionalized microbeads into the chamber array were described in our previous report (Zhang et al., 2012). Briefly, each beads loading channel containing two disconnected channel segments was aligned with corresponding chamber under a microscope to construct a bead immobilization unit. When the two disconnected segments were aligned over the chamber, gaps of two different sizes were formed. While the larger gap allowed beads flowing into the chamber position, the smaller gap prevented the beads from flowing out of the chamber. These channels were incubated with 1% bovine serum albumin for 1 h before use to minimize nonspecific adsorption of microbeads on channel walls. After the introduction of microbeads, the slab with bead loading channels was removed and bonded with another slab containing a simple micro-channel (i.e. the sampling channel) face-to-face to construct detection device. Since the channel depth of sampling channel was smaller than immobilized beads inside the arrayed chambers, fluidic flow generated during the sample loading would not flush away the beads.

2.3. Preparation and functionalization of gold nanoparticles (Au-NPs)

Au-NPs with narrow size distribution and good dispersion were prepared according to the reported method (Grabar et al., 1995). All glassware used in this preparation was thoroughly cleansed by aqua regia (three parts HCl, one part HNO_3), rinsed in doubly distilled water, and oven-dried prior to use. In a 250 mL Bunsen beaker, 100 mL of 0.01% $HAuCl_4$ in doubly distilled water was brought to a boil with vigorous stirring. To this solution 3 mL of 1% trisodium citrate was added. The solution turned to deep blue within 20 s, and the final color changed to wine-red after 60 s. Boiling was pursued for an additional 10 min before the heating source was removed, and the colloid solution was stirred for another 15 min. The resulting Au-NPs solution was stored in dark bottles at 4 °C and was used to prepare HRP-Ab_2-AuNPs conjugate.