Sensors and Actuators B 190 (2014) 737–744

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

Direct detection of prostate specific antigen by darkfield microscopy using single immunotargeting silver nanoparticle

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ARTICLE INFO

Article history:
Received 8 July 2013
Received in revised form 3 September 2013
Accepted 11 September 2013
Available online 20 September 2013

Keywords:
Silver nanoparticle
Prostate specific antigen
Darkfield microscopy
Single particle counting
Immunosensor

ABSTRACT

This work demonstrated a sensitive but direct method in detecting cancer biomarkers using dark field microscopy (DFM). Prostate-specific antigen (PSA), a well-known biomarker for prostate cancer, was chosen as the analyte to prove the concept. The immunoassay-based detection was performed in glass flow cell. Antibody-conjugated silver nanoparticles (AgNP-Ab) were served as the probe to capture PSA and then formed AgNP-Ab-PSA complexes on the surface of flow cell. The number of complexes is corresponding to the amount of PSA, which can be quantified by nanoparticle counting with DFM coupled with charge-coupled device (CCD) camera. The detection limit at 9 pM of this assay is well below the PSA threshold of prostate cancer patient, suggested the feasibility of our assay in diagnosis application.

1. Introduction

Previous studies found that the release of prostate specific antigen (PSA) in serum and semen is highly related to the growth of prostate tumor [1,2]. Hence, PSA is recognized as an oncological biomarker of PCa. Statistically, patients with PSA levels of 2–3 ng/mL (corresponding to 60–90 pM) in serum will have seven-times higher risk in suffering from prostate cancer within 10 years than those of less [3]. While the typical PSA cutoff level in serum regarding to suspicious PCa is 4 ng/mL (~120 pM), only patients detected with level higher than that will require follow-up diagnosis of prostatic biopsies for confirmation [4].

Among various kinds of PSA detection methods, immunoreaction is widely applied in both clinical applications and assay developments based on its direct immunochemical recognition approach and its high specificity [5]. For instance, enzyme-linked immunosorbent assay (ELISA) is a powerful technique for antigen quantification although considerations such as large sample consumption (~100 μL), laborious treatment steps, monoplexity in detection, low reproducibility and requirement of signal amplification using biochemical reaction have to be taken into account [6,7].

Fluorescence spectroscopy and microscopy [8,9], electrochemistry [10] and surface plasmon resonance (SPR) [11] assays were also developed for PSA quantification purposes. However, fluorescence and electrochemistry-based methods require labeling of target analyte with dyes and conductors for signal generation and amplification [9,10]. Sample loss during the modification steps may affect the turnout quantification results. Besides, prolonged exposure of fluorescence dye to excitation light source causes photobleaching and quenching of signals that may lead to false negative and underestimated results. Although SPR can provide a rapid, label-free and real time monitoring of the analyte by measuring change in refractive indices, high dependency on the physical refractive properties limits the types and sizes of analyte that can be detected by SPR and so restricts the choices of materials for biosensing platform [12,13]. A comparison among these methods is described in Table S1.

To overcome the limitation of conventional instrumental analysis, nanotechnology is now widely developed and applied in clinical and biomedical industry. Nanomaterials have good biocompatibility, large surface area to volume ratio and wide variety of chemical modifications possibility on their active surfaces [14,15]. These properties facilitate nanomaterials with applications of signal transducers, analyte-conjugating or capturing probes, nanocarriers for *in vivo* material transportation, etc. [16]. Among all types of nanomaterials, metallic gold and silver nanoparticles are commonly utilized due to their simple synthetic preparation in bulk amount, relatively high stability, unique optical properties and strong conjugation ability with biomolecules via electrostatic, Au/Ag-thiol or Au/Ag-amine interactions [17–19]. In addition, as there is no concern of photo-bleaching in nanomaterials, prolonged

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http://dx.doi.org/10.1016/j.snb.2013.09.057

light exposure that is forbidden in fluorescence analysis is hereby allowed in order to increase the signal to noise ratio of detection and improve assay sensitivity [20,21].

As a proof of concept, we proposed the use of silver nanoparticles (AgNPs) as a multifunctional nanomaterials that (i) conjugate on nanoparticle surface with the PSA-capturing antibody CHYH2, which serve as the immunochemical recognition counterpart of PSA; (ii) probe and capture the target antigen PSA which was electrostatically immobilized on surface of the glass flow cell for detection purposes; and (iii) serve as the signal transducer to scatter light as signals to be monitored and detected by the charge-coupled device (CCD) camera-conjugated dark field microscopy (DFM) system. Comparatively, silver nanoparticles (AgNP) demonstrated an enhanced extinction coefficient of approximately 3 times stronger than that of gold nanoparticles of same sizes [22], which gives a better signaling effect when applied as transducers under DFM. The nanoconjugate would capture the PSA on glass surface by immunoreaction and hence nanoparticles would be attached toward the glass surface. While the level of PSA is highly correlated to the number of nanoconjugates on the surface, by counting the number of AgNP with DFM, the amount of target PSA can thus be calibrated and determined. On the other hand, in order to illustrate the specificity of the assay, carcinoembryonic antigen (CEA) and Immunoglobulin G (IgG) were adopted as the negative control of the assay to test for the discrimination efficiency of the silver nanoprobe and the DFM system. Determination of PSA in normal donor serum sample was also demonstrated.

2. Materials and methods

2.1. Synthesis and characterization of silver nanoparticles

Citrate-stabilized silver nanoparticles (Citrate-AgNP) were synthesized based on the Lee-Meisel method [23]. Briefly, 1 mM silver nitrite solution was stirred and boiled with 1% sodium citrate tribasic dehydrate (≥99%, Sigma–Aldrich, St. Louis, USA) for 1 h until the solution turned greenish yellow in color. The UV–vis absorption spectrum of the silver particles was measured by a Cary 300 UV–vis spectrophotometer (Varian, Inc., Palo Alto, USA). The sizes and shapes of the nanoparticles were characterized by a Technai G2 Transmission Electron Microscope (FEI, Hillsboro, USA) with an acceleration voltage of 200 kV.

2.2. Preparation and optimization of silver nano-probes

The PSA-capturing silver nanoprobe was prepared by conjugation of silver nanoparticles with PSA-specific antibody via EDC/NHS chemistry. Briefly, the as-synthesized citrate-AgNP solution was first diluted with filtered distilled water. Then, excess amount of 1-mercaptoundecanoic acid (MUA, 98%, Sigma–Aldrich) was added to react covalently with the surface of the silver nanoparticles via Ag-S bond and displacement reaction. The solution was stirred for 2 h, and 1 μM of freshly prepared N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC, ≥97%, Sigma–Aldrich) and 100 μM of freshly prepared N-hydroxysuccinimide (NHS, ≥98%, Sigma–Aldrich) were then added into the MUA-AgNP solution and stirred for another 30 min. Finally, mouse anti-human PSA monoclonal antibody [Clone number: CHYH2] (Anogen, Ontario, Canada) was added to the activated AgNP, and the solution was rotated for 2 h at ambient temperature for conjugation of CHYH2 onto the nanoparticles surface and forming the nanoprobe CHYH2-AgNP. In order to avoid aggregation of nanoprobe under high ionic strength and also to block vacant sites on the particle surface from non-specific adsorption of other substances, thiolated methoxyl-polyethylene glycol (CH_3O-PEG-SH, 2000 Da, RAPP polymere, Tuebingen, Germany) was added to the CHYH2-AgNP solution. The dose of CH_3O-PEG-SH to be added into the nanoprobe was optimized by comparing the aggregation performance of nanoprobe under different ratio of [MUA]:[CH_3O-PEG-SH] applied, ranged from 1:19 to 19:1. The mixed solution with optimized amount of CH_3O-PEG-SH added was then incubated at room temperature for 30 min, and was subjected to centrifugation at 4 °C, 10,000 rpm for 30 min with the refrigerated microcentrifuge (LabnetPrism^{TM}R, Labnet, Edison, USA). The supernatant was removed, and the pellets were re-suspended in Tris buffer saline (TBS, contains 50 mM Tris–HCl (UltraPure, pH 8.0, Invitrogen, Carlsbad, USA) and 150 mM sodium chloride, filtered by 0.2 μm nylon filter prior to use) buffer for terminating the unreacted EDC/NHS surface and for storage.

2.3. Preparation of cover glasses

All cover glasses were pre-washed prior to experiments. Briefly, No. 1 22 × 22 mm and No. 1 22 × 30 mm microscopic cover slides (Menzel-Gläser, Braunschweig, Germany) were successively sonicated twice with filtered distilled water for 15 min and twice with absolute ethanol for 15 min. The slides were then rinsed with filtered distilled water and soaked in piranha solution (conc. H_2SO_4/30% H_2O_2, v/v 1:1) for 30 min, and then rinsed with filtered distilled water extensively. The piranha washing steps were repeated once, and the extensively rinsed slides were further sonicated twice in water for 30 min. The cleaned cover glasses were blown-dried completely with nitrogen.

2.4. Pretreatment of glass slides and preparation of flow cell

In order to increase the adsorption ability of the upper glass slides on the flow cells which will be imaged, the upper 22 × 22 mm glass slides were pre-treated with poly-L-lysine (PLL, M.W. 70k–150k, Sigma–Aldrich) to provide positive surface for antigen immobilization. Briefly, 20 μL of 0.1% PLL was added to the surface of glass slides and the droplet was spread over in order to cover the whole glass surface. The slide was then extensively rinsed with water after incubation of 5 min and then blown-dried under nitrogen before use. On the other hand, in order to reduce the non-specific adsorption of nanoprobes on the bottom glass slide, the bottom 22 × 30 mm glass slide were pre-coated with protein blocker Albumin from bovine serum (BSA, ≥98%, Sigma–Aldrich). The dose of BSA blocker to be applied was optimized by comparing the number of nano-probes non-specifically adsorbed onto the glass channel treated with 0, 0.25%, 0.5% and 1% BSA in 50 mM TBS solution. The BSA blocking condition with the lowest nanoprobes counts was considered providing the best blocking ability. Herein, 30 μL of BSA of optimal concentration was then added onto the bottom slides and spread over. The slides were rinsed with water after incubation of 15 min and blown-dry with nitrogen before use. Sealed flow cell was then prepared by combining the upper PLL-treated 22 × 22 mm cover glasses and the lower BSA-treated 22 × 30 mm cover glasses with double-sided adhesive tapes with a channel width of approximately 3 mm each (volume of each channel was estimated to be 6.6 μL).

2.5. Preparation of on-chip immunoassay

PSA immunoassay was established on-chip within the upper glass surface of channel. Briefly, 10 μL of 37 °C pre-warmed TBS buffer was flowed into each channel twice for conditioning of channels. The excess solution was removed at the end of each channel by Kimwipes based on capillary force. Then, 10 μL of target PSA of 0, 10, 50, 100, 150, 250, 500 pM and 1 nM respectively was added into individual channels and incubated for 15 min at ambient temperature to allow electrostatic immobilization of antigen onto the