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Triple signal amplification strategy based on size and shape transformation of ultrasmall sub-10 nm gold nanoparticles tag towards sensitivity improvement of electrochemical immunosensors

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ABSTRACT

Triple signal amplification strategy was designed to enhance the electrochemical detection sensitivity of immunosensing platform based on gold nanoparticles (AuNP) tagging. The strategy described herein includes the stepwise enlargement of AuNP tag. The first step was achieved by reducing gold chloride ion in presence of AuNP acting as nucleation seeds. In this step, size of AuNP was increased by the growth of gold shell over small AuNP tag. The second step was accomplished by initiating the growth of spiky AuNP. This particular step will offer a large increase of AuNP size and surface area due to the change of nanoparticles shape. Finally, the silver enhancement step was used to provide an electroactive layer for electrochemical detection. The sandwich immunoassay was carried out using prostate specific antigen (PSA), as a model analyte. Precursors and enhancement process, which were commonly used for spiky AuNP synthesis, were adapted and optimized to achieve the highest electrochemical signal. Since the spiky AuNP enhancement step is established and reported here for the first time, the effect of relevant experimental conditions, i.e. concentration and ratio of Au and Ag ions, reducing agents, and reaction time, were thoroughly examined. Size of AuNP tag and enhancing steps were also confirmed to have the most influence on the successful enhancement process. By understanding the valuable insight of this approach, detection sensitivity of applicable AuNP-tagged immunoassay can be significantly improved. Based on an electrochemical immunosensor demonstrated here, the triple signal amplification leads to over 260-fold of signal increase in comparison with conventional silver enhancement process. Therefore, this approach would be promising strategy for improving detection limit of AuNP-tagged sensing platform.

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1. Introduction

Ultrasensitive detection of nucleic acid or protein biomarkers has been considered the most critical tool in clinical diagnostics. Considering low concentration and small variation in expression of biomarker level, the development of sensitive, accurate, reliable, and cost-effective measurement is particularly important. Among several analytical techniques, electrochemical method has become an attractive choice of detection due to the advantages of being highly sensitive, high throughput, portable and applicable to point-of-care detection. Generally the development of biosensors toward high sensitivity mainly focuses on two key components; (1) electrode and (2) signaling probe. Highly conductive nanomaterials such as graphene, carbon nanotubes, and AuNP are typically used for modification of the former [1–4], while bio-conjugated enzymes, electroactive molecules and nanoparticles are employed as the latter. The biorecognition events such as antibody-antigen interaction, nucleic acid-nucleic acid hybridization generates an electrochemical signal through the availability of signaling probes. Many efforts have been made to amplify the signal by increasing the amount of signaling probes per single biorecognition event. Two main approaches have been widely used. The first method involved the incorporation of nanomaterials to maximize the loading of electrochemical labels. Carbon nanotubes (CNT), carbon nanohorn (CNH), graphenes (GP), gold nanorods (AuNR), and polymer beads have been demonstrated as carriers for high loading of enzyme and redox molecules [5–11]. This approach leads to highly amplified signal comparing to the conventional single-label probe; however, larger carrier has been reported to create steric hindrance causing poor binding interaction and inversely affect the detection signal [12]. The second method focuses on sensitivity improvement after completion of biorecognition event. In this case, the

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amplification performance depended on the ability to multiply the signaling probes. Indeed, without the signal amplification, AuNP labeling failed to compete with enzyme labeling because enzyme posses a very high turnover number resulting in high sensitivity. However, the interference from dissolved oxygen reduction limited practical application of enzyme labels, in particular horseradish peroxidase (HRP) enzyme [13,14]. Due to the aforementioned drawbacks of enzyme label, several signal amplification techniques of AuNP label have been developed [15–23]. AuNP-induced metal deposition, first pioneering by Wang et al. [24], is the most commonly used. Sensitivity improvement based on this principle can be accomplished through enzymatic or non-enzymatic catalytic reactions. For example, ultrasensitive multiplex detection of tumor markers (carcinoembryonic antigen, CEA and alpha-fetoprotein, AFP) was developed using AuNP-induced silver deposition [15]. Silver stripping peak was measured and analyzed using linear sweep voltammetry (LSV). Similar enhancing protocol was applied to CEA detection; though, polybead carried AuNP was used as labeling probe instead of colloidal AuNP for the purpose of high loading AuNP [8]. It has been recognized that silver enhancement typically involved high background current originated from an uncontrollable metallocatalytic reaction. Therefore, hydrazine-loaded AuNP was later reported for detection of human epidermal growth factor receptor 2 (HER2) over expressing on breast cancer cell. The hydrazine reductant was directly attached on the AuNP probe to avoid the nonspecific deposition of silver nanoparticles (AgNP) on the sensor surface [23]. Apart from AuNP-induced Ag deposition, Au deposition has also been studied [16,21,25,26]. Unfortunately for electrochemical detection, direct oxidative measurement of gold required high oxidation potential and harsh acidic conditions resulting in fewer applications developed to date.

In addition to non-enzymatic reaction, enzyme-catalyzed Au or Ag deposition was incorporated into the signal amplification. For example, alkaline phosphatase (ALP) was loaded onto irregular-shaped AuNP. The resulting complex was conjugated with anti-AFP antibody and used as signaling probe. Upon signal enhancement process, *p*-aminophenyl phosphate (PNNP) and silver nitrate ($AgNO_3$) were added. ALP then catalyzed conversion of PNNP to a *p*-aminophenol (p-AP) which acting as reducing agent for Ag deposition [27]. Combination of enzymatic and non-enzymatic silver enhancement was demonstrated via successive amplification step. The first step involved ALP-catalyzed silver deposition using ascorbic acid 2-phosphate (AA-p) as enzyme substrate. Followed by the second step of AgNP-induced Ag deposition via addition of $AgNO_3$ and hydroquinone [17]. Recently, enzyme-catalyzed gold deposition was established using glucose oxidase (GOD)-loaded gold nanorods (AuNR) as labeling probe. Pre-oxidation of deposited Au with subsequent Au stripping analysis was done for quantitative measurement of CEA [10]. The AuNP-induced Au and Ag depositions were not only coupled to electrochemical stripping analysis, but also been explored for quantitative analysis using dot-blot immunoassay [28,29], inductively coupled plasma mass spectrometry (ICPMS) [22], chemiluminescence immunoassay [26], and UV–vis absorption [21]. As noted above, remarkable signal enhancement associated with the use of nanoparticles probes coupled with different techniques have been highly acknowledged. Interestingly, Au and Ag enhancement exploited in those systems always relied on the standard Au and Ag enhancers.

In this paper, stepwise enhancement of AuNP tag of sandwich-based immunosensor was investigated using PSA as a model analyte. The enhancement step related to transformation of labeling tag from spherical AuNP to spiky AuNP was first introduced. Studies of chemical compositions of enhancer solutions and reaction conditions were performed. This information is critically important due to the fact that there is no prior knowledge on spiky gold enhancer solution, which is suitable for signal amplification of AuNP tag. The use of ultra-small AuNP tag (7 nm diameter) in combination with specific amplification steps was performed to investigate its effect on signal amplification. TEM analysis was used to physically characterize the transformation of AuNP size and shape. Finally, sensitivity improvement of the proposed strategy was demonstrated with respect to conventional single step enhancement coupled with regular size AuNP tag (26 nm diameter).

2. Experimental

2.1. Chemicals and materials

A mouse monoclonal antibody pair; anti-human prostate specific antigen (PSA) capture antibody (Ab1, clone no. CHYH1) and anti-PSA detection antibody (Ab2, clone no. CHYH2), and PSA standards in calf serum were obtained from Anogen/Yes Biotech Lab, Ltd. Carbon nanotube powder (CNT) was obtained from Bayer ®. Glutaric dialdehyde (GD, 25% aqueous solution, purified) was purchased from Thermo Fisher Scientific. Nafion 117® solution (purum, 5%) was purchased from Fluka. Chitosan (CS, ≥75% deacetylation), bovine serum albumin (BSA), gold(III) chloride hydrate ($HAuCl_4$, 99.999% trace metals basis, hexadecyltrimethylammonium bromide (CTAB, BioXtra, ≥99%), L-ascorbic acid (AA, 99%), hydroquinone (HQN, ReagentPlus®, ≥99%), hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$, ReagentPlus®, 99%), sodium borohydride ($NaBH_4$), sodium carbonate (Na_2CO_3) and sodium chloride (NaCl), were obtained from Sigma-Aldrich Chemical Co. Ltd. Silver nitrate ($AgNO_3$, AnalaR NORMAPUR®) was purchased from VWR International, Pty Ltd. Trisodium citrate was obtained from Riedel-deHaën. Tween-20 was purchased from Bio-Rad Laboratories, Inc. Screen-printed carbon electrodes (SPCEs) containing carbon working and counter electrodes and a Ag/AgCl reference electrode, were purchased from CHInstrument and used as received.

The buffers used in all experiments were as follow: (A) working solution; 0.01 M phosphate buffered saline, pH 7.4 (PBS). (B) washing solution; 0.01 M PBS containing 0.05% (w/v) Tween20. (C) blocking buffer; 0.01 M PBS containing 5% (w/v) BSA. The blocking buffer was stored at 4 °C and used within a week, and (D) citrate buffer (0.1 M, pH 3.5) were used for preparation of silver enhancer solution. All other reagents are analytical grade and used as purchased without further purification. Deionized (DI) water of specific resistance 18 MΩ was used throughout the experiment.

All enhancer solutions were separately prepared in 2 parts (A and B) and thoroughly mixed on the working electrode to locally activate the reactions. For the first Au enlargement step, enhancer solution A composes of 2.0 mM $HAuCl_4$ and 2.5 mM CTAB and enhancer solution B is 10 mM AA solution in PBS buffer. For the spiky Au enhancement step, enhancer solution A composes of 2.0 mM $HAuCl_4$ and 0.2 mM $AgNO_3$ and enhancer solution B is 10 mM $NH_2OH{\cdot}HCl$ in DI water (optimized conditions). To prepare silver enhancer solution, a mixture of citrate buffer and DI water at 1:1 vol ratio was used as diluent. The silver ion solution (2 mM $AgNO_3$) and reducing agent (50 mM HQN) were used as enhancer solution A and B, respectively. All enhancer solutions were freshly prepared prior to use.

2.2. Synthesis of gold nanoparticles (AuNP)

Two sizes (7 and 26 nm diameter) of AuNP were synthesized and used to prepare labeling probes. For small AuNP synthesis, 0.5 mL of an aqueous solution of 10 mM $HAuCl_4$ was added to 18.4 mL of DI water containing 0.5 mL of 10 mM sodium citrate. The solution was kept cold using an ice bath for 30 min prior to reaction. Then, 0.6 mL of a freshly prepared $NaBH_4$ solution was quickly injected

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