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Signal amplification for multianalyte electrochemical immunoassay with bidirectional stripping voltammetry using metal-enriched polymer nanolabels

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ABSTRACT

A novel bidirectional (anodic and cathodic) stripping voltammetric immunoassay (SVI) was designed for the simultaneous determination of multiple model cancer biomarkers (AFP, CEA and CA19-9) in a single run, based on the integration of Envision complex loaded metal nanoparticles as signal tags and immunomagnetic beads as capture probes. The Envision complex, which is a long branching polymer consisting of numerous secondary antibodies and horseradish peroxidase (HRP), was employed for signal amplification by labeling corresponding detection antibodies and further loading metal nanoparticles (CdS, PbS and gold) to prepare distinguishable metal signal tags. Herein, the generation of immunosensing probes involved the co-immobilization of three types of primary anti-AFP, anti-CEA, and anti-CA19-9 antibodies onto a single magnetic Dynabead. After a two-binding step sandwich-type immunoassay, the Envision/CdS, Envision/PbS and Envision/Au signal tags were introduced onto the surface of the Dynabeads. The subsequent bidirectional voltammetric analysis of stripping metal components from immunocomplexes for quantification of tumor biomarkers was performed in a microcell with minimum capacity of 50 μL. Experimental results showed the immunoassay enabled the simultaneous determination of multiple biomarkers over a broad range of concentrations (AFP, 1 pg mL^{-1}–50 ng mL^{-1}; CEA, 1 pg mL^{-1}–50 ng mL^{-1}; CA19-9, 5 pg mL^{-1}–100 ng mL^{-1}) with detection limits reaching 0.02 pg mL^{-1} for AFP, 0.05 pg mL^{-1} for CEA, and 0.3 pg mL^{-1} for CA19-9. The results indicated that the proposed bidirectional multiplexed immunoassay can increase the number of analytes by SVI and has high sensitivity, excellent stability, and great promise for applications in clinical cancer diagnosis.

1. Introduction

Cancer is one of the greatest threats to human health and early determination of cancer biomarkers is of great importance in clinical cancer diagnosis [1]. To meet requirements for monitoring trace biomarkers, many researchers have focused on detection of a single cancer biomarker over recent decades [2–4]. However, this approach does not take into account the complexity of the process of carcinogenesis. Many biomarkers are not specific to a particular cancer [5,6]. Therefore, the development of methods for the simultaneous determination of multiple tumor markers in a single assay has attracted much attention. Compared with single-analyte assays, simultaneous multianalyte immunoassays (SMIAs) have numerous advantages. For example, SMIAs require a shorter analytical time, smaller sample volume and reduced detection costs per assay [7–9]. Moreover, SMIAs provide improved specificity and accuracy for diagnosis.

Recently, numerous electrochemical immunoassays and immunosensors for the quantitative detection of multiple analytes have been reported because they have special merits including high sensitivity, inherent simplicity, and low cost [10–13]. Nowadays, most researchers focus on mutilabeling protocols to establish optimal SMIAs. Hence, different signal tags based on enzymes [14], redox tags [15] and metal labels [16,17] have been developed for this immunoassay system. Among these labeling

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methods, metal tags have attracted considerable interest due to the well-defined and sharp stripping voltammetric peak at different positions for these metal nanoparticle tracers from the corresponding signal tags. An anodic stripping voltammetry (ASV) measurement involves two steps: deposition and stripping procedure. Firstly, the analytes to be determined were deposited onto the working electrode at a cathodic potential. Then the potential moved in the anodic direction to strip this material from the electrode. And the stripping peak current is proportional to the concentration of analyte [18–21]. Recently, Tang et al. reported the development of an electrochemical immunoassay for multiplexed detection using PAMAM dendrimer-metal sulfide quantum dots nanolabels as signal tags [22]. Zhu et al. described an electrochemical immunoassay based on the use of metal-ion functionalized titanium phosphate nanospheres as signal tags with an extremely lower detection limit of pg/mL [23]. Kong et al. devised an electrochemical immunosensor for the simultaneous determination of two biomarkers using CdS/DNA and PbS/DNA nanochains as signal tags [24]. For a cathodic stripping voltammetry (CSV) measurement, the analytes were oxidized at an anodic potential, and the potential moved in the cathodic direction to strip this material from the electrode. And the stripping peak current is also proportional to the concentration of material. In general, CSV is employed to determine Au element. Leng et al. reported a multiplex immunoassay on screen printed carbon electrodes to detect two biomarkers using gold nanoparticles (Au NP) as an electrochemical label [25]. These successful developments in the simultaneous determination of multiple biomarkers employ anodic or cathodic stripping voltammetry, which is designated as a single-sweep voltammetry. However, the signal tags employed for detection in single-sweep voltammetry are limited, which also limits the number of detectable analytes. Consequently, there is an urgent requirement for the development of more electrochemical labels for the determination of a greater number of biomarkers in a single detection procedure. Therefore, we designed a novel SMIA based on several metal labels using anodic and cathodic stripping voltammetry, which was designated as a bidirectional voltammetry.

Moreover, the highly sensitive detection of trace target analytes has been required in early clinical diagnosis [26,27]. In order to enhance the sensitivity significantly, various polymerization-based amplification systems have been built as matrix by immobilizing signal source material. Up to now, a considerable number of biocompatible polymer materials have been used as carriers to increase the upload of electrochemical tags for detection of DNA and tumor markers due to its easy tuning of the amount of the functional polymer chains and adjustment of the spatial distribution of probe species [28–32]. Recently, Yuan et al. developed a polymerization-based amplification strategy for the detection of human immunoglobulin G antigens using the poly(glycidyl methacrylate) as a carrier to load CdTe quantum dots [33]. We have previously devised an electrochemical immunosensor for the sensitive detection of AFP using Envision complex on a gold electrode [34]. Envision complex, as a polymeric material, can exhibit excellent biocompatibility and water-solubility without decorating for the immobilization of signal substances. It is a polymeric conjugate consisting of numerous anti-antibodies which takes the primary antibody as antigen and horseradish peroxidase (HRP) coupled to a dextran backbone [35]. In this study, Envision signal amplification was based on the use of HRP as a mediated-carrier to provide a large number of enzyme sites for coupling of Au, CdS and PbS nanoparticles.

Magnetic beads have emerged as a powerful tool for fast separation and effective enriching when labeled with antibody to prepare capture probes in the field of electrochemical immunoassays. They significantly reduce the assay time, provide a large immobilization area, minimize matrix effects and favor analytical procedures more applicable to higher sample throughput and automation [36–40]. Moreover, the use of magnetic beads as a reaction matrix not only improves the performance of immunological processes but also avoids electrode fouling [41]. Kong reported a novel multianalyte electrochemical immunoassay for the simultaneous detection of dual proteins based on magnetic beads as immunosensing probes [24].

In this study, we designed a novel signal amplification and bidirectional stripping voltammetric immunoassay for the simultaneous detection of three tumor markers (CEA, AFP, and CA19-9 used as models) using Envision/metal nanolabels as distinguishable signal tags. Three primary antibodies were co-immobilized onto the surface of Dynabeads, which were used for capture and enrichment of the three target biomarkers. We prepared three signal tags, anti-AFP_2/Envision/CdS anti-CEA_2/Envision/PbS, and anti-CA19-9_2/Envision/Au, using Envision binding with three corresponding secondary antibodies as carriers prior to loading the three different types of metal nanoparticles (CdS, PbS and Au). After a typical sandwich immunoreaction, the immunocomplexes formed were quantified using anodic and cathodic stripping voltammetry. The study provides a reliable method to monitor multiple tumor markers.

2. Experiment

2.1. Reagents and apparatus

CEA, AFP, CA19-9, CA125, CA153, and human immunoglobulin G (IgG) standard grade antigens were purchased from Biocell (Zhengzhou, China). Mouse monoclonal primary antibody anti-AFP, anti-CEA, anti-CA19-9 and mouse monoclonal second antibody anti-AFP, anti-CEA, anti-CA19-9 were obtained from Linc-Bio Science Co. Ltd. (Shanghai, China). Envision™ Detection Kit was purchased from Gene Tech Company Limited (Shanghai, China). Bovine serum albumin (BSA) was purchased from Ding Guo Biotechnology Company (Beijing, China) 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and Human Serum Albumin (HSA) were purchased from Sigma–Aldrich. Mercaptoacetic acid and $HAuCl_4$ were purchased from Aladin Ltd. (Shanghai, China). 0.05 M PBS (containing 0.05 M NaH_2PO_4 and Na_2HPO_4), PBST consisted of a PBS (pH 7.4) with 0.05% (v/v) Tween 20. Dynabeads Myone™ Tosylactivated were purchased from Invitrogen (Dynal AS, Oslo, Norway). $CdCl_2$, Na_2S, $Pb(NO_3)_2$, NaOH, sodium citrate, bismuth nitrate, and all other chemicals were of analytical reagent grade, and doubly distilled water was used in all the experiments.

All electrochemical immunoassay measurements were performed on a CHI 620D Electrochemical Workstation (Shanghai, China). A glassy carbon electrode (GCE, 3 mm diameter) was used as working electrode, with Ag/AgCl and platinum wire acted as the reference electrode and auxiliary electrode, respectively. The UV–vis spectra were carried out using a TU-1901 spectrophotometer (Beijing Purkinje General Instrument Co. Ltd., China). The transmission electron microscopic (TEM) image was obtained with a H600 transmission electron microscope (Hitachi, Japan). Scanning electron micrographs (SEM) were obtained with a S3400N scanning electron microscope (Hitachi, Japan).

2.2. Preparation of the electrochemical reaction microcell

To reduce the volume of the sample and miniaturize the device, a novel electrochemical reaction microcell was designed based on a conventional three-electrode system comprising a glassy carbon electrode (GCE, 3 mm diameter) as a working electrode, a small