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Electrochemical coding for multiplexed immunoassays of biomarkers based on bio-based polymer-nanotags

De Wang [a], Tianhua Li [a], Ning Gan [a,*], Huairong Zhang [a], Nengbing Long [a], Futao Hu [a], Yuting Cao [a], Qianli Jiang [b,**], Shan Jiang [b]

[a] State Key Laboratory Base of Novel Functional Materials and Preparation Science, Faculty of Materials Science and Chemical Engineering, Ningbo University, Ningbo 315211, PR China
[b] The Department of Hemabology, Southern Medical University, Guangzhou, Guangdong 510515, PR China

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ABSTRACT

A novel sandwich-type electrochemical multiplexed immunoassay was designed for simultaneous determination of alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA) cancer biomarkers by using bio-based polymer-nanotags as signal probes and dual antibodies labeled magnetic beads as capture probes. This signal probes were prepared by co-immobilizing encoded metallic apoferritin (Cd-Apo and Pb-Apo) and primary antibodies (anti-AFP and anti-CEA) on poly-L-lysine (PLL) via gold nanoparticles (AuNPs). The preparation procedures were through self-assembly technology without using coupling agent. After a sandwich-type immunoreaction, the polymer-nanotags were captured to the surface of Dynabeads. The subsequent electrochemical stripping analysis of the metal components from the nanocomposite provide a means for discriminating dual targets based on the peak potential of Cd and Pb. The currents of Cd and Pb were proportional to the concentration of AFP and CEA, respectively. Experimental results showed the immunoassay enabled the simultaneous determination of AFP and CEA in a single run with dynamic ranges of 0.01-50 ng mL^{-1}. And the detection limits of AFP and CEA were 4 pg mL^{-1} (S/N = 3), respectively. This proposed multiplexed immunoassay is simple, sensitive and environment-friendly. More importantly, this proposal was employed in real serum samples to detect two tumor markers at the same time. It can be applied for clinical screening of biomarkers.

1. Introduction

Recently, various analytical techniques have been developed for simultaneous multianalyte immunoassay of tumor markers based on antigen-antibody highly specific recognition [1–4]. Compared with the conventional single-analyte assay, simultaneous determination of multiple targets of interest has stimulated intense research, due to its remarkable advantages such as improved specificity and accuracy for diagnosis, shortened analytical time, high sample throughput, low sample requirement and reduced cost per assay [5–7]. In conclusion, multiplexed electrochemical immunoassay has become one of the leading trends, owing to its easy miniaturization and automated detection, high sensitivity, low cost, and good portability [8,9].

For the successful development of simultaneous determination of multianalyte in a single run, the key is searching some distinguishable signal tags. The introduction of nanomaterials, which enhance response, sensitivity, and stability, provides an excitingly new possibility for fabricating such above signal tags [10–13]. Currently, quantum dots (QDs) were widely applied in the fabrication of metallic encoded labels to achieve simultaneous determination of multiple targets. Tang et al. designed a new multianalyte electrochemical immunoassay for the detection of multiplex biomarkers using PAMAM dendrimer-metal sulfide (QD) nanolabels as distinguishable signal tags [14]. Wu et al. reported an ultrasensitive multiplexing immunosensor using graphene as a sensoring platform and different QD-coated silica nanoparticles as tracing tags [15]. However, the synthesis of high-quality and uniform-size QDs is often a time-consuming process and requires harsh preparative conditions [16,17]. Moreover, the operation of adding strong acid is necessary before electrochemical detection, in order to release metal ions. Thus, the challenge still exits to develop new strategies for simple preparation of environment-friendly and biocompatible nanotags to achieve multiplexed electrochemical immunoassays.

* Corresponding author. Tel.: +86 574 87609987; fax: +86 574 87609987.
** Corresponding author.
E-mail addresses: ganning@nbu.edu.cn (N. Gan), jiangqianlid@aliyun.com (Q. Jiang).

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Apoferritin (Apo) is a hollow cage-like spherical protein with a outer diameter of 12 nm and an inner cavity diameter of 8 nm [18,19]. Apo has a high coding capacity with cadmium and lead ions. The metal ions (M), such as Cd and Pb ions, can be easily incorporated into and released from the cavity by adjusting pH. Moreover, it is biocompatible without toxicity. Recently, Chen et al. reported an electrochemical immunoassay using protein cage templated lead phosphate for the quantification of phosphorylated p53 [19]. Wang et al. have also designed an electrochemical immunosensor for the detection of avian leukosis virus *subgroup J* using graphene quantum dots (GQDs) and Apo-encapsulated Copper (Cu-Apo) nanoparticles for signal amplification [20]. Herein, Apo was used to encapsulate metal ions to prepare the encoded nanotags, which were designated as Cd-Apo and Pb-Apo respectively.

In order to enhance the sensitivity of multiplex immunoassay, some bio-based polymers were extensively used as matrix to immobilize nanolabel for signal amplification. Poly-L-lysine (PLL), a linear biopolymer with plentiful active amino groups and good solubility, can be employed as a carrier to load nanoparticles [21–23]. Gold nanoparticles (Au NPs) are considered as excellent nanomaterial due to their favorable biocompatibility and binding affinity [24,25]. Thus, the PLL-Au composite, which has large amount of active sites to immobilize proteins, can be used for directly immobilizing Apo nanoparticles and antibodies (Ab) to prepare signal tags and enhance electrochemical responses. Moreover, the entire process is simple through self-assembly technology and environment-friendly without using linking reagents. In this work, we prepared a kind of novel polymer-nanotags (PLL-Au-Cd-Apo and PLL-Au-Pb-Apo) by immobilizing encoded nanotags (Cd-Apo and Pb-Apo) on PLL-Au.

In this paper, a novel multiplex electrochemical immunoassay was designed for simultaneous determination of alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA) using PLL-Au-Cd-Apo-anti-AFP and PLL-Au-Pb-Apo-anti-CEA polymer-nanotags. Dual primary antibodies were co-immobilized on the immunized magnetic beads (Dynabeads), which were used for capturing target biomarkers. With a sandwich-type immunoassay, the resulted immunocomplex were formed on the surface of the capture probes. Dual analytes were quantitatively analyzed by square wave voltammetry (SWV) after metal ions were released from immunocomplex. Moreover, this proposed strategy was employed for simultaneous determination of AFP and CEA in human serum samples, whose outcomes were well consistent with conventional enzyme-linked immunosorbent assay (ELISA).

2. Experiment

2.1. Reagents and Apparatus

Dynabeads Myone™ Tosylactivated was purchased from Invitrogen (Dynal AS, Oslo, Norway). Apoferritin was purchased from Sigma. AFP, CEA, Immunoglobulin G (IgG), CA125 and Human Serum Albumin (HAS) standard grade antigens were purchased from Biocell Biotechnol. Co. Ltd. (Zhengzhou, China). PLL (molecular weight = 70–150 kD) was purchased from Sigma-Aldrich (St Louis, MO, USA). Mouse monoclonal primary antibody anti-AFP, anti-CEA and mouse monoclonal second antibody anti-AFP, anti-CEA were obtained from Linc-Bio Science Co. Ltd (Shanghai, China). Bovine serum albumin (BSA) was purchased from Ding Guo Biotechnology Company (Beijing, China). $HAuCl_4$ were purchased from Aladin Ltd (Shanghai, China). $CdCl_2$, $Pb(NO_3)_2$, NaOH, sodium citrate, bismuth nitrate, sodium acetate anhydrous (NaAc) and all other chemicals were of analytical reagent grade, and doubly distilled water was used in all the experiments. HAc-NaAc buffer solution (0.1 mol L^{-1}, pH = 4.5) was used as the supporting electrolyte. The washing/storage buffer was prepared with Phosphate-buffered saline (PBS, pH 7.4, 0.5 mM) containing 0.1% BSA and 0.05 Tween-20. Clinical serum samples were available from by Nanfang Hospital of Nanfang Medical University.

All electrochemical immunoassay measurements were performed on a CHI 842 C 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 Shimazu UV-1800 spectroscopy (Shimadzu, Japan). The transmission electron microscopic (TEM) image was obtained with a H600 transmission electron microscope (Hitachi, Japan). Scanning electron micrographs (SEM) were obtained with a S3400 N scanning electron microscope (Hitachi, Japan). Infrared spectra were recorded with a Nicolet 6700 FT-IR spectrophotometer (Nicolet, USA).

2.2. Preparation of Dynabeads-antibodies conjuates

The preparation procedure of Dynabeads-antibodies conjuates (designated as Dynabeads-Ab1) was carried out similar to our previous report [26]. In brief, 100 μL of Dynabeads (100 mg mL^{-1}) solution was washed with washing buffer and collected by magnetic separation in the pretreatment. Following that, the Dynabeads were incubated with 1.0 mL PBS containing 0.2 mg mL^{-1} anti-AFP_1 and 0.2 mg mL^{-1} anti-CEA_1 for 6 h at 4 °C. The obtained Dynabeads-Ab1 conjugates were collected by magnetic separation and washed with washing buffer for three times. Finally, the obtained conjugates were dispersed into 1.0 mL storage buffer and stored at 4 °C for further use.

2.3. Preparation of M-Apo nanoparticles

The M-Apo nanoparticles were prepared according to the following steps [27,28]. A 10-μL aliquot of Apo solution (25 mg mL^{-1}) was introduced into 4.0 mL PBS (0.2 M, pH 7.4). after the solution pH was adjusted to 2 and was stirred for 30 min at this pH, 50-μL aliquot of 0.1 M metal ions (Cd^{2+}, Pb^{2+}) was drop-by-drop added into above solution under stirring for 1 h. The pH was then adjusted to 8.5 by 0.1 M NaOH and kept stirring for 3 h. The above mixture was centrifuged at 6000 rpm for 10 min. The resulted precipitate was washed three time with Tris–HCl (0.1 M, pH 8.0) using Amicon filter (MWCO = 25000). Afterward, the obtained M-Apo nanoparticles were dispersed into a 1.0 mL Tris–HCl solution.

2.4. Preparation of distinguishable polymer-nanotags

Au NPs were prepared via citrate reduction according to the previous literature [29]. Briefly, 100 mL, 10% $HAuCl_4$ was injected into flask and heated to boiling under vigorously stirring. Next, 2.5 mL, 1% sodium citrate was rapidly introduced into above flask. The color of solution turned red slowly and was kept boiling until the color did not change. After that, the solution was cooled to room temperature and the obtained Au NPs (0.1 g mL^{-1}) was stored at 4 °C until use.

PLL-Au nanocomposites were synthesized according the following steps. 10 μL of PLL (5.0 mg mL^{-1}) was added into 200 μL Au nanoparticles while stirring. PLL contains abundant active amino groups. Massive Au NPs were conjugated to PLL to form PLL-Au nanocomposites, which is attributed to the strong coordinating capability between–NH_2 and Au NPs. After centrifugation at 8000 rpm for 10 min, the obtained PLL-Au nanocomposites were dispersed in 1 mL of doubly distilled water for further use. 100 μL of M-Apo (15.0 mg mL^{-1}) was added into the above