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Enhanced conductometric immunoassay for hepatitis B surface antigen using double-codified nanogold particles as labels

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ABSTRACT

A new conductometric immunoassay for hepatitis B surface antigen (HBsAg) was developed based bioelectrocatalytic reaction on a microcomb-type electrode by using double-codified nanogold particles as labels. This microcomb-type electrode was fabricated on an interdigitated transducer covered with a well-ordered *anti*-HBs/protein A/nanogold architecture. The double-codified nanogold particles were prepared by using nanogold-labeled *anti*-HBs antibodies conjugated with horseradish peroxidase (HRP). Sandwich-type immunoassay protocol was successfully introduced for the detection of HBsAg. The formation of the immunocomplex changed the direct electrical communication between the carried HRP and the electrode, and thus local conductivity variations could be assayed based on the bioelectrocatalytic reaction of the carried HRP in 0.01 M PBS (pH 7.0) containing 60 μM H_2O_2, 0.08 M KI and 0.1 M NaCl. Under optimized conditions, the linear range obtained by using HRP-conjugated *anti*-HBs as secondary antibodies was 1.5–450 ng/mL HBsAg, while the assay sensitivity by using double-codified nanogold particles could be further increased to 0.01 ng/mL with the linear range from 0.1 to 600 ng/mL HBsAg. The developed immunoassay method showed good precision, high sensitivity, acceptable stability and reproducibility, and could be used for the detection of real sample with consistent results in comparison with those obtained by the ELISA method.

1. Introduction

Immunoassays, based on highly specific molecular recognition of antigens by antibodies, have become the main analytical methods in clinical and biochemical analyses and in other areas such as environmental control, food quality control, etc. [1,2]. Despite many advances in this field, it is still a challenge to find new approaches that could improve the simplicity, selectivity, and sensitivity of immunoassays [3]. So, great efforts have been made worldwide to develop and improve immunoassays with the aim of making portable and affordable devices. Conductometric immunosensors, with high sensitivity, low cost, low power requirements and high compatibility with advanced micromachining technologies, have been achieved extensive attentions [4]. Furthermore, the conductometric immunosensors are suitable for miniaturization and large-scale production, without reference electrode and with low driving voltage [5]. Hnaiein et al. reported a new approach for immunoassays based on magnetite nanoparticles for Escherichia coli detection using conductometric measurements [6]. The nanoparticles-based conductometric immunosensors open a new avenue and platform in clinical conductometric immunoassays.

For the development of a successful conductometric immunosensor, the signal amplification of conductometric responses is very critical. The emerging research field of conductometric enzyme immunosensors provides excitingly new possibilities for advanced development of new analytical tools and instrumentation for bioanalytical and biotechnological applications [7]. One major merit of using bioactive enzyme is that conductometric enzyme immunosensors could detect products of enzymatic reactions due to increasing conductivity of the enzyme membrane [8]. Horseradish peroxidase (HRP) was largely used since it is well studied and is commercially available in a highly purified form. The catalytic reaction of HRP–H_2O_2–KI system has been developed for the detection of antigens in conductometric immunoassays, such as aflatoxin B_1 [8], staphylococcal enterotoxin B [9], and interleukin-6 [10]. In conductometric immunoassays, a large number of enzymatic reactions involve either consumption or production of charged species and therefore, lead to a change in ionic composition of the reacting solution.

In electrochemical immunoassays, sandwich-type protocol has the advantages of high specificity and sensitivity because of the

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use of a couple of match antibodies [11,12]. The mode is performed using antibodies or antigens with different labels. Zhu and co-workers reported a new method by using CdTe quantum dots as labels for electrochemical determination of protein with a detection limit of 5 pg/mL [13]. Lin introduced a disposable electrochemical immunoassay for the detection of IgG by using quantum dot (CdS@ZnS) as labels with a low detection limit of 10 pg/mL [14]. Recently, gold nanoparticle label is an ideal one in biotechnological systems due to its inherent advantages, such as easy preparation, good biocompatibility, and so on. Yang and co-workers described an ultrasensitive and simple electrochemical method for the fabrication of a sandwich-type heterogeneous electrochemical immunosensor. An IgG-nanocatalyst conjugate was prepared *via* direct adsorption of IgG on 10 nm gold nanoparticles [15]. However, there are little reports focusing on the conductometric immunoassays by using double-codified nanogold particles as secondary antibodies.

Hepatitis B is a serious disease caused by a virus that attacks the liver. The virus, which is called hepatitis B virus, can cause lifelong infection, cirrhosis (scarring) of the liver, liver cancer, liver failure, and death [16]. Hepatitis B surface antigen (HBsAg) is a major index of hepatitis B viruses [17]. Thus, the HBsAg detection is very helpful to clinical diagnoses. The aim of our present work was to develop a new conductometric immunosensor based on bioelectrocatalytic signal for the determination of HBsAg by using double-codified nanogold particles as secondary antibodies. The performance criteria of the conductometric enzyme immunosensors with specifically adapted characteristics are discussed as the following sections.

2. Experimental

2.1. Materials and reagents

HBsAg-ELISA kits, including 96 assay tubes precoated with mouse polyclonal *anti*-HBs, were purchased from China Zhengzhou Biocell Biotechnol. Co. Ltd. (dispatched from USA Sigma). The ELISA kits consisted of a series of HBsAg levels with various concentrations from 0 to 600 ng/mL. Chloroauric acid ($HAuCl_4$), sodium citrate, bovine serum albumin (BSA, 96–99%), and protein A (pA) were purchased from Sigma (USA). 0.01 M phosphate buffer solution (PBS) at various pH values was prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4 and then adjusting the pH with 0.01 NaOH and H_3PO_4. Hydrogen peroxide standardized by iodimetric titration, was freshly prepared in deionized water. 0.08 M stock solution of KI was prepared by dissolving in 0.01 M PBS (pH 7.0), immediately before use. Deionized and distilled water was used throughout the study. Clinical serum samples were provided from our Cancer Center of Chongqing Daping Hospital, China. All other reagents were of the best grade available and used as received.

The interdigitated conductometric transducers were purchased from the Institute of Chemo- and Biosensorics (Chongqing, China). Briefly, two identical pairs of Au interdigitated electrodes (150 nm thick) were assembled *via* the lift-off process on the Pyrex glass substrate (10 mm × 30 mm). A Ti intermediate layer of a 50 nm thick was utilized to improve the adhesion of Au to the substrate. Central part of the microelectrode was passivated by epoxy resin to define the electrode's working area. Both the digit width and interdigital distance were 10 μm, and their length was about 1 mm. Thus, the sensitive part of each electrode was about 1.0 mm^2. The schematic diagram of the interdigitated transducer is shown in Scheme 1a.

2.2. Preparation of 16 nm gold nanoparticles

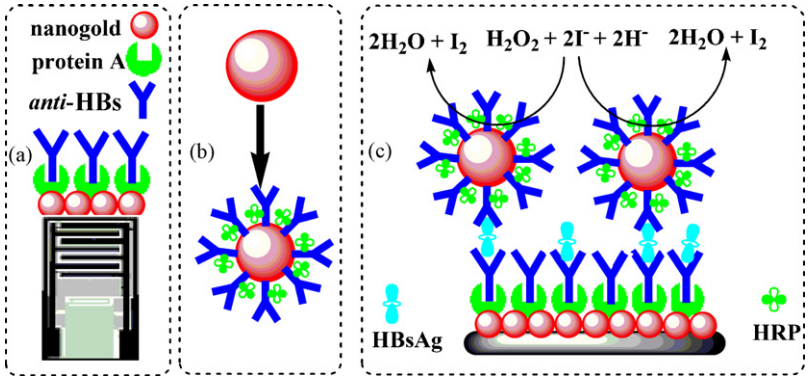
All glassware used in the following procedures was cleaned in a bath of $K_2Cr_2O_7$–H_2SO_4, rinsed thoroughly in double distilled water and dried in air. Gold colloids were prepared according to the literature [18]. Solution A: 1 mL of 1.0 wt% $HAuCl_4$ solution was added to 99 mL water. Solution B: 4 mL of 1.0 wt% sodium citrate solution. The two solutions were heated up to 60 °C respectively. During mixing, solution B was added to solution A quickly. The mixture was heated for 35 min subsequently. The solution color was claret. The mean size of the prepared gold colloids was about 16 nm, which was estimated from transmission electron microscopy (TEM) (data not shown).

2.3. Synthesis of double-codified nanogold particles

With gentle stirring, 1.5 mL of HRP-labeled *anti*-HBs antibodies was added to 100 mL gold colloids (pH 9.0). After overnight incubation at 4 °C, the mixture was centrifuged (13,000 rpm) at room temperature for 40 min. The red precipitation was resuspended in 10 mL of PBS (pH 7.4) solution containing 1.0 wt% BSA. The nanogold-labeled HRP–*anti*-HBs solution was stored at 4 °C until use (donated as *double-codified nanogold particles*, Scheme 1b).

2.4. Immunosensor fabrication

Prior to the bottom-up layer formation process, the interdigitated microelectrodes (IDME) were cleaned through immersing them in piranha solution for 5 min (*Caution: Piranha solution reacts violently with most organic materials and must be handled with extreme care.*). Nanogold particles were electrodeposited on the pretreated IDME by a potential-step electrolysis from +1.1 to 0 V in 0.5 M H_2SO_4 solution containing 1.0 mM $HAuCl_4$ with different pulse time, i.e., 10, 30 and 60 s. The nanogold deposited-IDME (denoted as nanogold/IDME) were taken out from the solution and thoroughly rinsed with water. Then, modification by protein A of



Scheme 1. (a) Schematic representation of an immunosensing layer, (b) double-codified nanogold particles, and (c) schematic view of sandwich-type conductometric detection of HBsAg.