Biosensors and Bioelectronics 59 (2014) 377–383

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Ultrasensitive enzyme-free electrochemical immunoassay for free thyroxine based on three dimensionally ordered macroporous chitosan–Au nanoparticles hybrid film

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ARTICLE INFO

Article history:
Received 2 January 2014
Received in revised form
12 March 2014
Accepted 31 March 2014
Available online 13 April 2014

Keywords:
Three dimensionally ordered macroporous materials
Chitosan–Au nanoparticles hybrid film
Magnetic multiwall carbon nanotubes
Free thyroxine
Sandwich-type electrochemical immunosensor

ABSTRACT

The measurement of free thyroxine concentration in serum is considered to be an essential indicator of thyroid function. Here, a novel enzyme-free sandwich electrochemical immunosensor for the detection of FT4 antigen based on the immobilization of primary antibody (Ab_1) on three dimensional ordered macroporous chitosan–Au nanoparticles hybrid (3DOM CS–AuNPs) film electrode, and magnetic multiwall carbon nanotubes (MMWCNTs) were used as label of secondary antibody (Ab_2). The 3DOM CS–AuNPs film electrode was constructed by one-step electrodeposition of CS–AuNPs composite onto Au electrode with silica opal template. MMWCNTs were prepared by chemical co-precipitation of Fe^{2+} and Fe^{3+} salts on carboxylated MWCNTs. $Ru(bpy)_3^{2+}$ labeled anti-FT4 ($Ru(bpy)_3^{2+}$–Ab_2) was covalently attached to MMWCNTs through the formation of amide bond between the carboxylic groups of MWCNTs and the amine groups of antibody. Under the optimal conditions, FT4 was detected in a concentration range from 0.71 fg mL^{-1} to 1.15 pg mL^{-1} with a correlation coefficient of 0.998 and a detection limit of 0.20 fg mL^{-1}. Moreover, the immunosensor showed excellent selectivity, good stability, satisfactory reproducibility and regeneration. Importantly, the developed method was used to assay clinical serum specimens, achieving a good relation with those obtained from the commercialized electrochemiluminescent method.

1. Introduction

Thyroid disease has been listed as the second largest disease of the endocrine field (Liao et al., 2013). Thyroxine (3,5,3′,5′-tetraiodothyronine, T4), with molecular weight of 777 Da, is the most commonly measured thyroid hormone for diagnosis of thyroid function (Islam et al., 2011). Normally, more than 99.9% of the T4 in blood is bound to carrier proteins, especially to thyroxine-binding globulin (TBG), and the free fraction of T4 (free T4, FT4) only accounts for about 0.03% (Wang et al., 2007). However, the FT4 level represents the biologically active form of total T4. Therefore, the measurement of serum FT4 could evaluate thyroid function better (Bayer and McDougall, 2006). Various methods have been employed to determine the FT4 level, such as radioimmunoassay (RIA) (Georgiou and Christofidis, 1996), chemiluminescence immunoassay (CLIA) (Dhatt et al., 2006), fluoroimmunoassay (FIA) (Stevenson et al., 1998), enzyme immunoassay (Kunst et al., 1988) and bioluminescent immunoassay (Frank et al., 2004). Regrettably, they have some limitations such as environmental pollution, poor reproducibility, high cost and needing time-consuming separations. Hence, there are several problems to overcome for a simple, highly sensitive, and reliable immunoassay for FT4 detection.

The sandwich-type immunosensor, due to its high sensitivity and selectivity, has been recently gained growing interest and found wide applications in the detection of tumor markers (Wu et al., 2013). In these methods, various enzyme biomolecules such as horseradish peroxidase (HRP) and alkaline phosphatase (ALP) were commonly used for the signal amplification (Yang et al., 2010; Yin et al., 2011). Although great achievements have been obtained in these enzyme-based immunoassay systems, the problems such as high cost and bad storage stability of the enzyme tags, especially the requirement of the deoxygenation process in the HRP-based electrochemical immunoassays, greatly limit their practical applications (Lai et al., 2013). To overcome these shortcomings, great attention has been focused on signal amplification procedure without the participation of enzymes (Tang et al., 2011a, 2011b).

Recently, nanostructured materials, not only as a good matrix for proteins but also as biological labels for immunoassay, have

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http://dx.doi.org/10.1016/j.bios.2014.03.068
0956-5663/

been developed rapidly to promote the progress of immunosensors due to their good biocompatibility, large surface area, excellent electrocatalytic activity, fascinating conductivity (Cao et al., 2013). Macroporous nanomaterials can provide a three dimensional (3D) network suited for the encapsulation of a variety of biomolecules. Recently, a series of inorganic porous materials have been proven to be promising as the immobilization matrices for antibody, including carbon nanospheres (Du et al., 2010), mesoporous silica NPs (Wei et al., 2010), and macroporous Au (Chen et al., 2008). In our previous works, we have constructed several electrochemical immunosensors on the basis of three dimensionally ordered macroporous (3DOM) Au film electrode (Chen et al., 2008; Chen et al., 2010). Uncoated Au nanoparticles (AuNPs), however, are sensitive to environmental factors, such as pH, temperature and solvents, because of the strong reactivity of the free electrons present on their surfaces (Boca et al., 2011). So, they easily tend to aggregate when used in such media. To address this challenge, protective substance, such as chitosan (CS), may be added to stabilize the AuNPs (Wang et al., 2013a, 2013b). CS, a natural polymer product, is derived from chitin via deacetylation with alkali. Its excellent film forming and adhesive ability, together with non-toxicity and biocompatibility make it a promising matrix in the fabrication of biosensors (Yang et al., 2009). For instance, Liu et al. (2010) developed an amperometric immunosensor based on CS–AuNPs composite film for the determination of α-1-fetoprotein. Yang's group electrochemically co-deposited hCG antibody with CS–AuNPs hybrid to construct an immunosensor for determining human serum chorionic gonadotrophin (hCG) (Yang et al., 2009). Masoomi et al. (2013) fabricated another immunosensor with loading anti-Aflatoxin B_1 on an AuNPs/CS modified glassy carbon electrode . To the best of our knowledge, there have been few reports on 3DOM materials made of natural polymers like polysaccharide. In our study, 3DOM CS–AuNPs composite film was prepared for the first time by a simple and controllable electrodeposition method and used as the support matrix for primary antibody (Ab_1).

Magnetic nano-materials have been widely applied in biological, medical and sensing fields due to their large surface area, high bioactivity and excellent stability (Liao et al., 2013). More importantly, it is convenient to separate them using an external magnetic field. However, there still some issues for the application of the magnetic nano-materials, such as lack of biocompatibility and potential damage to the biomolecules to some degrees (Liu et al., 2012; Liang et al., 2012). To address these problems, many researchers have focused on preparing novel magnetic nanocomposites with improved properties. Recently, Zhang and co-workers synthesized AuNPs-functionalized magnetic beads for fabricating a sensitive electrochemical immunoassay of thyroid-stimulating hormone (Zhang et al., 2012). Tang's group applied magneto-controlled graphene as immunosensing probe and multifunctional Au hollow nanospheres as distinguishable signal tags for an electrochemical immunoassay (Tang et al., 2011). Here, we have synthesized a new hybrid nanomaterial, magnetic multiwall carbon nanotubes (MMWCNTs), which not only offered high specific surface area and excellent electron transfer rate of MWCNTs, but also exhibited unique magnetic property. Therefore, it can be regarded as an ideal trace tag for loading a large amount of secondary antibody molecules (Ab_2) for fabricating an immunosensor with rapid separation and regeneration.

In this paper, we constructed an ultrasensitive enzyme-free sandwich-typed immunosensor for the determination of FT4, based on 3DOM CS–AuNPs film as the platform and MMWCNTs as the carrier for $Ru(bpy)_3^{2+}$–Ab_2. Here, 3DOM CS–AuNPs film was constructed by one-step electrochemical deposition in the mixture of tetrachloroauric (III) acid ($HAuCl_4$) and CS using silica template, which was tightly attached to the electrode surface and retained its natural properties. The AuNPs was effectively incorporated into CS to form a biocompatible sensing film. Both the large active surface area of 3DOM CS–AuNPs film and the streptavidin (SA)–biotin (bio) combination could increase the coupled amount of bio-Ab_1 on the SA modified 3DOM CS–AuNPs surface. Furthermore, greatly amplified sensitivity was achieved by using MMWCNTs as labels of Ab_2, which offered a large specific surface area and promoted electron transfer between the solution and the electrode.

2. Experimental section

2.1. Reagents

Au substrates were provided by Shanghai Institute of Microsystem and Information Technology (Shanghai, China). Bio-Ab_1 (2.5 ng mL^{-1}), FT4, and $Ru(bpy)_3^{2+}$–Ab_2 (50 ng mL^{-1}) were supplied by Roche Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA, 96–99%) and SA were purchased from Baoman Bio-tech Co., Ltd. (Shanghai, China). N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) was obtained from Aladdin Reagent Co., Ltd. (Shanghai, China). MWCNTs (OD: 10–20 nm; length: 10–30 μm; purity $>95\%$) were obtained from Nanjing Xianfeng Nano Co. (Nanjing, China). CS (MW: ca. 1×10^6, $>85\%$ deacetylation) was purchased from Sigma-Aldrich (Shanghai, China). 0.2 wt% CS aqueous solution was prepared by ultrasonically dissolving CS in 0.05 M acetic acid. Phosphate buffer saline (PBS, 0.1 M) with various pH values were prepared by mixing a stock standard solution of NaH_2PO_4 and Na_2HPO_4, and used as electrolyte for all electrochemistry measurement. The washing buffer (PBST) was prepared by adding 0.05% (w/v) Tween 20 in PBS (0.1 M, pH 7.5) solution. The supporting electrolyte of cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements was 0.1 M KCl containing 2 mM $[Fe(CN)_6]^{4-/3-}$ (1:1). The other chemicals were of analytical reagents grade and used without further purification. Double distilled water was used throughout the experiments. Human serum samples were obtained from the Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School and used as received.

2.2. Apparatus

Electrochemical experiments, including CV, EIS and differential pulse voltammetry (DPV) were performed with a CHI 660D electrochemical workstation (Shanghai CH Instruments Co.). A conventional three-electrode system comprised a platinum wire auxiliary electrode, a saturated calomel reference electrode (SCE) and a 3DOM CS–AuNPs film modified working electrode. All potentials here were referenced to the SCE. The morphologies of 3DOM CS–AuNPs film were characterized by field-emission scanning electron microscopy (FESEM, Hitachi S-4800). The structures and morphologies of MWCNTs and MMWCNTs were characterized by transmission electron microscopy (TEM, JEOL JEM-200CX).

2.3. Preparation of MMWCNTs

At first, carboxylated MWCNTs (c-MWCNTs) were produced according to the procedure described in the literature (Luo et al., 2011). Then, the c-MWCNTs were magnetized by co-precipitation of Fe_3O_4 NPs (Zhong et al., 2012). Under the N_2 atmosphere, 0.1 g of c-MWCNTs was dispersed into 50 mL of a solution containing 0.0378 g of $FeCl_3\cdot6H_2O$ and 0.0139 g of $FeCl_2\cdot4H_2O$. During the sonication, ammonium hydroxide ($NH_3\cdot H_2O$, 25% of ammonia) was added dropwise to regulate pH value to 10–11. After 30 min,