Article

Journal of
Nanoscience and Nanotechnology
Vol. 20, 7163–7168, 2020
www.aspbs.com/jnn

Quantitative Determination of Triiodothyronine by Electrochemical Impedance Spectroscopic Biosensor Using Gold Nanoparticle-Modified Electrode

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A label-free electrochemical impedimetric immunosensor for the detection of Triiodothyronine—a thyroid hormone that functions as the biomarker for monitoring for thyroid dysfunction was developed. The gold nanoparticle-modified electrode was employed to achieve the sensitive determination of Triiodothyronine at a low concentration level. The gold nanoparticle layer on the gold electrode was generated by chronoamperometry method and its resulting characteristics were investigated by scanning electron microscopy. Redox probe $[Fe(CN)_6]^{3-/4-}$ and electrochemical impedance spectroscopy was used for both evaluation of the immobilization of anti-Triiodothyronine antibody on the electrode surface and quantitative determination of target Triiodothyronine in different concentrations. The electrode with absorbed antibodies showed significant changes in charge transfer resistance upon binding the antigen, which resulted in an increase in normalized impedance change as the addition of antigen concentrations over a dynamic linear range of 0.01–100 ng/ml. These results indicated that the proposed immunosensor could be a potential alternative method for determination of Triiodothyronine in clinics with the advantage of low cost and less time-consuming.

Keywords: Triiodothyronine, Impedimetric Immunosensor, Gold Electrode, Gold Nanoparticles, Electrochemical Impedance Spectroscopy.

1. INTRODUCTION

Triiodothyroine (T3) is the one of the main thyorid hormones which is secreted by the thyroid gland—an organ with the shape of a butterfly that located in the base of the neck, approximately close to the first part of trachea [1, 2]. The majority of T3 circulating in the human blood extensively bound to binding-proteins such as thyroid binding globulin (TBG), albumin and pre-albumin [3, 4]. T3 plays a vital role in growth, maintenance of physiological function, energy homeostasis and are necessary for normal development [5, 6]. The concentration of T3 in human serum is relatively low which is in the range of 0.7–2.1 ng/ml or 1.1–2.8 nmol/l [7]. When T3 level is out of the aforementioned range, thyroid dysfunction or thyroid disease occurs. Thyroid dysfunction is a common clinical problem that has an effect on millions of individuals. The commonness of thyroid dysfunction is calculated approximately 1–5% in individuals with the age of over 60, it may be even higher depending on the gender and age of the population [6, 8]. It can be divided into two major types: Hyperthyroidism (too much thyorid hormone) and hypothyroidism (too little thyroid hormone) including several symptoms such as temperature intolerance, weight fluctuation, irregular heartbeat, tiredness, irritability, tremor [9, 10]. Therefore, the accurate assessment of thyroid function is of great importance for both the diagnosis and keeping track of thyroid diseases.

The conventional method for the determination of the concentration of T3 in unextracted serum is mostly radioimmunoassay (RIA), using a displacing agent such as 8-anilo-1-napthalene-sulfonic acid or salicylate to detach the hormone from binding proteins before measurement [11–13]. Besides, other types of immunoassay for T3 detection such as enzyme immunoassay [14], chemiluminescence [15] and electrochemiluminescence [16] immunoassays have also been reported. In addition, another T3 measurement by liquid chromatography—tandem mass spectrometry (LC-MS/MS) has been described with a precipitation step before introduction of the sample into the mass spectrometer [17, 18]. However, in terms of clinical practice, such methods require not only the use of enzymatic label or radioactive materials but also

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need intensive labor, complicated multistage processing, tedious and time consuming which make it less suitable for real time measurement. Therefore, newer platforms along with speed and simplicity but still obtaining enhanced analytical capability for low detection of T3 is highly desired.

In recent years, the development of electrochemical immunosensor has attracted great interest as a promising alternative to conventional immunoassay techniques [19]. Among these, affinity-binding based impedimetric biosensors are attracting attention and being investigated since they are direct and label-free sensor with respect to high sensitivity, rapid and precise response, portability and miniaturization, in which the interaction between probe (antibody) and target (antigen) is directly monitored and measured with no label requirement [20, 21]. Specifically, the formation of antibody-antigen on the electrode surface changes the impedance magnitude of the electrode-solution interface. This situation can be observed by enforcing a small AC sinusoidal voltage at a frequency to an electrochemical cell and monitoring the responding current, then the voltage–current ratio gives the impedance. This approach has been known as electrochemical impedance spectroscopy (EIS) [19]. It is a fast and powerful tool for the analysis of coupled processes such as electron transfer resistance and diffusion at the electrode-solution interface upon biorecognition occur at the modified surface [22–24].

In these approaches, the immobilization of the probe (antibodies) on the electrode is the most important factor in producing rapid response and developing highly sensitive immunosensors [23]. The biomolecules can be simply adsorbed on the gold electrode surface; however, the covalent linkage between bulk gold surface and biomolecules results in decrease of their biological activity despite their relatively good adsorption on the gold electrode [25]. Therefore, gold nanoparticles (AuNPs) are often utilized to modify the gold metal surface to maintain initial bioactivity of biological species [26, 27].

In this study, a label-free electrochemical impedimetric immunosensor for the quantitative detection of T3 was described. The flat AuNP-modified electrode was modified by generating a self-assembled mono (SAM) layer using (3-Aminopropyl) triethoxysilane (APTES). Imine reaction was applied to couple anti-T3 antibody with underlying AuNP-modified electrode. The anti-T3 Ab(casein)/GA/APTES/AuNP-modified electrode characteristics and its immuno-sensing ability towards quantitative detection of T3 were measured by EIS using the reversible redox couple $[Fe(CN)_6]^{3-/4-}$ as a redox probe.

2. EXPERIMENTAL DETAILS

2.1. Materials and Reagents

Pure ethanol (EtOH) ≥ 99.5%, (3-Aminopropyl) triethoxysilane (APTES) ≥ 98%, Potassium hydroxide (KOH) ≥ 85% ACS reagent, Glutaraldehyde (GA) solution 50 wt.% in H_2O, casein from bovine milk were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-T3 antibody (Anti-T3 Ab) was purchased from E ONE Laboratories (South Korea) and T3-BSA conjugate (Cat#C085, 5.14 mg/ml in PBS) was purchased from Cal-Bioreagents (San Mateo, CA, USA). Phosphate buffered saline (PBS, pH = 7.4) was obtained from Gibco (NY, USA). Deionized water (DIW) at 18.2-MΩ cm was purified using an Ace Water Treatment system (Tokyo, Japan). All chemical and biological reagents were of analytical grade which were used without further purification.

2.2. Measurement and Characterization

Scanning electron microscopy (SEM) measurements were performed by using a FE-SEM SIGMA HD (Carl Zeiss AG, Oberkochen, Germany) operated at a voltage of 5 kV with platinum thin film coating (∼3 nm) on the sample surface. The electrodeposition experiments were performed by using CHI660 Instruments (CH Instruments, Inc., Texas, USA) in a three-electrode configuration in which gold electrode, platinum wire and Ag/AgCl were used as working, counter and reference electrodes, respectively. The electrochemical conductivity of AuNP-modified electrode was evaluated with EIS measurement with redox probe was 1.0 mM ferricyanide/ferrocyanide $[Fe(CN)_6]^{3-/4-}$ in 0.1 M KCl solution. The initial potential for EIS was 0.01 V with a frequency range from 0.1 Hz to 1 MHz (10^6 Hz).

2.3. Electrodeposition of AuNPs Onto Gold Electrode

An electrochemical bath composed of tetrachloroauric acid ($HAuCl_4 \cdot 3H_2O$, 1 mM in DI water) was used for electrodeposition synthesis of AuNPs on the gold electrode. Electrode deposition was carried out by means of an amperometry technique. To control the loading amount onto the electrode surface, voltage was set to −0.9 V versus Ag/AgCl in the time of 120 seconds. After each eletrodeposition, the AuNP-modified electrodes were washed by DI water and slightly dried by N_2 gas. Impedance measurements of each step towards the development of the T3 immunosensor were performed using CHI660 Instruments (CH Instruments, Inc., Texas, USA) in a two-electrode configuration with Pt wire counter electrode.

2.4. Immobilization Protocol for T3 Impedimetric Biosensor

The AuNP-modified Au electrode was incubated with KOH for 10 minutes to generate the formation of hydroxyl (–OH) group on electrode surface by forming Au(I) hydroxide [28]. The –OH modified electrode was slightly washed with DI water, and then dried in N_2 gas. The electrodes were immersed in a solution of APTES 2% (v/v) in ethanol 5% (v/v) for antibody binding for 2 hours in

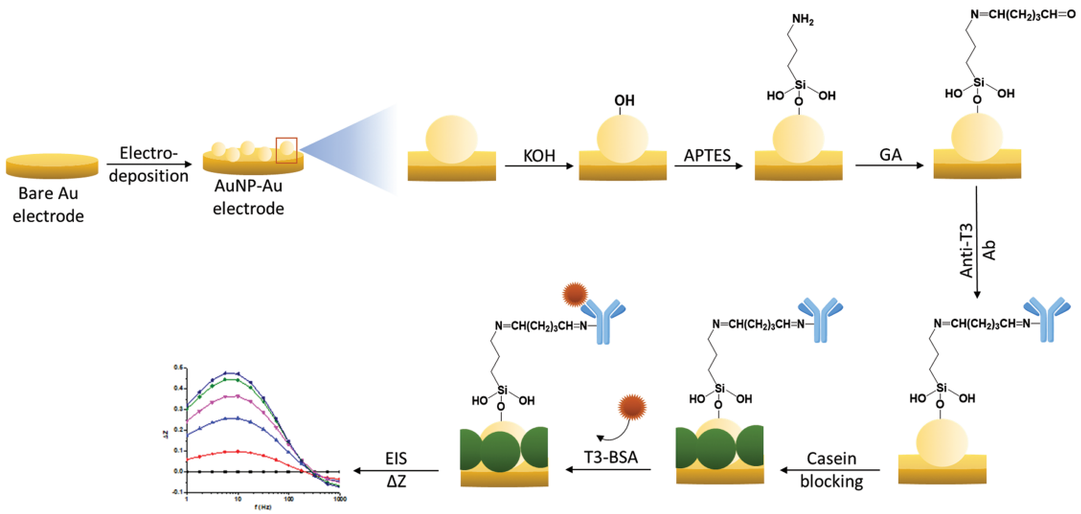

Figure 1. Schematic of the surface modification for AuNP-modified electrode and stepwise surface modification protocol for immuno-sensing process.

the incubator (37 °C), leaving a primary amine group on the surface. The activated surface was then gently washed with ethanol and DI water to remove successive APTES from the surface. Subsequently, the surface silanized with APTES was reacted with a 1% (v/v) solution of GA in PB buffer (pH = 6.0) for 90 minutes at room temperature, followed by rinsing with DI water and drying with N_2 gas. Then, 20 μl of anti-T3 Ab solution (10 μg/ml) was drop-casted onto the electrode to covalently bind anti-T3 Ab with the activated surface for 1 hour and kept in airtight chamber to prevent drying of the surface during binding. Afterwards, the electrodes were slightly washed with PBS 1× to remove any unbound anti-T3 Ab molecules. Anti-T3 Ab was immobilized by carrying out an imine linkage between the primary amine group of the antibody and the aldehyde group on the glutaraldehyde. Finally, to avoid nonspecific binding, casein (5 μg/ml) in PBS 1× was utilized, thus forming anti-T3 Ab(casein)/GA/APTES/AuNP-modified electrodes for detection of T3 (see Fig. 1). After each washing step, the changes in impedance magnitude ($|Z|$) were measured to confirm the successful reaction of reagents and antigen-antibody binding. Different concentrations of T3 were prepared in PBS 1× into different concentrations of 0.01, 0.1, 1, 10, 100 ng/ml, respectively.

3. RESULTS AND DISCUSSION

3.1. Characterization of GNP

SEM measurements were performed to determine the structure of the modified electrode area resulting from the electrodeposition of AuNPs material. The bare Au electrode showed a relatively smooth and homogeneous surface (Fig. 2(a)). Electrodeposition of AuNPs onto gold electrode resulted a layer of AuNPs with the size of <100 nm, which could be controlled by adjusting the deposition time and concentration of $HAuCl_4$ in solution (Fig. 2(b)). The AuNPs were formed depicting that they could increase the electrode's biocompatibility and conductivity.

3.2. EIS Measurements of Electrode Surface Modification Process

EIS is a non-destructive and rapid tool suitable for analyzing the interfacial characteristics of electrode surface modified by the development of electrode based impedimetric biosensors [29]. EIS spectra were perform and acquired for each step of modification process. At high frequencies, the process is limited by electron transfer of the redox species, meanwhile a diffusion-limited process is dominant the behavior of EIS signals in lower frequencies [24]. The measured impedance data were suitable to a modified Randles electrical equivalent circuit: the interfacial double layer capacitance (C_{DL}) was preferably modeled as a more complex constant phase element (CPE) because the surface of AuNPs modified electrode was rough; thereby having a higher real surface area. The equivalent circuit also includes the electron R_{CT}—the electron transfer resistance, R_S and Z_w—the ohmic resistance of the bulk solution and the diffusion of the redox species, neither being affected by target binding at the electrode surface [30]. R_{CT} and CPE related to the insulating features of the interface and can be sensitively measured by EIS.

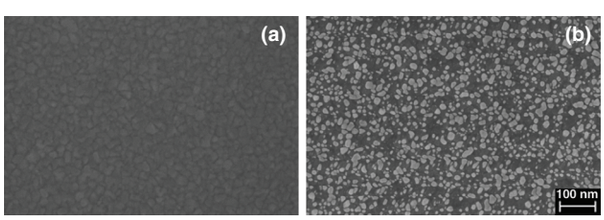

Figure 2. SEM image of various electrode surface: (a) Bare Au, (b) AuNPs-Au.

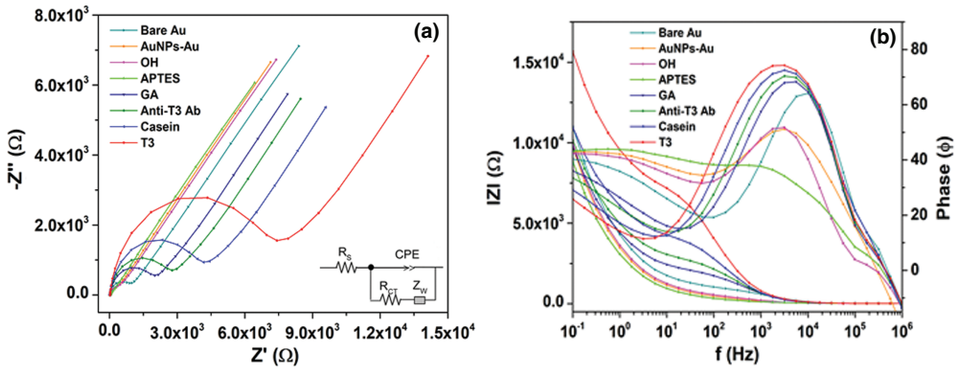

Figure 3. Nyquist (a) and bode (b) plots obtained after each step of the process towards developing T3 detection immunosensor.

Figure 3 showed the Nyquist and Bode plots for AuNP-modified electrode and subsequent modification with chemical linkers and biomolecule immobilizations towards T3 detection. Significant changes in impedance magnitude ($|Z|$) were observed after each step of the modification with KOH, APTES, GA, anti-T3 Ab and T3 which resulted in changes in R_{CT}. At first, the AuNP-modified electrode gives a very small circle domain, implicating a very low charge transfer resistance for the redox probe to go towards electrode surface (Fig. 3(a)). After forming a hydroxyl group layer by KOH, the impedance magnitude at 10 Hz slightly increased due to the hydroxyl (–OH) group which is negatively charged blocked the electron transfer of the negatively charged redox probe $[Fe(CN)_6]^{3-/4-}$. Later, APTES treatment was performed to make a self-assembled mono (SAM) layer on hydroxyl surface. APTES had a primary amine group that would be protonated and positively charged in an aqueos solution which resulted in a decrease of impedance magnitude because it worked as an electrostatic attraction with $[Fe(CN)_6]^{3-/4-}$ ions, allowed them reach towards electrode thus depicted low $|Z|$ [31]. Subsequently, GA was carried out to perform the reaction between APTES and GA which formed an imine. Herein, the terminal amine groups of APTES were displaced by the aldehydic group of GA which showed increment in $|Z|$ due to the negatively charged –CHO group and the length of GA chain that could partially block the electron transfer of the $[Fe(CN)_6]^{3-/4-}$ redox probe [31–33]. As the anti-T3 Ab is immobilized on the activated electrode by the covalent bonding between the aldehydic group with the primary amine group of the antibody, the increasing of Z occurs as the "interaction" between the antibody and redox couple could prevent the redox couple from reaching the electrode surface due to the blocking effect of the anti-T3 Ab. Finally, casein and T3 binding to anti-T3 Ab acted as an insulation layer which prevented the movement of $[Fe(CN)_6]^{3-/4-}$ towards the electrode surface [24, 33]. The surface state of immunosensor in different T3 concentrations led to different changes in $|Z|$, therefore, the change in impedance magnitude could be a detection parameter for sensitive determination of T3.

Overall, based on EIS signal with stepwise modification processes on AuNP-modified electrode, the immobilization of anti-T3 Ab onto the Au electrode was successful and ready to detect different concentrations of T3 in PBS 1× (pH = 7.4) solution. Next, changes in impedance magnitude were measured for a wide range of T3 concentration

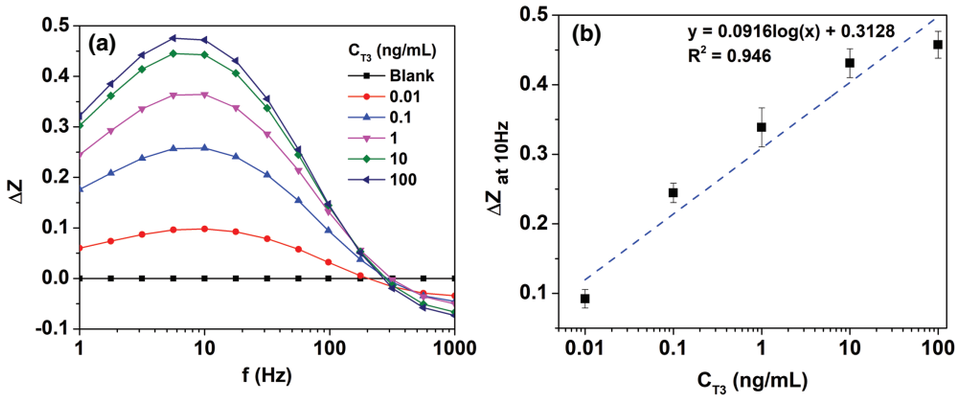

Figure 4. (a) Normalized impedance change (ΔZ) curves obtained for different concentrations of T3 (C_{T3}) in PBS 1× (pH = 7.4); (b) calibration plot for T3 impedimetric immunosensor obtained for various concentrations of C_{T3} in PBS 1× (pH = 7.4) at ΔZ (10 Hz).

from 0.01 ng/ml to 100 ng/ml using the developed impedimetric sensor.

3.3. Detection of T3 in PBS Using Developed Impedimetric Biosensor

To evaluate the immunochemical interaction between anti-T3 Ab and T3, anti-T3 Ab(casein)/GA/APTES/AuNP-modified electrode was exposed to various concentration of T3 (0, 0.01, 0.1, 1.0, 10, 100 ng/ml) in PBS 1× (pH = 7.4) solution. The measured impedance magnitude $|Z|$ were normalized and plotted for various concentrations, as shown in Figure 4(a). The normalized impedance magnitude clearly changed incrementally when increasing the T3 concentration. The normalized impedance magnitude (ΔZ) was defined as below [34]:

$$\Delta Z = \frac{|Z|_{\text{antigen}} - |Z|_{\text{antibody (blocking)}}}{|Z|_{\text{antibody (blocking)}}}$$

From the results shown as given in Figure 4(a), ΔZ in the low frequency region (particularly $f = 10$ Hz) changed the most in response to T3 concentrations. A calibration plot based on the change in ΔZ at 10 Hz after T3 binding was illustrated in Figure 4(b). A good linear relationship between ΔZ and the logarithm of T3 concentration from 0.01 ng/ml to 100 ng/ml was observed. Each data point in calibration plot represents three independent measurements and the reproducibility of the proposed impedance immunosensor was evaluated by measuring relative standard deviation. The coefficient of variations was found to be <9.8% when measuring electrodes prepared from independent sets under the same experimental conditions, indicating acceptable precision and fabrication reproducibility. Overall, the proposed AuNPs-modified electrode with EIS readout method could be employed to develop a label-free immunosensing platform of clinically relevant T3 levels.

4. CONCLUSION

A sensitive label-free impedimetric immunosensor for quantitative determination of T3 was developed. A controllable electrodeposition method of AuNPs onto the flat Au electrode by chronoamperometry was successfully achieved, thereby employing this platform to perform biomaterial immobilization and detection. This high-surface-area electrode indicated a simple method to enhance the immobilization of antibodies, which was the most important factor in developing the immunosensor for the quantitative determination of the target biomarker. The immunosensor proposed using this approach offered a linear range of 0.01–100 ng/ml of T3 and was able to reproducibly detect T3 at the pg/ml level without any labelling step for amplification. Thus, the presented immunosensing platform appeared to be having high potential and indicated the applicability that can be employed for further development of multi-detection for a comprehensive evaluation of thyroid hormones in clinical treatment.

Acknowledgments: This research was funded by National Research Foundation of Korea (Grant no. 2018K2A9A2A08000115) and Ministry of Trade, Industry, and Energy (Grant no. 10062995).

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Received: 19 July 2019. Revised/Accepted: 25 November 2019.