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Synthesis and electrochemical characterization of myoglobin-antibody protein immobilized self-assembled gold nanoparticles on ITO-glass plate

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ABSTRACT

We report a protein immobilized self-assembled monolayer (SAM) of gold nanoparticles (GNPs) on indium-tin-oxide (ITO) coated glass plate. The protein-antibody, Mb-Ab, was covalently immobilized over the self-assembly of GNPs through a mixed SAM of 11-mercapto undecanoic acid (MUA) and 3-mercapto propionic acid (MPA) via carbodiimide coupling reaction using *N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide (EDC) and *N*-hydroxy succinimide (NHS). The whole assembly was constructed on 0.25 cm^2 area of ITO-glass plate (Mb-Ab/MUA-MPA/GNPs/APTES/ITO-glass) and an impedimetric study was carried out for its application in myoglobin detection. This prototype assembly was characterized by scanning electron microscopy, atomic force microscopy and electrochemical techniques. The modified electrode showed an increased electron-transfer resistance on coupling with protein antigen, Mb-Ag, in the presence of a redox probe $[Fe(CN)_6]^{3-/4-}$. Its exhibits an electrochemical impedance response to protein myoglobin-antigen, Mb-Ag, concentration in a linear range from 0.01 μg to 1.65 μg mL^{-1} with a lowest detection limit of 1.4 ng mL^{-1}.

1. Introduction

Myoglobin, a non-enzymatic cardiac protein is being extensively used as diagnostic markers of acute myocardial infarction (AMI). As a cardiac biomarker, myoglobin is used in conjunction with troponin to help diagnose or rule out a heart attack. Due to its small size of 17.8 kDa, it is released into plasma in a significant amount within 3 h of the onset of AMI while the plasma concentrations usually return to normal within 24 h [1–3]. Normal serum myoglobin levels range from 30 to 90 ng mL^{-1}. After 1 h of the onset of myocardial infarction, serum myoglobin level can elevate to 200 ng mL^{-1} or even higher. During the peak hour, myoglobin level can be as high as 900 ng mL^{-1}. New biochemical diagnostic methods for AMI are being investigated in response to the requirement for superior diagnostic accuracy and rapidity and for improvements in the management of patients with chest discomfort [4,5].

The use of metal nanoparticles as catalysts in biosensors is due to their superior stability and complete recovery in biochemical redox processes. It also provides a biocompatible microenvironment for biomolecules and greatly increases the amount of immobilized biomolecules on the electrode surface.

Preparation of thin films by self-assembly technique is very important because the performance of the device is dependent on the characteristics of the molecular pattern formed on the substrate. It has been reported that the interaction between biomolecules and a solid surface with self-assembled specific functional groups have an ability to reduce the aggregation of biomolecules on the substrate [6]. The design and preparation of an optimum interface between the bio-components and the detector material is the key part of sensor development [7–9].

The various traditional methods such as radio immunoassay (RIA), chemiluminescent immunoassay, enzyme immunoassay are complicated multistage processes, tedious and time consuming [10–12]. The concept of direct label free sensors has advantages with respect to speed and simplicity in which the immune interaction between antibody and antigen is directly monitored. This technique can be used for highly insulating properties measurement of the self-assembled monolayer on semiconductors or on gold electrodes [13–17]. Unlabeled DNA and protein targets can be detected by monitoring changes in surface impedance when a target molecule binds to an immobilized probe [18]. When a target protein binds to the pre-functionalized probe surface, the impedance of the electrode–solution interface changes and this change is detected electrically over a range of measurement frequencies. Electrochemical impedance spectroscopy is a powerful technique for examining many chemical and physical processes in solutions as well as solids. For solution phase electrochemistry a complex sequence of coupled processes such as, electron transfer, mass transport and chemical reaction can all control or influence the output from an electrochemical measurement. Electrochemical

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impedance is usually measured by applying an AC potential to an electrochemical cell and measuring the current through the cell. Impedance measurements are divided into two categories namely non-Faradic and Faradic impedance. Electrochemical impedance spectroscopy (EIS) is a Faradic impedance technique and it is performed in the presence of a redox probe, whereas the non-Faradic impedance is conducted in the absence of any redox probe. This technique is regarded as an effective tool for sensing the formation of antigen–antibody interaction [19], biotin–adivin complexes [20] and oligonucleotide–DNA interaction [21] on the electrode surface by probing the features of the interfacial properties.

In this study, we describe an impedimetric study on a prototype of protein immobilized self-assembled GNPs on ITO-glass plate. The GNPs were self-assembled over a SAM of APTES modified surface of ITO-glass plate followed by surface modification with a mixed SAM (1:10) of MUA and MPA. The protein antibody, Mb-Ab, was immobilized over the surface modified GNPs through carbodiimide coupling reaction using the free carboxyl terminal group of alkanethiols. The each assembly step of the modified electrode (Mb-Ab/MUA-MPA/GNPs/APTES/ITO-glass) was characterized by cyclic voltammetry and electrochemical impedance spectroscopy in the presence of $[Fe(CN)_6]^{3-}$ as a redox probe. An impedimetric study was carried out on the modified electrode for its possible application in myoglobin detection in aqueous medium.

2. Materials and methods

2.1. Materials

Mb-Ab (Cat 4M23 MAb 4E2) and Mb-Ag (Cat 8M50) were obtained from Hytest (Turku, Finland). 3-Aminopropyltriethoxysilane (APTES) was purchased from Merck Chemicals (Germany). Tetrachloroauric (III) acid ($HAuCl_4$) was obtained from Himedia Pvt. Ltd., India for preparation of GNPs. 11-Mercapto undecanoic acid 95% (MUA), 3-mercapto propionic acid 99% (MPA), *N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide hydrochloride (EDC) and *N*-hydroxy succinimide 98% (NHS) were obtained from Sigma–Aldrich Chemicals. All other chemicals were of analytical grade and used without further purification.

2.2. Apparatus and sample preparation

Scanning electron micrographs (SEM) were obtained with a LEO 440 PC, UK based digital scanning electron micrograph at an acceleration voltage of 20.0 kV. Atomic force microscopy (AFM) images were obtained on a Nanoscope 5, VEECO Instrument Ltd., USA. Transmission electron microscopy (TEM) images were taken on high resolution TM model Technai G2 F30 S Twin, The Netherlands. Cyclic voltammetry and electrochemical impedance measurements were done on a PGSTAT302N, AUTOLAB instrument from Eco Chemie, The Netherlands. The impedance measurements were performed in the presence of a redox probe $[Fe(CN)_6]^{3-}$ at the scanning frequencies from 0.1 to 100,000 Hz.

The samples on ITO glass plates were taken as such for SEM and AFM micrographs, whereas for TEM image, the gold colloidal solution (3 μL) was taken on a copper grid. All measurements were carried out in a conventional three-electrode cell configuration consisting of a working electrode (Mb-Ab(BSA)/MUA-MPA/GNPs/APTES/ITO-glass), Ag/AgCl reference electrode and platinum foil as a counter electrode.

2.3. Fabrication of Mb-Ab(BSA)/MUA-MPA/GNPs/APTES/ITO-glass

The ITO coated glass plates (0.25 cm^2) were cleaned by sequential ultrasonic cleaning in soapy water (extran), acetone, ethanol, isopropyl alcohol and distilled water for 10 min each, and drying in vacuum. Then, the cleaned ITO-glass plates were exposed to oxygen plasma for 5 min in a plasma chamber. Finally the ITO-glass plates were once again washed with doubled distilled water and dried in vacuum. Cleaned ITO glass plates were immersed in 2% APTES solution prepared in ethanol for 1.5 h, under the ambient conditions, to form a SAM. The glass plates were then rinsed with ethanol in order to remove non-bonded APTES from the surface of the substrate and dried under N_2. Colloidal GNPs were prepared by citrate reduction of $HAuCl_4$ in aqueous solution with an average particle diameter of 10–12 nm [22]. The APTES modified ITO glass plates were then immersed in the above colloidal solution of GNPs for a period of 1 h, followed by washing with distilled water and dried under N_2 to form the GNPs/APTES/ITO glass plates. Colloidal GNPs obtained from $[AuCl_4]^-$ has an intrinsic fixed negative charge resulting from strongly adsorbed Cl^-/or a coating of $[AuCl_2]^-$ (produced by incomplete reduction of $[AuCl_4]^-$) [23]. The free NH_2 groups of APTES/ITO electrode get ionized in the pH 4–10 regime to form NH_3^+ and these positively charged amino groups are responsible for the electrostatic interaction with the negatively charged GNPs for the formation of the self-assembly of GNPs. Adsorption of GNPs by this method results in a monolayer coverage, further adsorption on the surface being limited by repulsion between the charged particles. The surface of GNPs was modified with a mixed SAM of MUA-MPA by immersing them in an ethanol solution of MUA and MPA (20 mM; 1:10; v/v) for 16 h. These were then washed in ethanol to remove the unbound MUA/MPA molecules and dried under N_2 flow. The combination of MPA and MUA was used to obtain a SAM not as compact as those formed with long-chain alkanethiols, thus allowing access of chemical species in solution, such as redox mediator, to the electrode surface. The mixture of MPA and MUA thus provide the characteristics of both, long and short-chain alkanethiol monolayers, which offers a better loading of proteins molecules, as reported earlier [24].

The above MUA-MPA/GNPs/APTES/ITO-glass plates were then further immersed in an aqueous solution containing 0.15 M EDC and 0.03 M NHS for 1 h, followed by washing with distilled water and dried under N_2 flow to obtain the NHS/MUA-MPA/GNPs/APTES/ITO-glass plates. The MUA-MPA/GNPs/APTES/ITO-glass plates were utilized for the immobilization of protein antibodies by immersing them in 100 μg mL^{-1} antibody, Mb-Ab in PBS, pH 7.4, for a period of 1.5 h. The excess antibodies were removed by rinsing with PBS and finally dried under N_2 flow, at room temperature. The Mb-Ab/MUA-MPA/GNPs/APTES/ITO-glass plates were further immersed in 1% BSA (W/V) solution to block the nonspecific binding sites both at the GNPs surface and the remaining unbound free carboxyl groups as well, followed by washing with distilled water, dried under N_2 flow and were stored at 4 °C. The stepwise construction of the prototype assembly is schematically represented in Fig. 1. The average size of the GNPs decorated over the APTES layer on ITO-glass is in the range of 10–12 nm as depicted in the TEM image (Fig. 2).

3. Results and discussion

3.1. Characterization of Mb-Ab/MUA-MPA/GNPs/APTES/ITO-glass

The physical morphology of prototype assembly before and after protein immobilization was characterized by using SEM and AFM images. The SEM micrograph of the GNPs modified ITO-glass plate shows a well distribution of GNPs over the entire surface of APTES layer on ITO-glass plate, at 20k× magnification (Fig. 3a). However, the SEM image of Mb-Ab/MUA-MPA/GNPs/APTES/ITO-glass taken at about same magnification shows aggregation of globular shaped