Electrochemical characterization of surface modified gold electrode for the detection of C-reactive protein

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Abstract
A label-free, electrochemical impedance electrode is reported by immobilizing protein antibody, αCRP-Ab, through a self assembled monolayer (SAM) of 11-mercaptoundecanoic acid (MUA) and 3-mercaptopropionic acid (MPA) via carbodiimide coupling reaction using N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride (EDC) and N-Hydroxy Succinimide (NHS) on gold (Au) electrode. The electrode (αCRP-Ab/NHS/MUA/MPA/Au) was characterized by scanning electron microscopy (SEM), Atomic force microscopy (AFM) and electrochemical techniques. The electrochemical performance of the electrode was studied by electrochemical impedance spectroscopy. The results showed an increased electron-transfer resistance with the immobilization of CRP antibody (αCRP-Ab) on the modified Au electrode and on their subsequent coupling with protein CRP antigen (αCRP-Ag) at the electrode surface in the presence of [Fe(CN)₆]³⁻⁻⁻⁻ as redox probe. The modified Au electrode exhibits an electrochemical impedance response to antigen, αCRP-Ag, concentration in a linear range from 45 ng to 5.84 µg/ml with a lowest detection limit of 30 ng/ml.

Keywords: Immunosensor, cyclic voltammetry, electrochemical impedance spectroscopy, covalent immobilization, immunoassay.

INTRODUCTION
The design and preparation of an optimum interface between the bio-components and the detector material is the key part of sensor development (Ahuja et al., 2008; Rajesh et al., 2009). Impedance biosensors are a class of electrical biosensors that show promise for point-of-care and other applications due to low cost, ease of miniaturization, and label-free operation. Unlabeled DNA and protein targets can be detected by monitoring changes in surface impedance when a target molecule binds to an immobilized probe (Daniels et al., 2007). 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.

C-reactive protein (CRP) is a protein found in the blood, the levels of which rise in response to inflammation (an acute-phase protein). CRP is synthesized by the liver in response to factors released by fat cells. In the body, CRP plays the important role of interacting with the complement system, an immunologic defense mechanism. CRP in human blood is a marker of cardiovascular diseases (Ridker et al., 1997). A person is at an average risk of cardiovascular disease when the CRP level in blood is between 1-3 mg/l. A CRP level above 3 mg/l predicts a high risk and below 1 mg/l is an indication of low risk of cardiovascular disease (Ridker et al., 1998). CRP is diagnosed with tests like enzyme-linked immunosorbent assay (ELISA), nephelometric and turbidimetric assays (Kushner et al., 2002).

Traditional methods such as radio immunoassay (RIA) and ELISA are complicated multistage processes, tedious and time consuming. The concept of direct label free immunoassays 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...
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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 (Kharotonov et al., 2000), biotin-avidin complexes (Athey et al., 1995) and oligonucleotide-DNA interaction (Bardia et al., 1999) on the electrode surface by probing the features of the interfacial properties.

In this study, we describe a label-free electrochemical impedance immunosensor for the quantitative detection of αCRP in aqueous solution. The surface area of the gold (Au) flat wire (0.08 cm²) was modified by forming a SAM of Mercaptoundecanoic acid (MUA) and 3-Mercapto Propionic acid (MPA) (1:9). The surface modifies gold electrode is subsequently immobilized with protein antibody, αCRP-Ab, through strong amide bonding by using carbodiimide coupling reagents N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride (EDC) and N-Hydroxy succinimide (NHS). The immobilization of antibody [αCRP-Ab] and interaction of antibody [αCRP-Ab]-antigen [αCRP-Ag] at the surface of the modified electrode was characterized by cyclic voltammetry and electrochemical impedance spectroscopy in the presence of [Fe (CN) 6]3-/4- as a redox probe.

MATERIALS AND METHODS

Materials

αCRP-mAb (Cat 4C28 mAbC2) and αCRP-Ag (Cat 8C72) were obtained from Hytest (Turku, Finland). 11-Mercapto undecanoic acid 95% (MUA), 3-Mercapto propanionic acid 99% (MPA), N-(3-Dimethylamino propyl)-N’-ethyl carbodiimide hydrochloride (EDC) and N-Hydroxy succinimide 98% (NHS) were obtained from Sigma-Aldrich chemicals. Other chemicals were of analytical grade and used without further purification.

Apparatus

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 VEECO/DICP2, USA scanning probe microscope. Cyclic voltammetry and electrochemical impedance measurements were done on a PGSTAT302N, AUTOLAB instrument from Eco Chemie, Netherlands. All measurements were carried out in a conventional three-electrode cell configuration consisting of a working electrode (αCRP-Ab(BSA)/NHS/MUA/MPA/Au), Ag/AgCl reference electrode and platinum wire as a counter electrode in PBS, pH 7.4 at about 25°C. A stirring bar and magnetic stirrer provided convective transport.

Figure 1: Schematic presentation of fabrication steps of surface modified Au electrode.

Fabrication of modified αCRP-Ab(BSA)/NHS/MUA/MPA/Au electrode

The flat gold wire (0.80 cm²) was cleaned by sequential ultrasonic cleaning in soapy water, acetone, ethanol, isopropyl alcohol and distilled water for 10 minutes each, and drying in vacuum. Then, the Au wire was immersed in 20 mM MUA/MPA (1:9, v/v) solution in ethanol for 16 hours and a SAM of MUA-MPA was obtained over the Au flat wire. The MUA-MPA/Au electrode was then washed in ethanol to remove the unbound MUA-MPA molecules and dried under N₂. The mixture of MPA and MUA has been used to obtain the surface structure in molecular order. The combination of MPA and MUA has been used to obtain the SAMs 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 (Campuzano et al., 2006). The MUA-MPA/Au wires were further immersed in NHS/EDC aqueous solution for 1 h, followed by washing in distilled water and dried under N₂ to obtain the NHS/MUA-MPA/Au electrodes. The NHS/MUA-MPA/Au electrodes were further immersed in 1% BSA solution to block the nonspecific
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binding sites at the surface of the electrode where antibodies, αCRP-Ab, were not bound, followed by washing with distilled water, dried under N₂ and were stored at 4°C. The above stated Au electrode modification steps are well represented in scheme 1.

RESULTS

Characterization of enzyme αCRP-Ab (BSA)/MUA-MPA/Au electrode

The αCRP-Ab (BSA)/MUA-MPA/Au electrode was characterized by cyclic voltammetry and electrochemical impedance spectroscopy. It was demonstrated by Hicks et al., (2002) and Horswell et al., (2003) that cyclic voltammetry and impedance spectroscopy are comparable for the determination of electron-transfer rate on modified electrodes. The changes in peak current and separation of peak potentials in voltammograms at different electrode surfaces are theoretically related to the electron transfer rate constant i.e. the electron-transfer resistance. All electrochemical measurements were performed in PBS solution, pH 7.4, containing 0.1 M KCl and 2 mM [Fe(CN)₆]³⁻/⁴⁻. The [Fe(CN)₆]³⁻⁺ probe was used as a marker to investigate the changes in electrode behavior after each surface modification step. The cyclic voltammograms of the Au electrode was measured before and after each step of surface modification with SAM of MUA-MPA, treatment with EDC and NHS and after the immobilization of protein antibody αCRP-Ab. In all the CV experiments, the 3rd cycle was considered since no significant changes were observed in the subsequent cycles. A quasi-reversible voltammogram with a separation of peak potential (ΔEp) of 90 mV was observed for bare Au flat wire, which upon modification with SAM of MUA-MPA shows a peak separation (ΔEp) of 142 mV between the cathodic and anodic waves of the redox probe. This widening in peak separation after the formation of MUA-MPA layer is because of the repulsive interaction of polyanions (COO⁻) with anionic probe ([Fe(CN)₆]³⁻⁺) at the surface interface. This confirms the formation of MUA-MPA layer at the surface of Au flat wire. The CV curve of NHS treated MUA-MPA/Au electrode also shows an increased peak separation of ΔEp 175 mV vs Ag/AgCl due to the formation polyanions of NHS layer over the electrode surface, which results in a repulsive interaction with anionic probe [Fe(CN) j]⁻⁻ at the surface interface. A further increase in the peak separation was observed after the immobilization of insulating protein antibody, αCRP-Ab at the surface of the modified electrode, as shown in Fig. 2 [Supplementary data].

Each step of above surface modification of the gold electrode towards the fabrication of αCRP-Ab (BSA)/MUA-MPA/Au electrode was further characterized by electrochemical impedance spectroscopy conducted in 0.1 M KCl solution containing 2 mM [Fe(CN)₆]³⁻ /[Fe(CN)₆]⁴⁻ (pH 7.4, 2 ml), at the scanning frequencies from 0.1 to 100,000 Hz. The impedance spectroscopy was represented as an equivalent circuit (Randles, 1947) as shown in the inset of Fig. 3 [Supplementary data]. Fig. 3 present the frequency-dependent impedance, also named Nyquist diagram, of the different steps of the surface modified flat Au wire tested in this experiment. On the x-axis the real part of the cell impedance is reported, while the imaginary part is on the y-axis. The impedance spectrum, which includes a semicircle portion at higher frequencies, corresponding to the electron-transfer limiting process and a linear part at the low frequencies resulting from the diffusion limiting step of the electrochemical process. The ohmic resistances of the electrolyte solution (Rₛ) and the Warburg impedance (Zw) represent the bulk properties of the electrolyte solution and diffusion features of the redox probe in solution, respectively. The diameter of the semicircle in the Nyquist plots represents the electron-transfer resistance of the layer, which can be used to describe the interface properties of the modified electrode.
The electron-transfer resistance values of each surface modified step of the Au electrode are listed in Table 1 [Supplementary data]. The bare Au flat wire shows an electron transfer resistance (R\text{et}) value of 135.3 kΩ. The R\text{et} value for MUA-MPA/Au electrode was increased to 156.29 kΩ, which indicates a perturbation to electronic transport at the electrode surface interface after the MUA-MPA modification of Au wire. In the later steps of treatment of MUA-MPA/Au electrode with NHS and immobilization of protein antibody αCRP-Ab and blocking of nonspecific sites with BSA over the modified electrode results in the formation of insulating layers at the electrode surface, as is evident with increased electron transfer resistance of 239.63 kΩ and 253.20 kΩ, respectively. These results of electrochemical impedance spectra of the assembled electrode are in conformity to a similar pattern of results obtained with cyclic voltammetry measurements, which further confirms the fabrication of the αCRP-Ab (BSA)/MUA-MPA/Au electrode.

The surface morphology of modified Au electrode before and after protein immobilization was characterized by using scanning electron micrographs and AFM images. The SEM micrograph of the flat Au wire shows a sheet like structure (Fig. 4a). However, after the protein immobilization the SEM micrograph of αCRP-Ab(BSA)/MUA-MPA/Au shows aggregation or globular shaped protein molecules distributed over the electrode surface (Fig. 4b). To further understand the surface morphology, AFM images were taken in a contact mode, for an EDC/NHS treated MUA-MPA/Au (Fig. 5a) and a protein immobilized αCRP-Ab(BSA)/MUA-MPA/Au electrode (Fig. 5b). One may notice that AFM image αCRP-Ab(BSA)/MUA-MPA/Au surface (Fig. 5b) exhibits a bunch of hair-like bushes of protein molecules well distributed over the surface in comparison to the non-protein immobilized Au surface (Fig. 5a), which exhibits almost a plane structural feature.

Electrochemical impedance response to antigen

Electrochemical impedance response of the αCRP-Ab(BSA)/MUA-MPA/Au electrode was measured after the addition of successive aliquots of different concentrations of protein antigen αCRP-Ag in 0.1 M KCl solution containing 2 mM [Fe(CN)\text{6}^3^-]/[Fe(CN)\text{6}^4^-] (pH 7.4, 2 ml), under a slow constant stirring of 100 rpm, at the scanning frequencies from 0.1 to 100,000 Hz. An electron-transfer resistance value observed in an impedance measurement conducted for the αCRP-Ab(BSA)/MUA-MPA/Au electrode in sample containing no antigen αCRP-Ag was taken as a blank sample response for the detection of antibody-antigen interaction upon subsequent addition of different concentrations of protein antigen αCRP-Ag. The Nyquist plots of impedance spectra of the modified Au electrode for different concentrations of antigen αCRP-Ag are shown in Fig 6 [Supplementary data]. The impedance response of the αCRP-Ab(BSA)/MUA-MPA/Au electrode to protein antibody is due to the antibody-antigen coupling (Sargent et al., 1999; Brillhart et al., 1991). The coupling of antigen to the immobilized antibody at the electrode surface acts as a kinetic barrier for the electron transfer. The modified Au electrode exhibits an increasing order of electron-transfer resistance with the increasing antibody-antigen coupling on the electrode surface, as is evident from the semicircle of the Nyquist plot, which kept increases with increasing concentration of added antigen, αCRP-Ag. The modified Au electrode displayed a well defined concentration dependence curve for antigen, αCRP-Ag. Fig 7 shows a linear relationship between the electron-transfer resistance and the logarithmic value of antigen, αCRP-Ag concentration in a range from 45 ng to 584 µg/ml with a correlation coefficient 0.998 (n=5). The sensitivity of the modified Au electrode towards antigen, αCRP-Ag concentration was found to be 3.33 kΩ per decade. Taking into account the blank and the signal fluctuation (noise), the lowest detection limit of the modified Au electrode was found to be 30 ng/ml. This linear range of detection for CRP concentration is much wider than the recently reported gold film based immunosensor, which shows a small impedance response beyond 10 ng of CRP (Chen et al., 2008). This may be due to high loading of protein antibodies over the modified flat thin gold wire. The reproducibility of the response of the calibration curve for 5 modified Au electrodes from the same batch of fabrication was observed within a limit of 12.5%.

The stability of the αCRP-Ab(BSA)/MUA-MPA/Au electrode was studied, under a storing temperature of about 25°C and 4-5°C, respectively. The stability of the modified Au electrode was determined by continuously monitoring the impedance response to 1.0 µg/ml antigen concentration for 3 months, under the identical experimental conditions. The impedance response of the modified Au electrode sharply decreases from its initial value for αCRP-Ag solution after 7 days when it was stored at about 25°C due to the inactivation of immobilized protein antibody, αCRP-Ab. However, the modified electrode could restore 85% of its initial CV and impedance response value up to 70 days under a storing condition of 4-5°C.

CONCLUSION

We have reported an electrochemical characterization of a surface modified Au flat wire as electrode for detection of CRP. Cyclic voltammetry and impedance spectroscopy was used to characterize the each step of Au substrate modification, immobilization of antibody.
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αCRP-Ab and antibody-antigen interaction at the electrode surface in the presence of a redox probe [Fe(CN)₆]³⁻/[Fe(CN)₆]⁴⁻. A linear relationship between the electron-transfer resistance and the logarithmic value of the antigen αCRP-Ag concentration was found to be in a wide linear range of 45 ng-5.84 µg/ml. The experiments are currently in progress to improve the shelf life of the modified Au electrode beyond 70 days. The main advantage of this modified Au wire lies in the simple method of fabrication and its application in a wide range of αCRP detection at higher concentration (µg) in aqueous solution.

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