Application of Self-Assembled Monolayer of 10-Carboxy-1-Decanethiol for Cholesterol Biosensor

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Abstract

Cholesterol oxidase (ChOx) has been immobilized on to 10-carboxy-1-decanethiol (CDT) self-assembled monolayer (SAM) prepared on gold (Au) substrate. These bioelectrodes have been characterized using contact angle (CA) measurements, electrochemical impedance spectroscopy (EIS) and atomic force microscopy (AFM) respectively. These ChOx/CDT/Au bioelectrodes have been utilized for the estimation of cholesterol using surface plasmon resonance (SPR), UV-visible and cyclic voltammetric techniques respectively. The linearity has been obtained as 50 to 500 mg/dl of cholesterol in solution with a sensitivity of 2.194 x 10^-4 Abs (mg/dl)^-1 and \( K_m \) value of 4.64 mg/dl. The stability and reusability of these bioelectrodes have been obtained as 3 months and 10-15 times, respectively.

Keywords: Cholesterol oxidase, cholesterol, biosensor, surface plasmon resonance (SPR), 10-carboxy-1-decanethiol (CDT)

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1 INTRODUCTION

Cholesterol is an important biological molecule that plays an important role in membrane structure and is a precursor for the synthesis of the steroid hormones and bile acids [1]. The level of cholesterol must be properly regulated in order to prevent over-accumulation and abnormal deposition of cholesterol-rich lipoproteins in coronary arteries. Such deposition eventually leads to coronary heart diseases, hypertension, atherosclerosis and cerebral thrombosis etc [2-3]. In order to regulate various metabolites concentrations in the body, biosensors have recently received much attention due to their faster response, miniaturized size, reliability and reproducible results for the desired metabolites [4-7]. In order to achieve higher accuracy, precision and reliability, the surface plasmon resonance (SPR) technique based biosensors have received much interest. SPR is a surface sensitive technique based on local refractive index (RI) change. It is directly proportional to the change in the mass of the biomolecules bound to an electrode surface [8-10].

Recently, it has been shown that SPR technique can be used for the quantification of a biochemical reaction between an immobilized enzyme and the substrate like glucose [11], fructose [12], urea [13], cholesterol [14] etc in the solution. Various cholesterol biosensors based on different matrices such as conducting polymers [15-16], sol-gels [17], and self-assembled monolayers [18] etc have been developed. Among these, self-assembled monolayers have been used for the anchoring of enzymes and other biomolecules to achieve a number of advantages such as high reproducibility, molecular level control, vicinity from the surface, surface modification, direct electron transfer etc [19-21]. Vidal et al. 2004 [22] have prepared self-assembled monolayers (SAMs) on platinum (Pt) surface and used the Prussian Blue (PB) layer and outer layer of Nafion (Nf) as a means of improving selectivity for Pt/SAM/Ppy-ChOx/Nf electrode. Moreover, Gobi and Mizutani 2001 [23] have developed a cholesterol biosensor based on layer-by-layer nano thin films of ChOx and poly (styrenesulfonate) on covalently immobilized...
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microperoxidase-11 (MP-11) on self-assembled monolayer of mercaptopropionic acid and amioethanethiol. Bokoch et al, 2004 [24] reported an amperometric cholesterol biosensor based on platinum electrodes modified with a self-assembled monolayer of thiolipid. Cholesterol oxidase was immobilized in electrode-supported lipid bilayer membranes. The results show that the electrodes retained about 5% of the original response about 60 days. Recently, Li et al.[25] fabricated a carbon nanotube modified screen-printed carbon electrodes based biosensor for monitoring total cholesterol in blood. The electrode had a linear range of 100-400 mg/dl for total cholesterol and storage stability of 2 months. The shelf-life of this cholesterol biosensor was about 25 days.

In the present paper, we report the immobilization of cholesterol oxidase onto 10-carboxy-1-decanethiol (CDT) SAM using electrostatic interactions. These prepared ChOx/CDT/Au electrodes have been characterized and used for estimation of cholesterol concentration in solution using SPR technique.

2 METHODS AND MATERIALS

2.1 Chemical and Reagents
10-Carboxy-1-decanethiol (CDT) was procured from Dojindo, Japan. K3Fe(CN)6, KCl and ethanol (99.5%) were procured from the Koso Chemical Co., Ltd Tokyo, Japan. Cholesterol oxidase (ChOx; EC 1.1.3.6) from Streptomyces species with specific activity of 24 units per milligram (U/mg) was obtained from Sigma-Aldrich (USA).

2.2 Preparation of gold substrate, CDT/Au & ChOx/ CDT/Au bioelectrode
Gold was deposited onto the desired glass substrates having dimensions of 1.3 cm x 3.9 cm (width x length) by vacuum evaporation technique. Prior to deposition of Au layer on the glass substrate, a layer of Cr was deposited with thickness of 20 nm. 10-Carboxy-1-decanethiol (CDT) SAM was formed by immersing pre-treated Au substrate into 10-Carboxy-1-decanethiol solution (1mM) in ethanol for about 24 hours at room temperature. 10 µl of cholesterol oxidase (freshly prepared) was physically adsorbed onto CDT/Au SAM using electrostatic interactions.

2.3 Characterization of CDT/Au & ChOx/CDT/Au bioelectrode
SAM modification and enzyme immobilization onto gold electrode were characterized using contact angle measurements (Sessile drop method) [26]. Atomic force microscopy (AFM) images obtained in air using Digital Instruments NanoScope IIIa in tapping mode. The enzyme activity measurements were carried out using UV-visible spectrophotometer (Model 160A, Shimadzu). Cyclic voltammetry studies were performed by the OMNI90 potentiostat (Cypress System) and electrochemical impedance spectroscopy (EIS) measurements were carried out in the frequency range of 0.01–10⁵ Hz on an Autolab Potentiostat/Galvanostat from Eco Chemie (Netherlands) using a three-electrodes cell in phosphate buffer saline solution (50 mM, pH 7.0 and 5 mM Fe(CN)63-/4-)

Cholesterol estimation was carried out using surface plasmon resonance (SPR) technique (Autolab SPR, Eco Chemie Netherlands) in the Kretschmann configuration. A linearly p-polarized laser (670 nm) was directed through a prism onto the gold electrode. The intensity of the reflected light as a function of time was measured over a range of 4000 millidegrees (m°). All experiments were carried out at 25°C. UV-visible and cyclic voltammetric techniques were used to verify the results obtained using SPR.

3 RESULTS AND DISCUSSION

3.1 Contact angle Studies
The change in the value of contact angle from 82.0° to 28.0° for bare Au substrate and CDT/Au, respectively [Fig.1], indicates the presence of hydrophilic nature due to SAM formation with highly polar terminal carboxylate group [27, 28].

![Figure 1: (A) Blank gold, (B) CDT/Au, (C) ChOx/CDT/Au bioelectrode](image)

Further, immobilization of enzyme, the contact angle changed to 21.0° for ChOx/CDT/Au bioelectrode indicating the ChOx immobilization on the CDT/Au bioelectrode. The marginal change in the value of the contact angle after enzyme immobilization is attributed to the similar nature (hydrophilic) of the enzyme [29].

3.2 AFM studies
Atomic force microscopy was used for the topographic characterization of CDT/Au and ChOx/CDT/Au bioelectrode, respectively (Fig. 2). Change in the morphology with uniformly distributed structures after ChOx binding confirmed the immobilization of ChOx [21].
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3.3 Electrochemical impedance spectroscopy (EIS)
EIS is an effective tool for studying the interface properties of surface-modified electrodes. The magnitude of the charge transfer resistance (semicircle diameter) ($R_{CT}$) depend on the dielectric and insulating features at the electrode/electrolyte interface [30]. In Fig. 3, the Nyquist diameter ($R_{CT}$) of the CDT/Au electrode (Curve (ii), $R_{CT}$ ca. 6009 Ω) was larger than the bare gold (Curve (i) $R_{CT}$ ca 448 Ω) indicating the CDT SAM formation. The increase in $R_{CT}$ could be attributed to the repulsion between the negative charge of COO$^-$ group of SAM and negative charge on redox probe present in the system [31]. After the immobilization of ChOx, the value of $R_{CT}$ ca. 2514 Ω (Curve (iii)) decreased due to reduced negative charge on the surface by enzyme (cholesterol oxidase) coverage and the presence of amino groups in the enzymes [32]. This decrease in the value of $R_{CT}$ confirmed cholesterol oxidase binding.

3.4 UV-Spectrophotometric studies
ChOx/CDT/Au bioelectrodes were studied for their photometric response as a function of cholesterol concentration (50-500 mg/dl) at wavelength of 500 nm. The following biochemical reactions occurred:

\[
\text{Cholesterol + } O_2 \rightarrow \text{Cholestene-3-one + } H_2O_2 \;
\]

\[
H_2O_2 + \text{o-dianisidine (reduced)} \rightarrow 2H_2O + \text{o-dianisidine (oxidised)} \;
\]

During experiments, ChOx/CDT/Au bioelectrode was dipped in the reaction mixture containing 3.0 ml phosphate buffer (pH 7.0, 5mM), 50 μl of dye (ODA), 50 μl of horseradish peroxidase and 100 μl of substrate (cholesterol) and was kept for about 3 minutes at 30°C for biochemical reaction (Eqs. 1&2). The linearity and low detection limit of these bioelectrodes were obtained as 50-500 mg/dl and 50 mg/dl, respectively. The values of sensitivity and the Michaelis-Menten constant ($K_m$) have been calculated and have been found to be 2.194 x10$^{-4}$ Abs (mg/dl)$^{-1}$ and 4.64 mg/dl, respectively.

The activity of the ChOx/CDT/Au bioelectrode was measured by UV-visible spectroscopy at different time intervals. It has been found that ChOx activity decreased to 75-70 % after 45 days and to 50 % after 75 days of the original activity measured on zero day (100%).

3.5 Cyclic Voltammetric Response
In the cyclic voltammetric (CV) studies of bare Au, CDT/Au and ChOx/CDT/Au bioelectrode, the peaks seen at 0.3 V and at 0.27 V exhibits oxidation and reduction, respectively, indicating the reversible diffusion-controlled profiles of electrons on surface. However, after the SAM formation, the decrease in the flow of electrons between the SAM and Au surface reveals SAM formation (data not shown).

Fig. 5 shows the amperometric response as a function of cholesterol concentration. The increase in the magnitude of the current with increase in cholesterol concentration is attributed to the increase in $H_2O_2$ concentration by enzymatic reaction, which on oxidation generates electrons and contributes to the increase in current as shown in Eq. 1 & 3.

\[
H_2O_2 \rightarrow 2H^+ + O_2 + 2e^- \;
\]
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3.6 Surface Plasmon Resonance (SPR) studies
During SPR experiments, the first 100 seconds were given for baseline correction with phosphate buffer (pH 7.0, 50 mM) after which cholesterol solution (50-500 mg/dl) was added to allow interaction with ChOx/CDT/Au bioelectrode during the next 300 seconds (association phase) after which the electrode was washed with phosphate buffer (50 mM, pH 7.0) to remove any extra cholesterol (dissociation phase). This replacement with phosphate buffer (50 mM, pH 7.0) brought the same condition before and after the association phase. Hence, the increase in the value of SPR angle corresponds to the amount of binding since all other parameters were constant (Fig 6). After completion of the dissociation phase, the surface was fully regenerated by rigorous washing in the regeneration phase. Figure 6 show the change in SPR angle with different stages after the addition of 400 mg/dl cholesterol.

The increase in value of the SPR angle with the increase in cholesterol concentration confirms the interaction of ChOx with cholesterol. For quantification, the shift in the SPR angle change is plotted as a function of cholesterol concentration (Figure 7). The figure 7 show the linear range (50-500 mg/dl) of detection for ChOx/CDT/Au bioelectrode.

Table 1: Comparison between the 10-carboxy-1-decanethiol (CDT) SAM based cholesterol biosensor with those reported in literature.

4 CONCLUSIONS
Cholesterol oxidase has been immobilized onto CDT SAM on a gold substrate. The ChOx/CDT/Au bioelectrode works linearly in the 50-500 mg/dl range for cholesterol solution. The sensitivity and the $K_m$ value of the ChOx/CDT/Au bioelectrode have been found to be 2.194 x10^{-4} \text{Abs (mg/dl)}^{-1} and 4.64 mg/dl, respectively. The reusability and stability of the bioelectrode have been found to be 10 to 15 times and about 3 months when stored at 4°C. The presence of interferents such as glucose, urea and uric acid does not affect the SPR response obtained for the cholesterol concentration.

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### Table I Characteristics of various cholesterol biosensors

<table>
<thead>
<tr>
<th>Immobilization Matrix</th>
<th>Sensing element</th>
<th>Immobilization Technique</th>
<th>Linearity</th>
<th>Detection limit</th>
<th>Transducer used</th>
<th>Shelf-life</th>
<th>Reusability</th>
<th>Sensitivity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino-Undecanethiol self-assembled monolayer</td>
<td>Cholesterol oxidase</td>
<td>Covalently</td>
<td>50-500 mg/dl</td>
<td>50mg/dl</td>
<td>Optical</td>
<td>10 weeks</td>
<td>20 times</td>
<td>1.23mV/mg dl⁻¹</td>
<td>[33]</td>
</tr>
<tr>
<td>Modified ODT</td>
<td>Cholesterol oxidase</td>
<td>Covalently</td>
<td>50-500 mg/dl</td>
<td>50mg/dl</td>
<td>Optical</td>
<td>2 months</td>
<td>&lt;15</td>
<td>-</td>
<td>[20]</td>
</tr>
<tr>
<td>Silicicsolgel/prussian blue/carbon</td>
<td>Cholesterol Oxidase</td>
<td>Entrapment</td>
<td>1x10⁻⁶-8x10⁻⁴</td>
<td>1.2x10⁻¹</td>
<td>Amperometric</td>
<td>35 days</td>
<td>-</td>
<td>0.329μA mM⁻¹</td>
<td>[17]</td>
</tr>
<tr>
<td>Pt/SAM/Prussian Blue (PB)</td>
<td>Cholesterol Oxidase</td>
<td>Entrapment</td>
<td>0.35mM</td>
<td>8μ M</td>
<td>Amperometric</td>
<td>25 days</td>
<td>-</td>
<td>-</td>
<td>[22]</td>
</tr>
<tr>
<td>Alkanethiol</td>
<td>Cholesterol oxidase and microperoxidase</td>
<td>Covalently</td>
<td>0.2-3.0 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[23]</td>
</tr>
<tr>
<td>Thiolipid SAM</td>
<td>Cholesterol Oxidase</td>
<td>Vesicle fusion</td>
<td>10-1000μM</td>
<td>1μ M</td>
<td>Amperometric</td>
<td>60 days</td>
<td>-</td>
<td>-</td>
<td>[24]</td>
</tr>
<tr>
<td>1-hexadecanethiol</td>
<td>Molecular imprinted layer</td>
<td>Template</td>
<td>66-700nM</td>
<td>-</td>
<td>Cyclic voltammetry</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[34]</td>
</tr>
<tr>
<td>Modified poly (3-hexithiophene)</td>
<td>Cholesterol oxidase</td>
<td>Covalently</td>
<td>50-500 mg/dl</td>
<td>50mg/dl</td>
<td>Optical</td>
<td>10 weeks</td>
<td>&lt;15</td>
<td>1.04 mV/ (mg/ dl)</td>
<td>[35]</td>
</tr>
<tr>
<td>Screen printed rhodium–graphite</td>
<td>Cytochrome P450ccc on gold nanoparticles</td>
<td>Drop cast</td>
<td>0.01 – 0.07 mM</td>
<td>0.01mM</td>
<td>Amperometric</td>
<td>-</td>
<td>-</td>
<td>0.13 μA μM⁻¹</td>
<td>[36]</td>
</tr>
<tr>
<td>10-Carboxy-1-Decanethiol</td>
<td>Cholesterol oxidase</td>
<td>Electrostatic interaction</td>
<td>10 to 500 mg/dl</td>
<td>10 mg/dl</td>
<td>Optical and Amperometric</td>
<td>3 months</td>
<td>10-15 times</td>
<td>2.194x10⁻⁴ Abs (mg/dl)³</td>
<td>Present work</td>
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