Immobilization of Glucose Oxidase on Gold Surface for Applications in Implantable Biosensors

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***Abstract*—Surface modification is an important step in developing nanoscale biosensors. The uniform and stable surface structure by forming self-assembled monolayers (SAM) makes it suitable for the development of biosensors. We have studied several surface modification methods on a gold electrode to covalently immobilize the glucose oxidase. The methods with SAM formation were more efficient for immobilizing glucose oxidase, as expected. Furthermore, the gold surface modified with MPA and the coupling reagents EDC and NHS was found to be the best method among the various matrices tested. With highly efficient modified matrix, we hope to develop a continuous glucose monitoring system in the future.**

***Index Terms*—microneedle, glucose oxidase, subcutaneous tissue, continuous glucose monitoring system**

I. Introduction

The self-monitoring of glucose has several major advancements in recent years. However, it still has some limitations such as the number of tests it permits per day due to the inconvenience caused by the painful finger-stick sampling. Moreover, the current sampling method also easily neglects the nighttime monitoring during sleep. Therefore, frequent measurements or continuous monitoring is required for detecting changes in glucose level. [1]. The continuous glucose monitoring system (CGMS) is a useful method in diabetes treatment with advantages over the conventional glucose measurement methods by offering a longer-term ongoing display of glucose levels [2], [3]. However, the CGMS commercially available at present are expensive. Therefore, there is a demand for cost-effective CGMS.

The CGMS consists of an implantable electrochemical biosensor containing a glucose-dependent enzyme immobilized on a microneedle generating glucose-dependent electrical currents. The microneedle can be inserted into subcutaneous tissue and connected to a transmitter and a separate receiver that displays the glucose profile [4], [5]. Subcutaneously implantable devices are commonly designed to operate for several days to continuously monitor the change in glucose level, after which they are replaced by the patient. The microneedle tracks blood glucose levels by measuring the glucose concentration in the interstitial fluid of the subcutaneous tissue.

In the last two decades, a wide range of possible implantable glucose electrochemical biosensors, based on different materials and membranes has been studied [1]. An ideal biosensor will be the one, which monitors blood glucose variations in real time throughout the day and over extended periods under harsh conditions. The size of the sensor must be of very small size and appropriate for easy implantation with minimal discomfort [6]. The immobilization of enzyme on the electrode surface is an important parameter in determining the performance of a biosensor. In this regard, the covalent binding of enzymes onto a modified electrode surface through self-assembled monolayers (SAM) has received considerable interest [7], [8]. SAMs are very useful for the developing nano-scale biosensor because they are easy to prepare on a metallic structure with various functional groups, which can be linked to macromolecules such as enzymes. SAMs of organosulfur compounds (thiols, disulfides, sulfides) attaches to the gold surface spontaneously from either the liquid or the vapor phase and it can easily be implemented in a laboratory. The SAMs formed on gold surface are widely used for enzyme immobilization in biosensor applications [9]. The SAMs acts as a shield for the enzymes on the electrode surface and prevent them from denaturation [10]. This property is particularly useful in fabricating biosensors for CGMS.

In general, the first-generation glucose sensors employ the flavoenzyme glucose oxidase (GOx) immobilized on a working electrode. The redox cofactor of GOx flavin adenine dinucleotide (FAD) catalyzes the oxidation of

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glucose to glucanolactone, as shown in equations (1) and (2) [11].

$$\text{Glucose} + \text{GOx(FAD)} \rightarrow \text{Glucanolactone} + \text{GOx}(FADH_2) \quad (1)$$

$$\text{GOx}(FADH_2) + O_2 \rightarrow \text{GOx(FAD)} + H_2O_2 \quad (2)$$

The H_2O_2 produced from the above reaction is assessed amperometrically on the surface of the working electrode via equation (3), which relates current to glucose concentrations obtained from a calibration plot.

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (3)$$

The second-generation biosensors have employed redox mediators to decrease the oxygen dependence for the catalysis of glucose. In the third generation, the GOx is covalently linked to the electrode surface through its redox cofactor [1]. First-generation glucose sensors are preferred over second and third generation sensors for implantable bionsensors, as the later two have not been tested *in vivo* [12]. To develop a first generation implantable device, a suitable matric to immobilize the GOx is necessary. In this study, we have compared several organosulfur SAMs for immobilization of glucose oxidase on Au electrode surface.

II. Materials and Methods

A. Materials

Glucose Oxidase (GOx) (type x-s from *Aspergillus niger*), 3-Mercaptopropoinic acid (MPA), N-(3-Dimethylamino-propyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), Polyallylamine were purchased from Sigma-Aldrich and used as received. Dithobis (succinimidyl propionate) (DSP) was purchased from Thermo Scientific. All other reagents used were of analytical grade. The supporting electrolyte 50 mM phosphate buffer solution (PBS) was prepared from Na_2HPO_4 and NaH_2PO_4. All the electrolyte solutions were purged with pre-purified N_2 gas for 15 min prior to electrochemical experiments unless otherwise specified.

B. Apparatus

The electrochemical measurements were carried out using CHI 1211B handheld potentiostat. Electrochemical studies were carried out in a conventional three electrode cell using Au electrode (CHI) as a working electrode (area 0.07 cm^2), saturated Ag/AgCl as a reference electrode and Pt wire as a counter electrode. EIM6ex ZAHNER (Kroanch, Germany) was used for electrochemical impedance spectroscopy (EIS) studies.

C. Immobilization of GOx on Modified Au Electrode

The Au electrode surface was cleaned by electrochemical oxidation/reduction in 0.05 M H_2SO_4 for 30 min from +1.5 to -0.3 V vs Ag/AgCl at 0.1 V s^{-1}. The electrode was then washed with water and ethanol. The Au electrode with enzyme-containing matrix was prepared with the following methods [13]-[15].

1) MPA modification

The layer of MPA on the Au electrode was prepared by immersing in an ethanol solution containing 1 mM MPA for 1 h. MPA-Au electrode was modified by different polymers, before using it to immobilize the GOx. In the first method, 1% chitosan in acetate buffer, pH 5.0 was added and kept for drying at room temperature. In the next method, 20 µL of polyallylamine solution (10 mg/mL) in PBS for 1 h and then washed with distilled water [13]. In the third method, 2 mM EDC and 5 mM NHS was drop casted on the electrode and kept for 50 min to activate the carboxyl group [14]. Then the electrodes were washed thoroughly with PBS. 10 µL of GOx in PBS at 5 mg/mL conc. was drop casted on the electrode and kept overnight at 4°C.

2) DSP modification

About 8 mg of DSP was dissolved in 1 mL of DMSO (20 mM). The Au electrode was immersed in 20 mM DSP in DMSO for 1 h at room temperature which will result in the absorption of, ostensibly, N-succinimidyl-3-thiopropionate (NSTP) [15]. The electrode was then rinsed thoroughly with acetone and finally with 5 mM phosphate buffer (pH 7.0). 10 µL of GOx was added DSP modified Au electrode and incubate at 4 °C for 2 h. The modified electrode was washed
with PBS and air dried and used for EIS and CV studies.

The EIS was obtained for the GOx immobilized electrodes in PBS containing 5 mM $Fe(CN)6^{3-/4-}$ in the frequency range of 100 mHz. The Randles' equivalent circuit model was used for explaining the EIS experimental data (Fig. 1). The circuit included the Warburg element to consider the diffusion controlled process at low frequency region. Nyquist plot was drawn with Imaginary resistance versus real resistance. The CV was measured in oxygenated PBS at 0.05 V s^{-1} with various glucose concentrations ranging from 0-14mM.

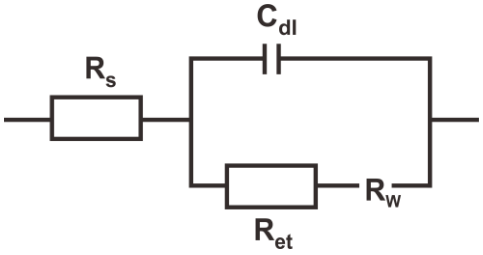

Figure 1. The Randles' equivalent circuit model with electron transfer resistance (R_{et}), double layer capacitance (C_{dl}), solution resistance (R_s) and Warburg element (R_W)

III. Results and Discussion

A. EIS of SAM Modified Au Electrodes

The electrochemical impedance spectroscopy (EIS) is used to measure the dielectric properties of a medium as a function of frequency. EIS is an effective method for studying the characteristics of SAM on Au electrode surface [16]. This study will give the impedance features presented as Nyquist plots (Z_{im} vs. Z_{re}), of electrodes at different modification steps. The GOx immobilization and its loading at various modified electrode surfaces have been investigated and significant differences were

observed in the impedance spectra. The various modified electrodes used in EIS are Au/MPA, Au/MPA/CS/GOx, Au/MPA/PAA/GOx, Au/DSP/GOx, and bare Au electrode was measured in PBS containing 5 mM $Fe(CN)_6^{3-/4-}$. The complex plane plots obtained for bare Au, SAM modified Au electrodes with different polymers and GOx are given in Fig. 2.

The bare Au electrode exhibited an almost straight line, which is characteristic of a diffusion limited electrochemical process. The electrode surface with SAM formed an insulating layer which acts as a barrier to the interfacial electron transfer. This is observed by the appearance of the small semicircular part of the spectrum.

The size of the respective semicircular element corresponds to the electron-transfer resistance (R_{et}) at the electrode surface. As shown in Fig. 2, an increase in semicircular diameter of the impedance spectrum was observed when incubating the Au-SAM modified electrode with GOx solution overnight indicating formation of a ferrocyanide transport-blocking layer on the electrode. In this study, EIS of GOx immobilized on MPA/EDC/NHS (Fig. 2c) and DSP (Fig. 2d) modified electrodes exhibited larger semicircle than other modified electrodes such as the MPA/PAA (Fig. 2a) and MPA/CS (Fig. 2b) electrodes.

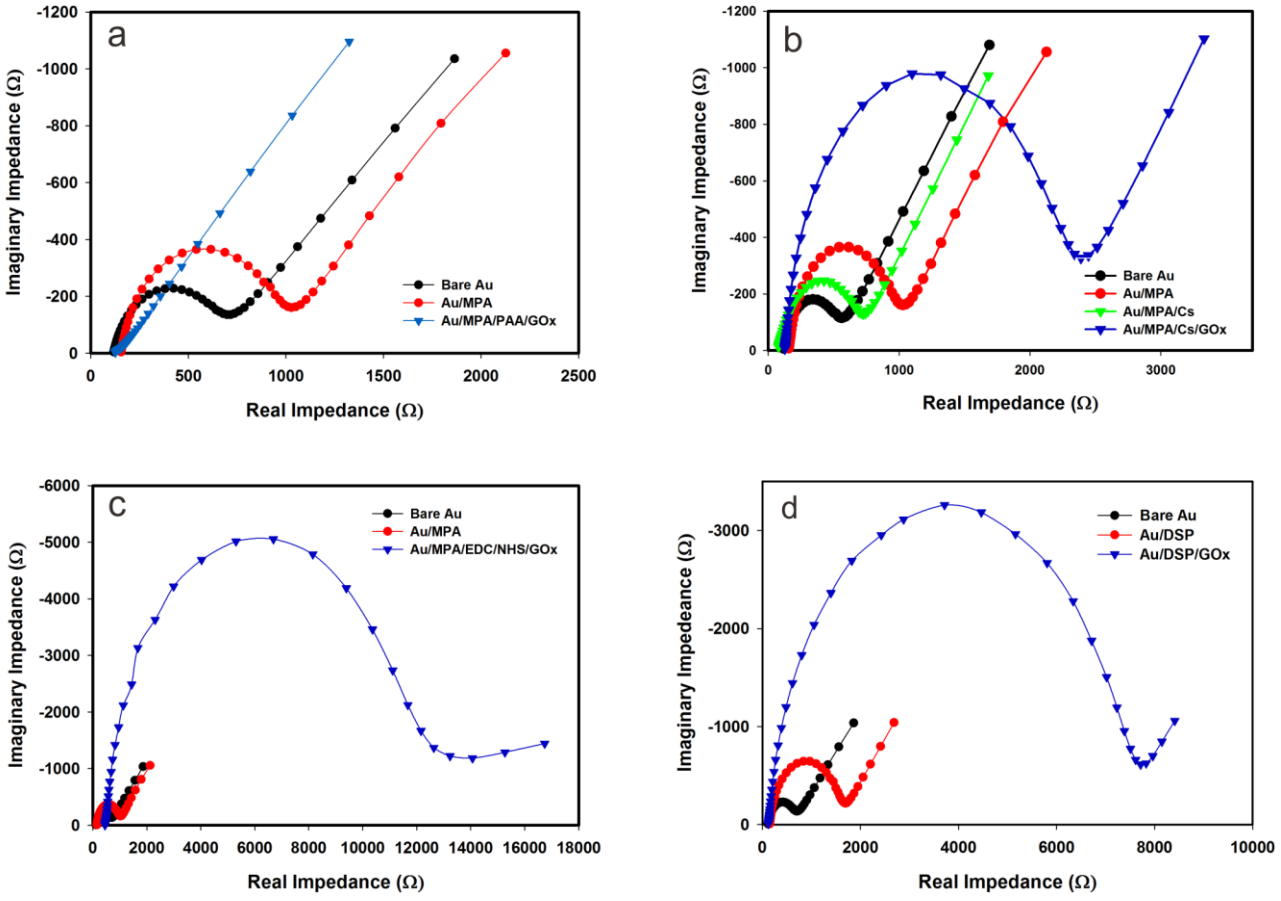

Figure 2. EIS of GOx immobilized on various SAM modified Au electrodes such as Au/MPA/PAA/GOx (a), Au/MPA/CS/GOx (b), Au/MPA/EDC/NHS/GOx (c) and Au/DSP/GOx (d)

This indicates that SAM formed by MPA and DSP on the Au electrode surface act as a large barrier to electron transfer occurs at the GOx modified Au electrode rather than bare Au electrode. This is revealed by the increase in diameter of the semicircle in EIS spectrum. The increased electron transfer resistance observed at GOx modified Au electrode could be due to the thick protein layer surrounding the FAD redox centre of GOx [1]. The EIS results are consistent with the CV experiments which demonstrate that more amount of GOx is well immobilized at the MPA/EDC/NHS/GOx and DSP/GOx electrodes. The EIS study reveals that GOx tightly binds to the Au electrode surface modified by MPA/EDC/NHS.

The upper limit on the density of enzyme molecules on the gold surface is determined by the geometric arrangement of the sulfuric groups and the nearest-neighbor distances between the metal atoms. In addition, the high degree of van-der Waals interactions with neighboring molecules determines majorly the macroscopic properties of the SAMs. And, the linkage of the enzymes is also affected by the overall macroscopic properties. It has been reported that the amine group present in the GOx couples with the acidic group of Au-MPA SAM, by forming imide group with the help of EDC and NHS [16]. So the MPA/EDC/NHS based SAM on Au surface can be a better matrix suitable for further

studies on fabricating implantable glucose sensor devices. The DSP used for SAM formation is insoluble in water, so DMSO was used to prepare the DSP solution for the electrode modification.

B. Electrochemical Detection of Glucose by SAM Modified Au Electrode

The electrochemical properties of the modified electrodes were investigated with a conventional three-electrode cell connected to the potentiostat/galvanostat. Fig. 3 shows the CV of modified electrodes with GOx in oxygen saturated PBS at 0.05 V s^{-1} scan rate with various concentrations of glucose. The cyclic voltammetry results show that the MPA modified electrodes showed higher electrocatalytic activity for glucose in the presence of oxygen. Thus, glucose was effectively oxidized at the composite film. This electrocatalytic response towards glucose is higher with MPA/EDC/NHS/GOx (Fig. 3) and DSP/GOx than that observed in MPA/CS/GOx and MPA/PAA/GOx (data not shown). This showed the efficient electrocatalytic ability of the SAM modified electrodes towards glucose oxidation as concomitant redox peaks were observed for the increased glucose concentration from 1-14 mM of glucose. The linear range of glucose detection is about 0-14 mM in the presence of oxygen (Fig. 4). There are reports about direct electrochemical behavior of GOx on modified glassy carbon electrodes, but there was no direct electrochemistry observed for GOx on Au electrodes in the presence of oxygenated PBS without mediator.

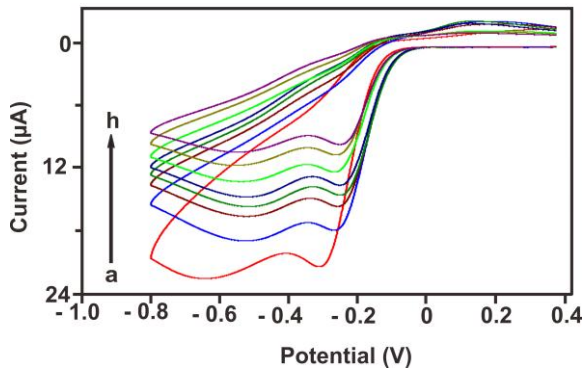

Figure 3. Cyclic voltammetry of Au/MPA/EDC/NHS/GOx electrode with 0-14 mM glucose (a to h) in oxygenated PBS.

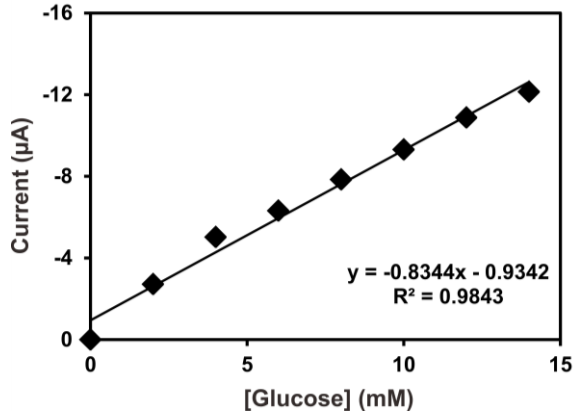

Figure 4. Calibration plot of glucose concentration (0 to 14 mM) vs. peak current.

IV. Conclusion

We demonstrated the GOx immobilization on Au surface by SAM formation. The cyclic voltammetry and the electrochemical impedance studies showed that GOx was successfully and stably immobilized onto Au-SAM electrode. The crosslinkers, EDC and NHS is likely to be involved in the tight binding of GOx to the modified electrode surface. The development of implantable glucose sensors for continuous glucose monitoring has to overcome several challenges including the long-term stability of the enzyme, oxygen deficiency, tissue inflammatory response, calibration, etc., The stability of the enzyme on the electrode surface can be enhanced by the SAM modified Au surface. Currently, the MPA based SAM formation is being applied to microneedle to immobilize GOx for implantable biosensor applications.

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