Iron Oxide Nanoparticles Reinforced Self-Assembled Monolayers of Cysteamine for Use in Enzymatic Biosensor Development

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ABSTRACT
Freshly prepared iron oxide nanoparticles were used to reinforce the cysteamine based self-assembled thiol monolayers attached via glutaraldehyde on gold-sheeted glass-plates which were also attached by covalent bonding with the enzyme, cholesterol oxidase. The prepared bioelectrode was well characterized and convincingly estimated the cholesterol concentrations in known test samples. The developed biosensor was found to have linearity from 50-400 mg/dL with the detection limit of 50 mg/dL. The shelf-life was found about 4 weeks with more than 30 times reuse capacity when stored at 4°C. It was observed that layering of nanoparticles reinforcement increased the shelf-life and over-all working stability of the developed biosensor.

KEYWORDS
Iron Oxide Nanoparticles (Fe$_2$O$_3$), Reinforced self-assembled monolayers (rSAM), Bioelectrode.

Biologically functional materials based sensors have recently gained much attention in estimating analytes of clinical importance due to their selectivity, accurate responses and faster time duration of sensitization linked readings. The biosensor templates have been designed using different cellular and subcellular functional materials namely enzymes, antibodies, DNA, section of tissues, micro-organisms and certain metabolites. The sensing components within the devices having abilities to real-time monitor the complex molecular species and bioprocesses with functional controls have been available lately. The evaluation of macromolecularly generated electrical signals in the miniaturized biotechnical device have diverse applications in biotechnology dealing with DNA and protein microarrays, biochips, microbial technologies, bioreactors, medicine, environmental biotechnology, clinical diagnostics, food analyses, pollution controls, biosafety and defense purposes. There have been intense developmental efforts to detect gases, chemicals, toxins and biomolecules of security and medicinal values using different enzyme types with an advantage over traditional detections in terms of time-consumption and in-situ micro chemical conditions representation. Various enzymes for different bio-conditions based analyses have limited stability and non-linear reactivities. However, Clark and Lyons [1] suggested immobilization of enzyme for analytical purposes by entrapping it by a semi-permeable membrane in conjunction with the indicator electrode. The solid support working as a matrix holds the sensitizing enzyme macromolecule. These macromolecules are highly sensitive and may undergo denaturation under certain conditions. Thus, the reactions involved in immobilization of these biomolecules on the desired electrode needs to be as mild as possible. A number of immobilization techniques and materials of biological and non-biological nature have been investigated for their biosensor applications [2, 3].

The spontaneous adsorption and organization of self-assembled monolayers (SAM) on various solid surfaces for use in matrix in macromolecular based biosensors has been the subject of numerous investigations during the last decade [4]. The most extensively studied systems are alkane-thiols on gold surfaces [5] wherein their organizations, properties and functions have been well-established by a large number of surface related techniques [6-10]. The advantage of this approach is a well-ordered and closely packed SAM formed by covalently linked thiol sulfur to the gold by simply immersing a gold sheet in a solution containing desired thiol compound. The SAM allows construction of a wide variety of functional layers on these metal surfaces with desired electrochemical properties [11]. Vidal et al. have studied the effects of SAM formations in improving stability of amperometric biosensor involving cholesterol as the sensitizing biomolecules [12]. The amphiphilic cholesterol molecule is an essential component of cell membrane and constituents of steroid hormones with properties to alter permeability and fluidity of the lipid bilayers [13-17]. It is important for synthesis of several hormones and is needed for vitamin D synthesis as well [18]. The cholesterol also plays important role in brain synapses, modulation of immune system, and
protections against cancers. The high level of cholesterol in blood, hypercholesterolemia, is a major risk factor for coronary heart disease, atherosclerosis, brain thrombosis and miscellaneous CVS disorders[19-21]. The estimation of cholesterol levels is crucial for the diagnosis and cure of several heart conditions[22]. Cholesterol levels are determined by estimating H2O2 obtained as a byproduct of reaction between cholesterol oxidase enzyme (ChOx) and cholesterol in the blood samples [23-27].

In the current work, cholesterol oxidase enzyme (ChOx) was covalently attached to cysteamine (Cyst) self assembled monolayer via glutaraldehyde to the gold–sheeted (Au) glass-plates. The SAM was reinforced by introduction of freshly prepared iron-oxide (Fe3O4) nanoparticles for increasing the shelf-life and over-all working stability of the designed electrodes. The cysteamine (Cyst) has thiol (SH) groups with affinity towards gold surface [28]. The free terminal NH2 group of cysteamine is available for further binding with the glutaraldehyde. The cross-linker glutaraldehyde is a bifunctional compound, aldehyde group at one end of the glutaraldehyde binds with the NH2 group of the cysteamine and the other aldehyde binds with the NH2 group of the enzyme, cholesterol oxidase. When SAM is dipped in nanoparticles solution it forms another layer of film on it. The confirmations came from FT-IR absorptions, SEM, cyclic Voltammetry studies, electrochemical Faradic Impedence spectra and contact angle studies for different set of forming layers and their electro-chemical properties, as applicable. The cyclic voltammogram of bare gold (Au) exhibited oxidation and reduction peaks at 0.246 V and at 0.270 V, respectively indicating a reversible diffusion-controlled profile. The addition of Cysteamine monolayer onto the gold electrode resulted in a small redox potential (0.157 V and 0.182 V) peak. The large current density of redox peak indicated that the redox couple is still easily accessible to the electrode surface. The assembly of cholesterol oxidase on the cysteamine monolayer resulted in lower amperometric responses. These results are consistent with the fact that further layer-by layer assembly on the electrode retards the interfacial electron-transfer kinetics of the redox probe, resulting in the perturbation of reversible behavior of the redox probe. The high value of amperometric current observed for Fe3O4-ChOx-Cyst-Au bioelectrode has been attributed to the fast electron transfer rate in the electrode due to conducting nature of IO nanoparticles. The Cyclic Voltammogram of ChO-Cyst-Au bioelectrode in PBS (pH 7.0) containing 5 mM FeCN6 -3/4- in the range of -0.6 to 0.8 V for cholesterol concentration with 50 mg/dL, 100 mg/dL, 200 mg/dL, 300 mg/dL and 400 mg/dL for the scan rate of 50 mV/s. It showed Cyclic Voltammogram of Fe3O4-ChOx-Cyst-Au bioelectrode in PBS (pH 7.0) containing 5 mM FeCN6 -3/4- in the range of -0.6 to 0.6 V for cholesterol concentration again at 50 mg/dL, 100 mg/dL, 200 mg/dL, 300 mg/dL and 400 mg/dL. In both the studies, various cyclic voltammogram were obtained for the bioelectrode as a function of the cholesterol concentration (50–400 mg/dL). The current increases with the increase in cholesterol concentration, indicating an increase in the H2O2 byproduct concentrations.

The electrochemical Faradic Impedence spectra in the Frequency range 0.01-105 Hz, represented by the Nyquist plots of the blank gold, Cyst-Au and ChO-Cyst-Au electrodes respectively in phosphate buffer saline (pH 7.0, 0.9% NaCl) containing 5 mM FeCN6 -3/4- as the redox probe, the blank gold electrode exhibited an (almost) straight line. The assembly of the cysteamine monolayer on the electrode surface generated a layer on the electrode that introduced a barrier to the interfacial electron-transfer and was reflected by the semicircle part of the spectra corresponding to a charge-transfer resistance of Rct at 4113.11 Ω. The impedance spectrum obtained after the immobilization of enzyme onto Cyst-Au SAM, and the value of Rct, was obtained as 7751.04 Ω. The increased value of Rct obtained for ChOx-Cyst-Au electrode indicated hindrance to the electron transfer thus, confirming the successful covalent binding of the NH2 group of enzyme (ChOx) with the carbonyls of linker glutaraldehyde.

The FTIR spectra of Cyst-Au confirmed the formation of cysteamine monolayer on the gold surface. Absorption bands at 517 cm-1 and 821 cm-1 in FTIR spectra of Fe3O4-ChOx-Cyst-Au indicated the formation of Fe3O4 nanoparticle film over the surface of cysteamine SAM. The 1271 cm-1 and 1471 cm-1 peaks were due to presence of C=N and C-N bonds in cysteamine. Also, the bands at 2836 cm-1 and 2903 cm-1 in FTIR spectra of ChOx-Cyst-Au and Fe3O4-ChOx-Cyst-Au were observed due to aldehyde group of cross-linker glutaraldehyde. The appearance of additional absorption bands at 1586 cm-1 and 1744 cm-1 in ChOx-Cyst-Au and Fe3O4-ChOx-Cyst-Au were due to amide bonds absorptions of the enzyme (ChOx).

The reinforced Fe3O4-ChOx-Cyst-Au bio-electrodes have shown high amperometric current and low electrochemical impedance which confirmed the fabrication of the layered biomolecular electrode, see also scanning electron micrographs (Fig.1).
The Fe$_2$O$_3$-ChOx-Cyst-Au electrode convincingly estimated the cholesterol concentration in known test samples over a period of four to six weeks and the biosensor was found to have linearity from 50-400 mg/dL with the detection limit of 50 mg/dL. The shelf-life was found about 4 weeks with 30-35 times reusability capacity when stored at 4°C. Thus, it was observed that the Fe$_2$O$_3$ nanoparticles reinforcement on SAM based electrode increased the shelf life and over-all working stability. Efforts are on to prepare other prototype reinforced SAM based bioelectrodes for different uses. The observation can be helpful in designing new and stable biosensors with use in diagnostic kits, imaging, environmental pollutants, food-allergy tests and security apparatuses dealing biochemical hazards provided appropriate SAM is used.

**EXPERIMENTAL**

**Preparation of gold substrate and NH$_2$ terminal-Cyst SAM**

The pre-cleaned gold plate was immersed in 1mM ethanol solution of cysteamine for 24 hours at room temperature. The SAM modified gold plates were rinsed with ethanol and water several times and dried.

**Immobilization of Cholesterol Oxidase on Cyst-Au Electrode**

The Cyst-Au SAM was dipping in 0.1% glutaraldehyde for about 3 hours and cholesterol oxidase was immobilized by dispensing and keeping dipped SAM overnight at 20°C. The resulting ChO-Cyst-Au electrodes were phosphate buffer washed to remove any unbound enzymes and were stored at 4°C.

**Preparation of Fe$_2$O$_3$ nanoparticles & Modification of Electrode**

The Fe$_2$O$_3$ nanoparticles were prepared by mixing ferrous chloride (10 mL, 3x10$^{-2}$ M) and ferric chloride (20 mL, 3x10$^{-2}$ M) aq. solutions to which triethyl amine (5 mL) was added drop-wise under continuous stirring until the black precipitate was obtained. The nanoparticles were collected with the help of a powerful magnet on the round-bottomed flask. The nanoparticles were washed several times with deionized pure water and dry and distilled methanol. The particles were kept in a vacuum oven at 40°C for drying for 3 hrs. A solution of Fe$_2$O$_3$ nanoparticles was prepared by dissolving Fe$_2$O$_3$ nanoparticles in Phosphate Buffer, pH 8 in ratio of 1:10. Kept Cyst-Au in this solution for overnight and then took out IO-Cyst-Au electrode at room temperature. The resulting electrodes were washed with phosphate buffer (50 mM, pH 7.0) to remove unbound iron nanoparticles enzyme.

**Immobilization of Cholesterol Oxidase & Characterization of Fe$_2$O$_3$-Cyst-Au electrode**

The Fe$_2$O$_3$-Cyst-Au electrode was modified with glutaraldehyde by dipping electrodes in 0.1% of glutaraldehyde for about 3 hours, after water wash ChOx was immobilized on glutaraldehyde modified Fe$_2$O$_3$-Cyst-Au electrode. The resulting Fe$_2$O$_3$-ChOx-Cyst-Au electrode was again washed with phosphate buffer (pH 7.0) to remove unbound enzymes and stored at 4°C. The contact angle measurements were also carried out to check the immobilization of enzyme by the Sessile drop method [29], using a drop shape analyzer (DSA 100). The electrochemical impedance measurements were recorded in frequency ranges of 0.01–10$^5$ Hz on potentiostat using three-electrode system in phosphate buffer saline (pH 7.0, 0.9% NaCl) containing 5mM Fe(CN)$_6^{3/-4}$. Cholesterol estimation was also carried out using cyclic Voltammetry. The Fe$_2$O$_3$:ChO-Cyst-Au was also visualized using Scanning Electron Microscopy.

**REFERENCES**

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