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Biosensor based on enzyme coupled PVC reaction cell for electrochemical measurement of serum total cholesterol

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ABSTRACT

Polyvinyl chloride (PVC) being chemically inert and corrosive resistant with ease in molding to various shapes and size, was used to fabricate enzyme reaction beaker which acts as electrochemical cell for three electrode-based system for measurement of serum total cholesterol. Inner bottom surface of PVC beaker (15 mL) was chemically modified for covalent immobilization of cholestryl esterase and cholesterol oxidase using glutaraldehyde as a coupling agent. HRP incorporated carbon paste working electrode was fabricated for amperometric measurement at a potential of −50 mV. The sensor showed optimum response within 20 s at pH 7.0 with incubation temperature of 45 °C. K_m and V_{max} for cholesteryl acetate were 760 mg dL^{-1} and 0.9 mA s^{-1}, respectively. The PVC-based electrochemical cell showed a correlation of 0.9916, with the values estimated with a commercially available Bayer's Enzo Kit. The reaction cell lost 50% of its initial activity during its regular use for 200 times over a period of 100 days when stored in 0.1 M sodium phosphate buffer, pH 7.0 at 4 °C. No metabolite interference was observed. The overall results strengthen our view that PVC can be used as a solid support for immobilization of enzymes and their applications in biochemical measurements.

1. Introduction

Enzyme-based diagnostic methods are being preferred over others because of their selectivity, sensitivity and greater accuracy [1–5]. However the use of free enzymes had limitations such as high cost due to single use and limited stability, which have been largely overcome by immobilization of enzyme onto insoluble support [6]. Co-immobilized enzymes form a single step procedure and also provides a close proximity between the enzymes, which lead to easy diffusion of intermediates between the enzymes in reaction medium. This leads to better efficiency of the co-immobilized enzymes as compared to the mixture of individually immobilized enzymes [7–10]. The nature of the support and immobilization procedures influences the behavior (stability and sensitivity) of the enzyme because the biotransformation equilibrium of the substrate occurs at the support-solution interface. A variety of support materials (organic and inorganic) has been used since long however problems like low stability to microbial attack, high cost of the support material or its immobilization procedure, leaching of immobilized enzymes, reduced substrate + enzyme complex formation due to steric hindrance, each time leads to a new hunt for an ideal support [11].

Immobilization and co-immobilization of enzymes led to the development of biosensors that have been increasingly used for routine and continuous monitoring of vital biochemical parameters. A number of amperometric cholesterol biosensors have been reported since 1982, employing cholesterol esterase, cholesterol oxidase and peroxidase immobilized onto octyl-agarose gel, activated with cyanogen bromide and placed in a reactor [3], enzyme electrode in floe injection system [12], nylon mesh over a platinum electrode [13], pyrrole membrane through electropolymerization and coupled with FIA for H_2O_2 analysis [14], single-use, reagentless, screen-printed strip [15], amino-undecanethiol self-assembled monolayer [16], carbon paste electrode modified with hydroxymethyl ferrocene and hydrogen peroxide [17] poly(2-hydroxyethyl methacrylate) (p(HEMA))/polypyrrole p(pyrrole), membrane [18], graphite–70% Teflon matrix with incorporated potassium ferrocyanide [19], ferrocene monocarboxylic acid co-entrapped in to polypyrrole film onto platinized PT electrode [20], electropolymerized layers of polypyrrole and poly-naphthalene derived polymers [21], deflavinated cholesterol oxidase (COD) and subsequent reconstituted apo-protein with a complexed flavin adenine

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dinucleotide (FAD) monolayer [22], layer of silicic sol–gel matrix on the top of Prussian Blue-modified glassy carbon electrode [23], photosensitive polymer on ultra-thin dialysis membrane [24], conducing polypryrrile (PPY) films using electrochemical entrapment technique [25], sol–gel chitosan/silica and multiwalled carbon nanotubes (MWCNTs) organic-inorganic hybrid composite material [26], porous silicon [27], CA membrane enzyme laminate [28]. Polyvenyl chloride (PVC) has been widely used in ion selective electrodes [29–31] and as a direct support for enzyme immobilization [32–35]. But due to low sensitivity and stability of ion selective membrane electrodes [29], charged surface derivatives of PVC membrane have been used in the fabrication of biosensors [29–31,34,35] and in other applications [32,33,36].

Sensitivity of enzymatic reaction must be correlated to sensitivity of transducer or biosensor's design, the best electrochemical transducer is amperometric electrode. In amperometric biosensors, hydrogen peroxide (H_2O_2), a by-product of analytical enzymatic reactions has been measured electrochemically by its direct oxidation under high potential (E) at the electrode as shown in the reaction [37]:

$$H_2O_2 \xrightarrow{E} O_2 + 2H + 2e^- \quad (1)$$

At high potential (E) others species or serum metabolites present in the sample act as interferents and do cause extra addition to the current [38]. Thus the use of amperometric biosensor involving horseradish peroxide (HRP) is an attractive alternative for the measurement of hydrogen peroxide. The direct mixing of HRP within the bulk of carbon paste has been proposed as well [39]. The location of the enzyme with in the conducting compost matrix forces a close electrical contact between the enzyme and the conducing site in the bulk and therefore on the surface of the electrode.

Taking in to account the above discussion, it was decided to design a working prototype in a way to exploit these properties of PVC, carbon powder, HRP by involving two principle enzymes cholestryl esterase and cholesterol oxidase, which could amount to sensitive, accurate and stable biosensor for total cholesterol determination. For this, cholesterol esterase and cholesterol oxidase have been covalently coupled onto inner bottom surface of PVC beaker using glutaraldehyde as covalent coupler. This PVC reaction beaker acts as a cell for three electrode system-based electrochemical measurements. The working electrode was fabricated by incorporation of HRP into carbon paste so as to enable efficient electron transfer.

2. Materials and methods

2.1. Chemicals and reagents

Cholesteryl esterase from *Pseudomonas species* (165.8 units/g), Triton X-100, cholesteryl acetate, glutaraldehyde (grade 1, 25% solution) and platinum powder were from Sigma Chemical Co., USA. Cholesterol oxidase from *Streptomycin* sp. (500 units/10 mg), HRP (80 U/mg) from SISCO Research Laboratory Pvt. Ltd., Mumbai. Carbon powder manufactured by Canon, Singapore and paraffine oil from Ranbaxy Fine Chemicals Ltd., New Delhi were used. Transparent PVC transparent beaker of 15 mL capacity was purchased from local market. All other chemicals were of AR grade.

2.2. Preparation of HRP incorporated carbon paste working electrode

Carbon powder (1.0 g) and NH_4Cl (0.2 mg) were mixed with paraffin oil in a ratio to obtain the consistency of paste. This paste was filled in a plastic hollow tube (2 cm length and 4 mm diameter) with one closed end, leaving an empty space of 5 mm in the top end (open end). 100 μL of HRP (0.5 mg), 25 μL of 15% BSA and 25 μL of 2.5% glutaraldehyde were mixed and allowed to react for 20 min. Carbon powder (0.5 g) and platinum powder (3 μg) were mixed well and added to HRP + BSA + gluteraldehyde mixture. To attain the paste, paraffin oil was added. This modified paste was filled into the empty part (open end) of the hollow plastic tube. This formed the body of the electrode. Electrical contact was obtained by inserting a silver wire into the carbon paste. The surface of the electrode was washed with buffer and stored at 4 °C when not in use.

2.3. Covalent coupling of cholestryl esterase and cholesterol oxidase on to PVC surface

Inner bottom surface of PVC beaker (15 mL) capacity was treated with 2 mL of fuming nitric acid at 30 °C for about 2 h. After that PVC beaker was washed first with running tap water and than with dou-

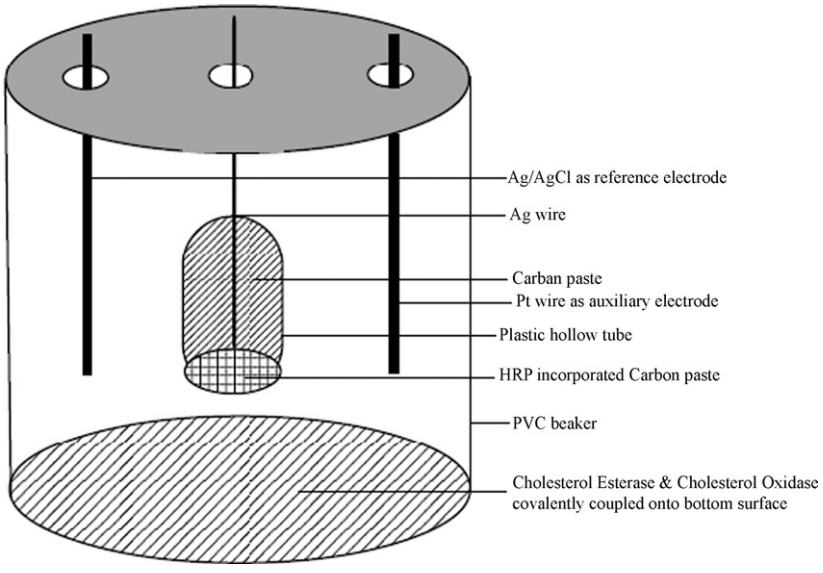

Fig. 1. Systematic diagram representing PVC electrochemical reaction cell.