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This study aims at fabricating multiwalled carbon nanotubes (MWCNTs) based enzymatic bioelectrode for total cholesterol estimation in human serum. For this purpose, a gold (Au) electrode was modified with MWCNTs uniformly dispersed in nafion (Nf) matrix. Cholesterol oxidase (ChOx) and cholesterol esterase (ChEt) were immobilised onto this Nf–MWCNTs film-modified Au electrode using layer-by-layer technique to fabricate the final bioelectrodes. The immobilisation of ChOx and ChEt onto the electrodes was demonstrated by scanning electron microscopy. The fabricated bioelectrodes were electrochemically characterised using cyclic voltammetry. The bioelectrodes offer reliable response characteristics towards cholesterol and stable electrochemical properties in terms of extended linear response range of 0.080–0.950 mM, detection limit up to 0.01 mM and optimum storage stability up to three weeks. Experimental results reveal that the fabricated bioelectrode offer optimum repeatability and reproducibility towards the cholesterol estimation and can also efficiently exclude interference by the commonly coexisting ascorbic acid, uric acid, lactic acid, glucose and urea, which is favourable for its efficient use in the highly selective analysis of total cholesterol in serum samples.

Keywords: cholesterol; bioelectrode; cholesterol oxidase; cholesterol esterase; multiwalled carbon nanotubes; nafion

1. Introduction

Over the past decade, there has been a huge interest in the development of different electrochemical cholesterol biosensors based on either cholesterol oxidase (ChOx) [1–7] or both ChOx and cholesterol esterase (ChEt) [8–11]. The majority of the cholesterol biosensors reported till date are based on the detection of electro-oxidation of hydrogen peroxide produced during the catalysis of cholesterol by ChOx. This requires a high anodic potential [12] that can induce simultaneous oxidation of other electrochemically active

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species present in the samples leading to false positive signals. Therefore, in recent years, cholesterol biosensor research is focused on the direct electron transfer between redox active site flavin adenine dinucleotide (FAD) of ChOx and electrode surfaces [13]. But deep embedment of FAD in the protein core prevents direct electrical communication between FAD and electrode [14,15]. In such cases, an electron transfer mediator is often used either in solution or coimmobilised with enzyme on electrode, to shuttle electrons between the redox centres of enzymes and the electrode. There are several reports on different types of electron transfer mediators immobilised with ChOx to fabricate cholesterol biosensors [16–19].

Carbon nanotubes (CNTs) have been demonstrated as a promising material for applications in various amperometric biosensors due to their unique chemical and electronic properties [20–27]. In such biosensing devices, they can be used for two purposes. Firstly, due to their high surface area, they can serve as immobilisation platforms for biomolecules. Secondly, due to their similarity in length scales with proteins and high electrical conductivity, they exhibit good electronic communication with active site of redox proteins [28,29], and therefore can mediate heterogeneous electron transfer between the active site of the redox enzymes and the electrode. They have been reported to decrease the overvoltage for the oxidation of substrate by enzyme and thereby prevent the interference caused by ascorbic acid, uric acid and hydrogen peroxide [30]. There are many reports on CNTs along with different immobilising supports having been used for the construction of cholesterol biosensors [1,31–36]. The major problem faced for developing CNT-based devices is their poor solubility in most solvents. Nafion (Nf), a perfluorinated ionomer, is widely used to solubilise CNTs. Apart from solubilising CNTs, Nf has other attractive features, such as good biocompatibility, optimum porosity and chemical inertness, which makes it an excellent support material to confine biomolecules at the electrode surface in the construction of various biosensors [37]. It is also known to exclude interference from different electroactive compounds present in physiological samples [38,39]. CNT/Nf nanocomposite electrodes were prepared and found to have excellent electrocatalytic activities and shown to minimise interference from non-target substances [40,41]. The aim of this study is to combine the features of Nf as a biocompatible enzyme immobilisation support and multiwalled carbon nanotubes (MWCNTs) as a mediator in electron transfer between the enzyme and the electrode, and developing an amperometric cholesterol biosensor for serum total cholesterol estimation through immobilisation of ChOx and ChEt in a Nf–MWCNTs matrix deposited on Au electrode.

2. Experimental procedure

2.1. *Chemicals and reagents*

ChOx (EC 1.1.3.6 from *Pseudomonas fluorescens*, 24 U mg^{-1} solid), ChEt (EC 3.1.1.13 from *Pseudomonas* sp., 1.47 U mg^{-1} solid), Nf (5wt %) and MWCNTs were bought from Sigma–Aldrich (USA). Cholesterol was purchased from TCI (Tokyo, Japan). Cholesterol estimation kit was obtained from Merck. All other chemicals were of analytical grade and used as received without further purification. Human serum samples from normal individuals were collected from the institute hospital.

Cholesterol stock solution of 20 mM was prepared in deionised (DI) water having 10% Triton X-100 and stored at 4°C. Stock solutions of ChOx (10 mg ml^{-1}) and ChEt

($1\,mg\,ml^{-1}$) were freshly prepared in 50 and 400 mM phosphate buffer (pH 7.0), respectively, prior to being used. All solutions were prepared with DI water from the Millipore water purification system (Millipore, Bedford, MA).

2.2. *Fabrication of bioelectrodes*

A gold (Au) electrode (diameter = 0.5 cm) was cleaned, first by polishing it with alumina powder, then washing it ultrasonically with 70% ethanol and water separately for 15 min each and finally drying in air. The cleaned Au electrode was dip coated three times with 1% Nf, with intermittent drying after each coating step. Nf dip coating is applied for the better attachment of the MWCNTs–Nf matrix onto the surface of the Au electrode. MWCNTs of 1 mg were dispersed in 100 µl of 5% Nf and sonicated for 20 min to obtain a stable homogeneous suspension. A 10 µl of this mixture was dropped on Nf-coated Au electrode and dried. For preparing Au–Nf–MWCNTs–ChOx–Nf bioelectrode, 12 µl of freshly prepared ChOx solution ($10\,mg\,ml^{-1}$) was dropped on Nf–MWCNTs-modified Au electrode, dried and covered with 8 µl of 5% Nf layer. Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf bioelectrode was prepared by applying an additional coating of 12 µl ChEt ($1\,mg\,ml^{-1}$) onto ChOx–Nf coating. Finally, 8 µl Nf (5%) was poured and dried. The fabricated bioelectrodes were immersed in 50 mM phosphate buffer solution (PBS) (pH 7) for 15 min prior to being used. When not in use, the bioelectrodes were stored in 50 mM PBS (pH 7) at 4°C.

2.3. *Apparatus and measurements*

Cyclic voltammetry (CV) was performed in a three-electrode configuration with an Autolab PGSTAT 1212 (Eco Chemie, The Netherlands). The working electrodes were Au–Nf–MWCNTs–ChOx–Nf and Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf bioelectrodes. Ag/AgCl (3 M KCl) and a platinum (Pt) rod served as reference and counter electrodes, respectively. PBS (50 mM, pH 7) was used as the supporting electrolyte. All potentials were measured and reported relative to the Ag/AgCl reference electrode. All experiments were performed at room temperature. Scanning electron microscopy (SEM) images were obtained on a scanning electron microscope (Leo 1430vp, Germany) using the following setting conditions: 15 KeV EHT, 50 µm aperture.

3. Results and discussion

3.1. *Morphological characterisation of the bioelectrodes using SEM*

Figure 1 shows the SEM images of Au–Nf, Au–Nf–MWCNTs and Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf electrodes revealing the stepwise changes in the electrode surface morphology at different stages of bioelectrode fabrication. As shown in figure, Au electrode modified with Nf shows the homogeneous surface (Figure 1(a)), whereas the SEM image of Au–Nf–MWCNTs electrode (Figure 1(b)) shows the granular porous morphology demonstrating the distribution of MWCNTs in Nf matrix on the electrode surface. When ChOx and ChEt were entrapped in this Nf–MWCNTs film to fabricate Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf bioelectrode, SEM image showed that the surface morphology changed into uniform globular and fibrillar structures (Figure 1(c)), attributed to the presence of ChOx and ChEt.

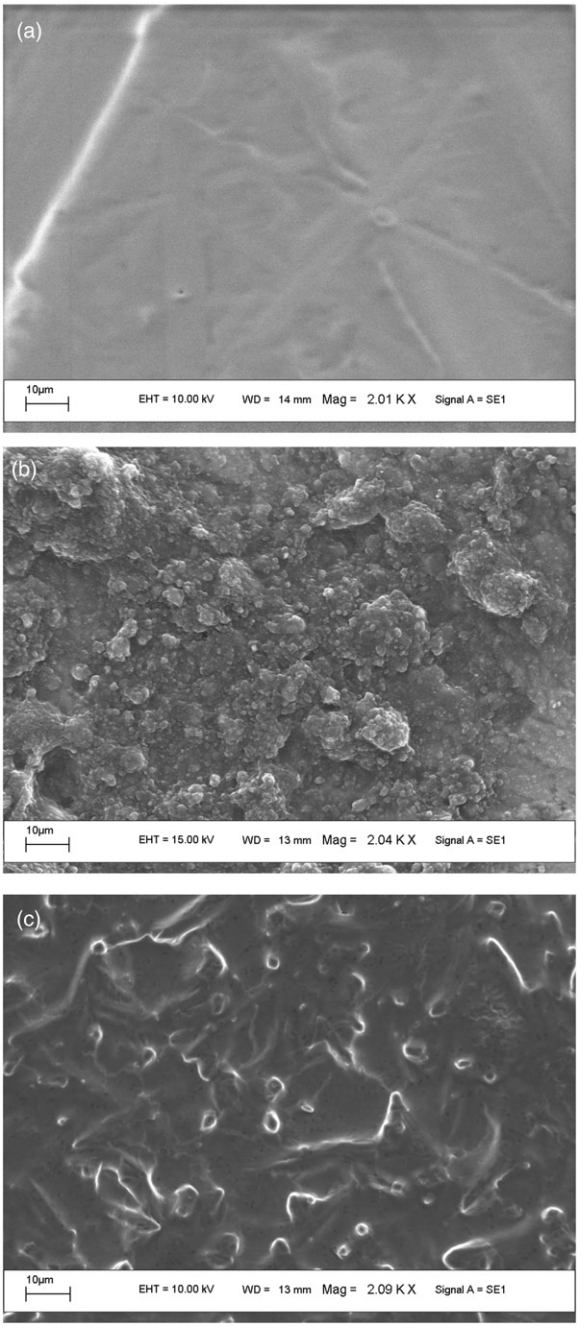

Figure 1. SEM images (a): Au–Nf, (b): Au–Nf–MWCNTs and (c): Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf electrodes.

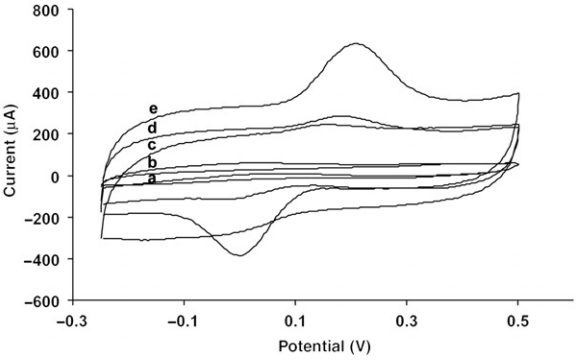

Figure 2. Cyclic voltammograms of Au (a), Au–Nf (b), Au–Nf–MWCNTs (c), and Au–Nf–MWCNTs–ChOx–Nf (d) recorded in PBS (50 mM, pH 7). Au–Nf–MWCNTs–ChOx–Nf (e) was recorded in the presence of 0.33 mM cholesterol in PBS (50 mM, pH 7). In all cases, a potential scan rate of $0.1\,V\,s^{-1}$ was used.

3.2. *Response mechanism of the fabricated bioelectrodes*

Figure 2 shows the results of CV studies carried out with Au, Au–Nf, Au–Nf–MWCNTs and Au–Nf–MWCNTs–ChOx–Nf electrodes in PBS (50 mM, pH 7). No major difference was observed among the cyclic voltammograms of the bare Au (curve a) and Au–Nf electrodes (curve b). When the Au electrode is modified with Nf–MWCNTs matrix, an increase in the background current intensity was observed, which is attributed to the increased electroactive surface area provided by MWCNTs. Also, a pair of redox peaks was observed at 0.153 and 0.002 V (curve c). These redox peaks are attributed to Nf–MWCNTs as these are absent in the CVs of Au and Au–Nf electrodes (curves a and b). Cyclic voltammogram for Au–Nf–MWCNTs–ChOx–Nf bioelectrode (curve d) shows a shift in the oxidation peak from 0.153 to 0.184 V. When the CV is taken with Nf–MWCNTs–ChOx–Nf bioelectrode in the presence of cholesterol, there is a further shift in the oxidation peak to 0.21 V and an increase in the oxidation current intensity at 0.21 V (curve f). Also, a CV was taken with Au–Nf–ChOx–Nf without the CNTs. In this case, the CV was similar to that of Au–Nf electrode (curve b) without any additional peak showing the absence of electronic communication between ChOx and the electrode in the absence of CNTs. For the visual clarity, the CV is not included in the figure. From these CV results, it may be concluded that MWCNTs play a role in the electro-catalytic activity of the ChOx. It may be proposed that during ChOx catalysis, the Nf–MWCNTs matrix accepts electrons from the reduced ChOx and subsequently gets oxidised at the electrode surface. This suggests that the ChOx gets electrically contacted with Nf–MWCNTs matrix. Based on the CV studies, the working mechanism of the fabricated bioelectrodes in detecting total cholesterol can be proposed as shown below:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{ChEt}} \text{cholesterol} + \text{fatty acids}, \tag{1}$$

$$\text{ChOx–FL}_{ox} + \text{cholesterol} \longrightarrow \text{ChOx–FL}_{red} + \text{4-cholestene-3-one}, \tag{2}$$

$$\text{ChOx–FL}_{red} + \text{MWCNT}_{S_{OX}} \longrightarrow \text{ChOx–FL}_{ox} + \text{MWCNT}_{S_{red}}, \quad (3)$$

$$\text{MWCNT}_{S_{red}} \longrightarrow \text{MWCNT}_{S_{OX}} + ne^{-}, \quad (4)$$

where ChOx–FL_{ox} and ChOx–FL_{red} represent the ChOxs having oxidised and reduced forms of FAD, respectively.

3.3. *Response characteristics of the fabricated bioelectrodes towards cholesterol*

The response characteristics of both Au–Nf–MWCNTs–ChOx–Nf and Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf bioelectrodes were determined to examine whether the incorporation of ChEt in the bioelectrode has any interference in the detection of cholesterol by ChOx. Figure 3(a) and 3(b) displays the amperometric response of Au–Nf–MWCNTs–ChOx–Nf and Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf bioelectrodes, respectively, for successive step changes of cholesterol concentrations in PBS (50 mM, pH 7) at an operating potential of 0.21 V. The response characteristics for both the electrodes are summarised in Table 1. For both the bioelectrodes, the magnitude of the anodic peak current at 0.21 V was found to increase linearly with increase in cholesterol concentration. It can be observed from Table 1 that the sensitivity of Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf is more than Au–Nf–MWCNTs–ChOx–Nf bioelectrode. This may be attributed to better substrate sequestration to the ChOx active site in the presence of ChEt. The increased sensitivity in turn led to smaller detection limit (DL) in case of Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf bioelectrode. The present system was observed to show a wider cholesterol response range when compared to some other reported range of 0.5–25 μM for ChOx and ChEt immobilised in laponite clay nanoparticle-poly(12-pyrrol-1-yldodecyl)triethylammonium tetrafluoroborate on Pt disc electrode [7,42] and 0.04–0.27 mM obtained when ChOx and horseradish peroxidase (HRP) were coimmobilised in tributylmethyl phosphonium chloride polymer membrane on pyrolitic graphite electrode [7,43] etc. The DL for the constructed bioelectrodes was determined from the expression $DL = 3 \times SD/\text{sensitivity}$ (where SD is the estimated standard deviation for the points used to construct the calibration curve and the sensitivity its slope). The calculated DL for our constructed bioelectrodes compares favourably with values reported for ChOx entrapped in ferrocene monocarboxylicacid-PPy/Pt/Pt electrode (0.012 mM) [44] and when ChOx covered in Nf was entrapped in PPy/PB/1-propanethiol (SAM)/Pt electrode (0.012 mM) [2,7].

3.4. *Interference, repeatability, reproducibility and long-term stability*

For exploring the efficient use of the fabricated Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf bioelectrode in the specific determination of total cholesterol in real samples, effects of some common interferents in cholesterol determination, such as glucose, ascorbic acid, uric acid, lactic acid and urea on the bioelectrode response have been studied. The current response at 0.21 V of the Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf bioelectrode in solutions containing equal concentration of cholesterol (0.33 mM) and each interferent (0.33 mM) has been determined. The bioelectrode response did not get significantly affected in the presence of either of these interferents (percentage error in all cases is less than 2%; Figure 4). Moreover, the actual level of these interferents in serum is usually very low

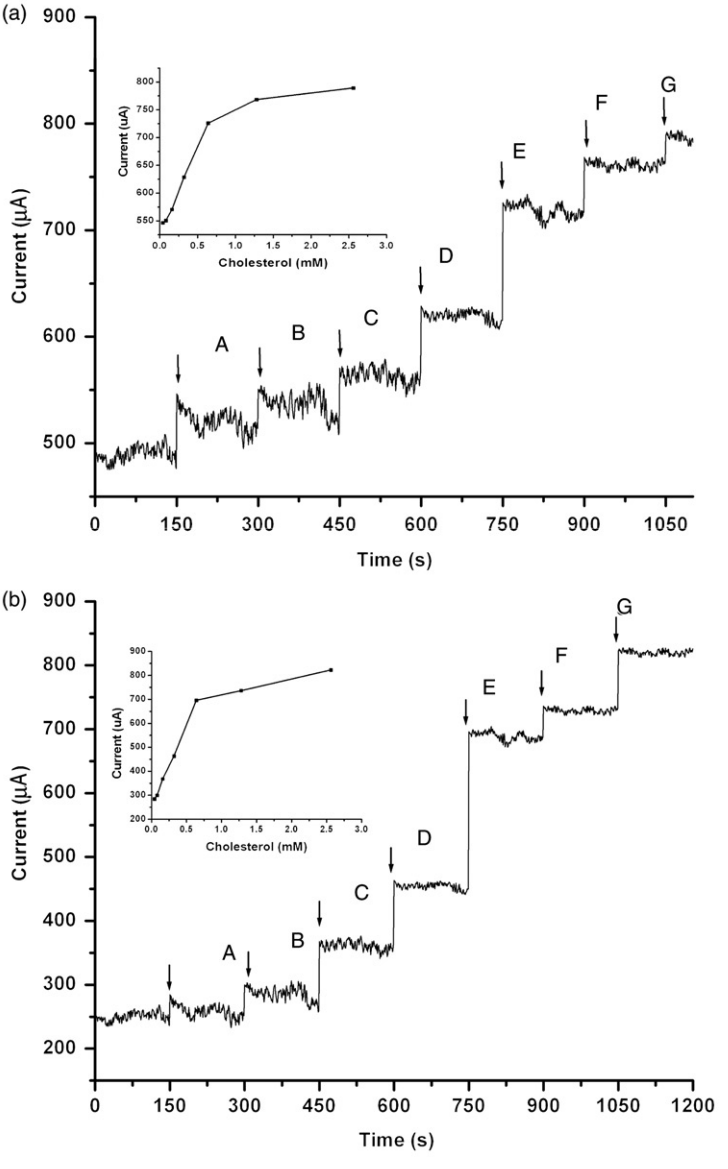

Figure 3. Chronoamperometric current responses of (a) Au–Nf–MWCNTs–ChOx–Nf and (b) Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf bioelectrodes for successive addition of (A) 0.04 mM, (B) 0.08 mM, (C) 0.16 mM, (D) 0.32 mM, (E) 0.64 mM, (F) 1.28 mM and (G) 2.56 mM cholesterol.
Note: Inset of both (a) and (b) shows calibration plots for the respective bioelectrodes.

as compared to the concentration used for this study, so interference from these compounds is negligible when the biosensor is used for analysing real serum samples. The optimum selectivity of the bioelectrode towards cholesterol may be attributed to two factors. Firstly, the working potential chosen is 0.21 V. At such a lower potential,

Table 1. Response characteristics of bioelectrodes.

	Bioelectrode	
Response characteristics	Au–Nf–MWCNTs –ChOx—Nf	Au–Nf–MWCNTs –ChOx–Nf –ChEt–Nf
Linear range	0.080–0.950 mM	0.080–0.645 mM
DL	0.025 mM	0.010 mM
Sensitivity	308.90 μA mM^{-1}	690.13 μA mM^{-1}
SD limit	2.676 μA	2.150 μA

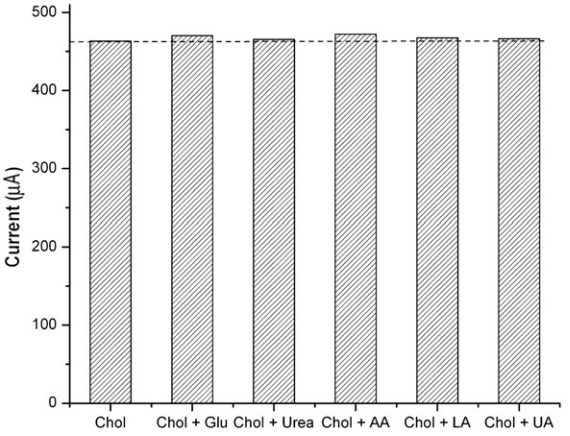

Figure 4. Effect of potential interfering agents on the Au–Nf–MWCNTs–ChOx–Nf–ChEt–NF bioelectrode response. Chol, cholesterol; AA, ascorbic acid; LA, lactic acid; and UA, uric acid.

the contribution of many interferents towards current response may get substantially minimised. Secondly, Nf polymer acts as a selective barrier for the restricted access of many interferents to the electrode surface by electrostatic repulsion [38,39]. The use of Nf polymer has been reported earlier also to decrease the interferences caused by compounds present in biological samples [45–47]. Based on these results, the fabricated bioelectrode can be proposed for use in physiological conditions.

The repeatability or operational stability of both the fabricated bioelectrodes was investigated from 16 successive measurements with 0.33 mM cholesterol during a period of 4 h. No change in the current response was observed till the last measurement. This high operational stability indicates that there was no enzyme leakage from the bioelectrodes. The fabrication reproducibility of both the bioelectrodes was estimated from the response to 0.33 mM cholesterol at three bioelectrodes prepared by the same procedure. The results showed an acceptable reproducibility with a relative SD of 2.6% for Au–Nf–MWCNTs–ChOx–Nf and 2.1% for Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf bioelectrodes. The storage stability of both the bioelectrodes was checked by carrying out voltammetric

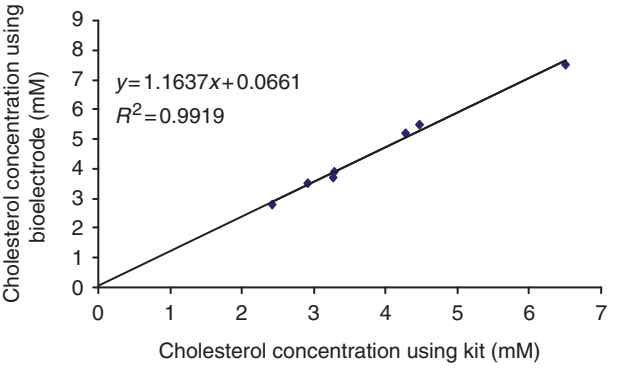

Figure 5. Correlation between the total cholesterol values determined by the enzymatic cholesterol kit and the present method.

measurements at the regular intervals of three days and it has been found that these electrodes retain about 90% of the original response even after three weeks when stored in PBS (pH 7) at 4°C. Such an excellent stability can be ascribed to the Nf–MWCNTs matrix which provides a suitable microenvironment for enzyme entrapment and also prevent enzyme from leaching.

3.5. *Real sample analysis*

For practical applications, the analysis of total cholesterol in serum samples is important. The practical usability of the fabricated Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf bioelectrode was judged by the determination of total cholesterol content in human serum samples. Five serum samples were first analysed using enzymatic cholesterol estimation kit (CHOD-PAP method) using three enzymes, ChEt, ChOx and peroxidase, and the chromogenic reagents p-hydroxybenzenesulphonate and 4-aminoantipyrine. Then, the same five serum samples were analysed using the Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf bioelectrode by diluting the samples with a dilution factor of 30 to make the concentrations fall in the linear range of the bioelectrode. The current response against each serum sample at 0.21 V was measured. The cholesterol concentration was then determined by interpolation on the linear region of the calibration curve (Figure 3(b) inset). The values obtained with the bioelectrode were compared graphically with those obtained with the enzymatic cholesterol estimation kit as shown in Figure 5. A straight line was observed with a correlation coefficient of 0.9919 and a regression equation of bioelectrode response = 1.1637× Kit response + 0.0661. These results imply a reasonable agreement between both the methods of analysis for serum cholesterol, and verify the reliability of the responses obtained using the Au–Nf–MWCNTs–ChOx–Nf–ChEt–Nf bioelectrode.

4. Conclusions

In this study, a bioelectrode was fabricated for total cholesterol estimation in serum by entrapping ChOx and ChEt onto Nf–MWCNTs hybrid matrix deposited on an Au electrode. MWCNTs in combination with Nf are used for the first time as the matrix for

the immobilisation of ChOx and ChEt for a total cholesterol estimation in human serum. It is found that the granular morphology of Nf–MWCNTs provides a better biocompatible environment for the enzyme and the MWCNTs provide an enhanced electronic communication between the ChOx and Au electrode for a highly sensitive and selective estimation of cholesterol. The bioelectrode exhibited good response characteristics with a proper linearity, good sensitivity, high selectivity and high operational and storage stability. Further investigation on the exact mechanism of electronic communication between the redox centre of the ChOx and the Nf-MWCNTs matrix may provide useful information for optimising the fabrication procedure to develop a bioelectrode of commercial importance.

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