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An electrochemical biosensor for 3-hydroxybutyrate detection based on screen-printed electrode modified by coenzyme functionalized carbon nanotubes

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Abstract 3-Hydroxybutyrate, one of the main blood ketone bodies, has been considered as a critical indicator for diagnosis of diabetic ketoacidosis. Biosensors designed for detection of 3-hydroxybutyrate with advantages of precision, easiness and speedy performance have attracted increasing attention. This study attempted to develop a 3-hydroxybutyrate dehydrogenase-based biosensor in which single-walled carbon nanotubes (SWCNT) was used in order to immobilize the cofactor, NAD^+, on the surface of screen-printed electrode. The formation of NAD^+–SWCNT conjugates was assessed by electrochemistry and electron microscopy. Cyclic voltammetry was used to analyze the performance of this biosensor electrochemically. The considerable shelf life and reliability of the proposed biosensor to analyze real sample was confirmed by this method. The reduction in the over potential of electrochemical oxidation of NADH to −0.15 V can be mentioned as a prominent feature of this biosensor. This biosensor can detect 3-hydroxybutyrate in the linear range of 0.01–0.1 mM with the low detection limit of 0.009 mM. Simultaneous application of screen-printed electrode and SWCNT has made the biosensor distinguished which can open new prospects for detection of other clinically significant metabolites.

Keywords Biosensor · 3-Hydroxybutyrate · Single walled carbon nanotubes · Screen-printed electrode

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Introduction

Biosensors, especially electrochemical biosensors, have received substantial attention because of their capability of rapid online analysis and high sensitivity. The specific properties of biological components such as enzymes and proteins for recognition and the advantages of electrochemical methods have drawn considerable interests in the development of electrochemical biosensors [1]. The application of biosensors in medicine was coincided with the development of an oxygen electrode for detection of glucose in 1960s [2]. Since then many efforts have been performed to develop different types of biosensors in order to manage diseases [3]. In recent years, the control of glucose and ketone bodies in diabetic patients has been the main purpose of biosensing technology. Blood ketone testing can be considered as a useful way to distinguish between simple hyperglycaemia and the potentially life-threatening ketoacidosis. The levels of 3-hydroxybutyrate (3-HB), one of the major ketone compounds in blood are defined between 1 and 3 mM in hyperglycemia and above 3 mM in diabetic ketoacidosis (DKA), while healthy individuals are recognized by 3-HB concentration below 1 mM. Furthermore, the ratio between 3-HB and acetoacetate—another major ketone body in blood—becomes as high as 10:1 in DKA. Therefore, rapid determination of 3-HB is of great importance for the management of DKA [4, 5].

Conventional methods for detection of 3-HB including chromatography, isotopic and spectrophotometric assays are very time consuming requiring expensive instruments and experienced technicians [6–8]. Considering such drawbacks and the alluring advantages of biosensors in detection of different types of metabolites, growing attention has been attracted to biosensing technology. The amperometric biosensors which have been developed for

electrochemical detection of 3-HB mostly depend on the enzymatic reaction of 3-HB dehydrogenase [9–12]. Like other dehydrogenase-based biosensors, 3-HB biosensors commonly use pyridine-coenzyme [$NAD(P)^+$] as the electron acceptor. Such biosensors are based on the anodic detection of NADH which results in the depletion of oxygen in enzymatic reaction and prevents the interference of oxygen in detection steps [13, 14]. Detection of NADH oxidation on electrode surface was indirectly evaluated by integration of chemical electron transfer mediators with biosensing process. However, interference by electroactive substances and enzyme inhibition by mediator are among drawbacks of such indirect detection method [9, 11]. On the other hand, direct detection of NADH oxidation was performed through an iridium-modified sensor prototype [12]. However, this method required complicated procedure, equipped laboratories and professional staff. Effective regeneration of the cofactor NAD^+ from its reduced form, NADH, was assessed by development of a bienzyme biosensor. This biosensor was constructed by coimmobilization of hydroxybutyrate dehydrogenase with salicylate hydroxylase in front of a Clark electrode [10]. Since this method was based on the application of two enzymes, the complexity and cost of system were increased. The inevitable loss in sensitivity of system caused by enzyme instability over the time can be considered as another disadvantage of such system.

Much effort has been devoted to the development of bioanalytical devices modified by different types of nanomaterials [15]. In electrochemical biosensors, the integration of nanomaterials with bare carbon electrodes suffering from low conductivity can facilitate detection by increasing the sensitivity and stability of system [16–18]. There has been growing interest to improve performances of biosensors employing carbon nanotubes (CNTs) in detection of NADH and hydrogen peroxide. Low limit of detection and signal enhancement can be achieved by application of CNT on electrode surface providing high surface area, low overvoltage, and rapid electrode kinetics. However, the low solubility of CNT in solvents makes its widespread utilization confined. Several methods for covalent and non-covalent functionalization of CNT have been introduced in order to overcome such problem as well as to improve its electrochemical properties [19–21]. It is noteworthy that the functionalized CNT can also serve as a support for immobilization of biological components maintaining the higher stability of system [16, 22, 23].

In the present study, in order to create carboxylic acid groups, CNT was oxidized by acidic treatment as a widespread method for covalent functionalization. The covalent attachment of NAD^+ to CNT using the popular cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) [22] was performed to immobilize NAD^+ on the surface of screen-printed carbon electrode (SPCE). Afterwards, hydroxybutyrate dehydrogenase was deposited on the modified electrode. SPCE is a miniaturized sensor consisting of work, reference and auxiliary electrodes in a single chip. Low cost, reduced size and simplicity of use are among the advantages of such electrode manufactured by screen printing technology [24]. Moreover, the possibility of modification of electrode surface by nanoparticles, polymers and biomolecules is another prominent property of this type of sensor; makes it a good choice for biosensor fabrication in this study [25–28]. In order to detect 3-HB, SPCE was deployed along with single-walled carbon nanotubes (SWCNT) in our previous work [29], in which SWCNT was successfully used to facilitate electron transfer and reduce oxidation potential of NADH. Herein, as an additional function, SWCNT is employed to help the maintenance of NAD^+ through the covalent attachment to CNT molecules. As a result, the addition of NAD^+ is not required for each electrochemical test, and only the first drop of NAD^+ conjugated with SWCNT on work electrode surface would be sufficient for several electrochemical tests. Scheme 1 illustrates the sequential steps in fabrication of biosensors.

Materials and methods

Chemicals

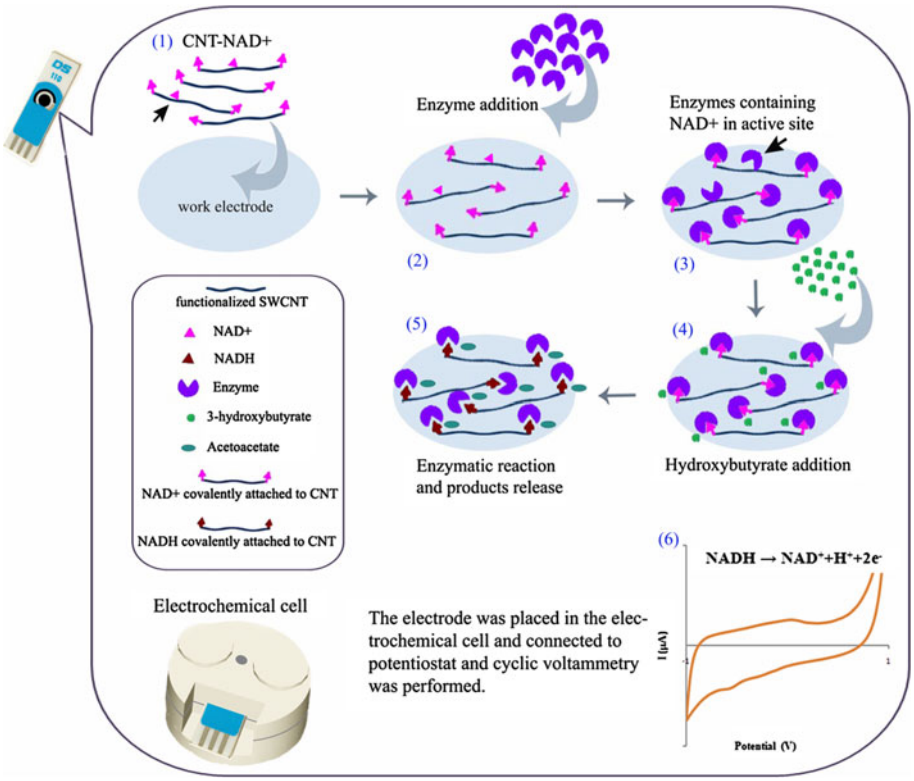
SWCNT with diameters of 1–2 nm were purchased from Neutrino. 3-HB dehydrogenase (400 U/mg), NAD^+, 3-HB, N-hydroxysuccinimide (NHS) and EDC were obtained from Sigma. All other employed chemicals were of analytical grade.

Preparation of functionalized SWCNTs

All details of functionalization of SWCNT have been provided in our previous report [29]. Briefly, the SWCNTs (5 mg) were sonicated in 10 ml of 65 % HNO_3 solution for 5 h at 40 °C. The content was centrifuged at 6,000 rpm for 10 min and repeatedly washed with distilled water and potassium phosphate buffer (pH 7). Washing was repeated till the pH of SWCNTs buffer remained stable at 7, then dried and stored until use. This procedure causes scission and carboxylation of SWCNTs.

Preparation of NAD^+–SWCNT conjugate

NAD^+–SWCNT conjugate was prepared by the formation of covalent bonds under the help of EDC and NHS. Briefly, functionalized SWCNTs were dispersed in dimethylformamide (2 mg/ml) and sonicated for 30 min at room temperature. 5 mg NHS and 1 mg EDC were added to 200 µl



Scheme 1 Schematic illustration of biosensor: CNT and NAD^+ were chemically attached to each other and coated on work electrode. Enzyme was deposited on electrode surface and addition of 3-hydroxybutyrate caused enzymatic reaction. Electrochemical measurement was performed by connection of electrode to potentiostat and is based on oxidation of NADH which causes a peak in voltammogram. *Arrows* on step 1 and 3 show the other possible attachments which are not favourable for biosensor development

SWCNT solution and stirred for 3 h in refrigerator. 300 µl of 30 mM NAD^+ solution was mixed with the mentioned solution and stirred for 20 h. In order to remove excess NAD^+, the solution was centrifuged in 13,200 rpm for 10 min at 4 °C and washed twice with phosphate buffer (pH 7.5). The NAD^+–SWCNT conjugates were stored in refrigerator while not in use.

Preparation of 3-HB biosensor

The biosensor was prepared by casting 4 µl of NAD^+–SWCNT conjugates dispersed in phosphate buffer (pH 7.5) on electrode surface. The resulting electrode was dried at 4 °C and then 1.2 U 3-HB dehydrogenase was totally coated onto NAD^+–SWCNT–SPE and kept at 4 °C in order to dry. To ensure the complete accessibility of NAD^+ for enzyme molecules, 2 µl of NAD^+–SWCNT was dropped again on enzyme layer.

Instrumentation and electrochemical measurements

Fourier-transformed infrared (FTIR) spectra was recorded with Thermo Nicolet Nexus 870 FTIR spectrophotometer and Eurosonic 4D was used for sonochemical functionalization of SWCNT. A computer controlled DropSens µ400 potentiostat and DropSens screen-printed electrodes including round-shaped graphite (GE) working electrode (3 mm diameter), an Ag/AgCl reference electrode and a graphite auxiliary electrode (DropSens, Spain) were used for electrochemical measurements and biosensor construction. The modified electrode was placed in a single Teflon-based electrochemical cell with 5 mm diameter (5–100 µl volume) in the sensing area (Scheme 1) and 50 µl of standard solution or real sample was dropped to cover the entire three-electrode zone of the electrode for electrochemical study. Potassium phosphate buffer (pH 7.5, 0.1 M) was used for recording background response. Cyclic voltammetry was performed without further addition of NAD^+ solution in potential range of 1 to −1 V and the scan rate of 50 mV/s. All the electrochemical measurements were performed in triplicates and at room temperature.

The long term storage stability of biosensor was investigated with one concentration of 3-HB by cyclic voltammetry during 180 days and after each experiment, the sensors were washed with buffer solution and stored at 4 °C [30].

Results and discussion

Preparation of SWCNT–NAD^+ conjugate

Chemical modification of CNT to create reactive groups on its surface is a prerequisite for covalent immobilization of any molecule. Acidic treatment of CNTs as the most popular method for functionalization is being performed by various protocols including different combinations of

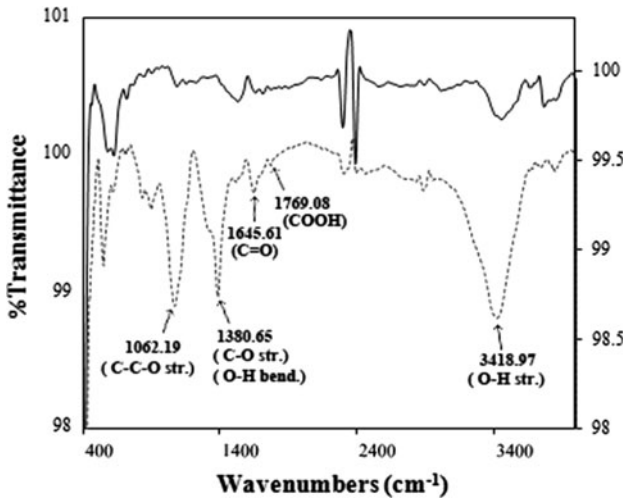


Fig. 1 FTIR spectra of single-walled carbon nanotubes before (*solid line*) and after functionalization (*dotted line*)

acidic solutions [31]. In this study, nitric acid was used for sonochemical treatment of SWCNTs. The FTIR spectra of SWCNTs treated with nitric acid (Fig. 1) exhibited bands at 3418, 1380 and 1645 cm^{-1}. The appearance of band in 1,645 cm^{-1} indicated the formation of C=O groups preferentially at the ends of the tubes. The bands existing at 3,418 and 1,380 can be attributed to the O–H stretching in carboxylic acid or alcoholic groups and the interaction of O–H bending and C–O stretching in phenol groups, respectively. The existence of the mentioned bands confirmed carboxyl functionalization of SWCNT which is necessary for covalent attachment of NAD^+ [32, 33].

Numerous advantages of electrochemical oxidation of NADH on CNT-modified electrodes including decreasing the overpotential of NADH oxidation and avoiding electrode surface fouling has led to wide application of CNT in dehydrogenase-based biosensors [34]. However, the high majority of these studies have just employed free form of NAD^+ in solution. The reports corresponding to direct attachment of NAD^+ to CNT are very rare and involve non-covalent attachment of NAD^+ to multi-walled carbon nanotubes (MWCNT) by Zhou et al. [35] and another recent study in which NAD^+ was covalently attached to MWCNT by the formation of amide bonds under the help of EDC and NHS [36]. The latter study benefitted from the advantages of coenzyme functionalized materials. Accordingly, we used this method to develop the NAD^+–SWCNT conjugates and overcome not only the problem of high overpotential of NADH oxidation, but also the costs resulting from the excess use of NAD^+. After acidic treatment, the SWCNTs have many functional groups such as –COOH and –OH and they have been used for immobilization [37, 38]. According to recent electrochemical studies, SWCNTs have shown to be capable to promote certain types of electron-transfer reactions as well as to improve electrocatalytic activity.

The shape and high surface area of SWCNTs introduce beneficial effect in terms of branched electrical conductivity coupled with increased electrode surface area. After purification, the SWCNTs were negatively charged [39]. Therefore, they can be easily assembled on the surface of positively charged NAD^+. Given strong adsorptive ability of SWCNTs, the biomolecules can be stably adsorbed on their surface.

Electron microscopy

The morphology of functionalized SWCNT and functionalized SWCNT in conjugation with NAD^+ (SWCNT–NAD^+) were characterized by SEM (Fig. 2). The SEM images indicate that after introduction of NAD^+ molecules, SWCNT exhibits much rougher surface and larger diameter as the clear results of modification of the tubes. The application of EDC for coupling of CNT with other molecules can result in aggregation of tubes as it was discussed previously [40]. The apparent aggregation of SWCNT–NAD^+ suggests that EDC serves as a coupling agent, which can facilitate the attachment of NAD^+ to CNT.

Towards electrochemical biosensing

As shown in Scheme 1, the detecting enzyme, 3-HB dehydrogenase, was dropped on the surface of SWCNT–NAD^+ modified electrode to develop a 3-HB biosensor. We expected a large number of efficient linkages, despite the fact that the physical attachment of NAD^+ to CNT molecules (step 1) and unfavorable attachment of enzyme to SWCNT–NAD^+ conjugates (step 3) were inevitable. In addition to SWCNT–NAD^+ conjugates, employing screen-printed electrodes is another strong point which makes this biosensor economically beneficial. In comparison to other types of electrodes, particularly oxygen electrodes, screen-printed electrodes are easy to use and stable over a long time. This provides the possibility of using a single electrode for several tests.

Figure 3a, shows the cyclic voltammograms of 3 mM 3-HB in the buffer solution, using the bare GE (curve a) and GE–SWCNT (curve c) electrodes, respectively. After the consecutive application of a triangular potential program in between -1, 1 V, no detectable accumulation of 3-HB on the bare GE electrode has been observed (curve b). However, using GE–SWCNT electrode, 3-HB represented two well-defined peaks in the voltammogram with a higher current (curve d). The current of the redox couple of 3-HB dramatically increased, indicating that the SWCNT immobilization on GE electrode can enrich the interaction with variation of 3-HB concentrations.

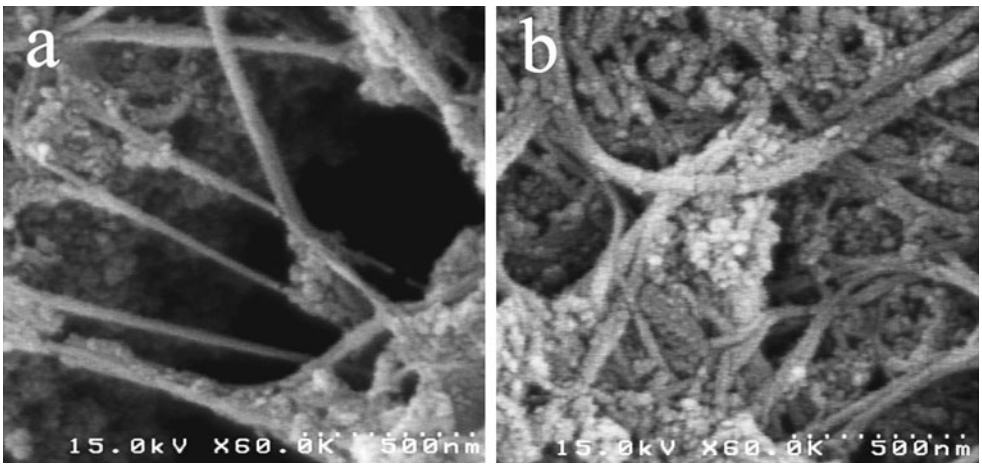


Fig. 2 **a** SEM images of functionalized single-walled carbon nanotubes and **b** functionalized single-walled carbon nanotubes in conjugation with NAD^+. Conditions: accelerating voltage 15 kV, magnification ×60,000

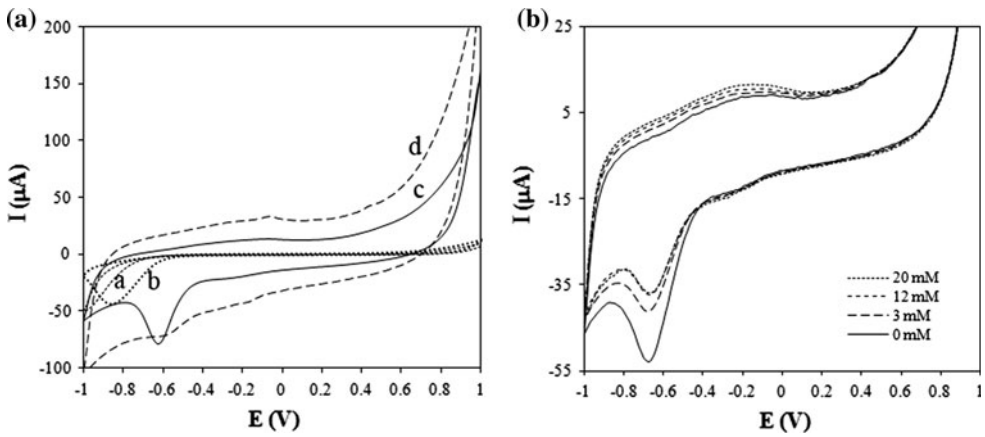


Fig. 3 **a** Cyclic voltammetry of 3-HB on bare GE and CNT/GE. *Curve a* and *b* corresponds to cyclic voltammetry of bare GE in absence and presence of 3-HB, respectively, while *curve c* and *d* show voltammograms of CNT/GE in absence and presence of 3-HB, respectively. **b** Cyclic voltammograms for the biosensor in 0.1 M phosphate buffer (pH 7.5) containing different concentrations of 3-hydroxybutyrate (0, 3, 12 and 20 mM). Scan rate 50 mV s^{-1}

Cyclic voltammetry of this biosensor in the presence of different concentrations of 3-HB confirmed the efficiency of linkages and good performance of biosensor (Fig. 3b). The reduction of coenzyme occurred in the cathodic half cycle with a current peak position at about −0.65 mV. The current of peak at −0.15 V, which corresponds to the potential of NADH oxidation, increased with the concentration of 3-HB in solution due to the formation of higher amount of NADH as the result of enzymatic reaction. To reduce the high potential of NADH oxidation in dehydrogenase-based biosensors, different electrode types and a wide range of electron transfer mediators were examined. The overpotential of NADH oxidation varies, depending on the type of electrode and modifications introduced on the surface of electrode [34]. While the NADH oxidation potential with an unmodified glassy carbon electrode was reported to be +0.82 V, the oxidation peak was shifted to +0.33 and +0.36 V by modification of electrode with multi-walled and single-walled CNT, respectively [41]. A decrease in the oxidation potential of NADH to −0.07 V was reported recently in a study of CNT functionalization for NADH detection employing carbon electrode and Ag/AgCl/3 M NaCl as the working and the reference electrode, respectively [42]. The potential of NADH oxidation has been reduced in different studies using other modifications on electrode surface demanding complicated processes and equipped laboratories.

According to cyclic voltammograms depicted in Fig. 3b, the concentration of NADH must be enriched on the GE–SWCNT electrode surface with respect to the lower concentration of 3-HB in solution. The diffusion coefficient of NADH must be decreased in the case of using GE–SWCNT electrode, because the (C. $D^{1/2}$) product remains the same. Considering the fact that mass transfer process should not depend on the target surface, it can be deduced that the mass transport process by diffusion is occurred in the thin layer of swollen SWCNT on the electrode surface, which has higher viscosity with respect to the bulk of the solution. It means that due to the interaction of SWCNT with NADH, the concentration of NADH is increased on the SWCNT layer/solution interface, and NADH diffuses through the SWCNT layer intercalating directly next to the

GE surface. Therefore, it can be concluded that the adsorbed SWCNT on the GE electrode surface most probably cannot act as an electron transfer mediator. Hence, because of the adsorption of SWCNT through the interaction of exposed base and/or external COOH groups, it is randomly adsorbed on the surface influenced by the length of the GE surface.

The shift in the potential of oxidation peak indicates that compared with the oxidized form of coenzyme (NAD^+), the reduced form (NADH) interacts more strongly with SWCNT. Since the peak potential separation of redox transition of NADH is not altered using GE–SWCNT electrode, the heterogeneous charge transfer kinetics do not change on the modified surface. Considering the intercalative nature of 3-HB as well as the more positive value of E^0_{Surf} compared with the value of E^0_{bulk}, intercalative attractions and stacking interactions of 3-HB between the base pairs of SWCNT can overcome the electrostatic attractions in long term [12].

To obtain the final concentrations of 0–3 mM, the serial concentrations of standard solutions were prepared by diluting the known concentrations of 3-HB with normal serum samples. The calibration curve for 3-HB concentrations can be observed in Fig. 4 in which the curve has a linear increasing trend from 0.01 to 0.1 mM. The high sensitivity for detection of low concentrations of 3-HB can be considered as an advantage of this biosensor. The detection limit of biosensor was calculated through the equation of $3S_b/k$, while k is the sensitivity of biosensor and S_b is the standard deviation of sequential blank measurements using buffer solution with no 3-HB [35]. Through biosensor response to eight blank samples, the detection limit was calculated to be 0.009 mM (84.15, 84, 83.97, 83.77, 83.56, 83.12, 82.9 and 82.61 μA). This would be quite appropriate for electrochemical measurements considering previous 3-HB biosensors with detection limits of 3.9 and 14 μM [10, 11].

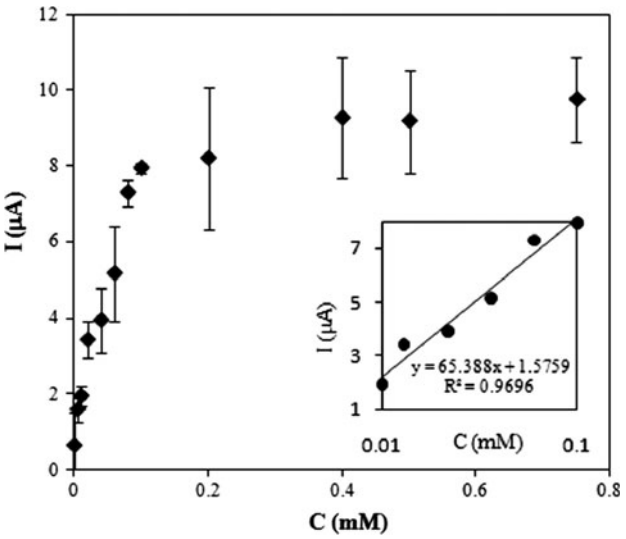


Fig. 4 Calibration curve of biosensor in presence of different concentrations of 3-hydroxybutyrate (0.01–0.1 mM). *Inset* shows the linear range of 3-HB detection (0.01–0.1 mM)

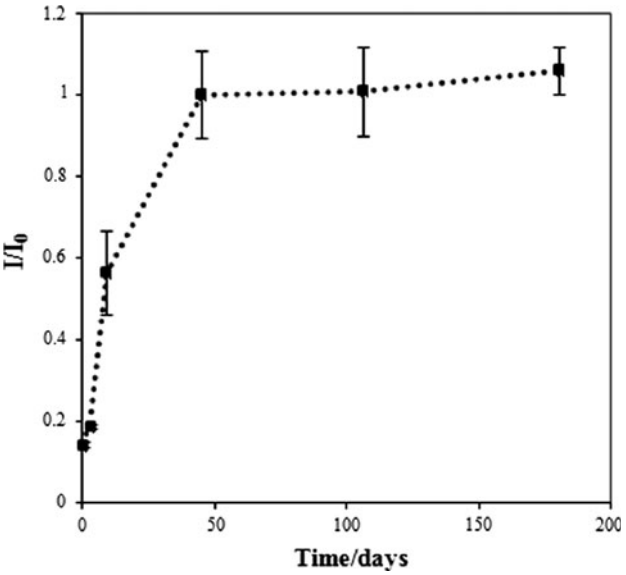


Fig. 5 Long term storage stability of the studied biosensor. Passing of time has positive effect on biosensor reaching from the relative current of 0.2 to 1 in approximately 40 days. The current obtained at day 40 was considered as I_0 (maximum response)

Stability and reproducibility of biosensor

Figure 5 illustrates the long term stability of this biosensor after storage for a period of 180 days. Furthermore, this graph can be considered as an evidence for the stability of SWCNT–NAD^+ attachments. Following an increase in the response of biosensor until the day 40, a stable trend can be seen in biosensor response. The proposed biosensor with significant long-term storage stability can be used for more than 6 months without any considerable loss of sensitivity. Regardless of a biosensor designed by Forrow et al. [9] with the shelf life of 18 months, other developed 3-HB biosensors could be used for <2 months and could not be considered for long term use.

To investigate the reproducibility of this biosensor, electrochemical response was measured at low (0.1 mM) and high (3 mM) concentrations of 3-HB in eight independent analytical runs. This biosensor with percent coefficient of variation (%CV) of 4.75, 7.62 %, respectively for the two mentioned concentrations of 3-HB displayed a good reproducibility for determination of 3-HB.

In order to evaluate the reliability of this biosensor, the concentration of 3-HB in 5 serum samples diluted by phosphate buffer (20 times) were detected through the proposed

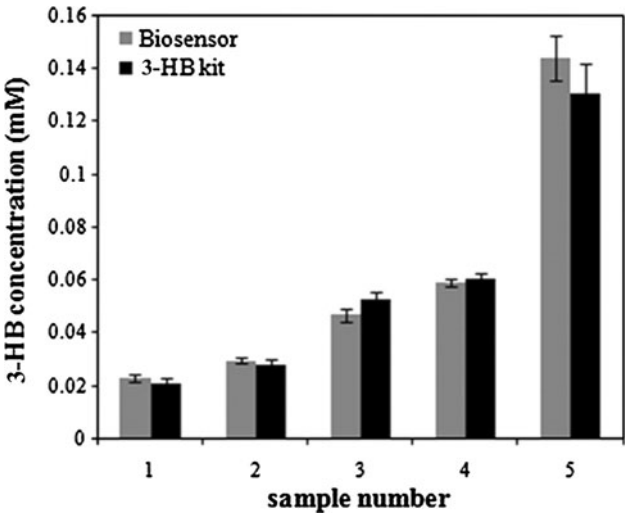


Fig. 6 Comparison of measured 3-HB concentration by β-hydroxybutyrate assay kit with 3-HB biosensor in different samples. The compatibility of the response of β-hydroxybutyrate assay kit with those of our biosensor revealed the good performance of them for analytical measurements of serum 3-HB. The amount of serum used in cell was 2.5 µl in overall volume of 50 µl. Thus, the concentration of 3-HB obtained from biosensor measurement should be multiplied by 20

biosensor and BioVision β-hydroxybutyrate assay kit (Fig. 6). Comparing the results revealed that the two mentioned methods were in good agreement and the performance of this biosensor in real samples can be acceptable.

Specificity of biosensor

Since the presence of interfering compounds can affect the precise determination of target analyte, the specificity of the present biosensor was assessed in the absence and presence of some possible natural interfering substances such as ascorbic acid, uric acid and a mixture of L-amino acids. The response to blank sample containing L-amino acids was fairly the same as what observed in their absence. Although the existence of a mixture of 0.5 mM L-amino acids in different 3-HB concentrations caused a fluctuation of 1–2.5 %, it can be ignored due to its slight effect on final response. The interference of ascorbic acid (0.1 mM) and uric acid (0.5 mM) with different concentrations of 3-HB was studied separately. The response increased by 17 % in blank test as well as different concentrations of 3-HB. Considering the fact that the existence of these acids in 3-HB sample can cause some perturbation in final results, further studies should be carried out regarding such problem.

Conclusion

In current work, an electrochemical biosensor was developed for detection of 3-HB. The proposed biosensor benefitted from the electrochemical advantages of single-walled CNT. Lower potential of NADH oxidation, higher storage stability and lowering the detection limit were achieved through application of such nanotubes. By taking the advantage of covalent linkage of NAD^+ to CNT, further addition of NAD^+ to reaction cell is not required in each measurement, which can be economically considerable. The agreement of the results revealed by this biosensor with a 3-HB detection kit confirmed its great potential for diagnostic purposes.

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