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Flow Injection Phosphate Biosensor Based on PyOx-MWCNTs Film on a Glassy Carbon Electrode Using FFT Continuous Cyclic Voltammetry

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A new flow injection enzymatic biosensor was designed for selective determination of phosphate ions. Pyruvate oxidase (PyOX) and multiwall cabon nanotubes (MWCNTs) were immobilized on the surface of a glassy carbon electrode by the nafion polymeric layer. The measurement method was based on fast Fourier transformation continuous cyclic voltammetry (FFTCCV) in which the charge under the peak calculated in a specific potential range. The characterization of the biosensor was studied by scaning electron microscopy (SEM) method. Experimental parameters affect the sensitivity of the biosensors, such as amounts of the cofactors, pH, and the pyruvate concentration, were optimized. Also the effect of scan rate, solution flow rate and buffer pH were studied. The results show that, the linear response range of the proposed biosensor was 1 to 100 µM and the detection limit was calculated 0.1 µM at a signal-to-noise ratio of 3. The biosensor showed good reproducibility and selectivity towards the other interfering anions. The long-term storage stability of the phosphate biosensor was studied.

Keywords: phosphate, biosensor, multiwall carbon nanotube, FFT Cyclic voltammetry, pyruvate oxidase

1. INTRODUCTION

In biological systems, phosphorus is found as a free phosphate ion in the solutions and is called inorganic phosphate (Pi), to distinguish it from phosphates bound in various phosphate esters. At

physiological pH (7.4), it primarily consists of a mixture of HPO_4^{2-} and $H_2PO_4^-$ ions. However, phosphates are most commonly found in the form of adenosine phosphates, (AMP, ADP and ATP) and in DNA and RNA and can be released by the hydrolysis of ATP or ADP.

The determination of the inorganic phosphate ion is especially important in the field of environmental, clinical, and food analysis. Determination of Pi concentration in body fluids is essential for the diagnosis of hyperparathyroidism, Vitamin D deficiency, and fanconi syndrome [1]. Hence, the need for a selective and sensitive method to determine the low level concentration of Pi in water and even in a single cell is obvious.

Multiwall carbon nanotubes (MWCNTs) can be used as a suitable intermediates between electrodes and enzymes. Recently, multiwall carbon nanotubes have been used in various areas of electrochemistry including biosensors, due to their high surface area, high surface/volume ratio, good electrical conductivity and significant mechanical strength [2-4]. MWCNTs in a suspension individually can be cytotoxic but cytotoxicity can be avoided by immobilizing on surfaces or within composites [5,6]. Hence, in the forms of paste, composite, or films, they cannot be toxic.

In order to prevent loss of the enzyme molecules and to improve the anti interfering ability of a biosensor, nafion films have been used widely in construction of biosensors. Nafion, due to its easy fabrication, good electrical conductivity, high chemical stability and good biocompatibility, has been used as a protective coating material for enzyme immobilization.

This work introduces a new flow injection electrochemical biosensor for determination of phosphate ions combine with fast Fourier transform continuous cyclic voltammetry (FFTCCV) technique [7-11]. To the best of our knowledge, this is the first application of FFTCCV method for phosphate ions biosensor based on enzyme (PyOx). In addition, the fabrication, characterization and analytical performance of the biosensor based on PyOx immobilized onto a glassy carbon (GC) by MWCNTs and nafion was investigated.

The biosensor was used to detect enzymatically generated H_2O_2 in a phosphate solution according to the following reactions:

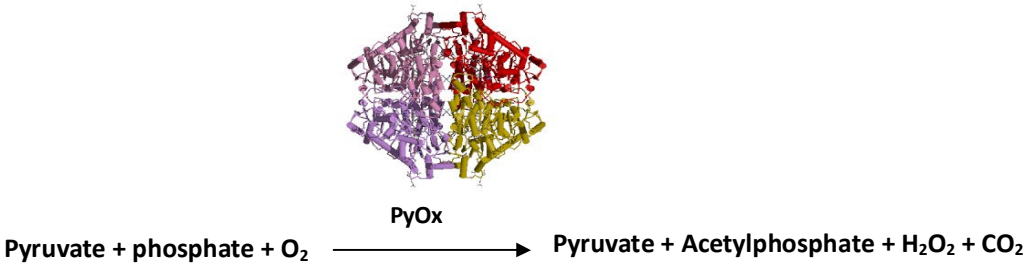


Figure 1. Schematic diagram of pyruvate oxidase (PyOx) enzymatic reaction

As shown in Fig. 1, the reaction is phosphate dependent. Thus, PyOx can be applied to inorganic phosphate analysis. The quantification of inorganic phosphate ions can be achieved via

electrochemical detection of the enzymatically liberated H_2O_2. The experimental parameters, which can affect the sensor performance, were optimized and the electrochemical characteristics of the sensor were described. The validity of the proposed phosphate biosensor was checked by measuring the amount of phosphate in a human serum sample.

2. MATERIALS AND METHODS

2.1. Reagents

Sodium dihydrogenphosphate, sodium pyruvate citric acid were obtained from Merck Co. Thiamine pyrophosphate chloride (TPP), flavin adenine dinucleotide (FAD), and N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid (HEPES) were all purchased from Sigma Co. Nafion (5 wt.%) solution in a mixture of lower aliphatic alcohols and water was obtained from Aldrich Co. Pyruvate oxidase (EC. 1.2.3.3, 100 units mg–l) from aerococcus species was also purchased from Sigma Co. MWCNTs, 10–20 nm were obtained from Shenzhen Nanotech Port Ltd. Co (Shengzhen, China). Doubly distilled water was used during the experiments.

2.2. Preparation of Nafion/PyOx -MWCNT/GC electrode

Glassy carbon electrodes (5 mm-diameter) were polished first with 1.0, and 0.05-μm alumina slurry. After rinsing the surface with doubly distilled water, they were sonicated in absolute ethanol and doubly distilled water for about 5 min, respectively.

Then a 20 μL suspension of PyOx and MWNT (v/v = 1:1) was dropped onto the surface of a cleaned glassy carbon electrode with a microsyringe and allowed to dry at ambient temperature. Finally, 1 μL of Nafion (5 wt.%) was casted and used as a net to hold the PyOx-MWCNTs on the electrode surface stably. The solvent was allowed to evaporate before use. The final electrode is taken as the Nafion/PyOx-MWCNTs/GC electrode. All resulting electrodes were stored at 4 °C when not in use.

2.3. Instrumentation

For the electrochemical FFTCV measurements a homemade potentiostat, that was connected to a PC PIV was used. The potentiostat was connected with an analog to digital (A/D) data acquisition board (PCL-818H, Advantech Co.). In the Electrochemical system, generating the analog waveform and acquiring current was by A/D board.

The potential waveform was repeatedly applied to the working electrode and then the data was acquired, and stored by the software. In the measurements, the data acquisition requirements electrochemical software was developed using Delphi 6.0. Also, in this electrochemical setup, the data could be processed and plotted in real time, or the stored data could be loaded and reanalyzed the voltammograms.

2.4. Flow Injection Setup

All of the flow injection analysis use for measurements, the equipment was integrated with an eight roller peristaltic pump (Ultratech Labs Co., Iran) and a four way injection valve (Supelco Rheodyne Model 5020) with 200 µL sample injection loop. The analyte solutions were introduced into the sample loop by means of a plastic syringe. The electrochemical cell used in flow injection analysis is shown in Fig. 2. A solution of pyruvate, TPP, FAD, and Mg^{2+} was used as eluent. 0.1 M citrate buffer solution was used to maintain the pH of the eluent 5.5.

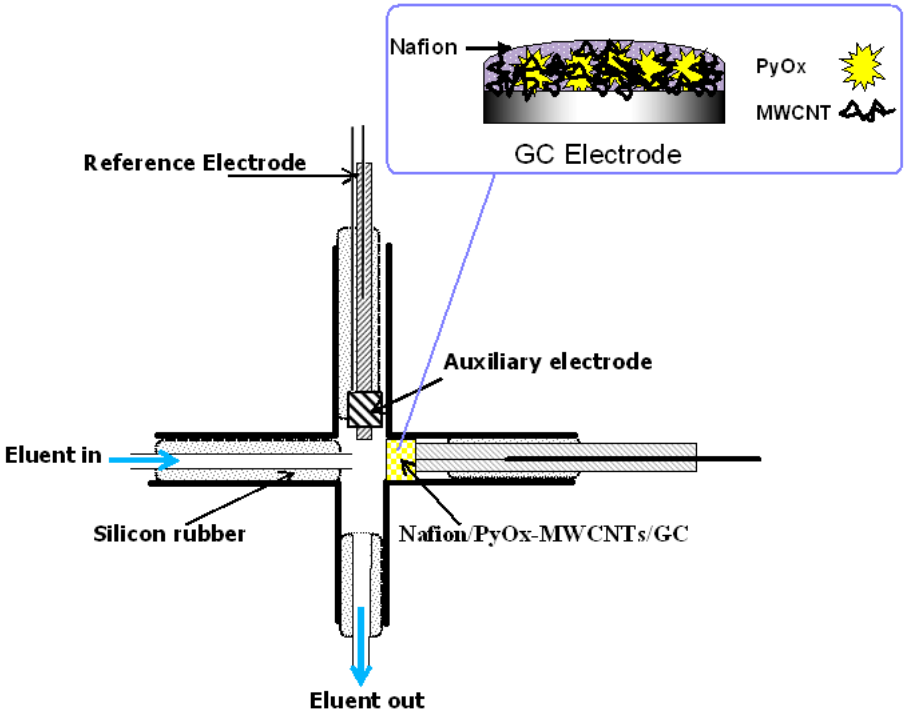


Figure 2. The diagram of phosphate ions biosensor and the electrochemical cell used in flow injection analysis

3. RESULTS AND DISCUSSION

In FFTCV measurement, the sensitivity of the detection is related to its physical morphology of surface of the biosensor electrode. Therefore in order to study the electrode, SEM of the electrode was prepared.

Fig. 3. shows the typical SEM images of Nafion/PyOx-MWCNTs/GC electrode surface.

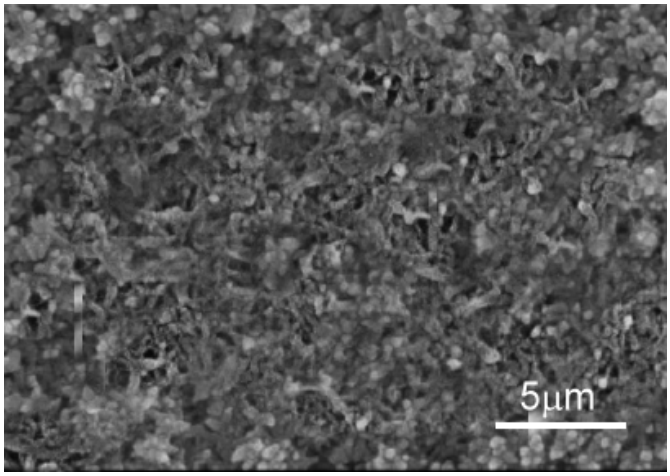


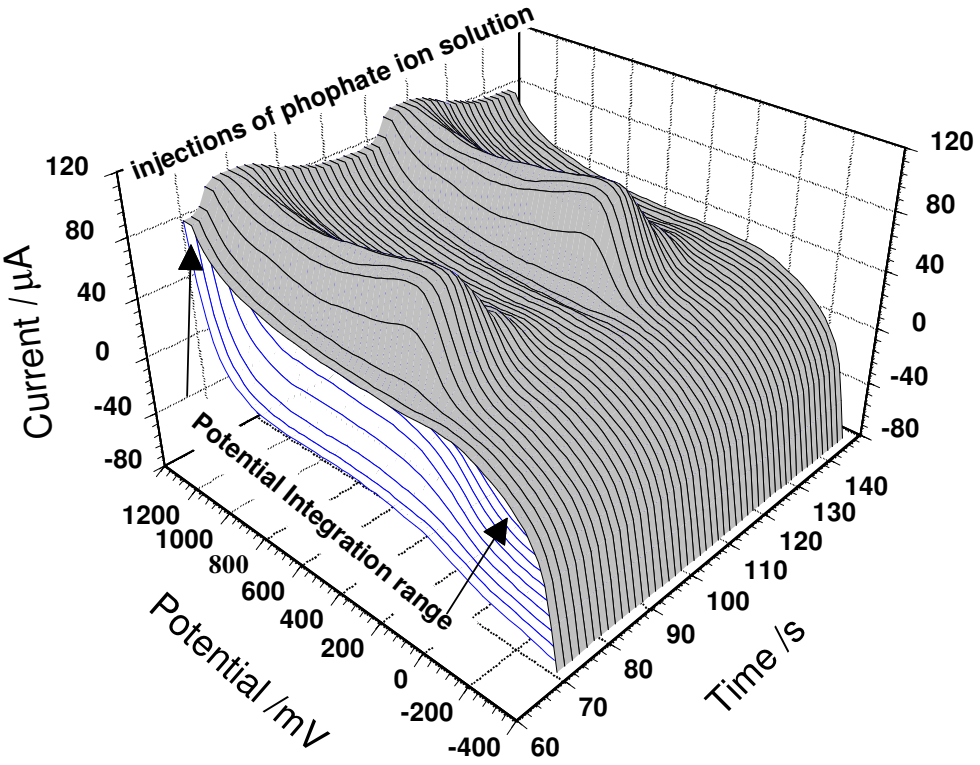
Figure 3. SEM image of the electrode PyOx-MWCNT/GC

For the measurements of phosphate ion, a FIA system was design and 200 μL of various concentration of phosphate ion were injected into the system. Fig. 4a demonstrates typical FFT cyclic voltammograms of the PyOx-MWCNTs/GC electrode in the potential range of -400 to 1400 mV at potential sweep rate is 6 Vs^{-1}. In the three dimensional graph, the time axis represents the time passing between the beginning of the flow injection experiment and the beginning of a particular sweep (i.e. it represents a quantity proportional to the sweep number) [14-17]. In Fig. 4b, the typical FIA response based on signal processing, in which the biosensor signal is calculated based on the background subtraction and current integration at the range 0-1200 mV is shown.

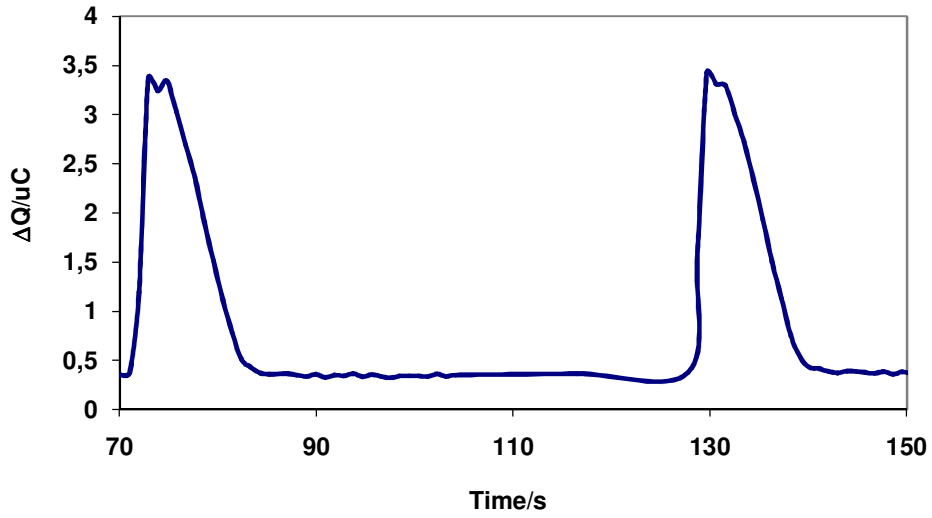
The figure shows that before injection (in absent of phosphate ions) there is no significant changes in the voltammograms, but by injection of 200 μL of 1.0×10^{-4} M phosphate ions in 20 mM HEPES buffer solution buffer at pH=5.5, a signal appears at potential 400 mV. Injection of phosphate ions sample into flowing eleuent, a well-defined catalytic peak was developed as a consequence of the PyOx catalytic oxidation of phosphate ions, and the electrocatalytic current increases with the increase of the concentration of phosphate ions in 20 mM HEPES buffer solution. The increase in the current of the electrode at potential 400 mV is due to the production of H_2O_2 during the oxidation of phosphate ions at the biosensor surface, by PyOx enzyme. Nevertheless, it can be suggested that the attachment of the PyOx to high surface area of PyOx-MWCNTs/GC nanoparticles facilitates a higher rate of direct electron transfer between the active sites of immobilized PyOx, which, can increase the peak current at the recorded cyclic voltammograms when the sample was injected.

Once FFTCCV is used to monitor a flowing system, phosphate ions electrochemical processes will cause a measurable change in the peak current at the voltammograms. According to Fig. 1, the electrochemical reaction for the detection of phosphate ions in presence of PyOx is proposed and the enzymatic reaction which produce H_2O_2.

In this detection method the current passing through the electrode was sampled only during the potential ramp. This data processing operation was carried out simultaneously with data acquisition during flow injection experiments.



A



B

Figure 4. A) FFT cyclic voltamograms of the Nafion/PyOx-MWCNT/GC electrode without (in absent) and with injection of 200 µL of 1.0×10^{-4} M phosphate ions in 20 mM HEPES at pH=5.5 in the potential range of -400 to 1400 mV at 6 V/s. and the potential Integration range for the current. B) the calculated response of the biosensor based on Eq.2.

The result of the integration is shown in Fig. 4b. The response was calculated as;

$$\Delta Q = Q - Q_0$$

where Q is the electrical charge obtained by integration of cyclic voltammetric curves between 0 and 1200 mV in the chatodic scan, and Q_0 represents Q in the absence of the adsorbent. The peaks in Fig. 4b is due to two consecutive injections of the same sample. The integration of net current changes is applied over the selected scanned potential range. In this method, ΔQ is calculated based on the all-current changes at the CV. A total absolute difference function (ΔQ) can be calculated by using the following equation:

$$\Delta Q\ (s\tau) = \Delta t\left[\sum_{E=E_i}^{E=E_f} \left| i(s,E) - i(s_r,E) \right|\right]$$

Where, s is the sweep number, τ is the time period between subsequent potential scan, Δt is the time difference between two subsequent points on the cyclic voltammograms, i (s, E) represents the current of the cyclic voltammograms recorded during the s-th scan and i (s_r, E) is the reference current of the cyclic voltammograms. E_i and E_f are the initial and the final potential, respectively, for integrating of current. This integration range for the current is shown in Fig. 3. The reference cyclic voltammogram was obtained by averaging a 5 to 10 cyclic voltammograms, recorded at the beginning of the experiment (i.e. before injection of the analyte). In addition, the results show that with increasing the concentration of phosphate ions in the injected sample, ΔQ increases. This confirms a fast electrocatalytic and electron exchange behavior of modified electrode at high potential sweep rates.

3.1. Optimizing the experimental parameters

In this method, sensitivity of the measurement mainly depends on the potential sweep rate and eluent solution flow rate, which is mainly due to kinetic factors of the electrode processes, and instrumental limitations [12-21]. From this point of view, it is necessary to exam, the sensitivity of the biosensor to the applied potential sweep rate. In this direction, the influence of the scan rates and the elunet flow rate on the sensitivity of the detector response, at scan rates (from 0.5 to 12 V/s) and the eluent flow rate (0.5 to 3 mL/min), were investigated. And the responses of the detector were recorded. The acquired results from injecting solutions of 1.0×10^{-4} M of phosphate ions are presented in Fig. 6.

As is show in the figure, the biosensor exhibits the maximum sensitivity (or signal) at 6 V/s of scan rate and 1.5 mL/min of the eluent flow rate. The effects of the sweep rate on the detection performance can be taken into consideration from three different characteristics; speed of data acquisition, kinetic factors of electrochemical processes at the electrode surface, and the flow rate of the eluent, which controls the time retention of the solution sample zone in the electrochemical cell.

The main reason for lower sensitivity of the biosensor at higher scan rates is limitation in the rate of electron transfer of electrochemical processes of PyOx with phosphate ions

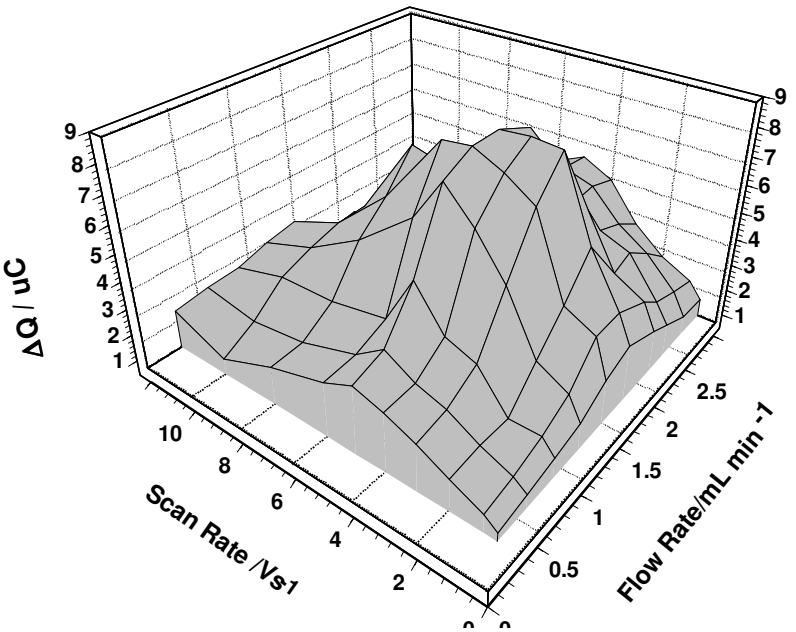


Figure 6. Effect of the sweep rate and effect of flow rate on the response of the Nafion/PyOx-MWCNTs modified GC electrode to injections of 1.0×10^{-4} M phosphate ions

3.2. Effect of pH value on the response current of the biosensor

Investigation of the effect of the pH value on the performance of the biosensor is of great importance, because the activity of the immobilized PyOx is pH dependent. The response current of the enzyme electrode increases in the pH range 5.0–7.0, the current response decreases and the background current increased at higher pH values. The signal/noise shows the maximum value at pH 5.5.

3.3. Calibration curve and biosensor characterization

Fig. 7 shows typical FFTCV response curves of in o.01 M CT at the biosensor electrodes. The regression equation is ΔQ (μC) = 0.0649 c (μM), + 0.0194 with R = 0.996. The ΔQ responses were obtained for standard solution of phosphate ions (from 1.0 to 1000.0 μM in 20 mM HEPES solution, pH=5.5). The results shown in this figure represent the integrated signal for 3 to 5 consecutive flow injections of the standard solution. A correlation coefficient of R=0.996 with %R.S.D. values ranging from 0.27–4.1% across the concentration range studied were obtained following linear regression analysis.

As mentioned above the electrode response could be expressed in various ways as peak heights or peak areas in the FFTCV. For this reason, the magnitude of the flow-injection response depends on the choice of the data processing methods.

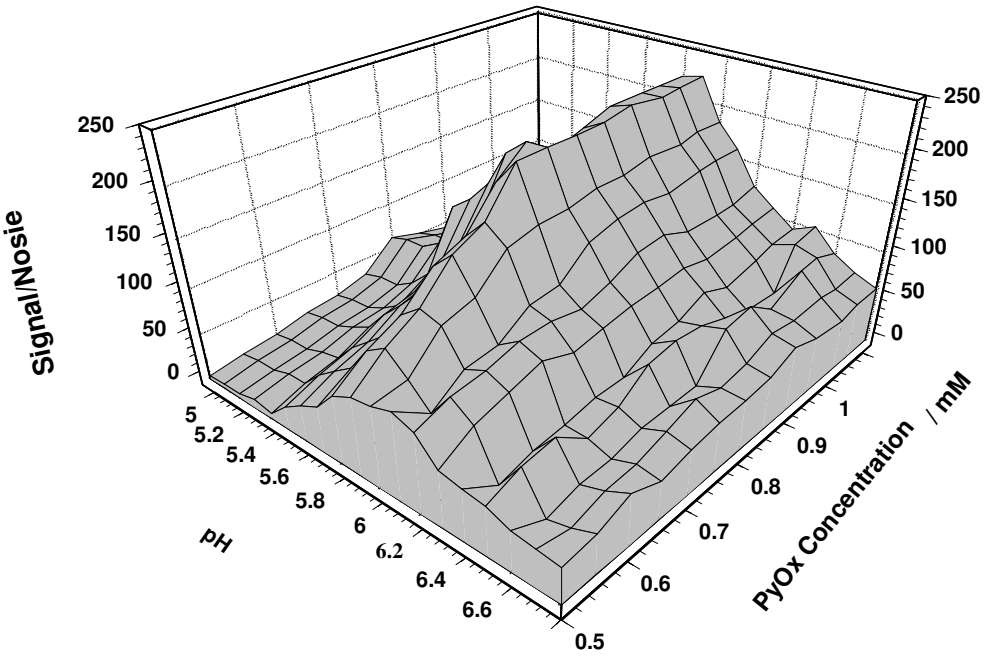


Figure 6. The effect of pH and PyOx concentration on response of the biosensor

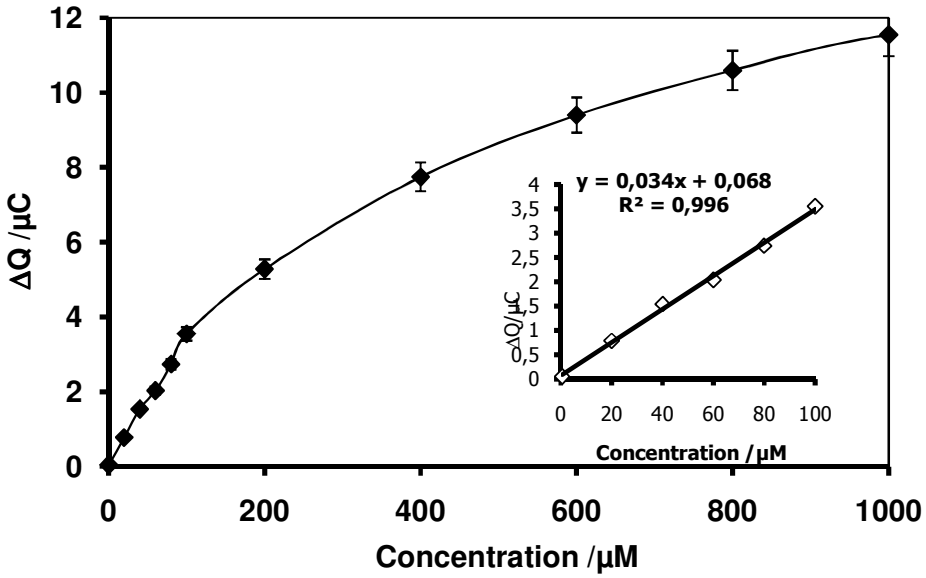


Figure 7. The calibration curve for phosphate ions determination

The linearity was evaluated by linear regression analysis, which calculated by the least square regression method. The detection limit, estimated based on signal to noise ratio (S/N=3), was found to be 0.10±0.01 μM. The long-term storage stability of the sensor was tested for 40 days. The sensitivity retained 93.7% of initial sensitivity up to 50 days which gradually decreases afterwards might be due to the loss of the catalytic activity.

4. CONCLUSIONS

In this work, a highly sensitive phosphate ions biosensor has been fabricated by modifying the GC electrode surface with PyOx/MWCNTs. To the best of our knowledge, the method is the first time that a very high-sensitivity and low detection limit flow injection analysis that is used for phosphate ions biosensors based on PyOx -MWCNTs/GC electrode. The sensitivity retained 93.7% of initial sensitivity up to 50 days which gradually decreases afterwards might be due to the loss of the catalytic activity. A good producible sensitivity, response time less than 10 s and detection limit of 0.1 μM was observed from the fabricated biosensor. The long-term storage stability of the sensor was tested for 40 days.

References

1. H. Kawasaki, K. Sato, J. Ogawa, Y. Hasegawa and H. Yuki, *Anal. Biochem.* 182 (1989) 366.
2. J. Zhong, L. Song, J. Meng, B. Gao, W.S. Chu, H.Y. Xu, Y. Luo, J.H. Guo, A. Marcelli, S.S. Xie and Z.Y. Wu, *Carbon* 47 (2009) 967.
3. M. Siswana, K.I. Ozoemena, T. Nyokong, *Sensors* 8 (2008) 5096.
4. P.M. Ajayan, *Chem. Rev.* 99 (1999) 1787.
5. M. A. Hussain, M. A. Kabir and A. K. Sood, *Curr. Sci.* 96 (2009) 664.
6. Z. G. Zhu, W. H. Song, K. Burugapalli, F. Moussy, Y. L. Li, and X. H. Zhong, *Nanotechnology* (2010) 21.
7. P. Norouzi, M. R. Ganjali, B. Larijani, A. Mirabi-Semnakolaii, F. S. Mirnaghi, and A. Mohammadi, *Pharmazie* 63 (2008) 633.
8. P. Norouzi, H. Rashedi, T. Mirzaei Garakani, R. Mirshafian and M. R. Ganjali, *Int. J. Electrochem. Sci.* 5 (2010) 377.
9. P. Daneshgar, P. Norouzi, F. Dousty, M. R. Ganjali, and A. A. Moosavi-Movahedi, *Curr. Pharm. Anal.* 5 (2009) 246.
10. P. Norouzi, M. R. Ganjali, S. Shirvani-Arani, and A. Mohammadi, *J. Pharm. Sci.* 95 (2007) 893.
11. M. R. Pourjavid, P. Norouzi, and M. R. Ganjali, *Int. J. Electrochem. Sci.* 4 (2009) 923.
12. P. Norouzi, M. R. Ganjali, M. Zare, and A. Mohammadi, *J. Pharm. Sci.* 96 (2007) 2009.
13. P. Norouzi, M. Qomi, A. Nemati, and M. R. Ganjali, *Int. J. Electrochem Sci.* 4 (2009) 1248.
14. P. Norouzi, B. Larijani, M. Ezoddin and M. R. Ganjali, *Mater. Sci. Eng. C* 28 (2008) 87.
15. M. R. Ganjali, P. Norouzi, R. Dinarvand, R. Farrokhi, and A. A. Moosavi-Movahedi, *Mater. Sci. Eng. C* 28 (2008) 1311.
16. P. Norouzi, M. R. Ganjali, and L. Hajiaghababaei, Anal. Lett., 39 (2006) 1941.
17. P. Norouzi, G. R. Nabi Bidhendi, M.R. Ganjali, A. Sepehri, M. Ghorbani, *Microchim. Acta,* 152 (2005) 123.
18. P. Norouzi, M. R. Ganjali, T. Alizadeh, and P. Daneshgar, *Electroanalysis*, 18 (2006) 947.

19. M. R. Ganjali, P. Norouzi, M. Ghorbani, and A. Sepehri, *Talanta*, 66 (2005) 1225.
20. M. R. Ganjali, P. Norouzi, S. Shirvani-Arani, and A. Mohammadi, *Russian J. Electrochem.* 44 (2008) 158.
21. M. R. Ganjali, P. Norouzi, and M. Zare, *Russian J. Electrochem.* 44 (2008) 1135.