Amperometric Biosensor based on Prussian Blue Nanoparticle-modified Screen Printed Electrode for Estimation of Glucose-6-phosphate

Suchanda Banerjee[a], Priyabrata Sarkar[a*], Anthony P.F. Turner[b]

[a] *Department of Polymer Science and Technology, University of Calcutta, 92 A.P.C. Road, Kolkata 700009, India*

[b] *Biosensors & Bioelectronics Centre, IFM-Linköping University, S-58183, Linköping, Sweden*

Short title: Amperometric Sensor for Glucose-6-phosphate

Subject Category: Special Topics

---

* Corresponding author. Tel.: 0091 3324852975 ; Fax.: 0091 33 24852976.

E-mail address: sarkarpriya@gmail.com (Priyabrata Sarkar)

ABSTRACT

Glucose-6-phosphate (G6P) plays an important role in carbohydrate metabolism of all living organisms. Compared to the conventional analytical methods available for estimation of G6P, the biosensors having relative simplicity, specificity, low-cost and fast response time are a promising alternative. We have reported a G6P biosensor based on screen-printed electrode utilizing Prussian Blue (PB) nanoparticles and enzymes, glucose-6-phosphate dehydrogenase and glutathione reductase. The PB nanoparticles acted as a mediator and thereby enhanced the rate of electron transfer in a bi-enzymatic reaction. The Fourier transform infrared spectroscopy and energy-dispersive X-ray spectroscopy study confirmed the formation of PB, whereas, the atomic forced microscopy revealed that PB nanoparticles were about 25-30 nm in diameter. Various optimization studies, such as pH, enzyme and cofactor loading, etc. were conducted to obtain maximum amperometric responses for G6P measurement. The developed G6P biosensor showed a broad linear response in the range of 0.01-1.25 mM with a detection limit of 2.3 μM and sensitivity of 63.3 μA/mM at a signal-to-noise ratio of 3 within 15 s at an applied working potential of -100 mV. The proposed G6P biosensor also exhibited good stability, excellent anti-interference ability and worked well for serum samples.

Keywords:

Glucose-6-phosphate

Prussian Blue nanoparticles

Screen-printed electrode

Amperometry

Introduction

Glucose-6-phosphate (G6P)[1] plays a major role in the carbohydrate metabolism of all living organisms. G6P participates in numerous catabolic pathways to yield adenosine triphosphate or nicotinamide adenine dinucleotide phosphate (NADPH). Monitoring of G6P concentration in blood or human tissue is particularly important since it can directly reflect the relative activity of several enzymes associated with numerous catabolic pathways, such as glucose-6-phosphate dehydrogenase (G6PDH), phosphoglucomutase, hexokinase, phosphoglucose isomerase, etc. G6P level in blood reflects onset of many diseases associated with G6PDH deficiency such as hemolytic anemia, neonatal jaundice, etc. It is also related to the regulations of few other enzymes, such as glycogen synthase, protein kinase, etc. [1-6]. The expected concentration of G6P in human serum generally varies in the range of 50 – 70 μM [1]. Therefore, highly sensitive and rapid methods are required for monitoring G6P level in human blood. The conventional analytical methods available for measurement of G6P concentration in blood mainly consist of radioactive, chromatographic and spectroscopic methods [1, 7, 8].

---

[1] *Abbreviations used:* G6P, glucose-6-phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; G6PDH, glucose-6-phosphate dehydrogenase; SPE, screen-printed electrode; $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate; GR, glutathione reductase; PB, Prussian Blue; H_2O_2, hydrogen peroxide; AFM, atomic forced microscopy; FTIR, Fourier transform infrared spectroscopy, EDX, energy-dispersive X-ray spectroscopy, UV–vis, ultaviolet-visible; WE, working electrode; w/v, weight by volume; v/v, volume by volume; % RSD, relative standard deviation.

However, these conventional methods are time consuming, require costly reagents, sample preparation and highly trained professionals. On the other hand biosensors exhibit high sensitivity, accuracy, rapid detection time and because of their low cost, could be employed as an alternative method for estimation of G6P [9].

Recently, several groups have reported development of electrochemical sensors for monitoring G6P concentration. Cui *et al.* have reported the development of an amperometric G6P biosensor by coimmobilization of *p*-hydroxybenzoate hydroxylase and G6PDH on a screen-printed electrode (SPE). [9]. Cui *et al.* have developed another bienzyme-based Clark-type electrode for determination of G6P using G6PDH and salicylate hydroxylase. The enzymes were entrapped on a Teflon membrane [10]. Tzang *et al.* have developed a voltammetric biosensor for estimation of G6P based on electrocatalytic oxidation of β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), using electropolymerized 3, 4-dihydroxybenzaldehyde modified glassy carbon electrode [11]. Aoki *et al*. have reported an amperometric biosensor for G6P using G6PDH from *B. stearothermophilus* immobilised on a porous platinum black electrode and the sensor was thermostable up to 60^oC [12]. Suye *et al.* have presented an amperometric biosensor using two enzymes, glutathione reductase (GR) and G6PDH on a chemically modified carbon electrode with oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) and polymerized mediator polyethyleneimine ferrocene [13]. Bassi *et al.* have proposed an amperometric carbon paste biosensor for G6P monitoring, which is based on entrapped Mg^{2+} ions, G6PDH, $NADP^+$ polyethylenimine and an electroactive mediator, tetracyanoquinodimethane. [14]

The applications of different nanoparticles have also played an important role in development of various electrochemical sensors. Nanoparticles have unique physical and chemical properties such as large surface-to-volume ratio, increased surface activity and catalytic efficiency that provide a platform for fabrication of novel electrochemical sensors [15, 16]. Prussian Blue (PB) nanoparticle is one such example that has been widely used in development of various biosensors [17]. PB is a typical metal hexacyanoferrate [18] with well-known electrochromic [19], electrochemical [20], photophysical [21], and magnetic properties [22]. PB is widely used in the field of electroanalytical chemistry and it is commonly known as "artificial enzyme peroxidase" as PB can catalyze electrochemical reduction of hydrogen peroxide (H_2O_2) at lower potential [23]. It has also been known as an effective mediator for carbon-based amperometric biosensors and possesses characteristics of an ideal mediator such as, low redox potential, enhance electron transfer rate and good stability [24]. PB modified working electrodes have been used for development of several biosensors such as glucose, ascorbic acid, catecholamine, cysteine, glutamate, lactate, cholesterol, galactose, acytelcholine, amino acid, alcohol, etc. [17]. Compared to bulk PB, PB nanoparticles play a major role in development of several more recent biosensors [17] because of their increased electrocatalytic effect arising from a large surface-to-volume ratio, high surface activity and fast electron transfer [25, 26].

The aim of this work was to develop an amperometric sensor for G6P measurement which possesses properties such as low cost, good stability, easy modification technique, rapid response time, low detection limit and excellent anti-interference ability against major blood components. Also, in this study, nanoparticles were used for the first time for biosensing of G6P concentration. The amperometric sensor for G6P measurement was fabricated by immobilizing

PB nanoparticles and two other enzymes, G6PDH and GR on the surface of SPE. Therefore, the G6P biosensor comprises of a bi-enzymatic reaction where electron transfer rate is usually slow. Hence, PB nanoparticle was used in this study to enhance the electrochemical response of the bi-enzymatic reaction by accelerating the electron transfer between the electrodes and the enzymes [27]. In this study, PB nanoparticles were synthesized by co-precipitation method and characterized by atomic forced microscopy (AFM), Fourier transform infrared spectroscopy (FTIR), energy-dispersive X-ray spectroscopy (EDX) and ultraviolet-visible (UV–vis) spectra. The major achievements in this study were high selectivity, low detection limit, good stability and fast amperometric responses to G6P. The modification of the sensor was simple, didn't require any sample preparation and the measurement could be done at ambient room temperature.

Materials and methods

Chemicals and Reagents

Rabbit serum was purchased from Himedia. Potassium ferricyanide ($K_3Fe(CN)_6$), G6P, GR (EC 1.8.1.7, from wheat grem), G6PDH (EC 1.1.1.49, from *Leuconostoc mesenteroides*) and $NADP^+$ were purchased from Sigma whereas H_2O_2, iron(III) chloride ($FeCl_3$), gelatin, gluteraldehyde and concentrated hydrochloric acid (HCl) were purchased from E-merck (India). For study of pH effect, 100 mM Tris-HCl buffer of pH 4-9 was used whereas for all other experiments, 100 mM Tris-HCl buffer of pH 7 (121.1 g of Tris base in 500 ml water and pH was adjusted by 100 mM HCl and then volume was adjusted to 1000 ml) was used. In all experiments,

18.2 MOhm Milli-Q (Millipore India Ltd.) water was used for preparing buffer and other reagents and kept in pre cleaned glass bottles.

Instrumentation

EDX studies were performed using Quanta 200 (FEI, USA). AFM studies were done using RTESPA silicon tip (VEECO, USA). A Cecil spectrophotometer (CE7200 CECIL) was used to record UV–vis spectra of PB nanoparticles in a wavelength range of 400–900 nm. FTIR spectra were recorded using IR 782 spectrometer (Perkin Elmer, USA).

All cyclic voltammetric measurements and amperometric experiments were carried out using AUTOLAB electrochemical analyzer (PGSTAT 12 Ecochemie B.V., Netherlands). The terminals of the working (WE), reference and counter electrodes of the AUTOLAB electrochemical analyzer were connected to the respective terminals of the disposable SPE system via standard connectors and all data processing and experimental controls were driven through the GPES 4.9 software installed on a computer interfaced with the electrochemical analyzer.

Disposable SPE system had a carbon WE, carbon CE and silver/silver chloride RE. SPEs were printed onto a 250 μm thick polyester sheet (Cadillac Plastic Ltd., Swindon, UK) using a DEK 248 screen-printing machine (DEK Printing Machines Ltd., Waymouth, U.K.) The detailed description of SPE system is given elsewhere [28]. A single disposable SPE system is for one-time use only.

Methodology

The proposed G6P biosensing system involves a bi-enzymatic reaction and therefore, modification of WE includes utilization of two enzymes, G6PDH and GR. Initially, in presence of G6P, enzyme G6PDH catalyzes the specific dehydrogenation of G6P consuming cofactor $NADP^+$ and produces 6-phosphogluconate and NADPH [13]. Again, in presence of GR, PB nanoparticles undergo redox reaction at WE surface and oxidize the product NADPH to $NADP^+$. The electron transfer rate is usually slow in a bi-enzymatic reaction mechanism. Therefore, to enhance the electron transfer rate between enzyme and electrodes, PB nanoparticle was used as a mediator. The PB nanoparticle also helps in regeneration of $NADP^+$ and lowers the redox potential, thereby increases the selectivity of the biosensor. This whole redox reaction involves electron transfer at the WE surface and thus, the response can be observed by amperometric measurements. The schematic diagram of the detection principle is shown in Figure 1.

Fig. 1. here

Preparation of PB nanoparticles

PB nanoparticles were synthesized by a co-precipitation method. The 0.01 M $K_3Fe(CN)_6$ solution was prepared in 0.01 M HCl solution and to it 0.01 M $FeCl_3$ solution was dispensed drop wise with continuous sonication in presence of excess of H_2O_2 [29]. A navy-blue precipitate was formed. The solution was stirred overnight and the resulting dark-blue colloidal

solution was centrifuged for 10 min at 10,000 rpm and the nanoparticles were washed with water several times and resuspended in water.

Modification of working electrode

PB nanoparticles and enzyme modified SPEs were prepared for estimation of G6P concentration by using layer-by-layer immobilization technique. Initially, 10 μl of PB (50 mg/ml) was dispensed on the WE and was allowed to dry at room temperature. Then, 20 μl of freshly prepared 0.25 mM $NADP^+$ and GR (0.6 U) in 100 mM Tris-HCl buffer (pH 8.0) was immobilized with 10 μl of 20% (w/v) gelatin and 2.5 μl of 12.5% (v/v) glutaraldehyde and was allowed to dry at 4^oC. After solidification of gelatin, G6PDH (1.8 U) in 100 mM Tris-HCl buffer (pH 7.0) was again immobilized on the top using gelatin and glutaraldehyde as described above and was again dried. The immobilization procedure was carried out in an ice bath and this whole process took less than 10 min. The modified electrodes were kept at 4^oC until further use [30]. This layer-by-layer immobilization was done to eliminate the effects of interfering agents.

Optimization study

Various optimization studies such as, working potential, pH, cofactor ($NADP^+$) and enzyme (GR and G6PDH) loadings were performed to obtain maximum amperometric response during measurement of G6P. Interference study was also performed with different major blood constituents such as glucose (5 mM), uric acid (0.1 mM), urea (0.5 mM) and L-cysteine (0.5 mM), to determine the selectivity of the PB nanoparticles-modified SPE.

Electrochemical measurement of G6P

The modified SPE was connected to the respective terminals of the AUTOLAB electrochemical analyzer for measuring amperometric response. In all such experiments, 200 μl of 100 mM Tris-HCl buffer (pH 7.0) was initially dispensed on the modified SPE covering the three electrodes to connect the electrochemical cell. Amperometry was then performed at constant working potential (determined from cyclic voltammetric experiments). After equilibration, 50 μl of G6P solution of different concentration was added and responses were noted after 15 s of addition of analyte. The readings were corrected by deducting the response at equilibration and calibration curves were constructed.

To demonstrate the usefulness of the proposed G6P sensor, rabbit blood serum samples were spiked with known concentration of G6P (10, 50, 70, 100, 500 μM) and were used as analytes for the amperometric measurements using freshly prepared PB nanoparticle modified G6P sensor. The results were compared using the calibration curve. The storage stability of the G6P biosensor at 4^{o}C was checked periodically by comparing the variation in response of the biosensor against 0.5 mM of G6P concentration for 45 days. The biosensor was not suitable to store at ambient temperature due to loss of activity of the enzymes. It might be noted that a freshly prepared electrode would always be preferred and the whole modification process would take only a few minutes with previously prepared PB nanoparticles.

Results and discussion

Characterization of PB nanoparticles

The AFM study of PB nanoparticles is shown in Figure 2a. This showed that spherical PB nanoparticles were well dispersed in water. The size of PB nanoparticles was homogenous and the average diameter was about 25-30 nm. It was observed from the EDX result of PB nanoparticles (shown in Figure 2b) that potassium (K) and iron (Fe) were the major elements. The existence of Fe indicated the actual formation of PB. The formation of PB was also confirmed by the FTIR spectrum. Figure 2c depicts the IR spectra of the PB nanoparticles. The peak at 2080 cm^{-1} could be attributed to the CN stretching in the Fe^{2+}–CN–Fe^{3+} of PB, which was in well accordance with the literature [31]. The UV–vis absorption of PB nanoparticles suspended in aqueous solution is shown in Figure 2d. The PB nanoparticles showed a broad band λ_{max} at 710 nm due to inter metal charge-transfer band from Fe^{2+} to Fe^{3+} in PB nanoparticles, which was in accordance with the previously reported literature [32].

Fig. 2. here

Optimization study for amperometric measurent of G6P

Working potential

The working potential for amperometric measurement of the proposed G6P biosensor was determined by performing various cyclic voltammetric studies (scan rate: 50 mV/s). The results of cyclic voltammetric studies are shown in Figure 3.

Fig. 3. here

The cyclic voltammogram of unmodified SPE (blank) did not give any reduction or oxidation peak in the presence of G6P (not shown in figure). It was also observed that the cyclic voltammogram of the enzyme and cofactor modified SPE (without PB nanoparticles) did not give any remarkable redox peak in presence of G6P. This is due to the fact that the electron transfer rate is very slow in bi-enzymatic reaction system. However, PB nanoparticle and enzyme modified SPE showed well-defined anodic peak even in absence of G6P in the system, as PB nanoparticles underwent redox reaction. The anodic peak (at -100 mV) of PB nanoparticles increased remarkably when 50 μM of G6P was added on the PB nanoparticle and enzyme modified SPE system and the anodic peak at -100 mV increased by three folds (obtained by comparing the anodic peak currents of PB nanoparticle modified SPE in absence and presence of 50 μM of G6P) and produced further enhanced peak with 100 μM of G6P. The cyclic voltammetric study implied that the PB nanoparticles acted as a mediator in the slow bi-enzymatic reaction system and the tendency of PB nanoparticles undergoing redox reaction

helped increase the electron transfer between enzymes and electrode. The optimum value of the working potential was chosen to be -100 mV from cyclic voltammetric studies. Thus, the role of PB nanoparticles was to enhance the electrochemical response of a bi-enzymatic reaction, thereby increasing the sensitivity of the G6P sensor.

Enzyme and cofactor loading

For improvement of the biosensor performance, both the enzymes and cofactor $NADP^+$ should be sufficient so as to obtain a broad linear response range. For optimum activity of a biosensor, various loadings of enzymes (G6PDH and GR) and cofactor ($NADP^+$) were determined. For optimization of enzyme loading, the biosensor response for 1.25 mM of G6P with various loadings of the two enzymes, G6PDH and GR was investigated and summarized in Table 1. Firstly, the amount of G6PDH were varied with amount of GR constant (1.8 U) and then amount of GR was varied and with G6PDH amount was kept constant (1.8 U). The amount of G6PDH was varied from 0.3 U to 1.8 U, whereas the loading of GR was varied from 0.15 U to 1.8 U. The amperometric response increased with increasing amount of the enzymes (G6PDH and GR) and maximum amperometric response was obtained with a loading of 1.8 U of G6PDH and 0.6 U of GR. This combination of enzyme was used in modification of SPE for all further experiments.

Table 1 here

To optimize the amount of cofactor $NADP^+$, the biosensor response to 1.25 mM of G6P with various $NADP^+$ loadings were monitored. The response increased with increasing $NADP^+$ and became saturated at 0.25 mM $NADP^+$ (shown in Figure 4). Thus, 0.25 mM $NADP^+$ was used as standard cofactor in modification of SPE for further experiments.

Fig. 4. here

Working pH

The pH of the buffer is essential to determine the sensitivity of the biosensors since the activity of enzymes (such as G6PDH and GR) and the stability of PB nanoparticles are dependent on the pH of the system [27]. To determine the effect of change in pH, 1.0 mM of G6P was used. The optimum activity of the sensor was achieved in the pH range of 6.5 to 7.5 (Figure 5). Both acidic and strong alkaline environment would decrease the activity of the enzyme and stability of PB nanoparticles respectively, leading to decrease in electrochemical response. Thus, 100 mM Tris-HCl buffer (pH 7.0) was chosen for the determination of G6P by using PB nanoparticle-modified SPE. All the electrochemical measurements were performed at ambient room temperature.

Fig. 5. here

Amperometric measurement of G6P

Once the optimal conditions such as working potential, pH, cofactor and enzyme loading were determined, the amperometric measurements were carried out for different concentrations of G6P. The responses using PB nanoparticle-modified SPEs for different concentrations of G6P in the range of 0.01 – 1.25 mM are shown in Figure 6.

Fig. 6. here

The amperometric response increased with increase in G6P concentration and linear response of the sensor was obtained in a concentration range 0.01 to 1.25 mM of G6P (slope: 63.3 μA/mM, R^2 = 0.997). The limit of detection (three times the standard deviation of the response of blank/slope) [33, 34] of G6P was determined as 2.3 μM (at signal-to-noise ratio [S/N] = 3), which is much lower than the concentration of G6P available in human blood serum (50 – 70 μM).

Reproducibility

The reproducibility of the proposed G6P electrochemical sensor was determined by measuring the amperometric responses of three independent sets of modified electrodes for different concentrations of G6P. This was evaluated in terms of relative standard deviation (% RSD) and was found to be 4.8% for three independent sets of experiments.

Interference study

The interference study was performed to assess the selectivity of the proposed sensor. The G6P sensor exhibited excellent anti-interferences ability with various major blood components such as glucose (5 mM), uric acid (0.1 mM), urea (0.5 mM) and L-cysteine (0.5 mM). Figure 7 depicts the effect of these compounds on amperometric response. The response of G6P was remarkably high compared to the negligible current responses of other blood components.

Fig. 7. here

The interference due to above mentioned components could be effectively eliminated by selection of a lower applied potential of –100 mV. The layer-by-layer immobilization of enzymes, cofactor and mediator was done to ensure that the interfering agents were not able to come in direct contact with the PB nanoparticles. Also, from reported literature, it was observed that these interfering components could be electrocatalytically oxidized or reduced by Prussian Blue only in presence of specific enzymes or modifications. For example, glucose and uric acid get oxidized in presence of glucose oxidase and uricase respectively, whereas, Prussian Blue was used to determine the product hydrogen peroxide as it is known as “artificial enzyme peroxidase” [35, 36]. For detection of L-cysteine, the PB modified electrode either requires over potential of ~0.9 V, otherwise needs to be modified with nano structured gold and palladium [37] or F-doped tin oxide thin film [38] etc. Therefore, the proposed G6P biosensor was highly selective.

Analysis of G6P in blood serum

The application of the proposed G6P biosensor in real sample analysis was investigated by measurement of G6P concentration in rabbit blood serum. For this study, rabbit blood serum samples were spiked with known concentration of G6P (10, 50, 70, 100, 500 μM) and amperometric measurements were done using G6P biosensor. Table 2 shows the results of G6P content in blood serum obtained using PB nanoparticle-modified G6P sensor, which is in well agreement with the added amount of G6P in serum samples. The recovery value of the spiked serum sample ranged from 94 – 105.2%. This study indicates that the proposed G6P sensor is almost free from interferences present in blood serum and can be effectively used for analysis of G6P content in blood serum.

Table 2 here

Storage stability

The modified electrodes were stored in 100 mM Tris-HCl buffer (pH – 7.0) at 4^{o}C. The stability was investigated by comparing the change of its amperometric response to 0.5 mM of G6P. It could be seen from Figure 8 that the response dropped to 95% on 25th day. The reduction in stability of sensor is possibly due to gradual reduction in activities of the enzymes G6PDH and GR with time.

Fig. 8. here

Comparison of results

The performance of the new sensory system was compared with other G6P sensing systems reported in literature in light of technology, limit of detection, detection range and stability (Table 3). It was observed that most of the reported G6P amperometric sensors suffer few major inherited drawbacks i.e. small detection ranges, less stability and time consuming modification procedures whereas in the proposed sensor modification procedure took less than 10 min. The G6P sensor also showed a broad detection range and lower detection limit compared to several reported G6P sensors. Also, the PB nanoparticle-modified G6P sensor showed good stability and exhibited excellent anti-interference property. Thus, it is clear that the present method could overcome many disadvantages of the reported ones.

Table 3 here

Conclusions

In this study, we have demonstrated a new analytical approach for measurement of G6P using PB nanoparticles, G6PDH, GR and $NADP^+$ modified SPE. The methods of preparation of PB nanoparticles by co-precipitation and modification technique of SPE were very simple and less time consuming. The nanoparticles with narrow size distribution were produced without agglomeration and demonstrated by various characterization studies. The use of PB nanoparticles enhanced the response by more than three folds as compared to only enzyme-modified electrodes. The cyclic voltammetric studies showed that PB nanoparticles served as mediator and enhanced

the response of bi-enzymatic reaction which in turn increased sensitivity (detection limit 6.3 μM) of the biosensor. The optimization studies of cofactor and enzyme loading showed that the amount of G6PDH, GR and $NADP^+$ necessary per electrode was very low. This reduced the cost of the sensor considerably. Also, the present procedure of G6P measurement did not require any sample preparation and all the measurement could be done at ambient temperature. The biosensor could exhibit reproducible results even in presence of many interfering substances such as glucose, ascorbic acid, uric acid, urea and L-cysteine etc. This was mainly due to low optimized working potential (-100 mV), which enhanced the selectivity of the sensor for G6P. The sensor also exhibited good stability at 4^0C and could be stored in buffer for several days. However, the modification electrode would take only a few minutes and hence a freshly modified electrode would always be preferred for the measurement. The proposed method could be a viable alternative to costly clinical estimation of G6P in blood serum.

Acknowledgments

The author SB is grateful to Council of Scientific and Industrial Research, India for providing fellowship support (09/028(0772)/2010-EMR-I) and the author PS is indebted to Department of Science and Technology for financial support through Sensor-Hub at CGCRI, Kolkata.

References

[1] H.N. Kirkman, G.F. Gaetani, Regulation of glucose-6-phosphate dehydrogenase from human erythrocytes, J. Biol. Chem. 261 (1986) 4033–4038.

[2] M.A. Coevoet, J.F. Hervagault, Irreversible metabolic transitions: the glucose 6-phosphate metabolism in yeast cell-free extracts, Biochem. Biophys. Res. Commun. 234 (1997) 162–166.

[3] B.M . Hogema, J.C. Arents, T. Inada, H. Aiba, K. Van-Dam, P.W. Postma, Catabolite repression byglucose-6-phosphate, gluconate and lactose in *Escherichia coli*, Mol. Microbiol. 24 (1997) 857–867.

[4] C. VillarPalasi, J.J. Guinovart, The role of glucose 6-phosphate in the control of glycogen synthase, FASEB J. 11 (1997) 544–588

[5] L.I. Sergeeva, D. Vreugdenhil, In situ staining of activities of enzymes involved in carbohydrate metabolism in plant tissues, J. Exp. Bot. 53 (2002) 361–370.

[6] L.H. Bellaver, N.M.B. De-Carvalho, J. Abrahao-Neto, A.K. Gombert, Ethanol formation and enzyme activities around glucose-6-phosphate in *Kluyveromyces marxianus* CBS 6556 exposed to glucose or lactose excess, Fems Yeast Res. 4 (2004) 691–698.

[7] M. Sapag-Hagar, R. Lagunas, A. Sols, Apparent unbalance between the activities of 6-phosphogluconate and glucose-6-phosphate dehydrogenase in rat liver, Biochem. Biophys. Res. Commun. 50 (1973) 179–185.

[8] G. Banhegyi, P. Marcolongo, R. Fulceri, C. Hinds, A. Burchell, A. Benedetti, Demonstration of a Metabolically Active Glucose-6-phosphate Pool in the Lumen of Liver Microsomal Vesicles, J. Biol. Chem. 272 (1997) 13584-13590.

[9] Y. Cui, J. P. Barford, R. Renneberg, Development of a glucose-6-phosphate biosensor based on coimmobilized *p*-hydroxybenzoate hydroxylase and glucose-6-phosphate dehydrogenase, Biosens. Bioelectron. 22 (2007) 2754–2758.

[10] Y. Cui, J.P. Barford, R. Renneberg, Development of an interference-free biosensor for glucose-6-phosphate using a bienzyme-based Clark-type electrode, Sensors Actuat. B-Chem. 123 (2007) 696–700.

[11] C. H. Tzang, R. Yuan, M. Yang, Voltammetric biosensors for the determination of formate and glucose-6-phosphate based on the measurement of dehydrogenase-generated NADH and NADPH, Biosens. Bioelectron. 16 (2001) 211–219.

[12] K. Aoki, H. Suzuki, Y. Ishimaru, S. Toyamaa, Y. Ikariyama, T. Iida, Thermophilic glucokinase-based sensors for the detection of various saccharides and glycosides, SensorsActuat. B-Chem. 108 (2005) 727–732.

[13] S. Suye, H. Zheng, H. Okada, T. Hori, Assembly of alternating polymerized mediator, polymerized coenzyme, and enzyme modified electrode by layer-by-layer adsorption technique, Sensors Actuat. B-Chem. 108 (2005) 671–675.

[14] A.S. Bassi, D. Tang, M.A. Bergougnou, Amperometric Biosensor for Glucose-6-Phosphate Monitoring Based on Entrapped Glucose-6-Phosphate Dehydrogenase, Mg2+ Ions, Anal. Biochem. 268 (1999) 223–228.

[15] Chen G, Liang Z, Li G, Progress of electrochemical biosensors fabricated with nanomaterials. Acta Biophysica Sinica 26 (2010) 711-725.

[16] G. Li in C.S.S.R Kumar (Eds.), Nanotechnologies for Life Sciences, Wiley-VCH, 2007, pp. 278-310.

[17] F. Ricci, G. Palleschi, Sensor and biosensor preparation, optimisation and applications of Prussian Blue modified electrodes, Biosens. Bioelectron. 21 (2005) 389-407.

[18] A.A. Karyakin, E.E. Karyakina, Prussian blue-based "artificial peroxidase" as a transducer for hydrogen peroxide detection. Application to biosensors, Sensors Actuat. B-Chem. 57 (1999) 268-273.

[19] P.J. Kulesza, K. Miecznikowski, M. Chojak, M.A. Malik, S. Zamponi, R. Marassi, Electrochromic features of hybrid films composed of polyaniline and metal hexacyanoferrate, Electrochim. Acta. 46 (2001) 4371-4378.

[20] K. Itaya, I. Uchida, V.D. Neff, Electrochemistry of polynuclear transition metal cyanides: Prussian blue and its analogues, Acc. Chem. Res. 19 (1986) 162-168.

[21] M. Kaneko, S. Hara, A. Yamada, A Photoresponsive Graphite Electrode Coated with Prussian Blue, J. Electroanal. Chem. 194 (1985) 165-168.

[22] C. Mingotaud, C. Lafuente, J. Amiell, P. Delhaes, Ferromagnetic Langmuir-Blodgett film based on Prussian Blue, Langmuir 15 (1999) 289-292.

[23] A.A. Karyakin, E.E. Karyakina, L. Gorton, Amperometric biosensor for glutamate using Prussian Blue based “artificial peroxidase” as a transducer for hydrogen peroxide, Anal. Chem. 72 (2000) 1720-1723.

[24] A. Chaubey, B.D. Malhotra, Mediated biosensors, Biosens. Bioelectron. 17 (2002) 441-456.

[25] W. Zhao, J.J. Xu, , C.G. Shi, H.Y. Chen, Multilayer membranes via layer by-layer deposition of organic polymer protected prussian blue nanoparticles and glucose oxidase for glucose biosensing, Langmuir 21(2005) 9630–9634.

[26] S.Q. Liu, J.J. Xu, H.Y. Chen, Electrochemical behavior of nanosized Prussian blue self-assembled on Au electrode surface, Electrochem. Commun. 4 (2002) 421–425.

494 [27] J. P. Li, T. Z. Peng, Y. Q. Peng, A Cholesterol Biosensor Based on Entrapment of
495 Cholesterol Oxidase in a Silicic Sol-Gel Matrix at a Prussian Blue Modified Electrode,
496 Electroanalysis 15 (2003) 1031-1037.
497 [28] P. Sarkar, S. Banerjee, Electrochemical sensing systems for arsenate estimation by
498 oxidation of L-cysteine, Ecotox. Environ. Safe. 73 (2010) 1495-1501.
499 [29] J. Chen, Y. Miao, X. Wu, Immobilization of Prussian Blue Nanoparticles onto Thiol SAM
500 Modified Au Electrodes for Analysis of DL-Homocysteine, Colloid J. 69 (2007) 660–665.
501 [30] S. Banerjee, P. Sarkar, Amperometric Detection of Hexavalent Chromium with L-DOPA
502 Modified Screen Printed Electrode, Sensor Lett. 9 (2011) 1–6.
503 [31] Q. Zhang, L. Zhang, J. Li, Fabrication and electrochemical study of monodisperse and size
504 controlled Prussian blue nanoparticles protected by biocompatible polymer, Electrochim. Acta
505 53 (2008) 3050–3055.
506 [32] F. Shiba, Preparation of monodisperse Prussian blue nanoparticles via reduction process
507 with citric acid, Colloids and Surfaces A: Physicochem. Eng. Aspects, 366 (2010) 178–182.
508 [33] M.F. Bergamini, D.P. Santos, M.V.B. Zanoni, Development of a voltammetric sensor for
509 chromium(VI) determination in wastewater sample, Sensors Actuat. B-Chem. 123 (2007) 902-
510 908.
511 [34] J.C. Miller, J.N. Miller in Statistics for Analytical Chemistry, 3rd ed., Ellis Horwood
512 Limited, Chichester, 1993, pp. 110-117.
513 [35] J. Zhu, Z. Zhu, Z. Lai, R. Wang, X. Guo, X. Wu, G. Zhang, Z. Zhang, Y. Wang, Z. Chen,
514 Planar amperometric glucose sensor based on glucose oxidase immobilized by chitosan film on
515 prussian blue layer, Sensors 2 (2002) 127-136.

516 [36] D. Iveković, M. Japec, M. Solar, N. Živković, Amperometric uric acid biosensor with
517 improved analytical performances based on alkaline-atable H_2O_2 transducer, Int. J. Electrochem.
518 Sci. 7 (2012) 3252-3264.
519 [37] P.C. Pandey, A.K. Pandey, D.S. Chauhan, Nanocomposite of Prussian blue based sensor
520 for l-cysteine: Synergetic effect of nanostructured gold and palladium on electrocatalysis,
521 Electrochim. Acta. 74 (2012) 23-.31
522 [38] L. Chena, K. Ho, Multimode optoelectrochemical detection of cysteine based on an
523 electrochromic Prussian blue electrode, Sensors Actuat. B-Chem. 130 (2008) 418-424.
524

List of Tables

Figure Legends

Fig. 1. Schematic diagram of enzyme, cofactor and PB nanoparticles mediated redox reaction of G6P at the WE surface

Fig. 2. (a) AFM image (b) EDX pattern (c) FTIR spectra (d) UV-vis absorption spectra of PB nanoparticles

Fig. 3. Cyclic voltammogram of (a) enzyme-modified SPE in presence of G6P (b) PB nanoparticle-modified SPE in absence of G6P (c) PB nanoparticle and enzyme-modified SPE in presence of 0.05 mM G6P (d) PB nanoparticle and enzyme-modified SPE in presence of 0.1 mM G6P (supporting electrolyte: 100 mM Tris-HCl buffer (pH 7.0); scan rate: 50 mV/s).

Fig. 4. Amperometric response of the PB nanoparticle and enzyme-modified SPE to 1.25 mM of G6P for various loadings of cofactor $NADP^+$ ranging from 0.05 to 1.25 mM $NADP^+$ (n=3) (sensor: 1.8 U G6PDH, 0.6 U GR and 0.5 mg PB nanoparticles; working potential: -100mV vs. Ag/AgCl; supporting electrolyte: 100 mM Tris-HCl (pH 7.0) buffer).

Fig. 5. Amperometric response of the PB nanoparticle and enzyme-modified SPE to 1 mM of G6P at various pH ranging from 4 to 9 (n=3) (sensor: 1.8 U G6PDH, 0.6 U GR, 0.25 mM $NADP^+$ and 0.5 mg PB nanoparticles; working potential: -100mV vs. Ag/AgCl; supporting electrolyte: 100 mM Tris-HCl buffer).

Fig. 6. Calibration curve for G6P estimation showing amperometric response of PB nanoparticles and enzyme-modified SPE with G6P concentrations ranging from 0.01 to 1.25 mM (n=3) (sensor: 1.8 U G6PDH, 0.6 U GR, 0.25 mM $NADP^+$ and 0.5 mg PB nanoparticles; working potential: -100mV vs. Ag/AgCl; supporting electrolyte: 100 mM Tris-HCl (pH 7.0) buffer).

Fig. 7. Amperometric study to determine the effect of glucose (5 mM), uric acid (0.1 mM), urea (0.5 mM) and L-cysteine (0.5 mM) on estimation of G6P (0.25 mM) using PB nanoparticles and enzyme-modified SPE. (All analytical conditions were same as Fig. 6.).

Fig. 8. Storage stability of PB nanoparticles and enzyme-modified G6P sensor stored at 4^oC and treated with 100 mM Tris-HCl buffer (pH 7.0) just before use (n=3). The G6P biosensor performance was tested using 0.5 mM solution of G6P and the relative response (%) was calculated by normalizing the signal to the maximum signal obtained on the first day of measurement. (All analytical conditions were the same as Fig. 6.).