Immobilization of Glucose Oxidase and Acetylcholinesterase onto Modified Polyamide Sorbent

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ABSTRACT: Two types of polyamide (PA) sorbents with high specific area were prepared. The effects of solvent type, concentrations of formic acid, and polymer on the porosity characteristics were studied. The sorbent with the highest specific area was obtained by using C_2H_5OH—HCOOH solvent (60% HCOOH) and the rest of the experiments were carried out with this type of sorbent. The possibility of applying the PA sorbent as carrier for immobilization of glucose oxidase (GOD) and acetylcholinesterase (AChE) was investigated. In order to increase the active groups content (necessary for enzyme immobilization), the sorbent was modified with dimethylaminoethylmethacrylate (DMAEM) and 2-acrylamido-2-methylpropensulfonic acid. The amount of the active groups introduced during the modification and the degree of hydrophilicity were determined. The quantity of bound protein and relative activity of GOD and AChE immobilized onto unmodified and modified sorbents were studied. Optimum pH and temperature of the immobilized GOD and AChE were also determined. The influence of three phosphoroorganic compounds on the activity of the immobilized AChE was investigated. Tetrachlorvinvos was found to be the strongest inhibitor, while AChE immobilized onto PA sorbent modified with DMAEM showed the highest stability. The possibility of using immobilized GOD and AChE in a flow-injection system for determination of the concentrations of glucose and phosphoroorganic compounds was studied. © 1998 John Wiley & Sons, Inc. J Appl Polym Sci 68: 323–329, 1998

Key words: immobilization, glucose oxidase, acetylcholinesterase, polyamide sorbent

INTRODUCTION

The advantages of immobilization of enzymes onto solid supports are known to be due mainly to the possibility of running enzymatic reactions continuously and saving time by avoiding the purification step for removing enzyme from the product stream. In addition, sometimes the immobilized enzymes will show prolonged activity in comparison with the free enzymes.[1]

Polymer sorbents in granular form are widely used as carriers for immobilization. They are characterized by good chemical stability, defined porosity, and intensive mass exchange leading to good interaction between enzyme and substrate in continuous product flow.

The various agarose polymers most widely used as matrices appear particularly ideal for immobilization of enzymes due to their high degree of porosity.[2] The acrylic polymers are also useful. The latter have good chemical and mechanical stability and are not susceptible to microbial attack.[3,4]

The use of polyamide (PA) sorbents for enzyme immobilization is also well known.[5] The authors carried out the immobilization by glutaraldehyde and carbodiimide onto unmodified PA sorbent.

In the present work, PA sorbent was prepared in granule form, chemically modified, and used as carrier for the immobilization of glucose oxidase

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Table I Basic Porosity Characteristics of PA Sorbent

	Sorbent Type I			Sorbent Type II		
No	Concn. of Alcoholic Solution of HCOOH (%)	Specific Area (m^2/g)	Average Radius by Area (Å)	Concn. of Aqueous Solution of HCOOH (%)	Specific Area (m^2/g)	Average Radius by Area (Å)
1.	50	20.070	603	50	8.362	4120
2.	60	19.428	807	60	7.560	4358
3.	70	17.323	1214	70	4.248	4470
4.	80	16.784	1282	80	2.445	9402
5.	90	16.184	2259	90	2.374	10200
6.	100	15.742	2793	100	—	—

(GOD) and acetylcholinesterase (AChE). The latter was used in a flow-injection system (FIS) to determine the concentration of glucose and phosphoroorganic compounds (POC).

EXPERIMENTAL

Materials

PA sorbent obtained from PA-6 (product of Vida Co., Vidin, Bulgaria) was used as carrier for the immobilization. The PA sorbent was modified with dimethylaminoethylmethacrylate (DMAEM) and 2-acrylamido-2-methylpropensulfonic acid (AMPSA; Fluka Chemie AG, Buchs, Switzerland).

GOD with enzyme activity of 50,000 U/mg protein, from Serva, Heidelburg, Germany, and horse blood plasma containing AChE with enzyme activity of 10 U/mg (product of the Institute of Veterinary Medicine, Polvdiv, Bulgaria) were used.

The following POC were used to determine the inhibition effect on AChE activity: tetrachlorvinvos, phenitrothion, and phozalone (Fluka).

Preparation of PA Sorbent

The PA sorbent was obtained by dissolving 10 g PA-6 in a 100-mL alcoholic (type I) or aqueous (type II) solution of formic acid with concentrations 50–100% and precipitating in a water–alcohol mixture (1 : 1), pH 7. The basic porosity characteristics of the sorbent were determined by mercury porosimetry (Porometer 1500 - Carlo Erba).

Chemical Modification of the PA Sorbent with DMAEM and AMPSA

The chemical modification with DMAEM and AMPSA was carried out by a method described in our earlier work.[6] The tertiary amino groups obtained by the modification with DMAEM were quaternized with 50% solution of benzylchloride in ethanol at 50°C for 7 h.

The ionogenic groups content was determined quantitatively by residual titration in a heterogeneous medium.[7] The degree of hydrophilicity was measured by the weight difference between the water-swollen and dry sorbent (water content) per unit of sorbent weight.[8]

Immobilization of GOD and AChE

Unmodified and modified PA sorbents were washed with 0.1*M* phosphate buffer, pH 5.8, and immersed in a 0.1% solution of GOD or 0.3% solution of AChE in 0.1*M* phosphate buffer, pH 5.8.

Table II Basic Characteristics of Modified PA Sorbent

No	Modifying Agent	Active Group Type	Amount of Active Groups (mequiv/g)	Degree of Hydrophilicity (%)
1	AMPSA	Sulfo	0.649	77.39
2	DMAEM	Quaternary amino	0.459	73.91

Table III Basic Characteristics of GOD and AChE Immobilized onto PA Sorbent

		Immobilized GOD		Immobilized AChE	
No	Sorbent	Bound Protein (mg/g)	Relative Activity (%)	Bound Protein (mg/g)	Relative Activity (%)
1	Unmodified	0.05	22.54	0.06	23.1
2	Modified with AMPSA	0.06	22.9	0.07	25.4
3	Modified with DMAEM	0.07	23.24	0.08	25.9

The immobilization was carried out at 4°C for 16 h. Then the PA sorbent containing GOD or AChE was washed thoroughly with distilled water and 0.1*M* phosphate buffer, pH 5.8.

The bound protein was measured by the method of Lowry and colleagues.[9] Enzyme activities of bound and free GOD and AChE were determined by a method described earlier.[10,11]

Determination of the Inhibition Effect of POC on AChE Activity

First, the activity of the immobilized AChE (U_0) was determined. Then PA sorbent with bound AChE was immersed in 0.1 and 1.0 mg/L POC for 30 min (static incubation method) and once again the enzyme activity was measured (U_1). The inhibition effect (l) of POC was calculated as the difference between these two activities:

$$l(\%) = \frac{U_0 - U_1}{U_0} \times 100$$

Flow-injection System with Immobilized GOD and AChE

A single-channel FIS was used,[12] comprising a peristaltic pump (DP2-2, VEB MLM Labortechnik, Germany); a rotation injector manufactured according to Ruzicka[13] with sample volume 100 μL: a universal polarograph OA-102 (Radelkis, Budapest, Hungary); and a pH meter OP-211/1 (Radelkis).

The flow carrier and the substrate studied were thermostated in a VB HLW thermostat (Merck, Darmstadt, Germany).

The enzyme reactor was made of polymethylmethacrylate with inner diameter of 6 mm and length 20 mm. PA sorbent with bound GOD or AChE was placed in the reactor.

Glucose concentration was measured by using bound GOD. The hydrogen peroxide obtained from the enzyme–substrate reaction was determined amperometrically with platinum and large-area saturated calomel electrodes (Universal Polarograph OA-102).

The concentrations of acetylcholine and POC were determined with immobilized AChE. The acetic acid obtained from the enzyme–substrate reaction was determined with pH meter and glass and reference Ag/AgCl electrodes.

RESULTS AND DISCUSSION

Two types of PA sorbents with high specific area were used. They were obtained by dissolving PA in alcoholic (type I) and aqueous (type II) solutions of formic acid with concentrations of 50–100%, followed by precipitation in a water–alcoholic mixture with pH 7. Polymer concentration was varied to find that fine disperse precipitate was obtained at polymer concentrations of 10 mass %. Particle size was from 0.25 to 0.5 mm.

The basic porosity characteristics of both sorbents are presented in Table I, determined by mercury porosimetry (Porometer 1500 - Carlo Erba). The results show that sorbent with higher specific area (15–20 m^2/g) was obtained with solvent C_2H_5OH—HCOOH.

The effect of formic acid concentration on the sorbent specific area was studied (Table I). It can be seen from Table I that both sorbents showed highest specific area when a 50% solution of formic acid was used. As reported in our earlier paper,[14] the most suitable PA sorbents for enzyme immobilization are those obtained with solvent C_2H_5OH—HCOOH (type I) in a 40 : 60 ratio. Sorbents of the same type obtained with 50% HCOOH gave unsatisfactory results, probably due to small pore size which decreases the enzyme–substrate reaction rate. All other experiments were carried out with PA sorbent obtained

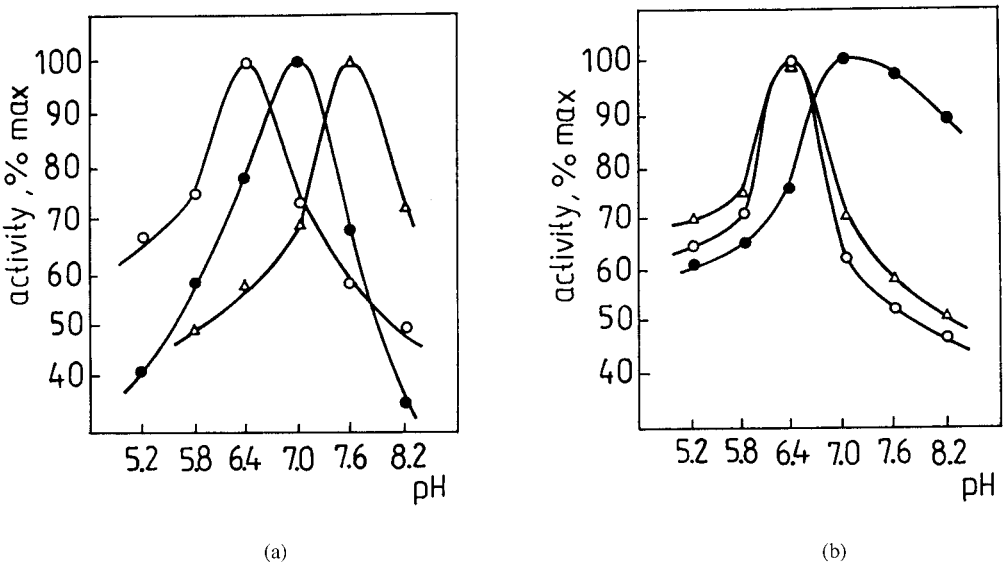

Figure 1 Effect of pH on the activity of GOD (a) and AChE (b) immobilized onto unmodified PA sorbent (○) and onto PA sorbent modified with AMPSA (●) and DMAEM (△). Activities were measured at pH from 5.0 to 8.5 and 28°C for 5 min; substrate concentration: 0.5 wt % acetylcholine and 18 wt % glucose.

with solvent C_2H_5OH—HCOOH at 60% formic acid.

The possibilities for application of PA sorbent as a carrier for immobilization of enzymes was studied. In order to increase the amount of active groups necessary for enzyme immobilization, PA sorbent was modified with AMPSA and DMAEM. The modification with AMPSA gives sulpho groups; and that with DMAEM yields tertiary amino groups, which were further quaternized. Table II shows the amount of active groups in modified sorbents. For the calculation of the amount of sulpho and quaternary amino groups, the amount of amino and carboxylic groups in the initial (unmodified) sorbent was taken into account.

The degree of hydrophilicity of the unmodified sorbents was measured to be 69% while that of the modified sorbent was found to increase (Table II).

GOD and AChE were immobilized onto the unmodified and modified PA sorbents. The enzymes were bound by physical adsorption onto unmodified sorbent, and by both physical adsorption and electrostatic attractive force (between the active groups of the carrier and amino and carboxylic groups of the enzyme) onto modified sorbent. The immobilization was carried out under the previously determined optimum conditions (time, pH, temperature, and concentrations of GOD and AChE) described above. Table III shows the amount of bound protein of immobilized GOD and AChE. Obviously, the modified sorbents bind more protein than do the unmodified ones. Also, the amount of bound AChE is higher than that of bound GOD on carriers used. The high affinity of the PA sorbents to binding GOD and AChE can be explained by their large specific area. Table III also shows the relative activities of the immobilized enzymes, which were found to be from 22 to 26%, and slightly higher for modified sorbents compared with the unmodified. The best characteristics possess GOD and AChE immobilized onto PA sorbent modified with DMAEM.

The pH optimum (pH_{opt}) of the free GOD was found to be 5.8—equal to pH values reported by other authors.[15] The pH_{opt} of GOD bound on PA sorbents is shifted to the alkali range compared with pH_{opt} of the free enzyme [Fig. 1(a)]. The shift is rather small for unmodified sorbent and significant for modified. For PA sorbent modified with AMPSA the shift is due to the excess sulfo groups which are not bound to GOD and do attract protons to sorbent surface, and the pH measurement in free solution gives higher values. For sorbents modified with DMAEM the amount of active quaternary amino groups is small and probably all of them are bound to GOD. However, this modification involves preliminary treatment with hydrochloric acid to obtain carboxylic groups in or-

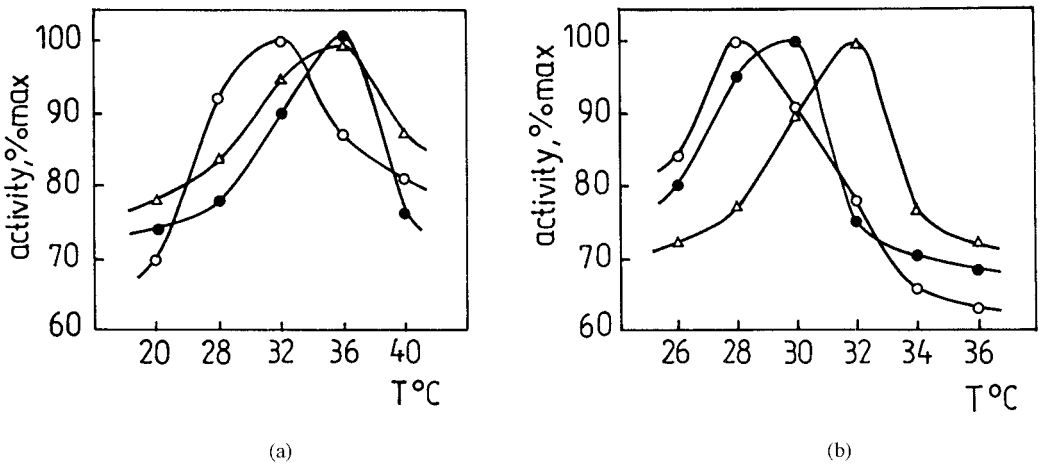

Figure 2 Effect of temperature on the activity of GOD (a) and AChE (b) immobilized onto unmodified PA sorbent (○) and onto PA sorbent modified with AMPSA (●) and DMAEM (△). Activities were measured at temperatures from 20 to 40°C and pH 5.8 for 5 min; substrate concentration: 0.5 wt % acetylcholine and 18 wt % glucose.

der to graft DMAEM.[6] Probably, the redundant carboxylic groups cause the shift in pH_{opt} of GOD immobilized onto PA sorbent modified with DMAEM to the alkali range.

The pH_{opt} of native AChE was found to be 5.8, which corresponds to data reported by other authors.[11] The pH_{opt} of immobilized AChE was determined [Fig. 1(b)]. It can be noted that pH_{opt} of AChE bound onto unmodified and modified-with-AMPSA PA sorbent are equal to those of bound GOD, which proves that the shift in pH_{opt} is due to the electric charge of the carrier. Only the shift for AChE-bound sorbent modified with DMAEM was smaller.

The temperature optima of free and immobilized GOD were measured. That of the native GOD was found to be 28°C while those of immobilized enzyme were 32°C (for unmodified carriers) and 36°C (for modified) [Fig. 2(a)].

The temperature optima (T_{opt}) of native and bound AChE were also determined. The temperature optimum of the native AChE was 28°C. The highest shift of T_{opt} of the bound AChE was measured for PA sorbent modified with DMAEM [from 28 to 32°C, Fig. 2(b)].

The stabilities of free and bound GOD and AChE were studied. After two months the activities of free and immobilized GOD decreased as follows: for free GOD, to 92% of initial activity; for GOD immobilized onto PA sorbent modified with AMPSA, to 93.5%; and modified with DMAEM, to 95.5%. For this period the activities of free and immobilized AChE also decreased: for free AChE, to 93.2% of initial activity; for AChE immobilized onto PA sorbent modified with AMPSA, to 94.5%; and modified with DMAEM, to 96.2%. It may be noted that the enzymes immobilized onto PA sorbent modified with DMAEM are more stable. This is probably due to the higher amount of enzyme bound onto this carrier.

Considering the results obtained, it may be concluded that PA sorbents, especially those modified with DMAEM, are suitable carriers for immobilization of GOD and AChE. Important advantages of these granular carriers are their highly devel-

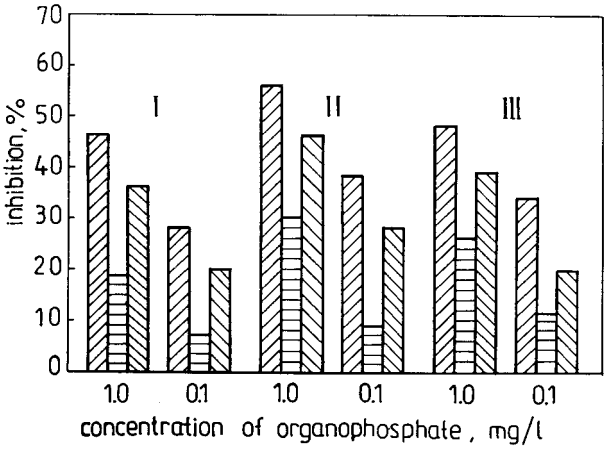

Figure 3 Inhibition effects of tetrachlorvinvos (▨), phenitrotion (▤), and phozalon (▧) on the activity of AChE immobilized onto PA sorbent modified with DMAEM (I) and AMPSA (II) and onto unmodified PA sorbent (III).

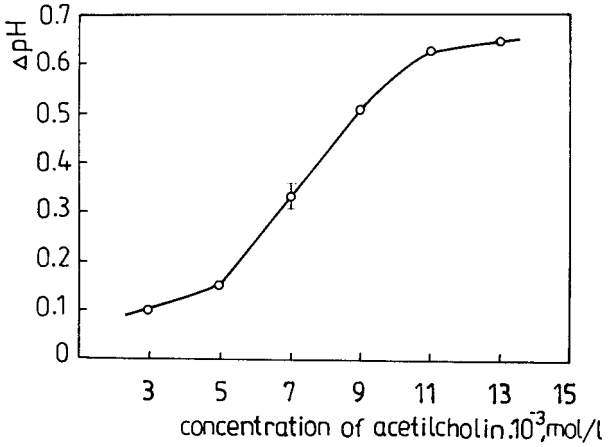

Figure 4 Calibration curve for acetylcholine. Injected volume, 100 μL; flow carrier, distilled water (pH 7, optimum flux 1 mL/min); temperature 28°C. The experimental points represent averages of three measurements. Standard deviations are shown as error bars.

oped specific area and defined porosity leading to intensive mass exchange and good interaction between enzyme and substrate in continuous flow.

It is well known[16–18] that POC are inhibitors of AChE and reduce its activity. Thus there is a possibility of determining pesticide (POC) concentration by measuring the reduction of enzyme activity. Several POC were studied: tetrachlorvinvos, phenitrotion, and phozalon, in concentrations of 0.1 and 1.0 mg/L. Then AChE bound onto unmodified (III) and modified with DMAEM (I) and AMPSA (II) PA sorbent was immersed into these solutions (incubation method). The strongest inhibitor of AChE was found to be tetrachlorvinvos (Fig. 3). The lowest inhibition effect was measured with AChE bound onto sorbents modified with DMAEM. This is probably due to the better stability of the AChE immobilized in this carrier. The concentration limit determined for tetrachlorvinvos was 0.0005 mg/L; for phenitrothion, 0.001 mg/L; and for phozalon, 0.008 mg/L. The results obtained show that on the basis of AChE-bound PA sorbents, accurate enzyme sensors can be prepared for determination of POC.

For this purpose, a FIS was designed, including a minireactor (20 mm long, 6 mm inner diameter) for the bound AChE. Flow-injection analysis was carried out under certain optimum conditions: pH 7, temperature 28°C, flux 1 mL/min. Distilled water with pH 7 was used as flow carrier. Samples of 100 μL substrate (acetylcholine) with concentration (3×10^{-3}) from 3×10^{-3} to 13×10^{-3} mol/L divided by (13×10^{-3}) mol/L were injected in the flow carrier and passed through the immobilized AChE. The products of the enzyme–substrate reaction (choline and acetic acid) were fed into a flow-through electrochemical cell equipped with a pH meter. The measured pH value depends on the substrate concentration.

Figure 4 shows the calibration curve obtained by injection of 100 μL solution of acetylcholine in the flow carrier. It can be seen that the linear region of the calibration curve is within the concentration interval from 0.005 to 0.01 mol/L. The concentration interval obtained is comparable to other similar methods employing pH detection.[19] It means that the immobilized AChE can be used to determine the concentration of acetylcholine. In addition, the enzyme can be used to detect AChE inhibitors, such as pesticides.

The possibility of using the FIS for determination of POC (AChE inhibitors) concentration was studied by injecting tetrachlorvinvos of certain concentration together with substrate solution (100 μL) into the FIS. The values of pH resulting from the enzyme–substrate reaction in the presence of POC were measured. The POC concentration was determined from the difference between the pH values measured with pure acetylcholine and an acetylcholine–tetrachlorvinvos mixture.

It can be seen from Figure 5 that the dependence is linear for tetrachlorvinvos concentrations from 0.05 to 0.15 mg/L. This method exhibits detection limits comparable with other flow-injection systems with pH detection.[19] However, it has lower sensitivity than spectrometric[20] and fluo-

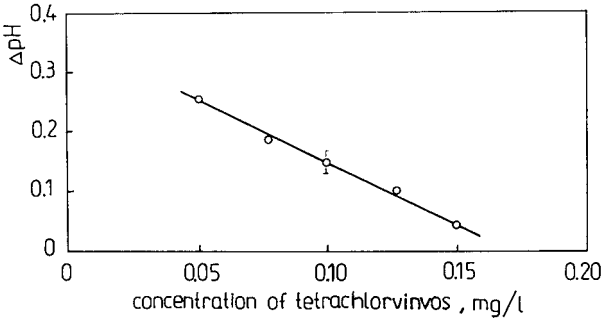

Figure 5 Calibration curves for tetrachlorvinvos determined in the presence of substrate (0.01 mol/L); injected volume, 100 μL (volume ratio acetylcholin : tetrachlorvinvos = 1 : 1); flow carrier, distilled water (pH 7, optimum flux 1 mL/min); temperature 28°C. The experimental points represent averages of three measurements. Standard deviations are shown as error bars.

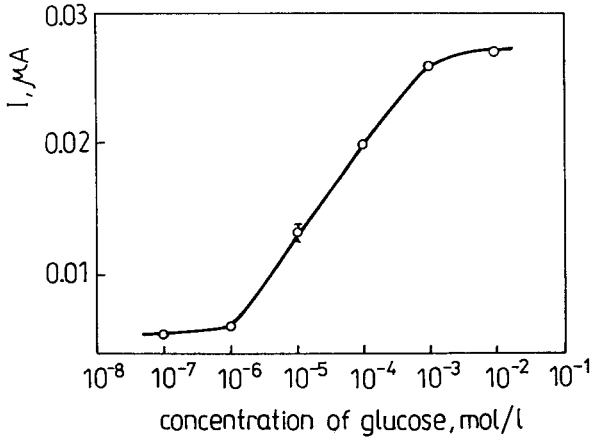

Figure 6 Calibration curves of 3-D-glucose. Injected volume, 100 μL; flow carrier, 0.1*M* potassium phosphate buffer (pH 5.8, optimum flux 1 mL/min); temperature 28°C; applied potential, 1.05 V (normal calomel electrode). The experimental points represent averages of three measurements. Standard deviations are shown as error bars.

rimetric[21] flow-through systems. Although the tetrachlorvinvos limit concentration for flow-injection analysis is higher than that of the static incubation method (described previously), the flow-injection analysis is faster and can be used successfully as a basis for the construction of continuously working monitoring systems for pesticides.

The same FIS but with amperometric detection[12] and miniflow reactor containing immobilized GOD was used to measure glucose concentration. The analysis was carried out under optimum conditions: pH 5.8, temperature 28°C, flux 1 mL/min. The flow carrier used was 0.1*M* phosphate buffer with pH 5.8. Samples of 100-μL glucose solution with concentration from 1×10^{-7} to 1×10^{-2} mol/L were injected into the flow carrier and passed through the immobilized GOD. The hydrogen peroxide obtained from the enzyme oxidation of the glucose was determined amperometrically by using an indicator Pt electrode and saturated calomel electrode. Optimum potential of 1.05 V was applied. The amperometric detection was used to measure the change of the cathode current (i, μA) with the variation of the substrate concentration.

From Figure 6 it can be seen that the linear range of the calibration curve is in the concentration interval from 5×10^{-6} to 5×10^{-3} mol/L. The working interval obtained is wide and can be used to measure lower glucose concentrations, compared with other similar methods.[12,22] The results obtained show that GOD bound onto PA sorbents can be used successfully to design a FIS for determination of glucose in biological liquids.

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