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Flow electrochemical biosensors based on enzymatic porous reactor and tubular detector of silver solid amalgam☆

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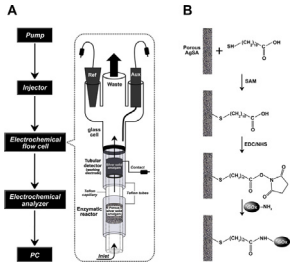
HIGHLIGHTS

- Flow amperometric enzymatic biosensor was constructed.
- The biosensor is based on a reactor of a novel material – porous silver solid amalgam.
- Tubular amalgam detector was used for determination of decrease of O_2 concentration.
- Covalent bonds amalgam–thiol–enzyme contributed to the sensor long-term stability.
- LOD of glucose was 0.01 mmol L^{-1} with RSD = 1.3% (n = 11).

GRAPHICAL ABSTRACT



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ABSTRACT

A flow amperometric enzymatic biosensor for the determination of glucose was constructed. The biosensor consists of a flow reactor based on porous silver solid amalgam (AgSA) and a flow tubular detector based on compact AgSA. The preparation of the sensor and the determination of glucose occurred in three steps. First, a self-assembled monolayer of 11-mercaptoundecanoic acid (MUA) was formed at the porous surface of the reactor. Second, enzyme glucose oxidase (GOx) was covalently immobilized at MUA-layer using N-ethyl-N′-(3-dimethylaminopropyl) carboimide and N-hydroxysuccinimide chemistry. Finally, a decrease of oxygen concentration (directly proportional to the concentration of glucose) during enzymatic reaction was amperometrically measured on the tubular detector under flow injection conditions. The following parameters of glucose determination were optimized with respect to amperometric response: composition of the mobile phase, its concentration, the potential of detection and the flow rate. The calibration curve of glucose was linear in the concentration range of 0.02–0.80 mmol L^{-1} with detection limit of 0.01 mmol L^{-1}. The content of glucose in the sample of honey was determined as 35.5 ± 1.0 mass % (number of the repeated measurements n = 7; standard deviation SD = 1.2%; relative standard deviation RSD = 3.2%) which corresponds well with the declared values. The tested biosensor proved good long-term stability (77% of the current response of glucose was retained after 35 days).

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Nomenclature

$\bar{X}$	average response
AcB	acetate buffer
AgSA	silver solid amalgam
AUX	auxiliary electrode
E_d	detection potential
ED	electrochemical detection
EDC	N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride
FA	flow analysis
FIA	flow injection analysis
GOx	glucose oxidase from *Aspergillus niger*
I_p	peak current
LOD	limit of detection
LOQ	limit of quantification
MES	2-(N-morpholino)ethanesulfonic acid
ML	monolayer
MP	mobile phase
MUA	11-mercaptoundecanoic acid
NHS	N-hydroxysuccinimide
PB	phosphate buffer
R	correlation coefficient
Ref	reference electrode
RSD	relative standard deviation
SAM	self-assembled monolayer
SD	standard deviation
TD	tubular detector
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol
t_{scan}	duration of one amperometric record, time scan
v	flow rate
V_{inj}	injecting volume

1. Introduction

Electrochemical detection (ED) in flow analysis (FA) is widely used especially in connection with biosensors [1–4]. Detection (working) electrode is a basic element and the sole source of analytical signal in electrochemical methods. Depending on the properties of the analyte and on conditions of measurements a suitable electrode material must be chosen. Electrodes of gold, platinum or of other, mostly noble metals, are used for work in positive potential range and for analyte oxidation. Electrodes of various types of carbon are suitable not only for measurements at positive potentials, but also at moderately negative ones. The best possibilities for performing electroreduction processes, particularly at high negative potentials, provide mercury and amalgam electrodes. High hydrogen overvoltage on these materials allows to work in aqueous solutions up to −2 V [5,6]. Incorporation of pure mercury electrodes in the flow systems is complicated due to the mechanical instability of the mercury drop, and therefore the Hg-electrodes are practically not used for this purpose [7]. Construction of solid [5,8–10] and paste [6,11,12] amalgam electrodes is not different from electrodes of other materials suitable for FA. The very useful advantage of solid amalgams is the possibility of preparing electrodes of required size and shape. The silver solid amalgam (AgSA) detectors were used for work in wall-jet [13,14] and thin-layer [13] arrangement of an electrolytic cell. Introduction of tubular AgSA-detector (TD) substantially simplified the design of flow electrochemical cell and increased the reliability and reproducibility of measurement [15].

Amalgam electrodes were shown to be a suitable base for thiol monolayers (ML) [16,17], on which different types of biosensors can be created. If the thiol chemisorbed at amalgam surface contains carboxylic group, it is possible to create an amide bond between —COOH groups of thiol and —NH_2 groups of a suitable protein by EDC/NHS chemistry [18–22]. This protein can be, for example, avidin or streptavidin, and the prepared biosensor can be used for selective measurement of (strept)avidin–biotin interactions [17]. Similar binding enzyme or antibody to thiol allows preparing enzymatic or immunological biosensor. Sensitivity of the enzyme biosensor depends on the area covered by enzyme (e.g., the use of porous gold substrate leads to a 11.4-fold increase of thiol adsorption and a 3.3-fold increase of protein adsorption in comparison with gold surfaces which were deposited on bare quartz crystals by electron beam evaporation [23]). Detection electrodes have a relatively small surface which can be enlarged by the use of an enzymatic reactor (ER). The active surface of ER is much larger than that of detector and this ER is commonly placed in flow system in front of the detector. The detector then registers either some product of enzymatic reaction or, e.g., decrease of oxygen concentration in the solution. Large surface of the enzyme reactor can be provided by a porous carbon felt [24], small particles of organofunctionalized silica [25], aminoaryl [3] or aminopropyl [26,27] controlled pore glass, by the immobilizing agent Eupergit® C 250 L (oxirane acrylic beads) [28], chitosan porous beads [29,30], porous agarose beads [31], nylon membrane coated with a thin layer of gold [32] or by microporous gold [33]. Another overview about utilized reactors and biosensors is given in paper [34]. We have prepared silver solid amalgam as a porous material and we have found no information on the use of this material for biosensors preparation.

The aim of this study was construction and testing of the enzyme biosensor based on porous reactor of silver solid amalgam in the flow system. Long-term stability of the biosensor should ensure anchoring the enzyme to the surface of the amalgam by covalent bonds. The used biosensor preparation procedure should be universal and should be suitable for binding various proteins or other compounds containing —NH_2 group.

2. Experimental

2.1. Reagents and solutions

Glucose oxidase (GOx, EC 1.1.3.4; 147.900 U mg^{-1}) from *Aspergillus niger*, D-(+)-glucose (≥99.5%), 11-mercaptoundecanoic acid (MUA, 99%), N-hydroxysuccinimide (NHS, ≥97%), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, ≥98.0%) and (2-(N-morpholino)ethanesulfonic acid (MES, ≥99%) were purchased from Sigma–Aldrich. All other chemicals were of p.a. grade.

The stock solution of GOx (2.0 mg mL^{-1}) was prepared in 0.10 mol L^{-1} phosphate buffer (PB) of pH 7.1 and was stored at −20 °C when not in use. Glucose stock solution (100 mmol L^{-1}) was prepared in the used mobile phase (MP) [0.10 mol L^{-1} acetate buffer (AcB); 1.00 mmol L^{-1} Na_2EDTA; pH 6.50]. Solution of MUA was prepared as [0.01 mol L^{-1} MUA; 0.01 mol L^{-1} $HClO_4$; 70% C_2H_5OH]. Activated solution for amide coupling between —COOH groups of MUA and —NH_2 groups of GOx contained [0.04 mol L^{-1} EDC; 0.10 mol L^{-1} NHS; 0.10 mol L^{-1} MES; 0.15 mol L^{-1} NaCl; pH 5.0] and it was freshly prepared before each use.

For the determination of glucose in honey, the procedure was as follows: one drop of the honey (weight about 0.1 g with 0.1 mg precision) was dissolved in 10 mL H_2O and 0.1 mL of this solution was added to 1.9 mL of the mobile phase. Sample of the honey prepared in this way was then analyzed by FIA-ED (preparation of the reactor and detector is described in Section 2.3).

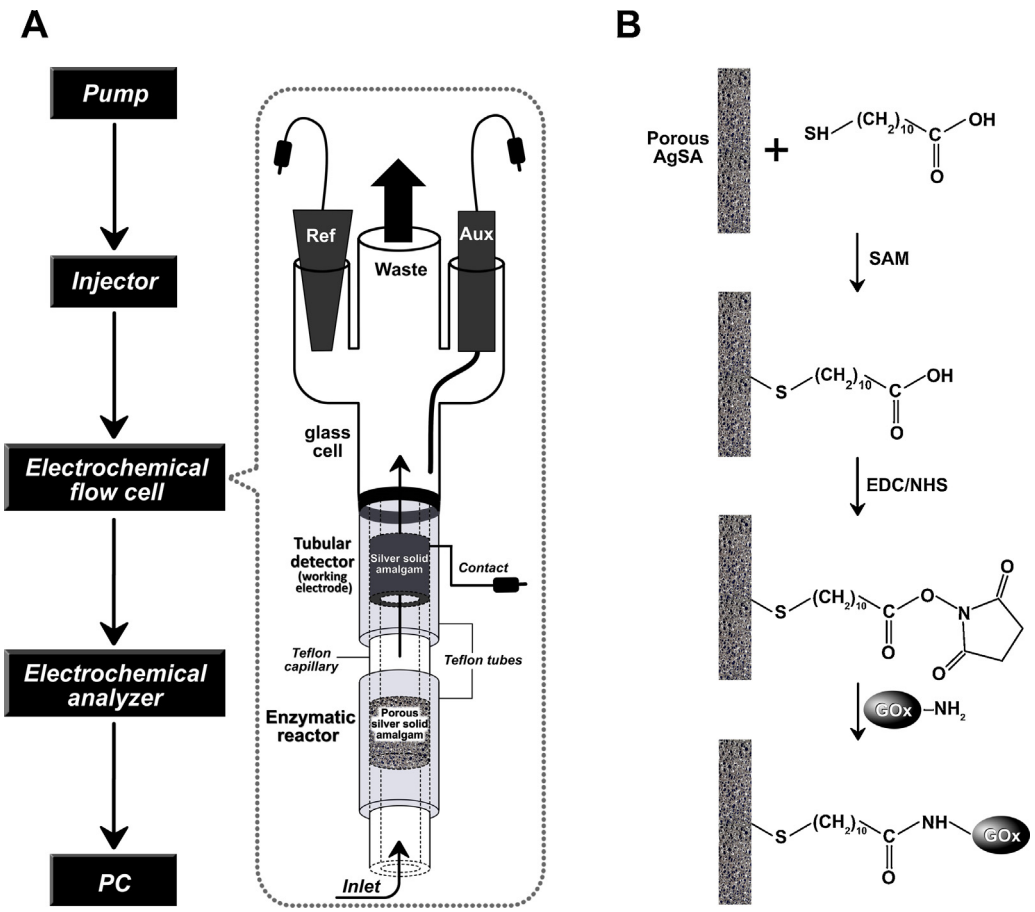

Fig. 1. (A) Scheme of FIA-ED. (B) Modification process of the reactor based on porous silver solid amalgam. EDC – N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride; NHS – (2-(N-morpholino)ethanesulfonic acid); GOx – glucose oxidase; Ref – reference electrode; Aux – auxiliary electrode.

2.2. Apparatus and methods

The determination of glucose by amperometric detection of oxygen concentration was carried out at room temperature using computer-controlled analyzer with MultiElchem v. 2.3 software (J. Heyrovský Institute of Physical Chemistry of AS CR, v.v.i.) and of the electrochemical stand (Polaro-Sensors, Czech Republic). The three-electrode system (Fig. 1A) consisted of tubular detector silver solid amalgam as a working electrode (TD, laboratory-made, inner diameter 0.5 mm, length 6.0 mm), the saturated calomel electrode based on silver paste amalgam (laboratory-made, it has the same potential as a saturated calomel electrode) as the reference electrode [35] and platinum wire as the auxiliary electrode. Immobilization of GOx was realized in the flow porous reactor based on solid silver amalgam (without electric contact). After the measurement, the tubular detector was kept in deionized water at room temperature and the reactor with immobilized enzyme was stored in [0.10 mol L^{-1} AcB; 1 mmol L^{-1} Na_2EDTA; pH 6.5] at 4 °C.

The FIA-ED system consisted of a linear syringe pump NE-1000 (New Era Pump Systems, USA) and sample injector Upchurch Scientific Valve V-451 (USA) with laboratory-made injecting loops of 50 μL (V_{inj}).

The limit of detection (LOD) was calculated according to IUPAC using three times the standard deviation corresponding to the lowest point of concentration dependence and the limit of quantification (LOQ) using ten times the standard deviation.

2.3. Construction and preparation of the reactor and tubular detector

A piece of Teflon capillary with the same outer diameter (OD, 1.59 mm) as the inlet Teflon capillary (see Fig. 1A) was inserted into the Teflon tube (length 22 mm, inner diameter 1.59 mm) in the place of the planned beginning of the silver solid amalgam column. A silver powder (particles size −100 mesh, Alfa Aesar, Germany) was gradually added into the upper hole of the tube and was mildly pressed by another piece of the Teflon capillary. As soon as the length of the silver powder column was 10 mm (or other if it was needed), it was rinsed 3 times by 2 mol L^{-1} $HClO_4$ and then mercury was added into this tube to convert silver powder into amalgam. After about 5 min, an excess of mercury was carefully removed from the reactor. At both ends of the Teflon tube, two 6–8 mm long silicone tubes were fixed. These tubes seal the inlet and outlet capillary with the reactor body. The prepared porous reactor can be used immediately, although it is preferable to let the solid amalgam to be stabilized until the next day. Porosity of the reactor depends on the size of the silver particles and on the degree with which the powder was compressed before amalgamation. Incorporation of the reactor into the flow system is shown in Fig. 1A.

Preparation of the tubular detector is described in detail in our previous paper [15]. Briefly, a silver powder was strongly pressed into the Teflon tube (inner diameter 1.5 mm, outer diameter 2.1 mm) and a platinum contact was fixed in this silver powder column. A hole of the same diameter as the inner diameter of the inlet capillary (0.5 mm) was formed along the central part of the

silver column. Into the Teflon tube with the silver column mercury was added and after about two hours an excess of mercury was removed. The piece of the inlet capillary was fixed by epoxy resin with the Teflon tube to be in touch with the silver column. On outside of the amalgam column, 2 mm long piece of the Teflon capillary (inner diameter 0.5 mm) was glued. The inner diameter of the used TD is the same (0.5 mm) as the inner diameter of connecting capillaries. Solid amalgam is a continuation of the inlet capillary and hence, here is a minimal impact to a mobile phase flow and to an injection zone.

Detector was cleaned in electrochemical way before each measurement. This regeneration process is experimentally checked for every type of measurements and it should provide a good reproducibility. In this work, the potential of −0.1 V was imposed on the detector for 10 s at first and then the potential was switched to −1.1 V for 10 s. Such detector regeneration ensured the reproducibility at the level of 1–2% for real samples of honey.

2.4. Preparation of the biosensor

The preparation of the biosensor consisted of two steps (Fig. 1B). First, a self-assembled monolayer of MUA was formed at the reactor and second, an enzyme GOx was covalently immobilized in MUA-layer using EDC/NHS chemistry. Each solution was flown through the reactor for a specified time, thereby ensuring reproducibility of the reactor preparation. In general, one reactor could be used for repetitive preparation of the biosensor, where many other enzymes could be attached.

Concentration of glucose in the model/real sample was measured amperometrically in the detector under flow injection analysis conditions from the decrease of oxygen concentration (directly proportional to the concentration of glucose) during enzymatic reaction:

$$\text{Glucose} + \text{GOx(FAD)} \rightarrow \text{gluconic acid} + \text{GOx(FADH}_2\text{)}$$

$$\text{GOx(FADH}_2\text{)} + \text{O}_2 \rightarrow \text{GOx(FAD)} + \text{H}_2\text{O}_2$$

where FAD is flavine adenine dinucleotide.

Prior to the self-assembled monolayer (SAM) formation, reactor was cleaned with 2.0 mol L^{-1} $HClO_4$ for about 15 min, followed by drying with nitrogen for 15 min. Next, 1 mL of the solution [0.01 mol L^{-1} MUA; 0.01 mol L^{-1} $HClO_4$, 70% EtOH] was passed through reactor with flow rate $v = 0.016$ mL min^{-1} for 30 min at room temperature to ensure reliable SAM formation. To remove all unbound and physisorbed MUA molecules, the reactor was rinsed for 5 min with EtOH. It is known that some proteins denature when they get into contact with metal surface. Chemisorbed thiol monolayer not only prevents a contact of enzyme with the metal surface but it (electro)chemically blocks this surface. This substantially reduces releasing of mercury and silver cations which can be inhibitors of the enzymatic reaction.

To create a stable amide bond between thiol and the enzyme by coupling, —COOH groups of thiol must be activated by EDC/NHS chemistry. Carboxylic groups of MUA can react to NHS in the presence of carbodiimide such as EDC, resulting in a NHS ester which may then react with amine groups of GOx. Therfore, for activation of —COOH groups of MUA, at first the reactor was washed 5 min with [0.10 mol L^{-1} MES, 0.15 mol L^{-1} NaCl; pH 5.0] and then, 1 mL of freshly prepared solution of [0.04 mol L^{-1} EDC, 0.10 mol L^{-1} NHS, 0.10 mol L^{-1} MES, 0.15 mol L^{-1} NaCl; pH 5.0] was passed through the reactor with $v = 0.020$ mL min^{-1} for 30 min. The reactor was then washed with 0.20 mol L^{-1} PB (pH 7.1) for 5 min. Stability of the created NHS-ester is dependent on the acidity of the solution. The half-time of the ester hydrolysis is 4–5 h at pH 7.0 and 10 min at pH 8.6 [24]. Anchoring of GOx was carried out in 1 mL of 2.0 mg mL^{-1} GOx in 0.10 mol L^{-1} PB (pH 7.1) for 60 min with $v = 0.016$ mL min^{-1}, when GOx was covalently attached at the activated MUA monolayer. The GOx modified reactor was then thoroughly rinsed with mobile phase used for determination of glucose – [0.10 mol L^{-1} AcB; 1 mmol L^{-1} Na_2EDTA; pH 6.5]. The function of Na_2EDTA in MP is bonding silver and mercury cations which often deactivate enzymes.

The described procedure should be universal and it was successfully used, e.g., for deposition of cholesterol oxidase at the surface of the porous AgSA-reactor.

3. Results and discussion

To test the proposed amalgam reactor, glucose oxidase was selected as one of the most studied and stable enzymes. During the enzymatic oxidation of glucose, oxygen is consumed for the conversion of enzyme to its original active state. Decrease of O_2 concentration in the mobile phase, which is proportional to the glucose concentration, can be registered electrochemically. The electrochemical reduction of oxygen usually proceeds via two well-separated two-electron steps [36,37]. The first step is due to the reduction of oxygen to hydrogen peroxide:

$$\text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2\text{O}_2\text{(acid solution)}$$

or

$$\text{O}_2 + 2\text{H}_2\text{O} + 2\text{e}^- \rightarrow \text{H}_2\text{O}_2 + 2\text{OH}^-\text{(neutral or alkaline solution)}$$

The second step corresponds to the reduction of the hydrogen peroxide:

$$\text{H}_2\text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow 2\text{H}_2\text{O(acid solution)}$$

or

$$\text{H}_2\text{O}_2 + 2\text{e}^- \rightarrow 2\text{OH}^-\text{(alkalinesolution)}$$

The half-wave potentials of these steps are approximately −0.1 and −0.9 V (vs. saturated calomel electrode) [37]. It is seen that oxygen can be determined according to the first two-electron step at low negative potentials (if platinum, gold or other detection electrodes with low hydrogen overvoltage are used) or by the overall four-electron step at potentials about −1 V or higher (with mercury or amalgam electrodes). In practice, this leads to double increase of peak current (sensitivity of measurements).

By the above mentioned enzymatic reaction, when one oxygen molecule is consumed, one molecule of hydrogen peroxide is created. Oxygen and hydrogen peroxide are reduced electrochemically in accordance with the above described scheme. If the enzyme has been immobilized at the detector (working electrode) surface, it is possible to register the reduction or oxidation of hydrogen peroxide (which is normally done for such type of biosensors). In our biosensor there is a much larger distance between the immobilized enzyme and the detector. During the path between reactor and detector, in our opinion, H_2O_2 (oxidizing agent) chemically reacts with that glucose (reducing agent) which did not react with the enzyme. In this way, peroxide is converted into water which is electrochemically inactive at the applied detection potential of −1.1 V. If peroxide was not chemically neutralized, the peak current would correspond to 2-electron reaction, because the decrease of oxygen concentration in the solution (decrease of the current) corresponds to the 4-electron reaction and the increase of the current due to the reduction of H_2O_2 is the 2-electron reaction. We cannot yet determine how many electrons correspond to a summary electrochemical reaction and it is possible that we register the peak which is the result of several of these processes.

From voltammetric/amperometric point of view, the silver solid amalgam detector really is here the working electrode though in the

special tube form. We have used the term "detector" because it is widely spread in flow methods. Amalgam detector/working electrode in this work is the place where an electrochemical reaction is performed, namely it is hydrogen peroxide reduction. Enzymatic reaction is taking place separately in the reactor and hence the term "biosensor" (in this work) should include both reactor and detector.

Various aqueous solutions were tested to find the optimal composition and concentration of the mobile phase. 0.1 mol L^{-1} acetate buffer of pH 5.5–6.5 was shown as best. Silver and mercury cations are inhibitors of glucose oxidase, and hence the strongly complexing Na_2EDTA was added in mobile phase. The chosen and used MP composition was: [0.1 mol L^{-1} AcB; 1 mmol L^{-1} Na_2EDTA; pH 6.5].

The common measurement procedure was as follows: (i) amperometric registration of the detector current response in the solution of the mobile phase. The measured current corresponds to reduction of oxygen dissolved in the mobile phase; (ii) injection of the solution containing glucose and performing another amperometric record. Glucose reacts with GOx in the reactor and oxygen is consumed from the mobile phase. This decrease of oxygen concentration in the mobile phase causes as the decrease of current and it is graphically represented as a reverse peak. The difference between the reduction current of oxygen in MP and the same current in MP + glucose is proportional to the glucose concentration in the sample solution (in a certain concentration range).

3.1. Effect of detection potential on the response of the biosensor

Effect of detection potential on the biosensor response is shown in Fig. 2A. As can be seen, the peak current increases with increasing negative potential to −1 V, and then it is practically unchanged up to −1.5 V. At higher potentials, the repeatability of measurements was substantially worse probably due to the evolution of hydrogen which forms microbubbles on the surface of the detector and uncontrollably changes the active area of the detector. It is necessary to mention that the increase in potential leads to an increase in the baseline current as well. This phenomenon does not affect negatively the sensitivity of measurements because the noise of the whole system is aproximately the same and the current baseline is subtracted from the current peak. Detection potential −1.1 V was chosen as the optimum for maximum sensitivity and for the best repeatability of measurements (see below). This detection potential was used for all further experiments.

3.2. Effect of the flow rate on the response of the biosensor

The speed of movement of the solution through the flow system significantly affects the efficiency of the enzymatic reactor and of the detector response. The enzymatic reaction has a certain rate, depending on the time of contact of the enzyme with the substrate. It can be assumed that the lower is the flow, the higher decrease of oxygen concentration in the solution will be observed. The flow rate (v) also affects the response of the detector, and for different substances such dependence may have the opposite effect [15]. The data in Fig. 2B show a complex effect of the flow rate on the reactor and detector. The maximum peak current was observed at $v = 0.04$ mL min^{-1} (scan time $t_{scan} = 360$ s). Only at the lowest tested rate ($v = 0.02$ mL min^{-1}; $t_{scan} = 600$ s) the response is diminished, but also repeatability and the peak shape significantly deteriorated probably due to an unstable flow. To select the optimum flow rate some compromise was sought between the biosensor response and the duration of a single measurement. Recovery time of the enzyme activity after each glucose measurement had also to be taken into account. It was found that if the time between the injections of glucose was less than 220–240 s, the biosensor response gradually decreased. Scan time 240 s is sufficient for reliable recording of the whole peak at $v = 0.1$ mL min^{-1}, and therefore this flow rate was chosen as optimal and used for further measurements.

3.3. Precision of measurements

Repeatability was determined by evaluation of peak heights (I_p) of 11 replicate injections of model samples of glucose ($c = 4.0 \times 10^{-4}$ mol L^{-1}). The biosensor provided average response −2.093 μA with confidence interval of 0.025 μA, standard deviation 0.038 μA and relative standard deviation 1.83%.

In order to determine the intra-days reproducibility of the biosensor, 5 repetitive measurements of 4.0×10^{-4} mol L^{-1} model sample of glucose were performed every 5 days. When the reactor with the immobilized enzyme was not used, it was stored in microtube with mobile phase at 4 °C. The results of this monitoring indicated that 77% of the current response of glucose was retained after 35 days. Nevertheless, despite this decrease of the response the biosensor could be successfully used further, but for evaluation of the data a standard addition method or comparison the results of measurements of samples with the measurement of the glucose standard solution should be applied.

3.4. Relation between biosensor current response and glucose concentration

Under optimum conditions (MP [0.10 mol L^{-1} AcB; 0.001 mol L^{-1} Na_2EDTA; pH 6.5]; $E_d = -1.10$ V; $v = 0.10$ mL min^{-1}; $V_{inj} = 50$ μL), the calibration dependence was measured over the concentration range 2.0×10^{-5}–2.0×10^{-3} mol L^{-1}. The peak current I_p was a linear function of concentration ($I_p = -0.04$ to $-5.03c$; $R^2 = 0.9995$) in the range $2 \times 10^{-5} - 8 \times 10^{-4}$ mol L^{-1}. The lower limit of detection was 1.0×10^{-5} mol L^{-1}. FIA-ED recordings and calibration graph are depicted in Fig. 2C. Very good parameters of the linearity confirm that the measurement conditions were chosen correctly, and that this biosensor is suitable for measuring the concentration (content) of glucose in real samples.

3.5. Biosensor selectivity and interferences

The possible interfering compounds commonly present in honey are carbohydrates, proteins, amino acids, carboxylic acids (e.g., gluconic acid, citric acid, succinic acid), vitamins (e.g., C, B_1, B_2) or minerals (e.g., Ca^{2+}, Mg^{2+}, K^+, Zn^{2+}). Apart of glucose the most often represented carbohydrates in honey are fructose, maltose and sucrose [38]. Three repeated injections of 1.0×10^{-3} mol L^{-1} solutions of these compounds were carried out. No changes of the baseline were observed which confirms the high selectivity of GOx. Widely spread ascorbic acid is reduced at −1.7 V and it cannot influence the measurement of glucose which takes place at −1.1 V. The total concentration of the rest of potential organic interferences mentioned above is low [39] and they are primarily oxidizable species. The trace concentrations of reducible heavy metal cations are then eliminated in presence of strong chelating agent Na_2EDTA in mobile phase.

3.6. Practical application: determination of glucose in a commercial honey

Determination of glucose in real samples was the next step in the biosensor testing process. In this paper, a commercial honey with declared glucose concentration 32–37 mass % was used. Concentration of glucose in honey depends on many factors and it often ranges from 30 to 40 mass %. Literature describes the determination of glucose in honey by kits in comparison with electrochemical biosensors. There are some results of the compared analyses of

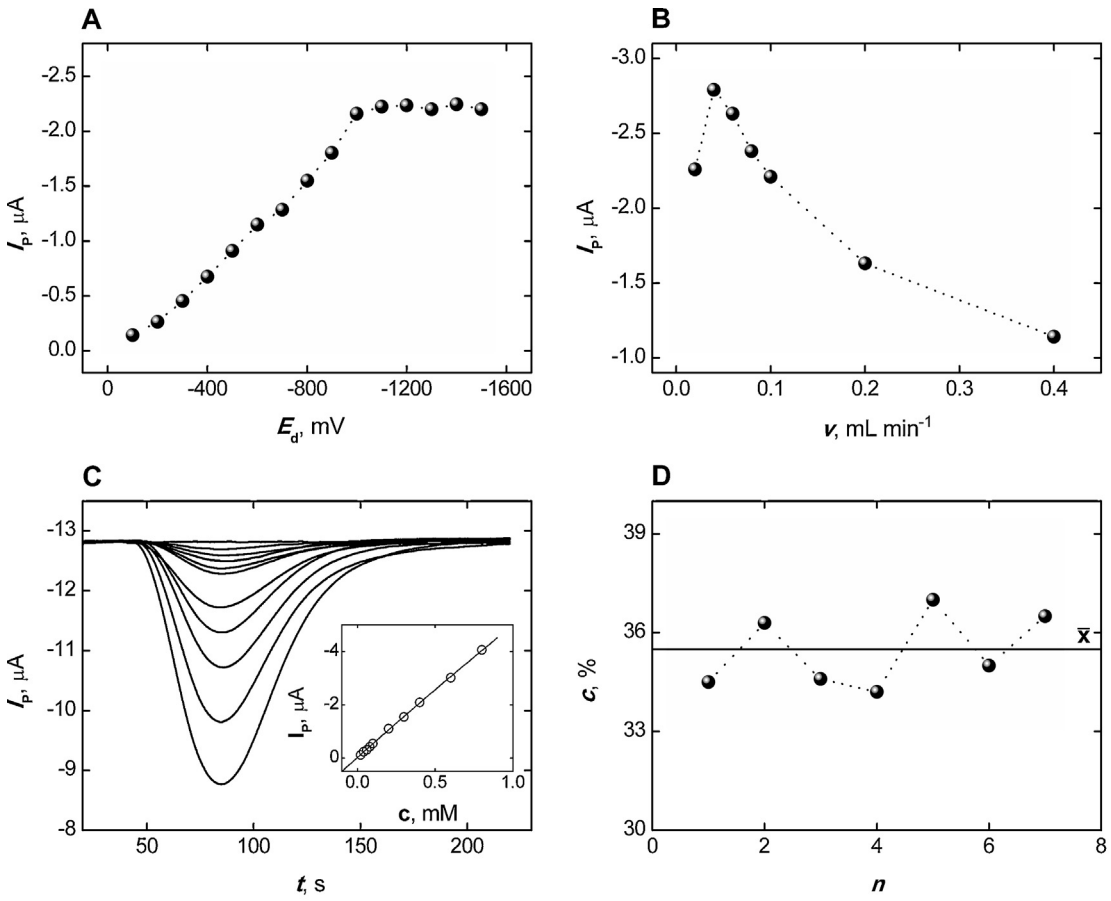

Fig. 2. (A) Dependence of the peak current I_p of glucose in model sample on the detection potential E_d. (B) Dependence of I_p on the flow rate v of the mobile phase for determination of glucose in model sample. (C) FIA-ED recordings of glucose in the mobile phase and in the model samples in concentration range 2.0×10^{-5}–2.0×10^{-3} mol L^{-1} (the calibration graph is in inset). (D) Reproducibility of the determination of glucose in seven samples of the same honey. Experimental conditions: (A–D) MP [0.10 M AcB; 0.001 M Na_2EDTA; pH 6.5]; V_{inj} = 50 μL. (A) c(glucose) = 4.0×10^{-4} mol L^{-1}; v = 0.10 mL min^{-1}. (B) c(glucose) = 1.0×10^{-3} mol L^{-1}; E_d = −1.10 V. (C) E_d = −1.10 V; v = 0.10 mL min^{-1}. (D) c(glucose) = 4.0×10^{-4} mol L^{-1}; E_d = −1.10 V; v = 0.10 mL min^{-1}.

glucose in various honeys by two independent methods: by colorimetric glucose assay kit 35.0 mass % and by electrochemical biosensor 34 ± 1 mass % [40]; by glucose assay kit 35.1 mass % and by electrochemical biosensor 31.6 mass % [41]; by enzyme kit 39 ± 4 mass % and by electrochemical biosensor 36 ± 2 mass % [42].

In our method, seven samples of the same honey were analyzed using the proposed biosensor. Concentration of glucose 35.5 ± 1.0 mass % (n = 7; SD = 1.2%; RSD = 3.2%; see Fig. 2D) was determined. This value was obtained by comparing the results of measurements of the honey samples with measurement of the glucose standard solution (5.0×10^{-4} mol L^{-1}) in the same mobile phase. The results show that the suggested method is simple and quick and the obtained concentration of glucose corresponds well with the declared and literature values.

4. Conclusions

In this work, the enzymatic porous reactor based on silver solid amalgam was constructed and tested. A large surface of the porous reactor ensured a high efficiency of the enzymatic biosensor and the covalent bonds amalgam–thiol–enzyme effectively contributed to long-term stability. Connecting the reactor directly before the tubular detector allows a precise determination of decrease of oxygen concentration during enzymatic reaction. High hydrogen overvoltage of the silver solid amalgam detector enables to apply detection potential more negative than −1.0 V which increases the sensitivity of oxygen measurement.

Under optimum conditions the lower limit of detection of glucose was 1.0×10^{-5} mol L^{-1} with RSD of the measurements repeatability 1.3% (n = 11). Calibration dependence of glucose was linear over the range 2.0×10^{-5}–2.0×10^{-3} mol L^{-1} (R^2 = 0.9995). The low limit of detection and the obtained linear range of concentration dependence allow working with smaller amounts of samples containing glucose (honey, blood, juices, etc.). If glucose concentration in the sample is higher than the linear range, the sample in most cases can be diluted by a mobile phase. The proposed biosensor was successfully used for determination of glucose in commercial honey as a real sample.

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