Flow-injection Measurements with Miniaturised Amperometric Biosensors with Supported Bilayer Lipid Membranes

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Introduction

The use of artificially self-assembled lipid membranes seems to be a very attractive method for design of chemical and biochemical sensors and detectors. In numerous studies this possibility was already explored for producing free-suspended bilayer membrane (BLM) systems for analytical applications [1-7], filter supported sensors [8,9], as well as solid-supported systems [10-19]. The incorporation in the structure of these membranes suitable ion-carriers [2,12,15], proteins [3,4,8,17], or attachment to their surface suitable biomolecules [11,13, 14,19] provides chemical or biochemical sensing systems of satisfactory dynamic properties, selectivity and sensitivity to various analytes. For practical applications of such sensors a sufficient long-term stability, repeatability of signal, reproducibility of their preparation and satisfactory selectivity is indispensable. These practical aspects from the point of view of real analytical applications were rarely discussed so far in cited above papers.

Due to very simple preparation and easy handling especially solid-supported systems (s-BLM) offer more advantages than other configurations. The used solid supports can serve directly as convenient internal electrochemical detection system where amperometric, potentiometric or conductimetric measurements can be employed. Such design facilitates easy manipulation either with sensor itself or with solutions, where measurements are carried out.

With a very delicate structure of BLM even when it is formed on solid-support, each transfer of BLM sensor from one solution to another is a potential danger of mechanical destroying of BLM layer. For this reason is seems to be particularly favourable to use this kind of device in measuring system where all steps such as preliminary conditioning of sensor, calibration, and measurements in analysed samples can be performed with transfer of sensor between solutions. This type of handling the most easily can be realized in flow-injection analysis (FIA) system, where sensor is permanently conditioned in appropriate buffering solution, whereas analytical signals are recorded as transient response is segments of analyzed solution injected to the measuring set-up. With expense of decrease of absolute magnitude of

measured signal, in comparison to equilibrium measurements, a significant improvement of precision of measurements and stability of measuring conditions can be attained.

FIA measurements with BLM sensors have been already reported in several papers. Study of interaction of lipid membrane with valinomycin was carried out using thin-layer flow cell with free-suspended BLM [1]. At flow-rate 0.7 ml/min the recording of single response to valinomycin injection took about 30 min, and the system was extremely sensitive to pressure fluctuations caused by peristaltic pump. In a very similar system the interaction of aflatoxin with BLM was also investigated [4]. Very fast and short FIA signal of duration below 10 s was attributed to alterations of the electrostatic field at the surface of BLM due to the adsorption of analyte. The same behaviour in FIA measurements was observed for several herbicides using filter-supported BLM [9]. In yet another case a cell with filter supported BLMs incorporating enzymes for determination of several substrates of hydrolytic enzymes was employed [8]. Hydronium ions produced by the enzymatic reaction caused also very fast dynamic alterations of electrostatic fields and phase structure of BLMs, and as result ion current transients of about 10 s were obtained of magnitude proportional to concentration of substrates. In all these cases measured signals were in picoampere range.

In this work an attempt was made to determine the possibility of the use in FIA measurements solid-supported BLM biosensors with enzyme attached to the outer BLM surface. For this purpose the earlier developed miniaturised amperometric glucose biosensor was used, where on the steel disk surface BLM was formed from biotinylated lipid, to which conjugate of avidin with glucose oxidase (GOD) was attached [19]. The aim of this work was to examine analytical functional properties of such system.

EXPERIMENTAL

Apparatus

All measurements concerning the preparation and non-flow measurements with glucose BLM biosensor were carried out in conventional three electrode system using computerized electrochemical set-up AUTOLAB from Eco Chemie (Utrecht, Netherlands) with reference Ag/AgCl electrode and Pt foil auxiliary electrode.

The flow-injection set-up used in this study consisted of a multichannel peristaltic pump Minipuls 2 from Gilson (Viliers-le-Bel, France), a rotary injection valve Rheodyne model 5020 (Cotati, CA, USA) with 100 µl loop, a voltammograph CV-37 from Bioanalytical Systems (West Lafayette, IN, USA) and a strip chart recorder Kipp and Zonen type BD 111 (Delft, Netherlands). A two-line flow-injection system was used, where the sample was injected into a stream of deionized water, which was merged with a stream of 50 mM Tris·HCl and KCl buffer of pH 7.0. A large volume wall-jet cell arrangement was used in flow measurements (Fig. 1).

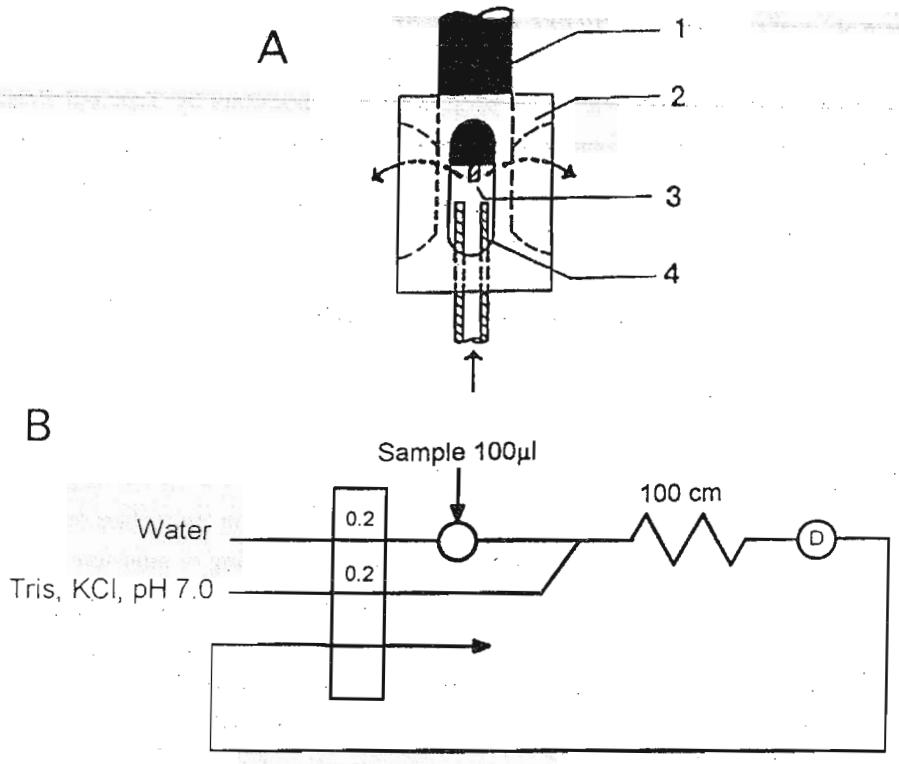

Fig. 1. A - Wall-jet detector arrangement with BLM miniature glucose sensitive biosensor:
1 - teflon-coated stainless steel wire in Perspex holder;
2 - wall-jet cap;
3 - sensing electrode surface with BLM formed;
4 - flow direction from FIA manifold.
B - Scheme of FIA two-line manifold.

As working electrodes in the design of biosensor, Teflon coated stainless steel wire of 0.33 mm diameter from Leico Industries (New York, USA) was used, together with a Pt foil auxiliary and silver/silver chloride K 801 from Radiometer (Copenhagen, Denmark) was used as a reference electrode.

Reagents

For the preparation of glucose biosensor *N*-(biotinoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (biotin DHPE) from Molecular Probes Europe (Leiden, Netherlands) was used. Avidin-glucose oxidase conjugate was prepared using avidin, and glucose oxidase of activity 138 U/mg from Sigma as described earlier [14].

Tris(hydroxymethyl)aminomethane (SIGMA 7-9®) was purchased from Sigma. All other reagents used were of analytical grade from POCh (Gliwice, Poland).

Preparation of s-BLM-based glucose biosensor

Glucose *s*-BLM biosensor was prepared according to procedure by Tien and Salamon [22] by immersing freshly cut tip of the working electrode in lipid solution for 1 min, and then in avidin-GOD conjugate also for 1 min. It was stored between measurements in buffer Tris·HCl/KCl in refrigerator.

RESULTS AND DISCUSSION

Detector and FIA system configuration

With regard for the shape and size of biosensor used, the most proper configuration of the flow detector was wall-jet cell with free outflow of the solution from the surface of miniature disc biosensor (Fig. 1A). Such a cell is easy to assemble and use. Placing of miniature biosensor in it does not create any danger of mechanical damage of BLM structure on the working detector surface. It was successfully applied by numerous authors in measurements with amperometric as well as potentiometric detection, and also used in HPLC measurements. For tested flow-rates ranged from 0.5 to 2.0 ml/min the optimal distance between the end of outlet tube and the electrode surface was 2.0 mm. The composition of external solution did not influence the magnitude of recorded signal when this distance was used and the stable base line was obtained, too. Stainless steel wire of 0.33 mm diameter was easy to place it in perspex body following by BLM formation on its surface and then to fit the wall-jet cap on the ready electrode holder.

The measurements were carried out in two-line FIA manifold (Fig. 1B) where sample of 100 µl volume was injected into the carrier stream of distilled water merged with buffer stream (50 mM Tris in 50 mM KCl solution, pH 7.0).

As in the case of any amperometric detection, the main parameter influencing the height of FIA signal was the flow-rate. In the used FIA manifold the additional limitation was high mechanical sensitivity of bilayer lipid membrane formed. The increase of flow-rate favoured the transport of analyte towards biosensor surface but also decreased the time of interaction between the sample segment reaching the surface of the detector and the sensing layer of biosensor containing an enzyme. In the tested range of total flow-rate from 0.5 to 2.0 ml/min the maximum signal was at 1 ml/min (i.e. 0.5 ml/min in each stream). The dependence of the signal height and the total flow-rate is shown in Fig.2.

Response characteristics in FIA system

The shape of the signals obtained in FIA manifold is presented in Fig.3 for glucose concentration in standard samples in the range from 2 to 20 mM. The precision for multiple

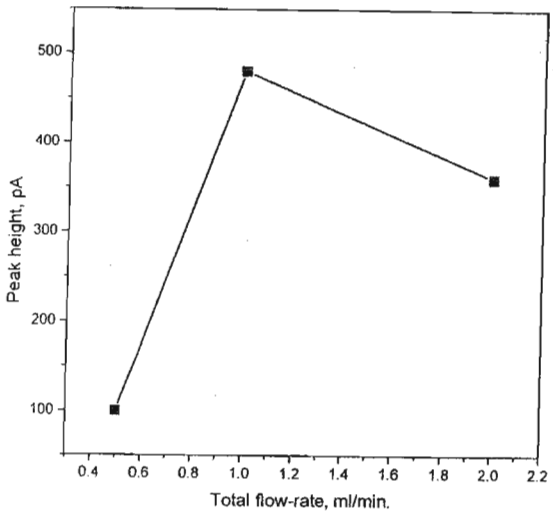

Fig. 2. The dependence of signal height on total flow-rate for BLM glucose sensitive miniaturized biosensor obtained in FIA manifold.

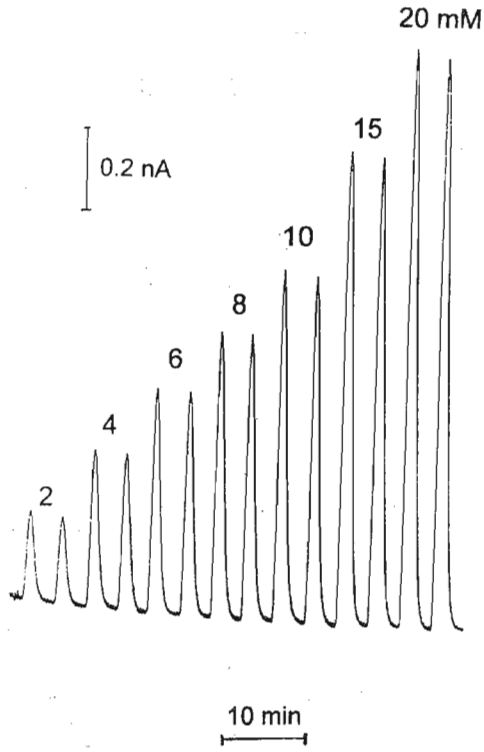

Fig. 3. Flow-injection response recorded for BLM glucose biosensor at total flow-rate 1 ml/min.

injection of the same standard solution was from 1 to 3 % estimated as RSD (n = 5-8). The limit of detection for recorded noise level was 0.3 mM of glucose (S/N = 3).

The essential advantage of FIA measurement was the possibility of avoiding of biosensor transfer from one standard solution to another. Such a procedure favoured the stable operation of the whole measurement system and did not expose the sensitive biosensor surface (BLM) to risk of accidental disturbance. Because of the transient character of FIA signal the sensitivity of the measurement is lower than obtained for the equilibrium measurement (Fig.4B). The recording for the same biosensor obtained in equilibrium conditions (Fig.4A) clearly shows that after the time of the FIA peak maximum reaching (40 s) only 75 % of equilibrium signal was obtained. Its further decrease should be mainly attributed to the dispersion in FIA manifold.

As mentioned earlier in free-suspended BLM systems where the signal is form only due to disturbance of electrostatic field at the surface of membrane, the width of the peak starting from the base line until it is reached again was in the range from few seconds [7] to 30 minutes [1]. The values ranged in seconds were also reported for interaction of herbicides with filter supported BLM [9] and for filter supported BLM with incorporated enzyme [8]. The

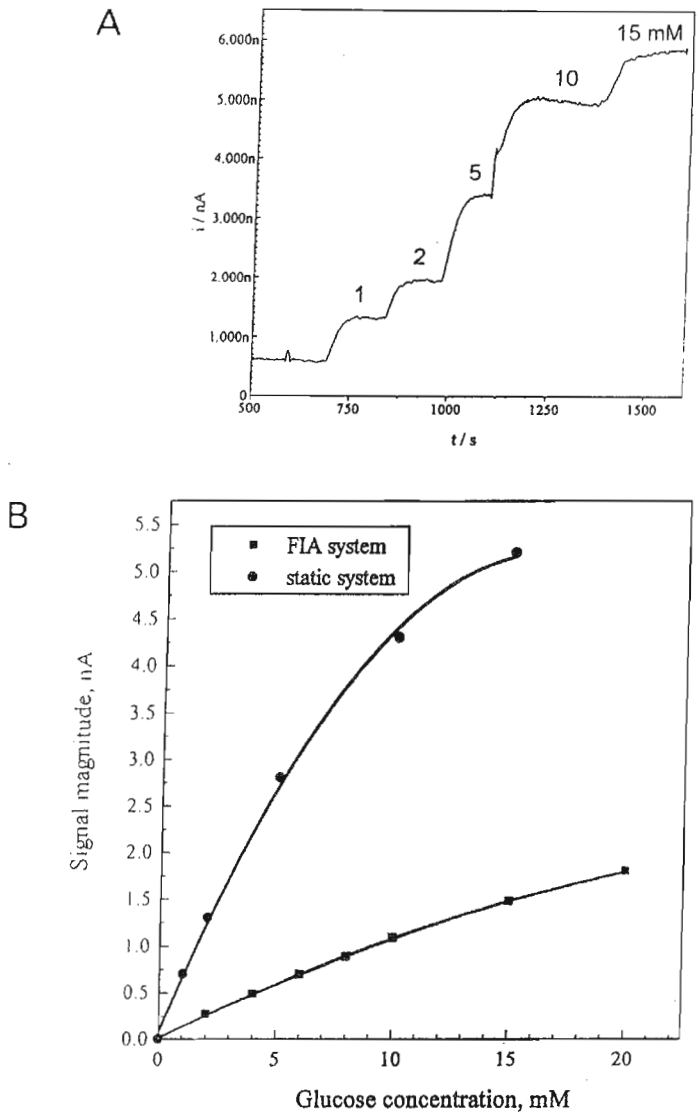

Fig. 4. A - The recording of equilibrium signals for BLM-GOD biosensor in Tris/KCl buffer of pH 7.0. Concentration of glucose standards shown in the Figure.
B - Comparison of calibration plots obtained in FIA and equilibrium conditions.

mechanism of glucose detection in the biosensor described in this work is based on the diffusion of hydrogen peroxide formed as a product of enzymatic reaction in BLM with immobilized GOD to the electrode surface and subsequent anodic oxidation. For the glucose concentration in millimolar range the maximum of the peak was attained after *ca.* 40 sec. and the return to the base line after 2 min. Hence the total time of the peak formation was *ca.* 3 min. enabling the sample throughput of about 20 h^{-1}.

During enzymatic reaction catalyzed by GOD also gluconic acid is formed what can be utilized for the potentiometric following of the process, *e.g.* [23]. However, no fast spikes of

the signal were observed in this case after several seconds since injection as it was reported for FIA response of BLM loaded with hydrolytic enzymes [8].

Biosensor properties

BLM based biosensors are extremely interesting systems from the point of view of biophysics of formation of membrane system, which mimics the natural lipid membranes and the electric signals obtained as a result of various chemical interactions. The measurement system of analytical utility should be characterized by satisfying long-term stability and selectivity in the case of numerous known and applied designs. There are only few works among reported here concerning metal supported BLMs where one can find some discussion on this subject. The long-term stability of gramicidine-based biosensor for ammonia was only 48 h [15], and that of valinomycin based one sensitive to K^+ ion was evaluated as 120 h [12]. The stability of BLMs for herbicides [18], and cyanide [17] was better than 48 h. BLM-based biosensor for xanthine with streptavidin was stable during 5 days but the same system with GOD showed 70 % of initial sensitivity after two weeks [11].

The stability of BLM-based biosensor depends on the stability of chemical components forming bilayer structure, the way how it is used, the chemical conditions and the temperature of storage between measurements. In this study the stability was tested by comparison of parameters of calibration curves performed in FIA system during successive days of biosensor operation in the glucose concentration range from 1 to 20 mM. Between measurements the biosensor was stored in Tris/KCl buffer at room temperature. The bisoensor stored at thistemperature, showed the continuous decrease of sensitivity and after 4 days it was only less than 40 % of initial value, although during the whole period its response was fairly linear up to 16 mM of glucose concentration (Fig. 5A). When stored at low temperature during the first 8 days even the slow increase of the sensitivity of the response was observed (Fig. 5B). After 13 days of the biosensor functioning, despite non-linear response, the sensitivity decreased down to 15 % of the maximum sensitivity. Practically when stored in refrigerator the BLM-based glucose biosensor can be used in FIA system during one week without any loss of sensitivity.

A significant question to answer is whether the main reason of the decrease of biosensor sensitivity is low stability of avidin-GOD conjugate, or biotinylated lipid, deactivation of enzyme or gradual destruction of lipid bilayer connected with continuous decrease of the amount of immobilized conjugate. In order to answer this question the sensitivity of few biosensors obtained from the same batch of biotinylated lipid and of two freshly prepared lots of avidin-GOD conjugate according to the earlier procedure [14] was examined. The former studies on avidin-GOD [14] and avidin-urease [19] conjugate have showed very limited repeatability of preparation of such biosensors. The initial sensitivity may vary even few times. The data obtained in our study support this observation (Fig. 6). No regularity was also obtained for the dependence of the initial sensitivity on the time of avidin-GOD conjugate or

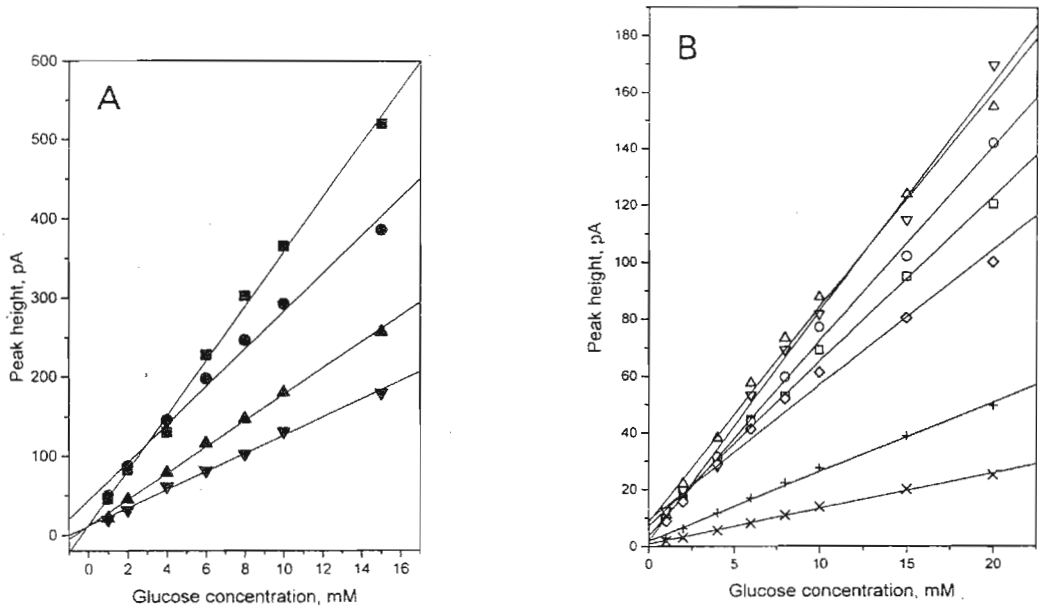

Fig. 5. Calibration plots obtained in FIA system for BLM glucose biosensor stored at room temperature (A) and in refrigerator (B) in Tris/KCl buffer.

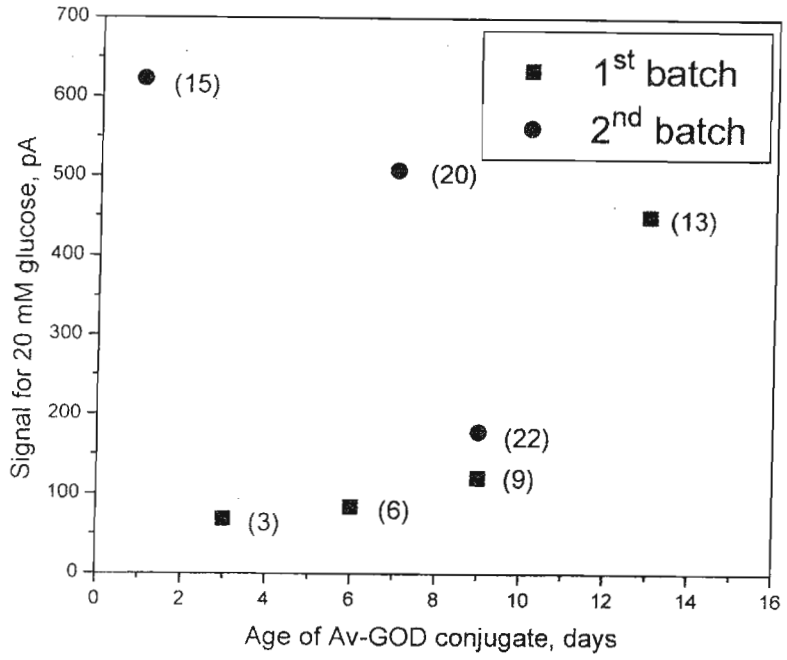

Fig. 6. Magnitude of flow-injection signal height for 20 mM glucose for BLM glucose biosensors prepared from different batches of avidin-GOD conjugate and the same biotinylated phospholipid. Numbers in parentheses indicate the age of lipid batch (in days).

Table 1. Relative response of glucose BLM biosensor on steel support in FIA system compared to 20 mM glucose for sensor configuration with working electrode surface uncovered and covered with Nafion layer.

Interferent	Relative response, %	
	Without Nafion	With Nafion
0.2 mM ascorbic acid	112	16
1.0 mM uric acid	116	94
0.2 mM paracetamol	8.3	6.4

biotinylated lipid ageing. However, this experiment does not decide whether the reason of the sensitivity loss during time is a result of enzyme deactivation or the slow destruction of BLM's structure causing the progressive loss of conjugate form BLM surface..

In the case of every biosensor with immobilized enzyme form oxidase group not containing any mediator when amperometric detection of hydrogen peroxide formed as a product of enzymatic reaction is carried out one can expect serious interference from electroactive components of the matrix of analyzed sample, which can be oxidized at the applied potential. In the above described biosensors for glucose based on BLMs with streptavidin [11], or xanthine [13] conjugates, only strong interference from ascorbic acid was observed and not from uric acid. In our study the strong interference from ascorbic acid was confirmed, but also uric acid interfered (Table 1). The modification of stainless steel electrode surface with the layer of cation exchanger Nafion before BLM was formed essentially reduced the interference of ascorbic acid which at pH 7 occurs only in anionic form. However this procedure decreased the uric acid interference only slightly. Since protonation constants of tested acids are almost the same (pK_a values for ascorbic and uric acid are 4.10 and 3.89 [24], respectively), and both exist at pH 7.0 in anionic form, the explanation of such different behaviour of both acids towards BLM-based biosensor requires further studies. According to our previous work on lactate biosensors the effective removal of urate interferences can be obtained using the additional polypyrrole layer [25].

CONCLUSIONS

s-BLM-based glucose biosensors can be successfully applied in FIA system. The proper storage conditions allow for about one week use without any loss of sensitivity. Dynamic properties are good enough to obtain the sample throughput 20 h^{-1} in FIA measurements. The biosensor response is linear up to 20 mM of glucose with detection limit on submillimolar level. The interferences of anionic forms of electroactive components of the sample can be removed with additional layer of cationic exchanger Nafion.

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