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Biosensor for dopamine based on stabilized lipid films with incorporated resorcin[4]arene receptor

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Abstract

This work reports a technique for the stabilization after storage in air of a lipid film with incorporated resorcin[4]arene receptor based biosensor for dopamine. Microporous filters composed of glass fibers (nominal pore sizes, 0.7 and 1.0 μm) were used as supports for the formation and stabilization of these devices and the lipid film is formed on the filter by polymerization prior its use. Methacrylic acid was the functional monomer, ethylene glycol dimethacrylate was the crosslinker and 2,2′ -azobis-(2-methylpropionitrile) was the initiator. The stability of the lipid films by incorporation of a receptor for the preparation of stabilized lipid film biosensor is studied throughout this work. The response towards dopamine of the present stabilized for repetitive uses lipid membrane biosensor composed of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidic acid was compared with planar freely suspended bilayer lipid membranes (BLMs). The stabilized lipid membranes provided similar artificial ion gating events as BLMs in the form of transient signals and can function for repetitive uses after storage in air. However, the response of the stabilized lipid films was slower than that of the freely suspended BLMs. This will allow the practical use of the techniques for chemical sensing based on lipid films and commercialization of these devices, because it is now possible to prepare stabilized lipid film based biosensors and store them in the air.

Keywords: Biosensor; Lipid membranes; Stabilization; Dopamine

1. Introduction

Perturbation of the structure of artificial lipid membranes can be monitored by electrochemical methods, and this offers opportunities for development of chemically selective biosensors [1]. A large number of biochemical reactions based on enzyme-substrate, antibody–antigen, lectin–saccharide and hormone–receptor interactions have been monitored by observation of the transmembrane ion current [2]. Electrochemical methods offer the simplest route for signal detection than other techniques such as optical, etc., techniques. Optical and differential scanning calorimetric methods have been widely used to explore the mechanism of signal generation.

Significant progress has recently been achieved in the design, analytical applications and stabilization of biosensors based on lipid films. This type of biosensor provides a generic method for transduction of selective binding events into an analytical signal, and offers advantages such as high sensitivity and fast response times. In addition, lipid films can be excellent host matrices for the maintenance of the activity of many biochemically selective species, such as enzymes, antibodies and molecular receptors. The analytical utility of these devices has recently been demonstrated in a number of experiments [3,4]. A recent review article provides the analytical applications of lipid-based biosensors [2].

The inherent fragility of freely suspended bilayer lipid membranes (BLMs) remains a major obstacle preventing the use of BLMs as practical biosensors. The membranes collapse in response to even weak mechanical or electrical shock. Bilayer lipid membranes that were stable to mechanical and electrical shock for periods of time greater than 48 h were reported in literature [5–10]. Self-assembled BLMs at the surfaces of freshly cleaved metal electrodes have been reported by a number of groups to be stable for uses in biological matrices [11–13]. For example, such metal-supported lipid films were found to be stable in urine and human serum for more than 5 h [12,13]. Microfiltration filters have been examined as mechanical supports for planar BLMs for flow through experiments [14]. However,

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in almost all reports about stabilized BLMs, the important criterion of stability of these membranes that is storage in air for repetitive uses has not been reported in literature up to date. No reports were given up to date that describe the use of lipid films based biosensors after storage in air. This has prohibited the scale-up production of these biosensors for possible commercialization.

This paper describes the design of the formation and use after storage in air of a stabilized lipid film based biosensor with incorporated with incorporated resorcin[4]arene receptor by polymerization on microporous filtering media such as glass fibers. The novelty of the present paper is the stabilization of lipid films after storage in air with an incorporated receptor. A technique for the preparation of polymerized filter-supported micro-BLMs and incorporation of the receptor is described herein, and is investigated in terms of simplicity for membrane preparation and for stability of BLMs after storage in air when are immersed in electrolyte solution. The response towards dopamine of these stabilized lipid membrane devices composed of dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidic acid (DPPA) was compared with planar freely suspended BLMs. These stabilized lipid films provide similar response to freely suspended BLMs (i.e., artificial ion gating events in the form of transient signals) and can function for repetitive uses after storing in air. However, the time response was larger as compared with the freely suspended BLMs. The present technique will make possible the practical use of techniques based on lipid films for chemical sensing and allow commercialization of these devices.

2. Experimental

2.1. Materials and equipment

Dipalmitoyl phosphatidylcholine (C18:0) (DPPC) and dipalmitoyl phosphatidic acid (DPPA) were supplied by Sigma (St. Louis, MO) and were used as lipids for the formation of BLMs and monolayer membranes. Dopamine was also purchased from Sigma. The functional monomer, methacrylic acid, and the crosslinker, ethylene glycol dimethacrylate, were both supplied by Aldrich (Aldrich-Chemie, Steinheim, Germany). The initiator, 2,2′-azobis-(2-methylpropionitrile) (AIBN), was supplied by Merck KgaA (Darmstadt, Germany). Water was purified by passage through a Milli-Q cartridge filtering system (Milli-Q, Millipore, El Paso, TX, USA) and had minimum resistivity of 18 MΩ cm). All other chemicals were of analytical-reagent grade. The filters and (nominal) pore sizes used were glass microfiber (0.7 and 1.0 μm, Whatmam Scientific, Kent, UK). The receptor molecule (2, 8, 14, 20-tetraundecylpirogallol[4]arene) was synthesized as follows: 3.2 ml of 12 M HCl was added into a 20-ml solution of pyrogallol and dodecanal in ethanol containing 1.26 g of pyrogallol and 1.84 g of dodecanal at 0 °C under argon. The mixture was refluxed for 6 h and the precipitate was filtered, washed with ethanol, recrystallized from acetonitrile and dried at 80 °C in vacuo. The yield (1.4 g) was a pink powder with melting point of 273 °C. ^{1}H NMR [$(CD_3)_2CO$] analysis has given the following results (ppm relative to TMS): δ 0.90 (t, 12H, Me), 1.30 (bs, 72H, CH_2), 2.35 (m, 8H, CH_2), 4.33 (t, 4H, methane), 7.15 (s, 4H, ArH), 7.24 (s, 4H, OH), 8.20 (s, 8H, OH). Water was purified by passage through a Milli-Q cartridge filtering system (Milli-Q, Millipore) and had minimum resistivity of 18 MΩ cm). All other chemicals were of analytical-reagent grade.

The apparatus for the formation of stabilized lipid films consisted of two plexiglass chambers separated by a Saran-Wrap™ (PVDC; DowBrands, Indianapolis, IN) partition of a thickness of ca. 10 μm). This apparatus has been described previously [15]. The lipid film is formed on a microporous filter by polymerization prior its use. Methacrylic acid was the functional monomer for the polymerization, ethylene glycol dimethacrylate was the crosslinker and 2,2′-azobis-(2-methylpropionitrile) (AIBN) was the initiator.

A Ag/AgCl reference electrode was placed in the circular hole and an external 25 mV d.c. voltage was applied across the lipid membrane between the two reference electrodes. A digital electrometer (Model 614, Keithley Instruments, Cleveland, OH) was used as a current-to-voltage converter. The electrochemical cell and electronic equipment were isolated in a grounded Faraday cage.

2.2. Procedures

Stabilized lipid films were prepared by polymerization as follows [16,17]: 5 mg of a mixed lipid powder comtaining 65% (w/w) of DPPC, 35% (w/w) DPPA and 0.26 mg of receptor were mixed with 0.070 ml of methacrylic acid, 0.8 ml of ethylene glycol dimethacrylate, 8 mg of 2,2′-azobis-(2-methylpropionitrile) and 1.0 ml of acetonitrile. Methacrylic acid was the functional monomer for the polymerization ethylene glycol dimethacrylate was the crosslinker and 2,2′-azobis-(2-methylpropionitrile) (AIBN) was the initiator. The mixture was sparged with nitrogen for about 1 min and sonicated for 30 min. This mixture could be stored in the refrigerator. For the preparation of the stabilized lipid films, 0.15 ml of this mixture was spread on the microfilter and was left at 60 °C for 12 h. The polymerization is described in Fig. 1. This microporous filter disk (diameter of approximately 9 mm) with the stabilized lipid film was placed between the two plastic layers, with the filter centered on the 0.32 mm orifice.

The Saran Wrap partition with the filter in place was then clamped between the plexiglass chambers. The partition extended beyond the limits of the edges of the chambers so that no ion current leakage could occur around partition. The presence of the lipid membrane was verified by the magnitude of the ion current obtained. When the ion

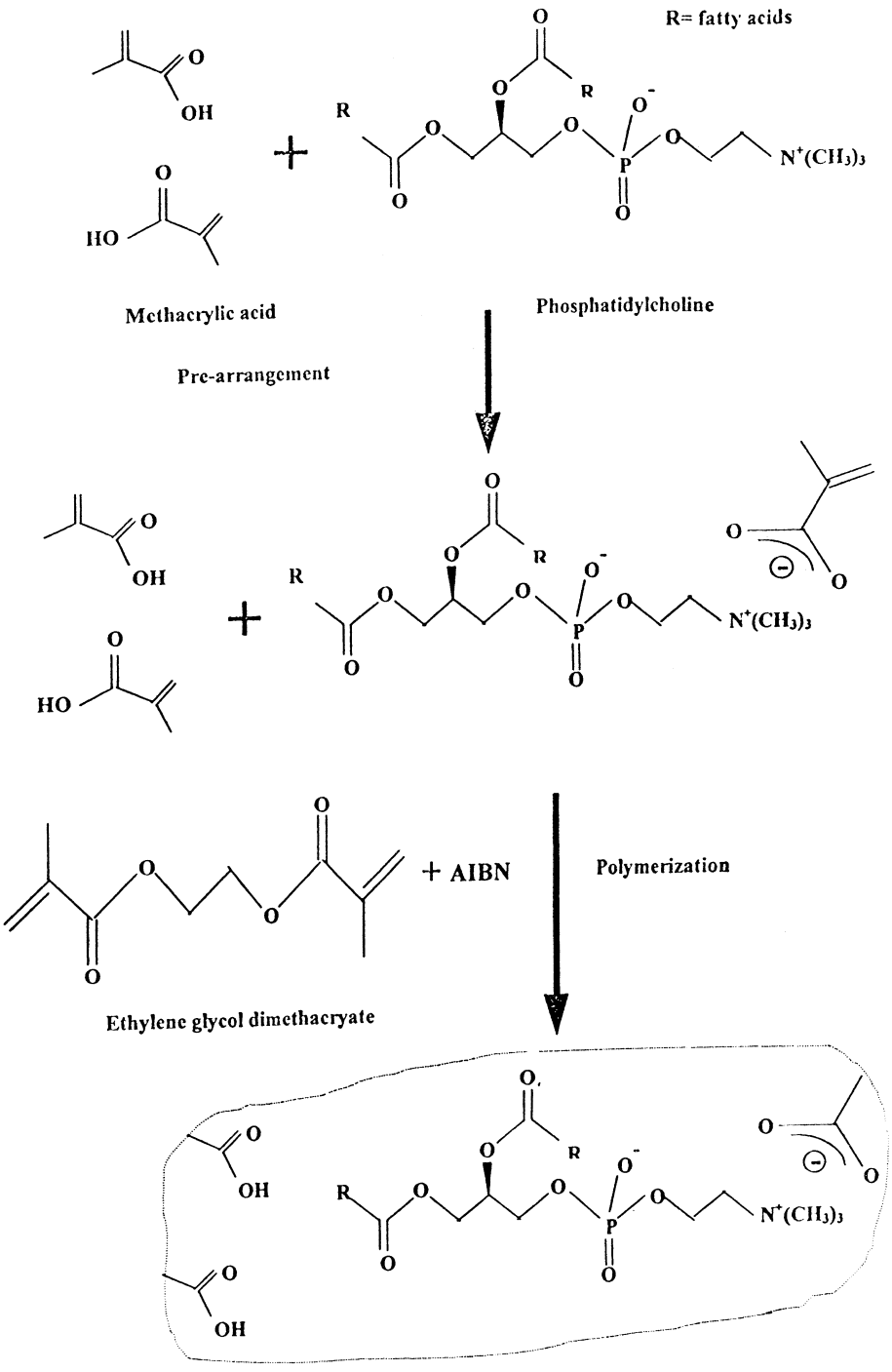

Fig. 1. A schematic version of polymerization stage and preparation of polymerized lipid membranes.

current stabilized (over a period of less than 5 min), the solution of dopamine was injected in one solution compartment using continuous gentle stirring. All experiments were done at 25 ± 1 °C. These membranes were stable in storage in air for repetitive uses. The lipid films were supported in a 0.1 M KCl electrolyte solution. The stock aqueous solutions of dopamine were 0.0100 and 0.0010 M. The dilute dopamine solutions were prepared daily just before use.

3. Results and discussion

The aim of this work was to establish a reliable method for preparation of stabilized in storage in air of lipid membranes with incorporated receptor that could be used after storage outside the electrolyte solution. The preparation of such stabilized in the air lipid films with incorporated receptor for repetitive uses has not been reported in literature up to date.

In this work aspects of filter-supported BLMs [14] were combined with molecular imprinted polymer [16] containing lipid to provide a basis for the preparation of these stabilized lipid films. Accordingly, an amount of 5 mg of lipid was found adequate for the formation of polymerized filter-supported BLMs. Over 99% of attempts of lipid film formation were successful and it was found that these lipid films could be reused after use and storage outside the electrolyte solution, making it unnecessary to reconstruct another lipid membrane.

The glass microfiber filters covered the standard 0.32 mm aperture that we have commonly used to prepare filter supported lipid membranes for flow through experiments [14]. These devices have been extensively studied and details of preparation of these devices are given elsewhere [14]. However, an important aspect of these biosensors for possible commercialization (i.e., stability of the membrane outside the electrolyte solution) has not been successful up to date. It has been found that these membranes can only be stable outside the electrolyte solution for only 10 min or less [18]. It was found that the electrolyte solution could be brought below the level of the elliptical hole for periods of only 10 min or less, and then could be raised again without failure of the BLMs. Such an experiment done over periods of more than 10 min would lead to disruption of the BLM network, likely due to loss of hydration at the membrane/electrolyte interface.

The observed stability of these lipid films for storage in air was a result of the use of supports with reduced diameters of aperture size [14] and polymerization structure of these films. Similar polymerization has been described in literature for preparation of molecular imprinted polymer [16]. The lipid is probably enclosed in the polymer through electrostatic forces [17]. These forces retain the lipid for multiple uses after storage in air and at the same time allow response similar to freely suspended BLMs.

Typical values for the specific resistance of the lipid membranes used in our studies were about 10^7 Ω cm^2. These results were calculated from the steady-state values of current when applying voltage increments of 20 mV in the range 0–300 mV. These values are similar to freely suspended BLMs. Measurements of membrane capacitance could indicate whether these films have a bilayer structure and will provide a value of membrane thickness [15]. However, the exact value of membrane capacitance in our case cannot be calculated because it is unknown whether a single lipid membrane occupies the total area of a filter paper, or whether an undefined number of micro-BLMs cover only the apertures of the filter.

The thickness of the polymerized filter-supported lipid films and whether these films were bimolecular was estimated by conductance alterations that were induced when the channel-forming agent gramicidin D was added to the electrolyte [15]. Injections of gramicidin-D at various concentration levels 1.0, 10 and 100 μM were made in the electrolyte solution, but the conductance did not increase. It is known that gramicidin does not induce conductance changes if the lipid membrane is thicker than one bilayer. These experiments have shown that the polymerized filter-supported lipid films do not have a bimolecular structure, but most probably consist of a multilayer of lipid. The structure of the present polymerized filter-supported lipid films is under further investigation by the use of scanning electron microscopy experiments.

The chemical response characteristics of these polymerized filter-supported BLMs were compared with those of freely suspended BLMs using dopamine. The insertion of a receptor in BLMs may increase the selectivity or sensitivity of the device [2]. Recent report has explored electrochemically the interactions of dopamine with surface-stabilized bilayer lipid membranes (s-BLMs) that could be used for the direct sensing of dopamine [19]. s-BLMs supported on metal wire were used to construct a minisensor which rapidly and sensitive responded to dopamine [19]. The interactions of dopamine with these membranes were found to be electrochemically be transduced by s-BLMs observed as ion current increases which reproducibly appeared within a few seconds after exposure of the lipid membrane to dopamine. The magnitude of the current signal increases was related to the concentration of dopamine in bulk solution in the micromolar range (0.65–6.5 μM).

Fig. 2 shows a representative experimental result obtained with injections of dopamine by using the present stabilized lipid films. It can be seen that the lipid membranes shows a response towards dopamine similar to that of freely

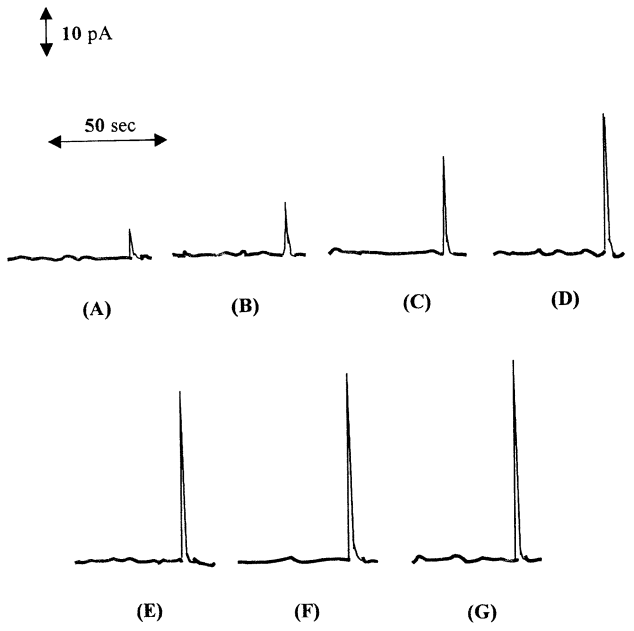

Fig. 2. Typical recordings of the stabilized lipid film biosensor responses to dopamine concentration changes in electrolyte solution. Dopamine concentration (μM) in solution is: (A) 0.20; (B) 0.50; (C) 1.00; (D) 1.5; (E) 2.0; (F) 3.0; and (G) 4.0. The electrolyte solution was 0.1 M KCl. The injection of samples was made at the beginning of each recording. Experiments were done at 25 ± 1 °C.

suspended BLMs (i.e., transient ion current signals) [19]. The time delay for the appearance of the transient currents using these polymerized lipid films was, however, longer than that of freely suspended BLMs (i.e., 46 ± 4 s, $N = 11$, range 43–52 s). The results have indicated that the analytically useful concentration range for dopamine determination is between 0.2 and 2.0 μM and the current was also related to dopamine concentration [Fig. 3, ΔI (pA) = 16.8 C (μM) + 2.30, $r^2 = 0.998$]. The detection limit (based on the lowest concentration that could be measured) was 0.1 μM. The calibration graph declines from linearity above ca. 2.5 μM concentration of dopamine probably due to saturation of the sensor in dopamine. This does not limit in practical terms the working concentration of the device because the linear working range is one decade of dopamine and the relationship between signal and concentration is linear and not logarithmic relationship as it is noted in ion selective electrodes. The reproducibility of chemical sensing of dopamine by the use of polymerized filter-supported lipid films is between ± 4% to ± 7% (e.g., RSD = 4.6% for 1 μM of dopamine, $N = 5$, see Fig. 3). Dopamine additions have been made at different times after membrane formation and stabilization. Only after addition of dopamine were the transient currents of Fig. 2 observed.

The polymerized filter-supported BLMs used in our experiments provide the major advantage that they could be reused after storage in air. Such lipid membranes were stored in air for more than a month and provided stability and response to dopamine. For example, after 1 month in air, the reproducibility varied between ± 5% and ± 7%. A main aspect of the polymerization step was that the polymerization should take place in 12 h. Times of polymerization less than 12 h (i.e., 4 and 8 h) provided polymers with reduced stability in electrolyte solution and were prone to breakage after a mechanical or electrical shock.

The operational stability of the present biosensor was found not to practically depend on analyte concentration, continuous/sequential contact with the analyte solution, temperature up to about 37 ± 1 °C, pH (in the range 3–8) presence of alcohol up to 60% w/w. Other organic solvents could not be tried because of the response of the BLM electrochemistry (i.e., measurement of ionic current in organic solvents is impossible). The stability of the sensor was strongly dependent whether the polymerized lipid membrane was horizontal or vertical. An ammonium gas electrode body was used to prepare a device that may be used as an electrode in which the ultrafiltration membrane with the polymerized containing the lipid membrane was placed at the bottom of the electrode and the internal filling solution was the same as the electrolyte solution. It was found that the lipid membrane was stable for about half an hour.

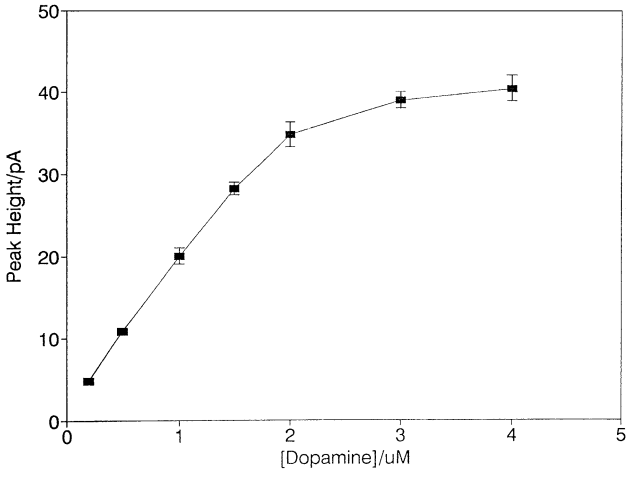

Fig. 3. Calibration of dopamine determination. The results are mean of five determinations.

A biosensor, in general, needs chemical selectivity of analytes against interfering substances, which is usually implemented by incorporating a receptor in the supporting membranes. This is true also in the case of a lipid membrane based biosensors and in principle the incorporation of receptor molecules in the lipid film provides the chemical selectivity or it may even increase the sensitivity of the method [2]. Therefore, interference studies were performed with the present sensor in the presence of the selective receptor in a competitive study (i.e. both dopamine and interferent together in solution) and included investigation of most commonly found compounds in a wide range of real samples, i.e., commercial pharmaceutical preparations, foods, biofluids, etc. The compounds examined as interferents were: maltodextrin (15 000 + 1), dextrose (15 000 + 1), fructofuranose (15 000 + 1), ascorbic aid (7500 + 1), lactose (1500 + 1), sorbitol (1500 + 1), manitol (1500 + 1), glucose (1500 + 1), leucine (1500 + 1), carboxymethylcellulose (150 + 1), glycine (1500 + 1), calcium phosphate (150 + 1), tartrate, citrate, bicarbonate and benzoate ions (1500 + 1), calcium silicate (1500 + 1), caffeine (1500 + 1), urea (15 000 + 1), uric acid (150 + 1) and aspartame (1500 + 1). No significant interferences were noticed from the presence of these compounds (i.e., the relative error in all the cases was less than 5%) (Table 1).

To adapt the above electrochemical sensor of dopamine in real samples of human biofluids, matrix effects due to proteins have to be investigated and possible interferences to be eliminated. Control experiments using a buffered bovine serum albumin solution of concentration similar to that found in human biofluids (6–8% w/v in human serum and 50–140 mg/100 ml in urine) were performed. The results have shown that no interference is observed for concentrations of albumin up to 322 mg/100 ml. For larger concentrations, interference from albumin in the form of random ion current transients of no discrete pulse height due to protein adsorption in BLMs occurred after 2.5 min from the injection of albumin in the electrolyte solution. Therefore, the present sensor could be used for the rapid detection of dopamine in human urine. However, if the sample was human serum, the sensor cannot be used for

Table 1
Results of interference studies

Dopamine (μM)	Peak height (pA)	Interferent	Peak height (pA)
1.00	19.1 ± 1.0	7.5 μM ascorbic acid	20.0 ± 1.0
1.00	19.1 ± 1.0	300 mM ascorbic acid	19.7 ± 0.9
1.00	19.1 ± 1.0	15 mM maltodextrin	19.2 ± 0.5
1.00	19.1 ± 1.0	15 mM dextrose	19.0 ± 0.6
1.00	19.1 ± 1.0	15 mM fructofuranose	19.2 ± 0.3
1.00	19.1 ± 1.0	1.5 mM lactose	18.5 ± 0.4
1.00	19.1 ± 1.0	1.5 mM sorbitol	18.8 ± 1.0
1.00	19.1 ± 1.0	1.5 mM manitol	18.9 ± 0.4
1.00	19.1 ± 1.0	1.5 mM glucose	18.6 ± 1.0
1.00	19.1 ± 1.0	1.5 mM leucine	19.3 ± 0.7
1.00	19.1 ± 1.0	150 μM carboxymethylcellulose	19.5 ± 0.6
1.00	19.1 ± 1.0	1.5 mM glycine	18.7 ± 0.9
1.00	19.1 ± 1.0	150 μM calcium phosphate	18.0 ± 0.8
1.00	19.1 ± 1.0	1.5 mM tartrate	18.7 ± 0.2
1.00	19.1 ± 1.0	1.5 mM citrate	18.6 ± 1.1
1.00	19.1 ± 1.0	1.5 mM bicarbonate	18.9 ± 1.0
1.00	19.1 ± 1.0	1.5 mM benzoate	18.7 ± 1.2
1.00	19.1 ± 1.0	1.5 mM calcium silicate	20.0 ± 1.6
1.00	19.1 ± 1.0	1.5 mM caffeine	18.3 ± 1.0
1.00	19.1 ± 1.0	15 mM urea	18.0 ± 1.1
1.00	19.1 ± 1.0	150 μM uric acid	18.2 ± 0.3
1.00	19.1 ± 1.0	1.5 mM aspartame	18.4 ± 0.7

many repetitive uses due to the interferences from proteins. Research is therefore now targeted to use the present sensor in flow injection systems that eliminate the interferences from proteineous molecules [14].

Dipalmitoyl lecithin throughout all our studies for the preparation of polymerized lipid films because this lipid is characterized and very stable, whereas egg lecithin exhibits an instability in air explosion for storage in air for long periods of time (i.e., oxidation of unsaturated lipids). We did not notice any change of resistivity of these polymerized films composed of DPPC after long storage in air. Therefore, presently BLMs composed of DPPC were selected for our experiments.

4. Conclusions

Our present results indicate the preparation of a mini-sensor based on polymerized stabilized lipid film with incorporated receptor having extremely fast response times (speeds of a few s) for the rapid screening of dopamine that potentially could be commercialized. The use of micro-porous filters to prepare stabilized lipid films goes back to 1970s [20] and their use for flow injection experiments back in 1995 [14,21,22]. However, the use of molecular imprinted technology that was recently reported in literature (1999) [16] has made possible the preparation of stabilized lipid films that could be stored outside the electrolyte solution (i.e., in the air) for periods of time more than one month. The results have shown that these polymerized stabilized lipid films can be reused after storage in air even after a period of a month and can be reproducibly fabricated with simplicity and low cost. The present technique now opens the route for commercialization of lipid film based biosensors. The technique can be used as a stabilized sensor for the rapid detection of dopamine, and keeps prospects for potential applications for the selective continuous monitoring of dopamine in urine of athletes by using filter supported BLMs [14,21,22]. Research is therefore targeted to the application of the present technique in real samples that may contain proteins, such as urine of athletes.

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