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Optical portable biosensors based on stabilized lipid membrane for the rapid detection of doping materials in human urine

Dimitrios P. Nikolelis [a,*], Garyfallia Raftopoulou [a], Petros Chatzigeorgiou [b], Georgia-Paraskevi Nikoleli [a], Kyriakos Viras [b]

[a] *Laboratory of Environmental Chemistry, Department of Chemistry, University of Athens, Panepistimiopolis-Kouponia, 15771 Athens, Greece*
[b] *Laboratory of Physical Chemistry, Department of Chemistry, University of Athens, Panepistimiopolis-Kouponia, 15771 Athens, Greece*

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Abstract

This work reports a technique for the construction of portable devices that are based on stabilized lipid membrane with an incorporated artificial receptor that can be used for the rapid optical detection of doping materials in human urine. The artificial receptors are incorporated within the lipid mixture prior to polymerization. Microporous filters composed of glass fibers were used as supports for the stabilization of these sensors. The lipid film was formed on the filter by polymerization using UV irradiation 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 polymerization is completed within 4 h and the artificial receptors retain their activity. These devices can be used as portable sensors because they provided a simple screening sensitive spot optical test for the rapid one-shot detection of dopamine and ephedrine in human urine. It was now possible to have quantitative data based on a calibration graph. A quantitative method for the detection of dopamine or ephedrine in real samples of urine that can be complimentary to HPLC methods is provided in the present paper. An investigation of the mechanism of signal generation for the "switch on" and "switch off" the fluorescence signal is made herein. The construction of these devices will allow the practical use of the techniques for chemical sensing based on lipid membranes to prepare portable chips/biosensors for the detection of toxicants in foods or pollutants in the field and commercialization of these devices.

Keywords: Optical biosensors; Lipid membranes; Polymerization; Stabilization; UV irradiation; Dopamine; Ephedrine; Human urine

1. Introduction

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. Lipid bilayer membranes have been studied over recent years as a promising assay format for the construction of biosensors. 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 [1–4].

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. A paper appeared in the literature that describes the design of the formation and use, after storage in air of stabilized lipid film-based biosensors by polymerization on microporous filtering media, such as glass fibers by heating at 60–80 °C [5]. The function of these biosensors for repetitive uses after storing in air was also recently investigated [6]. However, in this recent report, the enzyme was incorporated in the lipid film after its formation by polymerization. In one of our recent papers, the polymerization process took place by using UV irradiation instead of

* Corresponding author. Tel.: +30 21089 57817; fax: +30 21072 74577.
E-mail address: Nikolelis@chem.uoa.gr (D.P. Nikolelis).

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heating the lipid mixture at 60 °C [7]. This process retained the activity of an enzyme (i.e., acetylcholinesterase), whereas heating may deactivate it. This method for preparation of stabilized lipid membranes was also studied using Raman spectroscopy [7]. The results have indicated that the polymerization is completed within 4 h and the mechanism of polymerization was investigated. This makes possible the practical use of techniques-based lipid films for chemical sensing, because it will allow incorporation of ion-channel receptors in these lipid films and the preparation of portable devices for the rapid optical sensing of toxicants in environmental, food and clinical samples.

The present work reports a technique for the construction of portable devices that are based on stabilized lipid membrane with an incorporated artificial receptor (i.e., hydroxy or permethoxy calixarene derivative) that can be used for the rapid optical detection of doping materials in human urine. The artificial receptors are incorporated within the lipid mixture prior to polymerization. The polymerization process takes place by using UV irradiation instead of heating at 60 °C. Microporous filters composed of glass fibers were used as supports for the stabilization of these sensors. The lipid film was formed on the filter by polymerization, using UV irradiation prior to its use. Methacrylic acid was the functional monomer, ethylene glycol dimethacrylate was the crosslinker and 2,2′-azobis-(2-methylpropionitrile) was the initiator. The polymerization is completed within 4 h and the artificial receptors retain their activity. These devices provided a simple screening sensitive spot optical test for the rapid one-shot detection of dopamine and ephedrine (using the hydroxy and permethoxy receptors, respectively, in the lipid films during polymerisation) in human urine. The lipid films without the receptors provided fluorescence under a UV lamp. The use of the receptors in these films quenched this fluorescence and the color became similar to that of the filters without the lipid films. A drop of urine containing dopamine or ephedrine provided a "switching on" of the fluorescence which allows the rapid detection of these stimulants in human urine at the levels of 10^{-9} M concentrations. The technique is now based on a calibration graph and therefore as a quantitative method for the detection of dopamine or ephedrine in real samples of urine is provided herein that can be complimentary to HPLC methods. The mechanism of signal generation was investigated in the present paper by using differential scanning calorimetric (DSC) measurements. The construction of these devices allows the practical use of the techniques for chemical sensing, based on lipid membranes to prepare portable devices for the optical detection of toxicants in environmental, clinical samples or foods in the field, and further commercialization of these devices.

2. Experimental

2.1. Materials and equipment

Dipalmitoyl phosphatidylcholine (C16:0) (DPPC) and dipalmitoyl phosphatidic acid (DPPA) were supplied by Sigma Chemical Co. (St. Louis, MO, USA) and were used as lipids for the formation of the lipid films. Methacrylic acid and ethylene glycol dimethacrylate was purchased from Aldrich (Aldrich–Chemie, Steinheim, Germany). The initiator, 2,2′-azobis-(2-methylpropionitrile) (AIBN), was supplied by Merck KgaA (Darmstadt, Germany). Dopamine hydrochloride was supplied from Sigma, whereas ephedrine hydrochloride was supplied from Aldrich. 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 Ltd., Kent, UK).

The preparation of the receptor molecule having the chemical structure 2,8,14,20-tetraundecylpirogallol[4]arene was previously reported [8]. The preparation of the permethoxy derivative of this receptor was also reported in one of our previous paper [9].

The fluorescence was measured with a Perkin-Elmer Model 612 double beam fluorescence spectrophotometer using excitation beam 276 nm. The calibration graph for the determination of dopamine was constructed by using the 2,8,14,20-tetraundecylpirogallol[4]arene receptor, whereas for the determination of ephedrine the permethoxy derivative of this receptor was used. The emission beam for the determination of both compounds was 471 nm.

A Perkin-Elmer differential scanning calorimeter (Model DSC-4) was used for the DSC experiments; the thermograms were processed by means of the Thermal Analysis Data Station (TADS) of the DSC-7.

2.2. Procedures

Stabilized lipid films with incorporated receptor were prepared by polymerization with a procedure similar to that previously described [7–9]. A 5 mg sample of a mixed lipid powder containing 65% (w/w) DPPC, 35% (w/w) DPPA and 0.26 mg of receptor was mixed with 0.070 mL of methacrylic acid, 0.8 mL of ethylene glycol dimethacrylate, 8 mg of AIBN and 1.0 mL of acetonitrile. The mixture was sparged with nitrogen for ∼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 microporous filter disk (diameter of ∼9 mm). The polymerization took place by using UV irradiation instead of the thermal polymerization [7].

The stock aqueous solutions of dopamine and ephedrine were 0.0100 and 0.0010 M. More dilute solutions were prepared daily, just before use. The solutions were placed on the center of the microfilter disk with the lipid film, using a microsyringe. The fluorescence spectrum was measured by cutting the microfiber disk in half and placing it at 45° angle to the incident radiation, vertical to the bottom of the cell of the fluorometer. All experiments were done at 25 ± 1 °C.

Solid DPPC and a piece of the filter with the stabilized lipid film (by cutting the microfiber disk and placing it in the DCS cell) with and without the receptors were used directly for the DSC experiments. The pan of the DSC instrument was hermetically sealed. The samples were scanned between 20 and 80 °C with a scanning rate of 2.5 °C min^{-1}.

3. Results and discussion

The preparation of stabilized in the air lipid films for repetitive uses has been reported in literature [5,6]; however, these works did not incorporate any protein or receptor during the polymerization process, because the polymerization was made by heating at 60–80 °C. This process may deactivate an enzyme or a receptor that is incorporated in the lipid mixture, and for this reason the enzyme (i.e., acetylcholinesterase) was incorporated after polymerization [6].

Raman spectroscopy has also provided information on the mechanism of polymerization and how the lipid film is attached to the polymer [7]. The lipid is attached to the polymer through electrostatic bonding [7]. The peak at 1690 cm^{-1} (that corresponds to the C=O stretching of the methycrylate) was decreased with time, showing that the C=O bond is altered to $C–O^-$; therefore, there is a formation of electrostatic bonding between the $C–O^-$ and $–NHR_3^+$ of phosphatidylcholine. There was also a shift of wavenumber of the peak at 1176–1195 cm^{-1} that showed a strong electrostatic interaction between those two groups. These forces retain the lipid for multiple uses after storage in air and at the same time allow response similar to free suspended BLMs [7,10]. The enzyme in our recent work [7] was incorporated during the preparation of these polymerized lipid films and the results have shown that no denaturation of the enzyme has occurred.

These stabilized lipid films supported on a polymer and prepared as previously described [7] were presently used as optical detectors for the rapid analysis of dopamine or ephedrine. Our recent papers [8,9] described the preparation of a sensor based on a lipid film supported on a polymer, with incorporated receptors, and that is stable in air; this could be used as a simple optical test for the rapid screening detection of dopamine or ephedrine. The results have shown that these lipid films supported on a polymer can be reused after storage in air even after a period of a couple of months (in some cases the polymer is stable even for periods of 6 months) and can be reproducibly fabricated with simplicity and low cost. It was also observed that the colors of the filters remain stable for periods of more than 2 months. This method was faster and had a lower cost than the one based on chromatographic techniques, and could be used as a rapid detector complimentary to these methods in the case of doping of athletes. However, our previous methods provided both a semi-quantitative test and the determination were not based on a calibration graph. In our present results a calibration graph was obtained for each of these doping materials, and therefore a quantitative technique is described herein. Presently also lipid films composed of DPPC and containing DPPA were selected for our experiments.

The present lipid films were found to show fluorescence emission using a UV lamp. The fluorescence emission spectrum of the filters with the polymer containing the lipid film has two main peaks (397 and 471 nm). When either receptor is incorporated in the lipid film structure, the fluorescence emission is quenched. Note that the filters do not provide any fluorescence [8]. An excitation beam of 276 nm was used because this wavelength was the maximum of absorption in UV.

The UV spectra of the filters with the polymer containing the lipid film with incorporated permethoxy receptor having a drop of aqueous solution of ephedrine was previously presented and was similar to that of dopamine [9]. The absorption maximum also appeared at 276 nm; therefore, an excitation beam of 276 nm was used for the present measurements of ephedrine. Two main peaks appeared in the fluorescence emission spectrum of the filters with the polymer containing the lipid film, at 397 and 471 nm. When the permethoxy derivative of the resorcin[4]arene receptor is incorporated into the lipid film structure, the fluorescence emission was quenched [9]. The emission spectrum of the filters was previously provided [8,9] and the filters did not provide any fluorescence. The fluorescence emission spectrum of the methacrylate polymer containing the lipid film with incorporated receptor having a drop of ephedrine provides two-emission maximum (397 and 471 nm). The emission maximum at 471 nm was presently selected in both determinations of dopamine or ephedrine due to the fact that this maximum was the largest between the two peaks. The polymerization could be seen optically under a mercury lamp with naked eye similar to those previously described [8,9]. The filters scatter the light (because the color is the same as that of the mercury lamp), whereas the polymer with incorporated lipids have a different color (bluish). The fluorescence emission is due to the formation of an electrostatic bond between the amino group of the lipid film with the carbonyl group of methacrylic acid during the polymerization stage [7] that provides a polymer that is rigid, and this is the reason of its fluorescence emission.

When the receptor molecules are incorporated in the structure of polymerised lipid films, the polymer with the lipid film becomes more flexible due to the incorporation of the receptor in the structure of the polymer [11–13]. The incorporation of a receptor alters the phase structure of the lipid to more fluid. It is well known that a complex formation between the receptor and dopamine takes place through hydrogen bonding [13–15] and the structure of the film becomes again less fluid [12,16]. The mechanism of signal generation was presently studied using DSC experiments. The phase transition temperature (T_m) of the DPPC was found to be 62 ± 1.0 °C (Fig. 1A). Note that the T_m of DPPC liposomes is 42 °C. However, presently DPPC in lipid films is polymerized and therefore exists in the solid state. When the permethoxy receptor was incorporated within the lipid film, the phase transition temperature was decreased to 56 ± 1.0 °C (Fig. 1B). These results show that the fluidity of the lipid film is increased and therefore the fluorescence is expected to be decreased or disappear. In our case, the benzene ring of ephedrine is intercalated within the cavity of the receptor molecule (Fig. 2) due to the hydrophobic interactions of the benzene rings of the receptor and ephedrine, and the formation of pseudo hydrogen bonding between the hydrogen of the –OH or =NH groups of ephedrine and the oxygen of the methoxy group of the receptor. This results again in an increase of the phase transition of the lipid film in the presence of ephedrine to 61 ± 1 °C (depending on ephedrine concentration, presently a concentration of 1×10^{-3} M of ephedrine was used) (Fig. 1C) which shows that the lipid film again becomes more crystalline and as a result the fluorescence is again “switched on”.

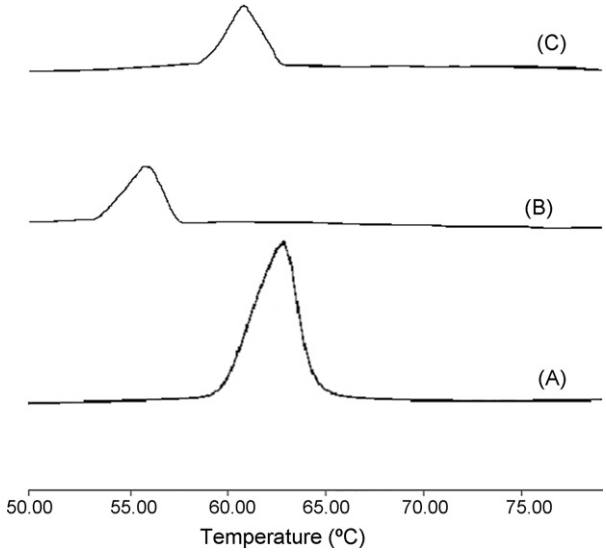

Fig. 1. DSC thermographs of DPPC (A) with incorporated ephedrine receptor (B) and DPPC with incorporated permethoxy receptor having a drop of ephedrine 1×10^{-3} M (C).

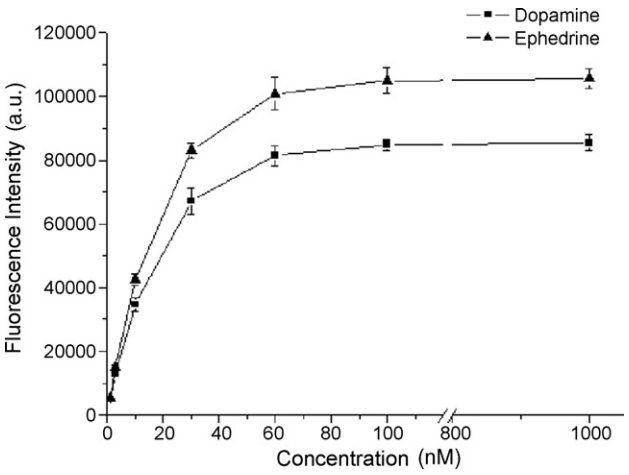

Fig. 3. Calibration graph for dopamine and ephedrine. The graph of dopamine was made using the 2,8,14,20-tetraundecylpirogallol[4]arene, whereas the graph of ephedrine was made with lipid films with incorporated permethoxy receptor.

Indeed, when the dopamine or ephedrine receptor molecules are incorporated within the lipid film structure, this results in the quenching of the fluorescence emission. The color of the polymer with the lipid film containing the receptor is again purple under a UV lamp, similar to that of the glass fiber filters [8,9]. When a drop of a sample of human urine containing the stimulant is deposited on the filter with the polymerised lipid film, the fluorescence is again "switched on" and the color becomes again blue in the spot where the drop of real samples of urine spiked dopamine was deposited. Results of such detection using various concentrations of dopamine (i.e., 10^{-7} and 10^{-8} M) were previously reported. Similar results were obtained when the permethoxy receptor was incorporated within the lipid film before polymerization in the absence or presence of ephedrine, in which the fluorescence is "switched off" or "switched on", respectively. Real samples of urine were spiked with different concentrations of ephedrine and provided again fluorescence (i.e., 10^{-9}, 10^{-8} and 10^{-7} M, see Ref. [9]). The advantage of the present detection is that this "switching on" or "switching off" of the fluorescence can be seen with naked eye and offers a simple detection route with detection limits down to 10^{-9} M concentration levels. Blank experiments, i.e., a drop of real sample of urine without spiked with dopamine or ephedrine did not provide "switching on" of the fluorescence.

The above results only provide a semi-quantitative method for the rapid detection of these doping materials that can be used as a portable simple screening optical device/sensor. However, in order to obtain more quantitative data, the fluorescence intensity was quantitatively measured using a Perkin-Elmer Model 612 double beam fluorescence spectrophotometer using excitation beam 276 nm and emission at 471 nm. Fig. 3 shows the calibration graphs for ephedrine and dopamine.

Each graph was obtained in the presence of each receptor. The 2,8,14,20-tetraundecylpirogallol[4]arene receptor was used for the determination of dopamine, whereas the permethoxy receptor for the determination of ephedrine. The amounts of the receptors are given in the Section 2. These graphs are similar to Langmuir adsorption isotherm. The concentration range that can be determined is between 0 and 100 nM. Lipid-based sensors were previously investigated for use as detectors for the rapid repetitive analysis of dopamine, adrenaline and ephedrine [16]. It was found that the resorcin[4]arene receptor has good selectivity toward dopamine against adrenaline and ephedrine [17]. The clear selectivity pattern was attributed to "molecular recognition" involving cavity-shape fitting and hydrogen bonding interactions, as common for resorcinol-derived calixarenes

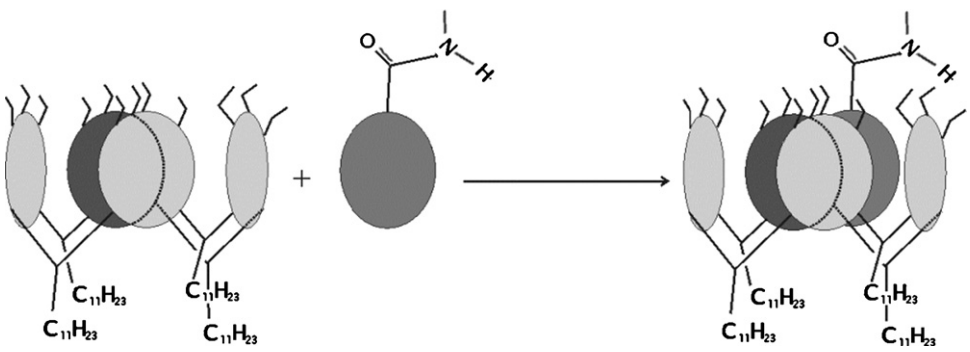

Fig. 2. A schematic illustration of the interaction between receptor and analyte.

[14,15]. On the basis of investigations that exploited the selectivity of the receptor toward these catecholamines [17], the selectivity coefficients as proposed by Wang [18] were calculated and found to be 11.5 for dopamine and 0.64 for ephedrine if adrenaline is the primary species.

Interference studies were done with the present sensor. These experiments included investigation of most commonly found compounds in real samples of human urine (ascorbic aid, glucose, leucine, glycine, tartrate, citrate, bicarbonate and caffeine). No significant interferences were noticed from the presence of these compounds (i.e., the relative error in all the cases was less than 5%). The matrix effects that were due to proteins were also investigated in these reports. Urine protein concentrations are between 100 and 200 mg L^{-1} of urine corresponding to about 40–150 mg/daily [19]. The most common protein in urine is albumin (i.e., concentration of albumin in urine is between 25 and 55 mg L^{-1} depending on the age and sex) [19]. No interference was observed for concentrations of albumin up to 3.22 g L^{-1}. For larger concentrations, interference from albumin was observed. Therefore, these results have shown that the present sensor could be used for the rapid detection of dopamine or ephedrine in human urine. The interference results are summarized as follows: A drop of sample containing ascorbic aid, glucose, leucine, glycine, tartrate, citrate, bicarbonate, caffeine and albumin at concentrations up to 3.22 g L^{-1} did not provide any fluorescence. These results and the results obtained using the above blank experiment shows that the present technique can be applied in human urine without interferences from the matrix.

The present paper describes the preparation of a sensor based on a lipid film supported on a polymer with incorporated receptor and that is stable in air, which can be used as a simple optical test for the rapid screening detection of dopamine and potentially could be commercialized. The results have shown that these lipid films supported on a polymer can be reused after storage in air even after a period of a month and can be reproducibly fabricated with simplicity and low cost. Note that there are a large number of analytical methods for determining urinary catecholamines in healthy subjects. These techniques mainly include HPLC methods [20–22]. The present method is faster and has a lower cost than the one based on chromatographic techniques and can be used complimentary to these methods. The present method can be applied to the rapid detection (i.e., in less than 1 min) of this stimulating compound in human urine and therefore can be used as a rapid detector in the case of doping of athletes.

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Biographies

Dimitrios P. Nikolelis received his PhD from University of Athens in 1976 and he is a prof. of environmental chemistry in Department of Chemistry, University of Athens. His current interests include: development of portable biosensors for the rapid detection of toxicants in environmental, food and other samples, lipid films, nanotechnology, electrochemistry, spectrophotometry, HPLC and environmental analysis.

Garyfallia Raftopoulou received her MSc from University of Athens in 2004 and she is a graduate student in Department of Chemistry, University of Athens. Her current interests include the development of biosensors for the detection of toxicants in foods.

Petros Chatzigeorgiou received his MSc from University of Athens in 2007 and he is a graduate student in Department of Chemistry, University of Athens. His interests include: vibrational spectroscopy and thermal properties of bioactive materials.

Georgia-Paraskevi Nikoleli received her BSc from University of Athens in 2007 and she is an MSc student in Department of Chemistry, University of Athens. Her interests include: environmental and food analysis and nutrition.

Kyriakos Viras received his PhD from University of Athens in 1978 and he is a prof. of physical chemistry in Department of Chemistry, University of Athens. His current interests include: vibrational spectroscopy and thermal properties of polymers.