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The Study on the Properties of Self-Assembled Bimolecular Layers at the Gold Electrode with Incorporated Calixarenes for Dopamine and Epinephrine Detection

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Abstract: Supported self-assembled lipid films (sBLM) on a gold electrode have been used as support for calix[4]arene (C1) and calix[6]arene (C2). The biosensing ability of such structures for dopamine (DA) and epinephrine (EP), was studied by electrochemical impedance spectroscopy (EIS). We showed that calixarenes selectively discriminate DA and EP. The sensitivity of detection by means of capacitance changes of EP was higher for C1 in comparison with C2 and reached 30 $\mu mol \cdot L^{-1}$. DA has been detected with much lower sensitivity (approx. 100 μM) that did not depend on the type of calixarene used. However, sensitivity of detection of DA by means of charge transfer resistance, R_{ct}, determined from impedance spectra was much higher, reaching 1.0 $\mu mol \cdot L^{-1}$.

Keywords: Self-assembled monolayers**,**; Calixarene; Electrochemical impedance spectroscopy; Dopamine; Epinephrine.

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Introduction

Over the last decade, self assembled monolayers (SAMs) and supported bilayer lipid membranes (sBLMs) have attracted the attention of several research groups interested in their application in different fields of biotechnology such as pharmacy, biomedicine, food and environmental technologies [1,2]. SAM provides the means for the surface modification of biomaterials [3,4]. The design flexibilty of SAMs technigue allows for the immobilization of biological macromolecules on the solid supports (Au, Ag, glassy carbon etc.) [5]. Among different kinds of SAMs, the monolayers formed by alkanethiols are widely used and well established because of the very good affinity between the Au surface and the alkanethiols which is due to the fact that the sulphur head groups generally bind as a thiolate, at three-fold hollow sits at the Au (1 1 1) crystal lattice [6].

The calixarene are macrocyclic compounds with typical cavity that can selectively bind certain ligands [7]. Calixarenes can be immobilized on the surface through aliphatic chains or thiol groups [8], or can be incorporated into the filter supported stabilized lipid films [9]. Currently a large variety of calixarenes that selectively bind various ligands have been synthesized [10]. For example, Oshima *et al.* [11] synthesized various calixarene carboxylic acid derivatives and shown their effectivity in extraction of catecholamines and cytochrome Nikolelis *et al.* have used resorcin[4]arene entrapped into the filter supported lipid membrane for effective detection of dopamine [9,12].

Dopamine (DA) and epinephrine (EP) belong to the class of catecholamine and serve as neurotransmitters. Dopamine plays a significant role in the central nervous, renal, hormonal and cardivascular systems. It can influence the heart rate and the blood pressure. Similarly, the epinephrine is a hormone which can also increase heart rate and affects the contraction of blood vessels. It is secreted from the adrenal gland in the blood stream, and thus prepares the body for response in the stress conditions [13]. Considering the aspect of significance of DA and EP it is therefore important to develop the method for detection of both compounds.

The biosensor technology is rather promising for detection catecholamines. However, so far the focus has been mostly on electrochemical detection of these compounds. This is due to the fact that catecholamines are easily oxidized at various metallic surface, such are gold, platinum, glassy carbon and others (see [8] for more details).

Another approach of catecholamine determination by sBLM biosensors modified by calixarenes is based on measurement transient current [10,11] or fluorescence [10]. The sensitivity of detection of DA and EP was, however, typically in 0.2-1.0 μM range. Substantial improvement of detection (limit of detection 20 nM) was made by means of specifically prepared composite electrodes based on zirconium phosphate silica gel [14]. Recently the thickness shear mode acoustic method (TSM) has been applied for detection dopamine [8].

In this work, we used novel approach in preparing the biosensor based on supported lipid films. The sensing layer has been formed by liposome fusion at the surface of gold electrode modified by octadecane thiol. The small unilamellar liposomes from soybean phosphatidylcholine (SBPC) were modified with two types of calixarenes: calix[4]arene and calix[6]arene. The detection of DA and EP has been made by electrochemical impedance spectroscopy (EIS). The integrity of the sBLM was checked by cyclic voltammetry and by EIS at presence of redox probe $[Fe(CN)_6]^{3-/4-}$. This approach brought about an appreciable improvement in the limit of which is as alow as 50 pM

Experimental

Chemicals and Reagents

All chemicals used for the preparation of stock and standard solutions were analytical reagent grade and purchased from Merck (Darmstadt, Germany), or Sigma-Aldrich (USA). McIlvaine buffer (pH 5) was used as supporting electrolyte, which was prepared using 10.3 mL of 0.2 M NaH_2PO_4 and 9.7 mL of 0.1 M acetic acid solution. We also used 0.05 M Na_2HPO_4 buffer (pH 6.5). In this case, the pH was adjusted by 2 M $HClO_4$ and measured by a glass electrode callibrated with a set of commercially available standard buffers. Soybean phosphatidylcholine (SBPC, ≥ 99%, Serva, Switzerland), dopamine and epinephrine hydrochloride (Sigma-Aldrich), were used as recived. Calix[4]arene (C1) and calix[6]arene carboxylic acid derivatives (C2) have been synthesized as described in Refs. [10,15], respectively.

All solutions were prepared with deionized water (18 MΩ cm^{-1} resistivity; MiliQ, Millipore, USA). Electrode cleaning kit from CH Instruments Inc. (USA) was used for gold electrode mechanical polishing.

Apparatus

A modular electrochemical system AUTOLAB equipped with PGSTAT-30 (EcoChemie, The Netherlands) was used in combination with a control by GPES 4.9 and FRA 4.9 softwares (EcoChemie, The Netherlands). The three-electrode cell compartment was an integral part of this assembly.

The electrochemical cell was fitted with an Ag|AgCl|KCl (3 M) reference electrode (connected via 0.1 M KNO_3 bridge). A 1 mm diameter platinum wire was used as a counter elctrode. Measurements were carried out at room temperature (20± 1 ^oC).

Preparation of Biosensors

Different molar ratios (10:1, 100:1) of SBPC to calix[n]arene (n = 4 and 6) suspension were prepared as follows. 10 mg of the SBPC was dissolved in a small quantity of chloroform and 2 mg/mL calix[n]arene solution prepared in chloroform has been added to the SBPC solution in a volume of 500 µl or 50 µl, respectively, so the molar ratios mentioned above were obtained. The solution has been dried under nitrogen in order to obtain thin layer on the wall of the glass flask. A 5 mL aliquot of electrolyte (100 mM NaCl, 2 mM imidazole and 1.5 mM $CaCl_2.2H_2O$; pH 7.2) was then added, and after 30 min incubation, the mixture was ultrasonicated for 20 min with a sonicator (VWR Ultrasonic cleaner USC300T) in a water bath at room temperature (approx. 20 ^oC).

The sensing layer was assembled on the gold electrode surface as follows. First the gold electrode (3.14 mm^2 active area) was carefully cleaned by immersion in hot "piranha" solution; i.e., 3 mL of concentrated H_2SO_4 to which 1 mL of 30% H_2O_2 has been stepwisely added. (**Caution:** *"Piranha" solution reacts violently with most organic materials and must be handled with extreme care !)*

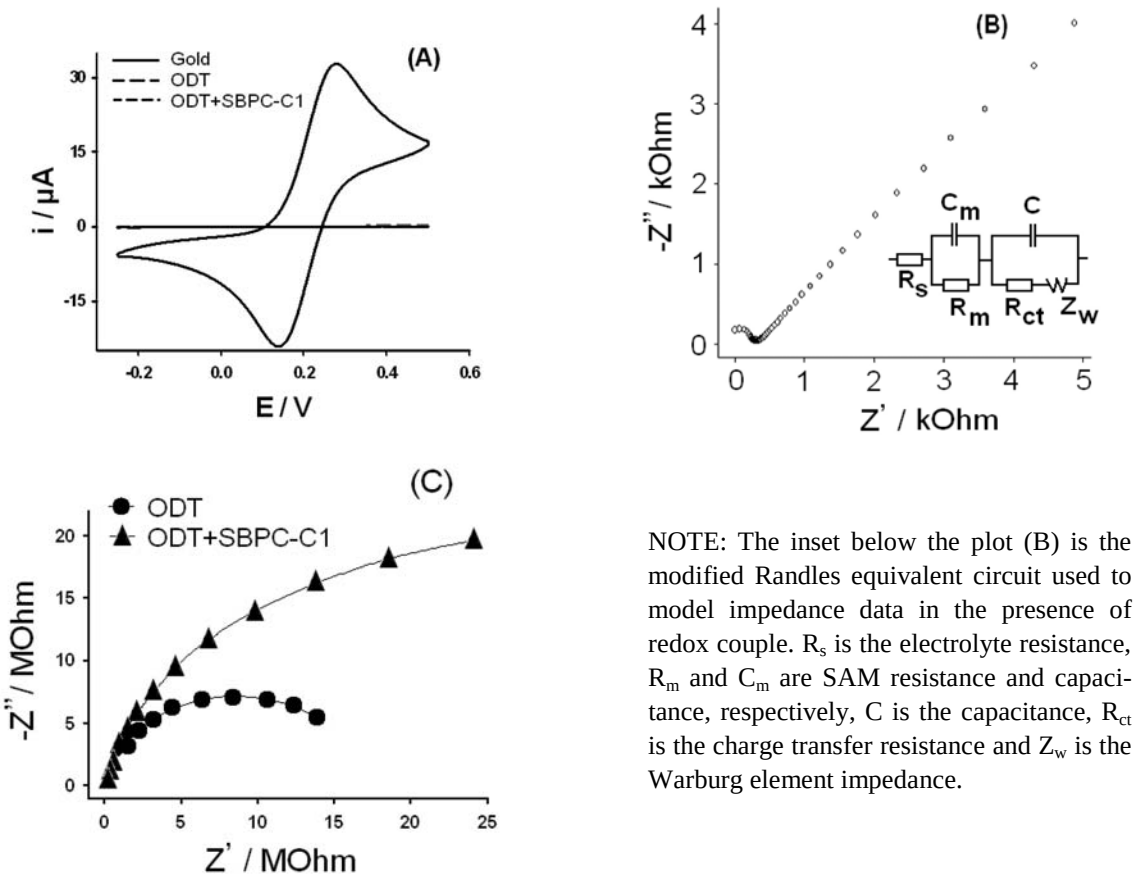
Afterwards, the electrode was rinsed with absolute ethanol and dried in the stream of nitrogen. The electrode was then placed in a 1 mM solution of octadecanethiol (ODT) in absolute ethanol for at least 12 hrs and then washed with absolute ethanol. Finally, the ODT-modified electrode was placed in the SBPC-calixarene suspension for 90 min and washed with McIlvaine buffer.

Results and Discussion

In the first series of experiments we studied the integrity of the self-assembled layers at the gold electrode by staircase cyclic voltammetry (SCV) in the presence of a redox probe (5 mM $K_4[Fe(CN)_6]$). At he bare gold electrode well-resolved reduction and oxidation peaks with a peak separation of 136 mV resulted (Fig. 1A). Coverage of the electrode by SAM of ODT resulted in disappearance of redox peaks, which is evidence on restricted access of the redox probe to the gold surface. Further slight decrease of the current has been observed after addition of the calixarene-SBPC monolayer (Fig. 1A). In order to obtain additional information on the electrical properties of the SAM, we used EIS under the same conditions as for SCV. The Nyquist plots for bare gold, ODT-SAM covered surface and with the additional calixarene-SBPC monolayer are presented on Fig. 1 B and C, respectively.

The impedance properties of the SAM studied can be analyzed with help of the modified Randles equivalent circuit (Fig. 1B, inset) [16]. It can be seen that Nyquist plot of the bare gold electrode consists of semicircle at higher frequencies and linear part at lower frequencies. The diameter of semicircle is proportional to the charge transfer resistance, R_{ct}, while the linear part corresponding to the mass transfer to the electrode surface, which can be determined by Warburg impedance. The Nyquist plot of SAM modified gold surface differ substantially from those of bare gold electrode. We can see that the diameters of semicircles for SAM layers are larger which evidence on higher charge transfer resistance due to hydrophobic barrier. Interestingly the charge transfer resistance is higher for the SAM composed of ODT layer in comparison with those covered in addition by SBPC-calixarene monolayer. It is likely that due to presence of positively charged choline groups in lecithin molecules, the redox probe can easier penetrate across the sBLM and reach the electrode surface, which is reflected in enhancement of charge transfer.

In order to analyze the interaction of DA and EP with the calixarene-based biosensors, we prepared the sensors composed of SAM contained lipid monolayer with two molar ratios of SBPC to calixarenes C1 and C2: 100:1 and 10:1 and have measured the impedance spectra of the sensors at different concentrations of DA and EP. We have been interested in the sensor response depending on the type of calixarene used. The Nyquist plots of the sensor for the molar ratio SBPC:calixarene = 10:1 for C1 and C2 calixarenes and for different concentrations of dopamine are presented on Figure 2 A and B, respectively.



NOTE: The inset below the plot (B) is the modified Randles equivalent circuit used to model impedance data in the presence of redox couple. R_s is the electrolyte resistance, R_m and C_m are SAM resistance and capacitance, respectively, C is the capacitance, R_{ct} is the charge transfer resistance and Z_w is the Warburg element impedance.

Figure 1. (**A**) *Cyclic voltammograms of bare gold and those modified by ODT and sBLM with calixarenes in SBPC:C1 molar ratio 100:1.* Exp. conditions: SCV; 0.05 M phosphate buffer (pH 6.5) + 5 mM $K_4[Fe(CN)_6]$; v = 0.1 Vs^{-1}. Nyquist plot for (B) bare gold electrode and (C) gold electrode modified by ODT and sBLM with calixarenes in SBPC:C1 molar ratio 100:1.

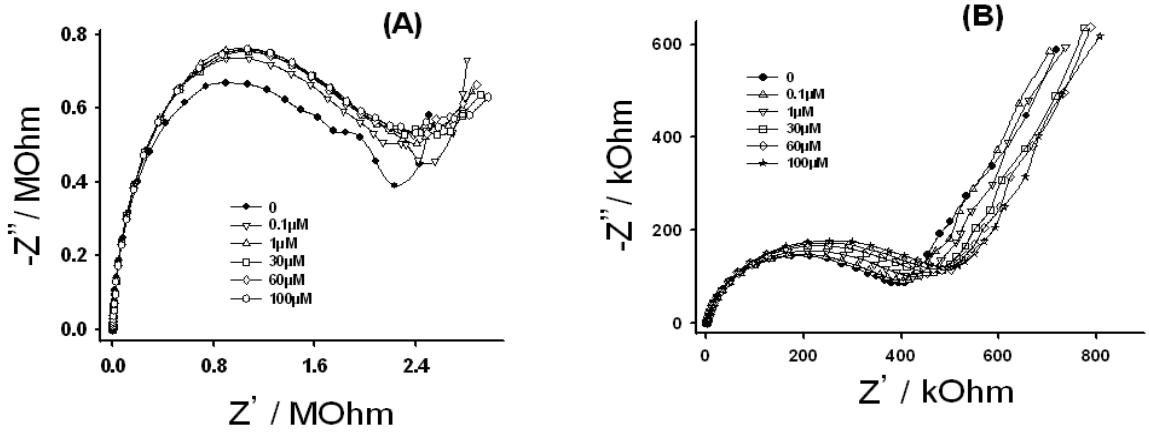


Figure 2. *Nyquist plots for the biosensor based on SBPC-calixarene SAM at a gold electrode covered by ODT at presence of different concentrations of DA in McIlvaine buffer* (pH 5.0). Molar ratio of SBPC:calixarene=10:1. **(A)** C1; **(B)** C2.

For both calixarenes the Nyquist plot displays a semicircle and linear part. The analysis of the Nyquist plot using the modified Randles equivalent circuit (Fig. 1B, inset) shows, that the charge transfer resistance R_{ct} for C1 is approx. 2 MOhm, while around 400 kOhm was typical for C2. Thus, the charge transfer through C2 based layer is easier in comparison with those of C1. We assume that the C1 based SAM is more compact which may be due to the presence of four dodecane hydrophobic chains at C1 [10], while much shorter butyl chains are present in C2 [11]. Addition of DA, however, resulted in increase of the R_{ct} value in both cases. This can be due to the fact that DA is incorporated into the calixarenes by its positively charged NH_3^+ group, while two hydroxyl groups remain in the buffer. Dissociation of these groups impart a negative charge to the sensor surface, which cause repealing of the anionic redox probe $[Fe(CN)_6]^{-3/-4}$ and hence the increase of the charge transfer resistance takes place. Qualitatively similar results were obtained also at presence of EP. The R_{ct} value is rather sensitive to the DA. The significant changes of charge transfer resistance were obtained even at as low as 1 μM of DA.

The analysis of Nyquist plot for various concentrations of DA and EP suggests also that the changes of membrane capacitance, C_m, took place in response to the interaction of these compounds with the sensor. Therefore in the next series of experiments we analyzed the response of the capacitance of the sensor following addition of DA or EP. However, these experiments were performed without a redox probe. The changes of the capacitance of the sensing layer composed of SBPC: C1 in a molar ratio of 10:1 following addition of DA and EP is presented in Fig. 3.

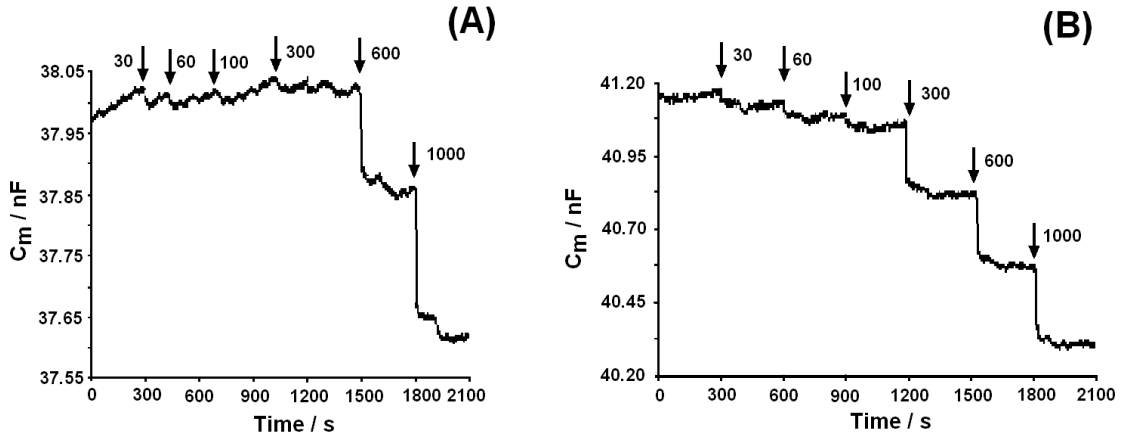


Figure 3. *The changes in the capacitance of the sensing layer composed of SBPC:C1 in a molar ratio 10:1 with successive addition of DA* (A) *or EP* (B)*;* McIlvaine buffer (pH 5).

It can be seen that with increasing concentration of DA and EP the sensor capacitance has tendency to decrease. We assume that this is due to the increase of the thickness of the SAM layer as a result of analyte binding to calixarene. The changes of dielectric permittivity, which also could affect the electrical capacitance, are probably less significant. If the changes of capacitance could be due to changes of dielectric permittivity than we should observe increase and not decrease of the capacitance. The specific capacitance of the SAM composed of C1 and C2 in SBPC:calixarene molar ratio 10:1 was 1.15±0.30 μF/cm^2 and 1.13±0.30 μF/cm^2. Thus there were no significant differences in the capacitance of the SAM composed of C1 and C2. At the same time the obtained values of specific capacitance are in good agreement with those for the SAM of sBLM reported earlier [17]. The plot of relative changes of the sensor capacitance as a function of DA or EP concentrations for sensor composed of either C1 or C2 is presented on Fig. 4.

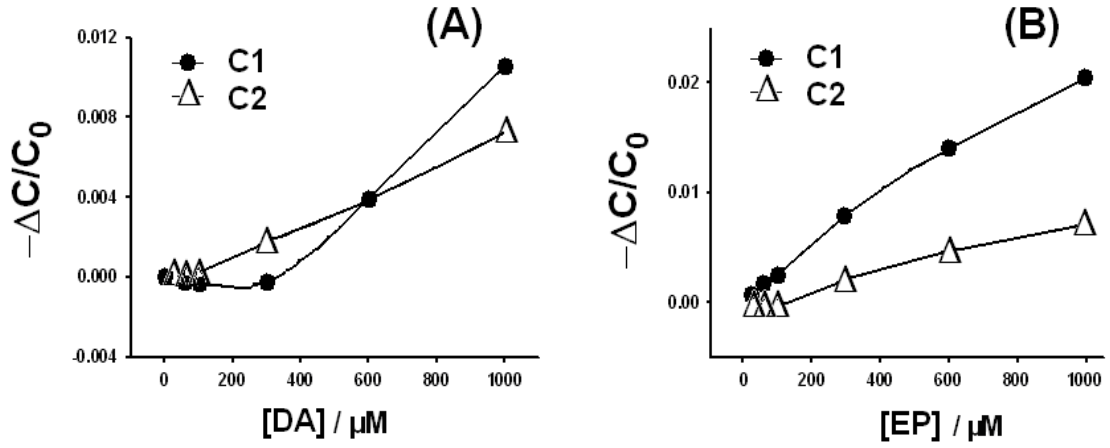


Figure 4. *Representative plot of the relative changes of the sensor capacitance ΔC/C$_0$* (ΔC=C-C$_0$, where C$_0$ is the capacitance prior the addition of analyte and C is that measured at a certain analyte concentration) *as a function of DA* (A) *and EP* (B) *concentration* for a biosensor composed of either C1 or C2 in a calixarene:SBPC molar ratio 1:10. The standard error of capacitance was below10%.

It can be seen that DA does not interact selectively with C1 or C2, while EP more selectively interacts with C1 in comparison with C2. Considering that significant sensor response has been noticed over 30 μM for EP and over 100 μM for DA we can also conclude that the sensitivity of EP detection according to capacitive changes was higher in comparison with those of DA.

The sensitivity of the capacitive sensor based on calixarenes is lower in comparison with those reported earlier. However, the method of formation SAM and impedance analysis may be useful for monitoring the properties of the sensor and in selection of suitable calixarenes. It should be also note that further effort is required for testing the biosensor in real samples as far as interference of interferences of ascorbic acid, uric acid and other biogenic concomitants is concerned. Particular attention should be paid also To possible non-specific interactions of DA and EP with lipid layers in the absence of calixarenes. Experimental work addressing these issues works is in progress.

Conclusions

In this article, we have shown that the SAM composed of SBPC and calixarenes can be advantageously used for preparation of sensing layer for detection of some catecholamines, such as dopamine and epinephrine. The limit of detection using the capacity changes as response signal is in the range approx. 30 μM EP and 100 μM DA, but more sensitive detection (of about 1 $\mu mol \cdot L^{-1}$) can be reached by means of charge-transfer resistance measurements.

Also, the methodology emphasised here may be useful for assessing the effectivity of the calixarenes- ligands interactions, as well as for selection of suitable calixarenes during the sensor development.

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