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A minisensor for the rapid screening of atenolol in pharmaceutical preparations based on surface-stabilized bilayer lipid membranes with incorporated DNA

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Abstract

This work describes an electrochemical technique that is suitable for the rapid and sensitive screening of atenolol based on surface-stabilized bilayer lipid membranes (s-BLMs) composed from egg phosphatidylcholine (PC). The interactions of atenolol with s-BLMs produced electrochemical ion current increases that reproducible appeared within a few seconds after the exposure of the membranes to the drug. The current signal increase was related to the concentration of atenolol in bulk solution in the micromolar range. The present lipid film-based sensor provided fast response (i.e. on the order of a few seconds) to alterations of atenolol concentration (20 to 200 μM) in electrolyte solution. ssDNA incorporated into s-BLMs can interact with atenolol, and decreased the detection limit of this drug by one order of magnitude. The oligomers used were single stranded deoxyribonucleic acids: thymidylic acid icosanucleotide terminated with a C-16 alkyl chain to assist incorporation into s-BLMs (5′-hexadecyl-deoxythymidylic acid icosanucleotide, dT_{20}-C_{16}). The electrochemical transduction of the interactions of atenolol with s-BLMs was applied in the determination of these compounds in pharmaceutical preparations by using the present minisensor.

Keywords: Biosensors; Electrochemistry; Surface-stabilized bilayer lipid membranes; Atenolol; ssDNA

1. Introduction

Atenolol [4-[2-hydroxy-3-isopropyl-aminopropoxy]-phenyl-acetamide] belongs to the category of β-blockers and, more specifically, it is a hydrophillic β1-receptor blocking agent. This β-andrenoceptor blocking drug is of therapeutic value in the treatment of various cardiovascular disorders, such as angina pectoris, cardiac arrhythmia and hypertension [1]. Most of the β-andrenoceptor blocking drugs in use are aryloxypropanolamines (AOPAs). Atenolol is an amino-alcohol and induces a chiral center. β-blockers are exceptionally toxic and most of them have a narrow therapeutic range; the differences between the lowest therapeutic and the highest tolerable doses are small [2]. Gas chromatography (GC) with mass spectrometry or electron capture detector has extensively been used for the determination of atenolol [2,3]. High performance liquid chromatography (HPLC) has also been extensively used for the determination of atenolol [4,5]. Chromatographic methods however require expensive instrumentation and are often complicated and tedious, for example, in particular require a derivatization step. Finally, immunoassays have been used for the determination of this β-adrenoceptor blocking agent [6]. Due to the importance of atenolol, it is interesting to develop a rapid screening method for its determination in pharmaceutical preparations.

The use of lipid films for the rapid detection or continuous monitoring of a wide range of compounds in foods and environmental pollutants was recently provided in a review article [7]. The present paper explores electrochemically the interactions of atenolol with surface-stabilized bilayer lipid membranes (s-BLMs) that can be used for the direct sensing of atenolol. s-BLMs supported on metal wire [8–10] were used to construct the present minisensor which rapidly and sensitive responds to atenolol. Such studies of specific interactions of atenolol with lipid membranes have not been reported in the literature up to date. It is well known that a

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wide range of drugs interact with lipid membranes (for example, see [Ref. 7]). The interactions of atenolol with these membranes were found to be electrochemically transduced by s-BLMs observed as ion current increases that reproducibly appeared within a few seconds after the exposure of the lipid membrane to atenolol. The magnitude of the current signal increase was related to the concentration of atenolol in bulk solution in the micromolar range. The present lipid film-based sensor provided fast response (i.e. on the order of a few seconds) to alterations of atenolol concentration (20–200 μM) in electrolyte solution. The present s-BLM-based sensor was found to be stable for long periods of time (over 48 h). The sensor can be easily constructed, at low cost (and therefore can be used as a disposable sensor) with fast response times on the order of a few seconds. We have recently demonstrated that biosensors based on s-BLMs with incorporated DNA can be employed to decrease the detection limit of hydrazines and aflatoxin M1 [11,12]. The oligomers used were single stranded deoxyribonucleic acid: thymidylic acid icosanucleotide terminated with a C-16 alkyl chain to assist incorporation into s-BLMs (dT_{20}-C_{16}). We also presently demonstrate that ssDNA incorporated into s-BLMs displays a similar analytically useful tool to decrease the detection limit of atenolol by one order of magnitude.

The application of the present electrochemical transduction for the determination of atenolol in pharmaceutical preparations was investigated. The results have shown that the recovery of the atenolol in these real samples varied between 97% to 103%.

2. Experimental

2.1. Reagents and apparatus

Lyophilized egg phosphatidylcholine (egg PC; Sigma, St. Louis, MO) was used for the formation of BLMs. Atenolol was kindly donated by GAP Pharmaceuticals, (Athens, Greece). The chemicals and basic techniques used for the oligonucleotide synthesis have been previously reported [13,14]. Silver wire (diameters 0.5 to 1 mm) was obtained from Aldrich Chemical (Milwaukee, WI, USA). 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.

A two electrode configuration was used in our experiments with s-BLMs and consisted of a sensing electrode (i.e. silver wire coated with a BLM) and an Ag/AgCl electrode acting as a reference. Cyanoacrylate glue (Colle Cyano Gel′ Glue™, Continent, France) was used to isolate the silver wire acting as a sensing electrode. A DC potential of 25 mV was applied between the electrodes and the ionic current through the BLM was measured with an electrometer (Model 614, Keithley Instruments, Cleveland, OH, USA). The electrochemical cell and sensitive electronic equipment were placed in a grounded Faraday cage. The sensing electrode was connected to the power supply that provided the 25-mV, whereas the reference electrode was connected to the electrometer. The electrometer was grounded through the Faraday cage, and therefore, the applied potential at the sensing electrode was positive to a virtual ground (i.e. electrometer). The same electrometer was used as a current-to-voltage converter so that the current will respond to be recorded.

A Perkin-Elmer differential scanning calorimeter (Model DSC-7) 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

Self-assembled BLMs were constructed according to established techniques [8–10]. Solutions of PC (2.5 mg ml^{-1}) in *n*-hexane were used for the formation of metal-supported BLMs. A lipid layer was deposited onto a nascent metallic surface cut with a scalpel just before or while immersing the metal wire into the lipid solution. The wire with the lipid layer was subsequently immersed into a 0.1 M KCl aqueous solution, and ionic current was stabilized over a period of 10 to 15 min depending on the diameter of the silver wire.

The oligonucleotide used was 5′-hexadecyl-deoxythymidylic acid icosanucleotide (dT_{20}-C_{16}), and was synthesized as previously reported [13–16]. The concentration of the stock solution of the oligonucleotide in normal saline that was used to treat s-BLMs was 6.35 mg ml^{-1}.

Calibrations of the response of the s-BLMs (without or with incorporated ssDNA) in the presence of atenolol were subsequently done by stepwise additions of 7.5 mM atenolol standard solution added to the KCl electrolyte while continuously stirring. Once the calibration graph or its equation is set up, the unknown atenolol concentration of a solution can be independently determined using a fresh BLM on a nascent metallic surface and the procedure of immersing the wire with BLM into a KCl solution, etc. is repeated. All experiments were performed at 25 ± 1 °C.

Differential Scanning Calorimetric (DSC) experiments were performed as previously described [17]. Lipid vesicles composed of dipalmitoylphosphatidylcholine (DPPC; Sigma) with a concentration of 2.5 mg ml^{-1} were used for the DSC experiments. The lipid was dissolved in *n*-hexane and the organic solvent was evaporated under a stream of nitrogen gas. The lipid was resuspended by sonication with a 0.1 M KCl electrolyte buffered solution, at a temperature above the estimated phase transition, T_m, of the vesicles (i.e. 40 °C in our experiments). The suspensions had a concentration of 2.5 mg ml^{-1} and were left refrigerated overnight. An amount of 20 μl of lipid suspension was withdrawn using a calibrated microsyringe and mixed in an aluminum pan with 1 μl of atenolol solution (3.8 or 7.85

mM) or 1 μl of ethanol. The pan was hermetically sealed. Vesicles were scanned between 30 and 45 °C with a scanning rate of 2 °C min^{-1} using a DSC-4 differential scanning calorimeter (electrolyte buffer solution was used as the control). Scanning was initiated after 5 min after mixing of the lipid suspension with atenolol solution to ascertain adsorption of this compound in BLMs. Similar experiments were done by mixing 20 μl of lipid suspension with 1 μl of atenolol solution (1.82 or 7.22 μM) and 1 μl of dT_{20}-C_{16} (13 μg ml^{-1}). The DSC curves were analyzed using a Thermal Analysis Data Station (TADS) of the DSC-4.

3. Results and discussion

3.1. Interaction of atenolol with s-BLMs

The method of formation of metal supported stabilized BLMs as reported by Tien et al. [8–10] was chosen among various techniques owing to the simplicity and low cost of BLM preparation. This method of formation of s-BLMs was recently used in the development of biosensors for the detection of wide range of chemical compounds (7).

Fig. 1 shows recordings of responses of the s-BLM-based sensor towards atenolol in electrolyte solution. It can be seen that the response times (to establish 99% of steady-state current) of this sensing device is on the order of a few seconds. The observed decay in response over the first fraction of seconds would be indicating that diffusion control is being established, and/or that membrane charging is occurring.

The current values are linearly related to atenolol concentration within the range of 20–200 μM [ΔI (nA) = 11.1 C (μM) − 108.3, $r^2 = 0.992$] as it is seen in Fig. 2. This regression equation results from linear regression of the current responses that were obtained (from Fig. 1) to stepwise atenolol concentration changes in electrolyte solution. The value of r (that is 0.996 and therefore approaches the value of one) shows that there is a good linearity of the graph of Fig. 2. The detection limit presently obtained (for S/N = 3 and for noise levels of 7 nA) is 15 μM. A number of BLM-based sensors (independently prepared according to procedure) were used to determine the reproducibility of fabrication. These experiments have indicated that the reproducibility is on the order of ± 4% to 8% ($N = 5$, 95% confidence limits, Fig. 2).

The longevity of the present sensors exceeded 48 h; this time is sufficient for calibration that can be achieved in less than an hour. The ionic current through BLMs stabilized within 10–15 min after immersion of the metal wire with the lipid coating into the electrolyte solution. The volume of the cell that can be used can be as small as 1 ml. The rapid addition and mixing of the atenolol can be made in less than 5 s (as observed with injections of an indicator, i.e. methyl orange) [18,19]. A fresh sensing membrane must be prepared for each calibration or determination of unknown to insure that surface contamination and memory effects are completely avoided. However, this is not of primary concern owing to the simple, cheap and fast method of preparation of this minisensor, having a relatively good reproducibly of fabrication and can therefore be used as a disposable sensor.

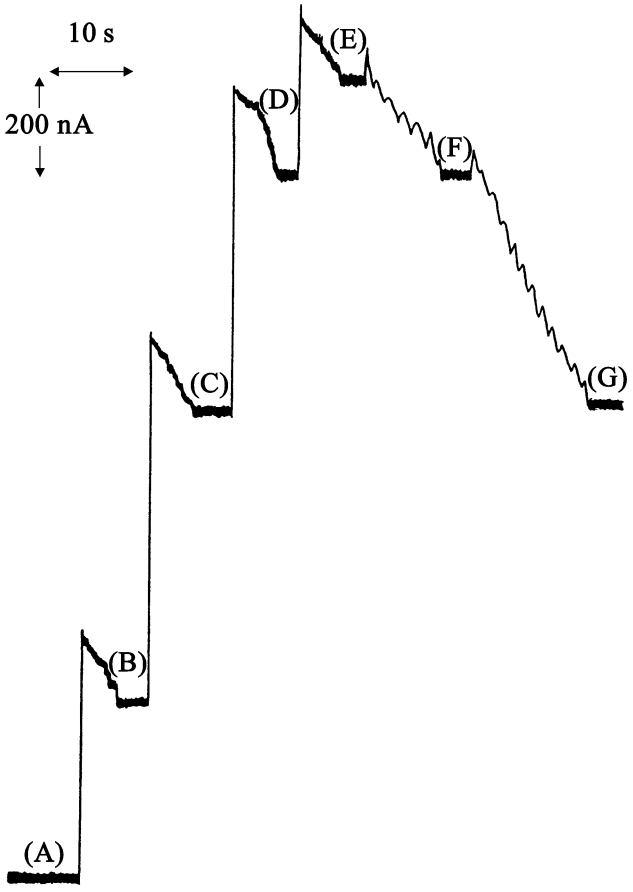

Fig. 1. Typical recordings of the BLM-based minisensor responses to stepwise atenolol concentration changes in electrolyte solution. Atenolol concentration (μM) in solution is: (A) 30.0; (B) 60.0; (C) 105; (D) 150; (E) 190; (F) 150; (G) 105. (A) to (F) represent the forward ion current response for increasing atenolol concentration in solution, whereas (F) and (G) represent the response during consecutive dilutions of atenolol solution with the background electrolyte solution (0.1 M KCl).

The pH effect on the signal magnitude was examined in the pH range of 3.0–9.0 [20] in which the bathing solution was buffered with 10 mM HEPES. The signal was found to be constant within experimental uncertainty for the pH range examined. PC is an amphiphile molecule above pH 3.0 [20] and atenolol is a very weak acid [21] and at any value above 3.0 is a neutral compound.

Differential Scanning Calorimetric studies were performed to study the mechanism of signal generation. Results using differential scanning calorimetry have shown that the addition of atenolol did not change the transition temperature, T_m, of vesicles composed of DPPC. These results exhibit that atenolol does not form hydrogen bonding with the lipid but it partitions into the lipid membrane with the lipophilic part which in agreement with no change in the

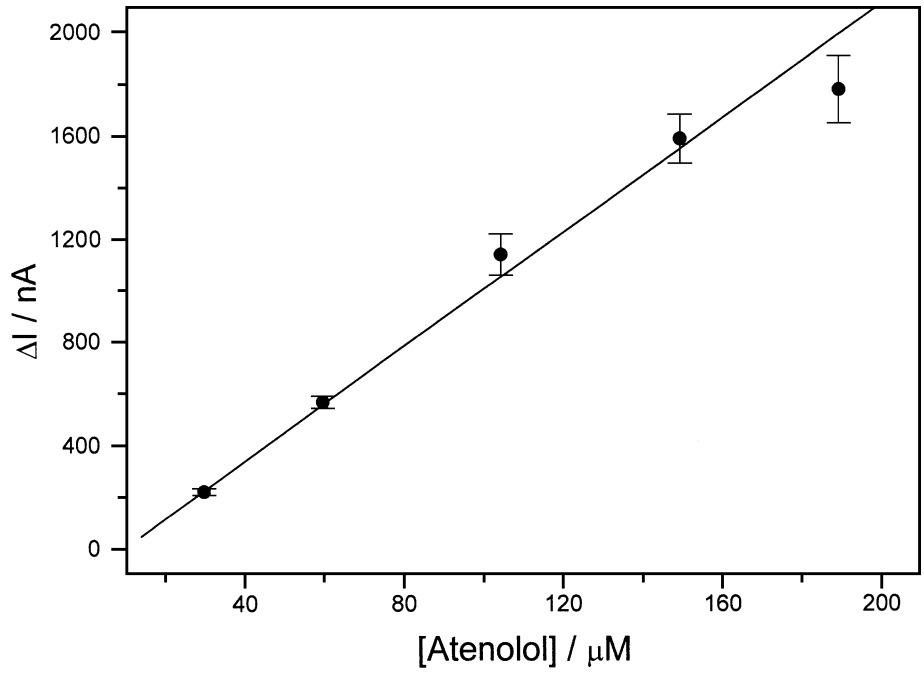

Fig. 2. Calibration of atenolol determination in the absence of DNA. Each point represents the mean of five determinations.

transition temperature [22]. The polar groups of the compound exert into the aqueous solution and alter the surface potential of the lipid film that results into alterations of the ion current. The mechanism of the signal generation is under further investigation.

3.2. Interactions of atenolol with ssDNA-modified s-BLMs

The ssDNA is modified to contain a C16 hydrocarbon chain that helps assure incorporation into a lipid membrane. The selection of dT_{20}-C_{16} as a modifying agent was made on the basis of experience and knowledge of the kinetics and adsorption characteristics of this material onto lipid membranes from earlier work [23,24]. Our experimental work has also included investigation of adsorption of dT_{20}-C_{16}, and mixed base pair sequences up to 25mer in length [25]. These sequences adsorb to membranes, although apparently with different partition equilibria than for dT_{20}-C_{16}. The dT_{20}-C_{16} provides a high degree of electrostatic modification of lipid membranes as seen by ion current changes, and is relatively strongly adsorbed as determined by washing experiments that show negligible loss of material over time courses [12]. Long strands of native DNA and unmodified (no C16 tail) DNA have not been used since preliminary results show that they are more weakly adsorbed than short strands of DNA with a C16 tail [23–25]. There has been no evidence of interaction between ssDNA and atenolol. There were no reports of specific interactions of atenolol with DNA in the literature up to date. Therefore, it is assumed that ssDNA does not act as a selective receptor for atenolol. Rather, the presence of the ssDNA alters the electrochemical sensitivity of the BLM so that perturbations of the charge and structure of the membrane are amplified to provide greater changes in signal (ion current).

ssDNA incorporated in s-BLMs was used as a "receptor" for the detection of atenolol. Experiments were performed where injections of various concentrations of atenolol in the bulk electrolyte solution were done after the incubation and stabilization of s-BLMs with 6.35 ng/ml dT_{20}-C_{16} in saline solution. The addition of the oligomer resulted in an increase of the ion current to values that were consistent with the previous publications (i.e. about 250 nA) [23–25]. Some typical results of ion current increases with ssDNA concentration obtained herein are presented in Fig. 3. The subsequent addition of atenolol standard solution resulted in further increases of the ion current when the ssDNA concentration was kept constant during a set of experiments. The response time was 18–20 s and the increases of the ion current were linearly related to atenolol concentration [ΔI (nA) = 15.9 C (μM) + 100, $r^2 = 0.999$] for 6.35 ng/ml dT_{20}-C_{16}. The detection limit that was established (for S/N = 3 and for noise levels of 7 nA) was 1.8 μM. The detection limit was 10 fold lower than that observed in the absence of ssDNA. It is possible that the electrostatic interactions of ssDNA with s-BLMs are modified by atenolol, resulting in alterations of surface potential and structure at the membrane surface.

Optimization of analytical signal was achieved by the use of different oligomer concentrations that were used to treat s-BLMs (e.g. 6.35 and 19.0 ng/ml). Larger concentrations of the oligomer (i.e. more than 19.0 ng/ml) resulted in substantially increased background ion current, and limited of the useful range of the analytical signals.

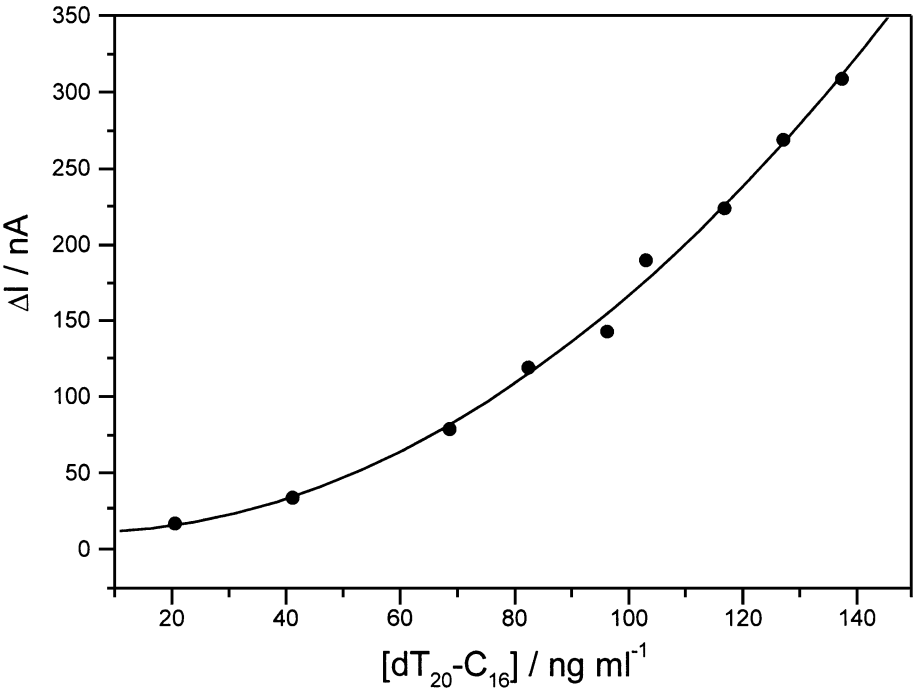

Fig. 3. Calibration of oligonucleotide using 0.1 M KCl electrolyte solution s-BLMs composed from PC.

The results obtained indicate that the ion current values are linearly related to atenolol concentration [ΔI (nA) = 15.9 C (μM) + 100, $r^2 = 0.999$ and ΔI (nA) = 0.958 C (μM) + 17.2, $r^2 = 0.990$ for 6.35 and 19.0 ng/ml dT_{20}-C_{16}, respectively].

The incorporation of dT_{20}-C_{16} in BLMs therefore lowered the detection limit for the detection of atenolol by one order of magnitude as compared with the detection limit obtained in the absence of DNA. The incorporation of C16-ssDNA provides a method to modulate surface charge to achieve better analytical results.

3.3. Interference studies and application of the method for the determination of atenolol in pharmaceutical preparations

Interference studies were done with the present sensor in a competitive study, i.e. both atenolol and interferent together in solution in ratio 1:1. These interferences included investigation of most commonly found compounds in pharmaceutical preparations containing atenolol such as magnesium stearate, macrocrystalline cellulose, povidone and sodium starch glycolate. No significant interferences were noticed from the presence of these compounds.

The potential application of the present interactions of atenolol with BLMs was investigated by quantification and recovery experiments of atenolol in pharmaceutical preparations. The quantification of atenolol in Ternomin (“Cana”, Athens, Greece) tablets containing 25, 50 and 100 mg of atenolol provided the results given in Table 1. The results of quantification obtained have shown good agreement with the percent of atenolol given by the manufacturer (Table 1). The recovery studies were made by spiking a known amount of atenolol in a solution of Tenormin. The recovery ranged between ca. 97% and 103% which shows no interferences from the matrix effects.

In conclusion, our present results indicated that a minisensor based on stabilized BLMs, having extremely fast response times (speeds of a few seconds) for the rapid screening of atenolol and practically no interferences from atenolol or other constituents of atenolol, can be reproducibly fabricated with simplicity and low cost. Furthermore, the present results indicate that s-BLMs that contain single stranded DNA oligomers can cause a 10-fold decrease of the detection limit of atenolol. The technique keeps prospects of selective determination of atenolol in pharmaceutical preparations and for the continuous monitoring and analysis of this compound using filter-supported BLMs.

Table 1
Quantification of atenolol in Tenormin tablets (the results presented herein are random from a large number of experiments)

Atenolol given by the manufacturer (mg)	Atenolol found (mg)
25	25.4
50	51.2
100	99.2

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