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Planar bilayer membranes from photoactivable phospholipids

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Planar bilayer membranes formed from photoactivable phospholipids have been characterized by low frequency voltametry. Cyclic voltametric measurements were applied for simultaneous registration of planar membrane conductivity and capacitance. The procedure has been utilized to characterize the formation and stability of planar bilayer membranes. Bilayer membranes were formed from *N'*-(1,2-dimyristoyl-*sn*-glycero-3-phosphoethyl)-*N*-((*m*-3-trifluoromethyldiazirine)phenyl)thiourea (C_{14}-PED), a head-group photosensitive phospholipid. In situ photoactivation of C_{14}-PED at wavelengths $\geq$ 320 nm altered neither the mean conductivity nor the capacitance of the bilayer. Ionophore (valinomycin) and ion channel (gramicidin) activities were not impaired upon photoactivation. In contrast, bilayer membranes formed from 1,2-bis(hexadeca-2,4-dienoyl)-*sn*-glycero-3-phosphocholine (C_{16}-DENPC) revealed short life times. In situ photopolymerization of the diene fatty acids significantly increased the membrane conductivity or led to membrane rupture.

Introduction

Molecular lipid layer stabilization and covalent coupling of macromolecules to lipid monolayers, bilayer membranes or solid surfaces have wide reaching implications in biotechnology. Immobilization of surface-reactive biocomponents may prove advantageous for the construction of efficient biosensors [1,2]. Formation of covalent bonds between lipid layers and supporting surfaces will yield thermodynamically defined interfaces and simultaneously improve the system stability. Both Langmuir-Blodgett films or layers of biocatalysts have been immobilized on piezoelectric quartz or semiconductor surfaces by adsorption [3] or by chemical coupling [4]. Timed initiation of biomolecule binding and area-selective immobilization may advantageously be realized by non-invasive, lightinduced processes. Reaction conditions for both photo-coupling and photopolymerization are compatible with integrated circuit technology.

Before initiating studies with the ultimate goal to photo-immobilize monomolecular layers of biocatalysts on chemically stabilized lipid layers, it is required to document that photoactivable lipid components form planar (bi)layers. Furthermore, possible light dependent effects on bilayer structure and membrane function need thorough characterization. To date, stable vesicular membranes have been prepared with photoactivable phospholipids containing diene- or diynoyl fatty acids and stabilization of monolayers and vesicles has been achieved by photopolymerization [5,6]. For photoselective surface coupling C_{14}-PED, a carbene generating, head-group photoactivable, phospholipid has been selected. External ligand and/or neighboring lipid coupling via photogenerated carbenes provides a new dimension to the immobilization of molecular layers since photogenerated carbenes do not require defined functional groups for ligand coupling [7].

In the present study the conductivity and capacitance of planar bilayers have been monitored and visualized simultaneously by low frequency cyclic voltametry. Planar bilayers were formed from C_{14}-PED, a phospholipid analogue which contains a photoactivable diazirine head group or from the photoactivable diene phospholipid C_{16}-DENPC which leads

Abbreviations: DENPC, 1,2-bis(2,4-hexadecadienoyl)-*sn*-glycero-3-phosphorylcholine; C_{14}-PED, *N'*-(1,2-dimyristoyl-*sn*-glycero-3-phosphoethyl)-*N*-((*m*-3-trifluoromethyldiazirine)phenyl)thiourea; POPE, 1-palmitoyl-2-oleyl-L-α-phosphatidylethanolamine; POPG, 1-palmitoyl-2-oleyl-L-α-phospho-DL-glycerol; PC, phosphatidylcholine; Pipes, piperazine-1,4-bis(2-ethanesulfonic acid); C_m, planar membrane capacitance.

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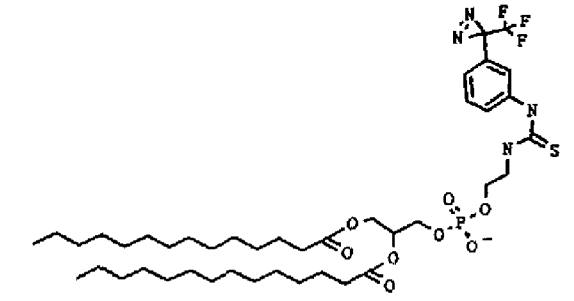
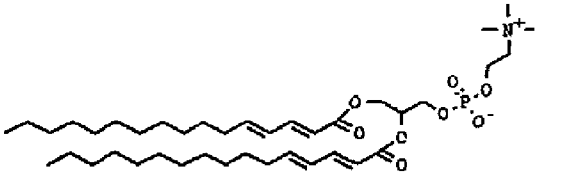


C_{14}-PED



C_{16}-DENPC

Fig. 1. Chemical structures of the photoactivable phospholipids C_{14}-PED and C_{16}-DENPC.

to lipid polymerization (Fig. 1). Macroscopic electrical properties of the bilayers, their thinning, photoinduced stabilization, and their ability to incorporate valinomycin and gramicidin were investigated.

Materials and Methods

Materials

C_{14}-PED was prepared by thiocarbamoylation of 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine with trifluoromethyl-(*m*-isothiocyanophenyl)diazirine [8,9]. C_{16}-DENPC was synthesized as previously described [10]. Synthetic lipids POPG, POPE, glyceryl monooleate, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine and soybean PC were purchased from Avanti Polar Lipids. HPLC purified 1,2-diacylglyceryl-3-*O*-4'-(*N,N,N*-trimethyl)homoserine, a betaine ether lipid isolated from *Ochromonas danica* [11] was gift of G. Vogel. Hexane and hexadecane (puriss.) were obtained from Fluka. Valinomycin and gramicidin A (*Bacillus brevis*) were purchased from Sigma.

BLM instrumentation

A planar bilayer membrane apparatus has been constructed for the in situ photoactivation of bilayer lipid components. Teflon cells, prepared according to Schindler [12] were furnished with a quartz window (5 mm diameter). Individual cell compartments (total volume 1.5 ml) were separated by a teflon foil (6 μm Poly(tetrafluoro ethylene), Elring). Foils were punctured by electrical discharges (10 kV) yielding uniformly sized apertures ($(2-4)\cdot 10^{-4}$ cm^2, 80–120 μm diameter). Aperture size and rim smoothness were controlled by light microscopy.

Bilayer conductivity and capacitance measurements were carried out at ambient temperature ($\approx$ 21°C). Electrical signals were preamplified by a current to voltage converter (OPA 128 Burr Brown; feedback resistance = $3\cdot 10^9$ Ω, Eltec), with the converter adjacent to the Ag/AgCl electrodes [12]. Preamplifier and teflon cells were kept in a Faraday cage mounted on a vibration absorbing base. Signals were amplified and displayed on a digital oscilloscope (Leader LBO-5825). Electrical measurements were calibrated with standard electronic components.

Planar bilayer formation

Phospholipids were dissolved in hexane (1% w/v), C_{16}-DENPC in hexane/ethanol (95:5, v/v) at 45°C. Monolayers were formed by spreading 10 μl of the dissolved lipid onto the aqueous buffer surface of each cell compartment. The buffer contained 0.5 M KCl, 1 mM Pipes (pH 6.2). Planar bilayers were formed by apposition of two monolayers, achieved by elevation of the buffer levels in the contiguous teflon cells [12]. Valinomycin and gramicidin, dissolved in ethanol at 20 μg/ml and 2 ng/ml, respectively, were added to gently stirred aqueous phases after formation of the planar bilayer membrane.

Planar bilayer photoactivation

Formed planar bilayer membranes were irradiated with filtered light from a high pressure vapor mercury lamp (Osram 350 W) operating with a controlled 200 W output. Irradiation times are given in the legends. Emitted light was filtered with a 230–420 nm band pass UG 5 filter (Schott) for C_{16}-DENPC polymerization. For C_{14}-PED activation a 320 nm cut off filter (Corning) and saturated $CuSO_4$ (1 cm) were used.

Results and Discussion

Low frequency cyclic voltametry of planar bilayers

In cyclic voltametry the potential of the electrode is swept between pre-set limits and the resulting current is recorded as a function of the applied potential. In electrical terms, a bilayer membrane behaves like a linear system. The total current (I_{tot}) flowing between the electrodes is the sum of the conductive current (I_1) through the bilayer and the capacitive current (I_2) [13]. By applying a triangular wave form potential ($d\Delta V/dt$ = constant) the terms I_1 and I_2 can be distinguished ($I_{tot} = I_1 + I_2 = G_m \cdot \Delta V + (d\Delta V/dt)\cdot C_m$). Simultaneous recording of the applied potential on the *X*-axis and the current I_{tot} on the *Y*-axis yields a parallelogram, whose slope corresponds to the conductance of

the membrane (G_m). The height of the parallelogram equals $2 \cdot I_2$, from which the membrane capacitance (C_m) is calculated.

The procedure provides facile electrical characterization of planar bilayer membranes and allows continuous monitoring of their stability. Low-frequency (6 Hz) voltametric measurements were carried out by applying a sawtooth voltage with an amplitude of 40 mV (peak to peak) to a preformed C_{14}-PED membrane (Fig. 2A). A current-voltage recording of a newly formed bilayer is shown in Fig. 2B. Upon thinning of the membrane the electrical capacitance increased within few minutes from 0.23 $\mu F/cm^2$ to a steady-state capacitance of 0.73 $\mu F/cm^2$. A kinetic record of membrane conductivity is shown in Fig. 3. Following addition of valinomycin to C_{14}-PED bilayer membranes the conductivity increased with time. High sensitivity conductance measurements were obtained from the same membrane by changing the instrumental settings. The transbilayer current was registered with a constant voltage (± 20 mV, 40 mV). In Table I the electrical properties of planar membranes formed from C_{14}-PED and C_{16}-DENPC, respectively, are compared with the values obtained for various lipids commonly utilized in planar bilayer studies.

Planar bilayer formed from photoactivable phospholipids

Phospholipids containing photosensitive functional groups, activable by irradiation above 320 nm, are most attractive for covalent surface coupling of biomolecules. C_{14}-PED is a phosphatidylethanolamine derived lipid carrying a diazirine substituted photoactivable head group (Fig. 1). Photoactivation of the diazirine at 350 nm generates a highly reactive carbene which inserts into available chemical bonds of neighboring lipids, proteins and/or buffer components. It has been shown, that photoactivation of C_{14}-PED vesicles leads to protein and intraliposomal lipid linking [8]. In addition, extensive reaction of photogenerated carbenes with water is presumed.

C_{14}-PED formed bilayer membranes (Fig. 4A) with a membrane conductivity comparable to soybean PC or synthetic POPE (Table I). Completion of photoactivation of the preformed C_{14}-PED bilayer has been ascertained by irradiating C_{14}-PED vesicles in the electrode chamber. Upon exposure, the diazirine absorbance decayed exponentially [14] with a half-life time $t_{1/2} = 250$ s. The halftime represents an upper limit for the photoactivation halftime of C_{14}-PED in planar membranes, where total absorbance and scattering effects are minute. Based on this result C_{14}-PED bilayers were routinely irradiated for at least 5 minutes. Photoactivation did not rupture the bilayer and the average conductivity and capacitance of the bilayer were not changed. Irradiated planar membranes remained stable for hours. They revealed, however, conductivity

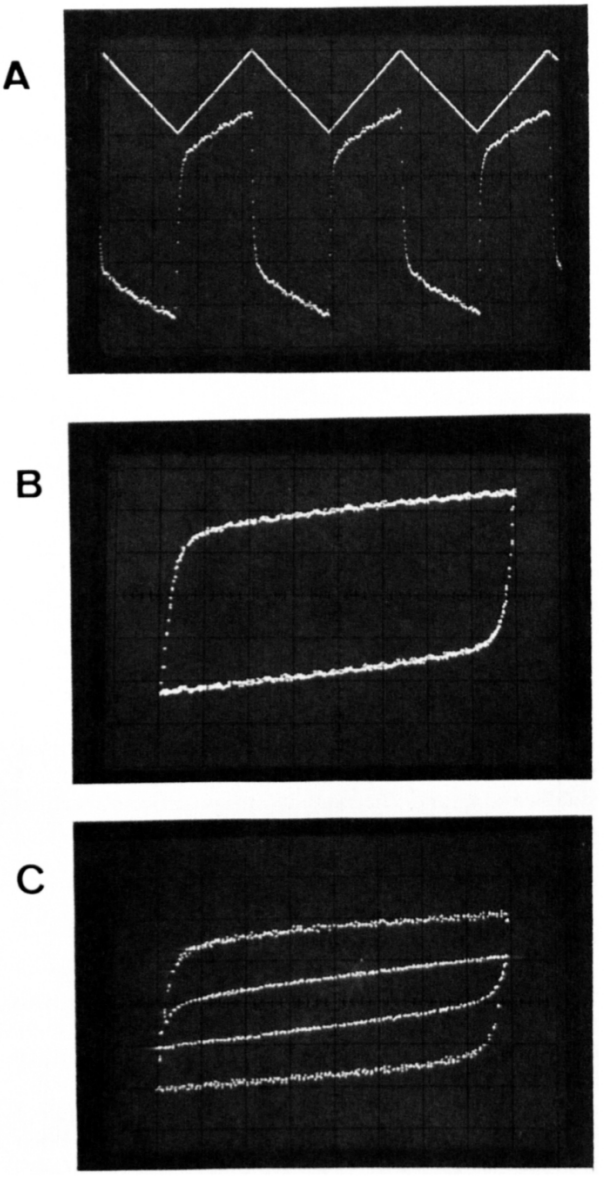


Fig. 2. Low-frequency voltametry of a planar bilayer membrane formed from the photoactivable phospholipid C_{14}-PED (bilayer surface $3.4 \cdot 10^{-4}$ cm^2). (A) Upper trace: voltage applied (*Y*-scale: 20 mV/div; *X*-scale: 50 ms/div). Lower trace: current waveform across the bilayer (*Y*-scale: 66 pA/div, *X*-scale: 50 ms/div). (B) Current-voltage *XY* recording of the bilayer described in (A) (*Y*-scale: 66 pA/div, *X*-scale: 5 mV/div). (C) Capacitance change during formation of the C_{14}-PED bilayer (thinning process). Inner trace membrane capacitance $C_m = 0.23$ $\mu F/cm^2$ measured after 0.2 min; outer trace $C_m = 0.73$ $\mu F/cm^2$ recorded after 10 min.

fluctuations of 0.5 $\mu S/cm^2$ over a mean conductivity of 1.4 $\mu S/cm^2$ (Fig. 4B). Conductivity fluctuations of similar extent have been reported by other investigators, who interpreted the phenomena in their system as

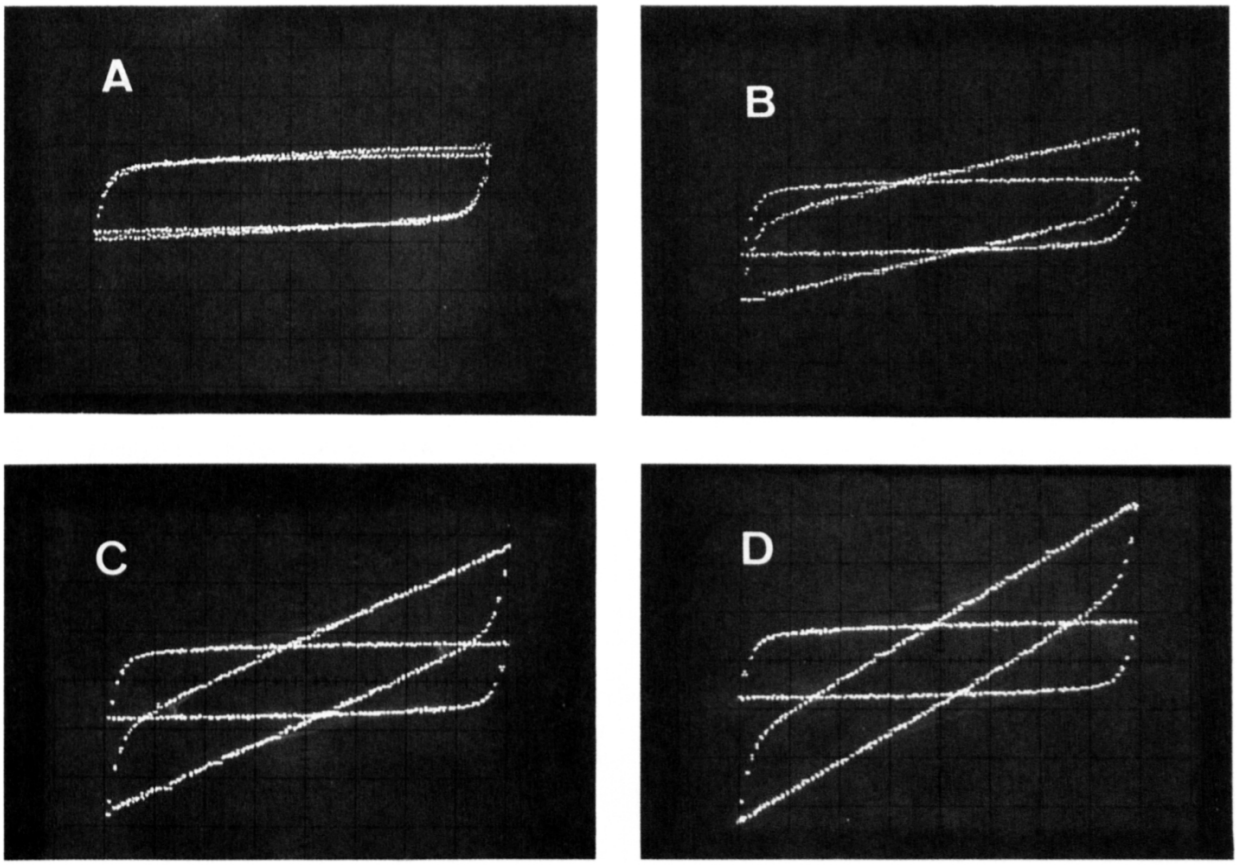


Fig. 3. Time dependent conductivity change of a valinomycin charged C_{14}-PED bilayer. Current-voltage *XY* recording (*Y*-scale: 167 pA/div. *X*-scale: 5 mV/div). A first trace was recorded 10 min after bilayer formation (2.4 $\mu S/cm^2$) and memorized. Superimposed traces were monitored after the addition of valinomycin. (A) Valinomycin (50 ng) was added to the same membrane (conductivity 6.8 $\mu S/cm^2$). (B) Record of the same membrane with 200 ng valinomycin after 1 min (23 $\mu S/cm^2$), (C) after 2 min (46 $\mu S/cm^2$) and (D) after 3 min (64 $\mu S/cm^2$).

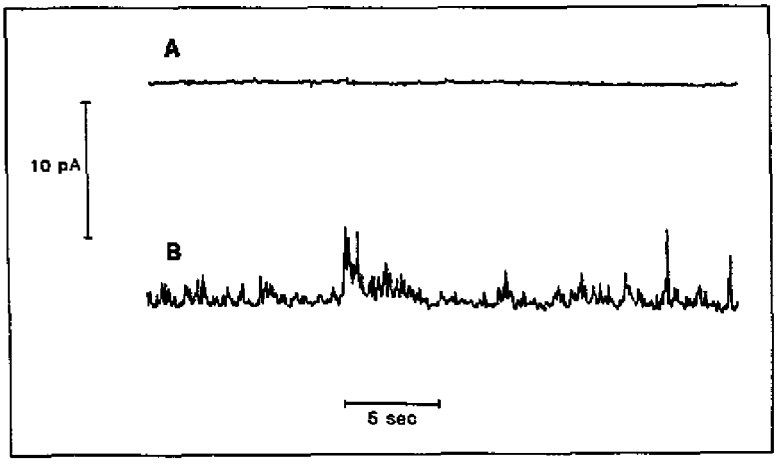


Fig. 4. Conductivity of a C_{14}-PED bilayer membrane. The membrane conductivity was registered with an applied 20 mV voltage (C_m = 1.4 $\mu S/cm^2$). A, before irradiation· B, after 10 min irradiation.

TABLE I

Planar bilayer capacitance and conductivity of photoactivable phospholipids

Planar bilayers have been formed from surface spread phospholipids and the electrical properties of C_{14}-PED and C_{16}-DENPC membranes were determined before and after photoactivation. Reference planar membranes have been formed with soybean PC, POPE, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine, glyceryl monooleate/hexadecane or POPE/POPG.

Lipid	Conductivity ($\mu S/cm^2$)	Capacitance ($\mu F/cm^2$)
C_{14}-PED before and after > 320 nm irradiation	1 – 6	0.75 ± 0.04
C_{16}-DENPC after 260 nm irradiation	1.5 – 5	0.39 ± 0.12
	12 – 25	0.43 ± 0.10
Soybean PC	5 – 12	0.74
POPE	3 – 12	0.70
Diphytanoyl PC	0.07 – 0.10	0.78
Glyceryl monooleate/hexadecane (1:1, w/w)	0.05	0.90
POPE/POPG (4:1, w/w)	≤ 0.015	0.97

either reorganization of the bilayer due to the presence of detergent or lysophospholipids [15], binding of proteins to the external lipid surface, or superposition of numerous channels [16,17]. In C_{14}-PED bilayers, binding of water to photogenerated carbenes alters the chemical properties of the membrane surface. Observed conductivity changes imply that ionophores are incorporated into the bilayer and indicate diffusion of the ionophores within bilayers (Figs. 3 and 5). Occurrence of global inter-lipid crosslinking is excluded since C_{14}-PED photoactivation did not inhibit ionophore diffusion within the membrane.

Planar bilayers, formed from the photopolymerizable diene phospholipid (C_{16}-DENPC) were generally short living, often less than 10 min. Attempts to photopolymerize planar bilayers in situ were either unsuccessful or lead to unstable membranes with increased conductivity (Table I). Observed effects are explained by the fact that polymerization of diene phospholipids leads to a decrease of the surface pressure [18] and lipid clustering. As a consequence apposed planar membranes rupture. In contrast, DENPC vesicles were stabilized upon photopolymerization. Solute permeability in crosslinked diene phospholipid vesicles was significantly decreased [5].

A second set of experiments indicated limited versatility of C_{16}-DENPC in conjunction with protein (channel) immobilization. As outlined for PED planar membranes, the half-life time of C_{16}-DENPC photopolymerization has been determined under conditions identical to planar C_{16}-DENPC membrane photopolymerization. C_{16}-DENPC vesicles were placed in the electrode chamber and the degree of polymerization was determined recording the 260 nm absorbance of withdrawn aliquots. Irradiation induced decrease of the 260 nm absorbance revealed a second order kinetic process [10] from which a half-life time of polymerization $t_{1/2} = 185$ s has been derived. Again, since this value was obtained by vesicle photoactivation, it represents an upper limit for the photoactivation kinetics of planar C_{16}-DENPC membranes. As mentioned above, photopolymerization of diene phospholipids requires irradiation at 260 nm where proteins with aromatic amino acid side chains are sensitive to photolysis [19]. Therefore, UV radiation effects on gramicidin A induced membrane conductivity were investigated. Planar bilayers were formed from 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine and gramicidin A was added. Utilizing identical irradiation conditions as for photopolymerization of planar C_{16}-DENPC membranes gramicidin A induced conductivity decayed with a half-life time $t_{1/2} = 190 \pm 30$ s. Similar rate constants were thus obtained for both C_{16}-DENPC photopolymerization and gramicidin photoinactivation. The experiment clearly documents limitations to the applicability of low wavelength photoactivation (dienes or acrylate functions) for membrane stabilization in the presence of proteins.

Gramicidin in C_{14}-PED bilayers

Gramicidin A formed channels in C_{14}-PED bilayer membranes (Fig. 5A). The average single-channel conductance was 61.9 ± 2.7 pS not regarding the 10 pS minor conductivity changes ('minis') [20]. Under identical experimental conditions (0.5 M KCl, 1 mM Pipes (pH 6.2), 40 mV voltage clamp, 20°C) gramicidin A revealed a channel conductance of 18.5 ± 1.5 pS in 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine. Nearly identical values have been reported by Urry et al. analyzing potassium conductivity in diphytanoylphosphocholine [23]. A conductance of 18.8 ± 1.5 pS has been obtained with DGTS a plant cell zwitterionic ether lipid which does not contain a phosphocholine head group. It has further been noted that the mean single-channel conductance of gramicidin A in C_{14}-PED bilayers exceeded 60 pS. This high value may in part be due to the net negative charge of C_{14}-PED and in part due to the aryl head group of the photoactivable phospholipid. Both properties may lead to concerted intermolecular interactions between gramicidin A an C_{14}-PED head groups. The membrane topology of gramicidin A has been established as N-terminal- N-terminal β-helical dimer with the tryptophan residues located in proximity of the membrane interface [22]. Molecular interactions between tryptophan side chains and the aromatic head group of photoactivated or inactivated PED may distinctly influence the energetics of cation

transport [21,23,24] and therewith modulate the magnitude of single-channel conductance.

Photoactivation of C_{14}-PED bilayers in the presence of gramicidin A showed gramicidin channel activity even after long term irradiation at wavelengths > 320 nm. Conductivity fluctuations appeared upon photoactivation and the rate of channel opening and closing was significantly increased (Fig. 5B). It has further been noted that gramicidin A forms channels even after extensive (40 min) photoactivation (Fig. 5C) implying its insertion and diffusion into the membrane. The average single-channel conductance of light activated C_{14}-PED bilayers was 44.6 ± 5.1 pS. The frequency of occurrence of gramicidin single-channel conductances in photoactivated or non-irradiated C_{14}-PED membranes are compiled in Fig. 6. The histogram shows a 17.5 pS shift in mean channel conductance. The net decrease is probably a consequence of structural changes occurring at the level of the bilayer surface.

The successful preparation of planar bilayer membranes implies that C_{14}-PED and C_{16}-DENPC spread on aqueous surfaces as monolayers. Both photoactivable lipids formed planar membranes upon monolayer apposition. Membrane stabilization and macroscopic effects induced by ionophores and channel forming antibiotics were monitored by low frequency cyclic voltametry, a procedure generally applicable for the survey of planar bilayer membranes. C_{14}-PED formed stable membranes and photoactivation did neither impair membrane stability nor the transmembrane function of channel forming gramicidin. Therewith, the carbene generating phospholipid has been characterized as a valuable bilayer forming amphiphile, applicable for surface linking of biomolecules.

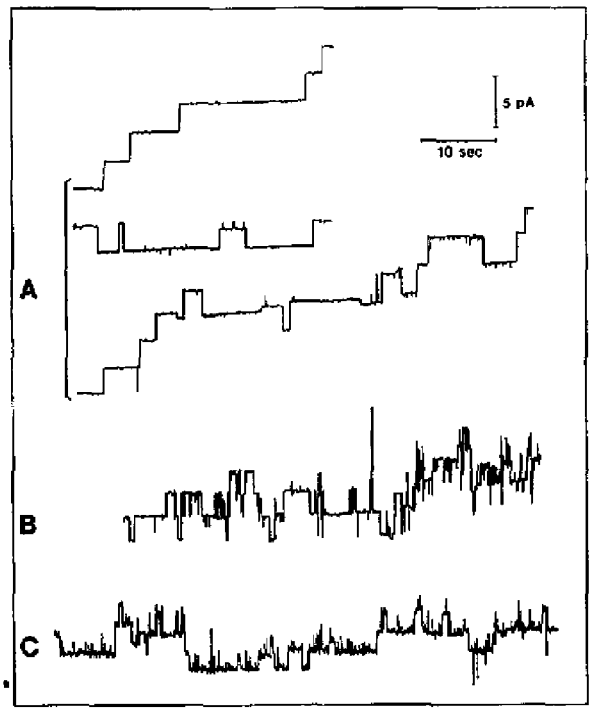


Fig. 5. Single-channel recording of gramicidin A in C_{14}-PED (applied voltage 40 mV). (A) Channel recording before irradiation. (B) Record of the same membrane after 5 min irradiation. (C) Gramicidin incorporated in C_{14}-PED bilayer which was photoactivated for 40 min.

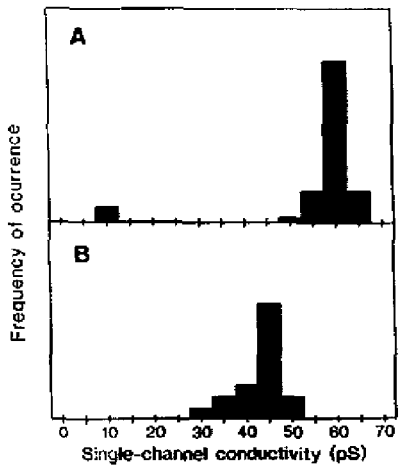


Fig. 6. Gramicidin channels in C_{14}-PED membranes. The frequency of occurrence has been determined from single-channel recordings. ≧ 50 channels were measured per experiment. Channel events are grouped in 5 pS steps. (A) C_{14}-PED bilayer without photoactivation; (B) incorporation of gramicidin after photoactivation (40 irradiation).

In contrast, diene lipids are less versatile.

Photopolymerization, although efficient, altered the membrane lipid coherence and photoeffects on biomolecules with functionally essential tryptophan residues became obvious. DENPC analogues, however, are known to tighten vesicular membranes upon polymerization. Providing low light intensity for photopolymerization and continuous adjustment of the surface pressure, DENPC may prove effective in forming chemically defined monomolecular interfaces. Within the scope of biosensor construction, the synthesis of lipid molecules is in progress providing both, the diene and the diazirine photoactivable functions.

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