*Biochimica et Biophysica Acta*, 426 (1976) 765—771


BBA Report

BBA 71246

# FUSION OF LIPOSOMES CONTAINING CONDUCTANCE PROBES WITH BLACK LIPID FILMS

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(Received November 25th, 1975)

## Summary

The fusion of liposomes with black lipid films was studied using gramicidin A and amphotericin B as conductance probes. Nonpolar alkyl solvents, which have been shown not to injure several membrane functions, facilitated fusion.

Recent publications report the fusion of liposomes with themselves, with planar black lipid films, or with biological membranes (see for example refs. 1—6). Except for a very few cases [1,5,6] it is probable that exchange of individual lipid molecules or lipid-like probes, or adherence of liposomes to the apposed surface, may be occurring instead of the putative fusion process. Distinguishing among these phenomena becomes important in attempts to mimic conditions for bioorganelle fusion, and also in the use of black lipid films as model membranes to which purified membrane proteins or protein-containing vesicles can be added. The interpretation of electrical measurements made in such systems depends on whether the protein or vesicles enter the film bilayer or simply adhere to it.

We report here some studies on the fusion of liposomes with black films, correlated with the facility of the same liposomes to fuse with themselves. Entry of the liposomes into films is traced by the increase in film conductance produced by the antibiotics gramicidin A or amphotericin B incorporated into the liposomes, while fusion of liposomes with themselves is observed by polarized light microscopy.

Egg lecithin was obtained from Lipid Products, Nutfield, Surrey; phosphatidylserine as bovine brain fraction III from Koch-Light; cholesterol from Nutritional Biochemicals; bacterial phosphatidylethanolamine from Supelco;

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gramicidin A from Koch-Light; and amphotericin B from Squibb. Other reagents were of analytical or better grade.

Multilayer liposomes of diameter > 1 $\mu$m (hereafter referred to as large) were made by dissolution of all components in chloroform/methanol or chloroform/ether, slow drying in vacuo at 30°C, and rehydration with hard shaking into solutions matching those used for film bathing solutions. Single-layer liposomes of about 300 Å diameter (hereafter referred to as small) were made by the method of Batzri and Korn [7]. Small liposomes were then centrifuged at 100 000 × $g$ for 1 h and the upper half of the solution employed. Both types of preparation were examined by light microscopy for uniform appearance and the absence of crystals or other evidence of phase separation. Final lipid concentrations were about 5 mM for large liposomes and 1—2 mM for small.

Fusion of large liposomes was followed with the aid of polarized light microscopy using a Reichert Zetopan microscope (700 or 1400 × magnification). Fusion of small (sub-microscopic) liposomes was indicated by the development of turbidity and sedimentation in the suspensions, and by the growth of liposomes to the threshold of visual resolution, at which stage fusing liposomes could be seen (liposome diameter about 1 $\mu$m).

Black lipid films were formed from decane solutions of lipids transferred by brush to Teflon supports [8]. Decane was chromatographed once on alumina grade II. Glass and Teflon ware were thoroughly pre-cleaned [9]. The film support and outer chamber were thoroughly rinsed with ethanol or methanol between films and the dry film hole reprimed with fresh lipid, in order to avoid carryover of material in the film border. Film quality and size were monitored optically and by capacitance measurements at 1000 Hz [10]. Gross film conductance changes were monitored using a voltage divider circuit in combination with an electrometer (Vibron 33B-2) and recorder as previously described (circuit time constant $\tau > 1$ s) [10]. Conductance kinetics were monitored using an operational amplifier circuit in the configuration previously described ($\tau < 0.1$ s) [9]. Voltages applied across films were from 20 to 150 mV; all experiments were performed at 21°C.

Unless otherwise noted, aqueous solutions were 0.1 M NaCl. Solutions for phosphatidylserine systems were buffered at pH 7.5 with 10 mM Tris·Cl. Film chambers were filled and overflowed to produce a clean surface through which liposomes were introduced. Fusing agents were normally introduced by saturating the aqueous phase bathing films or liposomes.

Films to which large liposomes were added were made across a horizontal hole, and liposome sedimentation and adhesion to films were followed by dark- and light-field transmitted and reflected light microscopy at low (138 ×) magnification. Films to which small single-layer liposomes were added were made on vertical supports and observed with a low-power telescopic arrangement. Liposomes were added via micropipette to the aqueous bathing solutions about 1 mm distant from the film.

Gramicidin A, which has been shown to form conducting channels across bilayer films [9], was one of two conductance probes employed here. The addition of large lecithin liposomes containing 3 mol% gramicidin A to lecithin films resulted in increased film conductance only when liposomes were visibly

immobilized on the film. After liposomes settled on the film, a period during which the conductance was constant was followed by a linear rise in conductance lasting several minutes. The rate of rise varied from $10^{-11}$ to $5 \cdot 10^{-9}$ $\Omega^{-1} \cdot \text{min}^{-1}$ per 50 $\mu$g of lipid added.

Decane has recently been noted to promote fusion of human erythrocytes (Dagger, F. and Haydon, D.A., personal communication). In the present study, in three out of four cases, addition of a "chaser" of 50 $\mu$l of decane-saturated solution (formed by briefly sonicating 0.2 ml of decane with 1 ml aqueous solution) after liposomes had adsorbed to the film, produced an immediate linear increase in the theretofore steady film conductance. An aliquot of solution removed from above the film showed, under 700 × magnification, actively fusing liposomes. A $\chi^2$ test on 11 films indicated that addition of decane was not, however, essential for fusion ($p > 0.8$). Control additions of lecithin liposomes, sonicated decane suspension, or the supernatant from a suspension of liposomes containing gramicidin A and centrifuged 12 min at 14 000 × $g$, did not alter film conductance.

In contrast to the infrequent responses obtained for the fusion of one or a few large liposomes with films, small liposomes containing gramicidin A gave results consistent with their greater numbers. Unlike the large liposomes, small liposomes produced initial conductance rises which accelerated in time until attaining maximum rate in 2 to 3 min.

The rate of fusion of lecithin liposomes with phosphatidylethanolamine films was found to be significantly higher than with lecithin or phosphatidylserine films (see Tables I and II). The highest rate of fusion is seen (Table II) for lecithin-phosphatidylserine liposomes added to phosphatidylserine-cholesterol films with fusing agent present. In this case, the increased surface tension resulting from incorporation of alkyl solvent into the liposomes apparently provides a driving force for fusion sufficient to overcome the charge repulsion of phosphatidylserine interfaces at pH 7.5.

TABLE I

Fusion of small lecithin liposomes with black films and with themselves. $F$ is the number of films showing conductance rises, followed after the slash by the number of films tested; $K$ is the initial rate of increase in film conductance per 20 $\mu$g lipid added (liposome mol ratio lecithin:gramicidin A, 100:1). Lecithin: phosphatidylethanolamine liposomes (mol ratio 5:2) were observed to fuse with themselves in 3 out of 4 cases with no added fusing agent.

| Film composition | No fusing agent | | Plus fusing agent | | |
|---|---|---|---|---|---|
| | $F$ | $K(\Omega^{-1} \cdot \text{min}^{-1})$ | $F$ | $K(\Omega^{-1} \cdot \text{min}^{-1})$ | Agent |
| Lecithin | 0/2 | | 2/2 | $1 \cdot 10^{9}$ | $C_{10}$ |
| Lecithin + satd. chol. | 1/1 | $8 \cdot 10^{-11}$ | 1/1 | $8 \cdot 10^{-10}$ | $C_6$ |
| Phosphatidylethanolamine | 4/4 | $5 \cdot 10^{-10}$ | 1/1 | $5 \cdot 10^{-9}$ | $C_6$ |
| Fusion of lecithin | 1/3 | | 2/2 | | $C_{10}$ |
| liposomes with | | | 4/4 | | $C_6$ |
| selves | | | 4/4 | | $C_5$ |

Conductances of phosphatidylserine films remained steady when exposed to lecithin-phosphatidylserine liposomes containing gramicidin A, but no fusing agent (Table II). This result indicates that no gramicidin A free in solution was available to the film. Similarly, the supernatant from a suspension of small lecithin-gramicidin A liposomes, fused by centrifugation for 1 h at

TABLE II

The effect of phospholipid charge on fusion of small gramicidin A-containing liposomes with black films (see Table I for explanation of entries).

| Film composition | Lecithin liposomes | | | | Lecithin:phosphatidylserine (3:1) liposomes | | |
|---|---|---|---|---|---|---|---|
| | No fusing agent | | Plus $C_6$ | | No fusing agent | Plus $C_6$ | |
| | $F$ | $K(\Omega^{-1}\cdot min^{-1})$ | $F$ | $K(\Omega^{-1}\cdot min^{-1})$ | $F$ | $F$ | $K(\Omega^{-1}\cdot min^{-1})$ |
| Phosphatidylserine | 2/2 | $5\cdot10^{-11}$ | — | — | 0/4 | — | |
| Phosphatidylserine:chol. (1:1) | 5/5 | $5\cdot10^{-11}$ | 1/1 | $1\cdot10^{-10}$ | 0/6 | 4/4 | $2\cdot10^{-10}$ |
| | | | | | | *3/3 | $1.3\cdot10^{-8}$ |
| Fusion of liposomes with selves | 1/3 | | 2/2 | | 0/4 | 2/2 | |
| | | | | | | *3/3 | |

*Aqueous solutions in contact with phase of excess fusing agent.

100 000 × $g$ with excess fusing agent present, had no effect on the conductance of lecithin films.

The presence of cholesterol (1:1 mole ratio in the decane film-forming solution) in the phosphatidylserine films did not inhibit fusion of liposomes (see Table II). Judging from black film systems of other compositions [11,12], the amount of cholesterol in the film is probably lower than in bulk solution.

Amphotericin B was also employed as a conductance probe on account of two useful properties: it has almost no effect on black film conductance unless introduced on both sides of the film, and unless the film contains a suitable sterol [13]. Thus, if liposomes containing cholesterol and amphotericin B are added near one side of a film lacking cholesterol, any rise in film conductance is likely to be caused by fusion of liposomes with the film, rather than by entry of stray antibiotic or by exchange of antibiotic between liposome and film without simultaneous exchange of cholesterol.

Results obtained by adding large lecithin liposomes containing cholesterol and amphotericin B to lecithin films are shown in Table III. The addition of alkyl solvent to the bathing solutions initiated linear rises or jumps in film conductance. In the absence of fusing agent, no conductance rises occurred. The barrier to fusion, overcome by the addition of fusing agent, is attributed to the presence of cholesterol in the liposomes [1]. Conductance rises were short-lived and decayed within a few minutes.

When small liposomes containing amphotericin B were added to films containing cholesterol, a series of small positive conductance jumps usually followed provided that fusing agent was present (see Table IV). These conductance jumps were each from $2 \cdot 10^{-11}$ to $2 \cdot 10^{-10}\ \Omega^{-1}$ in height; the conductance did not fall in the intervals between jumps.

At a mole ratio of lipid:antibiotic of 100:1, the number of antibiotic molecules per 1 $\mu$m large liposome (in the outermost layer) and per 300 Å small liposome are estimated to be more than $10^6$ and about 100, respectively. Hladky has estimated that only perhaps one in $10^8$ adsorbed gramicidin A molecules contributes to the conductance of a glycerylmonooleate film similar in thickness to those used herein, and the gramicidin A channels in this system exist for less than 1 s [14]. Hence, the smoothed conductances shown in Tables I and II are averaged film responses to a large number of

TABLE III

Fusion of large liposomes containing amphotericin B with lecithin films (see Table I for explanation of entries). Liposomes contained lecithin, cholesterol, and amphotericin B (mol ratios: 200:100:1). Solutions were 0.2 M NaCl with or without 15 mM $CaCl_2$. Films exhibited whether strictly linear conductance rises (about $10^{-9}\ \Omega^{-1} \cdot min^{-1}$) or rapid conductance jumps complete within seconds. Liposomes lacking amphotericin B or cholesterol did not affect film conductance.

| Liposomes added to: | Frequency of fusion $F$ | | |
|---|---|---|---|
| | Fusing agent | | |
| | None | $C_{10}$ | $C_6$ |
| One side of film | 0/3 | 6/9 | 2/2 |
| Both sides of film | 0/2 | 3/4 | — |
| Fusion of liposomes with selves | 0/3 | 2/2 | 5/5 |

small liposomes. The conductances rose slowly to a maximal rate which is consistent with the progressive arrival of small liposomes at the film from a source a small distance away. (When large liposomes had already settled on the film, the addition of fusing agent resulted in a linear rise in conductance).

In contrast, the step conductances typical of the data in Table IV appear to reflect the entry of only one or a few amphotericin B-containing conducting units into the films. The smoothed conductance rises were again, as in the case of gramicidin A-containing small liposomes, initially greater than linear.

In general, it was noted that the shorter alkanes ($C_5$ and $C_6$), which are more water-soluble than decane, provoked more extensive fusion of liposomes with themselves. Short incubations at temperatures of 30°C or above also accelerated the rate of fusion.

TABLE IV

The effect of small liposomes containing amphotericin B on phosphatidylserine:cholesterol films (mol ratio 1:1 in the film-forming solution). Bathing solutions were 0.1 M NaCl, 10 mM Tris·Cl, plus 10 mM $CaCl_2$, at pH 7.5. Smoothed conductance rises averaged $1.5 \cdot 10^{-9}\ \Omega^{-1} \cdot min^{-1}$ per 10μg lipid added. Discrete conductance jumps occurred whether liposomes were added to one side of the film or both. Control liposomes lacking cholesterol or amphotericin B did not affect film conductance; liposomes lacking phosphatidylserine behaved similarly to those containing phosphatidylserine.

| Liposome composition: Lecithin:phosphatidylserine: chol.:amphotericin B (mol ratio) | Fusing agent | | |
|---|---|---|---|
| | None | $C_6$, no excess present | $C_6$, excess present* |
| Liposomes added to one side of film | | | |
| 100:20:60:1 | 0/1 | 0/1 | 1/2 |
| 100:20:60:5 | 0/1 | 2/2 | 3/3 |
| Liposomes added to both sides of film | | | |
| 100:20:60:1 | 0/1 | 0/1 | 1/1 |
| 100:20:60:5 | 0/1 | 1/2 | 3/3 |
| Fusion of liposomes with selves | | | |
| 100:20:60:1 | 0/2 | 2/2 | 2/2 |
| 100:20:60:5 | 0/3 | 2/2 | 2/2 |

*Aqueous solutions in contact with phase of excess fusing agent.

Recently, a sizable number of purified membrane proteins have been solubilized in the form of lipid-protein vesicles. On the basis of the results herein and those of Dagger and Haydon, we suggest the utility of aliphatic hydrocarbons for fusing such vesicles with black films. Other types of fusing agents such as aliphatic alcohols [15] may inactivate membrane functions including ATPase activity of sarcoplasmic reticulum [16] and $(Na^+ + K^+)$-dependent ATPase [17]. The fusing agent lysolecithin [18] also inhibits many membrane functions [19,20], including normal fusion in myoblasts [21] In contrast, the solvents used herein are predicted to be fairly noninjurious to membrane function, in part because they are not amphipathic. They are not likely to displace essential lipid from membrane protein or to denature the protein. Dagger and Haydon (personal communication) have found that after human erythrocytes are equilibrated with decane-saturated aqueous solutions, ATP-dependent $Na^+$ transport and $SO_4^{2-}$ and $PO_4^{3-}$ permeabilities are within 30% of normal values; and Drabikowski and coworkers have found both ATPase activity and $Ca^{2+}$ transport in sacoplasmic reticulum to be unaffected by heptane [22].

The author thanks Dr. D.A. Haydon and Dr. S.B. Hladky for providing facilities and for critical advice. This work was supported by American Cancer Society postdoctoral fellowship PF-810.

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