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CHLOROPHYLLS AS PROBES FOR MEMBRANE FUSION

POLYMYXIN B-INDUCED FUSION OF LIPOSOMES

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Chlorophylls *a* and *b* exhibited efficient energy transfer when incorporated into liposomes, at concentrations greater than 2% of total lipids. The efficiency of energy transfer decreased when lower pigment concentrations were used. This phenomenon was utilized to construct an assay for membrane fusion. Liposomes containing photosynthetic pigments at 2% of total lipids were induced to fuse in the presence of excess non-pigmented liposomes. The fusion with the non-pigmented liposomes caused dilution of the pigments resulting in lower efficiency of energy transfer. The assay was applied successfully to cation-induced fusion of acidic liposomes. The extent of fusion depended both on cations and on the phosphatidylethanolamine content. The assay was used to demonstrate that the antibiotic polymyxin B was capable of inducing fusion. The efficiency of polymyxin B-induced fusion was extremely high, and on charge ratio basis it exceeded even that of polylysine. Acidic liposomes containing chlorophylls were incubated with Ehrlich ascites tumour cells in the presence of either Ca^{2+} or polylysine. This led to tight binding of liposomes to cells. However, the efficiency of energy transfer between the chlorophylls, associated with the cells, did not change. This result excluded fusion or net transfer of lipids as major modes of liposome-cell interaction.

Introduction

Fusion of phospholipid vesicles has been studied for several years [1]. Since fusion involves mixing of vesicles' contents and membrane components, assays have been devised accordingly. The use of Tb^{3+} and dipicolinic acid as a fluorescent couple [2] and the luciferin-luciferase system [3] were used among others as fusion assays involving mixing of vesicles' contents. Although being extremely sensitive these assays are limited to fusion not involving change of vesicle permeability.

Changing the respiratory control of cytochrome oxidase by fusion of vesicles with others containing a proton channel [4] is an example of an assay using the mixing of membrane components. Papahadjopoulos et al. [5] detected fusion of liposomes consisting of different phospholipids by the changes in the thermotropic phase transitions. Other methods use energy transfer couples either by increasing the yield of energy transfer [6,7] or by decreasing the yield of energy transfer [8,9]. The fluorescent probes used in these assays were synthetic fluorescent derivatives of natural phospholipids. The fluorescent groups were present on the surface of the membrane.

In the present work, it is suggested that chloro-

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Abbreviations: CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid.

phylls are highly suitable as a fluorescent couple allowing assay of fusion. A low concentration of a crude extract of pigments from a higher plant incorporated into liposomes served as a probe for the latter's fusion with either other liposomes or with cells. The assay has been utilized to demonstrate that the antibiotic, polymyxin B is probably one of the most efficient reagents capable of inducing fusion.

Materials and Methods

Cardiolipin and PC (type VII) were purchased from Sigma Chemicals Co. Polyaspartic acid, polylysine (30–70 kDa) and polymyxin B sulfate were purchased from Chemalog. PE was purified from soybean phospholipids as described [10]. All phospholipids proved to be at least 98% pure by thin-layer chromatography. Phospholipid concentration was determined [11] and expressed as mM P_i. Pigments were extracted from spinach leaves as described [12]. The chlorophylls and β-carotene were separated on Whatman 3 filter paper using 10% (v/v) acetone in light petrol ether as a solvent. Chlorophyll concentration was determined according to Arnon [13].

Sonicated liposomes were prepared as described [14] with the addition of either a crude spinach pigment extract (2% w/w of total lipids) or purified pigments i.e. chlorophyll *a*, chloropyll *b* and β-carotene. The concentration of pigmented liposomes did not exceed 12.5 mM P_i. The buffer used throughout the work consisted of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes, pH 7.6), 128 mM KCl and 32 mM NaCl.

Fluorescence of the sonicated samples was measured in an MPF-44B Perkin Elmer Spectrofluorimeter (excitation at 468 nm; emission at 658, 677 and 700 nm). The efficiency of energy transfer from chlorophyll *b* to chlorophyll *a* was calculated as follows $(F_{677} - F_{700})/(F_{658} - F_{700})$. In order to avoid distortion of results, slits for excitation and emission were kept narrower than 6 nm and 3 nm, respectively.

Fusion assay, based on dilution of chlorophylls was carried out as follows: Liposomes containing 2% (w/w) spinach pigment extract were incubated with 10-fold excess of non-pigmented liposomes of similar composition, at a final phospholipid concentration of 4 mM P_i, in the presence of divalent cations, polylysine or polymyxin B. After chelation of the cations and polylysine as previously described [15], the efficiency of energy transfer was measured. The final chlorophyll concentration was calculated using a calibration scale, similar to that shown in Fig. 2. The extent of fusion was defined as

$$\% \text{ fusion} = \left(\frac{C_o}{C_t} - 1\right) \cdot \frac{100}{D - 1}$$

C_0 being the initial chlorophyll concentration, C_t the concentration in the fused liposomes and D the maximal possible dilution, depending on the ratio of non-pigmented to pigmented liposomes.

The charge ratio was given as the number of cation charges divided by the number of charges on the liposomes. The charge ratios were calculated, assuming that, at the pH used, cardiolipin, polylysine and polymyxin B were fully charged and PE was not charged. The distribution of the acidic phospholipids between the layers was not taken into account.

Samples were prepared for electron microscopic observation, according to already published procedures, by negative staining [14] or thin sectioning [15].

Results

No energy transfer from chlorophyll *b* to chlorophyll *a* was observed when liposomes containing chlorophyll *b* were incubated with liposomes containing chlorophyll *a* (Fig. 1). When both chlorophylls were present in the same liposomes at concentrations of over 2% of total lipids, the energy transfer was efficient and most of the light was emitted at wavelengths characteristic of chlorophyll *a* (Fig. 1). At lower concentrations, the energy transfer was lower and at high dilutions no energy transfer was observed and the emission spectrum was identical to that of liposomes containing only cvhlorophyll *b* (Fig. 1).

This allowed assay of actual concentration of chlorophylls in the liposomes with no interference of other membranes present in the medium. A calibration scale of energy transfer as a function of chlorophyll concentration is presented in Fig. 2. It

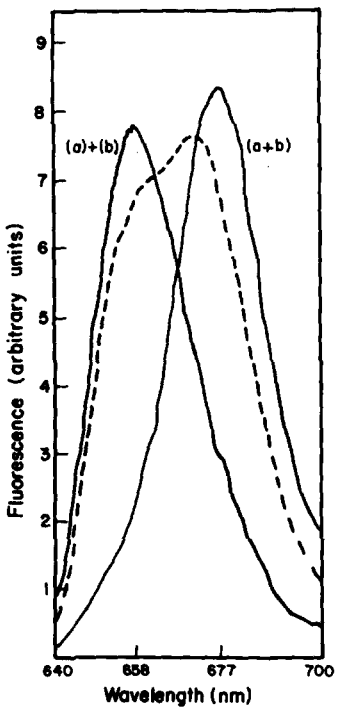


Fig. 1. Emission spectra of pigmented liposomes. All liposomes contained PC/CL (70:30, molar ratio) with the addition of pigments. The following liposome populations were illuminated at 468 nm, which is preferentially absorbed by chlorophyll *b* and the emission spectra were recorded: (a+b), spinach pigment extract (2% of total lipids); ------, spinach pigment extract (0.4%); (a)+(b), a mixture of equal amounts of liposomes containing chlorophyll *a* (1.35%) and liposomes containing chlorophyll *b* (0.65%).

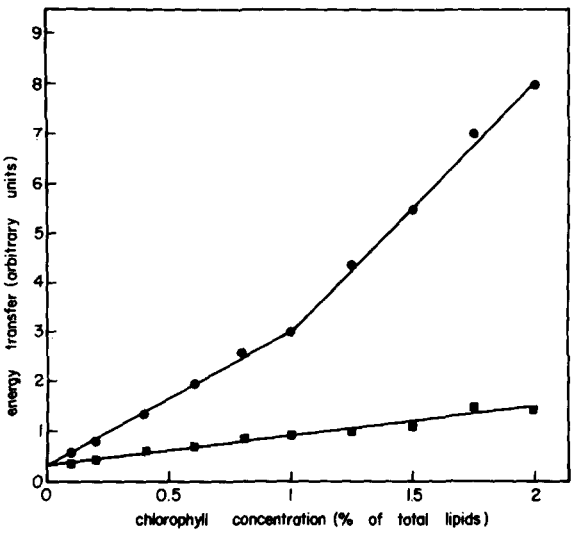


Fig. 2. Calibration of chlorophyll concentration in liposomes. Liposomes were prepared to contain PC:CL (70:30, molar ratio) and various concentrations of spinach pigments extract. The efficiency of energy transfer was recorded for each sample.

is evident that chlorophylls *a* and *b* at a molar ratio of 2, which is similar to that observed in higher plants, proved suitable for this assay. Mixtures containing chlorophylls *a* and *b* at lower molar ratios can be used but the sensitivity of the assay is remarkably smaller already at a ratio of 0.9 (Fig. 2). At the concentration range used, even a 10-fold molar excess of β-carotene incorporated together with chlorophyll *a* into liposomes did not promote energy transfer in contrast to the high yield of direct energy transfer from carotenes to chlorophyll observed in the highly organized thylakoids [16]. As a result it was possible to use a crude spinach pigment extract for the routine assay of fusion described here.

Fusion of membranes could be assayed as dilution of chlorophylls by fusion with non-pigmented liposomes. In order to test the validity of the assay, it was applied to an already reported fusion system [14,15].

Liposomes consisting of 2% spinach pigment extract in PC, PE and cardiolipin at a molar ratio of 1:6:3 or 5:2:3, respectively, were incubated with similar non-pigmented liposomes in the presence of various concentration of $MgCl_2$, $CaCl_2$ or polylysine (30–70 kDa). The extent of fusion depended characteristically on the cation up to a concentration beyond which 100% fusion was achieved (Fig. 3). Polylysine induced 100% fusion already at a charge ratio (cation to phospholipid) of 2, while both divalent cations did so only at ratios greater than 15. PE-poor (20 mol%) liposomes fused to a lesser extent (Fig. 3). When liposomes devoid of PE were used, no significant effect was expected [14] or observed with $CaCl_2$. Polylysine caused precipitation of these liposomes. However, upon its removal the suspension regained its clarity and only a low level of fusion was revealed by the assay of chlorophyll dilution.

The extent of energy transfer was not affected by addition of cations to a suspension containing only pigmented liposomes. The change in energy transfer efficiency was also not due to increased

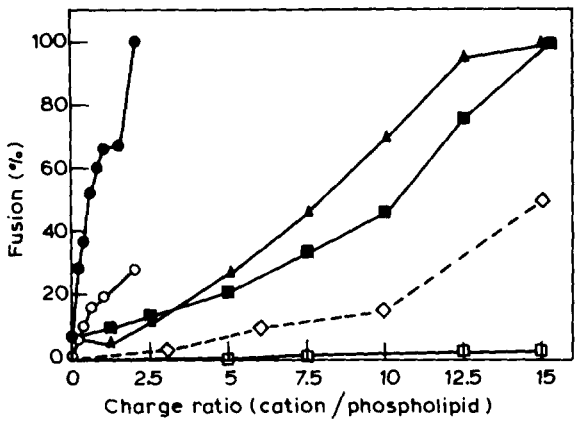


Fig. 3. Cation- and polycation-induced fusion. Liposomes were prepared consisting of 2% spinach pigment extract and PC:PE:CL at molar ratios of 1:6:3 (■, ▲, ●), 5:2:3 (◇) or PC:CL (□, ○). They were incubated with 10 fold excess of similar non-pigmented liposomes in the presence of varying concentrations of $MgCl_2$ (■), $CaCl_2$ (▲, ◇, □) or polylysine (●, ○). The extent of fusion was calculated in each sample as described under Materials and Methods.

turbidity of the suspension. Non-pigmented liposomes were induced to fuse with $CaCl_2$ which subsequently was removed by chelation. The turbid suspension was added to pigmented liposomes without an effect on the energy transfer in the latter. The efficiency of energy transfer did not change in the pH range of 4.6–8.5. The fusion assay was applied successfully to liposomes containing a wide variety of acidic phospholipids. The assay could not be applied to dicetyl phosphate, since commercially available dicetyl phosphate catalyzed destruction of the pigments. On the other hand, the detergents, cholate, deoxycholate, Triton X-100 or SDS did not destroy the pigments after incubation for an hour.

The assay was applied to interaction of liposomes with cells. PE-rich acidic liposomes containing chlorophylls were incubated with Ehrlich ascites tumor cells in the presence of $CaCl_2$ or polylysine. The cells were washed of excess liposomes and assayed for energy transfer between the chlorophylls. Although the cell fraction was fluorescent, no reduction in energy transfer was observed indicating that the vesicles remained intact, either adsorbed to the cells or endocytosized (Table I).

TABLE I

INTERACTION OF LIPOSOMES WITH CELLS

Liposomes consisting of PC:PE:CL (1:6:3, molar ratio) and 2% spinach pigment extract were incubated with or without Ehrlich ascites tumour cells (1 μmol $P_i/10^8$ cells) in the absence and presence of $CaCl_2$ (20 mM) or polylysine (250 μg/ml). After chelation of the cations the cells were washed and the efficiency of energy transfer (arbitrary units) was measured in all the cell fractions.

	$-Ca^{2+}$	$+Ca^{2+}$	−Poly-lysine	+Poly-lysine
Pigmented liposomes	5.18	5.2	5.2	5.17
Pigmented liposomes + cells	5.21	5.19	5.18	5.17

The antibiotic polymyxin B efficiently induced fusion of liposomes. As shown in Fig. 4, already at low polymyxin to phospholipids ratio it caused an increase in turbidity of a suspension of PE-rich liposomes. Since polymyxin could not be removed, the aggregates could not be dissipated and already at a charge ratio of over 1, the liposome suspen-

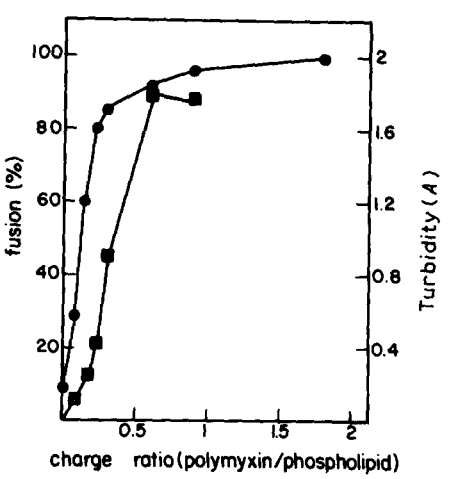
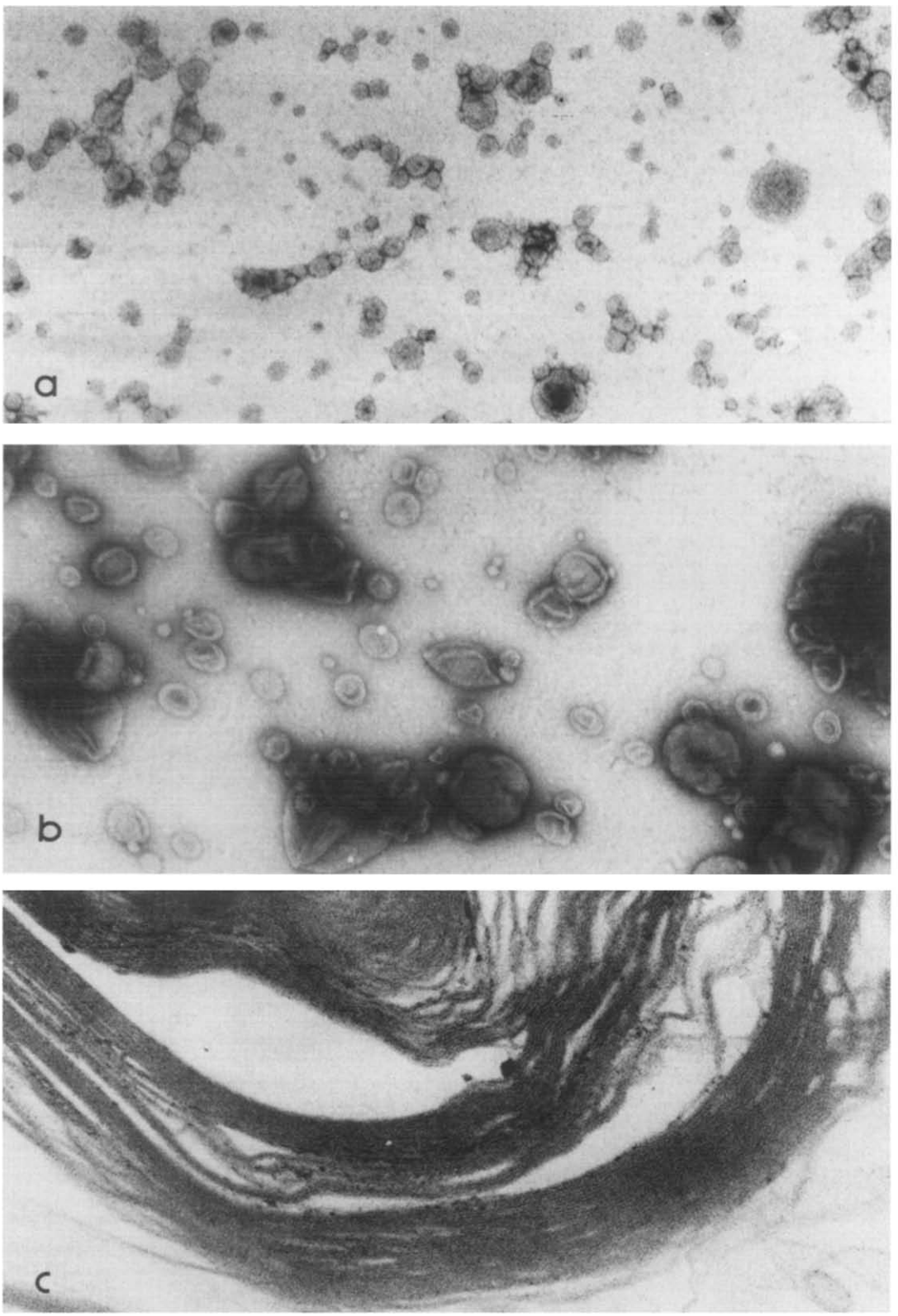


Fig. 4. Polymyxin B-induced fusion. Liposomes consisting of PC:PE:CL (1:6:3, molar ratio) and 2% spinach pigment extract were incubated with 10-fold excess of similar non-pigmented liposomes in the presence of varying amounts of polymyxin B. After 1-h incubation the turbidity of the suspension was recorded (■). In addition, the yield of energy transfer was determined and the extent of fusion was calculated (●).


a
b
c

sion precipitated. Fusion of the liposomes was assayed as dilution of chlorophylls. Polymyxin was even more efficient than polylysine and already at a change ratio of 0.2, almost complete intermixing of the membranes occurred. The efficiency of polymyxin B in inducing fusion was also demonstrated by the use of electron microscopy. Sonicated, small PE-rich acidic liposomes (Fig. 5a) were induced by 0.2 mM polymyxin B to fuse into larger vesicles with diameters up to 0.3 μm. When 0.3 mM polymyxin B (approx. charge ratio of 1) was used, extremely large, densely packed multilamellar structures were formed (Fig. 5c).

Discussion

Dilution of chlorophylls by fusion of pigmented vesicles with non-pigmented ones us a reliable and easy assay of fusion. A suitable plant extract can be prepared cheaply by a rapid procedure. In contrast to other probes whose fluorescent moiety protrudes out of the bilayer [6,7], the hydrophobic chlorophylls are buried within the membranes, their transfer or exchange among membranes does not seem to contribute to reduction in energy transfer. Chlorophylls exit from pigmented liposomes trapped in a dialysis bag to non-pigmented liposomes present outside the bag at a rate even slower than the exit rate of radioactive triacylglycerols (unpublished results). Polylysine caused massive aggregation and precipitation of PE-devoid liposomes but only a slight change in the yield of energy transfer, proving that the effect is not due to transfer between tightly aggregated liposomes. This observation confirms that the phenomena of aggregation/precipitation and fusion are separable [17,18]. Liposomes tightly adsorbed to cells, remained intact and the chlorophylls were not transferred to cellular membranes. This is in contrast to certain derivatives of phospholipids that have been shown to be exchanged/transferred into cellular membranes [19]. The tight binding of liposomes to cells seems to be in conflict with the previously reported fusion of proteoliposomes with red blood cells [20]. We are investigating the possibility that presence of proteins facilitates vesicle-cell fusion while inhibiting liposome-liposome fusion [14]. In contrast to other assays that determine initial rate [2,3], the chlorophyll dilution assay determines the overall extent of the fusion. It can also be used to monitor the kinetics of fusion.

The term $((C_0/C_t)-1)$ does not arise arbitrarily from the need to obtain a fusion scale of 0–100%. It determines the average number of non-pigmented liposomes that have fused with each pigmented one, just as the term $D-1$ gives the ratio of non-pigmented to pigmented liposomes in a given assay. The resolution of the assay was determined by the site of $D-1$, i.e. by the ratio of the two liposome populations used.

The assay has been applied to an already well-characterized fusion system, namely cation or polycation-induced fusion of acidic liposomes containing phosphatidylethanolamine [14,15]. The results obtained with the chlorophyll-dilution system confirmed that polylysine was very efficient in inducing fusion and that the presence of phosphatidylethanolamine facilitated fusion. The presence of chlorophylls in the membrane did not affect the latter's fusion characteristics. Up to 10% fusion is observed even without cations in PE-rich liposomes. PE has long been known to confer instability on membranes [21]. One may therefore expect and observed occassional spontaneous fusion even in the absence of fusions inducers.

Alternatively, membrane fusion could be assayed as increase in energy transfer. Upon fusion of chlorophyll *a*-containing vesicles with chlorophyll *b*-containing vesicles, the energy transfer from chlorophyll *b* to chlorophyll *a* increased (unpublished observation). This type of assay was not adopted since it demanded extra steps for purification of the pigments but mainly because the resolution was comparatively poor.

Polymyxin B is an interesting antibiotic which bears five positive charges and a hydrophobic moiety capable of intercalating into membranes. Hartmann et al [22] have shown that it induces phase separation in phosphatidic acid containing membranes. Here we show that polymyxin is ex-

Fig. 5. Polymyxin-induced fusion studied by electron microscopy. Liposomes consisting of PC, PE and CL (1:6:3), 4 mM P_i final concentration, were incubated without (a) and with 0.2 (b) or 0.3 (c) mM polymyxin. After 1 h the samples were negatively stained (a, b) or processed by thin sectioning (c) for electron microscopic observation. Magnification: $\times$ 100000.

tremely efficient both on molar basis and even on charge ratio in inducing aggregation, precipitation and fusion of PE-rich membranes. The large, densely packed multilamellar structures formed upon fusion, induced by polymyxin B already at a charge ratio of 1, resemble those induced to form by Ca^{2+} at a charge ratio of 10 [14] and by polylysine at a charge ratio greater than 1 [15]. This drug should prove interesting in elucidating the mechanism of membrane fusion. Despite its high efficiency in inducing fusion, its damaging effect on membranes [23] may limit its application to cell-liposome fusion.

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