On the Interaction of Phospholipid Vesicles with Chaotropic Ions*

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The addition of a threshold amount of ClO_4^--ions induces a precipitation of dipalmitoylphosphatidylcholine vesicles predominantly in the crystalline state. Other anions show a similar but less pronounced behaviour. The effect follows the chaotropic order of the regarded anions. The influence of alkaline cations is described but difficult to characterize. Measurements on the mechanism of this precipitation reveal that the vesicles stay intact and that vesicle-vesicle fusion can almost be excluded in the time-domain studied (seconds to minutes). The flocculation of vesicles induced by the addition of chaotropic ions is assumed to be the origin for the observed precipitations.

Introduction

The self-aggregation of lipid-molecules in excess of water originates from the favourable hydrophobic interaction which overcomes any steric or electrostatic repulsions. The respective kind of assembly, *i.e.* micelles or bilayers, depends on the delicate balance between the various interactions involved. Especially steric hindrances by the shape of the molecules could play an important role in the final form of these aggregates. The following results are restricted to lecithins in excess of water where the formation of bilayers is favoured, the monomer- and micelle-concentrations are very small, and any non-bilayer structures can be ignored [1]. Under usual conditions lecithin-molecules spontaneously aggregate into bilayers which are further arranged into onion-like multilamellar structures commonly called liposomes. From these multilamellars it is possible to obtain unilamellar vesicles, frequently this is performed by sonifying a lipid dispersion. Unilamellar vesicles are only metastable. They ultimately and irreversibly form multilamellars. However, for a large number of studies vesicular ensembles are well suited, especially as the reassembly into liposomes is usually a slow process. Studies on vesicles and liposomes were initiated because the lipid bilayer forms the building-block of biological membranes.

The various lipid states are indicated in Fig. 1. Within the frame of Fig. 1 the addition of salts or other compounds could not only modify the dynamic equilibria but could also cause structural changes in the bilayers or micelles. The situation is rather complex because all interactions are interrelated. The structural changes might be induced by conformational changes of the lipid-molecules as consequence of *e.g.* the ion-binding to the zwitterionic polar headgroup of the lecithin-molecules [2]. As the major contribution of the bilayer-stability is thought to stem from the hydrophobic interaction we studied the influence of the solvent "structure" on some physical properties of vesicular ensembles. For that purpose ions known as "structure-breakers" [3] were added to vesicle solutions. The ion-lipid interaction is a major field of current research originating from
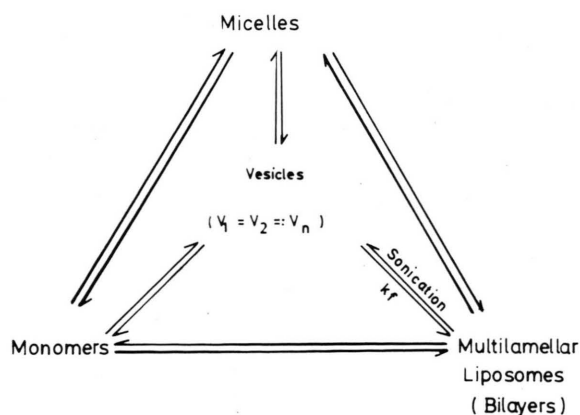
Fig. 1. Lipid states in excess of water. The V_i correspond to different kinds of vesicles which finally fuse (k_f) to form multilamellar liposomes.

the fact that fusion-processes can be promoted by certain ions [4, 5]. The ability to cause fusion between negatively charged lipid vesicles is especially pronounced for Ca-ions [4, 5]. For the zwitterionic lecithin-bilayers the binding of cations is small [6, 7]. Even the terminus "binding" might not be appropriate. However, the presence of bi- or trivalent cations can lead to an increase of the phase-transition temperatur (T_c) [8−10] and to a conformational change of the polar headgroup [2]. Ions could not only directly bind to the zwitterionic polar headgroups but could also alter the water-structure or dehydrate the membrane [11] and thus bear a more indirect influence on the membrane.

Studies of the influence of structure-breaking ions on various aspects of vesicular lipid bilayers are almost absent in the literature except for a few cases [9, 10, 12]. Calorimetric and surface tension investigations on mono- and multilamellar systems show small shifts of the phase-transition profile at rather high salt concentrations [9, 10], and the observed trends can be correlated with the chaotropic character of the ions studied. Very recently the interaction of poly(ethylene glycol) with phospholipid vesicles was discussed in terms of a steric exclusion mechanism where a decrease of the strength of the hydrophobic interaction could finally lead to the observed lipid-exchange and/or membrane fusion [13]. Similar arguments might hold for other "mediated" fusion processes [14].

Some years ago we found during our annealing studies [15] that below T_c the formation of vesicles was favoured with increasing charge of the cations while the kind of anions (Cl^-, NO_3^-, $H_2PO_4^-$, CH_3COO^-) did hardly show any influence on the parameter investigated [15]. We began our present studies with ClO_4^- which is known as potent structural breaker and which is even widely used to induce conformational changes in proteins, polypeptides or polyaminoacids [16]. Detailed investigations of ClO_4^--effects on phospholipid vesicles are to the best of our knowledge not available. Measurements on the phase-transition profile of lecithin vesicles as function of the $NaClO_4$ concentration failed above 10 mM $NaClO_4$ because lipid material started to precipitate during the experiments. Thus most of our present studies were performed in order to understand the kinetics and kind of aggregate-formation induced by the addition of ClO_4^- or other chaotropic ions.

Experiments

Materials

Inorganic salts and Triton X-100 were from E. Merck, Darmstadt. 1,6-Diphenylhexatrien and tris-(Hydroxymethylaminomethane) (Tris) were from Fluka, Buchs and Serva, Heidelberg, respectively. Uridine 5'-(γ-1-(5-sulfonic acid)-naphthylamido) triphosphate (γAmNS-UTP) was synthesized according to [25]. Venom phosphodiesterase from Crotalus atrox (E.C. 3.1.4.1) was purchased from Sigma, München. Sephadex G 10, G 25 and Sepharose 4B used for column chromatography were from Pharmacia Fine Chemicals. β,γ-Dipalmitoyl-α-phosphatidylcholine (DPPC) was obtained from Calbiochem and was shown to be pure by thin-layer chromatography.

Buffer:

50 mM TrisHCl, 20 mM $CaCl_2$, pH 7 or 20 mM $CaCl_2$, 0.02% NaN_3 at neutral pH.

Vesicle preparation

5−20 mg DPPC were sonified (MSE soniprep 150) in 2 ml buffer for 5 min above the phase transition temperature (T_c) usually at medium power. The resultant stock-solution (typically bluish) was diluted for appropriate transmittance reading and incubated 5 min at 55 °C prior to use. For measurements of the fluorescence anisotropy 5 µl DPH (in the mM concentration range) dissolved in tetrahydrofuran were pipetted into a conical centrifuge-tube. After vacuum-evaporation of the tetrahydrofuran the weighed amounts of DPPC and 2 ml buffer were added. The solution was sonified below the phase-transition temperature in intervals of 30 s for 5 min. The DPH:DPPC ratio was about 1:1000. One half of the unannealed vesicles was dialysed against a buffer solution containing additionally various amounts of $NaClO_4$ for 24 h at room temperature. Finally, the dialysed and remaining vesicles were annealed for 5 min at 60 °C [15]. This procedure ensures that the vesicles with and without $NaClO_4$ are of the same size to start with. It can be assumed that the Na^+-, ClO_4^--ions pass the unannealed bilayer and are thus equally distributed within the intra- and extravesicular milieu. Any addition of $NaClO_4$ before the sonication period could lead to a different size distribution compared

with a separate and NaClO₄-free preparation of vesicles.

Measurements

The fluorescence anisotropy *r* was measured with a SPF 500 ratio spectrofluorometer (American Instrument Company) with polarisation accessory. The temperature of the thermostated cuvette was slowly increased ($\sim 0.3°$/min) and directly read in the cuvette by a copper/constantan thermocouple attached to a digitalthermometer (Newport Laboratories). For transmission measurements a Beckman UV 5260 spectrometer was used. The stopped-flow equipment consists of the following arrangement: the light (300 W Xenon (Schoeffel Instrument) or 30 W tungsten (Zeiss)) passes through a monochromator (Schoeffel Instrument) and is focussed into the observation chamber of the stopped-flow apparatus (Sigma-Biochem, Puchheim-München) which works almost pressureless except during the short driving-periods of the pistons. The vertical arrangement of the driving-syringes minimizes the interference with unavoidable small air-bubbles. Absorption, fluorescence or light scattering measurements are possible. The data from the photomultiplier (EMI 9558 QA or RCA PF 1039) are digitized (ANDI, Zahner Elektronik, Kronach) and fed into a 8032 CBM computer. Minimal time-resolution is 0.2 ms for a 12 bit data-point.

Results and Discussion

a) Phase-transitions

Bilayers of saturated lecithin-molecules show a crystalline to liquid-crystalline phase transition. In multilamellar system the maintransition at T_c is precluded by the so-called pretransition. The three phases are characterized with increasing temperature by $L_{\beta'}$, $P_{\beta'}$ and L_α [17]. In small vesicles curvature effects become important and any rippled structures ($P_{\beta'}$) are difficult to envisage [19]. Thus the main transition is usually broader than in liposomes, and small unilamellar vesicles do not show a pronounced pretransition if a pretransition exists at all.

Phase transitions can conveniently be measured by several techniques. We have used the temperature-dependent fluorescence anisotropy of diphenylhexatrien (DPH) embedded in the lipid bilayer [18].

With increasing temperature the fluorescence anisotropy *r* decreases in a sigmoid manner by passing the phase-transition. The method is rather sensitive and easily applicable, however, a quantification in terms of microscopic parameters is not unambiguous and still in discussion. In the present case we did not observe any differences in the phase-transition profiles for vesicles composed of dipalmitoylphosphatidylcholine (DPPC) without and with the addition of 10 mM NaClO₄ (Fig. 2). Above 10 mM NaClO₄ on both sides of the membrane the vesicle solution became turbid. DPH is located in the hydrophobic core of the bilayer. Studies with other marker-molecules located at the membrane-water interface also failed above 10 mM NaClO₄ because of a precipitation.

b) Vesicular precipitation

Kinetic investigations on the onset of the precipitation were performed in order to understand the underlying phenomena of the aggregation process. Two principle questions were raised concerning the salt-specificity and the mechanism of the observed aggregation.
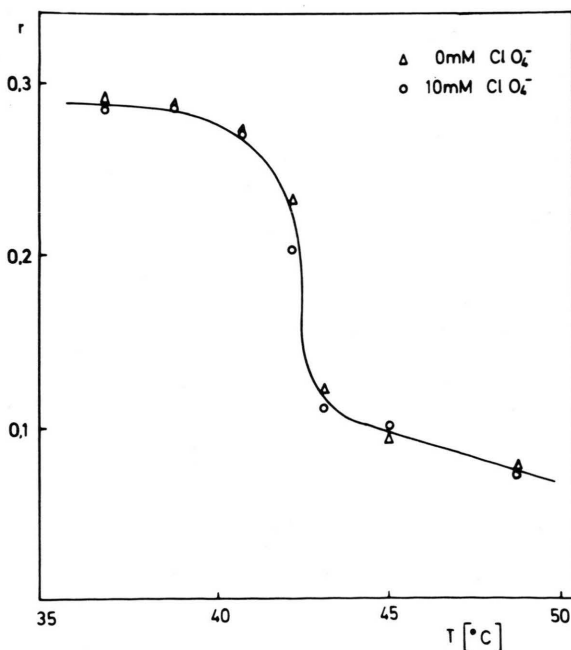

Fig. 2. Fluorescence anisotropy *r* of diphenylhexatrien (DPH) as function of temperature *T*. $\lambda_{ex} = 360$ nm, $\lambda_{em} = 428$ nm. DPPC vesicle, DPPC:DPH = 1000:1.

Ion-specificity

Various anions as Na-salts of equal concentrations were mixed with the lecithin vesicles while the turbidity or intensity of the scattered light were monitored. The results of a characteristic set of experiments are presented in Fig. 3. Except for the additional salts the syringes contained the same buffer. The mixing of the two solutions (vesicle in buffer and buffer plus salts) creates an osmotic gradient across the membrane. However, unilamellar vesicles do not act as osmometers in contrast to liposomes [20] or erythrocytes [21]. This becomes evident from the NaCl experiment where the light scattering or turbidity signal is not affected by the extravesicular addition of 0.2 M NaCl. The resultant effects are therefore clearly not caused by the osmotic gradient accompanying the mixing-process. The tendency to induce a precipitation of the lecithin vesicle solution increases in the order (Fig. 3)

$$HCOO^- < Cl^- < CH_3COO^- < NO_3^-$$
$$< N_3^- < Br^- < SCN^- < I^- < ClO_4^-.$$
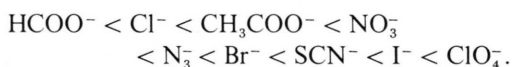
This order of anions can favourably be compared with the structure-breaking tendency [3] with $SCN^- < Cl^- < NO_3^- < Br^- < I^- < ClO_4^-$. We have further added 5% palmitic acid in order to put a negative charge on the DPPC-vesicles but we obtained almost the same tendency as above. Measure-
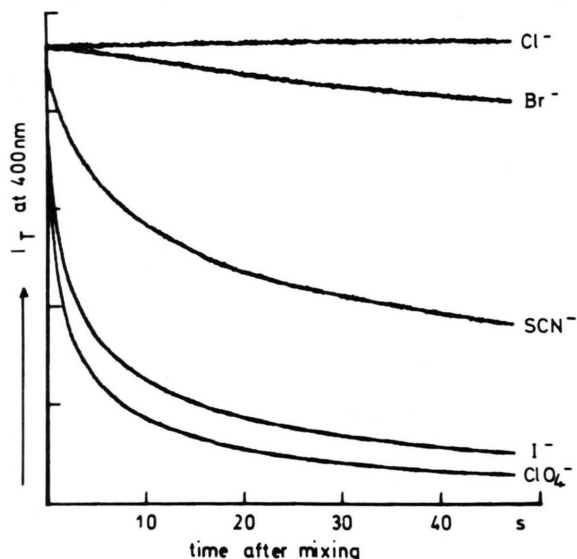

Fig. 4. NaClO$_4$-concentration dependence of the light scattering increase at 600 nm. Initial slope v_0 *vs* NaClO$_4$-concentration. DPPC vesicles, room temperature.

ments on the cation-specificity were performed with XBr salts which should show well observable tendencies according to Fig. 3. We found that the capability to induce a turbidity-increase of the vesicular solution decreases in the series $Na^+ > K^+ > Rb^+ > Cs^+$, *i.e.* a decrease with increasing ion-radius. Li^+ behaved untypically and possibly caused a precipitation of larger particles. The addition of 5% palmitic acid changed the tendency. Now Li^+ showed hardly any effect; Na^+, K^+ and Rb^+ had the same but rather small trend, and Cs^+ had the strongest but still medium tendency accompanied with a precipitation of larger particles. The results on the cation-specificity are not as straight-forward as the results on anions. All investigations on the salt-specificity were performed below T_c (41.5 °C for DPPC) at room-temperature.

NaClO$_4$ concentration dependence (extravesicular)

The variation of the ClO$_4^-$-concentration revealed that a threshold concentration of NaClO$_4$ (70–80 mM in the present case) exists (Fig. 4). Above this concentration precipitation becomes visible in the time-range studied (seconds). At values above 250 mM NaClO$_4$ in the extravesicular milieu there seems to be a plateau. However, both the threshold-value and the beginning of the plateau might depend on the lipid to salt ratio.

Vesicle concentration dependence

The results of the vesicle concentration dependence at constant NaClO$_4$ concentration are indicated in Fig. 5. Clearly there is a non-linear dependence of the initial slope of the light scattering signal as function of the concentration of vesicles. A log-log-plot of the data of Fig. 5 revealed almost a

Fig. 3. Anion-specificity of the aggregation-effect. Transmitted light I_T at 400 nm *vs* time after mixing. DPPC vesicles. Final salt (NaX) concentration 200 mM, room-temperature.
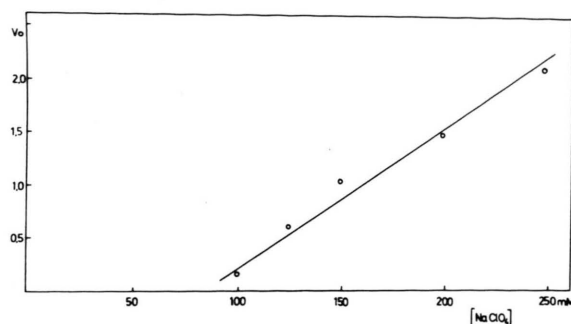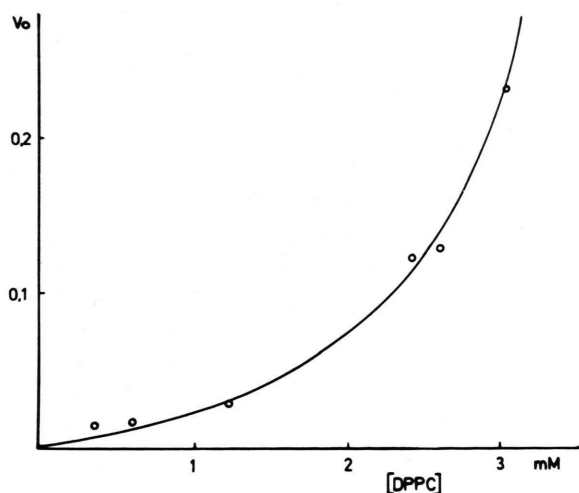
Fig. 5. Vesicle-concentration dependence of the light scattering increase at 600 nm. Initial slope v_0 vs DPPC concentration. All samples were obtained from one stock-solution, thus the same size-distribution of the vesicles is guaranteed. $NaClO_4$ 150 mM, room-temperature.

slope of two which is consistent with a mechanism mediated by vesicle-vesicle collisions [22, 23]. This second order behaviour rules out any lipid-transfer as origin for the observed turbidity increase.

Temperature dependence

Temperatur-dependent studies indicate a decrease of the ClO_4^--induced vesicle aggregation with increasing temperature. In the liquid-crystalline phase the observed effects, i.e. an increase of the turbidity by the addition of a threshold amount of ClO_4^- ions, are almost temperature-independent. Astonishingly, there is no pronounced change at the phase-transition temperature. However, it was noticed that the approximation of the initial slope by a polynomial function of second order showed larger errors around the transition temperature.

Fusion or aggregation (flocculation, coagulation)

ClO_4^--ions above a threshold concentration induce an increase of the vesicular turbidity. A lipid-transfer mechanism can be excluded on the basis of Fig. 5. Thus it may be asked whether this ClO_4^--effect or more general the effect of chaotropic ions on vesicle solutions remains at the step of aggregation (flocculation) or proceeds via fusion to the formation of larger vesicles or multilamellars. In the following some pieces of evidence are put together which can

exclude vesicle-vesicle fusion as origin for the observed turbidity-increase of vesicle solutions.

Reversibility: The observed effect induced by chaotropic ions is reversible. Samples which had been exposed to the salt-action were submitted to prolonged dialysis against the pure buffer. Table I documents that the salt-action is almost reversible according to the transmission readings.

Vesicle integrity: the vesicles stay intact during the course of the observed aggregation. This behavior was probed by a special test-kit consisting of a fluorescent nucleotide-analog (γAmNS-UTP) and a phosphodiesterase. One substance was intravasicularly encapsulated and the second added to the extravesicular milieu. If during the period of precipitation vesicles are broken down the enzyme gets access to the substrate and catalyses the splitting of α-β-phosphoryl-bond. At the same time the fluorescence intensity should increase because the reaction product — the pyrophosphate adduct of the 1-amino-naphthalene sulfonate — shows a higher quantum yield than the educt where the fluorescence is intramolecularly quenched by the uracil-base [25]. The results of these experiments indicated that the intra- and extravesicular compartments remained separated during the $NaClO_4$-induced vesicle precipitation. Only if the vesicles were destroyed by the addition of Triton X-100 [24] the encapsulated substrate (enzyme) got into contact with the enzyme (substrate) and an increase of the fluorescence intensity was observed. Preliminary experiments where both the substrate and the enzyme were in separate vesicles seem to rule out any union of vesicles as consequence of the $NaClO_4$ addition. Fusion processes are evidently not the origin of the observed turbidity increase.

Electronmicrographs: Negatively stained vesicles with and without $NaClO_4$ show differences. The

Table I: Dialysis-experiments on the reversibility of the salt-action on DPPC vesicles. Transmission after the addition of 200 mM salt (final concentration) and after prolonged dialysis against the pure buffer.

Salt 200 mM	Transmission at 400 nm	
	Before dialysis	After dialysis 22 h at 44 °C
Buffer (control)	64.0	60.0
$NaClO_4$	7.8	42.3
NaI	11.7	37.2
NaBr	13.5	52.3

ClO_4^- containing solutions reveal larger amounts of aggregates and the vesicular surfaces turn form spherical to a more polyhedral form.

Conclusions

In the preceding sections we described the tendency of certain ions to induce aggregation phenomena in vesicular systems. This tendency is ion-specific and follows the chaotropic character of the ions studied. There seems to be a threshold-concentration. The passing of this concentration in the extravesicular medium leads to a process of rapid aggregation which shows second order kinetics with respect to the vesicle concentration. With increasing temperature the observed ion-effect decreases, thus the backward reaction (redispersion) is obviously favoured with increasing temperature. Preliminary experiments do not reveal pronounced changes by passing the phase transition of the respective lipid. The vesicles stay intact and evidently keep their individuality, i.e. induced fusion processes can almost be excluded. The observed aggregation is almost reversible.

In terms of the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (for application to vesicular systems see [14, 26, 27]) the described ion-effect leads to changes of the intervesicular energy of interaction, i.e. the action of the chaotropic ions can deepen the secondary minimum or decrease the barrier between the two energy-minima. In the first case flocculation results, in the second-one coagulates with close intervesicular distances are stabilized. Vesicular fusion is supposed to proceed from these coagulates [14, 27, 28]. The indicated temperature-dependence and the reversibility seem to favour the vesicle flocculation as origin for the observed turbidity increase. The kinetic experiments were performed in the time-period of seconds and with the respective ions only in the extravesicular medium. Under those conditions flocculation proceeds within seconds. A precipitation of vesicles seems to start at lower $NaClO_4$-concentration if the salt is present on both sides of the membrane. Whether the induced flocculation leads to vesicle-vesicle fusions on a longer timescale is under current investigation.

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