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ORIGINAL CONTRIBUTION

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Cation-mediated interaction of dextran sulfate with phospholipid vesicles: binding, vesicle surface polarity, leakage and fusion

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Abstract The interaction of dextran sulfate (DS) with dimyristoylphosphatidylcholine (DMPC) large unilamellar vesicles was investigated. DS of different molecular weights (1, 8, 40 and 500 kDa) and divalent cations (Ca^{2+}, Mg^{2+} and Mn^{2+}) and the trivalent cation La^{3+} were used in the experiments. Binding of DS was studied by use of the microelectrophoresis and monolayer technique. Binding depends strongly on cation and NaCl concentrations in the medium and does not occur in the absence of multivalent cations. Binding is modulated by the molecular weight of the polymers; DS with lower molecular weights lead to less negative zeta potentials at identical concentrations. A comparable monomer of DS, glucose-6-sulfate, does not change the zeta potential of DMPC vesicles. Monolayer experiments revealed a decrease in surface pressure after addition of multivalent cations and DS, indicating a stronger interaction of the cation–polymer complex with the phosphatidylcholine headgroups than its penetration into the phospholipid (PL) bilayer. The cation-mediated binding of DS to the vesicles leads to aggregation of the vesicles. The tendency to promote aggregation of DMPC vesicles is $La^{3+} > Ca^{2+} > Mn^{2+} \geq Mg^{2+}$. The aggregated vesicles show a stacklike arrangement of the bilayers as shown by freeze-fracture electron microscopy. The strong aggregation is accompanied by lipid mixing measured by the 1,4-nitrobenzo-2-oxa-1,3-diazole–phosphatidylethanolamine (PE)/lissamine rhodamine B sulfonyl-PE assay. At low ionic strength substantial lipid mixing can be observed in the previously mentioned order of the cations. This lipid mixing is accompanied by an increase in the permeability of the vesicles as revealed by the 1-aminonaphthalene-3,6,8-trisulfonic acid/*p*-xylenebis (pyridiium bromide) assay. The extent of leakage is determined by the cation used and the DS molecular weight. These interaction processes between the opposing bilayers are connected with a decrease in the water content in the gap between the opposing PL bilayers. As a measure for the change of the polar properties of the vesicle surface the shift of the emission wavelength of the fluorescent probe dansylphosphatidylethanolamine was measured. The effectiveness of divalent/trivalent cations to decrease the surface dielectric constant of DMPC vesicles also followed the sequence of ions as found for binding, PL mixing and leakage. The results are discussed in terms of the changed hydration at the bilayer surface induced by DS in the presence of multivalent ions.

Key words Dextran sulfate · Liposomes · Dimyristoylphosphatidylcholine · Ca^{2+} · Fusion

Introduction

Glycosaminoglycans (GAG) are important components of the extracellular matrix. The study of the interaction of GAG with surfaces and their influence on the physicochemical properties of phospholipid (PL) membranes has been the subject of several studies in the last few years [1–13]. Because most biological surfaces are negatively charged at physiological pH due to the dissociation of polar surface groups, electrostatic attraction of these anionic polymers should be less probable. However, it could be shown that GAG and the homologous molecule dextran sulfate (DS) can bind to the surface of cells [11], lipoproteins [14] and viruses [7]. The binding of GAG to virus and lipoprotein surfaces occurs partially by electrostatic interaction with positively charged domains on the surface [7, 10, 15]. It was shown that Ca^{2+} ions and PLs are involved in the binding [6]. Ca^{2+} concentrations of about 2 mM occur in the extracellular space, which is more than 3 orders of magnitude higher than the intracellular concentration. The physiological role of extracellular Ca^{2+} for processes occuring at extracellular surfaces is far less recognized than for intracellular Ca^{2+}.This extracellular Ca^{2+} is of great importance for the adsorption of GAG because bridges between the zwitterionic PLs in the vesicle surface and the polymer can be formed. The binding of GAG to the vesicle surface is accompanied by the aggregation of the particles at subcritical GAG concentrations. The aggregation of PL vesicles [2, 5] and lipoproteins [12] induced by divalent cations and GAG has been known for a long time. Nevertheless, knowledge of the molecular mechanisms of the interaction is limited. Structural changes [6] and changes in the thermodynamic parameters of PL vesicles have been reported in recent articles [3, 13].

Cevc [28] developed a unified theory using the effective interfacial hydration of the PL headgroups for the description of colloidal and physicochemical properties of PL bilayer systems. This hydration is primarily a function of surface polarity. On this basis, Ohki and Arnold [16] applied fluorescence methods for the description of ion-induced aggregation and fusion of negatively charged phosphatidylserine vesicles. It was shown that the surface dielectric constant decreases drastically if water-poor contacts of the surfaces are formed in the aggregation state. The different actions of cations on the aggregation could be described. The authors were also able to explain the combined effect of poly(ethylene glycol) and cations on the aggregation. However, multivalent cations fail to induce aggregation of phosphatidylcholine (PC) vesicles, except at concentrations higher than 0.1 M. Aggregation of PC vesicles is only possible in the presence of negatively charged polymers/multivalent cations or by the poly(ethylene glycol) method. On the other hand, di- and trivalent cations bind to PC membranes [32] and induce a change in the interbilayer separation as shown by Lis et al. [33] using X-ray techniques.

Materials and methods

Dimyristoylphosphatidylcholine (DMPC) was purchased from Sigma. The purity was checked by thin-layer chromatography. The fluorophore-labelled phospholipid 1,2 diacyl-*sn*-glycero-3-phospho-*N*-[5-dimethylaminonaphthalene-1-sulfonyl]ethanolamine (DPE) was from Sigma, 1-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (NBD–PE) and lissamine rhodamine B sulfonyl (Rh–PE) were from Avanti Polar Lipids (USA).

DS with a molecular weight of 1 kDa was from Pfeifer & Langen, Germany, DS with molecular weights of 8 and 40 kDa was obtained from ICN Biochemicals (USA) and DS with a molecular weight of 500 kDa was obtained from Pharmacia (Sweden). Glucose-6-sulfate was purchased from Sigma. 1-Aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and *p*-xylenebis(pyridiium bromide) (DPX) were obtained from Molecular Probes (USA).

Multilamellar DMPC liposomes (MLV) were prepared using the method of Bangham et al. [17]. The lipid was initially dried from chloroform, subsequently dispersed in the appropriate buffer solution and shaken at a temperature above the gel-to-liquid-crystalline transition temperature for 10 min.

Large unilamellar vesicles (LUV) were prepared by five freeze-thawing cycles of MLV followed by extrusion (5 times) through 0.1-μm Nucleopore filter membranes using an extruder (Lipex Biomembranes) at 30 °C. The size of the samples was checked by electron microscopy (see later) for representative experiments and routinely by photon-correlation spectroscopy using a Zetasizer 4 (Malvern, UK) using an AZ 110 chamber applying multimodal deconvolution of data.

In all experiments aqueous stock solutions of the cations and DS were used und added to achieve the appropriate concentrations in the samples.

PL mixing assay (NBD-PE/Rh-PE)

The mixing of PLs was followed by the fluorescence resonance energy transfer method, using NBD-PE and Rh-PE according to the method described in Ref. [18]. The vesicles were composed of DMPC and 1% (mol/mol) of both NBD-PE and Rh-PE. One part (0.05 μmol lipid) of the fluorophore-incorporated vesicles and two parts (0.1 μmol lipid) of the unlabelled vesicles were suspended in 2 ml of the appropriate buffer solution (with/without DS). The fluorescence measurements were carried out by exciting the mixture at 470 nm and recording the fluorescence in the wavelength range from 500 to 620 nm. The recording started after the system had almost reached equilibrium (about 1 min). The extent of PL mixing, M, was calculated from the fluorescence intensity of NBD-PE at 525 nm and Rh-PE at 578 nm:

$$M = (I_{525}/I_{578}) - (I^0_{525}/I^0_{578}) \ , \qquad (1)$$

where I is the fluorescence amplitude of the solution containing DS and I^0 is that of the solution without DS. The experiments were done at 35 °C. The 100% value of fusion was defined as the value obtained by solubilization of the vesicles in 0.2% (v/v) Triton X-100.

Measurements of the vesicle surface polarity

The vesicle surface polarity was measured by use of the Stokes shift of the fluorescent probe DPE according to the method described in Refs. [16, 19]. DPE was mixed with the PLs in chloroform at a

molar ratio of PL/DPE of 200–300. An aliquot of the vesicle stock solution was suspended in an appropriate buffer solution. The excitation wavelength was 340 nm and the emission was measured in the range 400–600 nm (Perkin-Elmer, LS-50).

Leakage measurements

The measurements of the release of vesicle contents were carried out using procedures based on the quenching of ANTS fluorescence by DPX [20]. For the preparation of vesicles containing both ANTS and DPX, the PLs were suspended in the appropriate buffer solution containing 1.25 mM ANTS, 4.5 mM DPX and 2.0 mM NaCl. The solution was titrated to 20 mosmol/l using a Knauer digital osmometer. LUV were produced as mentioned earlier. Free dyes were removed by passage through a Sephadex G-75 column. The excitation wavelength was set to 360 nm and the emission was measured above 530 nm using a Corning filter and the total emission attachment for the Perkin-Elmer LS-50 B spectrofluorimeter. The fluorescence intensity obtained from intact vesicles containing both ANTS and DPX was set for 0% leakage. 100% leakage was measured after addition of 0.1% (v/v). Intensities were corrected for light scattering, measured under the same conditions as fluorescence using unlabelled vesicles.

Monolayer experiments

Measurements of the surface tension were done on a Krüss K 12 D process tensiometer equipped with a thermostatted and stirring unit. An appropriate amount of PL in chloroform/hexane was added to achieve a film pressure of about 25 mN/m unless otherwise indicated. After a period of stabilization (about 30 min) DS and cation stock solutions were added using a Hamilton syringe and changes in surface tension were recorded while the subphase was stirred. Stable values of the surface tension were obtained after a measuring time of at least 1 h.

Microelectrophoresis and light scattering

The electrophoretic mobilities, u, of DMPC LUV were measured using a Malvern Zetasizer 4 device equipped with an AZ 104 cell. The cell was thermostatted (for all measurements 30 °C) and the electrodes were separated by semipermeable membranes from the sample compartment. The electrophoretic mobilities of the LUV were measured using a modulation frequency of 250 Hz and the electrode current was 2.5 mA for 0.01 M NaCl containing buffer to avoid electrode polarization. The viscosities of the samples containing liposomes, DS and the complexes were set to be the same as that of the pure buffer solution. Viscosity measurements using the highest concentrations of DS 500 kDa (1 g/l) did not indicate changes in viscosity with respect to the buffer solution.

Zeta potentials, ζ, of the DMPC LUV were calculated using the Helmholtz–Smoluchowski equation, modified for small particles

$$\zeta = 3u\eta/2\epsilon\epsilon_0 \quad . \tag{1}$$

90° light scattering was measured using the same sample. Multimodal analysis was used for the deconvolution of the data.

Freeze-fracture electron microscopy

For freeze-fracture experiments a higher lipid concentration (5 g/l) was used for vesicle preparation. Freshly extruded lipid suspensions were handled at 35 °C, sandwiched between copper specimen holders and quickly frozen by plunging into liquid propane cooled by liquid nitrogen. Freeze-fracture preparations were made with a BAL-TEC BAF 400 T freeze-fracture device by use of a double replica stage. For replication Pt(C) was evaporated under an angle of 35°. The thickness of the deposited Pt (about 2 nm) was controlled by quartz crystal monitoring. The replicas were cleaned in chloroform/methanol (1/1), placed on a uncoated Mesh 300 copper grid and examined in a LEO-EM 900 electron microscope.

Results

Laser electrophoresis

Changes in the zeta potentials of DMPC LUV are given as a function of DS of different molecular weight in the presence of different cations in Fig. 1. DMPC vesicles without addition of di- and trivalent cations show a zeta potential of nearly 0 mV. The addition of DS in the absence of divalent/trivalent cations in separate experiments does not change the zeta potential of the vesicles. La^{3+} binds to the DMPC vesicles and the zeta potential changes to about +15 mV at a concentration of 1 mM La^{3+}. Addition of 1 mM divalent cations did not change the zeta potential of the DMPC vesicles. In accordance with the Gouy–Chapman theory we found that the zeta potential of the DMPC vesicles after addition of ions and DS is strongly influenced by the ionic strength of the solution (data not shown). In order to obtain optimum experimental conditions, we carried out the measurements in solutions with low ionic

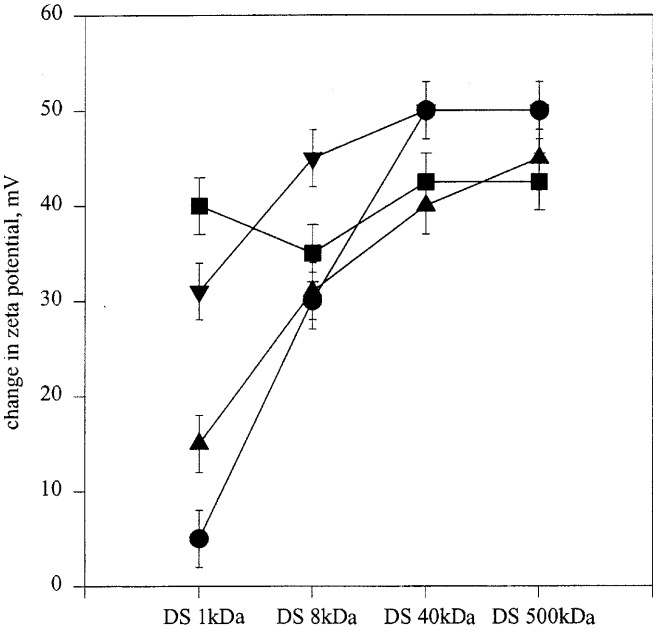


Fig. 1 Change in the zeta potentials of dimyristoylphosphatidylcholline (*DMPC*) large unilamellar vesicles (*LUV*) in the presence of 1 mM Ca^{2+} (●), La^{3+} (■), Mg^{2+} (▼) and Mn^{2+} (▲) after addition of 0.25 g/l dextran sulfate (*DS*) with different molecular weights in 10 mM *N*-(2-hydroxyethyl) piperazine-*N'*-ethanesulfonic acid (*HEPES*), 10 mM NaCl, pH 7.4, 30 °C. *Error bars* represent the standard deviation of the measurements

strength (10 mM NaCl). The titration of DS in the presence of divalent cations with DMPC vesicles results in negative zeta potentials. In the presence of Ca^{2+}, Mn^{2+} and Mg^{2+} the maximum zeta potential which can be reached is dependent on the molecular weight of DS used, i.e. DS with a higher molecular weight induces stronger changes in the zeta potential. The maximum changes in the zeta potential of DMPC vesicles were found for cation concentrations of 1 mM and about 0.25 g/l DS. Comparable results were obtained by Krumbiegel and Arnold [6] for Ca^{2+}/DS 500 kDa and DMPC. It should be noted that the calculation of the zeta potentials of liposomes bound to large polymers is hampered by the fact that the distance of the hydrodynamic plane of shear from the PL vesicle surface may be changed.

In contrast to the divalent cations, which do not change the zeta potential of DMPC LUV, La^{3+} increased the zeta potential to +15 mV. This is based on the higher binding constant to PC membranes as determined by several authors [22, 24]. Addition of identical DS concentrations (0.25 g/l) as used for the experiments with divalent cations results in a zeta potential of about −25 mV. Under the same experimental conditions the maximum zeta potential for divalent cations is about −40 mV.

In order to compare the effects of the DS polymer and the monomer, glucose-6-sulfate was added to DMPC LUV in the presence of 1 mM Ca^{2+}. The monomer does not induce a change in the zeta potential in the presence of 10 mM as well as 100 mM NaCl.

Size measurements (data not shown) indicate a strong increase in size (diameter above 1 μm) of the vesicles in the presence of multivalent cations and DS, indicating strong vesicle aggregation.

Monolayer experiments

A monolayer of DMPC was formed and the surface pressure was held constant at 25 mN/m at a temperature of 30 °C. In our experimental setup a stable surface pressure of a DMPC monolayer can be obtained after 30 min under temperature control. In order to measure changes in the lateral organization of the PL monolayer, 1 mM Ca^{2+} was added to a DMPC monolayer, this does not result in a change in the surface pressure. If, additionally, DS is added to the subphase, a decrease in film pressure can be observed. The decrease in the film pressure is given as a function of the concentration of Ca^{2+} in the presence of DS of different molecular weight in Fig. 2. Using DS 500 kDa (1 g/l) and Ca^{2+} concentrations of about 4 mM, the surface pressure is decreased by about 3.4 mN/m. Lower cation concentrations result in a reduced decrease in the surface pressure. DS with lower molecular weight than 500 kDa induces a lower decrease in the surface pressure, for example, the combination of DS 1 kDa (1 g/l) and 5 mM Ca^{2+} decreases the surface pressure in the order of about 1 mN/m.

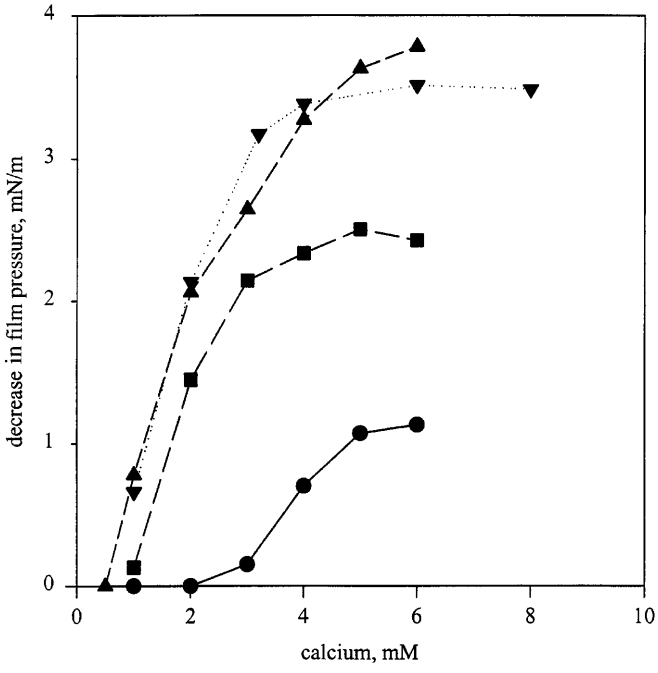


Fig. 2 Decrease in the surface pressure of a DMPC monolayer in the presence of 1 g/l DS 1 kDa (●), DS 8 kDa (■), DS 40 kDa (▼) and DS 500 kDa (▲) induced by addition of Ca^{2+}. The initial film pressure was set to 25 mN/m; buffer: 10 mM HEPES, 10 mM NaCl, pH 7.4, 30 °C

Comparable concentrations of glucose-6-sulfate (1 g/l) and calcium (4 mM) did not change the surface pressure of a DMPC monolayer.

As shown in Fig. 3, if 1 mM La^{3+} is present in the subphase, the addition of 1 g/l DS with different molecular weights is followed by a strong decrease in film pressure of about 4–9 mN/m depending on the initial film pressure. At comparable conditions (1 mM of the cation) the decrease in film pressure after DS addition is much higher for La^{3+} (4–5 mN/m) in comparison to Ca^{2+} (around 1 mN/m). In agreement with the results obtained with Ca^{2+}, the addition of 1 g/l glucose-6-sulfate does not change the film pressure at different initial film pressures.

Electron microscopy

Replicas of freeze-fracture preparations are shown for DMPC LUV in Fig. 4. The vesicles were prepared by extrusion through 200-nm Nucleopore filter membranes.

The fracture faces of freshly prepared LUV frozen from a 35 °C exhibit a facetlike texture formed by

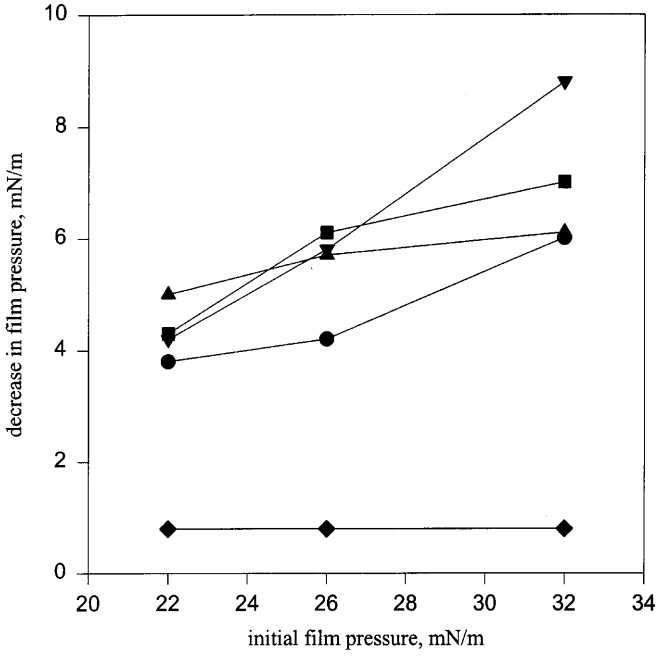


Fig. 3 Decrease in the surface pressure of a DMPC monolayer in the presence of 1 g/l DS 1 kDa (●), DS 8 kDa (■), DS 40 kDa (▼), DS 500 kDa (▲) and glucose-6-sulfate (◆) induced by the addition of 1 mM La^{3+}. The initial film pressure was varied in the range between 22 and 32 mN/m; buffer: 10 mM HEPES, 10 mM NaCl, pH 7.4, 30 °C

cooling during quick freezing (Fig. 4A). No aggregation behaviour was observed. After addition of DS 500 kDa again no aggregation of LUV occurred, but small particle structures of about 8 nm in diameter appeared in the medium between the vesicles (Fig. 4B).

In contrast, after addition of DS 500 kDa and Ca^{2+} large aggregates of LUVs were formed, indicated also by a visible increase in the turbidity of the suspension. After addition of a more moderate Ca^{2+} concentration of 1 mM mainly aggregates of tightly packed vesicles (Fig. 4C) and more seldom larger fusion products were found (Fig. 4D). After addition of a higher Ca^{2+} concentration of 10 mM, besides aggregated, tightly packed LUVs additionally multilayered stacks of extended lamellae and MLV were formed (Fig. 4E). Multilayered stacks of lamellae and MLV were more frequent after incubation of the sample at 35 °C overnight (Fig. 4F), showing that the fusion process ran over a long time.

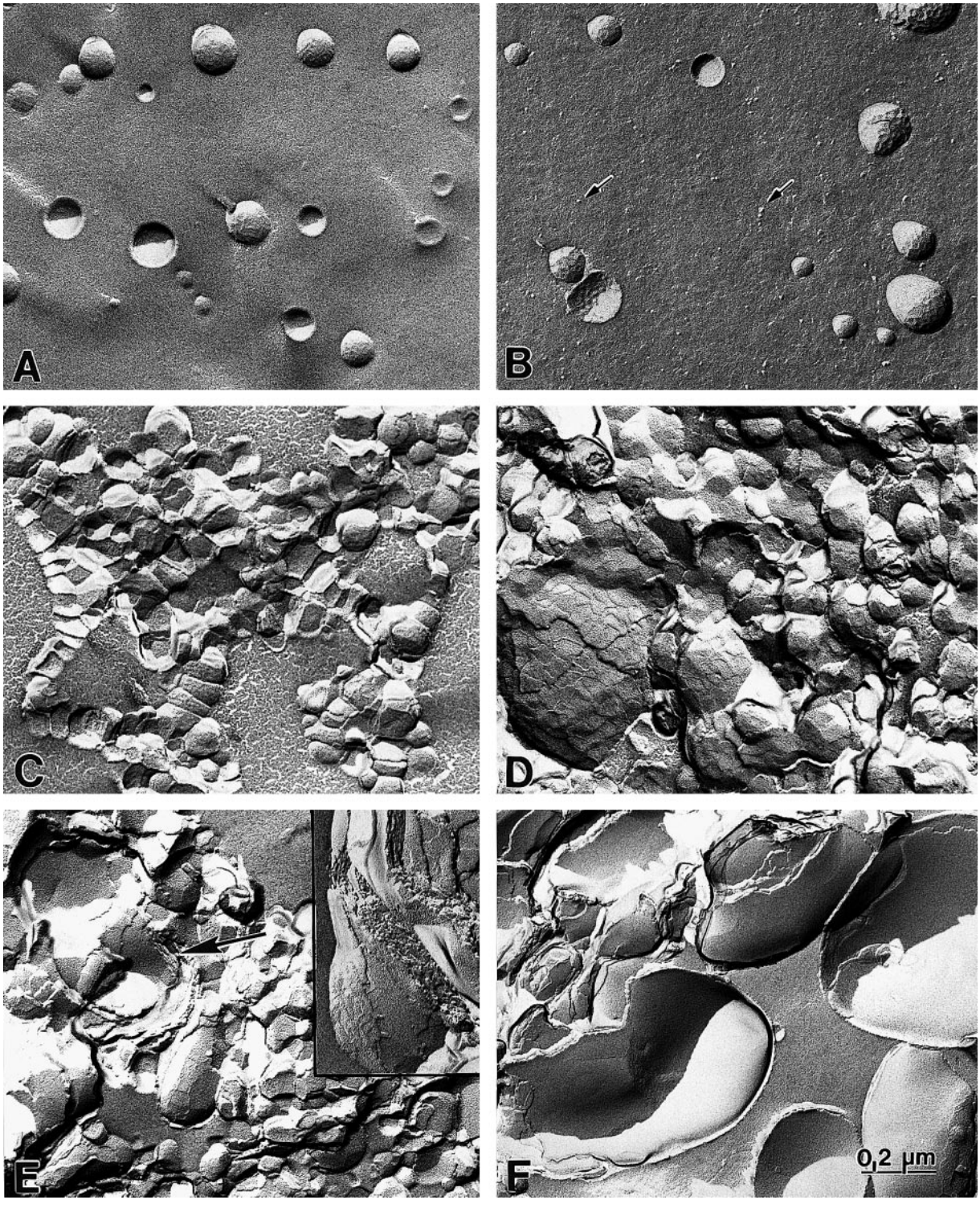
Fluorescence measurements

A shift of the emission peak of DPE embedded in DMPC LUV to lower wavelength was measured after DS was added in the presence of di- and trivalent cations. In previous reports it was shown that binding of di- and trivalent cations can shift the emission maximum of DPE embedded in negatively charged PL matrices to lower wavelengths. Using neutral vesicles (PC or PE) no changes in the DPE emission peak position were detected. The dependence of the maximum shifts of the positions of the emission maxima of the fluorescent probe DPE on a fixed DS concentration and 1 mM of the cations used is given in Fig. 5. A temperature of 35 °C was chosen because differential scanning calorimetry (DSC) measurements have shown that this temperature is above the phase transition in the presence of DS and divalent/trivalent cations [13]. Normally, the position of the emission peak of DPE embedded in DMPC LUV without any addition is about 507 nm. As shown by Steffan et al. [13] the shift of the DPE emission peak is dependent on the NaCl concentration.

The addition of 1 mM La^{3+} in the presence of 0.25 g/l DS 1 kDa, 8 kDa, 40 kDa and 500 kDa shifts the DPE emission peak to lower wavelength by about 15, 13, 10 and 16 nm, respectively. In the presence of DS 500 kDa 1 mM Ca^{2+} induces a maximum shift of 3 nm (0.05 g/l DS 500 kDa). Ca^{2+} and DS with lower molecular weights induce shifts lower than 2 nm. Mg^{2+} does not induce a considerable shift of the DPE emission peak for all DS. In the presence of DS Mn^{2+} induces a shift of the DPE emission peak: 3 nm for DS 1 kDa, 5 nm for DS 8 kDa, 4 nm for DS 40 kDa and 6 nm for DS 500 kDa. All experiments were conducted up to a DS concentration of 5 g/l; the shift of the emission peak did not increase further compared to the values shown in Fig. 5.

It should be noted that a decrease in the sample temperature has a strong influence on the fluorescence properties. For temperatures below the phase transition of the DMPC/cation/DS system larger blueshifts of the DPE emission peak were observed, which should be taken into consideration if our results are compared with the data given by Steffan et al. [13].

We have found that the strong aggregation of the vesicles induced by cations and DS can be accompanied by vesicle fusion. The maximum extents of the PL mixing (NBD-PE/Rh-PE assay) of DMPC LUV (diameter 0.4 μm) after addition of DS 1 kDa, 8 kDa, 40 kDa and 500 kDa in the presence of tri- and divalent cations are given in Fig. 6. By measuring time traces of the NBD fluorescence intensity, one can observe that the mixing process is very fast (about 10 s). For this reason we monitored the final PL mixing extents after at least 1 min. Using La^{3+} very high PL mixing extents (about 20–40%) can be achieved for DS with high molecular weight (DS 8, 40 and 500 kDa) at concentrations of 0.25 g/l, whereas DS with a molecular weight of 1 kDa at a concentration of about 0.25 g/l induces PL mixing extents of about 12%. Ca^{2+} is less effective and PL maximum mixing extents of 6% were observed in the presence of DS 500 kDa (at 0.25 g/l). DS with lower



molecular weights induced smaller extents of PL mixing (DS 40 kDa about 3%, DS 8 kDa about 2%, DS 1 kDa ineffective). In the presence of Mg^{2+} and Mn^{2+} DS does not induce substantial PL mixing (less than 3%) regardless of molecular weight.

The maximum extent of release of ANTS/DPX from DMPC vesicles is given in Fig. 7 for a given concentration (1 mM) of the cations used and DS (0.25 g/l) of different molecular weights. The fluorescence intensities were corrected for scattered light (complex formation of

◀

Fig. 4A–F Freeze-fracture electron micrographs of DMPC LUV prepared by extrusion through a Nucleopore filter and frozen from about 35 °C. Buffer: 10 mM HEPES, 10 mM NaCl, pH 7.4. **A** LUV resulting from pores of 100 nm in diameter without DS and Ca^{2+} addition. Textures on the fracture faces are caused during quick freezing. **B** LUV as in **A** after addition of 0.2 g/l DS 500 kDa and 1 mM Ca^{2+}. No aggregation of vesicles occurred. Small particles of about 8 nm in diameter (*arrows*) in the surrounding of the vesicles correspond to DS. **C**, **D** LUV as in **A** after addition of 1 g/l DS 500 kDa and 1 mM Ca^{2+}. Large complexes of aggregated vesicles predominate. **C** Aggregated vesicles were slightly flattened and often arranged in rows. **D** Additionally large fusion products were found. **E**, **F** LUV as in **A** after addition of 1 g/l DS 500 kDa and 10 mM Ca^{2+}. **E** Immediately after mixing of LUV, DS and Ca^{2+} besides aggregated vesicles multilayered stacks of lamellae (*arrow* and *inset*) were present. **F** After storage of the samples at 35 °C overnight fusion continued as indicated by many enlarged fusion products. The *bar* represents 200 nm

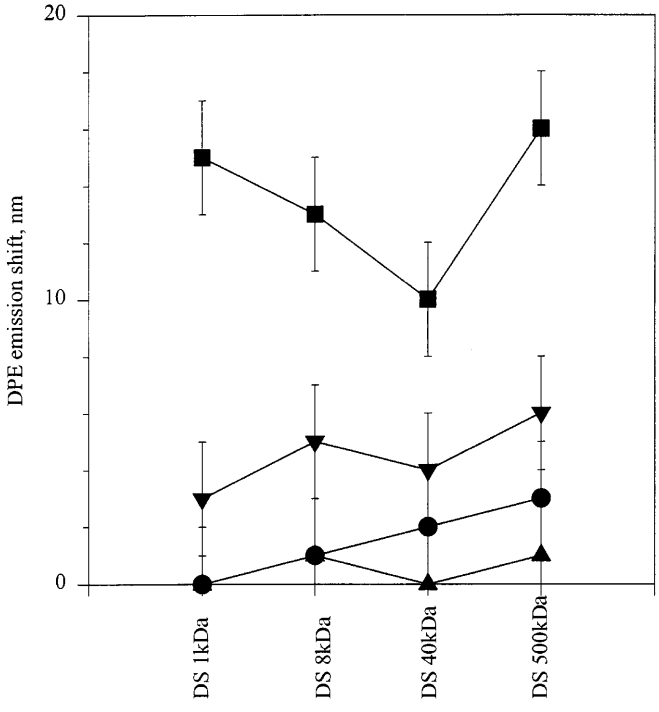


Fig. 5 Change in the position of the fluorescence emission maximum of dansylphosphatidylethanolamine (*DPE*) incorporated in DMPC LUV (diameter 0.4 μm) in the presence of 1 mM Ca^{2+} (●), La^{3+} (■), Mg^{2+} (▲) and Mn^{2+} (▼) as a function of DS molecular weight at a concentration of 0.25 g/l. 0.08 mM DMPC, 10 mol% DPE, 10 mM HEPES, 10 mM NaCl, pH 7.4, 35°C. The *error bars* represent the inaccuracy of the determination of maximum peak position

PC/cation/DS). The release of ANTS/DPX from the vesicles is only observable if both ions and DS are present: the addition of one partner is insufficient for inducing leakage. Using 1 mM of the tri- and divalent cations and 0.25 g/l DS of different molecular weights the DMPC LUV were very leaky. The extent of leakage was the highest in the presence of La^{3+} (71% leakage

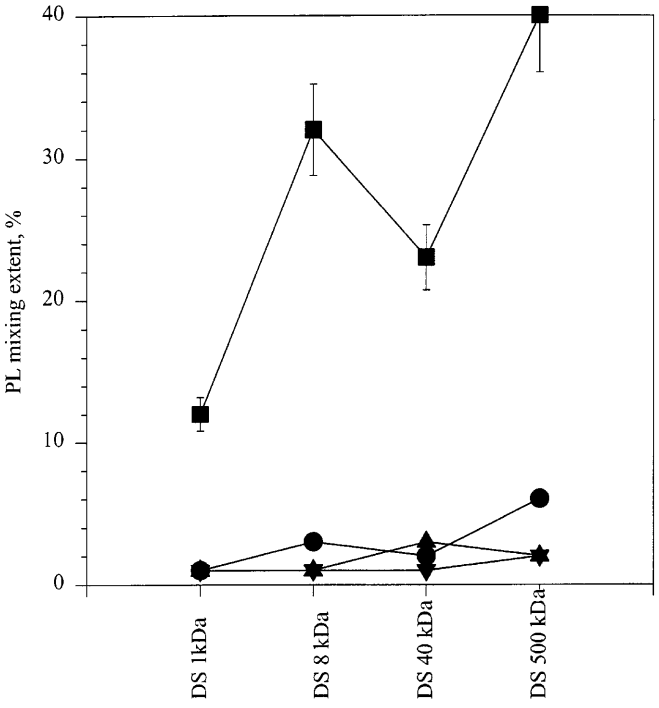


Fig. 6 Phospholipid (*PL*) mixing extent, 1,4-nitrobenzo-2-oxa-1,3-diazole–phosphatidylethanolamine (*PE*)/lissamine rhodamine B sulfonl–PE assay, of DMPC LUV (diameter 0.4 μm) induced by 1 mM Ca^{2+} (●), La^{3+} (■), Mg^{2+} (▲) and Mn^{2+} (▼) at 0.25 g/l DS of different molecular weights. 10 mM HEPES, 10 mM NaCl, pH 7.4, 35 °C

for DS 1 kDa, 54% for DS 8 kDa, 50% for DS 40 kDa and 54% for DS 500 kDa). The leakage extents for Ca^{2+}, Mn^{2+} and Mg^{2+} were in the range from 10 to 47%.

Discussion

The addition of DS and divalent/trivalent cations to DMPC LUV results in a binding of the polymers to the vesicle surface as demonstrated by laser electrophoresis measurements. The molecular weight of DS determines the maximum zeta potential of the DMPC/cation/DS aggregates. The higher the molecular weight of DS the more negative the electrophoretic mobility (EPM) of the aggregates. Using the experimental data one can deduce that at comparable molecular weight and concentrations of DS the magnitude of interaction with DMPC is influenced by cations in the order $La^{3+} > Ca^{2+} \geq Mg^{2+} \geq Mn^{2+}$. Previous binding studies revealed intrinsic binding constants of divalent/trivalent cations to PC membranes in the range 0.1–3.0 l/mol [22, 23] in the order $Ln^{3+} > Ca^{2+} = Mg^{2+}$ [24]. The binding of divalent cations to GAG was determined for chondroitin-4-sulfate: Ba > Sr > Ca > Mg; dermatan sulfate: Ba = Sr = Ca = Mg; heparin: Ca > Ba > Sr > Mg [25]. It is difficult

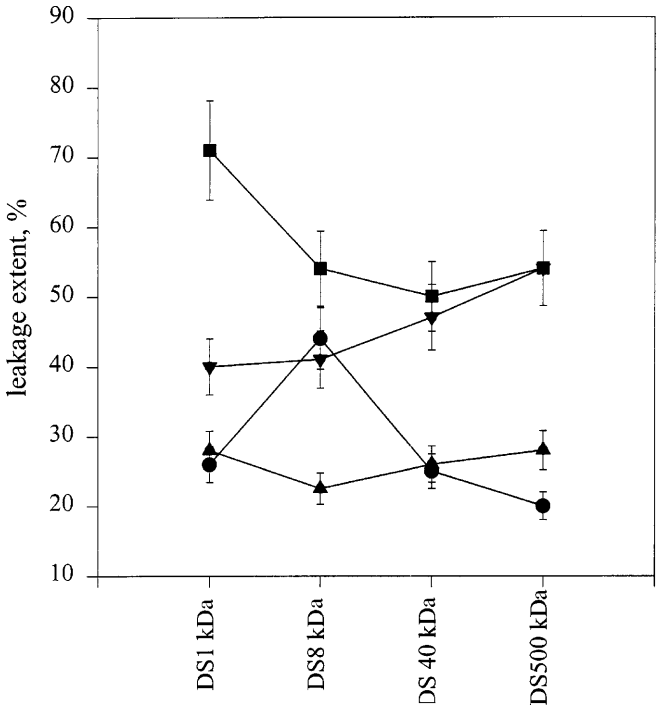


Fig. 7 Extent of leakage of DMPC LUV measured by the 1-aminonaphthalene-3,6,8-trisulfonic acid/*p*-xylenebis(pyridiium bromide) assay induced by 1 mM Ca^{2+} (●), La^{3+} (■), Mg^{2+} (▲) and Mn^{2+} (▼) and 0.25 g/l DS of different molecular weights. 10 mM HEPES, 10 mM NaCl, pH 7.4, 35 °C

to calculate binding constants from our EPM data because the binding process of this three-component system is much more complex. Huster and Arnold [31] determined an apparent Ca^{2+} binding constant of about 89 M^{-1} in the DMPC/DS 500 kDa/Ca^{2+} system using ion-selective electrodes. The apparent binding constant of Ca^{2+} to DMPC was determined to be 37 M^{-1}.

Strong shifts of the PL phase-transition temperature to higher values for the DMPC/di- and trivalent cations/DS system were observed by Steffan et al. [13]. It is known that the chain melting properties are regulated by the surface of the PL bilayer [26, 27]. These effects must be mediated by the binding of the polyanions to the polar headgroup. The shift of the phase-transition temperature due to changes in the polar region can result from different contributions such as the headgroup hydration, lipid–lipid bonds, etc. [27]. One contribution could arise from interlipid bonds formed by the polyanion. A network of the adsorbed polymers on the bilayer surface could restrict the motional freedom of the lipids. The measured decrease in film pressure of a DMPC monolayer after addition of DS and cations is a result of the restricted lateral mobility of the PLs. Interface and lattice properties, as well as lateral packing of PL monolayers, are strongly influenced by interaction with cationic polyelectrolytes [34].

Using freeze-fracture electron microscopy and light scattering it was demonstrated that DMPC LUV of high homogeneity in size form large associations of aggregated vesicles after addition of divalent/trivalent cations and DS. Depending on the DS used and the cation concentration two types of bilayers could be observed within these associations. One type are more or less loosely aggregated liposomes nearly unchanged in diameter and the other type are enlarged fusion products in the shape of multilamellar stacks/MLV. When frozen from the pretransition temperature of hydrated DMPC the fracture faces of the multilamellar stacks/MLV do not exhibit ripple structures (not shown here), indicating that the bilayers within the multilamellar structures are very tightly connected. The simultaneous existence of two types of bilayers is in good agreement with the appearence of two peaks in the DSC measurements as shown by Steffan et al. [13].

As measured by the shift of the emission wavelength of the fluorescent probe DPE the DMPC vesicles aggregated in the presence of DS and the cations exhibit a decrease in surface polarity depending on the molecular weight of DS. Although Ca^{2+} and other divalent cations can reduce slightly the surface dielectric constant of PC bilayers in the presence of DS, a strong reduction in the surface dielectric constant was only observed for La^{3+}. This result supports other findings that divalent cations do not remove PC-bound water thoroughly enough to produce the unhydrous DS-mediated cation-PC complexes.

As described by Krumbiegel and Arnold [6] the binding of GAG to DMPC vesicles is dependent on the phase state of the PL matrix; in the fluid crystalline state less GAG is bound to the PL surface compared in to the gel state. Using the DPE shift we found further evidence for this observation. If the sample (DMPC/cation/DS) is examined below the phase-transition temperature (data not given) the measured blueshift of DPE is increased compared to the data discussed earlier. This indicates a higher dehydration of the PC surfaces under these conditions.

In a recent article by Steffan et al. [13] the effect of ionic strength on the water content near the DMPC head group was measured in the presence of DS and divalent cations. The authors also found that low ionic strength conditions result in more dehydrated regions near the PL surface.

The PL mixing of PC small unilamellar vesicles induced by Ca^{2+} and DS 500 kDa was described by Arnold et al. [4] and Bichenkov et al. [29] using the NBD-PE/Rh-PE assay. In both reports an increase in PL mixing at 1 g/l DS 500 kDa in the presence of 1.6–2 mM Ca^{2+} was described. In these articles the increase in the NBD-PE fluorescence was only shown without calibration of the data, for example, to the effect of Triton X-100. So, a direct comparison is not possible. In

this article, the studies were extended to the action of various cations as well as DS of different chain length. The extent of PL mixing of DMPC LUV decreases in the order $La^{3+} > Ca^{2+} > Mn^{2+} \geq Mg^{2+}$ at constant DS concentration. Keeping the cation concentration constant, the ability of DS for inducing PL mixing increases with its chain length or with the number of sulfated groups. The variation in DPE emission shift and PL mixing extents obtained for DS of different molecular weights may be explained by the unequal density of sulfate groups of the distinct polymer.

The PL mixing process is accompanied by a destabilization of the vesicle bilayer as shown by the ANTS/DPX leakage assay. As shown for fusion processes of negatively charged PL vesicles induced by divalent cations, leakage is usually connected with these interaction processes. We conclude from time-dependent measurements that both processes (PL mixing and leakage) have the same rate constant and seem to be very fast (about 10 s).

Taking into consideration the light scattering measurements and the results obtained by the emission spectrum shift of DPE and PL mixing data, one can conclude that strong aggregation is not necessarily connected with strong contact of the vesicles leading to PL mixing of the vesicles and their surface dehydration. On the other hand, the strong binding of DS in the presence of divalent/trivalent cations leads to destabilization of the PL membranes. Our results indicate that destabilization is not necessarily connected with a dehydration of the PL vesicle surface.

All DS of different molecular weight can bind in the presence of tri- and divalent cations to neutral DMPC vesicles. Comparing the different cations by their ability to induce membrane interactions, it seems that La^{3+} can induce aggregation, PL mixing, a shift in DPE fluorescence and vesicle content leakage. Ca^{2+}, Mn^{2+} and Mg^{2+} do not induce substantial PL mixing and DPE shift, but aggregation and leakage did occur. This leads to the conclusion that Ca^{2+}, Mn^{2+} and Mg^{2+} in the presence of DS exert a weaker influence on the interaction forces of the DMPC vesicles than La^{3+}. The divalent cations are not able to bind as strongly as La^{3+}, thus preventing the formation of anhydrous water regions in the contact zone where the polymers are sandwiched between the bilayers. Therefore, it seems that a major function of charged polymers in DMPC liposome interaction is the promotion of liposome aggregation by reducing the repulsive forces as the hydration repulsion. Further studies are required to elucidate the additional molecular events of membrane destabilization. It is possible that lipid reorganization leads to the fusion or mixing of membranes at points where a close contact of PL membranes is mediated by the polymer. These contact points are also areas for pore formation as shown in our experiments.

In a recent article of Huster et al. [30] it was shown that the Ca^{2+}-mediated binding of DS to DMPC membranes results in a decrease in the average area per lipid molecule. The modifications of the interactions between neighbouring PL molecules due to DS binding via Ca^{2+} occurs at the lipid headgroups since DS does not penetrate the hydrophobic core of the membrane.

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