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A polycation causes migration of negatively charged phospholipids from the inner to outer leaflet of the liposomal membrane

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Abstract

Aggregation of the negatively charged liposomes caused by the addition of the linear polycation, poly-*N*-ethyl-4-vinylpyridine bromide, was studied. At the point of maximal size and zero electrophoretic mobility of aggregates, the concentration of positive charges brought in by the adsorbed polycation was found to be equal to the total concentration of negative charges both on the outer and inner surface of the lipid bilayer. Since polycation saturation of the liposomal negative charges was found to occur without disruption of the membrane, it was concluded that the polycation induced migration of negatively charged phospholipid molecules from the inner to outer leaflet of the bilayer.

Key words: Polycation-liposome interaction; Transmembrane lipid migration

1. Introduction

Since the early work of Hammes and Schullery [1], interaction of synthetic polyelectrolytes with liposomes has been mostly investigated in terms of membrane aggregation and fusion [2–8]. In the literature, no detailed information is available about macromolecule distribution between liposomes and solution, and about the composition and structure of polymer–liposome aggregates.

The essence of this work is to obtain such information studying the interaction of negatively charged PC-CL liposomes with PEVP.

Synthetic polyelectrolytes interacting with cells are known to induce some important physical chemical and biological effects such as clustering of membrane proteins, increase of ionic permeability of the membrane, cell activation, etc. [9–11]. So, the liposome–PEVP system can be regarded as a model to clarify the details of interaction of synthetic polycations with the cell membrane.

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Abbreviations: PC, phosphatidyl, choline; CL, cardiolipin; PA, phosphatidic acid; PS, phosphatidyl serine; PEVP, poly-*N*-ethyl-4-vinylpyridinium bromide; PAA, polyacrylic acid; CF, 5(6)-carboxyfluorescein; EPM, electrophoretic mobility; DP, degree of polymerization.

2. Experimental

PEVP was prepared in accordance with [12]. Fraction $M = 2.3 \times 10^5$ (DP = 1100) was used. PAA, $M = 5 \times 10^3$ (50% aqueous solution) was obtained from Aldrich, USA. The concentration of both polyelectrolytes is given in repeating charged units per liter.

PC was obtained from the Kharkov Bacterial Preparation Institute, Ukrainia; CL, PA and PS from Sigma, USA. Thin-layer chromatography of these lipids showed virtual purity. CF of analytical grade (Serva, Germany) was used without further purification.

To prepare PC-CL liposomes, the following procedure was used. First, corresponding amounts of lipid solutions in methanol or chloroform were mixed in a flask. Then, the solvent was carefully evaporated under vacuum. Thin layer of lipid mixture was dispersed in 2 ml borate buffer and then sonicated in an ultrasonic homogenizer 'UZDN-2T' (Russia) for 9 min (3 × 3 min) under icecooling. The liposome sample thus obtained was separated from titanium dust by centrifugation and used within one day. The average diameter of liposomes was found to be 40–60 nm. Fluorescent dye-loaded liposomes were obtained by the same technique, but borate buffer containing 0.1 mg/ml CF was used. Excess dye not loaded into the inner part of liposomes was separated by gel filtration.

The particle size was measured by photon correlation spectroscopy with Autosizer 2c (Malvern, UK). This method was also used to determine the concentration of the liposome suspension after gel filtration. To perform the latter measurements, a light scattering intensity calibration curve was obtained using initial liposome suspensions of predefined concentrations. EPM measurements were performed using the laser microelectrophoresis method with Zetasizer 2c (Malvern, UK).

Sedimentation analysis was done with a Spinco E analytical scanning ultracentrifuge E (Beckman, USA), fluorescent analysis with an F-4000 fluorescence spectrophotometer (Hitachi, Japan). Absorbance spectra were recorded using a 150/20 spectrophotometer (Hitachi, Japan). pH values were measured using a PHM83 potentiometer with a standard glass electrode G2040C (Radiometer, Denmark). The experiments were performed in borate buffer solution, 10^{-3} M, sodium tetraborate of analytical grade, pH 9.18. All the solutions were prepared using bidistilled water and further purified by passing it through Milli-Q water system (Millipore, USA).

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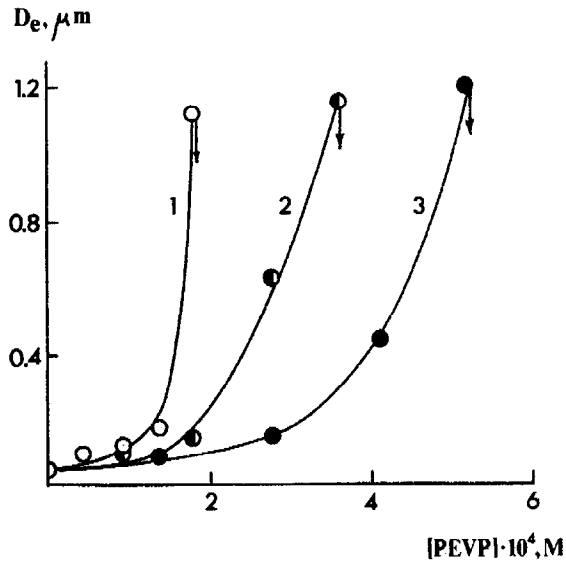

Fig. 1. Dependence of the liposome-PEVP aggregate size on the PEVP concentration. Liposome concentration: 1 mg/ml (the sum of all the constituents). Concentration of the cardiolipin negative charge: 1.5×10^{-4} (1), 3×10^{-4} (2) and 4.5×10^{-4} M (3). The points indicated by arrows correspond to precipitation of PEVP-liposome complexes.

3. Results and discussion

We found that interaction of PEVP with negatively charged PC-CL liposomes resulted in a changing of particle size as it was recently observed for all the studied polycations [1–8]. As the PEVP concentration increases, the size of particles increases continuously and when, reaching a maximal value (Fig. 1), sedimentation of particles occurs (marked with arrows). It follows from the figure that the sharp enlargement and sedimentation of particles depends linearly on the amount of the charged lipid constituent.

Loss of stability was reasonably assumed to be due to neutralization of the liposome surface charge by adsorbed polycations. Actually, EPM measurements confirm that a dramatic increase in particle size takes place at EPM values of about 0 (Fig. 2). EPM of colloidal particles is known to be determined by the surface charge value. In the case of liposomes, it must be determined by an amount of ionogenic groups on the outside of the lipid bilayer membrane. So, naturally it is to be expected that the EPM value will reach zero when all charges at the outside of the membrane are neutralized by adsorbed polycations. However, to our great surprise, the amount of positive charges introduced by polycations or at a zero EPM value exceeded the amount of negative charges in the outer leaflet of the bilayer, and it was found to be equal to the total amount of negative charges in both leaflets of the bilayer.

It is very important that the same result can be obtained when substituting CL with another negatively charged lipid. We carried out experiments similar to these described above with liposomes composed of a mixture of PC and PA, or PS. In both cases, in order to neutralize the liposome surface charge and cause liposomes to enlarge and precipitate, an amount of PEVP equal to the total amount of negatively charged lipid on both leaflets of the bilayer, was required (results are not given).

Four different reasons for this can be assumed.

1. First of all, it is natural to assume that the observed picture is the result of non-complete binding of PEVP to liposomes. This means that constant of formation of the PEVP–liposome complex is not high enough and therefore, only part of initial amount of the polycation interacts with liposomes, while the other part remains free in solution. According to this scheme, neutralization of the liposome surface charge would require more polycation compared to the complete binding case.

To verify the assumption, we studied the completeness of PEVP binding with liposomes by means of the ultracentrifugation technique. The results of the experiment are shown in Fig. 3. One can see that complete adsorption of the polycation was observed up to a PEVP concentration of 4×10^{-4} M. The polycation can be recorded in a continuous phase only at higher concentrations.

2. The second reason might be the disruption of the liposome membrane by adsorbed polycations. In this case, all the charged lipids, placed both on the outer and inner leaflets of the membrane, could form ionic bonds with units of the polyaction chain, and the registered aggregates could be formed from fragments of the original membrane.

To check this possibility, the following procedure was used. First, liposomes, containing a water-soluble fluorescent dye inside, were prepared. Then, the liposomes

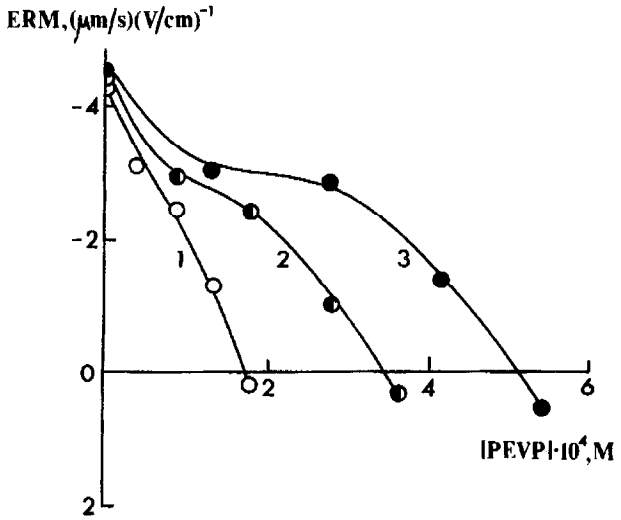

Fig. 2. Dependence of the EPM of liposome–PEVP aggregates on the PEVP concentration. All conditions as in Fig. 1.

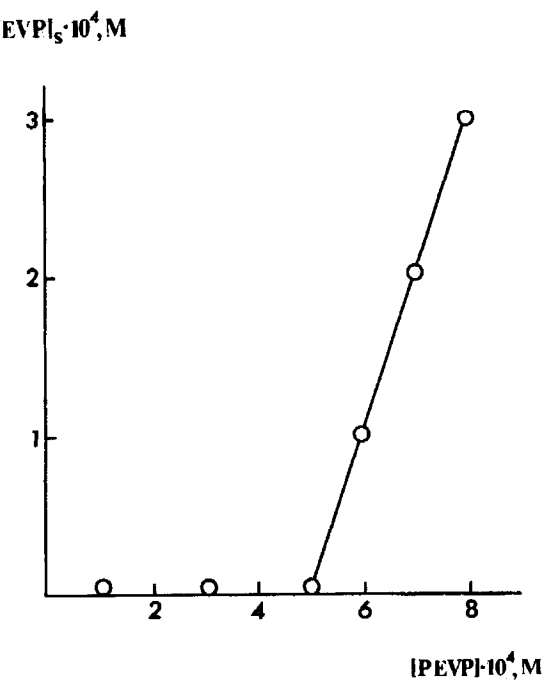

Fig. 3. Dependence of concentration of PEVP unbound to liposomes on the total PEVP concentration. Concentration of cardiolipin negative charge: 3×10^{-4} M. All other conditions as in Fig. 1.

were mixed with PEVP and passed over a Sephadex G-25 column. It was found that only one peak corresponding to the dye-loaded liposomes occurred on the cromatographic curve, and no free dye was detected. This means that the integrity of liposomes included into complex with PEVP remained unchanged.

Moreover, as PAA is known to form strong complexes with PEVP [13], addition of PAA solution to the aggregates at a PEVP/PAA molar ratio of 1:10 causes disaggregation of liposome clusters and the formation of a suspension composed of liposomes of the initial size.

These results prove that the including of PC-CL liposomes into the clusters does not change the integrity of the lipid bilayer.

3. Another reason might be the drastical asymmetry in distribution of charged lipids between the outer and inner leaflet of the bilayer. If charged lipids are concentrated on the outer leaflet, sedimentation of liposomes will occur when adsorbed polycations neutralize all the charges brought on by these molecules. This scheme is in a good agreement with the obtained results. However, it was previously found that in mixed liposomes, similar to these studied in the present work, negatively charged lipid molecules were practically uniformly distributed between both leaflets of the bilayer [14,15], or even concentrated on its inner leaflet [16].

4. The above data indicate that the phenomena observed can only be explained in terms of the hypothesis of the transbilayer migration of charged lipid molecules from the inner to outer leaflet of the bilayer. In the native liposome membrane, such a transfer is known to proceed very slowly [17]. At the same time, the flip-flop transfer is shown to be stimulated, by several orders of magnitude, by different procedures, for instance, by protein introduction in the membrane [18] and chemical modification of lipids, resulting in their asymmetrical distribution in the membrane [19]. Polycations, when adsorbed on the lipid membrane, could do it in the similar way.

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