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Interaction of a cationic polymer with negatively charged proteoliposomes

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Abstract

Proteoliposomes were prepared by making bilayer vesicles from neutral egg yolk lecithin and negatively charged α-chymotrypsin that had been previously stearoylated. Interaction of these proteoliposomes with a cationic polymer, poly-(*N*-ethyl-4-vinylpryidinium bromide) (PEVP) was examined. For comparison purposes, interaction of PEVP with egg lecithin vesicles containing an anionic phospholipid, cardiolipin, was also examined. Binding of PEVP to both types of vesicles was electrostatic in nature with the polymer manifesting a higher affinity to the cardiolipin relative to the enzyme. PEVP had no effect on the permeability of the bilayer membranes to sodium chloride. On the other hand, PEVP increased the transmembrane permeability of the nonionic anti-tumor drug, doxorubicin. The greater the negatively charged component in the membrane, the greater the PEVP effect. Polycation binding to the vesicles was accompanied by clustering of the stearoylated chymotrypsin (sCT) molecules within the membrane. This protein clustering is most likely responsible for the increase in the doxorubicin permeation. Enzymatic activity of the membrane-associated sCT remained unchanged upon PEVP binding. These findings seem relevant to the effects of polyelectrolytes on cellular membranes.

Keywords: Bilayer lipid membrane; α-Chymotrypsin; Doxorubicin; Permeability; Polycation; Proteoliposome

1. Introduction

Synthetic polyelectrolytes are widely used now in pharmacy and medicine as carriers for drug and gene delivery [1–5] and components of artificial vaccines [6,7]. This requires to study the behavior of polymers in biological environment and especially, the mechanism of their interaction with cells. Two main approaches on this point are discussed in the literature. The first is to use tissue cultures or fixed cells [8–12]. It was shown that adsorption of polycations on cell membranes could result in grave physiological effects: receptors clustering [7,13], changes in the mem-

Abbreviations: CT, α-chymotrypsin; sCT, stearoylated α-chymotrypsin; PEVP, poly-(*N*-ethyl-4-vinylpyridinium bromide); EL, egg yolk lecithin; CL^{2-}, cardiolipin; LPR, lipid-to-protein concentration ratio in liposome membrane; ATEE, *N*-acetyl-L-tyrosine ethyl ester; PMSF, phenylmethylsulfonyl fluoride; Dox, doxorubicin; STI, soybean trypsin inhibitor; NHSS, stearic acid *N*-hydroxysuccinimide ester; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; *v*, molar fraction of negatively charged groups in liposome membrane; $Z_{\pm}$, ratio of molar concentrations of polycation and negatively charged groups on liposome membrane

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brane permeability [7–10] and functioning of ion channels [11–14]. The second approach consists in using model systems mimicking some features of the cell membrane structure. The simplest but quite appropriate cell-mimetic object seems to be bilayer vesicles composed of phospholipid molecules (liposomes). A cell membrane usually carrying a net negative charge, the main attention of contributors was concentrated on the interaction of synthetic polycations with negatively charged liposomes. It was found that adsorption of polycations on the liposomal membrane could be accompanied by lateral lipid segregation [15–17], transmembrane migration of lipid molecules [18–21], incorporation of polyelectrolytes into the membrane [5,22–24], as well as fusion [25], aggregation and disruption of liposomes [21].

At the same time, a cell membrane contains, together with lipids, a considerable amount (up to 60 wt.%) of different proteins. It is known that membrane proteins can carry covalently attached fatty residues providing protein molecules with anchoring to the lipid bilayer [26–28]. In particular, post-translational fatty acylation of proteins often determines their localization in a plasma membrane and their functioning [29,30]. It is reasonable to assume that polycations, when adsorbed on the cell surface, can interact with cell membrane proteins thus influencing their biological activity. This aspect has been practically unstudied up to now, in spite of obvious importance for biomedical application of polyelectrolytes. In the present work negative protein-containing lipid vesicles (proteoliposomes) were prepared by attachment of hydrophobized α-chymotrypsin to a bilayer composed of egg yolk lecithin, and interaction of these vesicles with a cationic polymer, poly-(*N*-ethyl-4-vinylpyridinium bromide) (PEVP), was explored.

2. Experimental

2.1. Materials

α-Chymotrypsin (CT) (EC 3.4.21.1) was purchased from Sigma (St. Louis, USA); phosphatidylcholine (egg yolk lecithin, EL) and diphosphatidylglycerol (cardiolipin, CL^{2-}) were from JSC Biolek (Kharkov, Ukraine); deoxycholic acid, *N*-acetyl-L-tyrosine ethyl ester (ATEE), octyl-β-D-glucopyranoside, soybean trypsin inhibitor (STI), phenylmethylsulfonyl fluoride (PMSF) and perylene were from Serva (Heidelberg, Germany); trinitrobenzenesulfonic acid from Merck (Darmstadt, Germany), and doxorubicin (Dox) from Antibiotics Institute (Moscow, Russia). Stearic acid *N*-hydroxysuccinimide ester (NHSS) was synthesized according to [31].

2.2. Modification of α-chymotrypsin by stearic acid residues

α-Chymotrypsin amino groups were chemically modified by NHSS in the deoxycholate micelle solution following the procedure of Huang et al. [26]. Briefly, 16 mg of α-chymotrypsin was dissolved in 7 ml of 0.1 M borate buffer (pH 8) containing 10 mM *N*-acetyl-L-tyrosine and 3% (w/v) of deoxycholate. Seven hundred μl of NHSS solution in dioxane was then added, and the mixture was stirred for 45 min at room temperature. After that, the mixture was centrifuged to remove free stearic acid. Stearoylated α-chymotrypsin (sCT) was separated from the detergent and organic solvent by gel-filtration on Sephadex G-50 and lyophilized. The obtained protein preparations appeared to contain a considerable admixture of deoxycholate that was shown by potentiometric titration and thin-layer chromatography. Therefore, aqueous solution of hydrophobized protein with pH 3.5 was prepared, and then a protonated form of deoxycholic acid was extracted from the solution using an equal volume of cold ether, temperature being carefully maintained at 4°C. Aqueous protein solution and organic extract were separated rapidly by centrifugation at 10 000 rpm and 4°C using a J-21 centrifuge (Beckman, Fullerton, USA), the extraction procedure being repeated three times. The resulting aqueous solution was dialyzed extensively against 5 mM acetic acid solution with pH 3.5 and lyophilized. No traces of deoxycholic acid were detected in the final protein preparation either by potentiometric titration or by thin-layer chromatography. The final purification was performed at pH 3.5, because as shown in [33] the enzyme did not undergo autodigestion under these conditions.

The number of stearoyl residues covalently attached to CT was determined by spectrophotometric

titration of remaining protein amino groups with trinitrobenzenesulfonic acid [32], measuring optical density at $\lambda = 405$ nm with a Multiscan Plus microplate photometer (Huntville, Titertek, USA). The native CT was used as a control, assuming each protein molecule carries 16 amino groups [33]. CT concentrations were measured either spectrophotometrically at $\lambda = 280$ nm ($\varepsilon = 50\,000\ M^{-1}\,cm^{-1}$), or by using a Lowry method [34]. Both approaches were found to give the same results.

Modification of CT with stearoyl residues did not change an amount of acidic groups in enzyme molecule. By using a potentiometric titration of water solutions of native and hydrophobized CT with RTS-822 automatic titrator (Radiometer, Copenhagen, Denmark), the amount of titratable acidic groups in both cases was found to be equal to 14.

2.3. Unilamellar liposomes

To prepare EL and mixed EL/CL^{2-} unilamellar liposomes the following procedure was used. First ethanol solutions of EL or its binary mixtures with CL^{2-} (EL/CL^{2-} being 1:0.003, 0.99:0.01, 0.9:0.1 and 0.8:0.2) were put in a flask and the solvent was carefully evaporated under vacuum. A thin film of lipids was dispersed in a 0.1 M borate buffer (pH 8.5) with a 4700 Cole-Parmer ultrasonic homogenizer (Vernon Hills, USA) at 22 kHz (4×200 s). Double-distilled water was used treated additionally by passing it through a Milli-Q system for deep purification from organic impurities (Millipore, USA). Liposome samples thus obtained were separated from titanium dust by centrifugation and used within 1 day. The diameter of liposomes measured by photon correlation spectroscopy was in the 70–100 nm range.

EL liposomes with a fluorescent perylene probe incorporated into the bilayer were prepared using the same technique, perylene being added to EL ethanol solution at a perylene/EL molar ratio equal to 1:1000.

2.4. Attachment of stearoylated α-chymotrypsin to liposomes (proteoliposome preparation)

2.4.1. Sonication technique [21]

sCT water solutions were mixed with 10 mg/ml EL liposome suspension in 10 mM borate buffer, pH 8.5 so that the final protein concentrations varied from 25 to 200 μM and the lipid/protein molar ratio (LPR) from 200 to 25. The mixtures were then sonicated for 100 s at 16 kHz and 0°C. Thus prepared EL/sCT proteoliposomes were separated from unbound enzyme by gel-filtration on Sepharose CL-4B, 10 mM borate buffer being used as effluent. To calculate the amount of the enzyme attached to the outer leaflet of liposomal membrane, the data on enzymatic activity of intact proteoliposomes and the data on active sites titration of proteoliposomes with STI were used. Both approaches gave the same results. Proteoliposomes with LPR varying from 3000 to 300 were thus prepared. An average hydrodynamic diameter of proteoliposomes was found to be equal to 70–120 nm using photon correlation spectroscopy with an Autosizer 2c instrument (Malvern Instruments, Malvern, UK).

Unless otherwise stated, all EL/sCT proteoliposomes used in this work were prepared by sonication procedure.

EL/sCT proteoliposomes with fluorescent perylene probe attached to the bilayer were prepared according to the same protocol, perylene-labeled EL liposomes being used.

2.4.2. Detergent dialysis technique [35]

Seven mg of sCT and 10 mg of EL were dissolved in 1 ml of 3% (w/v) octyl-β-D-glucopyranoside solution in 10 mM borate buffer (pH 8), additionally containing 150 mM NaCl. The mixture was sonicated at 16 kHz and 4°C for 70 s and then was extensively dialyzed against the same buffer for 40 h. Protein unbound to liposomes was partially precipitated during dialysis procedure and then separated by centrifugation at 7000 rpm for 10 min. The supernatant solution, containing proteoliposomes, was additionally purified by gel-filtration on Sepharose CL-4B, equilibrated with the borate buffer solution. The amount of sCT in thus prepared proteoliposomes was determined by comparing the levels of enzyme catalytic activity in the initial detergent solution and in the proteoliposome preparation. In the special experiments it was found that neither EL nor octyl-β-D-glucopyranoside inhibited the enzymatic hydrolysis of ATEE. LPR value in thus prepared proteoliposomes was equal to 200–300, their hydrodynamic diameter being about 300 nm.

2.5. Enzyme catalytic activity determination

The catalytic activity of sCT in solution and in the membrane of proteoliposomes was determined using ATEE as a specific substrate. Rates of the enzymatic ATEE hydrolysis were measured potentiometrically in 20 mM KCl solution (pH 8.0) using an RTS-822 pH-stat (Radiometer, Copenhagen, Denmark).

2.6. Fluorescence spectra

Fluorescence intensity of sCT in solution and after its attachment to the membrane of liposomes were measured using an F-3000 spectrofluorimeter (Hitachi, Tokyo, Japan) at $\lambda_{em} = 340$ nm ($\lambda_{ex} = 280$ nm). Fluorescence intensity of a membrane probe perylene was measured with the same instrument at $\lambda_{em} = 446$ nm ($\lambda_{ex} = 339$ nm).

2.7. Polycation

PEVP was synthesized by radical polymerization of 4-vinylpyridine and further exhaustive ethylation of a narrow fraction of poly(4-vinylpyridine) with degree of polymerization (DP) equal to 1100 and 2100 as described in [36] and actually represented a copolymer containing about 5–7% of residual 4-vinylpyridine units. The PEVP composition was determined by IR spectroscopy measuring the ratio of optical densities at 1600 and 1640 cm^{-1} [37]. A sample of PEVP was dissolved in water and its concentration, expressed in moles of positive quaternized units per liter, was determined spectrophotometrically at 256 nm with $\varepsilon = 2900$ M^{-1} cm^{-1} [37].

2.8. Polycation-induced protein aggregation

PEVP-induced aggregation of protein molecules, distributed in the proteoliposomal membranes, was studied using a glutaraldehyde fixation procedure originally designed for controlling cell receptor clustering [38]. Proteoliposome suspension in 10 mM borate buffer (pH 8.5), containing 0.5 mg ml^{-1} of EL and 0.06 mg ml^{-1} (2.4 μM) of sCT, was treated with 5 mM PMSF solution for 10 min to avoid α-chymotrypsin autodigestion during the experiment. Then, PEVP was added so that its concentration achieved 33 μM. The mixture was incubated for 1 h at 25°C, then 1 mM glutaraldehyde was added, and the resulting mixture was incubated for half an hour, after which the enzyme was precipitated by addition of trichloroacetic acid up to final concentration of 10% (w/v). The obtained enzyme samples were washed with cold acetone and dissolved in 1% (w/v) SDS-containing buffer solution for electrophoresis. Control samples were also prepared using the above procedure, except addition of PEVP. The PEVP-treated and control samples were analyzed by the SDS–PAGE technique using 4–15% acrylamide gradients. The gels were silver-stained according to [39].

2.9. Kinetics of transmembrane doxorubicin permeation

Kinetics of transmembrane permeation of the antitumor drug Dox, characterized by a strong fluorescence, was investigated using the procedure described in [40]. Dox molecule contains an amino group with pK_a 8.6, so in neutral or slightly alkaline solutions a part of Dox molecules is non-charged and can incorporate into the liposome membrane. If the internal liposome cavity is loaded with an acidic buffer, Dox desorbs from the membrane and accumulates inside liposomes, that finally results in self-quenching of Dox fluorescence. This allows to follow the transmembrane Dox permeation by measuring the fluorescence intensity in the system [40].

According to this scheme, in the present work EL/CL^{2-} liposomes and EL/sCT proteoliposomes were prepared in 0.3 M Tris–citrate buffer solution at pH 5, using the sonication procedure described above, and then passed through a Sephadex G-50 column, equilibrated with 20 mM Tris–citrate buffer at pH 7.5, additionally containing 0.3 M sucrose for osmotic pressure compensation. Suspensions of pH-gradient EL/CL^{2-} liposomes and EL/sCT proteoliposomes with pH 5 inside vesicles and pH 7.5 in surrounding solution were thus prepared. Hydrodynamic diameter of such vesicles, measured by photon correlation spectroscopy, was in the 100–115 nm range.

Dox was added to pH-gradient liposome suspensions at 50 μM concentration which corresponded to the maximum Dox fluorescence intensity. When concentration of Dox inside liposomes exceeded 50 μM,

the fluorescence intensity in the system began decreasing due to the self-quenching effect, the process following the first-order kinetics. Importantly, the rate constant, calculated from the obtained kinetic curves, was in a good agreement with that evaluated by direct measurement of Dox concentration in liposomes after their separation from external free Dox and further surfactant-induced lysis. The PEVP-induced acceleration of the Dox transport was quantified as a k_p/k_0 ratio, where k_p and k_0 are the rate constant values in the presence and in the absence of PEVP, respectively.

3. Results and discussion

3.1. Attachment of α-chymotrypsin to the liposomal membrane

Under the chosen experimental conditions (20 mM KCl solution, pH 8), native CT did not bind to neutral EL liposomes at all either after incubation of the enzyme/liposome mixture for 4 h, or after sonication of the mixture for 100 s. This was proved by measuring the enzymatic activity of liposomes after their separation from unbound enzyme by gel-filtration. Incubation of hydrophobized enzyme with preformed EL liposomes was accompanied by only a slight increase in an amount of liposome-associated enzyme. At the same time, sonication of a mixture, containing EL liposomes and sCT, for 100 s resulted in association of the enzyme with liposomes, i.e., in EL/sCT proteoliposome formation.

As we have shown previously, enzyme acylation results in a decrease in the content of active sites in the enzyme preparation from 80% in the initial preparation to 55–60% in the modified sample. Therefore in this work, all measurements of enzyme quantity were referred to the catalytic activity of the modified enzyme. The location of enzyme molecules in EL/sCT proteoliposomes prepared by sonication technique was studied by titration of the enzyme active sites with STI. The liposomal membrane being impermeable for STI, the enzyme activity inhibited by STI could be definitely ascribed to enzyme molecules located on the outer side of the membrane. To estimate the total amount of liposome-bound enzyme molecules, proteoliposomes were destroyed by addition of 1% (w/v) Triton X-100 solution and the active sites were then titrated with STI according to [26]. It was found that addition of Triton X-100 resulted in nearly twice increase in the amount of the enzyme active sites accessible to STI. In addition, Triton-induced destruction of proteoliposomes led to a twice increase in the level of enzyme catalytic activity. So, one can conclude that in the original proteoliposomes a half of active sCT molecules bound to the outer leaflet of the membrane and only these molecules contributed into catalytic activity of the intact proteoliposomes. In other words, by measuring the catalytic activity of proteoliposomes, one can estimate the amount of sCT attached to the outer leaflet of the liposomal membrane.

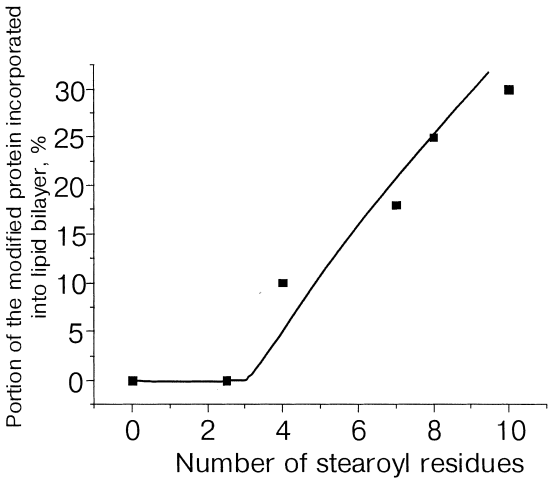

Fig. 1. Effect of the number of stearoyl residues in CT molecule (N) on the extent of CT bound to the outer leaflet of EL liposomes produced by sonication procedure. LPR = 2500. CT active site concentration 8.4 μM; 10 mM borate buffer, pH 8.5.

Modification of α-chymotrypsin by stearoyl residues provided it with affinity to the liposomal membrane probably due to an increase in the enzyme surface hydrophobicity. It should be noted that an appreciable association of the modified enzyme was registered only if the average number of stearoyl radicals per one enzyme molecule (N) exceeded 2 (Fig. 1). However, the fraction of the enzyme molecules bound to the liposomes did not exceed 30% (mol.) even at $N = 10$, and was not enhanced by increasing liposomes content up to 5000-fold molar excess of the lipid as regards to the enzyme. Therefore, it is reasonable to assume that not all modified protein molecules but only those with some favorable

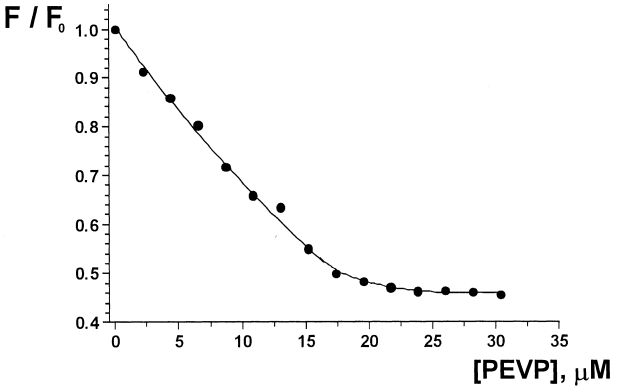

Fig. 2. Effect of PEVP on relative fluorescence intensity of EL/sCT proteoliposomes (CT modified by 10 stearoyl residues was used). [sCT] = 1 μM, corresponding to 14 μM concentration of the protein-negative groups; [EL] = 1 mg/ml; 10 mM borate buffer, pH 8.5.

location of stearoyl radicals, randomly attached to the protein amino groups, were able to bind effectively to the EL membrane.

3.2. Binding of a polycation to proteoliposomes

The isoelectric point (p*I*) of native CT is 7.8–8.3 [41]. However, acylation of CT amino groups changes a basic to acidic groups ratio in the protein molecule and results in a decrease in enzyme p*I* value. For example, modification of five amino groups in CT decreased its p*I* value down to 3.5–4.5 [42]. Therefore, interaction of cationic PEVP with EL/sCT proteoliposomes was studied at pH 8.5 when sCT molecules and the whole proteoliposomes were negatively charged. PEVP being an effective fluorescence quencher [51–53], the PEVP/proteoliposome interaction was followed by measuring the fluorescence intensity of tryptophan residues in sCT molecules. As follows from the data in Fig. 2, addition of PEVP solution to proteoliposome suspension resulted in a decrease in tryptophan fluorescence intensity, indicating that PEVP chains bound to the membrane sCT molecules obviously due to ionic interaction of PEVP positive units with sCT negative residues. Electrostatic nature of PEVP/protein binding in water solutions of these components has been earlier disclosed and described in detail [43–45].

It should be taken into account that PEVP, after binding to the membrane sCT, could either retain on the surface of proteoliposomes, or extract sCT molecules from the proteoliposomal membrane and transfer them in surrounding solution in the form of water-soluble polycation–protein complex. In the both cases, the PEVP-mediated quenching of the sCT tryptophan fluorescence should be observed. To distinguish between them, EL/sCT proteoliposomes with a fluorescent perylene probe incorporated into the bilayer were used. Being a highly hydrophobic substance, perylene incorporated into the internal lipophilic part of the liposomal membrane but did not associate with sCT molecules which were likely located at the water–membrane interface. As follows from the data in Fig. 3, addition of PEVP to a suspension of perylene-labeled proteoliposomes resulted in a considerable decrease in the probe fluorescence (Fig. 3, curve 1), positively indicating adsorption of PEVP on the proteoliposomal surface. For comparison, addition of PEVP to perylene-labeled neutral EL liposomes had an extremely slight effect on the fluorescence (Fig. 3, curve 2), thus showing a negligible PEVP binding to EL liposomes. These results allow to conclude that PEVP actually adsorbed on the surface of proteoliposomes and then did not move from there in a solution.

It has been shown earlier [26] that acylation of CT with stearic acid resulted in an increase in Michaelis constant (K_m) value, further attachment of sCT to the membrane of EL liposomes resulted in K_m decrease nearly down to the native CT level. At the same time, the catalytic constant (k_{cat}) was only

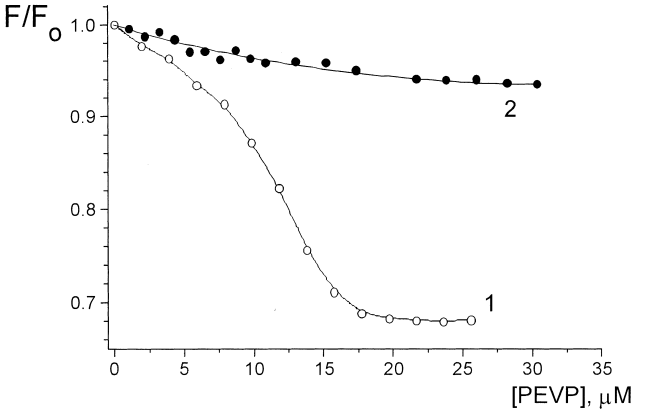

Fig. 3. Effect of PEVP of relative fluorescence intensity of perylene-labeled EL/sCT proteoliposomes (1) and EL liposomes (2). [sCT] = 1 μM, corresponding to 14 μM concentration of the protein-negative groups; [EL] = 1 mg/ml; 10 mM borate buffer, pH 8.5.

slightly affected as CT was hydrophobized and attached to the liposomal membrane.

Adsorption of PEVP on the surface of EL/sCT proteoliposomes was found not to influence either K_m or k_{cat} values of low molecular substrate ATEE hydrolysis. This means that PEVP adsorption did not affect either substrate-binding or catalytic sites of the membrane-attached sCT molecules.

3.3. Polycation-induced aggregation of protein molecules in proteoliposomes

It has been reported earlier that adsorption of multicharged species either on the cell or liposomal membrane surface can be accompanied by clustering of membrane proteins and lipids. To demonstrate this, flash-induced transient dichroism [46,47], fluorescence recovery after photo-bleaching [48], electron microscopy [7], and differential scanning calorimetry [21] techniques were used. In the present work, another approach was applied for the study of PEVP-induced clustering of hydrophobized protein molecules attached to the membrane of EL/sCT proteoliposomes. PEVP-induced sCT clusters were fixed by cross-linking with glutar aldehyde, separated from polycation and lipids, and then analyzed using SDS–PAGE. Two types of proteoliposomes were used with different sizes and LPR values. The first, prepared by the sonication procedure, were 70–80 nm in diameter, LPR value being equal to 3000. The second, prepared by the detergent dialysis technique, were 200–300 nm in diameter with LPR value equal to 300.

Before addition of PEVP, appropriate glutar aldehyde concentration was found in order to avoid cross-linking of the membrane-bound sCT molecules in the absence of PEVP, small sonicated proteoliposomes with LPR = 3000 being used. At a proper glutaraldehyde concentration, no clustering of sCT molecules in a PEVP-free suspension was observed: molecular mass (M_r) of particles, detected by SDS–PAGE, was between 20 000 and 30 000 kDa (Fig. 4, lane 1) that, within the accuracy of the gel-electrophoresis method, corresponded to M_r of individual sCT molecules. Treatment of both types of proteoliposomes by PEVP with DP = 2100 had quite different effects on arrangement of the membrane sCT. Large liposomes with a high sCT surface density

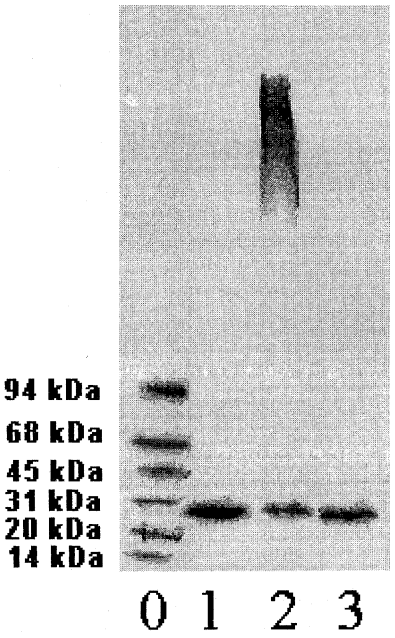

Fig. 4. Electrophoretic analysis of PEVP effect on clustering of sCT molecules attached to the membrane of EL/sCT proteoliposomes. Prior to electrophoretic separation the protein aggregates were fixed with glutaraldehyde. Protein standards (control) (0); proteoliposomes with LPR = 300 (1); proteoliposomes with LPR = 300 after incubation with PEVP (2); proteoliposomes with LPR = 3000 after incubation with PEVP (3).

(LPR = 300) formed huge protein clusters with M_r = 1500–2000 kDa (Fig. 4, lane 2), corresponded to 60–80 sCT molecules in each of them. On the contrary, small liposomes with a low sCT surface density (LPR = 3000) did not demonstrate sCT cluster formation (Fig. 4, lane 3).

It should be noted that interaction of PEVP with large proteoliposomes was accompanied by formation of liposome aggregates, their size achieving 800 nm. Therefore, clustering of sCT molecules, described above, could develop either within individual proteoliposomes or with the participation of several proteoliposomes, involved in such aggregates. In the both cases, lateral diffusion of sCT molecules most likely contributed in the sCT cluster formation. To clarify, which of the two clustering mechanisms mainly determine the whole process some additional studies are required. Anyway, a diminishing in the sCT surface density, when changing large proteoliposomes for small resulted in a decrease in an amount of sCT molecules involved in the sCT cluster formation. Obviously, in proteoliposomes with LPR = 3000

the sCT surface density turned out to be so low that sCT clustering process was completely suppressed.

3.4. Competitive interactions in polycation/protein/lipid ternary system

Binding of polycations to the cell surface is mediated by all negative membrane components: proteins, lipids and carbohydrates. In the present work, a comparative study of PEVP interaction with membrane-bound anionic proteins and negative lipids was undertaken. For this, two types of negative bilayer vesicles, EL/sCT proteoliposomes and mixed EL/CL^{2-} liposomes, were prepared. In the first the surface charge was exclusively originated from sCT anionic groups, and in the second from CL^{2-} phosphate groups. The mole fraction of negative groups in liposomes, ν, was calculated as $\nu = 14[\text{sCT}]/(14[\text{sCT}]+[\text{EL}])$ for EL/sCT proteoliposomes (each CT molecule carries 14 acidic groups [33]) and $\nu = 2[CL^{2-}]/(2[CL^{2-}]+[\text{EL}])$ for mixed EL/CL^{2-} liposomes, where [sCT], $[CL^{2-}]$ and [EL] are the molar concentrations of sCT, CL^{2-} and EL, respectively. In both proteoliposomes and mixed liposomes ν value was about 0.0025.

Addition of PEVP to proteoliposome suspension was accompanied by a quenching of sCT fluorescence (Fig. 5A, curve 1), that could be attributed to interaction of PEVP with sCT molecules, located on the outer leaflet of the proteoliposomal membrane, i.e., one half of the total sCT amount. Taking this into account, a fraction of the outer sCT molecules bound to adsorbed PEVP

$$\varphi = \frac{2(I - I_o)}{I_o} \quad (1)$$

was determined at different PEVP concentrations (Fig. 5B, curve 1). Assuming a simple equilibrium

$$P + Q \rightleftarrows PQ$$

between protein (P) and polycation-quencher (Q), resulting in PQ complex formation, an apparent dissociation constant was written as:

$$K_P = \frac{([P]_o - [PQ])([Q]_o - [PQ])}{[PQ]} \quad (2)$$

where $[P]_o$ is the concentration of anionic groups of

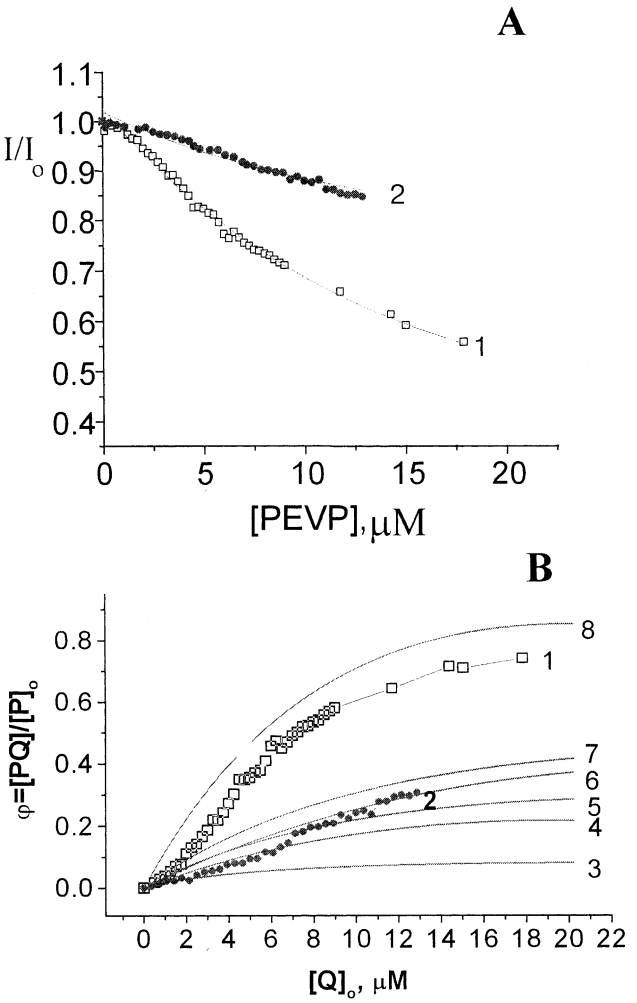

Fig. 5. (A) Effect of PEVP on relative fluorescence intensity of EL/sCT proteoliposomes before (1) and after (2) addition of EL/CL^{2-} liposomes. [sCT negative groups] = 2.1 μM, [CL^{2-} negative groups] = 2.8 μM, [EL] = 1 mg/ml; 10 mM borate, pH 8.5. (B) Portion of sCT bound to PEVP (φ) in EL/sCT proteoliposomes (1) and in the mixture of proteoliposomes with EL/CL^{2-} liposomes (2) vs. PEVP concentration $[Q]_o$. Theoretical calculation of φ at different $\beta = K_L/K_P$ values (β = 0.1–3, β = 0.3–4, β = 0.4–5, β = 0.45–6, β = 0.5–7, β = 1–8). K_P = 5.8 μM, at $[P]_o$ = 2.1 μM, $[L]_o$ = 2.8 μM.

sCT, located on the outer leaflet of the proteoliposomal membrane, equal to 7[sCT] (as mentioned above sCT was uniformly distributed between both leaflets of the proteoliposomal membrane), $[Q]_o$ is the concentration of PEVP-positive units, and [PQ] is the concentration of protein–polycation salt bonds. Eq. 2 was reduced to a quadratic form in [PQ]:

$$[PQ]^2-[PQ]([Q]_o+[P]_o+K_P)+P_oQ_o=0 \quad (3)$$

The root of Eq. 3

$$\frac{[PQ]}{[P]_o}=$$

$$\frac{[P]_o+K_P+[Q]_o-\sqrt{([Q]_o+[P]_o+K_P)^2-4[P]_o[Q]_o}}{2[P]_o} \quad (4)$$

was another representation of the above φ value, i.e.

$$\varphi=\frac{2(I-I_o)}{I_o}=\frac{[PQ]}{[P]_o} \quad (5)$$

Then, corresponding φ values from curve 1 (Fig. 5A) were substituted in Eq. 4, and a mean K_P value was determined, taking $[P]_o$ equal to 4.2 μM. The calculation gave $K_P = 5.8$ μM.

Then, PEVP was added to a mixture, composed of two types of negative vesicles: EL/sCT proteoliposomes and EL/CL^{2-} liposomes, and sCT fluorescence intensity was measured again (Fig. 5A, curve 2). One can see that the level of fluorescence intensity in the ternary system was higher than in the PEVP/proteoliposome binary system, indicating distribution of polycation between both types of vesicles. The fraction of the outer sCT molecules bound to adsorbed PEVP, $\varphi=(1-I/I_o)/0.5$, depending on PEVP concentration in the ternary system is shown in Fig. 5B (curve 2). As follows from the figure, admixing of EL/CL^{2-} liposomes to the proteoliposome suspension resulted in a decrease in the fraction of sCT electrostatically bound to PEVP.

In the ternary system, PEVP complexation can be obviously described by the two equilibria:

$$P+Q \rightleftarrows PQ$$

and

$$L+Q \rightleftarrows LQ,$$

where L symbolizes negative CL^{2-} molecules. The corresponding apparent dissociation constants are:

$$K_P=\frac{([P]_o-[PQ])([Q]_o-[PQ]-[LQ])}{[PQ]} \quad (6)$$

for the first equilibrium and

$$K_L=\frac{([L]_o-[LQ])([Q]_o-[PQ]-[LQ])}{[LQ]} \quad (7)$$

for the second. As shown earlier in [18–21] adsorption of PEVP on the surface of EL/CL^{-2} liposomes was accompanied by migration of CL^{-2} molecules from the inner to the outer leaflet of the liposomal membrane. As a result, all CL^{-2} molecules from both membrane leaflets formed ionic bonds with adsorbed PEVP. Therefore, $[L]_o$ in Eqs. 6 and 7 is the total concentration of negative CL^{-2} headgroups in the membrane of EL/CL^{-2} liposomes.

Assuming $K_L=\beta\times K_P$ and merging Eqs. 6 and 7, an Eq. 8 of the third degree in $[PQ]/[P]_o=\varphi$ was obtained:

$$\varphi^3(1-\beta)+$$

$$\varphi^2\left(2\beta-1+\frac{[L]_o}{[P]_o}-\frac{[Q]_o}{[P]_o}(1-\beta)-\frac{K_P}{[P]_o}(1-\beta)\right)-$$

$$\varphi\left(\beta+\frac{[L]_o}{[P]_o}+\frac{[Q]_o}{[P]_o}+\frac{\beta K_P}{[P]_o}\right)+\frac{\beta[Q]_o}{[P]_o}=0 \quad (8)$$

This equation was solved for φ at β values from 1 up to 0.1, taking $[P]_o = 2.1$, $[L]_o = 2.8$ and $K_P = 5.8$ μM, and corresponding φ–$[Q]_o$ curves 3–8 were constructed (Fig. 5B). As follows from the figure, the best fit to the experimental data was observed at $\beta = 0.45$. In other words, the apparent dissociation constant of PEVP–CL^{-2} complex was 2–2.5 times lower than that of the PEVP–sCT one. It apparently resulted from a difference in a structural coactivity of the components of both types of electrostatic complexes. As mentioned above, negative lipid molecules in the mixed liquid membrane are able to move within each membrane leaflet and also transfer between them. This allows the formation of lipid clusters with maximum number of polycation–lipid ionic contacts and optimum conformation of adsorbed polycation molecules [7,15–17], finally resulting in a better cooperative PEVP binding with CL^{-2} molecules as compared to membrane-associated sCT globules.

In the system described above, PEVP should make a choice between EL/CL^{2-} liposomes and sCT molecules already attached to the lipid bilayer. To some extent, it prefers the former. Now the question is what happens if PEVP is added to a mixture, com-

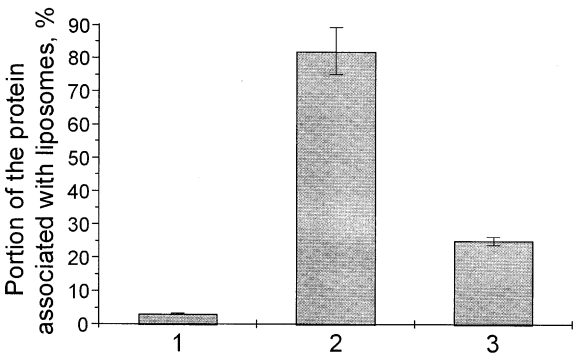

Fig. 6. Association of sCT with negative EL/CL^{2-} liposomes. sCT+liposomes (1), (1)+PEVP (2), (2)+NaCl (3).

posed of EL/CL^{2-} liposomes and sCT molecules originally unbound to the lipid bilayer?

As stated above, incubation of sCT with preformed neutral EL liposomes was accompanied only by a slight increase in amount of liposome-associated enzyme (shown by measuring the enzymatic activity of liposomes after their separation from unbound enzyme by gel-filtration). The same result was obtained for incubation of sCT with negative EL/CL^{2-} ($\nu = 0.12$) liposomes (Fig. 6, column 1). Then, the mixture of EL/CL^{2-} liposomes and sCT was prepared, and PEVP solution was added to this mixture so that PEVP/CL^{2-} charged group ratio ($Z_{\pm}$) equal to 1.5. This was accompanied by enlargement of particles in the system probably due to formation of PEVP/liposome/sCT ternary complex. The complex particles were centrifuged using a J-21 Beckman centrifuge, and then resuspended in 1 M NaCl solution. As the result a decrease in the particle size down to that of the initial liposome was observed. As shown in [18–21], the latter usually reflects a removal of a polycation from the negative liposomal surface because of shielding of polycation/liposome interaction with small counterions. So, it is reasonable to assume that addition of excessive amount of NaCl also made PEVP molecules to leave the surface of negative EL/CL^{2-} liposomes and caused disaggregation of the complex particles. By measuring the enzymatic activity of sCT, about 85% of sCT, initially added to EL/CL^{2-} liposomes, were found in the obtained suspension (Fig. 6, column 2). Liposomes were isolated by gel chromatography on Sepharose CL-4B in 0.7 M NaCl. Twenty-five percent of the initially added sCT was found in liposome fractions (Fig. 6, column 3), i.e., a significant part of sCT remained contacting liposomes after PEVP removal, most likely due to attachment of enzyme to the liposomal membrane. This approach might be used in a proteoliposome preparation practice to enhance the amount of protein incorporated into the lipid bilayer.

3.5. *Effect of polycation on the permeability of liposomal membranes*

There is a large body of evidence in the literature indicating that interaction of polycations with cells is accompanied by a leakage of potassium ions from cells in the surrounding solution and calcium ions from surrounding medium to cells [8–14]. This may result from influence of polycations on the packing of lipids and proteins, leading to formation of transient pores in a cell membrane, and/or their influence on the functioning of ion channels and transport systems [6].

It has been shown previously that PEVP has no effect on the permeability of EL/CL^{2-} liposomes towards a simple salt unless the content of negative CL^{2-} headgroups exceeds 20 mol%. Liposomes loaded with a concentrated NaCl solution were used, a salt leakage being controlled by conductivity of the surrounding solution [19–21]. In the present work, a similar approach was used to study an effect of PEVP on the permeability of EL/sCT proteoliposomes. For this, a suspension of EL/sCT proteoliposomes with $\nu = 0.12$, loaded with 0.15 M NaCl solution was prepared using detergent dialysis technique and mixed with PEVP solution. Adsorption of PEVP on the proteoliposomal membrane had no effect on the suspension conductivity during at least 1 h, indicating no NaCl leakage through the liposomal membrane. This result indicates that the above proteoliposomes are still not sufficient models to mimic an increase in ionic permeability observed upon interaction of polycations with membranes of the real cells [7–14].

Non-charged molecules can permeate through a cell membrane according to the mechanism proposed in [49,50]. Such substances are supposed to incorporate into the hydrophobic part of a cell membrane and then diffuse inside cells. This way is very sensitive to any rearrangements in the protein/lipid membrane. As shown previously the adsorption of poly-

cations on the surface of cells and liposomes induced lateral segregation of membrane proteins and lipids [7,18–21,47,48]. These structural rearrangements could change the membrane permeability with respect to non-charged molecules. So, we studied whether a cationic PEVP was able to influence the permeability of mixed EL/CL^{2-} liposomes and EL/sCT proteoliposomes towards a neutral Dox form. The technique based on the pH-induced uptake of Dox into liposomes was used [40]. Briefly, liposome suspensions were prepared with neutral aqueous buffer solution outside (pH 7) and acidic one inside (pH 4–5). Addition of a strong fluorophore Dox to these suspensions resulted in pH-gradient-induced uptake of noncharged Dox molecules inside liposomes and their protonation. When concentration of Dox inside liposomes exceeded the self-quenching concentration, the fluorescence intensity in the system decreased drastically, an effect that was monitored using fluorescence spectroscopy. This process, following the first order kinetics, reflected the transmembrane Dox permeation.

Kinetics of Dox permeation through the membrane of pH-gradient neutral EL liposomes is described by curve 1 in Fig. 7. In the case of pH-gradient EL/sCT proteoliposomes with LPR = 150, corresponded to $\nu = 0.085$, the same kinetics of Dox permeation was observed (Fig. 7, curve 2). Curves 3–5 describe the kinetics of Dox permeation in the presence of increasing concentration of PEVP, added

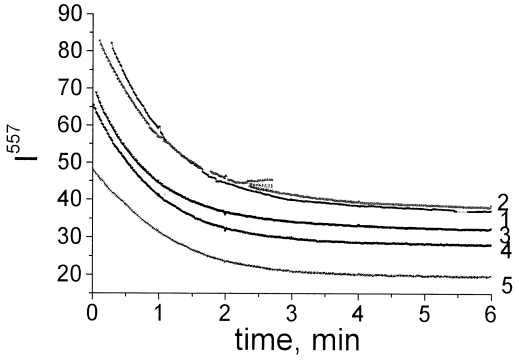

Fig. 7. Kinetics of Dox permeation through the membrane of pH-gradient liposomes. EL liposomes (1); EL/sCT proteoliposomes with 0.7 mol% of sCT (LPR = 150) (2); (2)+PEVP: [PEVP] = 6.2 μM ($Z_\pm = 0.5$) (3), 12.4 μM (4) ($Z_\pm = 1$), 31 μM (5) ($Z_\pm = 2.5$). External buffer: 20 mM Tris–citrate, pH 7.5, supplied with 0.3 M of sucrose; internal buffer: 0.3 M citrate–Tris, pH 5.0; 27°C.

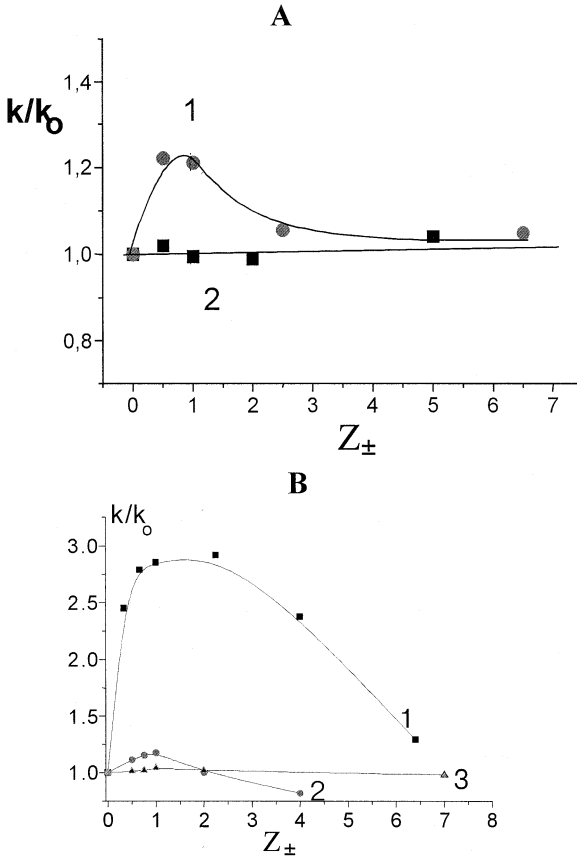

Fig. 8. Effect of PEVP on Dox permeation through the liposomal membrane as a k_p/k_0–$Z_\pm$ plot. (A) pH-gradient EL/sCT proteoliposomes with LPR = 150 (1) and 3000 (2), (B) pH-gradient EL/CL^{2-} liposomes, containing 20 (1), 10 (2) and 1 mol% of negative CL^{2-} groups (3). See the experimental conditions in the legend to Fig. 7.

to the proteoliposome suspension. PEVP being an effective fluorescence quencher, its addition was accompanied by some decrease both in initial and final levels of Dox fluorescence. The decrease could also result from aggregation of liposomes in contact with PEVP and increase in suspension turbidity. By analyzing the data of Fig. 7, the rate constant of Dox permeation were quantified. The effect of PEVP on this process is presented in Fig. 8A as a k_p/k_0–$Z_\pm$ plot, where k_p and k_0 are the rate constants in the presence and in the absence of PEVP, respectively, and $Z_\pm$ is a ratio between concentrations of positive PEVP units and negative sCT groups. One can see that the PEVP effect slightly increased upon $Z_\pm$ grew up and reached the maximum value $k_p/k_0 = 1.2$ at $Z_\pm = 1$ (curve 1). On further $Z_\pm$ rise, the PEVP effect

was eliminated. For comparison, Fig. 8A presents a k_p/k_0–$Z_\pm$ dependence for EL/sCT proteoliposomes with LPR = 3000, corresponding to $\nu = 0.0046$ (Fig. 8A, curve 2). In this case, no effect of PEVP on Dox permeation was detected.

Fig. 8B shows the effect of PEVP on the permeability of Dox through the membrane of mixed EL/CL^{2-} liposomes with $\nu = 0.2$ (curve 1), 0.1 (curve 2) and 0.01 (curve 3). It mainly follows the regularities found for PEVP/proteoliposome system. Actually, an increase in $Z_\pm$, i.e., PEVP concentration, led to rising in k_p/k_0 value in the case of liposomes with high CL^{2-} content (curves 1 and 2) and had no effect on k_p/k_0 value in the case of liposomes with low CL^{2-} content (curve 3). The maximum k_p/k_0 values were equal to 2.8 for liposomes with $\nu = 0.2$ and 1.2 for those with $\nu = 0.1$. In the both cases, the maximum PEVP effect was reached at $Z_\pm = 1$.

Thus, adsorption of PEVP of the surface of both EL/sCT proteoliposomes and mixed negative EL/CL^{2-} liposomes stimulated a transmembrane Dox permeation. This effect was observed in vesicles with high content of a negative membrane component, reaching the maximum at equimolar PEVP/sCT and PEVP/CL^{2-} ratios. As mentioned above, adsorption of polycations, including PEVP, on the surface of cells and liposomes could be accompanied by lateral segregation of membrane proteins and lipids [7,18–20,47,48]. PEVP-induced clustering of sCT molecules, attached to the membrane of proteoliposomes, was also demonstrated in the present work. Possible structural and biological consequences of the clustering effect are broadly discussed in the literature [51,52]. In particular, clusters themselves and the boundary between clusters and the surrounding bilayer are usually considered as membrane 'defects'. Formation of clusters, composed of anionic proteins and negatively charged lipids, could be a reason for the increase in the membrane microviscosity, induced by polycation binding [53]. Membrane defect formation is regarded as an important event in the cell fusion processes [25]. Along this line, we also assume that the boundaries of PEVP-induced protein and lipid clusters could be responsible for the acceleration of transmembrane Dox permeation. We found that the maximum acceleration of the Dox permeation is achieved at $Z_\pm = 1$, i.e., at a complete neutralization of negative sCT or lipid molecules by adsorbed PEVP. Obviously, the maximum size of clusters and their boundary length should be achieved just at this $Z_\pm$ value. Addition of an excess of PEVP to liposomes decreased its effect on Dox permeation. As shown in [54,55] an increase in an amount of PEVP adsorbed on a mixed bilayer membrane was accompanied by 'loosening' of the packing of the clusters formed by PEVP and a negative membrane component. Such structural rearrangements could affect the membrane permeability towards Dox molecules.

The above observations seem to favor the cluster hypothesis, although additional experiments have to be done to prove it.

4. Conclusions

The goal of the paper was to describe how a synthetic polycation could affect the structure of the negative lipid/protein bilayer membrane and functioning of the membrane protein. Binding of a polycation to the membrane was electrostatic in nature; polycation demonstrated higher affinity to negative lipid molecules than to negative enzyme globules incorporated into the lipid bilayer. A polycation had no effect on the permeability of the membrane towards a simple salt, but significantly increased the transmembrane permeability of the non-charged form of the anti-tumor drug doxorubicin. Polycation binding was accompanied by clustering of protein molecules in the membrane. This process was most likely responsible for the increase in the doxorubicin permeation. Functional activity of the membrane-associated enzyme molecules remained unchanged upon polycation adsorption.

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