



Historical perspective

## Multi-liposomal containers

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## ABSTRACT

Small unilamellar liposomes, 40–60 nm in diameter, composed of anionic diphosphatidylglycerol (cardiolipin,  $CL^{2-}$ ) or phosphatidylcerine ( $PS^{1-}$ ) and zwitter-ionic egg yolk lecithin (EL) or dipalmitoylphosphatidylcholine (DPPC), electrostatically complex with polystyrene microspheres, ca. 100 nm in diameter, grafted by polycationic chains (“spherical polycationic brushes”, SPBs). Polymer/liposome binding studies were carried out using electrophoretic mobility (EPM), dynamic light scattering (DLS), fluorescence, conductometry, differential scanning calorimetry (DSC), and cryogenic transmission electron microscopy (cryo-TEM) as the main analytical tools. By these means a remarkably detailed picture emerges of molecular events inside a membrane. The following are among the most important conclusions that arose from the experiments: (a) binding of liposomes to SPBs is accompanied by flip-flop of anionic lipids from the inner to the outer leaflet of the liposomal membrane along with lateral lipid segregation into “islands”. (b) The SPB-induced structural reorganization of the liposomal membrane, together with the geometry of anionic lipid molecules, determines the maximum molar fraction of anionic lipid (a key parameter designated as  $\nu$ ) that ensures the structural integrity of liposomes upon complexation:  $\nu = 0.3$  for liposomes with conically-shaped  $CL^{2-}$  and  $\nu = 0.5$  for liposomes with anionic cylindrically-shaped  $PS^{1-}$ . (c) The number of intact liposomes per SPB particle varies from 40 for ( $\nu = 0.1$ ) to 13 ( $\nu = 0.5$ ). (d) By using a mixture of liposomes with variety of encapsulated substances, multi-liposomal complexes can be prepared with a high loading capacity and a controlled ratio of the contents. (e) In order to make the mixed anionic liposomes pH-sensitive, they are additionally modified by 30 mol% of a morpholinocyclohexanol-based lipid that undergoes a conformational flip when changing pH. Being complexed with SPBs, such liposomes rapidly release their contents when the pH is reduced from 7.0 to 5.0. The results allow loaded liposomes to be concentrated within a rather small volume and, thereby, the preparation of multi-liposomal containers of promise in the drug delivery field.

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## 1. Introduction

Spherical bilayer lipid vesicles (liposomes) have been widely used as nano-scale containers for encapsulation, delivery and release of biologically active (medicinal) substances [1,2]. Hydrophobic guests can be incorporated within the liposomal membrane, whereas hydrophilic guests situate in the aqueous liposomal cavity [3,4]. A traditional approach, where individual drug-loaded liposomes operate independently, proved to be very fruitful in allowing enhanced bioavailability of poorly soluble drugs [2,5]; in protecting drugs against premature biodegradation [6,7]; in modifying the liposome surface by “vector” molecules and ensuring liposome binding to the target cells [8,9]; in increasing the circulation time of liposomes in the blood stream [2,10]; and in improving the drug uptake by the target cells [11]. The scientific and patent literature describes antitumor, antifungal, and antiviral liposomal drugs [2,12,13], among the most cited of which are liposomal forms of the antitumor antibiotic doxorubicin, Doxil/, Caelyx and DaunoXome [14–16]. However, other principal issues are still waiting for their solutions. Concerns remain over the limited capacity of conventional liposomal containers; over problems with multi-component drugs for combination therapy; and over the slow release of drugs in the area of therapeutic action [17–19].

Immobilization of liposomes on a suitable surface could result in a more capacious depot for biologically active compounds and an increase in their therapeutic effects. Liposomes were adsorbed onto mica [20], glass [21], silica [22], gold [23], polymers [24] and supported lipid bilayers [25]. Unfortunately, due to liposome–surface and liposome–liposome interactions, fusion and rupture vents are common during adsorption particularly at higher coverage. Successful attempts at intact liposome immobilization, described in the literature, include pre-modification of liposomes and/or surface. For example, liposomes and the surface were modified by complementary single-stranded oligonucleotide [26]. Immobilization of liposomes was accompanied by formation of a double-stranded oligonucleotide bridge which, by protecting liposomes from direct contact with the surface, allowed the liposomes to retain their integrity. Liposomes and the surface modified by poly(ethyleneglycol) chains with terminated biotin groups could be conjugated via addition of protein avidin and formation of liposome/biotin–avidin–biotin/surface bridges [27]. In another example, modification of liposomes by charged polymeric nanoparticles provided the electrostatic binding of the modified liposomes to the surface of polystyrene latex particles while, at the same time, prevented fusion of bound liposomes [28]. Plasma treatment of metal surfaces created carboxylic groups, which were used for covalent binding of amino-containing liposomes [29]. A simpler technology includes a preliminary aggregation of liposomes with subsequent immobilization of the aggregates [30]. However, the aggregation is often accompanied by liposome disruption and a premature loss of the encapsulated drug [31]. Additionally, the aggregation is difficult to control in that it leads to irreproducible multilayer liposome adsorption [30]. A need thus arises for carriers devoid of such stability problems.

In the present review, we describe electrostatic adsorption of anionic liposomes on the surface of polymeric microspheres with grafted polycationic chains, known as “spherical polycationic brushes” (SPBs). We show that the liposomes retain their integrity after adsorption and the resulting liposome/brush complex does not dissociate into its components in physiological solution with  $[\text{NaCl}] = 0.15 \text{ M}$ . By varying the anionic lipid fraction in the liposomal membrane, we can manipulate the amount of bound liposomes. By using liposomes with different entrapped contents, the multi-liposomal complexes with desirable content ratios can be prepared. Complexation decreases the cytotoxicity of the polycationic brushes to approximate that of the initial anionic liposomes. This makes the brush-based multi-liposomal containers promising in the drug delivery field.

## 2. Complexation of the polycationic brushes with conventional liposomes composed of anionic and electroneutral lipids

In order to prepare SPBs, cationic poly(trimethylaminoethylmethacrylate) ammonium chloride macromolecules were grafted from the surface of 100 nm monodispersed polystyrene latex particles as described elsewhere [32]. Dynamic light scattering showed a mean hydrodynamic diameter of the brush equal to 230 nm with a thickness of a cationic corona ( $L_c$ ) of  $(230 - 100) / 2 = 65 \text{ nm}$  (see structure in Fig. 1). Concentrations of SPBs are expressed as moles of cationic units per liter ( $[\text{SPB}^+]$ ) throughout the text. The brushes were coupled with liposomes composed of electrically neutral egg yolk lecithin (EL) and diphosphatidyl glycerol (cardiolipin,  $\text{CL}^{2-}$ ), a lipid bearing two anionic headgroups and four alkyl tails. Their chemical structures are shown in Fig. 2 (I and II). A molar content of negative  $\text{CL}^{2-}$  headgroups  $\nu = 2[\text{CL}^{2-}] / (2[\text{CL}^{2-}] + [\text{EL}])$  was specified from 0.05 up to 0.4. The bilayer membrane of these “liquid” liposomes is, at room temperature, in the fluid (or liquid crystalline) state. This fluid state is characterized by considerable lipid chain disorder and an ability of lipid molecules to migrate within each membrane leaflet (lateral mobility) and between them (transmembrane migration or flip-flop).

Binding of anionic  $\text{EL}/\text{CL}^{2-}$  liposomes to SPBs was monitored by a fluorescence method. SPB suspensions were mixed with increasing concentrations of  $\text{EL}/\text{CL}^{2-}$  liposomes whose membranes contained a fluorescence tag, N-fluorescein-iso-thiocyanatylidipalmitoylphosphatidylethanolamine (FITC-DPPE) shown in Fig. 2 (III). SPB/liposome complex particles were separated by centrifugation, and the fluorescence intensities of supernatants were measured. The amount of unbound liposomes vs. total amount of added liposomes was then plotted as for several values of  $\nu$  (0.05 to 0.4) (Fig. 3). It is seen that there are no unbound liposomes until the SPB particles are saturated, at a concentration designated as  $C_{\text{lip}}$ , beyond which the number of unbound

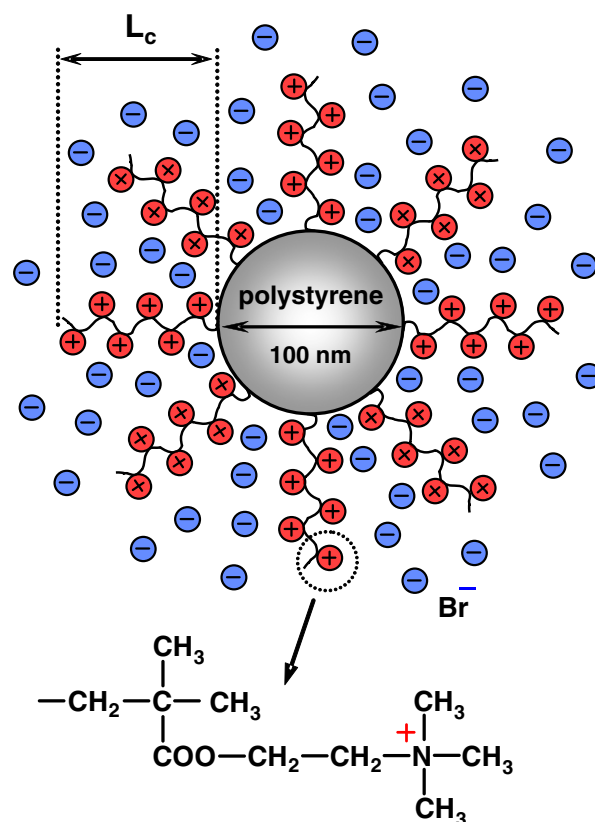


Fig. 1. The polycationic brush (schematical presentation).

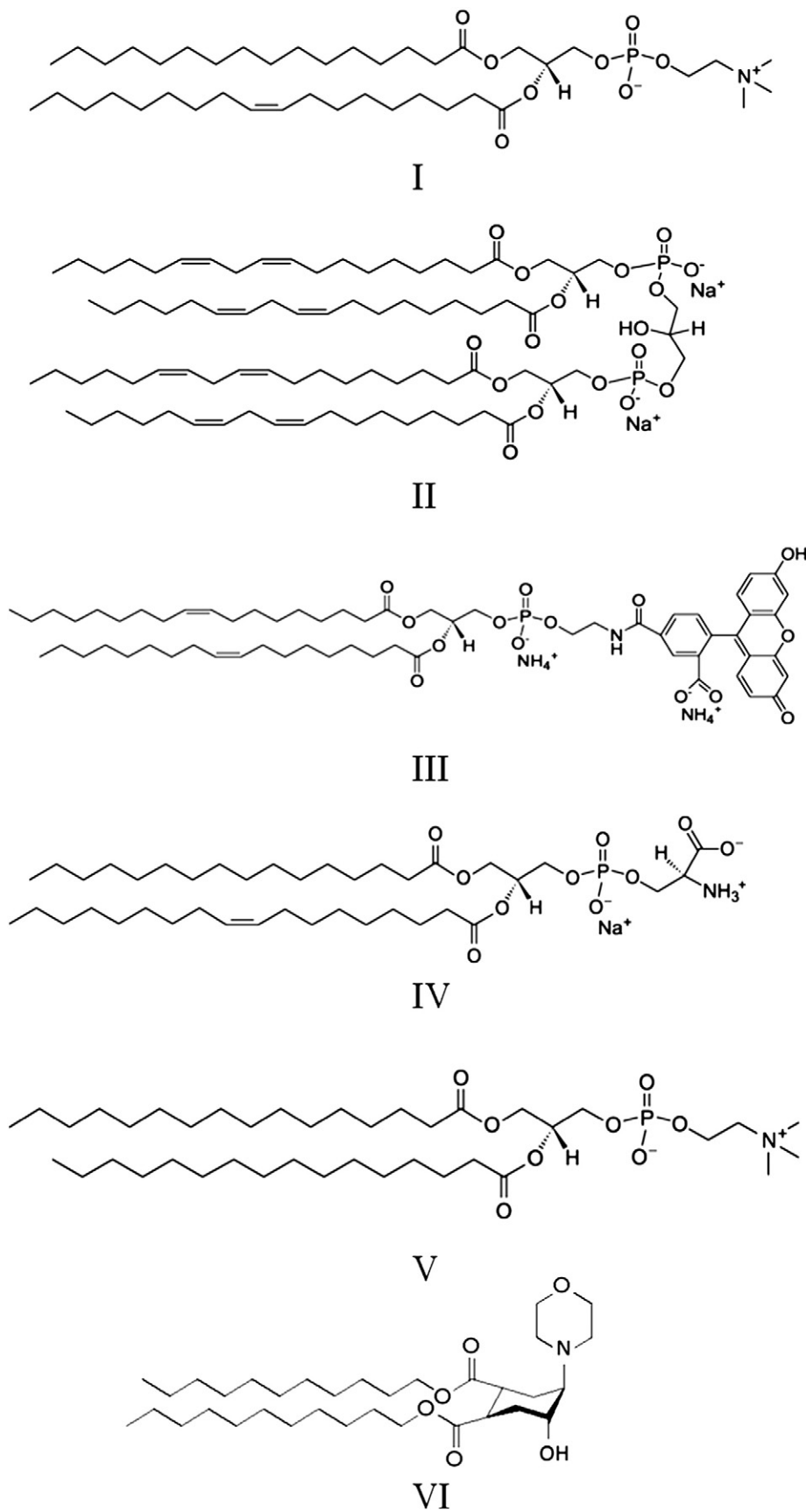
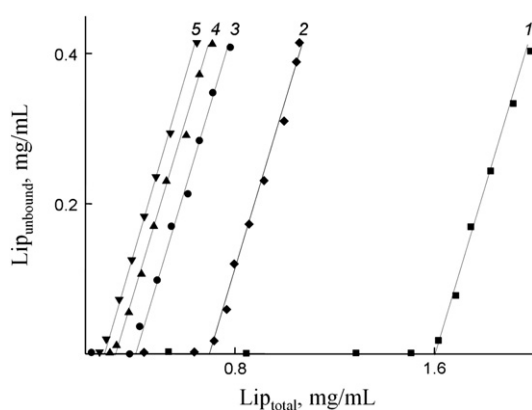


Fig. 2. Lipids (schematical presentation).



**Fig. 3.** Concentration of EL/CL<sup>2-</sup> liposomes unbound to SPB vs. the total liposome concentration.  $\nu = 0.05$  (1), 0.1 (2), 0.2 (3), 0.3 (4), and 0.4 (5). [SPB<sup>+</sup>] =  $1 \times 10^{-4}$  M; Borate buffer, pH 9, 25 °C. Reprinted with permission from A.V. Sybachin, O.V. Zaborova, M. Ballauff, E. Kesselman, J. Schmidt, Y. Talmon, F.M. Menger, and A.A. Yaroslavov. Composition and properties of complexes between spherical polycationic brushes and anionic liposome. *Langmuir: the ACS journal of surfaces and colloids*, 28(46):16108–16114, 2012. Copyright (2012) American Chemical Society.

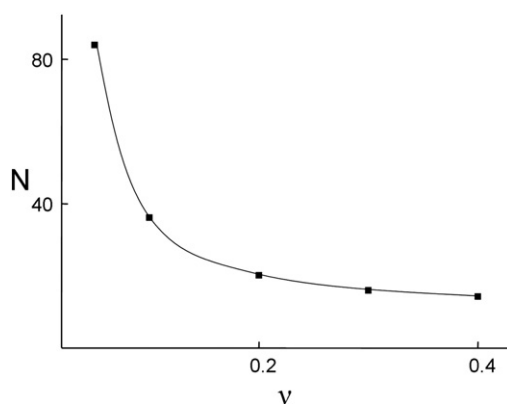
liposomes increases linearly. The greater the fraction of anionic lipid in the liposomes (i.e. the greater the  $\nu$  value), the smaller the lipid concentration needed to achieve saturation.

The experimentally determined  $C_{lip}$  values allowed the calculation of an ultimate number of EL/CL<sup>2-</sup> liposomes capable of binding to a single SPB particles [33] as:

$$N = \left( C_{lip} \times S_1 \times N_a \times D^3 \times \rho \right) / \left( 6C_{brush} \times d^2 \times M \right) \quad (1)$$

where  $C_{lip}$  is a lipid concentration at saturation,  $C_{brush}$  is a SPB concentration,  $D$  is a diameter of polystyrene core, 100 nm, and  $\rho$  is its density [34],  $d$  is a mean liposome diameter (50 nm),  $S_1$  is a mean surface area per one lipid molecule ( $0.7 \text{ nm}^2$ ) [35],  $M$  is the mean molecular weight of the lipid [35], and  $N_a$  is Avogadro's number. The calculations (Fig. 4) show a progressive decrease in  $N$  with an increase in  $\nu$ : from  $N = 80$  for  $\nu = 0.05$  down to  $N = 15$  for  $\nu = 0.4$ . In other words, the number of liposomes per particle decreases 5-fold when the molar fraction of anionic CL<sup>2-</sup> headgroups increases from 0.05 to 0.4.

A key question concerns the stability of SPB/liposome complexes to dissociation down to the initial components in aqueous salt solutions. Since cationic polymers are known to be effective fluorescence quenchers [36], the formation and dissociation of the complexes were

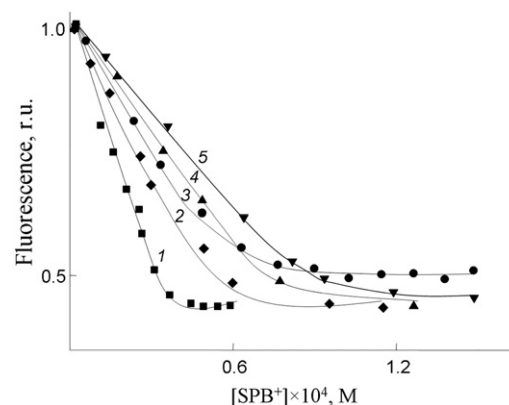


**Fig. 4.** Number of ultimately adsorbed EL/CL<sup>2-</sup> liposomes per one vs.  $\nu$ . Borate buffer, pH 9, 25 °C. Reprinted with permission from A.V. Sybachin, O.V. Zaborova, M. Ballauff, E. Kesselman, J. Schmidt, Y. Talmon, F.M. Menger, and A.A. Yaroslavov. Composition and properties of complexes between spherical polycationic brushes and anionic liposome. *Langmuir: the ACS journal of surfaces and colloids*, 28(46):16108–16114, 2012. Copyright (2012) American Chemical Society.

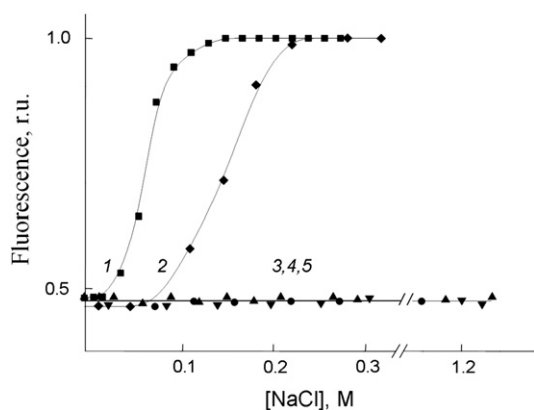
monitored by recording the fluorescence intensity of FITC-labeled lipid embedded in the liposomal membrane. Addition of the SPB suspension to the suspension of the labeled liposomes with different  $\nu$  values leads to a decrease in the FITC fluorescence (Fig. 5) as the SPB particles absorb the liposomes and quench their fluorescence. Subsequent addition of a NaCl solution to the SPB/liposome complex suspensions has different effects on the fluorescence depending on the CL<sup>2-</sup> content in the adsorbed liposomes. The complexes formed by the  $\nu = 0.05$  liposomes completely dissociate at [NaCl] = 0.15 M as revealed by the re-emerging fluorescence. The complexes of the  $\nu = 0.1$  liposomes are dissociated at [NaCl] = 0.25 M, while the liposomes with the  $\nu \geq 0.2$  form complexes that do not dissociate in solutions with a salt concentration even as high as 1.2 M (Fig. 6).

The irreversible liposome-to-SPB complexation could conceivably result from incorporation polycationic chain fragments into the liposomal membrane. Such incorporation should be accompanied by a formation of defects in the lipid bilayer and a subsequent leakage of liposomal contents into the surrounding solution. The integrity of EL/CL<sup>2-</sup> liposomes complexed with SPB was assessed conductometrically, using liposomes loaded by a 1 M NaCl solution. Violation of the liposome integrity led to salt release and an increase in the conductivity of the liposome/SPB suspension which was compared with the conductivity of a liposome suspension where liposomes had been completely destroyed in the presence of an excess of surfactant Triton X-100 that was taken as a 100% level. It was found that the addition of liposomes with  $\nu \leq 0.3$  to the SPB suspension does not affect the conductivity (Fig. 7 curves 1–4), while the liposomes with  $\nu = 0.4$  increase the conductivity (curve 5). In other words, the integrity of EL/CL<sup>2-</sup> liposomes with  $\nu \leq 0.3$  persists after the complexation. In contrast, a CL<sup>2-</sup> content of  $\nu = 0.4$  induces formation of defects in the liposomal membranes and, consequently, leakage of NaCl solution.

For comparison, we added the suspension of NaCl-loaded  $\nu = 0.1$  EL/CL<sup>2-</sup> liposomes to a suspension of 100 nm polystyrene latex particles without grafted polycationic chains but with cationic trimethylammonium groups attached to the particle surface. When the liposomes were added, the surface charge of latex particles decreased indicating liposome-to-latex complexation. However, the complexation was accompanied by an increase in the suspension conductivity that was obviously due to formation of defects in the liposomal membrane and a leakage of NaCl solution. This result shows a principal difference in the behavior of anionic liposomes bound to the two types of cationic colloidal particles: disruption of liposomes after their adsorption on the solid cationic surface of conventional latex particles vs. preservation of liposomes immobilized on the surface with



**Fig. 5.** Relative fluorescence intensity of labeled EL/CL<sup>2-</sup> liposomes vs SPB concentration.  $\nu = 0.05$  (1), 0.1 (2), 0.2 (3), 0.3 (4), and 0.4 (5). Total lipid concentration 1 mg/mL. Borate buffer, pH 9, 25 °C. Reprinted with permission from A.V. Sybachin, O.V. Zaborova, M. Ballauff, E. Kesselman, J. Schmidt, Y. Talmon, F.M. Menger, and A.A. Yaroslavov. Composition and properties of complexes between spherical polycationic brushes and anionic liposome. *Langmuir: the ACS journal of surfaces and colloids*, 28(46):16108–16114, 2012. Copyright (2012) American Chemical Society.



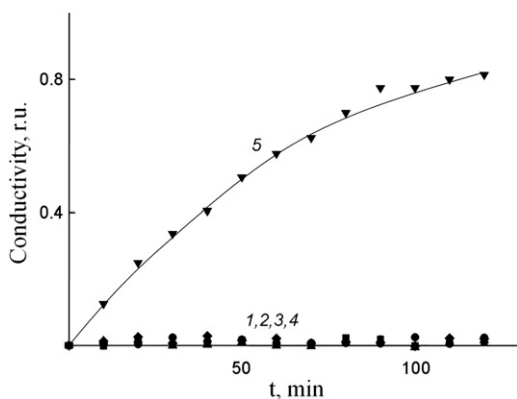
**Fig. 6.** Relative fluorescence intensity of labeled EL/CL<sup>2-</sup> liposomes complexed with SPB vs. NaCl concentration.  $\nu = 0.05$  (1), 0.1 (2), 0.2 (3), 0.3 (4), and 0.4 (5). [SPB<sup>+</sup>] =  $1 \times 10^{-4}$  M; total lipid concentration 1 mg/mL. Borate buffer, pH 9, 25 °C. Reprinted with permission from A.V. Sybachin, O.V. Zaborova, M. Ballauff, E. Kesselman, J. Schmidt, Y. Talmon, F.M. Menger, and A.A. Yaroslavov. Composition and properties of complexes between spherical polycationic brushes and anionic liposome. *Langmuir: the ACS journal of surfaces and colloids*, 28(46):16108–16114, 2012. Copyright (2012) American Chemical Society.

grafted polycationic chains. The difference may be related to the difference in the flexibility of the two cationic surfaces.

Additional information about the morphology of SPB/liposome complexes was obtained with the use of cryogenic transmission electron microscopy (cryo-TEM). This method was specifically developed for analyzing the morphology of soft objects, including liposomes, but one must bear in mind that EM involves sample drying that can change size and shape.

Fig. 8a shows a typical micrograph of a polycationic brush in a water-salt buffer solution; grafted polycationic chains are visible in the figure without additional contrast. A micrograph of the  $\nu = 0.1$  EL/CL<sup>2-</sup> liposomes (Fig. 8b) confirms their monolamellar structure; an average size of liposomes ( $50 \pm 10$  nm) is consistent with the size obtained by dynamic light scattering. SPB/liposome complex particles are represented in Fig. 8c. Single brushes are surrounded by several intact liposomes, thus corroborating the fluorescent and conductimetric data.

Replacement of CL<sup>2-</sup> in the liposomal membrane by another anionic lipid, phosphatidylserine (PS<sup>1-</sup>) (see chemical structure in Fig. 2, IV), does not affect the liposome-to-brush complexation. Binding of EL/PS<sup>1-</sup> liposomes to SPB is also accompanied by neutralization of the



**Fig. 7.** Time-dependence of relative conductivity of a complex suspension composed of SPB and NaCl-loaded EL/CL<sup>2-</sup> liposomes.  $\nu = 0.05$  (1), 0.1 (2), 0.2 (3), 0.3 (4), and 0.4 (5). [SPB<sup>+</sup>] =  $1 \times 10^{-4}$  M; total lipid concentration 1 mg/mL. Borate buffer, pH 9, 25 °C. Reprinted with permission from A.V. Sybachin, O.V. Zaborova, M. Ballauff, E. Kesselman, J. Schmidt, Y. Talmon, F.M. Menger, and A.A. Yaroslavov. Composition and properties of complexes between spherical polycationic brushes and anionic liposome. *Langmuir: the ACS journal of surfaces and colloids*, 28(46):16108–16114, 2012. Copyright (2012) American Chemical Society.

SPB surface charge, enlargement of dispersed particles, and quenching of the FITC fluorescence incorporated into the liposomal membrane. However the EL/PS<sup>1-</sup> liposomes showed a different behavior in water-salt solutions. Fig. 9 reflects how the fluorescence of the FITC-labeled lipid, quenched after the liposome complexation with SPB, and responds to the increase in the salt concentration in surrounding solution. The fluorescence recovers up to the initial level provided that the molar content of negative PS<sup>1-</sup> headgroups, namely  $\nu = [\text{PS}^{1-}] / ([\text{PS}^{1-}] + [\text{EL}])$ , does not exceed 0.5 (curves 1–5). The liposomes with a higher  $\nu$  give complexes which do not dissociate in a water-salt solution (curve 6). An ultimate number of EL/PS<sup>1-</sup> liposomes capable of binding to a single SPB particles was found to be 40 for the liposomes with  $\nu = 0.1$  and 13 for liposomes with  $\nu = 0.5$ .

The fluorescent data correlate well with the conductometric data on the integrity of the SPB-bound EL/PS<sup>1-</sup> liposomes. The NaCl solution does not leak from the SPB-bound liposomes with  $\nu \leq 0.5$  (Fig. 10, curves 1–5), while  $\nu = 0.54$  liposomes lose the salt solution (curve 6) thus again indicating the formation of defects in the liposomal membrane. We see a much faster NaCl leakage from  $\nu = 0.54$  EL/PS<sup>1-</sup> liposomes in comparison with a NaCl leakage from  $\nu = 0.4$  EL/CL<sup>2-</sup> liposomes (cf. curve 6 in Fig. 10 and curve 4 in Fig. 7). This might be related to the difference in the structure of the two defective liposomes: the destruction of the  $\nu = 0.54$  EL/CL<sup>2-</sup> bilayer and formation of isolated defects in the  $\nu = 0.4$  EL/PS<sup>1-</sup> bilayer.

A different structural stability of EL/PS<sup>1-</sup> and EL/CL<sup>2-</sup> liposomes after their complexation with SPBs might arise from a different geometry of anionic lipids, PS<sup>1-</sup> and CL<sup>2-</sup>. PS<sup>1-</sup> is cylindrically-shaped and geometrically complementary to the cylindrical neutral EL. Such geometric fit between the two lipids allows the 50% ( $\nu = 0.5$ ) anionic component of the bilayer before leakage sets in. In contrast to this, because of the non-complementary nature of the cone-shaped CL<sup>2-</sup> and the cylindrical EL, the integrity of EL/CL<sup>2-</sup> liposomes is maintained only as long as the CL<sup>2-</sup> content is less than  $\nu = 0.3$ .

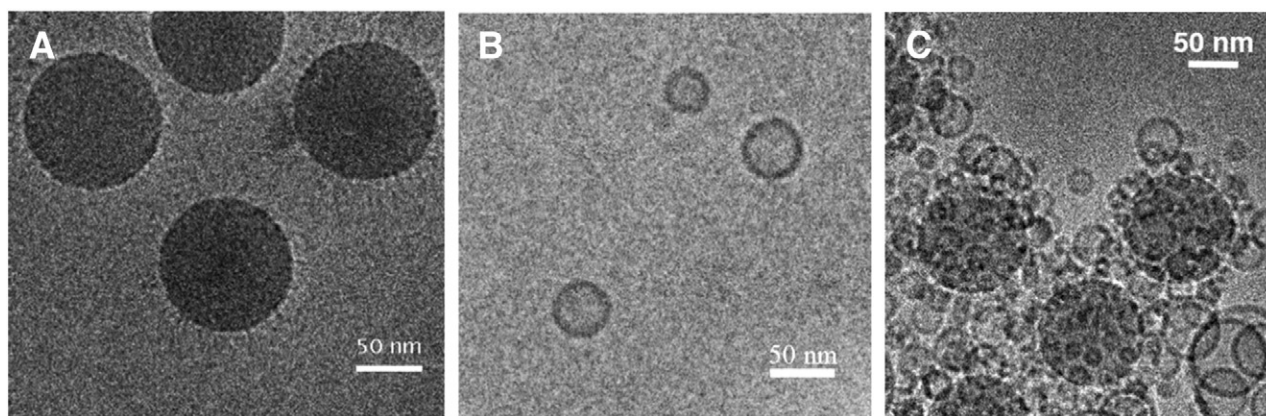
Thus, the replacement of “asymmetrical” CL<sup>2-</sup> for “symmetric” PS<sup>1-</sup> allows one to preserve the high adsorption capacity of SPB towards anionic liposomes while, concomitantly, to extend the interval of anionic lipid content that ensures the integrity of SPB-bound liposomes. The latter can be of critical point for creating multi-liposomal containers with a controllable rate of drug release.

### 3. Structural rearrangements in the membranes of adsorbed liposomes

The interaction of “liquid” anionic liposomes, whose membrane is in a liquid-crystalline state, with flexible linear cationic polymers can induce structural rearrangements in the liposomal membrane. These include an acceleration of the transmembrane migration of lipid molecules (flip-flop) and phase separation in the liposomal membrane (lateral lipid segregation) [37,38]. Similar structural rearrangements could be reasonably assumed to occur within the membrane of liquid anionic liposomes upon their binding to flexible poly(trimethylaminoethylmethacrylate) ammonium chloride grafted to polystyrene particles. These transformations were studied using laser microelectrophoresis and differential scanning calorimetry.

The first method follows the change in electrophoretic mobility (EPM) of SPBs when their surface is covered by anionic liposomes. Corresponding dependences for liquid EL/PS<sup>1-</sup> liposomes when the parameter  $\nu$  was varied from 0.1 to 0.54 are shown in Fig. 11. In all cases, the SPB surface charge goes down to zero when increasing liposome concentration, and it becomes negative in an excess of liposomes. A higher PS<sup>1-</sup> content in the liposomal membrane requires a lower liposome concentration for the SPB charge neutralization and renders a higher ultimate negative charge to the SPB-liposome complex.

Curve 1 in the figure describes the electrophoretic titration of SPB particles by a suspension of  $\nu = 0.1$  liposomes. Now we know that  $\nu = 0.1$  liposomes are quantitatively bound to SPBs (Fig. 3), and that



**Fig. 8.** Cryogenic transmission electron microscopy images of SPBs (a), EL/CL<sup>2-</sup> liposomes (b) and SPB/liposome complex. [SPB<sup>+</sup>] = 1 × 10<sup>-4</sup> M; ν = 0.1, 2 mg/mL lipid concentration. Reprinted with permission from A.V. Sybachin, O.V. Zaborova, M. Ballauff, E. Kesselman, J. Schmidt, Y. Talmon, F.M. Menger, and A.A. Yaroslavov. Composition and properties of complexes between spherical polycationic brushes and anionic liposome. *Langmuir: the ACS journal of surfaces and colloids*, 28(46):16108–16114, 2012. Copyright (2012) American Chemical Society.

they retain their integrity after complexation (Fig. 7). This means that at the point EPM = 0 the total positive SPB charge is numerically equal to the total negative charge located on the outer (facing to the SPB surface) leaflets of the complexed liposomes, as expressed in Eq. (2):

$$[\text{SPB}^+] = [\text{PS}^{1-}]_{\text{out, EPM}=0} \quad (2)$$

The fraction of PS<sup>1-</sup> molecules involved in electrostatic complexation with SPBs (γ) can be estimated from Eq. (3), where [PS<sup>1-</sup>]<sub>t, EPM = 0</sub> is the total concentration of PS<sup>1-</sup> in solution at EPM = 0:

$$\gamma = [\text{PS}^{1-}]_{\text{out, EPM}=0} / [\text{PS}^{1-}]_{\text{t, EPM}=0} = [\text{SPB}^+] / [\text{PS}^{1-}]_{\text{t, EPM}=0} \quad (3)$$

The calculation based on Eq. (3) gives γ = 0.94 × 10<sup>-4</sup> M / 1.01 × 10<sup>-4</sup> M ≈ 1. Since γ = 1, all PS<sup>1-</sup> molecules must shift from the inner leaflet to the outer leaflet as a result of electrostatic complexation with the SPB particles. For complexed EL/PS<sup>1-</sup> liposomes with ν = 0.1, we can write Eqs. (4) and (5), where [Lip]<sub>EPM = 0</sub> is a

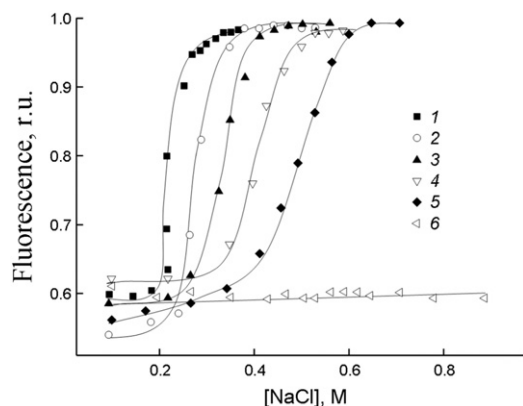
concentration of all membrane lipids (EL + PS<sup>1-</sup>) in solution at EPM = 0:

$$[\text{SPB}^+] = [\text{PS}^{1-}]_{\text{out, EPM}=0} = [\text{PS}^{1-}]_{\text{t, EPM}=0} = [\text{Lip}]_{\text{EPM}=0} \times \nu \quad (4)$$

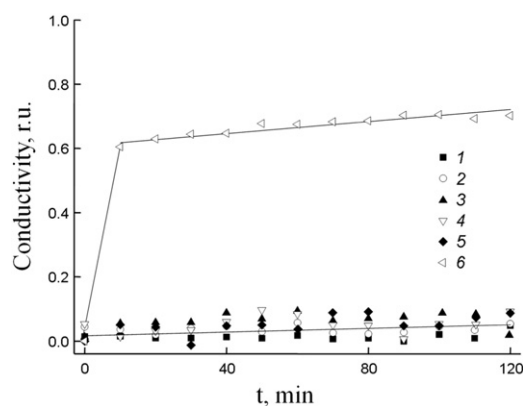
$$[\text{Lip}]_{\text{EPM}=0} = [\text{SPB}^+] / \nu \quad (5)$$

If the SPB-induced flip-flop of PS<sup>1-</sup> molecules occurs in liposomes with different PS<sup>1-</sup> contents, Eq. (5) should be satisfied for all studied systems. The data of Fig. 11, re-plotted according to Eq. (5), show linearity in the entire 1/ν interval (Fig. 12), thus providing evidence for SPB-induced flip-flop in all complexed EL/PS<sup>1-</sup> liposomes whatever their PS<sup>1-</sup> content. In summary, flexible polycation grafted onto a polymeric particle induces flip-flop of anionic lipids in the membrane of bound liquid liposomes.

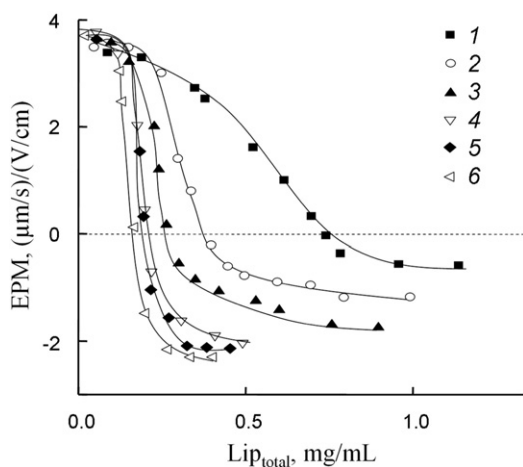
At a PS<sup>1-</sup> content in the liposomal membrane of ν ≤ 0.5, the SPB-induced lipid flip-flop need not affect the integrity of liposomes, because PS<sup>1-</sup> migration from the inner to the outer membrane leaflet can be compensated by migration of an equal number of neutral EL molecules in the opposite direction. Hence, the bilayer integrity can be sustained. At higher ν, the SPB-induced PS<sup>1-</sup> migration can no longer be compensated by EL back-migration, and this leads to formation of defects in the



**Fig. 9.** Relative fluorescence intensity of labeled EL/PS<sup>1-</sup> liposomes complexed with SPB vs. NaCl concentration. ν = 0.1 (1), 0.2 (2), 0.3 (3), 0.4 (4), 0.5 (5), and 0.54 (6). [SPB<sup>+</sup>] = 1 × 10<sup>-4</sup> (1), 2 × 10<sup>-4</sup> (2), 3 × 10<sup>-4</sup> (3), 4 × 10<sup>-4</sup> (4), 5 × 10<sup>-4</sup> (5) 6 × 10<sup>-4</sup> (6) M; total lipid concentration 1 mg/mL. Tris buffer pH 7, 25 °C. Reprinted with permission from A.V. Sybachin, O.V. Zaborova, V.N. Orlov, P.I. Semenyuk, M. Ballauff, E. Kesselman, J. Schmidt, Y. Talmon, F.M. Menger, and A.A. Yaroslavov and. Complexes between anionic liposomes and spherical polycationic brushes. An assembly of assemblies. *Langmuir: the ACS journal of surfaces and colloids*, 30(9):2441–2447, 2014. Copyright (2014) American Chemical Society.



**Fig. 10.** Time-dependence of relative conductivity of a complex suspension composed of SPB and NaCl-loaded EL/PS<sup>1-</sup> liposomes. ν = 0.1 (1), 0.2 (2), 0.3 (3), 0.4 (4), 0.5 (5), and 0.54 (6). [SPB<sup>+</sup>] = 1 × 10<sup>-4</sup> M; total lipid concentration 1 mg/mL. Tris buffer pH 7, 25 °C. Reprinted with permission from A.V. Sybachin, O.V. Zaborova, V.N. Orlov, P.I. Semenyuk, M. Ballauff, E. Kesselman, J. Schmidt, Y. Talmon, F.M. Menger, and A.A. Yaroslavov and. Complexes between anionic liposomes and spherical polycationic brushes. An assembly of assemblies. *Langmuir: the ACS journal of surfaces and colloids*, 30(9):2441–2447, 2014. Copyright (2014) American Chemical Society.

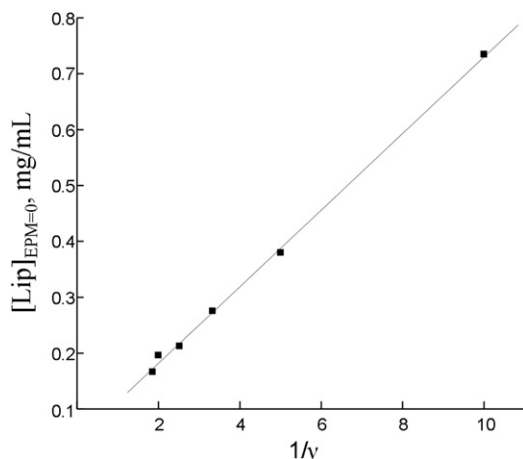


**Fig. 11.** Electrophoretic mobility of SPB/liposome complex vs. EL/PS<sup>1-</sup> liposome concentration.  $\nu = 0.1$  (1), 0.2 (2), 0.3 (3), 0.4 (4), 0.5 (5), 0.54 (6); [SPB<sup>+</sup>] =  $1 \times 10^{-4}$  M; Tris buffer pH 7, 25 °C. Reprinted with permission from A.V. Sybachin, O.V. Zaborova, V.N. Orlov, P.I. Semenyuk, M. Ballauff, E. Kesselman, J. Schmidt, Y. Talmon, F.M. Menger, and A.A. Yaroslavov and. Complexes between anionic liposomes and spherical polycationic brushes. An assembly of assemblies. *Langmuir: the ACS journal of surfaces and colloids*, 30(9):2441–2447, 2014. Copyright (2014) American Chemical Society.

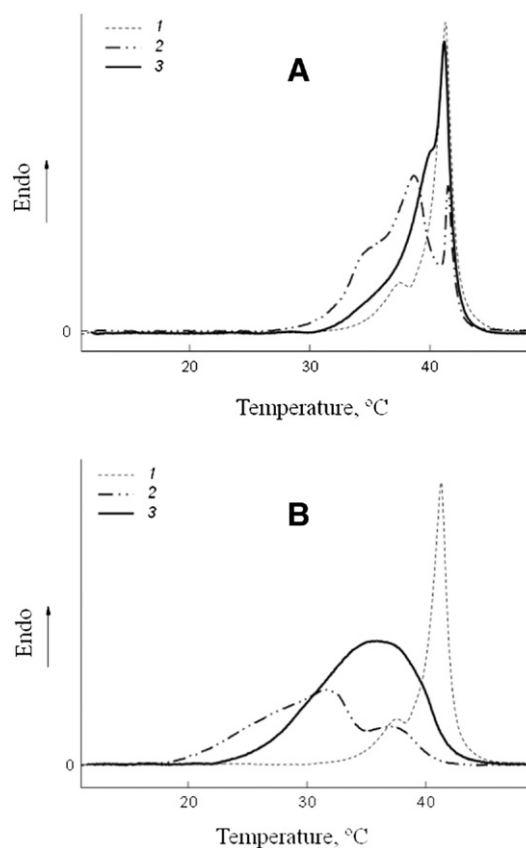
liposomal membrane with leakage of the contents as revealed from the conductivity data.

The second method, differential scanning calorimetry (DSC), detects the phase transitions in lipid bilayer, thus controlling the distribution of anionic lipids in the mixed liposomal membrane before and after binding of liposomes to SPBs. Lipid bilayers are characterized by phase transition temperatures (melting temperatures),  $T_m$ . Below  $T_m$ , the lipid bilayer is in the so-called gel state with the reduced mobility of the lipid molecules (“solid” liposomes). Above  $T_m$ , the bilayer enters the liquid-crystalline state with higher lipid mobility in lateral and trans-membrane directions (“liquid” liposomes). In our DSC experiments EL, which is actually a low- $T_m$  mixture of natural lipids with different melting temperatures, was replaced by a synthetic neutral dipalmitoylphosphatidylcholine (DPPC) (see structure in Fig. 2, V), with a melting point of 41 °C. Calorimetric curves for DPPC liposomes are represented in Fig. 13A (curve 1) and Fig. 13B (curve 1).

Calorimetric curve of  $\nu = 0.1$  DPPC/PS<sup>1-</sup> liposomes (curve 2 in Fig. 13A) shows a rather wide DSC profile with a melting peak at 39 °C



**Fig. 12.** EL/PS<sup>1-</sup> liposome concentrations in the electroneutral complexes with SPBs vs.  $1/\nu$ . [SPB<sup>+</sup>] =  $1 \times 10^{-4}$  M. Tris buffer pH 7, 25 °C. Reprinted with permission from A.V. Sybachin, O.V. Zaborova, V.N. Orlov, P.I. Semenyuk, M. Ballauff, E. Kesselman, J. Schmidt, Y. Talmon, F.M. Menger, and A.A. Yaroslavov and. Complexes between anionic liposomes and spherical polycationic brushes. An assembly of assemblies. *Langmuir: the ACS journal of surfaces and colloids*, 30(9):2441–2447, 2014. Copyright (2014) American Chemical Society.

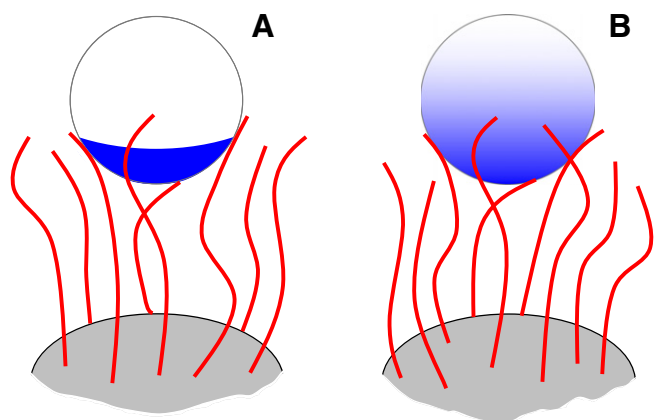


**Fig. 13.** Calorimetric curves of DPPC liposomes (1), DPPC/PS<sup>1-</sup> liposomes (2), and complexes of DPPC/PS<sup>1-</sup> liposomes with SPBs (3).  $\nu = 0.1$  (A) and  $\nu = 0.3$  (B). Tris buffer pH 7, 25 °C. Reprinted with permission from Yaroslavov, A. A., Sybachin, A. V., Zaborova, O. V., Orlov, V. N., Ballauff, M., Talmon, Y. and Menger, F. M., Lipid Segregation in Membranes of Anionic Liposomes Adsorbed onto Polycationic Brushes. *Chem. Eur. J.*, 19: 13674–13678, 2013. Copyright (2013) John Wiley & Sons.

and a shoulder at 35 °C, which reflect co-existence of two mixed DPPC/PS<sup>1-</sup> phases with different DPPC-to-PS<sup>1-</sup> ratios, and a sharp maximum at 41 °C corresponding obviously to pure DPPC domains. Addition of the  $\nu = 0.1$  liposomes to SPBs caused a shift to higher temperatures in the calorimetric curve (curve 3 in Fig. 13A). The transition profile became narrower with a maximum now at 41 °C, reasonably attributed to pure DPPC domains cleansed from PS<sup>1-</sup>, and a shoulder at 40 °C for DPPC domains with only a slight PS<sup>1-</sup> admixture.

An increase of the PS<sup>1-</sup> content in the liposomal membrane to  $\nu = 0.3$  led to a much broader calorimetric curve with two maxima at 32 and 38 °C (curve 2 in Fig. 13B) that reflect formation of mixed DPPC/PS<sup>1-</sup> domains with more PS<sup>1-</sup> in comparison with the DPPC/PS<sup>1-</sup> domains in the  $\nu = 0.1$  liposomes. Addition of SPBs to the  $\nu = 0.3$  liposomes had a slight effect on the width of the phase transition (curve 3 in Fig. 13B) which however now shows only one peak at 37 °C. The absence of a sharp peak at 41 °C definitely indicates no pure DPPC domains in the membrane of the  $\nu = 0.3$  liposomes complexed with SPB.

Distribution of the anionic lipid in the membrane of DPPC/PS<sup>1-</sup> liposomes complexed with SPB is schematically represented in Fig. 14. Complexation extracts the anionic PS<sup>1-</sup> lipids from the inner to the outer leaflets of the bilayer. At a low PS<sup>1-</sup> content ( $\nu = 0.1$ ), the majority of PS<sup>1-</sup> molecules concentrates within the membrane area facing the SPB surface (Fig. 14A). Few PS<sup>1-</sup> molecules remain on the opposite side of the adsorbed liposomes. In other words, the liposomal membrane, initially with a uniform distribution of neutral DPPC and anionic PS<sup>1-</sup>, is divided into two domains: one comprising pure DPPC and the other consisting of PS<sup>1-</sup> electrostatically bound to SPB. A small percentage of PS<sup>1-</sup> provides a low negative charge as observed by microelectrophoresis (Fig. 11, curve 1). When the content of PS<sup>1-</sup> increases up



**Fig. 14.** Possible PS<sup>1-</sup> distribution in liposomes adsorbed on SPB for  $\nu = 0.1$  (A) and  $\nu = 0.3$  (B) (schematic presentation).

to 0.3, a greater population of the PS<sup>1-</sup> locates at the outer lipid leaflet of adsorbed liposomes. However for steric reasons, only a part of PS<sup>1-</sup> molecules can be clustered in the membrane area facing the SPB surface (Fig. 14B). The remainder of the anionic lipid is distributed over the membrane, thus rendering the liposomes a higher negative charge in comparison with the  $\nu = 0.1$  liposomes (as the microelectrophoresis shows, see Fig. 11, curve 3). In this case, only mixed DPPC + PS<sup>1-</sup> domains with different DPPC-to-PS<sup>1-</sup> ratios and no pure DPPC domains are found in the membrane.

We see therefore significant structural rearrangements in the membrane of liquid anionic liposomes after their electrostatic complexation with the polycationic brushes. The complexation induces the trans-membrane migration of anionic lipids and formation of the anionic lipid clusters in the outer membrane leaflet. At high anionic lipid content, these structural metamorphoses are accompanied by disruption of the liposomal membrane and a leakage of encapsulated water-soluble compound. Thus, by combining a variety of physical methods one is able to obtain a detailed picture of molecular events occurring when a membrane engages in complexation.

#### 4. Complexation of the polycationic brushes with anionic liposomes loaded by different substances

In the previous sections we have discussed the electrostatic complexation of polycationic brushes with anionic liposomes loaded by a water solution of a simple salt, NaCl. However the liposome-to-brush complexation allows preparation of multi-liposomal containers, comprising liposomes with various guests, e.g. medications that are of interest to the comprehensive treatment of oncological diseases [39,40]. Liposome complexes with SPB can be prepared from liposomes with identical lipid composition but containing different hydrophilic (water-soluble) and/or hydrophobic (membrane-soluble) encapsulated substances. The latter do not contribute to the total negative charge of liposomes and therefore should not affect the ability of liposomes to

bind to the positively charged SPBs. If this is the case, we can hypothesize that the ratio between variably-loaded liposomes in the initial liposome mixture will equal to the ratio of liposome types in the adsorbed layer.

Three types of mixed DPPC/PS<sup>1-</sup> liposomes with  $\nu = 0.1$  were prepared (Fig. 15). The first was loaded with an aqueous solution of 7-hydroxyphenazine (lackmus) (LAC); the second type was a membrane-soluble hydrophobic dye, 1,1-dioctadecyl-3,3,3',3'-tetramethylene-carbocyanine perchlorate (DIL), embedded in the membrane; the third type was a liposome modified by dipalmitoyl-phosphatidylethanolamine covalently labeled with carboxyfluorescein (PECF). The liposomes thus contained different types of dyes: hydrophilic, hydrophobic and hydrophilic with a hydrophobic anchor.

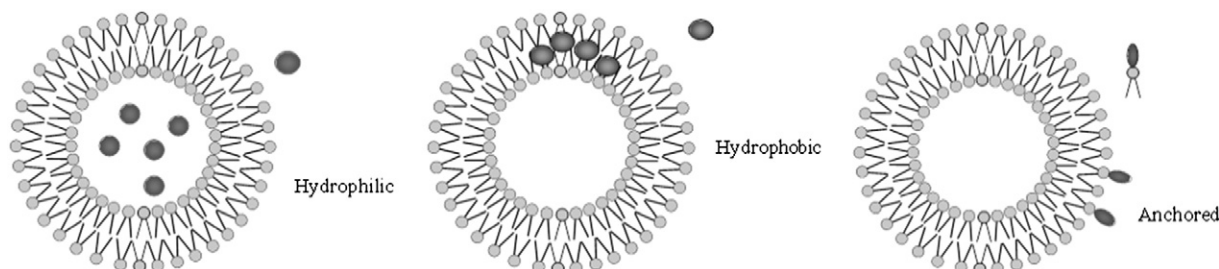
Three types of the labeled liposome suspensions were mixed in different weight ratios  $Q_{ini} = LAC_{ini}/DIL_{ini}/PECF_{ini}$ , and the resulting mixtures were added to a suspension of SPB, each time maintaining a double-excess of liposomes in comparison with their saturating concentration. After separation of the SPB-liposome complex by centrifugation, the supernatants were analyzed spectrophotometrically for the presence of the individual labeled liposomes. The LAC-loaded liposomes were detected at 586 nm, the LID-loaded liposomes at 551 nm, and PECF-loaded liposomes at 498 nm. The results were converted into concentrations of individual liposomes complexed with SPB and represented as a weight ratio  $Q_{comp} = LAC_{comp}/DIL_{comp}/PECF_{comp}$ . As follows from the data of Table 1,  $Q_{ini} \approx Q_{comp}$  for all liposome mixtures, in line with our expectations. The simple method for preparing multi-liposomal containers with controlled contents of encapsulated substances was thus experimentally verified.

#### 5. Complexation of the polycationic brushes with pH-sensitive anionic liposomes

We now discuss liposomal containers that release their biologically active contents only after they fall into slightly acidic media typical for inflamed tissues and tumors. Thus,  $\nu = 0.1$  EL/PS<sup>1-</sup> liposomes were prepared with 30 mol% of a morpholinocyclohexanol-based lipid (MOCH-lipid, Fig. 2 VI) embedded into the membrane. The MOCH-lipid undergoes a conformational flip when the pH is decreased from 7 to 5 as shown in Fig. 16 [38]. The simultaneous conformational change of many molecules disturbs the lipid packing and triggers a quick release of the contents from these pH-sensitive liposomes ("fliposomes") [41].

A suspension of EL/PS<sup>1-</sup>/MOCH (6/1/3) fliposomes was mixed with a SPB suspension in a pH 7 buffer solution. The dependence of the brush EPM on the fliposome concentration (curve 1 in Fig. 17) coincides with the EPM dependence of SPBs in the presence of the EL/PS<sup>1-</sup> (9/1) binary liposomes deprived of MOCH lipid (curve 2). Such a profile of the electrophoretic fliposome titration reflects their electrostatic complexation with the polycationic brushes and proves the absence of a contribution from MOCH amino groups to the complexation at pH 7.

The electrophoretic titration was then performed in a buffer solution with pH 5. Since there was no change in EPM of the brushes with increasing fliposome concentration (curve 3), the complexation was



**Fig. 15.** Schematic presentation of liposomes filled with hydrophilic, hydrophobic and anchored substances.



**Table 1**  
Complexation of polycationic brushes with labeled liposome mixtures.<sup>a</sup>

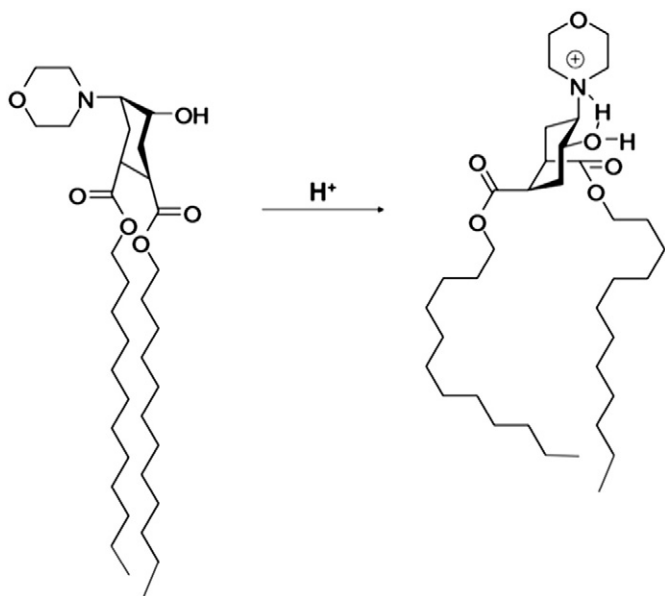
No.	$Q_{ini} = LAC_{ini}/DIL_{ini}/PECF_{ini}$	$Q_{comp} = LAC_{comp}/DIL_{comp}/PECF_{comp}$
1	0.66/1/1	0.6/1.15/1
2	0/1/2	0/1/1.85
3	0.1/1.5/1	0.16/1.4/1

<sup>a</sup>  $Q_{ini}$  is the ratio of differently-loaded liposomes prior to mixture with the SPB particles.  $Q_{comp}$  is the same ratio on the surface of the SPB particles.

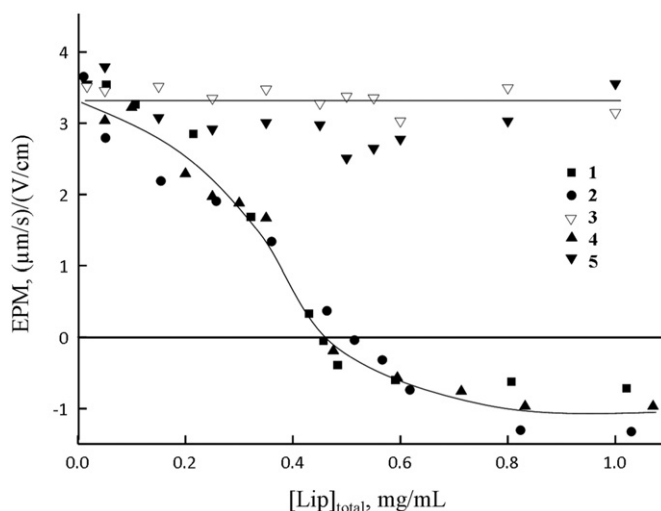
suppressed by the overall positive charge of the fliposomes owing to protonation of MOCH amino groups. In contrast, the anionic EL/PS<sup>1-</sup> binary liposomes, whose charge remained constant with a decrease in pH, adsorbed on the surface of cationic brushes in acidic solution. The EPM vs. liposome concentration plot at pH 5 (curve 4) overlaps with the plot for the same system obtained at pH 7 (curve 2).

The behavior of the brush-fliposome complex, prepared in a neutral solution and then moved to an acidic solution, is of particular interest. This experiment simulates the situation when multi-liposomal containers, injected into the bloodstream with pH close to neutral, reach an acidic region typical for inflamed tissues and tumors. Along this line, a series of complexes with different SPB/fliposome ratios was prepared in a pH 7 solution after which the pH of the samples was decreased down to 5. Only positive particles were detected in the pH-adjusted system (curve 5). This result might reflect either a positive charge of bare SPBs after desorption of the now positive fliposomes or, alternatively, a positive charge of the entire SPB/fliposome complex containing the protonated MOCH lipid. To distinguish between these two cases, the sizes of particles in the SPB/fliposome systems were measured with dynamic light scattering.

Addition of fliposomes to a SPB suspension at pH 7 led to formation of aggregates with a maximum size at 0.5 mg/mL lipid concentration (curve 1 in Fig. 18) which corresponds to the EPM = 0 on curve 1 in Fig. 17. No change in particle size was detected upon addition of fliposomes to SPB at pH 5 (curve 2 in Fig. 18), indicating an absence of complexation and aggregation in acidic solution. The light scattering data are in agreement with the above described EPM results. Curve 3 in Fig. 18 shows the sizes of SPB/fliposome complexes prepared in a pH 7 solution and then acidified down to pH 5. Acidification did not cause the size of complexes to decrease (cf. curve 1 and curve 3), thus reflecting the absence fliposomes removal from the SPB surface upon



**Fig. 16.** The protonation-triggered conformation switch in MOCH (schematic presentation). Reproduced from [38] with permission from The Royal Society of Chemistry.

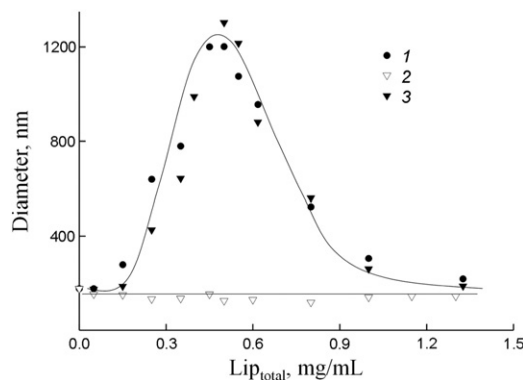


**Fig. 17.** EPM of SPB particles vs. liposome concentration. EL/PS<sup>1-</sup>/MOCH (6/1/3) liposomes, pH 7 (1) and 5 (3); EL/PS<sup>1-</sup> (9/1) liposomes, pH 7 (2) and 5 (4); EL/PS<sup>1-</sup>/MOCH (6/1/3) liposomes were complexed with SPBs at pH 7 and the resulting complexes were transferred to a pH 5 solution (5).  $[SPB^+] = 1 \times 10^{-4}$  M. Tris buffer pH 7, 25 °C. Reproduced from [38] with permission from The Royal Society of Chemistry.

acidification. In other words, the already adsorbed fliposomes do not leave the surface under acidic conditions, despite their cationic charge.

How could such behavior of the EL/PS<sup>1-</sup>/MOCH fliposomes could be explained in solutions with variable pH values? We have discussed above the binding of the  $\nu = 0.1$  EL/PS<sup>1-</sup> liposomes with SPBs followed by lateral lipid segregation and formation of the PS<sup>1-</sup> domains in the liposomal membrane facing the SPB surface as shown in Fig. 14A. It is reasonable to expect similar structural rearrangements in the membrane of the EL/PS<sup>1-</sup>/MOCH fliposomes at pH 7, namely clustering of PS<sup>1-</sup> molecules in areas of immediate contact with the brush surface while electroneutral EL and MOCH molecules are exposed to the external water. When pH is decreased down to 5, MOCH amino groups are protonated and generate a positive charge in the outer portion of adsorbed liposomes. However, liposomes remain held on the SPB surface due to strong pre-existing electrostatic interaction between the PS<sup>1-</sup> clusters and grafted polycation chains.

By means of the described centrifugation-and-fluorescence procedure, each polycationic brush was found to adsorb nearly 40  $\nu = 0.1$  EL/PS<sup>1-</sup>/MOCH fliposomes at pH 7. The same capacity was found for the brushes towards the  $\nu = 0.1$  EL/PS<sup>1-</sup> binary liposomes (see Fig. 4). This result, in agreement with the EPM-based conclusion about no effect of MOCH on the fliposome complexation at pH 7, provides a



**Fig. 18.** Hydrodynamic diameter of SPB particles vs. anionic liposome concentration. EL/PS<sup>1-</sup>/MOCH (6/1/3) liposomes, pH 7 (1) and 5 (2); EL/PS<sup>1-</sup>/MOCH (6/1/3) liposomes were complexed with SPBs at pH 7 and the resulting complexes were transferred to a pH 5 solution (3).  $[SPB^+] = 1 \times 10^{-4}$  M. Tris buffer pH 7, 25 °C. Reproduced from [38] with permission from The Royal Society of Chemistry.

simple way for electrostatic concentration of liposome molecules within a rather small volume.

In order to test whether EL/PS<sup>1-</sup>/MOCH liposomes release their contents in response to acidification of the solution, conductometry was again applied. In a control experiment, when the NaCl-loaded liposomes were added to a SPB suspension at pH 7, the conductivity did not change within 2 h after component mixing. In other words, the integrity of liposomes was retained after their binding to the brushes. Acidification of the external solution down to pH 5 led to a rapid increase in the conductivity, e.g. up to 50% for the first 15 s. For the initial liposomes in the absence of the brushes a much slower NaCl leakage was observed, e.g. only 12.5% for the first 10 min after acidification. We see, therefore, that the liposomes bound to the polycationic brushes release their contents when the pH decreases from 7 to 5. Moreover, the rate of the release from the multi-liposomal complexes is much faster than from the initial (non-complexed) liposomes.

## 6. Cytotoxicity of the brush-liposome complexes

Finally, the cytotoxicity of SPB/liposome complexes towards breast adenocarcinoma cells (MCF7) was estimated, using a methyl-tetrazolium blue assay. SPBs were complexed with the  $\nu = 0.1$  EL/PS<sup>1-</sup> liposomes so that two types of the complexes were prepared: unsaturated with 15 liposomes per one SPB and EPM = 2.75 ( $\mu\text{m/s}$ ) / (V/cm) and saturated with 40 liposomes per one SPB and EPM = -0.56 ( $\mu\text{m/s}$ ) / (V/cm). According to the standard procedures [42], a series of the anionic SPB/liposome complex suspensions with different complex concentrations was prepared. Each sample was incubated with cells for 1 h, washed from complex, and incubated in a complex-free medium for 1 day after which a dye solution was added. The dye penetrated into living cells was converted into formazan via red-ox enzymes and precipitated as dark-blue crystals, whereas no transformation of the dye occurred in dead cells. The sample was dissolved in dimethylsulfoxide, and the optical density was measured at 550 nm and compared with a calibration curve in the absence of complex. The procedure was then repeated with the cationic SPB/liposome complex suspension and two control samples: a suspension of EL/PS<sup>1-</sup> liposomes and a polycationic brush suspension. All runs were carried out in quadruplicate. The cytotoxicity was defined as the additive concentration at which 50% viability of cells was detected (EC50). A lower EC50 value corresponded to a higher cytotoxicity.

Fig. 19 shows the fraction of retaining living cells in the presence of liposomes, polycationic brushes, and brush-liposome complexes. The EL/PS<sup>1-</sup> liposomes showed low cytotoxicity (EC50 = 5 mg/mL) (curve 1), in accordance with the data of other groups [42,43]. For the brushes (curve 2), a much higher cytotoxicity was observed (EC50 =

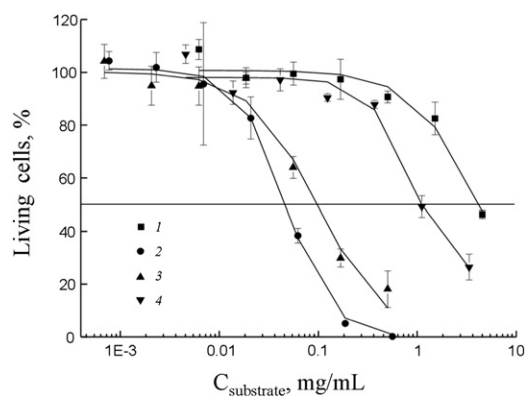


Fig. 19. Fraction of retaining living cells vs. concentration of EL/PS<sup>1-</sup> liposomes (1), SPBs (2), unsaturated cationic SPB/liposome complex (3), and saturated anionic SPB/liposome complex (4).

0.06 mg/mL), apparently resulting from their ability to adsorb on the negative cell surface and affect the cell functioning. The cytotoxicity of brush-liposome complexes was dependent on their surface charge. The unsaturated cationic complex (curve 3) showed the cytotoxicity comparable with that for the brushes (EC50 = 0.1 mg/mL), while the saturated anionic complex (curve 4) was closer to the free unbound liposomes (EC50 = 1.5 mg/mL). The toxic effect of the unsaturated cationic complex obviously resulted from its total positive charge which ensured the complex binding to cells and causing their death, similar to the toxic effect of original polycationic brushes. A lower cytotoxicity of the anionic saturated complexes in comparison with the original brushes was probably due to total covering of toxic cationic grafted chains by adsorbed liposomes.

## 7. Conclusions

Small unilamellar liposomes, 40–60 nm in diameter, composed of anionic and zwitter-ionic lipids electrostatically complex with polystyrene microspheres, ca. 100 nm in diameter, grafted by polycationic chains (“spherical polycationic brushes”, SPBs). The complexation induces flip-flop of anionic lipids from the inner to the outer leaflet of the liposomal membrane and lateral lipid segregation in the outer membrane leaflet. The structural reorganization of the membrane together with the geometry of anionic lipid molecules determines the maximum molar fraction of anionic lipid in the liposomal membrane that retains the integrity of complexed liposomes: 0.3 for liposomes with anionic conically-shaped cardiolipin and 0.5 for liposomes with anionic cylindrically-shaped phosphatidylserine. The number of intact liposomes per SPB particle varies from 40 ( $\nu = 0.1$ ) to 14 ( $\nu = 0.3$ ). By using a mixture of liposomes with variety of encapsulated substances, multi-liposomal complexes can be prepared with a high loading capacity and a controlled ratio of the contents. In order to make the mixed anionic liposomes pH-sensitive, they were additionally modified by 30 mol% of a MOCH lipid that undergoes a conformational flip when changing pH. Being complexed with SPBs, such liposomes rapidly release their contents when the pH is reduced from 7.0 to 5.0 owing to the MOCH conformational change that creates defects in the bilayer membrane. Remarkably, the liposomes complexed with SPB release their cargo much faster than the identical but uncomplexed liposomes. The results allow the creation of liposomes in high concentration within a rather small volume and, thereby, the preparation of multi-liposomal containers of promise in the drug delivery field.

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