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Complexes between Anionic Liposomes and Spherical Polycationic Brushes. An Assembly of Assemblies

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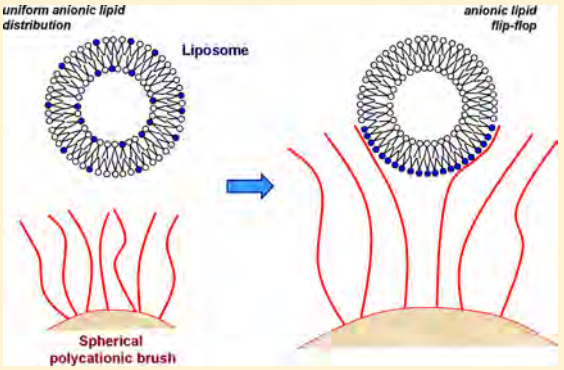
ABSTRACT: This paper has at its objective the assembling of liposomal assemblies onto nanoparticles. In this manner, one generates nanoparticles with a high loading capacity. Thus, spherical spherical polycationic "brushes" (SPBs) were synthesized by graft polymerizing a cationic monomer, (trimethylammonium)ethylmethacrylate chloride, onto the surface of monodisperse polystyrene particles, ca. 100 nm in diameter. These particles were complexed with small unilamellar anionic liposomes, 40−60 nm in diameter, composed of egg lecithin (EL) and anionic phosphatidylserine (PS^{1-}) in PS^{1-}/EL ratios from 0.10 to 0.54, a key parameter designated as ν. These complexes were then characterized according to electrophoretic mobility, dynamic light scattering, conductivity, fluorescence, and cryogenic transmission electron microscopy, with the following main conclusions: (a) All added liposomes are totally associated with SPBs up to a certain saturation concentration (specific for each ν value). (b) The number of liposomes per SPB particle varies from 40 (ν = 0.1) to 14 (ν = 0.5). (c) At sufficiently high liposome concentrations, the SPBs experience an overall change from positive to negative charge. (d) SPB complexes tend to aggregate when their initial positive charge has been precisely neutralized by the anionic liposomes. Aggregation is impeded by either positive charge at lower lipid concentrations, or negative charge at higher lipid concentrations. (e) The liposomes remain intact (i.e., do not leak) when associated with SPBs, at $\nu \leq 0.5$. (f) Complete SPB/liposome dissociation occurs at external [NaCl] = 0.3 M for ν = 0.1 and at 0.6 M for ν = 0.5. Liposomes with ν = 0.54 do not dissociate from the SPBs even in NaCl solutions up to 1.0 M. (g) Complexation of the PS^{1-}/EL liposomes to the SPBs induces flip-flop of PS^{1-} from the inner leaflet to the outer leaflet. (h) The differences in the ability of PS^{1-} (a cylindrical lipid) and CL^{2-} (a conical lipid) to create membranes defects are attributed to geometric factors.



INTRODUCTION

Spherical bilayer lipid vesicles (liposomes) are widely used for controllable encapsulation and release of drugs: hydrophilic compounds can be dissolved in the inner water cavity, while hydrophobic guests are incorporated into the lipid bilayer.[1−3] It has been shown that modifying the liposomal membranes with polymers imparts mechanical stability to the liposomes,[4] protects them against aggregation,[5] and enhances their circulation time.[1,6] Certain problems, for example, a relatively small capacity of conventional liposomes toward drugs,[7] are still awaiting solution.

Recently, we described electrostatic adsorption of anionic liposomes on the surface of colloidal particles onto which polycationic chains have been grafted ("spherical polycationic brushes", SPB).[8] This allows the assembly of multiple liposomes, each containing entrapped compounds, within a rather small volume. By altering the particular anionic lipid comprising the liposomal membrane, one can control the integrity of adsorbed liposomes and their adsorption/desorption properties. Liposomes, composed of doubly anionic diphosphatidylglycerol (cardiolipin, CL^{2-}) and zwitterionic egg yolk lecithin (EL), were found to form a stable layer and remain intact on SPB surfaces, provided the molar fraction of anionic CL^{2-} groups in the lipid mixture (ν) lies in the 0.2−0.3 range.[8] At lower ν, the liposomes desorb from the SPB surface in aqueous salt solutions, whereas at higher ν loss of encapsulated compound is observed.

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We have shown earlier that, along with other parameters, the integrity of anionic liposomes complexed with linear cationic polymers depends upon the geometry of the anionic lipid molecules.[9] Liposomes made with phosphatidylserine (PS^{1-}), a cylindrical anionic lipid with roughly equal cross sections for its polar group and alkyl chains, are more stable after complexation with polycations in comparison with liposomes containing CL^{2-}, a cone-shaped anionic lipid. This allows a wider range of PS^{1-} content under which the liposomes remain intact when bound to polycations. In the present Article, we quantify the complexation of SPB and mixed PS^{1-}/EL liposomes, assess the integrity of these SPB-bound liposomes, examine the stability of the complexes to dissociation in aqueous salt solutions, and visualize the complexation by cryogenic transmission electron microscopy (cryo-TEM).

EXPERIMENTAL SECTION

In order to prepare SPBs, cationic poly-(trimethylaminoethylmethacrylate) ammonium chloride macromolecules were grafted on the surface of 100 nm monodispersed polystyrene latex particles as described earlier.[10] The particles were analyzed by cleaving off the polycation chains by a strong base.[11] The molecular weight of the chains cleaved from the surface of the particles has been determined by viscosimetry. Dynamic light scattering showed a mean hydrodynamic diameter of the brush equal to 230 nm with a thickness of a cationic corona of (230 − 100)/2 = 65 nm. Zwitterionic phosphatidylcholine (egg lecithin, EL) (I), anionic phosphatidylserine (PS^{1-}) (II), *N*-fluoresceiniso-thiocyanyldipalmitoyl-phosphatidylethanolamine (FITC-DPPE) (III), and zwitterionic dipalmitoylphosphatidylcholine (IV) from Avanti were used as received (see structures in Figure 1).

Small unilamellar anionic liposomes, 40−60 nm in diameter, were prepared by the standard sonication procedure[12] after evaporating under vacuum a mixed EL/PS^{1-} chloroform solution. The resulting thin lipid film was dispersed in a TRIS buffer (pH 7, 10^{-2} M) for 400 s with a 4700 Cole-Parmer ultrasonic homogenizer. Liposome samples were separated from titanium dust by centrifugation for 5 min at 10 000 rpm, and used within 1 day. Liposomes with a molar fraction of anionic PS^{1-} head groups $\nu = [PS^{1-}]/([PS^{1-}] + [EL])$ from 0.10 up to 0.54 were thus obtained. For preparation of fluorescent-labeled liposomes, the sonication procedure was applied again but 0.1 wt % FITC-DPPE was added to the lipid mixture solution before chloroform evaporation.

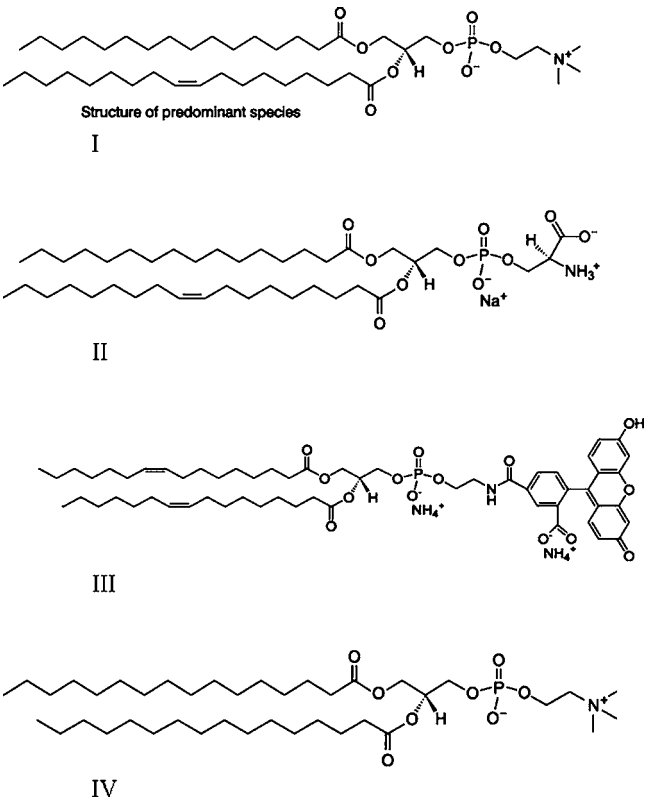

Figure 1. Lipids (schematical presentation).

The fluorescence intensity of FITC-labeled liposome suspensions was detected with a F-4000 Hitachi fluorescence (λ_{em} = 525 nm, λ_{ex} = 495 nm); their UV−vis spectra were recorded with a UV-mini 1240 Hitachi spectrophotometer.

The mean hydrodynamic diameters of SPBs, liposomes, and SPB/liposome complexes were determined by dynamic light scattering in a thermostatic cell with a Brookhaven Zeta Plus instrument. Electrophoretic mobility (EPM) of SPBs, liposomes, and complex particles was measured by laser microelectrophoresis in a thermostatic cell using a Brookhaven Zeta Plus instrument.

By measuring the conductivity of NaCl-loaded liposome suspensions with a CDM83 Radiometer conductometer,[8] permeability of the liposomal membranes toward a simple salt was assessed.

The SPB/liposome complexes were visualized by cryogenic transmission electron microscopy (cryo-TEM). A Philips CM120 or an FEI T12 transmission electron microscope was used as equipped with a Gatan 791 MultiScan cooled-CCD digitally recording camera (CM120) or Gatan US1000 high-resolution cooled-CCD digitally recording camera (T12). See ref 13 for details.

Double-distilled water was used for making solutions after additionally treating it with a Milli-Q Millipore system composed of ion-exchange and adsorption columns as well as a filter to remove large particles. Experiments were done at 20 °C when the membranes of EL/PS^{1-} liposomes were in the fluid (liquid-crystalline) state.[14]

RESULTS AND DISCUSSION

In this paper, we describe the interaction between cationic SPBs and anionic liposomes composed of zwitterionic egg lecithin (EL) and anionic phosphatidylserine (PS^{1-}) shown in Figure 1. The molar fraction of PS^{1-} in the liposomal membrane (a key parameter in this work designated as ν), varied from 0.10 up to 0.54, with an increase in the PS^{1-} content resulting in an enhanced negative charge on the liposomes. SPB-to-liposome complexation was always accompanied by neutralization of the SPB surface charge, as seen from the electrophoretic mobility (EPM) of liposome-bound SPBs (Figure 2). An overall change

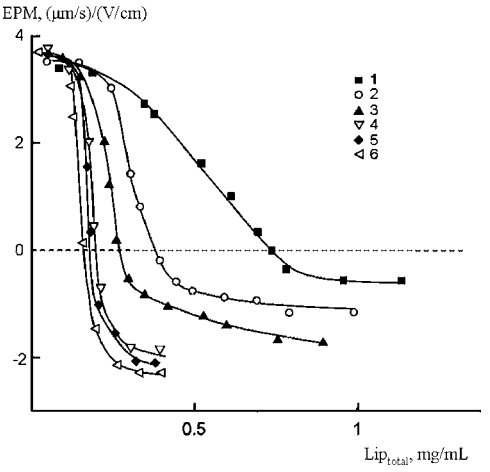

Figure 2. Dependence of SPB-EL/PS^{1-} complex EPM on liposome concentration. ν = 0.1 (1), 0.2 (2), 0.3 (3), 0.4 (4), 0.5 (5), 0.54 (6); SPB concentration 1×10^{-4} M; 10^{-2} M TRIS buffer, pH 7; 20 °C.

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from positive to negative charge occurs at sufficiently high liposome concentrations. Elevation of the PS^{1-} content progressively decreases the neutralizing gram-per-liter liposome concentration and, at the same time, generates a higher negative charge on the SPBs at excess PS^{1-}. For example, the ν = 0.10 liposomes neutralize the SPB charge at 0.74 mg/mL lipid, and produce a saturated layer on the SPB surface with EPM = −0.59 (μm/s)/(V/cm), whereas the ν = 0.54 liposomes neutralize the SPB charge at 0.18 mg/mL lipid, and produce a saturated layer with EPM = −2.27 (μm/s)/(V/cm).

The total surface charge of colloidal particles is an important factor that determines the stability of hydrophilic colloids against aggregation.[15] As expected, the size of SPB/liposome complex particles, determined by dynamic light scattering (Figure 3), shows the largest aggregates at EPM = 0 (cf. Figures 2 and 3); any charge, either positive or negative, on the individual SPB/liposome complexes inhibits the aggregation.

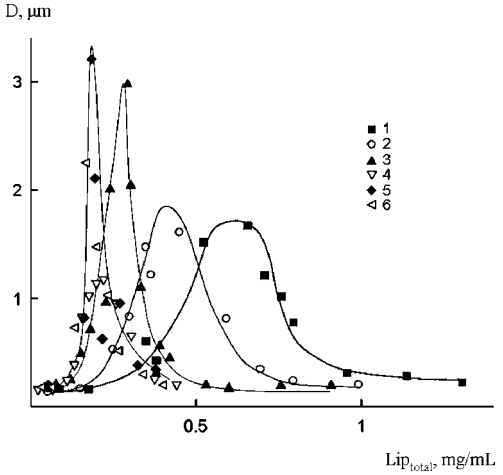

Figure 3. Dependence of SPB-EL/PS^{1-} complex size on liposome concentration. ν = 0.1 (1), 0.2 (2), 0.3 (3), 0.4 (4), 0.5 (5), 0.54 (6); SPB concentration 1 × 10^{-4} M; 10^{-2} M TRIS buffer, pH 7; 20 °C.

In order to estimate the number of liposomes bound to SPBs, the following procedure was used. A mixture of SPB suspension and a fluorescent-labeled liposome suspension was incubated for 5 min, and the resulting SPB/liposome complex was separated by centrifugation. The fluorescence intensity in the supernatant provided the concentration of unbound liposomes after using the corresponding calibration curve. A dependence of unbound liposome vs total liposome concentration (Figure 4) shows a complete binding of liposomes to SPBs up to a certain saturation concentration specific for each ν value and appearance of free (unbound) liposomes at higher concentrations.

The maximum number of liposomes capable of binding to a single SPB particle (N) was calculated from data of Figure 4[8] as:

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

where C_{lip} is the lipid concentration at saturation, C_{brush} is the SPB concentration, D is the diameter of the polystyrene core, 100 nm, and ρ is its density,[16] d is the mean liposome diameter (50 nm), S_1 is the mean surface area per one lipid molecule (0.7 nm^2),[14] M is the mean molecular weight of the lipid,[13] and N_a is Avogadro's number. The calculations (Figure 5) show a progressive decrease in N with an increase in ν: from N = 40

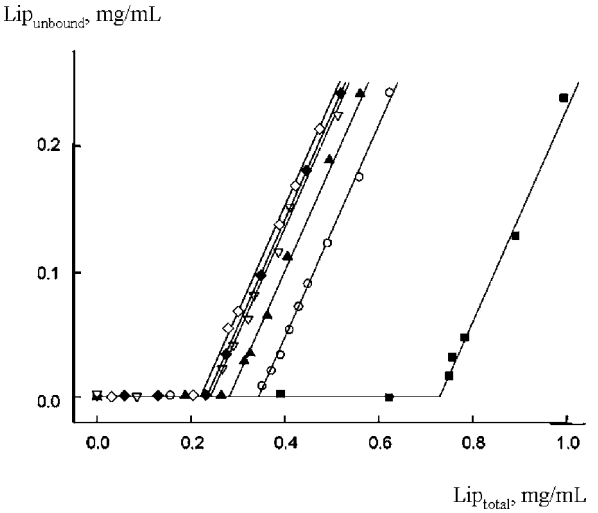

Figure 4. Dependence of concentration of liposomes unbound to SPB on the total liposome concentration. ν = 0.1 (1), 0.2 (2), 0.3 (3), 0.4 (4), 0.5 (5), 0.54 (6); SPB concentration 1 × 10^{-4} M; 10^{-2} M TRIS buffer, pH 7; 20 °C.

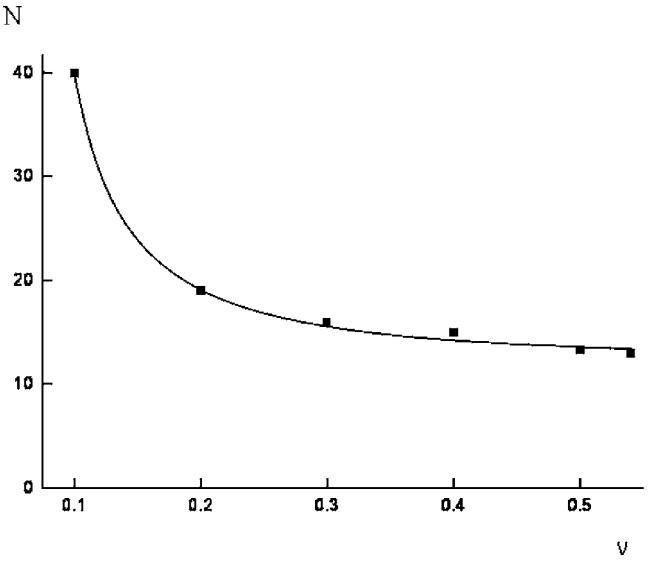

Figure 5. Dependence of the number of adsorbed liposomes per one SPB upon ν. 10^{-2} M TRIS buffer, pH 7; 20 °C.

for ν = 0.10 down to N = 13 for ν = 0.54. In other words, the number of liposomes per particle decreases 3-fold when the molar fraction of PS^{1-} increases from 0.10 to 0.54.

The integrity of liposomes after complexation is a question of particular interest. Do defects form in the liposomal membranes after the complexation, and, if so, at what PS^{1-} content does this occur? To address this question, the EL/PS^{1-} liposomes loaded with a 1 M NaCl solution were prepared. The release of NaCl from liposomes into surrounding solution was detected conductometrically and compared with a 100% conductivity of a suspension of NaCl-loaded liposomes completely destroyed when an excess of Triton X-100 surfactant was added. As follows from the data of Figure 6, addition of liposomes with $\nu \leq 0.5$ to the SPBs does not change the conductivity (curves 1−5), but the conductivity from liposomes with ν=0.54 rises sharply after being added to the SPB suspension (curve 6). Thus, complexation with SPBs has no effect on the integrity of EL/PS^{1-} liposomes with $\nu \leq 0.5$,

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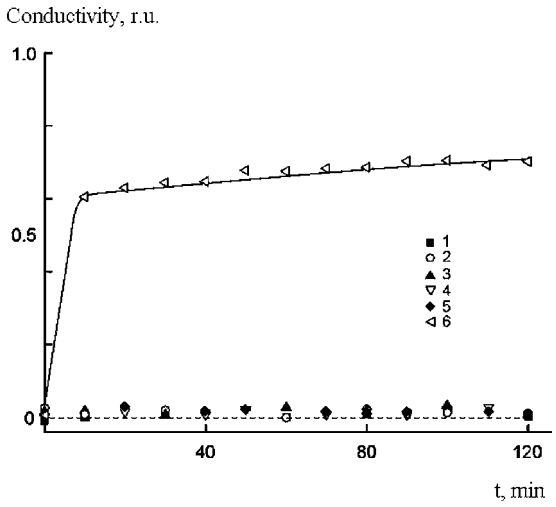

Figure 6. Time-dependence of relative conductivity of SPB–EL/PS^{1-} complex. ν = 0.1 (1), 0.2 (2), 0.3 (3), 0.4 (4), 0.5 (5), 0.54 (6); SPB concentration 1 × 10^{-4} M; 10^{-2} M TRIS buffer, pH 7; 20 °C.

but causes defect formation in the liposomal membrane with ν = 0.54.

The stability of SPB/liposome complexes in aqueous salt media was examined using a fluorescence method. Since cationic polymers are effective fluorescence quenchers,[17] complexation of cationic SPB with EL/PS^{1-} liposomes (and subsequent dissociation of SPB/liposome complexes) was accompanied by decrease (and recovery) of the fluorescence intensity of a FITC-labeled lipid embedded into the liposomal membrane. Complexation of SPBs with the FITC-labeled liposomes with any PS^{1-} content resulted in quenching of the FITC fluorescence (Figure 7). Addition of a NaCl solution

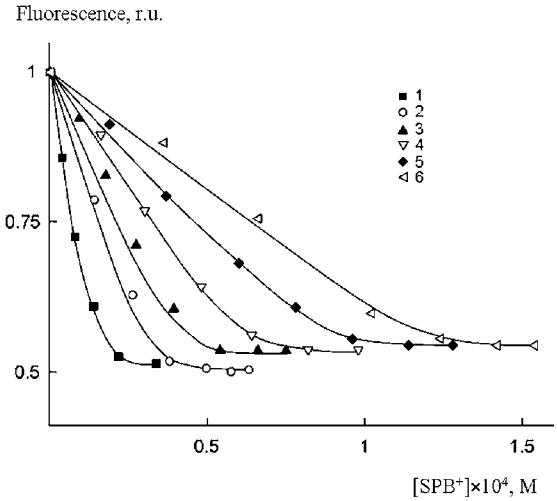

Figure 7. Dependence of relative fluorescence intensity of SPB–EL/PS^{1-} complex on SPB concentration. ν = 0.1 (1), 0.2 (2), 0.3 (3), 0.4 (4), 0.5 (5), 0.54 (6); liposome concentration 1 mg/mL; 10^{-2} M TRIS buffer, pH 7; 20 °C.

caused the resulting complex to dissociate down to individual components (i.e., SPBs and liposomes), unless the PS^{1-} content in the liposomal PS^{1-} membrane exceeded 0.5 (Figure 8). Complete dissociation depended on the PS^{1-} content and equaled [NaCl] = 0.3 M for complexes with ν = 0.1, and [NaCl] = 0.6 M for complexes with ν = 0.5. In contrast,

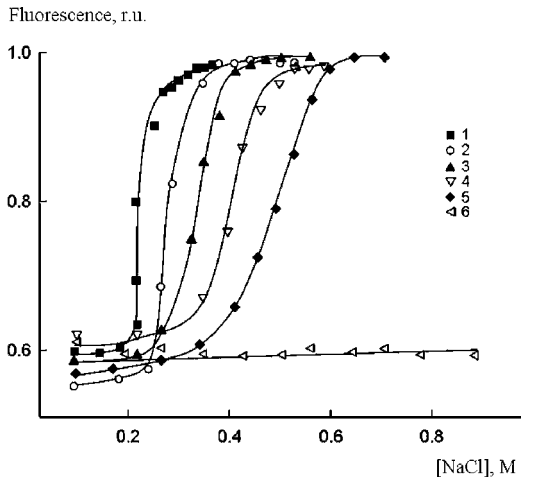

Figure 8. Dependence of relative fluorescence intensity of labeled EL/PS^{1-} contacting SPB on NaCl concentration. ν = 0.1 (1), 0.2 (2), 0.3 (3), 0.4 (4), 0.5 (5), 0.54 (6); 10^{-2} M TRIS buffer, pH 7; 20 °C.

complexes of liposomes with ν = 0.54 did not dissociate up to 1 M NaCl concentration. Thus, anionic EL/PS^{1-} liposomes with $\nu \geq 0.5$ were irreversibly bound to the polycationic brushes. We conjecture that the complex stability might in part result from incorporation of polycation segments into the defect areas of the liposomal membrane as displayed in the conductometric experiments.

EL/PS^{1-} liposomes display a different behavior from that described earlier for EL/CL^{2-} liposomes complexed with SPBs. The former retain their integrity, unless the content of PS^{1-} exceeds 0.5, while the latter retain their integrity unless the content of CL^{2-} exceeds only 0.3. The use of PS^{1-} instead of CL^{2-} allowed us to extend the range of anionic lipid content, thereby ensuring both the quantitative binding of anionic liposomes to SPBs and the integrity of SPB-bound liposomes.

The membrane of EL/PS^{1-} liposomes is in the liquid-crystalline state so that lipid molecules are able to move along each membrane leaflet and to pass from one leaflet to the other. This leads to uniform (50/50) distribution of the anionic PS^{1-} lipids between both membrane leaflets.[18] It has been shown earlier that binding of "liquid" liposomes to a flexible linear polycation induces lateral segregation and transmembrane migration (flip-flop) of anionic lipids. The cationic polymer, located on the external liposome surface, causes anionic lipids from both membrane leaflets to concentrate on the outer leaflet[19] and to cluster there due to multiple electrostatic polymer unit/lipid contacts. A similar structural rearrangement could be reasonably assumed within the membrane of liquid EL/PS^{1-} liposomes upon their binding with flexible poly(2-methylpropenoyloxyethyl) trimethylammonium chloride chains grafted to polystyrene particles.

Figure 2 was once again used in an attempt to test this assumption. Curve 1 in the figure refers to the electrophoretic titration of SPB particles by a suspension of ν = 0.10 liposomes. Now we have shown that the entire population of added ν = 0.10 liposomes are complexed with SPBs, and that they retain their integrity after complexation. This means that at the point where EPM=0 the total positive charge of the SPBs is numerically equal to the total negative charge located on the outer leaflets of the complexed liposomes, as expressed in eq 2:

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

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The fraction of PS^{1-} molecules involved in electrostatic complexation with SPBs (γ) was estimated from eq 3, where $[PS^{1-}]_{t,EPM=0}$ is the total concentration of PS^{1-} in solution at EPM = 0:

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

The calculation based on eq 3 gives $\gamma = 0.94 \times 10^{-4}$ M/1.01×10^{-4} M ≈ 1. This result can be interpreted only in terms of migration of all PS^{1-} molecules originally in the inner leaflet to the outer leaflet as a result of association with the SPB particles. For $\nu = 0.10$ EL/PS^{1-} membrane of complexed liposomes, we can write eqs 4 and 5, respectively, where $[Lip]_{EPM=0}$ is a concentration of all membrane lipids (EL + PS^{1-}) in solution at EPM = 0:

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

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

If the SPB-induced flip-flop of PS^{1-} molecules occurs in liposomes with different PS^{1-} contents, we should expect the validity of eq 5 for all studied systems. The data of Figure 2, replotted according to eq 5, shows linearity in the entire $1/\nu$ interval (Figure 9) that constitutes evidence for SPB-induced

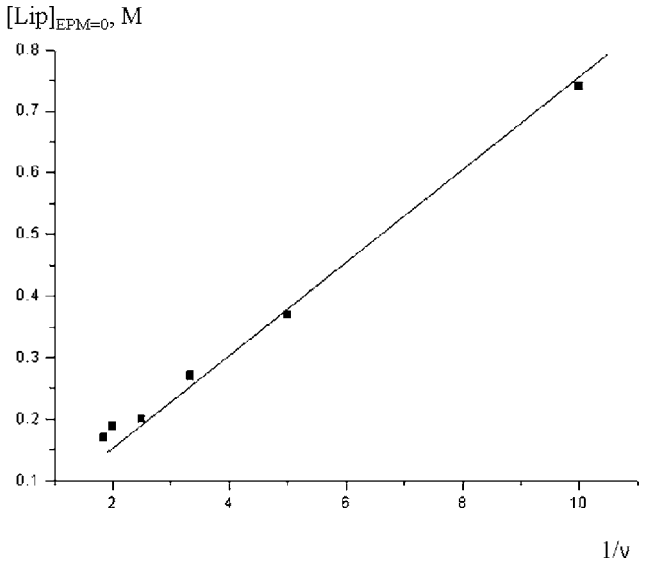

Figure 9. Dependence of liposomes concentrations in the electroneutral complexes with SPBs upon $1/\nu$. SPB concentration 1×10^{-4} M; 10^{-2} M TRIS buffer; pH 7; 20 °C.

flip-flop in all complexed EL/PS^{1-} liposomes whatever their PS^{1-} content. Earlier we demonstrated an ability of flexible linear polycations to extract anionic lipids from the inner to outer leaflet of the liquid liposomal membrane.[19] We now see that a flexible polycation grafted to a polymeric particle also leads to flip-flop of anionic lipids in the membrane of liquid liposomes.

At low content of PS^{1-}, the SPB-induced lipid flip-flop does not have an effect on the liposomal integrity since migration PS^{1-} from the inner to the outer membrane leaflets can be compensated by migration of an equal number of zwitterionic EL molecules in the opposite direction (from the outer to the inner leaflets), thus sustaining the bilayer integrity. The maximum PS^{1-} molar fraction ν that ensures the integrity of adsorbed liposomes is equal 0.5. At higher ν (0.54 in our experiments) the SPB-induced PS^{1-} migration can no longer be compensated by EL back-migration, leading to formation of defects in the lipid bilayer as was detected by the conductivity experiments.

Cryo-TEM technique allowed visualization of the SPB/liposome complexes. In these experiments, 50−200 nm EL/PS^{1-} liposomes were used to facilitate their experimental observation. Typical cryo-TEM micrigraphs for SPBs bound to liposomes with different PS^{1-} contents are given in Figure 10.

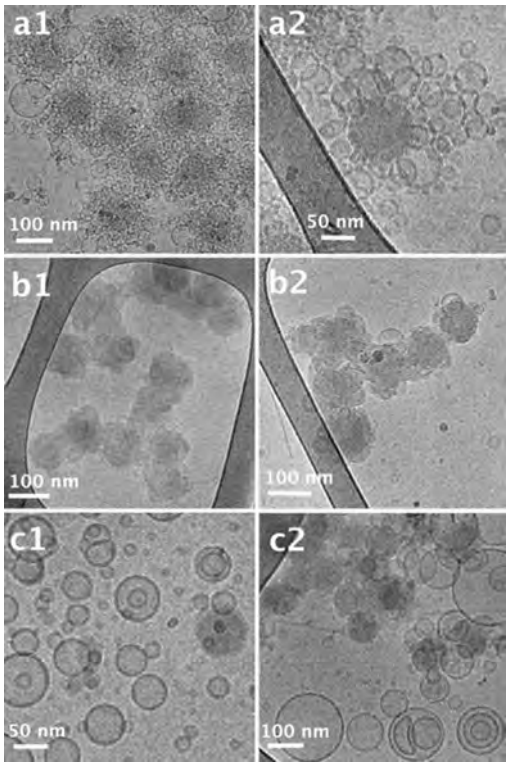

Figure 10. Cryogenic transmission electron microscopy images of mixed SPB+EL/PS^{1-} liposome suspensions. $[SPB^{+}] = 2 \times 10^{-4}$ M; liposome concentration 2 mg/mL for $\nu = 0.1$ (a), 1 mg/mL for $\nu = 0.3$ (b), and 0.75 mg/mL for $\nu = 0.5$ (c).

All micrographs display black SPB disks surrounded by black undisrupted liposome circles. Thus, the cryo-TEM data corroborate the integrity of liposomes with $\nu \leq 0.5$ after their binding to SPBs as shown above in the fluorescence and conductivity experiments and (b) reflect a progressive decrease in the number of complexed liposomes with elevation of the PS^{1-} content in the liposomal membrane, as was demonstrated in the fluorometric adsorption experiments.

Having characterized the complexes of PS^{1-}/EL liposomes with the cationic brush polymers, we can now compare these complexes with CL^{2-}/EL liposomes studied previously.[8] The comparison is interesting, because whereas PS^{1-} is cylindrically shaped, CL^{2-} (cardiolipin) is conically shaped,[20] and this shape difference has a big impact on properties.[9] Most prominently, PS^{1-}/EL liposomes complexed with cationic brushes can tolerate a 50% mol fraction of PS^{1-} ($\nu = 0.5$) before leaking. In contrast, liposomes with CL^{2-} as the ionic component remain

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intact upon complexation only when mol fraction of CL^{2-} is below 0.3. In other words, the noncomplementary nature of the cone-shaped CL^{2-} and the cylindrical EL does not perturb the polymer-complexed liposome integrity as long as the CL^{2-} content is less than $\nu = 0.3$. Above that value, complexation electrostatically attracts a CL^{2-} content in the outer membrane that is too high to prevent defects. The geometric fit between PS^{1-} and EL, both of them cylindrical, allows a much higher anionic component of the bilayer before leakage sets in.

We see therefore a number of common traits in behavior of liquid liposome complexes with a linear polycation and the polycationic brush. Both are formed when mixing aqueous solutions of the components and retain stable (do not dissociate) in a 0.15 M NaCl solution.[8,9] In both, flip-flop of anionic lipids occurs that leads to formation of defects in the lipid bilayer with a high content of anionic lipid. However, the complexes show morphologically different structures: several linear polycations are able to adsorb on the surface of a single liposome,[21] while each polycationic brush binds dozens of anionic liposomes. The latter allows concentration of liposomes within a rather small volume thus preparing multiliposomal containers promising in the drug delivery field.

CONCLUSIONS

Spherical polycationic "brushes" (SPBs) were synthesized by graft polymerizing a cationic monomer, (trimethylammonium)-ethylmethacrylate chloride, onto the surface of monodisperse polystyrene particles, ca. 100 nm in diameter. The resulting brushes were 230 nm in diameter, having a cationic polymer layer 65 nm thick. To these particles were complexed small unilamellar anionic liposomes, 40 - 60 nm in diameter, composed of egg lecithin (EL) and anionic phosphatidylserine (PS^{1-}) in PS^{1-}/EL ratios varying from 0.10 to 0.54, in 0.1 increments (a key parameter designated as ν. These complexes were then characterized according to their electrophoretic mobility, dynamic light scattering, conductivity, fluorescence, and electron microscopy with the following main conclusions: (a) All added liposomes are totally associated with SPBs up to a certain saturation concentration (specific for each ν value) above which they remain in the supernatant. (b) The number of liposomes per SPB particle varies from 40 ($\nu = 0.1$) to 14 ($\nu = 0.5$) in a TRIS buffer, pH = 7.0, 20 °C (the conditions of most of the experiments). (c) At sufficiently high liposome concentrations the SPBs experience an overall change from positive to negative charge. The higher the ν value the greater the negative charge on the particles at excess PS^{1-}. (d) SPB complexes tend to aggregate when their initial positive charge has been precisely neutralized by the anionic liposomes. Aggregation is impeded by either positive charge at lower lipid concentrations or negative charge at higher lipid concentrations. (e) Liposomes remain intact (i.e., do not leak) when associated with SPBs as long as ν is 0.5 or less. When the PS^{1-} content of the liposomes exceeds 50% (e.g., $\nu = 0.54$), the liposomes become leaky to encapsulated salt. (f) Complete SPB/liposome dissociation occurs at external [NaCl] = 0.3 M for $\nu = 0.1$ and at 0.6 M for $\nu = 0.5$. Liposomes with $\nu = 0.54$ do not dissociate from the SPBs even in NaCl solutions up to 1.0 M. (g) Complexation of the PS^{1-}/EL liposomes to the SPBs induces flip-flop of PS^{1-} from the inner leaflet to the outer leaflet whatever the PS^{1-} content. (h) Differences between PS^{1-} (a cylindrical lipid) and CL^{2-} (a conical lipid) in their ability to create membranes defects are attributed to geometric factors.[22]

AUTHOR INFORMATION

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. All authors contributed equally.

Notes

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