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# Lipid Segregation in Membranes of Anionic Liposomes Adsorbed onto Polycationic Brushes

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Spherical lipid-bilayer vesicles (liposomes) are a powerful tool in the study of membranes derived from both synthetic and cellular sources.<sup>[1,2]</sup> The response of liposomes to the binding of polymers, natural or otherwise, allows structural and functional manipulation of membrane properties. It has been found, for example, that adsorption of cationic polymers onto liposomes containing anionic lipids, admixed with an excess of electroneutral lipid, induces migration of the anionic lipids from the inner to the outer bilayer leaflet (“flip-flop”) followed by anionic lipid segregation.<sup>[3]</sup> The ultimate result is the formation of clusters (“domains”) of anionic lipids within the outer membrane leaflet, due to electrostatic contacts between the anionic head-groups and the adsorbed polycation units.<sup>[4]</sup> Overall positive or negative charge on the interfacial clusters causes them to move apart and minimize cluster/cluster repulsion.<sup>[5,6]</sup> In other words, clusters consist of anionic lipid islands with a polycationic coating embedded in a sea of electroneutral host lipids. Complete liposomal segregation into two microphases, namely polycation-bound anionic lipids and free electroneutral lipids, is observed even for liposomes with containing a large amount of anionic lipid.<sup>[7,8]</sup>

In the present Communication, we describe the structural rearrangements in membranes of anionic liposomes upon their electrostatic binding to polycationic chains covalently attached (grafted) onto polystyrene particles to create “spherical polycationic brushes” or SPBs. We show that liposome-to-brush complexation also induces lipid segregation in the liposomal membrane. However, the efficiency of the lipid segregation depends upon the amount of anionic lipid in the electroneutral lipid host. The greater the initial anionic lipid content in the liposomal membrane, the more the electroneutral lipid is involved in mixed anionic lipid/neutral lipid clusters. This allows a protective negative charge on the outer border of brush/liposome complexes, thus making them stable against aggregation and compatible with other negative components of biological surroundings. Structural information is further amplified by studying the integrity and morphology of liposome/SPB complexes as a function of liposome charge.

Polystyrene particles with covalently attached polycation chains, the SPBs, were synthesized by graft polymerization of a cationic monomer, (2-methylpropenyloxyethyl)trimethylammonium chloride, on the surface of monodisperse polystyrene (PS) latex particles about 100 nm in diameter (details of the SPB synthesis are in the Supporting Information, Figure S1). Dynamic light scattering revealed a mean hydrodynamic diameter of the brushes of 230 nm, giving a cationic corona thickness of 65 nm. The concentration of SPB is expressed in moles of cationic subunit per liter [SPB<sup>+</sup>].

In a typical experiment, a SPB suspension was mixed with a suspension of unilamellar anionic liposomes (ca. 50 nm) prepared by sonicating a lipid mixture composed of zwitterionic egg lecithin (EL) and anionic phosphatidylserine (PS<sup>-1</sup>). The molar fraction of PS<sup>-1</sup> in the PS<sup>-1</sup>/EL mixture, a key parameter designated hereafter as  $\nu$ , varied from 0.1 to 0.4 in 0.1 increments (details of liposome preparation are in the Supporting Information, Figure S2).

Liposome-to-SPB complexation was quantified as follows. EL/PS<sup>-1</sup> liposomes were prepared with 0.5 wt % of *N*-fluorescein-isothiocyanatyl dipalmitoylphosphatidylethanol amine, a fluorescent lipid incorporated into the bilayer. Then, a suspension of SPBs was mixed with a suspension of fluorescently labeled liposomes, and after 40 min, the SPB/liposome complex was removed by centrifugation. The fluorescence intensity in the supernatant provided the concentration of

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unbound liposomes with the aid of a calibration curve. The dependence of [unbound liposome] on [total liposome] as the PS<sup>-1</sup> molar fraction  $\nu$  increased from 0.1 to 0.4 is shown in Figure 1. Clearly, added liposomes are entirely complexed

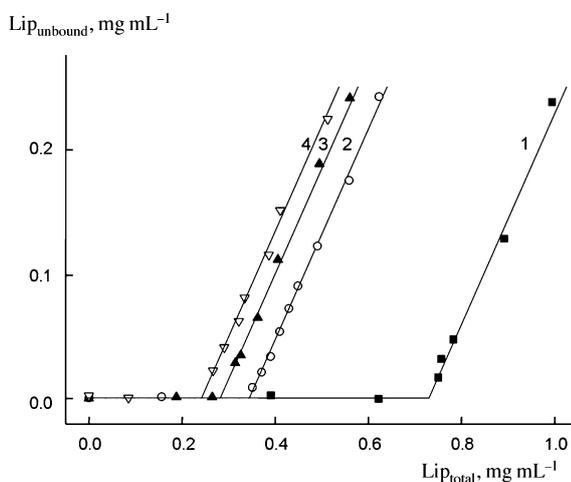


Figure 1. Concentration of PC/PS<sup>-1</sup> liposomes in the supernatant after separation of the SPB/liposome complex versus total liposome concentration.  $\nu=0.1$  (1), 0.2 (2), 0.3 (3), 0.4 (4). [SPB<sup>+</sup>]=10<sup>-4</sup>M.

to SPBs up to a certain concentration (specific for each  $\nu$  value). Above these liposome concentrations, the concentration of unbound liposomes in the supernatant rises rapidly. From the data of Figure 1, the number of liposomes ultimately binding to a single SPB particle ( $N$ ) could be calculated as a function of the molar fraction of PS<sup>-1</sup> (Figure 2; for details see the Supporting Information, Figure S4). It can be seen that there is a progressive decrease in  $N$  with an increase in  $\nu$  or, stated another way, the number of bound liposomes per SPB particle decreases with an increase in anionic charge on the liposome. For example,  $N=38$  for  $\nu=0.1$ , and  $N=15$  for  $\nu=0.4$ .

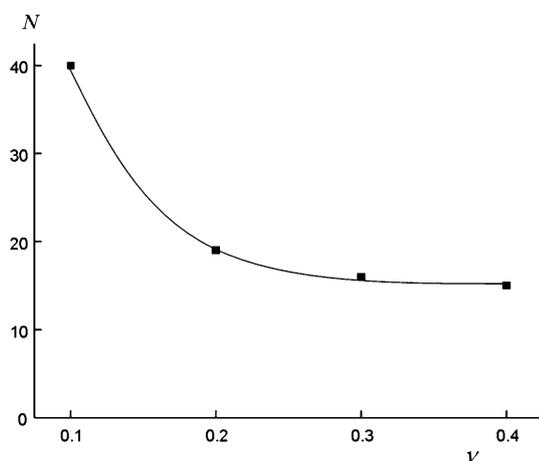


Figure 2. Maximum number of adsorbed PC/PS<sup>-1</sup> liposomes on the SPB particles versus  $\nu$  ( $\nu$  is the molar fraction of PS<sup>-1</sup> in the PC/PS<sup>-1</sup> mixture).

We have shown earlier<sup>[9]</sup> that liposomes, composed of EL and a doubly anionic phosphatidylglycerol (cardiolipin, CL<sup>-2</sup>), do not release encapsulated salt into the surrounding solution upon binding to SPBs. This proves that the EL/CL<sup>-2</sup> liposomes maintain their integrity after complexation with SPBs. A similar approach was used to monitor the integrity of SPB-bound EL/PS<sup>-1</sup> liposomes. Suspensions of EL/PS<sup>-1</sup> liposomes filled with a NaCl solution (1 M) were prepared with limiting  $\nu$  values of 0.1 and 0.4. Conductivity of the NaCl-loaded liposome suspensions was within an interval of (110±10)  $\mu\text{Sm cm}^{-1}$  and did not change during 1 h after preparation. When NaCl-loaded liposomes were mixed with a SPB suspension, the conductivity of (120±10)  $\mu\text{Sm cm}^{-1}$  was detected and remained unchanged for 1 h. Complete rupturing of the liposomes, when they were treated with a membrane-destructive Triton X-100 solution in control experiment, led to a conductivity of (220±10)  $\mu\text{Sm}$ . Thus, the anionic EL/PS<sup>-1</sup> liposomes with  $\nu$  from 0.1 up to 0.4 maintain their integrity (i.e., do not leak) when bound to SPB particles.

Complexation of anionic liposomes to cationic SPBs was accompanied by neutralization of the SPB surface charge, as detected by altered electrophoretic mobilities (EPM). Figure 3 shows how the EPM varies with liposomal concen-

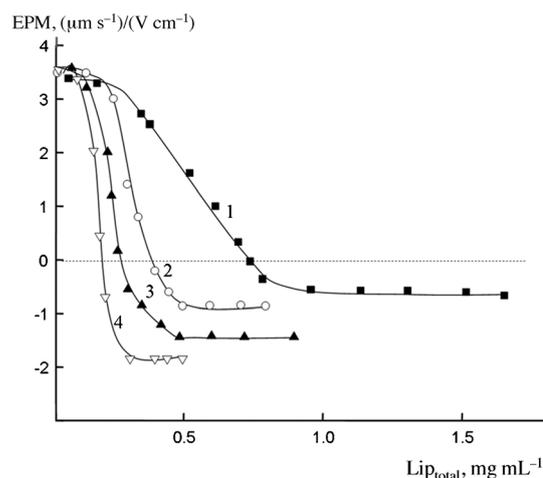


Figure 3. Electrophoretic mobility (EPM) of SPB particles versus PC/PS<sup>-1</sup> liposome concentration.  $\nu=0.1$  (1), 0.2 (2), 0.3 (3), 0.4 (4). [SPB<sup>+</sup>]=10<sup>-4</sup>M.

tration for liposomes with different PS<sup>-1</sup> contents at a constant brush concentration of 10<sup>-4</sup>M. In all cases, complexation led to a decrease in the SPB surface charge with an overall change from positive to negative charge at high liposome concentrations. As expected, the higher the PS<sup>-1</sup> molar fraction admixed with EL (namely  $\nu$ ), the lower the liposome concentration required to achieve SPB charge neutralization (EPM=0 in Figure 3). Concurrently, an increase in  $\nu$  made the charge of the complexes at saturation (i.e., the flat portion of the plots at the higher liposome concentrations) increasingly negative.

To summarize thus far: liposomes composed of EL/PS<sup>-1</sup> do not leak encapsulated NaCl when bound to SPBs. The number of liposomes binding to a single SPB particle ( $N$ ) decreases from about 38 for  $\nu=0.1$  to about 15 for  $\nu=0.4$  (see Figure 2). Since liposome size is nearly constant from  $\nu=0.1$  to  $\nu=0.4$ , the decrease in  $N$  with  $\nu$  can presumably be ascribed to electrostatic repulsion among the bound anionic liposomes. But EPM data in Figure 4 show that the liposomes do not cease their binding when the cationic charge on the SPB particles become precisely neutralized at EPM=0. Instead, the liposome/SPB complex takes on an anionic character with all values of  $\nu$ . Moreover, the greater the  $\nu$ , the greater the anionic charge at equilibrium (i.e., the region where the EPM plots level off). For example, when  $\nu=0.1$ , the minimum EPM=-0.7 units, whereas when  $\nu=0.4$ , the minimum EPM=-1.8 units. The remainder of the paper concerns itself with this key observation.

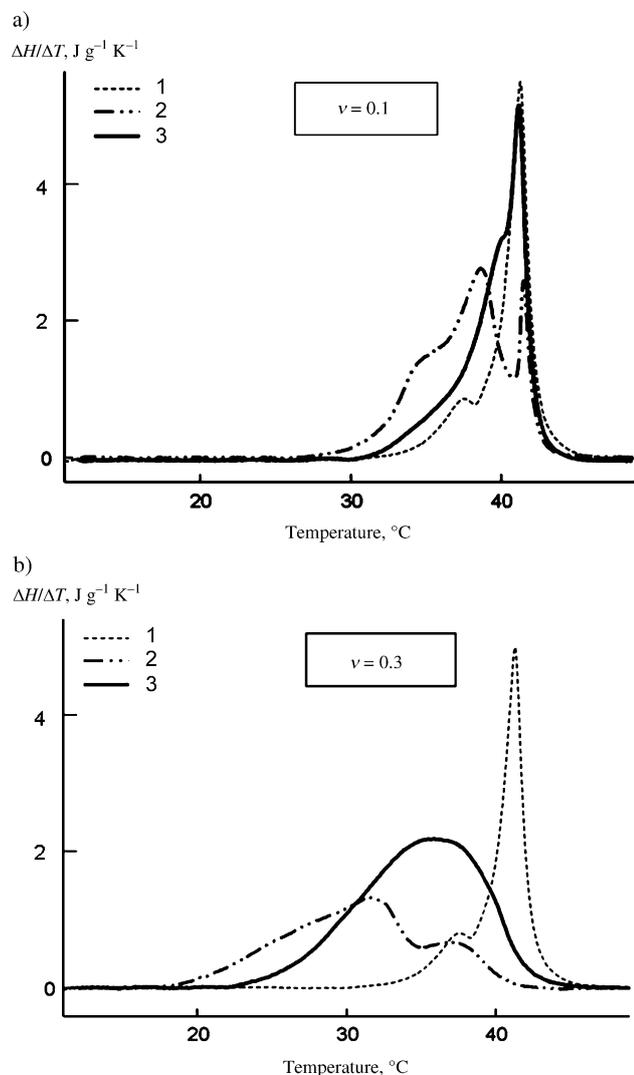


Figure 4. Calorimetric curves of DPPC liposomes (1), DPPC/PS<sup>-1</sup> liposomes (2), and complexes of DPPC/PS<sup>-1</sup> liposomes with SPBs (3) for a)  $\nu=0.1$  and b)  $\nu=0.3$ .

Complexation of mixed anionic/zwitterionic liposomes to conventional cationic polymers (i.e., polymers not grafted to colloidal particles) is accompanied by lipid segregation in the liposomal membrane. Two microphases then form, one composed of anionic lipids electrostatically bound to cationic macromolecules and other composed of zwitterionic lipids free of polycation. We hypothesize that the negative charge imparted to the liposome-covered SPBs might be related to lipid segregation in the liposomal membranes. In order to test this idea, structural organization within EL/PS<sup>-1</sup> bilayers before and after their binding to SPBs was examined using differential scanning calorimetry (DSC). This general approach has been widely used for assaying both trans-leaflet and lateral segregation (domain formation) in mixed lipid bilayers.<sup>[10]</sup> In our DSC experiments, EL was replaced with dipalmitoylphosphatidylcholine (DPPC), a zwitterionic lipid with a higher and more convenient phase transition temperature,  $T_m$ , of about 41°C.<sup>[7,10]</sup> Calorimetric curves for DPPC liposomes are represented in Figure 4a (curve 1) and Figure 4b (curve 1). In the absence of brushes, DPPC/PS<sup>-1</sup> liposomes with a PS<sup>-1</sup> molar fraction of  $\nu=0.1$  possess a rather wide DSC profile with an endothermic maximum at 39°C and a shoulder at 35°C in addition to a sharp maximum at 41°C (curve 2 in Figure 4a). The 39°C maximum and its shoulder likely reflect the co-existence of two mixed DPPC/PS<sup>-1</sup> phases having different DPPC-to-PS<sup>-1</sup> ratios, while the sharp maximum clearly corresponds to pure DPPC domains.

To prepare liposome/SPB samples for the DSC study, dispersions of SPBs and DPPC/PS<sup>-1</sup> liposomes were first pre-heated above the liposomal membrane phase-transition temperature, mixed at a ratio of [PS<sup>-1</sup>]/[SPB]=5 (corresponding to saturation of the SPB surface with liposomes), and finally cooled down to room temperature. As seen in curve 3 of Figure 4a, SPBs caused a shift to higher temperatures in the calorimetric curve for  $\nu=0.1$  liposomes. The transition profile became narrower with a maximum now at 41°C and a shoulder at 40°C. The main peak can reasonably be attributed to pure DPPC domains cleaned from PS<sup>-1</sup>, while the shoulder represents DPPC domains with only a slight PS<sup>-1</sup> admixture.

Increasing the PS<sup>-1</sup> percentage 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 Figure 4b). These curves reflect formation of mixed DPPC/PS<sup>-1</sup> domains with a greater PS<sup>-1</sup> content than with  $\nu=0.1$  (curve 2, Figure 4a). Although addition of SPBs to the liposome suspension had only a slight effect on the width of the phase transition (curve 3, Figure 4b), only one maximum at 37°C was now evident. Absence of a sharp peak at 41°C for the  $\nu=0.3$  liposomes complexed with SPB shows definitively that there exist no pure DPPC domains. The major portion of PS<sup>-1</sup> is distributed as mixed DPPC/PS<sup>-1</sup> regions.

Our explanation of the calorimetric and electrophoretic data relies on three important features of the liposome/polymer complexes: 1) The mean hydrodynamic liposome/polymer diameter of 40–60 nm exceeds by a wide margin the mean distance between the grafted polycationic chains of about

20 nm.<sup>[9]</sup> For this reason, the liposomes are assumed to adsorb primarily at the polycation/water interface (rest “on top of the polycation fingers”) rather than penetrate deeply into the grafted polycation layer. 2) The membranes of EL liposomes and DPPC liposomes above the  $T_m$  are in the liquid-crystalline state where lipid molecules are able to move both in lateral and transmembrane directions. It is well-documented that binding of EL/PS<sup>-1</sup> liposomes to polycations induces transmembrane migration (flip-flop) of anionic lipids from the inner to outer leaflets.<sup>[3,11,12]</sup> Owing to the ionic contacts between grafted polycationic chains and the external liposome surface, these outer anionic lipids tend to cluster in the outer leaflet. 3) A final relevant point is that migration of an anionic liposome in an EPM electric field depends only on the charge of the outer leaflet; anions in the inner leaflet do not contribute to migration because the corresponding anionic charge is neutralized by encapsulated counterions.

With these considerations in mind, the following model can be invoked. At a low PS<sup>-1</sup> molar fraction ( $\nu=0.1$ ), the majority of PS<sup>-1</sup> molecules in the complex reside in the portion of the liposome in contact with the brushes (Figure 5 a). Only a small percentage of the PS<sup>-1</sup> molecules will be found in the remainder of the liposome that is external to the brushes and that, consequently, is surrounded only by water. It is these relatively sparse PS<sup>-1</sup> molecules that impart the small negative charge seen in the EPM (curve 1, Figure 3). The nearly pure external DPPC is reflected in the DSC

(curve 3, Figure 4a). Now, when the molar fraction of PS<sup>-1</sup> admixed with DPPC is increased ( $\nu=0.3$ ), more of the PS<sup>-1</sup> population is located outside the confines of the SPB (Figure 5b). The ensuing electrostatic repulsion among the anionic liposomes decreases the number of adsorbed liposomes per SPB particle ( $N=38$  for  $\nu=0.1$  but  $N=17$  for  $\nu=0.3$ ). Despite the reduced number of liposomes per SPB particle with  $\nu=0.3$ , the resulting lower negative charge is more than compensated for by the enhanced population of external PS<sup>-1</sup>. This explains why the negative charge on the complexes increases with  $\nu$  (Figure 4) and no free DPPC is visible in the DSC (curve 3, Figure 4b).

The above results along with our previous work illustrate a principal difference between ordinary nongrafted cationic polymers and grafted cationic polymers when they bind to anionic liposomes. With the former, binding is accompanied by complete anionic lipid segregation into two types of domains: those enriched with anionic lipids and those totally free of anionic lipids. With the latter, binding causes only partial (incomplete) lipid segregation where domains coexist with different ratios between anionic and zwitterionic (electroneutral) lipids. Anionic liposomes can impart a negative charge to the complex surface that progressively increases as the anionic lipid content in the membrane increases. This increase can be ascribed to an increased population of anionic lipid in the non-SPB-surrounded portion of the bound liposomes. In the present paper, we described the structural reorganization in the mixed anionic liposomes electrostatically adsorbed on the surface of spherical polycationic brushes, but the behavior likely applies also to other geometries (e.g., planar surfaces with grafted polycations). However, this point was not investigated.

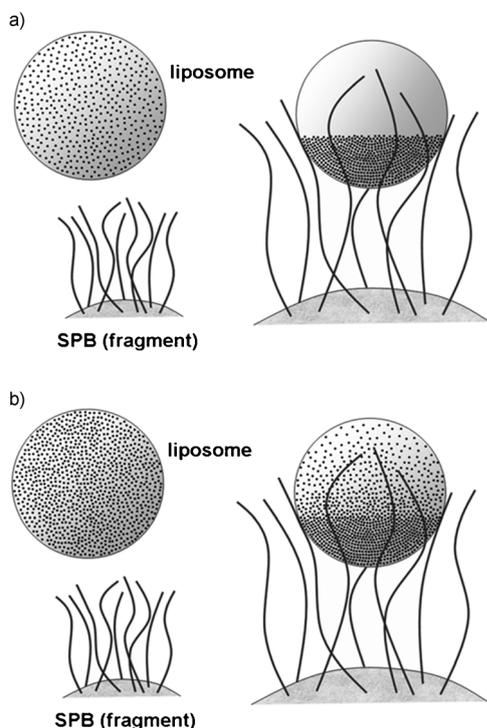


Figure 5. A schematic representation of liposomes with a) low and b) high anionic lipid content before (left) and after (right) complexation with cationic SPB particles. Low anionic lipid corresponds to  $\nu=0.1$ ; high anionic lipid corresponds to  $\nu=0.3$ .

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