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Biomembrane Sensitivity to Structural Changes in Bound Polymers

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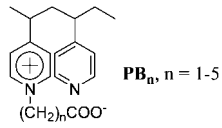
Cationic polymers bind strongly to negatively charged cell surfaces, thereby producing irreversible rearrangements in the protein/lipid bilayers. Antibacterial activity is one practical application of this behavior.[1] Bound polycations are also able to promote migration of lipid molecules between two liposomal leaflets ("flip-flop") and to induce lipid segregation into domains.[2−4] Such structural rearrangement in biological membranes often destroys cell integrity and function. From the point of view of drug delivery by direct contact, it is desirable for polymers to bind without actually disrupting cell integrity.[5] In an initial step toward this goal, we investigated a family of polybetaines (PB_n) drawn below, in which cationic and anionic units are covalently linked by short $-(CH_2)_n-$ spacers. These compounds were shown not to destroy model membranes while engaging in several different structure-sensitive mechanisms during polymer/bilayer interactions.



PB_n, n = 1-5

To obtain the series of PB_n polymers, a poly(4-vinylpyridine) (PVP) fraction with a degree of polymerization of 1100 was prepared and then quaternized with various ω-bromocarboxylic acids as described elsewhere.[6] The extent of quaternization for synthesized polymers, determined from the ratio of intensities at 1600 and 1640 cm^{-1} in the infrared,[7] averaged 81%. PB_n polymers were mixed at various concentrations with unilamellar ca. 50 nm liposomes, prepared using the standard ultrasonic procedure,[8] composed of zwitterionic dioleoylphosphatidylcholine (DOPC) admixed with anionic dioleoylphosphatidylglycerol ($DOPG^{1-}$) in a molar ratio of 4:1. Since quaternized pyridinium rings of PB_n polymers are known to be fluorescence quenchers,[9] PB_n-to-liposome interactions were assessed by incorporating 0.5 wt% of NBD-PC (Avanti 810130), a fluorescent phosphocholine, into the bilayer during the liposome preparation.

Figure 1a shows how the intensity of the NBD-PC fluorescence changes with increasing PB_n concentrations, $[PB_n]$ being expressed as moles of betaine units per liter. We see that PB_1 hardly affects the NBD-PC fluorescence intensity, whereas the four others (with $n = 2$, 3, 4, and 5) do quench the fluorescence. The different quenching efficiencies among the latter reflect the distance between the pyridinium ring and carboxylate and hence the availability of the pyridinium ring to quench the membrane-bound dye. Neither PB_2 nor PB_5 induce leakage or destruction of the liposomes because

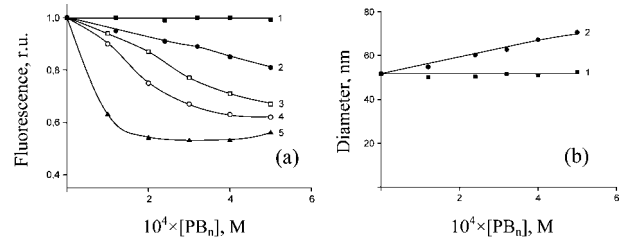


Figure 1. (a) Effect of polybetaines on the relative fluorescence intensity of labeled $DOPC/DOPG^{1-}$ liposomes. PB_1 (1), PB_2 (2), PB_3 (3), PB_4 (4), and PB_5 (5). Total lipid concentration 1 mg/mL, $[DOPG^{1-}] = 1.3 \times 10^{-4}$ M, 10^{-2} M phosphate buffer, pH 7.5. (b) Effect of polybetaine concentration on mean hydrodynamic diameter of $DOPC/DOPG^{1-}$ liposomes. PB_1 (1) and PB_2 (2). Total lipid concentration 1 mg/mL, $[DOPG^{1-}] = 1.3 \times 10^{-4}$ M, 10^{-2} M phosphate buffer, pH 7.5. No quenching was observed when PB_2 or PB_5 was added to NBD-PC-tagged neutral DOPC liposomes.

conductometry-detectable NaCl in the bulk solution was not observed when NaCl-loaded liposomes were exposed to the polymers under standard conditions.

The fluorescence data do not allow a definite decision as to whether or not PB_1 is a poor quencher or is simply unable to complex. To address the ambiguity, we measured the size of the liposomes in the presence of PB_1 with the aid of dynamic light scattering. Figure 1b shows no change in the liposome size up to $[PB_1] = 5 \times 10^{-4}$ M (curve 1), indicating no interaction between PB_1 and liposomes within this concentration range. For comparison, the size of liposomes with added PB_2 is also given in the same figure. In this case, an increase in a hydrodynamic diameter of liposomes from 51 up to 71 nm was registered, obviously reflecting PB_2 binding to liposomes in agreement with the fluorescence data. Thus, from the combination of the fluorescence and light scattering data, one can conclude that PB_1 does not bind to $DOPC/DOPG^{1-}$ liposomes, probably due to strong intrabetaine electrostatic interaction between the pyridinium cation and the proximate carboxyl anion. However, in polymers with $n \geq 2$, the positive betaine charges can function more independently so that binding to anionic $DOPC/DOPG^{1-}$ liposomes through formation of multiple pyridinium/$DOPG^{1-}$ "salt bridges" now becomes favorable as do, to an unknown extent, minor hydrophobic forces.

How does the lipid bilayer respond to complexation with PB_{2-5}? In particular, does the complexation affect structural reorganization of the biological membrane? The question was examined with differential scanning calorimetry (DSC) where the phase transitions in the membrane were analyzed before and after liposome binding to the polymers. This approach has been widely used for assaying lateral segregation (domain formation) in mixed lipid bilayers.[10] In our experiments DOPC was replaced with dipalmitoylphosphatidylcholine (DPPC), a zwitterionic lipid with a higher and more

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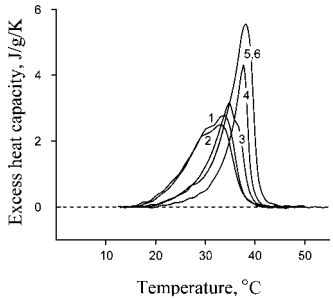

Figure 2. Calorimetric curves of DPPC/DOPG^{1-} liposomes (1), their complexes with PB_2 (2), PB_3 (3), PB_4 (4), and PB_5 (5), and DPPC liposomes (6). Total lipid concentration 1 mg/mL, [DOPG^{1-}] = 1.3×10^{-4} M, 10^{-2} M phosphate buffer, pH 7.5.

convenient phase-transition temperature, T_m, of ~41 °C.[11] The 4:1 binary DPPC/DOPG^{1-} liposomes were initially characterized by a rather wide transition profile with an endothermic maximum at 33.1 °C and a shoulder at 29.3 °C (curve 1 in Figure 2), apparently reflecting a coexistence of two types of mixed DPPC/DOPG^{1-} phases having different DPPC-to-DOPG^{1-} ratios. Previous laser electrophoretic mobility experiments[11] have shown that anionic lipids are distributed nearly equally between the two membrane leaflets.

To prepare PB_n/liposome samples for the DSC study, the polymer solutions and dispersions of 4:1 DPPC/DOPG^{1-} liposomes were preheated above the liposomal membrane phase-transition temperature, mixed at [PB_n]/[DOPG^{1-}] = 5, and then cooled to room temperature. PB_2 was found to have a negligible effect on the transition profile relative to liposomes with no added polymer (Figure 2, curve 2). PB_3, on the other hand, manifested a shift of the calorimetric curve to higher temperature; the transition profile became narrower while maintaining the presence of two-component phases with a maximum now at 34.6 °C and a shoulder at 36.5 °C (curve 3). In the presence of PB_4, only a single narrow peak in the calorimetric curve was found (curve 4), and its position at ~40 °C did not change further when PB_4 was replaced by PB_5 (curve 5). The narrow peak of PB_5 coincided with the peak characteristic of pure DPPC liposomes (curves 5 and 6, respectively).

The calorimetric results are informative. Thus, the identity of the PB_2 data to those from DPPC/ DOPG^{1-} liposomes without adsorbant signifies an absence of any polymer effect on the liposomal membrane organization. In contrast, PB_4 and PB_5 induced formation of DOPG^{1-} clusters within the liposomal membrane, thus "cleansing" the binary mixture from DOPG^{1-} and creating the domains of pure DPPC evident from the calorimetry. Flip-flop of DOPG^{1-} from the inner to outer leaflet must have been total because otherwise curve 5 would have manifested a peak or shoulder shifted to the left (to lower temperature) as occurs in curve 1. Since the PB_3 profile was intermediate between that of the PB_2 and PB_4/PB_5 situations, an incomplete microphase separation of DOPG^{1-} is observed. By this we mean that PB_3 can promote lateral segregation within the outer leaflet, giving rise to an altered DSC scan, but total flip-flop as with PB_4/PB_5 is absent.

Thus, the length of the $-(CH_2)_n-$ spacer in betaine units has a decisive effect on the ability of polybetaines to interact with anionic liposomes and to induce structural rearrangements within the liposomal membrane. PB_1 does not bind to liposomes and obviously cannot produce any change in the membrane organization. PB_2 does bind to the liposomes but induces no rearrangements within the liposomal membrane. PB_3 also binds to liposomes, thereby causing

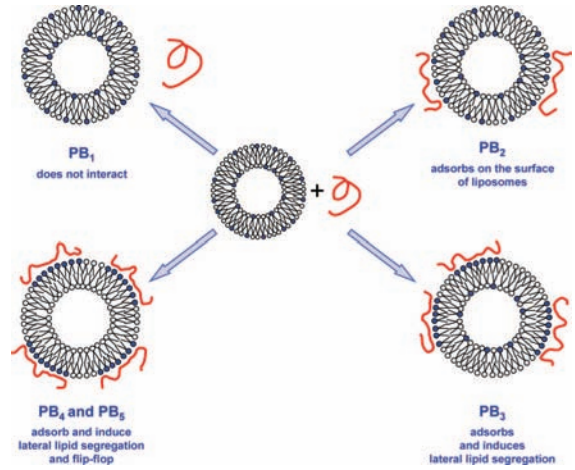

Figure 3. Events occurring after polymer/liposome interaction.

a partial segregation in the liposomal membrane in which only DOPG^{1-} molecules from the outer membrane leaflet are likely involved. Finally, adsorption of PB_4 and PB_5 causes all DOPG^{1-} molecules, from both membrane leaflets, to participate in an electrostatically imposed microphase separation via a flip-flop mechanism. The effects of subtle polymer changes on biomembrane morphology are summarized in Figure 3.

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