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Detection of surface charge-related properties in model membrane systems by aqueous two-phase partition

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Unilamellar vesicles composed of phosphatidylcholine (PC) and either phosphatidic acid (PA) or phosphatidylglycerol (PG) partition to the upper poly(ethylene glycol) (PEG)-rich phase of a charge-sensitive 5%:5% (w/w) PEG 8000/Dextran T-500 phase system containing 10 mM sodium phosphate at pH 7, consistent with the vesicles bearing a net negative charge. When prepared in the presence of a pH gradient (interior acidic), PC/PA vesicles exhibit an increased partition to the top PEG-rich phase, consistent with a redistribution of the PA from the inner to the outer monolayer of the vesicle bilayer. Conversely, when prepared in the presence of a pH gradient (interior basic), PC/PG vesicles exhibit a decreased top-phase partition, consistent with a redistribution of the PG from the outer to the inner monolayer of the vesicle bilayer. Unilamellar vesicles composed of PC and stearylamine partition to the lower dextran-rich phase of a 5%:5% (w/w) PEG 8000/Dextran T-500 phase system containing 10 mM sodium phosphate at pH 8.5, consistent with the vesicles bearing a net positive charge. When prepared in the presence of a pH gradient (interior acidic), conditions under which the stearylamine is trapped on the inner monolayer of the bilayer, the vesicles now partition predominantly to the interface in a manner similar to vesicles composed of PC alone. These results demonstrate that partitioning in aqueous two-phase polymer systems is a sensitive method for monitoring the asymmetry of charged lipids in model membrane systems and also suggests that partitioning in charge-sensitive systems depends only on the physical nature of the exterior surface of the membrane.

Introduction

Cells and subcellular organelles may be fractionated on the basis of charge by partitioning in 'charge-sensitive' aqueous polymer two-phase systems [1–3]. Such phase systems are obtained by mixing solutions of certain polymers, e.g., dextran and poly(ethylene glycol) (PEG), above critical concentrations. Incorporating ions that partition unevenly between the phases (e.g., phosphate) produces an electrostatic potential difference between the phases ($\Delta\Psi$). As phosphate favors the dextran-rich bottom phase, the PEG-rich top phase becomes relatively positive and attracts negatively charged particulates [4]. It is presumed that charges present only on the exterior surface of the cell are involved; however, the exact nature and location of these remains to be defined. Removal of externally exposed sialic acid from erythrocytes decreases top-phase partition [5], but surface differences due to ganglioside-linked sialic acid, which is not neuraminidase-susceptible and which is located close to the lipid bilayer, have been detected [6].

The difficulties inherent in studying biological surfaces complicate the interpretation of these results. Removal of the external sialic acid will undoubtedly alter the surface charge, but may also reasonably be expected to alter the segment density distribution, radius of gyration and separation of glycoproteins on the cell surface, as well as the hydrodynamic properties of the glycocalyx as a whole, effects which may in themselves influence partitioning behavior. Additionally, sialic acid is not the only charge-bearing constituent on the erythrocyte membrane. With regard to the dependence of surface free energy difference on $\Delta\Psi$, the interpretation of the contact angle measurements is complicated by difficulties in realistically modelling the charge distribution of the glycocalyx [2,7].

Abbreviations: PC, phosphatidylcholine; PA, phosphatidic acid; PG, phosphatidylglycerol; PEG, poly(ethylene glycol); SA, stearylamine.

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In this work we extend our previous studies of the partition of charged vesicles of defined size in two-phase systems [8] in order to critically address the issue of whether the polymer systems sample only the exterior surface charge of the membrane. The model systems we employ are unilamellar lipid vesicles composed of neutral and charged lipid species. By establishing a pH gradient across the vesicle bilayers, we are able to render the membranes asymmetrical with respect to composition [9,10] and thereby influence their partition behavior.

Materials and Methods

Preparation of phase system

A phase system of 5% (w/w) Dextran T-500 (Lot No. NC03572, Pharmacia) and 5% (w/w) poly(ethylene glycol), PEG 8000 (Lot No. 98874/2017, BDH) was prepared in 10 mM sodium phosphate at both pH 7 and pH 8.5. The phases were mixed and allowed to equilibrate at 25°C for several days. The PEG-rich top phase and the dextran-rich bottom phase were separated and stored at −20°C.

Preparation of lipid vesicles

Lipid vesicles were prepared by extrusion according to Hope et al. [11]. Egg phosphatidylcholine (PC), dioleoylphosphatidylglycerol (PG), and dioleoylphosphatidic acid (PA) were obtained from Avanti Polar Lipids (Birmingham, AL), and stearylamine from Sigma (St. Louis, MO) and were used without further purification. Lipids were combined in the appropriate molar ratios from stock solutions in chloroform, spiked with 1–2 μCi of [^{3}H]DPPC (NEN, Missasauga, ON) and solvent removed initially under nitrogen then by storage under reduced pressure (< 0.1 mmHg) for at least 2 h. Multilamellar vesicles (MLVs) with stearylamine were prepared by dispersing 25 μmol of lipid in 2 ml of 10 mM sodium phosphate pH 8.5 or 10 mM sodium citrate pH 4, by vortexing at room temperature. Unilamellar vesicles of average diameter 100 nm were prepared by extrusion of the MLVs through two stacked 0.1-micron filters (Nuclepore) under nitrogen pressure using the Extruder device (Lipex Biomembranes, Vancouver, BC). For vesicles prepared in pH 4 citrate, a pH gradient was established across the vesicle membrane by passing the vesicles down a Sephadex G50F column (Pharmacia, Baie d'Urfe, PQ) equilibrated with 0.01 M sodium phosphate pH 8.5. For PC/PG systems, MLVs were prepared in 300 mM sodium phosphate (pH 8), freeze-thawed from liquid nitrogen five times [11], extruded as above, and the external buffer exchanged by chromatography on Sephadex G50M using 200 mM sodium sulfate/20 mM sodium citrate (pH 4) as eluant. For PC/PA systems, MLVs were prepared in 150 mM citrate (pH 4), freeze-thawed five times from liquid nitrogen and external buffer exchanged for 10 mM sodium phosphate (pH 7) as above. For time-course measurements, vesicles with an applied pH gradient were incubated at either 25°C or 45°C for various times prior to partitioning. For construction of partition standard curves the external buffer was not exchanged.

Measurement of pH gradient

The pH gradient across the vesicle membrane was determined by monitoring the redistribution of [^{14}C]methylamine across the vesicle membrane. 1 μCi of [^{14}C]methylamine (NEN) was added to preformed vesicles (15 μmol lipid·ml^{-1} final concentration) in either 10 mM sodium phosphate pH 8.5 or in both the upper and lower phases of a 5% : 5% (w/w) PEG 8000/Dextran T-500 pH 8.5 phase system. At various times, 100-μl aliquots were removed and added to 1-ml tuberculin syringes packed with Sephadex G50F and equilibrated in the suspension buffer. After centrifugation (500 × g, 2 min) the eluant was counted and the pH gradient calculated as previously described [9].

Partition measurements

30 μl of vesicle preparation was added to 1.5 ml of top phase and 1.5 ml of bottom phase in a 10 × 75 mm plastic tube at 25°C. The contents were mixed by repeated inversion for 1 min. Triplicate 50 μl samples were removed for total counts. The tubes were allowed to stand at 25°C for 25 min then triplicate 50 μl samples of top phase and triplicate 20 μl samples of bottom phase removed for counting. Results are expressed as a percentage of the total counts added. The difference between the calculated total counts and the sum of the counts in the top and bottom phases was taken to represent the counts at the interface. No correction has been applied for counts adsorbed to the tube walls or air-water interface.

Results

Standard curves for the top phase partition of unilamellar vesicles containing various molar ratios of PG and PA in a 5% : 5% (w/w) PEG 8000/Dextran T-500, 10 mM sodium phosphate pH 7 phase system are shown in Fig. 1. In each case, there was a sigmoidal increase in the top phase partition with increasing amount of PA or PG with the region of greatest sensitivity between 3 and 6 mol% of the charged species. Note that for this phase system, the top phase partition of vesicles containing PG tended to be less than that for comparable vesicles containing PA, consistent with previous results [8].

On the basis of previous studies [9], it is assumed that for 100-nm vesicles, given an initially random distribution of the lipids, that 50% of the lipid will be present in the outer monolayer of the vesicle bilayer in the absence of a pH gradient. If all the PA in a vesicle

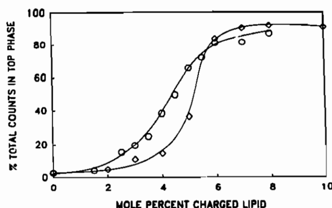

Fig. 1. Standard curves for the partition of PC/PA (○) and PC/PG (◇) 100-nm-diameter unilamellar vesicles in 5%:5% (w/w) PEG 8000/Dextran T-500, 10 mM sodium phosphate pH 7.2. Each data point represents the mean ± S.D. of triplicate measurements upon each preparation.

containing 2.5 mol% (total) PA were to redistribute to the outer monolayer, then it would be expected that the vesicle would partition as if the effective PA content were 5 mol%, assuming that the polymer system samples only the exterior charge. Initial experiments showed that vesicles composed of PC/PA (2.5 mol% PA) partitioned, mean ± S.D. (*N*), 24.9 ± 2.0(3)% to the top phase when prepared in the presence of a pH gradient (4 internal/7 external), but without incubation at elevated temperatures. When incubated at 45°C for 1 h prior to partitioning, the same vesicles now partitioned 75.9 ± 3.4(3)% to the top phase, i.e., they partitioned as if they were more negatively charged. The time course for the generation of asymmetry in PC/PA 100 nm vesicles containing 2.5 mol% PA in the presence of a pH gradient is shown in Fig. 2. Results are expressed in terms of the total amount of PA in the outer monolayer of the vesicle bilayer assuming that 2.5 mol% corresponds to

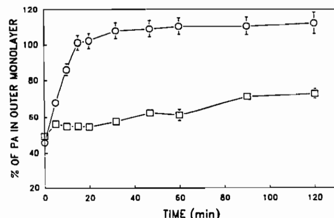

Fig. 2. Time course for the partition of PC/PA 100-nm vesicles containing 2.5 mol% PA, prepared in the presence of a pH gradient (pH 4 internal/pH 7 external), in 5%:5% (w/w) PEG 8000/Dextran T-500, 10 mM sodium phosphate pH 7.2 after incubation at 45°C (○) and 25°C (□). Values represent the mean ± S.D. of two separate vesicle preparations each measured in triplicate.

50% of the total PA. It is evident that following incubation at 45°C, the vesicles showed greatly increased partitioning to the upper phase, consistent with the vesicles bearing an increased negative charge. Assuming that this truly reflects the generation of a vesicles asymmetrical with respect to PA, these results would suggest that at 45°C the redistribution occurs within approximately 30 min and goes essentially to completion. Note that values in excess of 100% of the PA in the outer monolayer are presented, i.e., the vesicles partition as if they contain more than 5 mol% PA on their outer surface. This most probably reflects errors in vesicle composition, errors in interpolation from the standard curve near the plateau region (see Fig. 1), minor temperature variation during partitioning, variation between phase systems and non-specific adsorption of polymers to the vesicle surface amongst other factors. When incubated at 25°C, there was apparently a much slower redistribution of the charged species from the inner to the outer monolayer, with approximately 70% of the total PA being detected after 2 h of incubation. This result indicates that only a relatively minor correction need be applied to the data obtained at 45°C in order to account for the redistribution of the PA that occurs during the partition process itself, which is done at 25°C for 25 min. Controls demonstrated that for PC/PA (2.5% PA) vesicles prepared in the absence of a pH gradient (pH 7 internal and external) approximately 17.4 ± 2.1(3)% of the vesicles partitioned to the upper phase. When prepared in the presence of a pH gradient (pH 4 internal/pH 7 external) and partitioned immediately after elution from the column, 24.9 ± 2.0(3)% of the counts were in the top phase. This difference in partitioning presumably reflects the amount of PA that has redistributed across the vesicle bilayer in the presence of the pH gradient during the time they are partitioning in the polymer phase system. The difference corresponds to less than approximately 5% of the total PA. We have also observed that partitioning of charged asymmetrical vesicles is not significantly altered by prior incubation of the vesicles in the phase system for 25 min. This indicates that the polymers do not in themselves cause a redistribution of the lipids within the vesicle membranes.

A similar time-course for the redistribution of PG in PC/PG 100 nm vesicles containing 7 mol% PG, in the presence of a pH gradient (pH 8 internal/pH 4 external), is presented in Fig. 3. In this case we observed that after incubation at 45°C there was a rapid decrease in top-phase partitioning, indicating that the surface charge on the vesicles had decreased, consistent with a redistribution of the PG from the outer to the inner monolayer of the vesicle. Compared to PA (Fig. 2), it would appear that the redistribution occurs more slowly for PG, although it should be recognized that the amount of charged species present initially may be a contributory

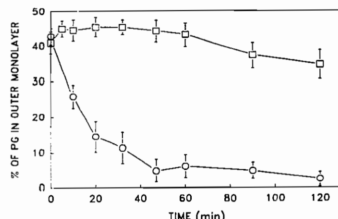

Fig. 3. Time course for the partition of PC/PG 100-nm vesicles containing 7 mol% PG, prepared in the presence of a pH gradient (pH 8 internal/pH 4 external), in 5%:5% (w/w) PEG 8000/Dextran T-500, 10 mM sodium phosphate pH 7.2 after incubation at 45°C (○) and 25°C (□). Values represent the mean ± S.D. of two separate vesicle preparations each measured in triplicate.

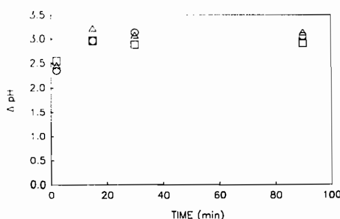

Fig. 5. Time-course for the formation and stability of the pH gradients in PC/stearylamine 100-nm unilamellar vesicles containing 5 mol% stearylamine prepared in the presence of a pH gradient (pH 4 internal/pH 8.5 external). Vesicles were diluted in 10 mM sodium phosphate pH 8.5 (○) or either the top (□) or bottom (Δ) phase of a 5%:5% (w/w) PEG 8000/Dextran T-500, 10 mM sodium phosphate pH 8.5 phase system, and pH gradients were measured as described in Materials and Methods.

factor. As with PA, it would appear that the redistribution goes very close to completion after 1 h or more, and that the rate of redistribution at 25°C is much lower than at 45°C.

The effect of stearylamine (SA) content on the partition of PC/SA vesicles in 5%:5% (w/w) PEG 8000/Dextran T-500, 10 mM phosphate pH 8.5 is shown in Fig. 4. We observed that in this phase system, vesicles composed of pure PC partitioned predominantly to the interface (75–80%). The effect of adding stearylamine was to favor partition to the lower negatively charged dextran phase, as expected, consistent with the results of Sharpe [12]. Previous studies have shown that if the pH gradient is dissipated, the pH-induced asymmetry of stearylamine across the membrane also decays [9]. We established that the pH gradient was stable in the presence of the polymer phases used for partition,

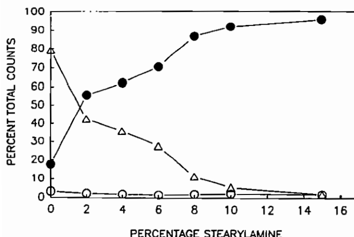

Fig. 4. Standard curve for the partition of PC/stearylamine 100 nm vesicles in 5%:5% (w/w) PEG 8000/Dextran T-500, 10 mM phosphate pH 8.5. Values represent the mean ± S.D. of samples measured in triplicate. Symbols represent counts in (○) top phase, (●) bottom phase and (Δ) at the interface.

according to Materials and Methods. As indicated in Fig. 5, the pH gradients, once established, were stable for at least 90 min. In the presence of a pH gradient (pH 4 internal/pH 8.5 external), we observed that the upper phase samples remained at 10 ± 2% of total counts, lower phase samples at 15 ± 2%, and the interface at 75 ± 4% of total counts between 30 and 60 min; i.e., the vesicles now partitioned as if they were composed solely of PC, consistent with a redistribution of the stearylamine from the outer to the inner monolayer of the vesicle bilayer.

Discussion

Water-soluble weak bases such as methylamine can permeate across a membrane in the neutral form to achieve transmembrane aqueous concentrations that satisfy the Henderson-Hasselbalch relationship and hence can be used to measure the pH gradient across a membrane [13]. Similarly it has been proposed that the neutral form of stearylamine can flip across the membrane, whereas the protonated form cannot flip as readily [9]. Thus, in the presence of a pH gradient (interior acidic with respect to the exterior) across the membrane, stearylamine will redistribute across the lipid bilayer so that the protonated form is localized primarily to the interior of the vesicle. If stearylamine does redistribute across the bilayer according to the Henderson-Hasselbalch relation, then for vesicles with a pH 4 internal and pH 8.5 external, the concentration of stearylamine on the outer surface should be reduced by a factor of 10^4 or more compared to similar vesicles without a pH gradient. By maintaining the exterior pH close to the pK_a of the amino group of the stearylamine, we weight the dissociation of the headgroup in

favor of the neutral species, and thus facilitate redistribution of the lipid across the vesicle bilayer.

For vesicles containing PA in the presence of an acidic pH gradient (pH 4 internal/pH 7 external), the PA behaves as a weak acid where the protonated, neutral form of the PA is more readily able to permeate across the membrane to the exterior surface, and so redistributes according to the applied pH gradient [10]. Similarly, PG moves down the reverse alkaline gradient (pH 8 internal/pH 4 external) to accumulate on the inner monolayer of the vesicle bilayer [10].

From the former, it is clear that two-phase aqueous polymer systems may be used to monitor the redistribution of charged lipids in model membrane systems. For the phase systems used in this study, partition was sensitive to small changes in composition within the range of approximately 2–6 mol% of the charged species. In order to make the systems more sensitive for vesicles containing smaller amounts of charged species, phase systems closer to the critical point should be used (unpublished observations). It should be appreciated that as systems closer to the critical point are used, other properties in addition to charge-related factors, such as interfacial tension, may be reflected to a greater extent by the partition. Conversely, using systems further from the critical point would extend the range of sensitivity. In addition to its sensitivity, phase partition is an attractive method because it may be applied to model systems containing any charged species. Further, the polymers do not dissipate pH gradients or in themselves cause redistribution of the lipids across the bilayer. The use of two-phase systems has the advantage that it does not require the covalent modification of the lipid in order to assay the lipid distribution [9] with the attendant concerns that the chemical assay procedure may itself influence the asymmetry. One disadvantage of the technique derives from the difficulty associated with the reproducible preparation of phase systems. Aside from errors in composition which become more important closer to the critical point, it is well recognized [2] that the phase properties depend upon both the molecular weight and polydispersity of the polymers used; it may be expected, therefore, that partition values will vary with different batches of the same polymer.

The results presented herein strongly indicate that the partition process is sensitive to charged components present only on the exterior of the membrane, consistent with previous interpretations [5,6], although it is clear that the partition process depends upon other factors as well as charge [2,3,12,14,15]. It is not obvious what aspect of the surface charge on the membrane the polymers sample. Despite reports that PEG interacts directly with model membranes [16], the weight of evidence would indicate a depletion or partial exclusion of the polymer from membrane surfaces [17–19], thus it is probable that the charge the phases are detecting does not correspond to the surface potential. Equally, studies on biological surfaces would indicate that the charge detected by the phase systems is not simply the zeta potential modulated by the surface excess free ions present within the shear plane [2,20]. We feel that it would be of value to extend these studies to compare the surface charges on model membrane systems by electrophoresis, as detected by polymer partition and by probes of surface potential such as TNS [21] or ^{2}H-NMR of suitably labelled phospholipids [22] in the presence of the phase systems.

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