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Aqueous two-phase polymer partitioning of lipid vesicles of defined size and composition

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The kinetics of the partitioning of lipid vesicles containing acidic phospholipids in aqueous two-phase polymer systems are dependent upon the vesicle size; the larger the vesicles, the more readily they adsorb to the interfaces between the two polymer phases and hence are cleared from the top phase as phase separation proceeds. The partitioning of neutral lipid vesicles is principally to the bulk interface and is the same in phase systems of both low and high electrostatic potential difference between the two phases ($\Delta\psi$). The incorporation of negatively charged lipids has two effects upon partition. First, vesicles with negatively charged lipids exhibit increased bottom phase partitioning in phases of low $\Delta\psi$ due to an enhanced wetting of the charged lipids by the lower phase. Second, the presence of a negatively charged group on the vesicle surface results in increased partition to the interface and top phase in phase systems of high $\Delta\psi$. Differences observed in the partition of vesicles containing various species of negatively charged lipid thus reflect a competition between these two opposing factors.

Introduction

Cells and organelles added to aqueous two-phase systems that are formed by mixtures of certain polymers (e.g., dextran and poly(ethylene glycol)) above critical concentrations partition between the two phases and the bulk interface separating them [1–3]. By manipulation of the composition of the phase system, selected features of the surfaces of the particulates can be made to dominate the partition behaviour and may be used for the analytical and preparative separation of the particulates on the basis of these properties. Ions that have different affinities for the two phases give rise to an electrostatic potential difference between the phases ($\Delta\psi$), which influences the partition of charged particles (charge-sensitive partitioning). Phase systems which contain salts that do not yield a $\Delta\psi$ give rise to non-charge-sensitive partitioning. Ligands to surface receptors linked covalently to either of the phase-forming polymers give affinity partition dependent on receptor status.

Understanding the mechanism and the molecular basis of the partitioning of cells and organelles is necessary if the full potential of phase partitioning for bioseparations of particulates is to be realized.

A thermodynamic model based on the Bronsted partition theory has been proposed by Albertsson (reviewed in Ref. 1) and extended and tested experimentally by Brooks and Sharp (described in Ref. 4). However, studies on the kinetics of cell partitioning [5–7] have revealed that non-thermodynamic factors also contribute to determining the effective partition coefficient.

When phase systems containing particulates are mixed and then allowed to phase separate, the particulates distribute between the microdomains of the bulk phase and the microinterfaces formed by the surfaces of droplets and streams, whose upward and downward motions and complex coalescence give rise to phase separation. Attachment to these microinterfaces is determined by the degree to which one phase preferentially wets the particle surface, as described by contact angle (equilibrium) measurements and by the (dynamic) effects of the complex motion and fluid shear

Abbreviations: MLV, multilamellar vesicle; DPPC, dipalmitoylphosphatidylcholine; LUV, large unilamellar vesicle; PC, phosphatidylcholine; PA, phosphatidic acid; PG, phosphatidylglycerol; PI, phosphatidylinositol; PEG, poly(ethylene glycol); QEL, quasielastic light scattering.

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stresses present during phase separation. Partitioning shows kinetics because of this close association with phase separation. Partition coefficients measured even when the bulk interface is clearly formed are unlikely to represent equilibrium values; for the purposes of cell separation and subfractionations by countercurrent distribution in two-phase systems such non-equilibrium conditions are required [8].

Within this context, and certainly relevant to the partitioning of subcellular organelles, it has been suggested that the association of particulate with an interface will be favoured when the magnitude of the free-energy change upon association with the interface is of the same order as the average thermal energy [4]. The change in surface free-energy will depend upon both the interfacial tension between the two phases and the surface area of the adsorbed particle; it is to be expected, therefore, that the kinetics of the partitioning of particulates in two-phase systems will be sensitive to the size of the particulate. The theoretical treatments of Albertsson and of Brooks and Sharp both predict an effect of particle size on partitioning.

At the molecular level, cell-surface sialic acid influences charge-sensitive partitioning [9]. In the particular case of charge-associated surface differences between rat red blood cells of different ages, ganglioside-linked sialic acid is responsible [10]. Non-charge-sensitive partitioning has received less attention. Correlations of partitioning with membrane lipid composition have been reported [11] and surface glycosylation, including sialylation, has an influence [11,12].

Although the modification of cell surfaces, either enzymatically or chemically, has given some insight into the molecular basis of partitioning, this approach is limited by the very complexity of cell membranes. By contrast, lipid vesicles represent attractive systems for modelling the partitioning of cells and subcellular organelles; the composition of the vesicle can be readily manipulated and precisely defined and the size of the vesicle controlled [13]. Previous studies have indicated that the charge on the lipid headgroup is dominant in determining the partitioning of lipid vesicles in phase systems of high $\Delta\psi$, although it is clear that other non-charge-dependent factors are important in the partition process [14–16].

However, lipid vesicles are not simply limited to use as model membranes. Their applications as drug delivery vehicles is now extensive. Partitioning provides rapid and sensitive measures of surface properties of lipid vesicles which may have importance for their use as pharmaceuticals.

In this study we have investigated the effects of vesicle size and lipid composition upon the partitioning of lipid vesicles in dextran-PEG phase systems of varying $\Delta\psi$. Our results indicate that the larger vesicles are cleared more rapidly to the interface than the smaller vesicles and that the incorporation of charged lipid species affects both charge-sensitive and non-charge-sensitive partitioning.

Materials and Methods

Preparation of lipid vesicles

Egg PC (EPC), egg PG (EPG) and egg PA (EPA) were obtained from Avanti Polar Lipids and were determined to be greater than 99% pure on the basis of two-dimensional thin-layer chromatography. All other lipids were obtained from Sigma. MLVs were prepared by dispersing 30 mg of a dry lipid film of the appropriate lipid mixture, labelled with 1 μCi of [^{3}H]DPPC (NEN, Canada) in 3 ml of 0.01 M sodium phosphate/150 mM NaCl (pH 6.8), by vortexing at room temperature. Large unilamellar vesicles were produced by repeated extrusion of the MLVs through polycarbonate filters [13] using the Extruder device (Lipex Biomembranes, Canada). Vesicles diameters were determined by QEL using a Nicomp model 270 particle analyzer.

Preparation of phase systems

Phase systems of 5% (w/w) Dextran T500 (Lot No. KL 38624, Pharmacia) and 5% (w/w) poly(ethylene glycol), PEG 6000 (Lot No. 9159110, BDH, U.K.), were prepared in (a) 0.01 M sodium phosphate/0.15 M NaCl (pH 6.8); (b) 0.068 M sodium phosphate/0.075 M NaCl (pH 6.8) and (c) 0.11 M sodium phosphate (pH 6.8), which have relatively low, medium and high $\Delta\psi$, respectively [17]. The phases were allowed to separate overnight at 25°C. The PEG-rich top phase and dextran-rich lower phase were separated and stored at −20°C for future use.

Partitioning

Lipid dispersion (50 μl) was applied to a 2 ml system (1 ml each of upper and lower phases) in a 50 × 10 mm tube, all equilibrated at 25°C. The phases were mixed for 1 min by repeated inversion, sampled in triplicate for total counts (50 μl), then the phases allowed to separate for a further 25 min at 25°C, prior to removal of triplicate samples from the top (50 μl) and bottom phases (20 μl) for counting. Results are expressed as a percentage of the total counts added. Counts adsorbed to the interface were calculated from the difference between the total counts added to a phase system and the sum of the counts in the top and bottom phases. No correction has been applied to include material adsorbed to the tube walls or air/water interface. The partition coefficient is defined as the top-phase counts divided by the remainder of the counts added.

Contact angle

Multilamellar vesicles were suspended in 0.15 M NaCl buffered with 0.01 M sodium phosphate (pH 6.8)

at a concentration of 2 mg/ml. 5 ml were filtered onto a 0.45 μm filter (Millipore) with gentle suction, ensuring that a thin layer of phase remained above the filter so that the vesicles did not dry out. The filter was cut into a 3 × 8 mm strip and placed in a 5 mm path-length cuvet containing top phase. The cuvet and contents were mounted vertically on a microscope stage. Drops of the bottom phase were delivered via Hamilton syringe onto the meniscus and allowed to settle onto the vesicle layer. Contact angles were measured on both sides of the drop by rotation of cross-wires in one eyepiece which was connected to a protractor scale. Bulk contact angle measurements on rough surfaces can have a number of sources of variability [18]. Consequently, the absolute values of contact angles obtained by this method must be viewed with reservations. These values do, however, provide an index of changes in the behaviour of surfaces with systematic variations in composition.

Results and Discussion

Fig. 1 shows the partitioning behaviour of both MLVs as well as LUVs prepared by sizing through 100 nm pore size filters, for each of EPC/EPG (6:4 mole ratio) and EPC/EPA (6:4 mole ratio) lipid mixtures, in phase systems of varying $\Delta\psi$. For a given $\Delta\psi$, MLVs exhibited decreased top- and bottom-phase partitioning and increased partitioning to the interface in comparison to LUVs, consistent with previous findings [14]. For both MLVs and LUVs, an increase in $\Delta\psi$ caused an expected increase in the top phase partitioning for these negatively charged vesicles [14–16]. It is also evident, most noticeably in the systems of lowest $\Delta\psi$, that EPC/EPA vesicles showed a greater bottom-phase partitioning than similar vesicles containing EPG. For EPC/EPA MLVs, it can be seen that in the systems of lowest $\Delta\psi$, approximately equal numbers of counts can be found in the bottom phase as in the interface. With increasing potential, approx. 40% of the total counts transfer from the bottom phase through to the interface and, to a much lesser extent, to the top phase. For EPC/EPG MLVs, by comparison, far more counts partition to the interface in the system of lowest $\Delta\psi$. The effect of increasing the potential is to pull approx. 35% of the total counts away from the interface into the top phase. Thus both the EPC/EPA and EPC/EPG MLVs are approximately equally responsive to the pres-

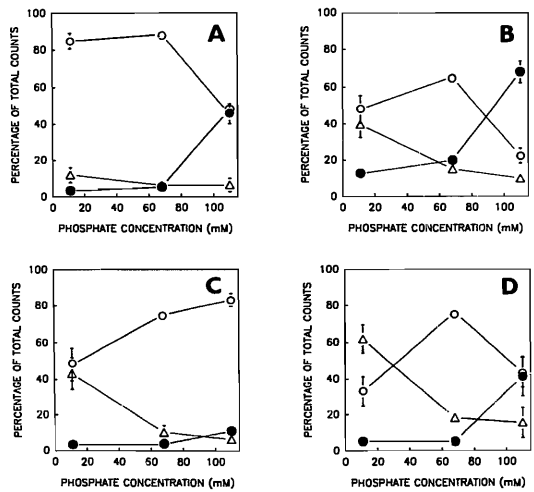

Fig. 1. Partition of (A) EPC/EPG (6:4) MLV, (B) EPC/EPG (6:4) 100 nm vesicles, (C) EPC/EPA (6:4) MLV and (D) EPC/EPA (6:4) 100 nm vesicles in three Dextran T500/PEG 6000 (5%/5%, w/w) systems prepared in (a) 0.01 M sodium phosphate/0.15 M NaCl (pH 6.8); (b) 0.068 M sodium phosphate/0.075 M NaCl (pH 6.8) and (c) 0.11 M sodium phosphate (pH 6.8) which have relatively low, medium and high $\Delta\psi$, respectively. Counts in the (●) top and (△) bottom phases and also at the (○) interface are shown as a percentage of the total counts at 25 min after mixing are shown versus the phosphate concentration of the phosphate-buffered saline (mean ± S.D. of three independent experiments).

TABLE I

Time-course for the partition of EPC/EPG and EPC/EPA vesicles

Values are expressed as percentage of total lipid in the top phase ± S.D., where (n) is the number of experiments. The phase system is Dextran T500/PEG 6000 (5%/5%, w/w) prepared in 0.11 M sodium phosphate (pH 6.8).

Filter pore-size (μm)	Time				
	13 min	25 min	45 min	120 min	> 12 h
EPC/EPG vesciles					
None (MLV)	43.6 ± 12.3 (4)	33.2 ± 9.7 (4)	20.2 ± 4.5 (4)	18.5 ± 4.8 (4)	3.0 ± 2.3 (4)
0.6	43.8 ± 4.8 (4)	32.0 ± 7.9 (4)	29.7 ± 5.6 (4)	26.2 ± 7.8 (4)	3.0 ± 1.4 (4)
0.4	43.4 ± 3.9 (4)	35.8 ± 3.9 (4)	35.5 ± 3.5 (4)	23.2 ± 2.2 (4)	4.6 ± 3.2 (4)
0.2	65.3 ± 12.6 (4)	58.4 ± 12.4 (4)	53.2 ± 13.9 (5)	36.8 ± 4.0 (4)	8.1 ± 5.9 (4)
0.1	69.6 ± 9.5 (4)	62.4 ± 8.7 (5)	61.6 ± 9.2 (5)	48.9 ± 2.3 (4)	30.4 ± 3.8 (4)
0.05	85.1 ± 9.8 (5)	84.6 ± 5.8 (5)	86.8 ± 7.7 (5)	78.0 ± 3.3 (4)	84.7 ± 4.9 (4)
EPC/EPA vesicles					
None (MLV)	20.9 ± 10.0 (4)	15.5 ± 8.1 (4)	12.1 ± 6.7 (4)	9.4 ± 3.2 (4)	4.4 ± 3.1 (4)
0.6	45.0 ± 5.0 (4)	44.4 ± 6.3 (4)	43.7 ± 6.6 (4)	40.9 ± 8.1 (4)	19.5 ± 2.6 (4)
0.4	49.4 ± 4.0 (4)	49.3 ± 7.7 (4)	43.6 ± 6.0 (4)	43.6 ± 11.5 (4)	19.5 ± 3.0 (4)
0.2	50.0 ± 7.2 (4)	48.0 ± 5.3 (4)	46.2 ± 5.6 (4)	46.7 ± 5.9 (4)	23.0 ± 4.5 (4)
0.1	59.3 ± 5.1 (4)	51.1 ± 10.6 (5)	54.9 ± 10.0 (4)	55.5 ± 10.6 (4)	28.2 ± 3.7 (4)
0.05	71.2 ± 6.7 (4)	77.2 ± 9.3 (5)	75.5 ± 10.1 (4)	80.4 ± 11.0 (4)	65.2 ± 6.6 (4)

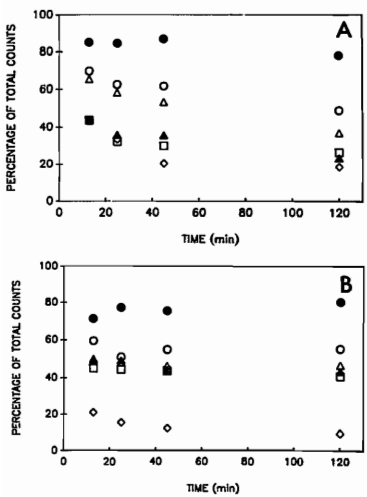

Fig. 2. The kinetics of the partition of (A) EPC/EPG (6:4) and (B) EPC/EPA (6:4) vesicles in a Dextran T500/PEG 6000 (5%/5%, w/w) system of high $\Delta\psi$. The filter pore-size diameter (nm) used for sizing of the vesicles was (●) 50, (○) 100, (◑) 200, (▲) 400 and (□) 600; (◇) corresponds to MLVs which were unsized. Top-phase counts as a percentage of the total counts added are shown versus time.

ence of an increased $\Delta\psi$. The differences in the observed partition behaviour in the system of highest $\Delta\psi$ do not, therefore, reflect any large differences in the vesicle surface charge as detected by the phase systems, but rather differences in the intrinsic wettability of the two surfaces.

Fig. 2 shows the effect of vesicle size upon the kinetics of partitioning of EPC/EPG (6 : 4) and EPC/EPA (6 : 4) vesicles in a phase system of high $\Delta\psi$. For visual clarity, error bars have been omitted from this figure, but partition values with standard deviations are listed in Table I.

It should be noted that for these phase systems, a well-defined macroscopic interface had formed 30 min after mixing the two phases, following addition of vesicles. It is evident from Fig. 2 that the rate of decrease in top-phase counts, for any given size of vesicles, was greatest over the time-period prior to formation of a visible interface. The kinetics of the partition process thus reflect the adsorption of the vesicles to the polymer interface [3–7].

From Table I it is clear that there is no appreciable difference in the rate of top-phase clearance of vesicles sized through 400 or 600 nm pore-size filters, at any time-point, for either EPC/EPA or EPC/EPG mixtures. For EPC/EPG vesicles, those sized through 400 nm pore-size filters partitioned similarly to MLVs. However, for EPC/EPA vesicles, those sized through 200–600 nm pore-size filters behaved similarly, whereas MLVs exhibited a relatively decreased top-phase partition, consistent with the results of Fig. 1. For both lipid mixtures there was a clear trend, most evident at the 12

h time-point (Table I), that smaller vesicles were cleared less rapidly to the interface. For vesicles sized through 50 nm filters, 85% (EPC/EPG) and 65% (EPC/EPA) of the total counts remained in thetop phase after 12 h.

The existence of vesicles in the top phase at times after macroscopic phase separation results from a combination of two general factors. First, microdroplets formed on mixing the two-phase system will settle slowly to the interface. Assuming that the presence of vesicles attached to such microdroplets does not markedly affect the coalescence of the microdroplets, then any vesicles thus attached will settle correspondingly slowly. Second, there are vesicles that exhibit an intrinsically lower affinity for the interface and so remain suspended in one phase. We make no distinction here between vesicles that simply do not attach to the initial microdroplets and vesicles, or aggregates of vesicles, that attach to the phase droplets, but are swept off by a combination of thermal motion and shear forces as the two phases demix.

Since the kinetic experiments (Fig. 2) were all performed in the same phase system (i.e., the interfacial tension was the same) and all samples were mixed identically, there is no reason to assume that there were any differences in the distribution of droplet sizes formed upon mixing the two phases that might affect the rate of settling. Further, there is no evidence that vesicles produced by the extrusion technique exhibit compositional heterogeneity that may affect the partitioning kinetics (unpublished observations).

Fig. 3 indicates that there is an approximately linear relationship between the natural logarithm of the partition coefficient and the EPC/EPG vesicle area at each of the time-points, at least for vesicles of 60–300 nm diameter. This would be consistent with the theory of Brooks and Sharp (reviewed in Ref. 4) and Albertsson's derivation from Bronsted partition theory [1] which indicates that the partition coefficient, K, should be exponentially dependent upon particle surface area.

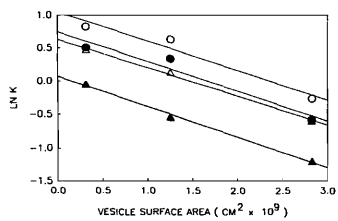

Fig. 3. Plot of the natural logarithm of the partition coefficient, K, versus vesicle surface area for vesicles sized through 100, 200 and 400 nm pore filters at (○) 13, (●) 25, (△) 45 and (▲) 120 min after mixing.

Even though both theoretical treatments are thermodynamic, they predict dynamic partitioning behaviour.

It has been estimated that for phase systems with an interfacial tension of $5 \cdot 10^{-3}$ erg/cm^2, particles with diameter less than approx. 30 nm will not absorb to the interface [4]. This is broadly consistent with our observation that vesicles sized through 50 nm pore-size filters (actual diameter 60 ± 20 nm by QEL) exhibited slow clearance from the top phase.

From the preceding, it is evident that there is a size dependency to the kinetics of the partitioning of lipid vesicles; thus, in examining the effects of lipid composition on partitioning, it is clearly important to compare vesicles of the same average diameter and size distribution. We present in Fig. 4 studies upon the effect of lipid composition on the partitioning of unilamellar vesicles produced by extrusion through 100 nm pore-size filters, in three phase systems of varying $\Delta\psi$.

Vesicles of egg PC alone partitioned predominantly (greater than 70%) to the interface in all three-phase systems, consistent with the lipid having no net charge. We noted that for both 100 nm diameter vesicles and also MLVs, settling of visible aggregates of vesicles to the interface occurred within 5 min after mixing the phases. Certainly part of the explanation of this is that neutral PC vesicles are known to be aggregated by the polymers at the concentrations used to form the two-phase system [19,20]. The incorporation of a charge lipid species in the PC vesicles rendered the partition sensitive to $\Delta\psi$. For negatively charged vesicles, the top-phase partition increased with increasing $\Delta\psi$ (top phase positive with respect to the lower phase), consistent with previous results [14,16]. For vesicles containing stearylamine, increased $\Delta\psi$ resulted in increased partitioning to the bottom phase as previously observed [16]. The differences in the observed partition amongst the various types of negatively charged vesicle arises from two contrasting features of the partition process. First, the incorporation of a negatively charged lipid increased the partition to the bottom phase in the phase system of lowest $\Delta\psi$. PI was the most effective at increasing bottom-phase partitioning, then in sequence, egg PA, egg PG and ganglioside. Second, as $\Delta\psi$ increases, partition moves in favour of the interface and then the top phase.

The observation that the partitioning of the positively charged stearylamine-containing vesicles in the phase system of low $\Delta\psi$ was the same as for egg PC alone (compare Fig. 4A and 4F), is consistent with this phase system having a negligible $\Delta\psi$ and also indicates that the enhanced bottom-phase partitioning observed for the negatively charged vesicles cannot be explained by simple charge considerations. The inference is that negatively charged vesicles exhibit enhanced wetting by the dextran-rich bottom phase or decreased PEG interaction, as compared with EPC. For example, note that

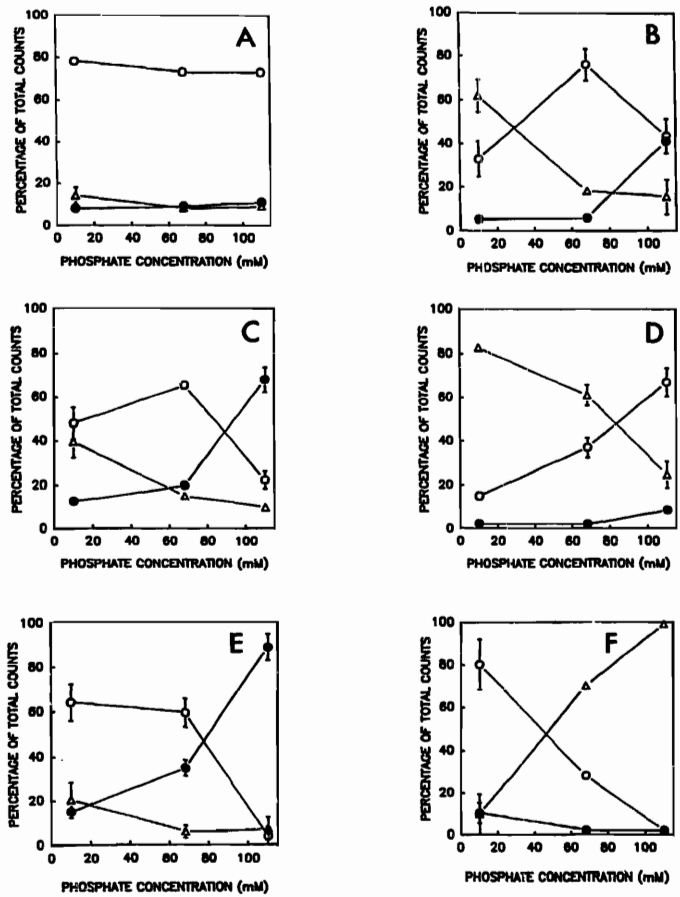

Fig. 4. Effects of composition on the partition of (A) EPC, (B) EPC/EPA (6:4), (C) EPC/EPG (6:4), (D) EPC/EPI (6:4), (E) EPC/ganglioside (6:4) and (F) EPC/stearylamine (6:4) 100 nm vesicles prepared in (a) 0.01 M sodium phosphate/0.15 M NaCl (pH 6.8); (b) 0.068 M sodium phosphate/0.075 M NaCl (pH 6.8) and (c) 0.11 M sodium phosphate (pH 6.8) which have relatively low, medium and high $\Delta\psi$, respectively. Counts in the (●) top and (△) bottom phases and also at the (○) interface at 25 min after mixing are shown versus the phosphate concentration of the phosphate-buffered saline (mean ± S.D. of five independent experiments).

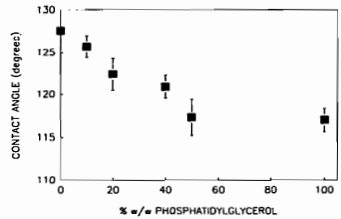

Fig. 5. Change in contact angle with EPG content for MLVs composed of EPC and EPG at 15°C (mean ± S.E.). Phase system is Dextran T500/PEG 6000 (5%/5%, w/w) prepared in 0.01 M sodium phosphate/0.15 M NaCl (pH 6.8) (low $\Delta\psi$).

the bottom-phase partition of the EPC/EPG vesicles was approx. 40% in the system of lowest $\Delta\psi$, compared with 15% for EPC alone. This enhanced wetting should be reflected as a decrease in the contact angle for EPG-containing vesicles as compared to EPC. Fig. 5 shows that this is the case.

While the interactions leading to this increased wetting are as yet undefined, it is a reasonable assumption that the extent to which the dextran can form hydrogen bonds with the headgroups of the phospholipids is in part responsible for this effect. On this basis it is again reasonable that PI with its polyhydroxylated inositol headgroup would be more effective than EPA or EPG at effecting transfer to the lower dextran-rich phase, although it is clear that other factors such as counterion binding to the zwitterionic phospholipid headgroups, the effect of the polymers on the dipole moments of the lipid headgroup, the steric exclusion effects and the hydration forces are all important.

In summary, we have shown that there is a size dependency to the partition of lipid vesicles and that the presence of charged species in the vesicles affects not only their response to $\Delta\psi$ but also their wetting characteristics. Clearly it would be of value to extend these studies to investigate the effects of temperature or the presence of chaotropes, on the partition behaviour of such vesicles. Equally, it is important to know the effects of the polymers on the pK_a of dissociable groups on the membrane surface before surface-charge effects can be rationalized.

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