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# Affinity partitioning of biotinylated mixed liposomes: effect of charge on biotin–NeutrAvidin interaction

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**Abstract**

The partitioning behaviour of biotinylated mixed liposomes in aqueous poly(ethylene glycol)/dextran two-phase systems containing NeutrAvidin–dextran suggests that the biotin–NeutrAvidin affinity interaction is charge dependent. Biotinylated phosphatidylcholine liposomes with a low negative surface charge distributed in the NeutrAvidin-containing bottom phase at neutral pH, but the introduction of additional negative charges by including phosphatidylserine or the surfactant sodium dodecylsulfate in the liposomes caused them to distribute in the poly(ethylene glycol)-rich top phase instead. By gradually lowering the pH of the affinity two-phase system below the isoelectric point (6.3) of NeutrAvidin, negatively charged phosphatidylserine/phosphatidylcholine liposomes increasingly were attracted by NeutrAvidin to the bottom phase. It is suggested that acidic amino acids present at the rim of the biotin-binding pocket of NeutrAvidin may interact electrostatically with charged residues of the closely apposed liposome surface affecting the affinity interaction. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Partitioning; Aqueous two-phase systems; Liposomes; Biotin; NeutrAvidin; Phospholipids

## 1. Introduction

Affinity partitioning in aqueous polymer two-phase systems is potentially a valuable tool for the rapid and highly selective purification of membranes under gentle conditions [1]. This technique was first exploited to enrich membranes from electric organs using cholinergic agonists as affinity ligands [2,3]. The technique also works well for the purification of animal plasma membranes in poly(ethylene glycol) (PEG)/dextran two-phase systems using the lectin wheat germ agglutinin coupled to dextran as affinity ligand [4,5].

Extending the technique to other ligands and membranes has proven difficult, however, perhaps largely due to insufficient knowledge on factors critical for the method to work. To examine such factors, we initiated studies [6] using liposomes as model membranes and biotin–avidin as an affinity couple having a particularly strong interaction. In these studies biotinylated phosphatidylcholine (PC) liposomes were redistributed by NeutrAvidin conjugated to dextran in a PEG/dextran two-phase system from the PEG-rich top phase to the dextran-rich bottom phase [6]. Approximately 1–2 biotin residues incorporated per liposome was sufficient for redistribution to take place and the interaction was enhanced when biotin was coupled to the lipid via an aminohexanoyl spacer arm. Contrary to this, isolated membrane fractions biotinylated in the same manner could not be reproducibly pulled from top to bottom phase by NeutrAvidin–dextran, necessitating further

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studies as to limiting factors of the affinity partitioning system.

As PC liposomes differ from the lipid component of membranes by being electroneutral at neutral pH, while membrane lipids together have a net negative charge, we have now extended the model experiments to biotinylated mixed liposomes to examine whether lipid charge influences the affinity partitioning process using the biotin–avidin affinity couple. It will be shown that the interaction between biotinylated liposomes and NeutrAvidin–dextran is critically dependent on the surface charge of both these components. This may have implications also in other studies based on interactions between biotin and avidin.

## 2. Experimental

### 2.1. Chemicals

Stock solutions in water of 20% (w/w) dextran T500 (Pharmacia Biotech, Sweden) and 40% PEG 3350 (Carbowax 3350; Union Carbide, Danbury, CT, USA) were prepared as described [7]. The dextran was freeze–dried from aqueous solution before use [5]. PC, phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), sodium dodecylsulfate (SDS) and dodecyltrimethylammonium bromide (DTAB) were obtained from Sigma (St. Louis, MO, USA). $^{3}$H-labelled PC was from Amersham Life Sciences (Little Chalfont, UK), immunopure NeutrAvidin and *N*-[6-({biotinoyl}amino)hexanoyl]dipalmitoyl-L-α-phosphatidylethanolamine (biotin-LC-DPPE) were from Pierce (Rockford, IL, USA) and 2,2,2-trifluoroethane sulfonyl chloride (tresyl chloride) was from Synthelec (Lund, Sweden). All other reagents were of analytical grade.

### 2.2. Preparation of liposomes

Small unilamellar vesicles of different lipid mixtures were prepared essentially as described [8]. A liposome preparation contained 3.96 mg of phospholipid and 0.04 mg biotin-LC-DPPE [both in chloroform–methanol (95:5, (v/v)] and 14 kBq $^{3}$H-phosphatidylcholine in a glass tube. The latter was added to monitor liposome partitioning radiometrically. After vortexing the mixture for 1 min, the solvent was evaporated under a stream of nitrogen and the sample further dried in vacuum overnight. The lipid film obtained was dispersed by vortexing in 0.2 ml of buffer (the same buffer as used for liposome partitioning) at 65°C, and the mixture was sonicated for 2 min in an ice-water bath with a Branson B-30 Sonifier (Branson Sonic Power, Danbury, CT, USA) equipped with a microtip (output setting 1, duty cycle 50%) to obtain small unilamellar vesicles. Sonication was for 30-s bursts with 10-s intervals to avoid undue heating. Surfactants (SDS or DTAB) included in the liposomes were added to the phospholipid mixture before evaporation.

### 2.3. Coupling of NeutrAvidin to dextran

Freeze–dried dextran was activated with tresyl chloride as described [5]. All organic solvents used in the activation procedure had been dried over molecular sieve and the glass material thoroughly dried in an oven to avoid inactivation of tresyl chloride. The tresyl–dextran was freeze–dried and stored at −20°C. It can be stored under these conditions for several months. The coupling step was done essentially as described [4,5]. Special attention was paid to add the dissolved NeutrAvidin dropwise to the dextran solution under vigorous vortexing to ensure optimum coupling. After repeated concentrations and redilutions with water using a Jumbosep centrifugal concentrator with a molecular mass cut-off of 100 000 (Pall Filtron, Northborough, MA, USA) to remove uncoupled NeutrAvidin and salts, the product was freeze–dried. The amount of NeutrAvidin coupled was determined by protein measurement [9] using NeutrAvidin as standard. Approximately 3 mg of NeutrAvidin were conjugated per g dextran.

### 2.4. Affinity two-phase partitioning

Affinity partitioning experiments were performed in two phase systems with a total mass of 1.0 g contained in 3-ml disposable plastic Ellerman tubes. Each system was prepared by dissolving the required amount of NeutrAvidin–dextran T500 in appropriate

amounts of PEG 3350, dextran T500, $Li_2SO_4$ and buffer stock solutions and water added to a total mass of 0.990 g. The system was mixed thoroughly and left to equilibrate at 4°C. Standard affinity systems contained (final concentrations) 10 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–NaOH, pH 7.5, 20 m*M* $Li_2SO_4$, 5.6% (w/w) each of PEG and dextran and 30 μg of NeutrAvidin bound to dextran. NeutrAvidin–dextran was omitted from blank systems. A 10-μl volume of liposome suspension containing 0.2 mg phospholipid was added to each system. The systems were incubated for 30 min at 4°C on a rocking table before phase separation, which was accelerated by gentle centrifugation. The phase boundaries were marked and each top phase was carefully removed to new tubes, leaving the bottom phase (including the interface). All operations were performed in a well-tempered cold room (4°C) as the partitioning process is strongly dependent on temperature.

### *2.5. Quantification of liposomes*

Liposomes in top and bottom phases were quantified radiometrically (Beckman liquid scintillator LS 1801, Beckman Instruments, CA, USA). To avoid quenching 100 μl of each phase was mixed with an equal volume of 10% SDS and incubated for 30 min prior to the addition of 7 ml Beckman ReadySafe scintillation cocktail.

## 3. Results

### *3.1. Affinity partitioning of mixed liposomes*

As was examined in detail earlier [6] addition of NeutrAvidin–dextran caused biotinylated PC liposomes to redistribute from the PEG-rich top phase to the dextran-rich bottom phase in an affinity two-phase system containing 5.6% each of PEG 3350 and dextran T500, 20 m*M* $Li_2SO_4$ and 30 μg/g phase system of NeutrAvidin coupled to dextran (cf. Fig. 1A). When biotinylated mixed liposomes containing increasing amounts of PS together with PC were partitioned in the same two-phase system, however, the fraction distributing in the bottom phase in the presence of NeutrAvidin–dextran decreased (Fig. 1A). Thus, more than 90% of PC liposomes and liposomes with an admixture of up to 4% (w/w) of PS were found in the bottom phase, but with 10% of PS and above 80–85% distributed in the top phase instead. Mixed biotinylated liposomes containing 10% (w/w) PG or PI together with PC partitioned in the top phase to the same extent as those containing 10% PS (not shown). In the absence of NeutrAvidin–dextran approximately 90% of the PS/PC liposomes distributed in the top phase at all lipid ratios tested (Fig. 1A), as did non-biotinylated liposomes both in the presence and absence of NeutrAvidin–dextran (not shown), indicating that distribution in the bottom phase was due to interactions between the biotin and NeutrAvidin moieties. The possibility that Neutr-

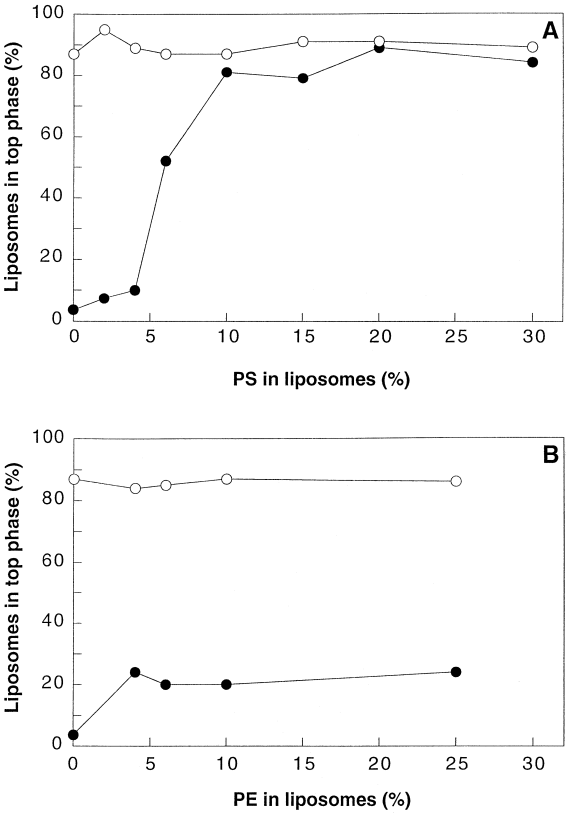


Fig. 1. Affinity partitioning of biotinylated PC liposomes containing increasing amounts of (A) PS or (B) PE. Standard 1-g two-phase systems were prepared (Section 2.4) with (closed circles) or without (open circles) 30 μg NeutrAvidin coupled to dextran. Liposomes contained the indicated amounts (by weight) of PS or PE together with PC.

Avidin–dextran repartitioned into the top phase in the presence of mixed PS/PC liposomes containing 10% PS, and that these therefore partitioned in the top phase, was also considered. As NeutrAvidin was detected (by protein measurement) only in the bottom phase after partitioning of these liposomes as well as of PC liposomes this possibility seems less likely.

A possible explanation for the different affinity of the mixed liposomes and pure PC ones might be charge differences; while PC is electroneutral at neutral pH, inclusion of PS, PG or PI in the liposome will introduce negative charges. One test of this possibility was to examine the affinity behaviour of mixed PE/PC liposomes where PE is electroneutral like PC and not negatively charged as PS. The results of such an experiment (Fig. 1B) shows that biotinylated mixed PE/PC liposomes with different contents of PE preferentially (approximately 80%) distributed in the bottom phase in a NeutrAvidin-dependent manner similar to pure PC liposomes, indicating that the behaviour of PC liposomes is not due to some specific surface property inflicted by the polar headgroup of PC affecting the affinity distribution. Instead the results reinforce the possibility that the introduction of negative charges in the liposomes weakens the interaction between biotin and NeutrAvidin in the partitioning system.

### *3.2. Effect of charged surfactants*

A further test to distinguish whether surface charge is a major interfering factor in affinity partitioning of liposomes using the biotin–NeutrAvidin affinity couple would be to incorporate charged molecules lacking the phospholipid polar headgroup, which might itself affect the partitioning process, into liposomes. To this end we used the charged surfactants DTAB and SDS which will readily insert into phospholipid bilayers. Negative charges were introduced into PC liposomes by adding SDS when forming the liposomes, while the net negative charge of mixed PS/PC liposomes was reduced by including DTAB. The surfactants were added in concentrations well below their critical micelle concentrations not to disrupt liposome structure.

The introduction of SDS into biotinylated PC liposomes changed their distribution from approximately 90% in the NeutrAvidin-containing bottom phase to 80% in the top phase both at an addition of 15 and 25 nmol of SDS to a standard affinity system containing 258 nmol PC (Fig. 2A). PS/PC mixed liposomes with a comparable fraction of charged lipids, i.e., containing 6% and 10% PS, partitioned to 50% and 90% in the top phase (Fig. 2B). Neutralising the negative charges by inclusion of DTAB brought these liposomes more into the bottom phase (Fig. 2B), although not to the same extent as PC liposomes as approximately 25% and 40%, respec-

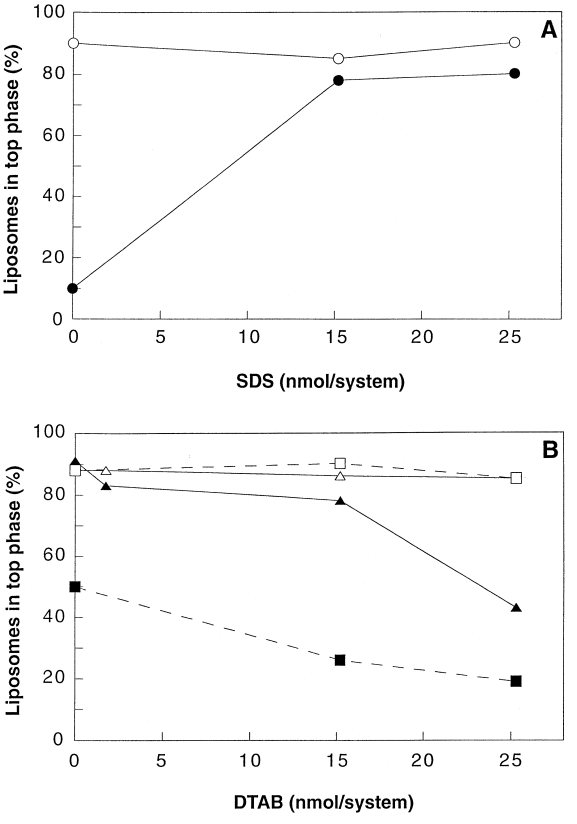


Fig. 2. Affinity partitioning of biotinylated liposomes containing added surfactants. Standard 1-g two-phase systems were prepared (Section 2.4) with (closed symbols) or without (open symbols) 30 μg NeutrAvidin coupled to dextran. (A) PC liposomes with added SDS. (B) Mixed PS/PC liposomes containing 6% (w/w) PS (squares) or 10% (w/w) PS (triangles) with added DTAB. Each system contained 258 nmol phospholipid and indicated amounts of surfactants.

tively, remained in the top phase at close to electroneutrality of included molecules.

The relationship between the distribution of biotinylated liposomes of different compositions and their calculated net surface charge is shown in Fig. 3 (a combined replot of the results presented in Figs. 1A and 2). To calculate the net surface charge the following assumptions were made: (1) all liposomes are of the same size as biotinylated PC liposomes, i.e., having a diameter of 20 nm with a particle mass of $1.88 \cdot 10^6$ and a 2:1 proportion of lipid molecules between the outer and inner liposome surfaces [6]. This assumption was based on the fact that the liposomes mainly contain PC and are therefore not likely to differ substantially in structure from biotinylated PC liposomes. (2) Different phospholipids and surfactants distribute randomly, and with the same density, between the inner and outer leaflets of the liposome bilayer excepting biotin-LC-DPPE which, due to steric reasons (protruding biotin residue and small size of liposome) is confined to the outer leaflet (cf. Ref. [6]).

Using these assumptions an admixture of 1% biotin-LC-DPPE (carrying one negative charge per molecule at neutral pH) gives PC liposomes a net surface charge of −17 (Fig. 3, cross in square; cf. Ref. [6] for calculations). Including PS in the liposomes increased their net negative charge with a simultaneous redistribution into the top phase (Fig. 3, squares; replot of Fig. 1A). The midpoint of this redistribution occurred at an admixture of 6% PS corresponding to a net surface charge of approximately −100 per liposome (closed circle in square), whereas maximum redistribution occurred at 10% PS (open circle in square). A similar dependency on net surface charge for the distribution was observed when PC liposomes were rendered more electronegative by inclusion of SDS (Fig. 3, crosses; replot of Fig. 2A) or PS/PC liposomes less electronegative by inclusion of DTAB [Fig. 3, closed (6% PS) and open (10% PS) circles; replots of Fig. 2B], although in these latter cases the redistribution between the phases occurred, as calculated using the above approximations, at a lower net negative surface charge than calculated for PS/PC liposomes.

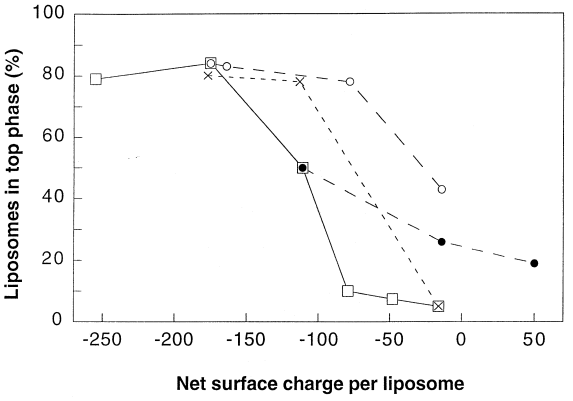


Fig. 3. The relationship between net surface charge of biotinylated liposomes and their distribution in 1-g affinity two-phase systems containing 30 μg NeutrAvidin coupled to dextran. Net surface charge was calculated using the approximations given in the text (Section 3.2). Open squares, PC liposomes with different amounts of PS (replot of Fig. 1A); crosses, PC liposomes with different amounts of SDS (replot of Fig. 2A); circles, mixed PS/PC liposomes containing 6% (w/w) PS (closed circles) or 10% PS (open circles) with different amounts of DTAB (replot of Fig. 2B). Combined symbols represent partitionings done with liposomes without added surfactants.

### 3.3. Effect of pH

An alternative test of whether charge affects the interaction between biotinylated liposomes and NeutrAvidin–dextran would be to vary the charge of NeutrAvidin rather than that of liposomes. The affinity distributions reported so far were performed at pH 7.5 where NeutrAvidin, with an isoelectric point (p*I*) of 6.3, has a net negative charge. To alter this, affinity distributions were instead performed below the p*I* of NeutrAvidin, in a pH interval where the biotinylated mixed PS/PC liposomes retain their net negative charge. Lowering the pH caused liposomes containing either 6% or 10% PS to redistribute increasingly into the NeutrAvidin-containing bottom phase (Fig. 4). While 50% and 80%, respectively distributed in the top phase at pH 7.5, close to 90% were found in the bottom phase at pH 5 (6% PS, closed triangles) or pH 4 (10% PS, closed squares). In comparison, more than 80% of the liposomes distributed in the top phase throughout the pH range examined in the absence of NeutrAvidin–dextran (open symbols). Thus, the NeutrAvidin-dependent affinity distribution of negatively charged biotinylated liposomes was strongly dependent on

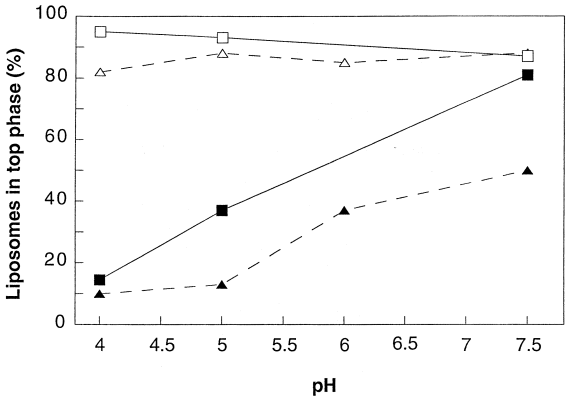


Fig. 4. Effect of pH on the partitioning of mixed biotinylated PS/PC liposomes. One-g systems were prepared as described in Section 2.4 with (closed symbols) or without (open symbols) 30 μg NeutrAvidin coupled to dextran. The liposomes contained 6% (w/w) PS (triangles) or 10% PS (squares). The buffers used were (final concentrations): pH 7.5, 10 m*M* HEPES–NaOH; pH 6.0, 5.0 and 4.0, 10 m*M* sodium citrate.

the pH of the two-phase system, and the interaction was favoured at pH values where NeutrAvidin had a net positive charge, i.e., below its p*I*.

## 4. Discussion

The experiments presented here were performed to elucidate basic factors affecting the affinity partitioning of membranes in aqueous two-phase systems. Conventional partitioning of membranes in such systems is influenced by several parameters, including the concentration and kind of two-phase polymers used and added salts [10,11]. The partitioning behaviour depends on surface properties of the membranes that are rather less well-defined. Additional factors to be defined are introduced in affinity partitioning, including interactions between the affinity couple utilised and, particularly when separating membranes, non-specific interactions between components in the membrane and the affinity ligand. In the present experiments liposomes were used as model membranes to avoid complications introduced by various membrane components, including proteins and the different composition and distribution of phospholipids in membranes, in order to be able to focus on the affinity event per se. Biotin–NeutrAvidin was used as the affinity couple because of the strong and well defined interaction between them, NeutrAvidin being a deglycosylated form of avidin with a p*I* of 6.3 retaining the strong binding to biotin [12] but having less non-specific interactions with other components (according to the manufacturer).

The results obtained indicate that charge is a limiting factor in the biotin–NeutrAvidin interaction, at least when this is exploited for affinity partitioning of liposomes. They also indicate that the charge of both participants in the affinity interaction, i.e., biotinylated liposomes and NeutrAvidin, can be altered in such a way as to enhance or weaken the interaction as monitored by the distribution of liposomes in the two-phase system. A sufficiently strong affinity to bring about NeutrAvidin-dependent redistribution of biotinylated liposomes from top to bottom phase prevailed when the liposomes had, at the most, few negative charges at the same time as the partitioning pH was kept above the p*I* of NeutrAvidin, i.e., this protein had a net negative charge (Fig. 3). NeutrAvidin with a net positive charge (below its p*I*), on the other hand, was able to attract liposomes with a high negative charge (Fig. 4).

The net surface charge of the liposomes was manipulated either by forming negatively charged mixed PS/PC liposomes or by inclusion of the positively or negatively charged surfactants DTAB or SDS in these mixed liposomes or in electroneutral PC ones. In all cases examined the distribution in the affinity two-phase system followed the charge restrictions outlined above, where a like net charge of liposomes and NeutrAvidin tended to decrease, and a different charge increase, the affinity interaction. The effect on liposome distribution when titrating liposome charge with the surfactants was different in extent from the expected when compared with PS (Fig. 3); negative charges introduced by SDS into PC liposomes had a greater effect, while neutralising the negative charges of PS in PS/PC liposomes with DTAB had a less pronounced one. This difference might in part be due to approximations made when calculating the liposome net surface charge (see Section 3.2), particularly regarding the transverse distribution across the phospholipid bilayer assumed for different phospholipids and surfactants. The

difference was quantitative rather than qualitative, however, and does not affect the general conclusion.

The detailed knowledge of the structure of the biotin binding site of avidin [13,14] offers an explanation of how the affinity partitioning process might be affected by liposome charge as well as by pH alterations. The avidin molecule forms a β-barrel structure made up of eight antiparallel β-strands. The biotin binding site is close to one end of the barrel, and forms a pocket whose entrance is surrounded by three polypeptide loops (loops 3, 5 and 7 in the structure). Interestingly, two of these loops contain acidic amino acids situated around the entrance to the biotin binding pocket, in loop 7 the residues $Asp_{105}$ and $Asp_{108}$ and in loop 5 $Glu_{74}$. In addition, biotin binds rather deeply in the pocket with the carboxyl group 10–12 Å below the protein surface formed by the loops. We found earlier [6] that the coupling of biotin to the phospholipid via an aminohexanoyl spacer arm greatly enhanced the affinity interaction between biotinylated PC liposomes and NeutrAvidin–dextran. This same spacer, with an arm length of 8–9 Å, was used also in the present investigation. Thus, when the biotin moiety of liposomes binds in the NeutrAvidin pocket the liposome and protein surfaces tend to be closely apposed as the liposome is comparatively large and cannot penetrate between the loops in the protein structure. As a consequence a weakening of the otherwise very strong binding between biotinylated liposomes and NeutrAvidin due to electrostatic repulsion would be expected when both components have the same charge. This was observed at pH 7.5, i.e., above the p*I* of NeutrAvidin, when increasing amounts of negatively charged PS or SDS were included together with electroneutral PC in the liposomes causing these to distribute in the top phase of the two-phase system away from the NeutrAvidin–dextran containing bottom phase (Figs. 1A and 2A). The acidic amino acid residues at the entrance of the biotin binding pocket would then be negatively charged, tending to repel these liposomes electrostatically. When the pH of the affinity two-phase system was decreased the mixed PS/PC liposomes retained their negative charge but increasingly distributed in the NeutrAvidin-containing bottom phase. This was presumably due to the gradual protonation of the carboxyl groups of the acidic amino acid residues at the rim of the biotin-binding pocket thereby decreasing the electrostatic repulsion between the affinity pair. The redistribution observed into the NeutrAvidin-containing bottom phase occurred at a pH well above the p$K_a$ value of ca. 4 of the side chain carboxyl groups of the free amino acids. This is to be expected as there is usually a charge shift of these groups to more alkaline values when the amino acids are incorporated into proteins.

The affinity system used tolerated some net negative charges in the liposomes at a pH above the p*I* of NeutrAvidin introduced by the affinity component biotin-LC-DPPE and of a limited amount of other negatively charged lipids. As a consequence, the effect of charge on the affinity interaction did not become apparent when the affinity partitioning of biotinylated PC liposomes was studied [6]. As was shown here the limitations of the affinity interaction could be overcome by altering the net charge of either of the affinity components. An alternative approach, suggested by the structure of the biotin-binding pocket of NeutrAvidin, would be to introduce a longer spacer arm when coupling biotin to phospholipid to avoid the close apposition between the liposome and NeutrAvidin surfaces on affinity binding. This would possibly allow affinity partitioning of negatively charged liposomes without pH restrictions, and would then also open up the possibility of using the biotin–NeutrAvidin affinity couple to examine critical factors in the affinity partitioning of membranes.

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