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Aqueous two-phase affinity partitioning of biotinylated liposomes using neutral avidin as affinity ligand

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Abstract

Biotinylated small unilamellar liposomes were affinity partitioned in an aqueous poly(ethylene glycol)–dextran two-phase system using avidin coupled to dextran as affinity ligand. In the absence of affinity ligand more than 90% of the liposomes partitioned in the poly(ethylene glycol)-rich top phase, whereas in its presence more than 95% partitioned in the dextran-rich bottom phase. For this redistribution to occur 10 m*M* and above of lithium sulphate, or other appropriate salts, had to be added to the two-phase system. Without added salt the liposomes with complexed avidin–dextran instead partitioned in the top phase. An extended mixing time for the system was required for maximum redistribution. Less than two biotin residues per liposome, coupled via a C_6 spacer arm, was required to redistribute the liposomes to the bottom phase.

Keywords: Partitioning; Affinity partitioning; Liposomes; Avidin–dextran ligands; Phospholipids

1. Introduction

Biological membranes are usually fractionated by centrifugation, often combining differential and density gradient centrifugations. The fractionation parameters, particle size and buoyant density, frequently overlap between membranes, however, resulting in incomplete separation. The adoption of alternative separation methods based on more specific factors therefore would be useful. Affinity partitioning in aqueous polymer two-phase systems [1] is one such potentially useful method in which a ligand conjugated to one of the phase polymers selectively causes a redistribution of the membranes of interest into the ligand-containing phase. The method is principally similar to affinity techniques in which the ligand is coupled to a solid-phase, but being advantageous by keeping the membranes in aqueous phase during the fractionation procedure.

So far affinity partitioning has been used to fractionate membranes [2–5] and cells [6–8] only in a few instances. In one case highly purified plasma membranes were obtained using a lectin as affinity ligand [4,5]. The lectin then was coupled to dextran, and selectively caused the redistribution of plasma membranes from the poly(ethylene glycol)-rich (PEG) top phase to the dextran-rich bottom phase in a PEG–dextran two-phase system, leaving other membranes in the top phase. Further attempts to extend the affinity method to other ligands and membranes have been less successful, however. Clearly, there are aspects of the affinity process that

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are not fully understood, impeding a more general use of the method for membrane purification. To better understand basic requirements for the method to work, we have now examined various separation parameters in a model system in which biotinylated small unilamellar phospholipid vesicles were affinity partitioned in a PEG–dextran two-phase system using avidin coupled to dextran as affinity ligand.

2. Experimental

2.1. Chemicals

Stock solutions in water of 20% (w/w) Dextran T500 (Pharmacia Biotech, Sollentuna, Sweden) and 40% (w/w) PEG 3350 (Carbowax 3350; Union Carbide, Danbury, CT, USA) were prepared as described [9]. The dextran was freeze-dried from aqueous solution before use [5]. Phosphatidylcholine (PC) from egg yolk was from Sigma (St. Louis, MO, USA) and ^{3}H-labelled PC from Amersham International (Little Chalfont, UK). Immunopure NeutrAvidin, *N*-(biotinoyl)dipalmitoyl-L-α-phosphatidylethanolamine (biotin-DPPE) and *N*-[6-(biotinoyl)amino)hexanoyl]dipalmitoyl-L-α-phosphatidylethanolamine (biotin-LC-DPPE) were from Pierce (Rockford, IL, USA) and 2,2,2-trifluoroethane sulphonyl chloride (tresyl chloride) was from Synthelec (Lund, Sweden). All other reagents were of analytical grade.

2.2. Preparation of liposomes

Small unilamellar vesicles were prepared essentially as described [10]. Non-biotinylated ones were prepared from PC only, whereas biotinylated liposomes also contained biotin-LC-DPPE or biotin-DPPE. A standard mixture for liposome preparation contained 3.96 mg PC and 0.04 mg biotin-LC-DPPE (i.e., 1%, w/w, biotin-LC-DPPE) dissolved in 1.2 ml chloroform–methanol (9:1, v/v) and 7–14 kBq of ^{3}H-labelled PC. The latter was added to monitor liposome partitioning radiometrically. The solvent was removed under a stream of nitrogen and the sample further dried in vacuum overnight. The lipid film was dispersed by vortexing in 0.2 ml 10 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)–NaOH, pH 7.5, at 65°C and sonicated for 2 min with a Branson B-30 Sonifier equipped with a microtip (output setting 1, duty cycle 50%) to obtain small unilamellar vesicles. Sonication was for 30-s bursts with 30-s intervals to avoid undue heating. The vesicles were used for partitioning experiments after a stabilisation period of 30 min at 65°C.

2.3. Coupling of avidin to dextran

Freeze-dried dextran [5] was activated with tresyl chloride essentially as described earlier [4,11]. All organic solvents used in the activation procedure had been dried over molecular sieve. NeutrAvidin was coupled to tresyl-dextran as in Refs. [4,5], but using 0.1 *M* $NaHCO_3$, adjusted to pH 7.5 with HCl, as coupling buffer. Excess tresyl groups were inactivated by the addition of 0.2 *M* Tris–HCl, pH 7.5. After repeated ultrafiltration in a Filtron Omegacell to remove uncoupled avidin and salts, the product was freeze-dried. The amount of avidin coupled was 4.5–4.9 mg per g freeze-dried product as determined according to Bradford [12] using NeutrAvidin as standard. This corresponds to a dextran–avidin average molar ratio of approximately 23:1. The number of biotin binding sites, analyzed as in [13], were about 3.5 per dextran-coupled avidin molecule.

2.4. Two-phase affinity partitioning

Affinity partitioning experiments were performed in two-phase systems with a total mass of 2.0 g contained in 3-ml disposable plastic Ellerman tubes. Each system was prepared by dissolving the required amount of avidin–dextran in appropriate amounts of buffer and salt stock solutions, and then adding PEG and dextran stock solutions to a final polymer concentration of 5.6% (w/w) each. The total mass of the system was balanced to 2.0 g with water. Each system was mixed thoroughly and left to equilibrate at 4°C overnight. Standard two-phase systems contained 10 m*M* HEPES–NaOH buffer, pH 7.5, 10 m*M* Li_2SO_4 and 90 μg of avidin bound to dextran. Avidin–dextran was omitted from blank systems.

Ten μl of liposome suspension containing 0.2 mg phospholipid was added to each system, and the systems were mixed thoroughly by vortexing for 20 s. The systems were agitated for 30 min before phase

separation, which was accelerated by gentle centrifugation (Wifug Doctor, 1250 rpm) for 5 min. Each top phase was carefully removed to new tubes, leaving bottom phase plus interface. All these operations were performed strictly at 4°C as the partitioning process is strongly dependent on temperature. Liposomes in both phases were quantitated radiometrically in Beckman ReadySafe scintillation cocktail. Samples of the bottom phase were treated with 5% sodium dodecyl sulphate prior to the addition of cocktail to avoid quenching.

3. Results and discussion

The partitioning of material in a conventional two-phase system is critically dependent on the composition of the system. The most important factors are the kinds of salts present and their concentrations, but the distribution of material is affected also by the molecular size of the phase polymers and their respective concentration [14]. The strategy was to select conditions under which liposomes would distribute in the PEG-rich top phase in a PEG–dextran two-phase system, whereas added avidin–dextran would cause a selective redistribution of biotinylated liposomes from the top phase to the dextran-rich bottom phase.

3.1. Salt dependency

In an affinity two-phase system containing 5.6% each of PEG and dextran and 10 m*M* HEPES–NaOH, but without added extra salt, approximately 95% of non-biotinylated liposomes distributed in the PEG-rich top phase (Fig. 1). Under these conditions, however, added avidin–dextran only caused a partial redistribution of biotinylated liposomes into the dextran-rich bottom phase, 80% of them remaining in the top phase. To attain a better redistribution appropriate salts had to be added to the system. Thus, the amount of biotinylated liposomes remaining in the top phase decreased to approximately 5% in the presence of 10 m*M* Li_2SO_4 and above (Fig. 1), whereas non-biotinylated liposomes, or biotinylated liposomes omitting avidin–dextran from the system, remained in the top phase. Avidin–

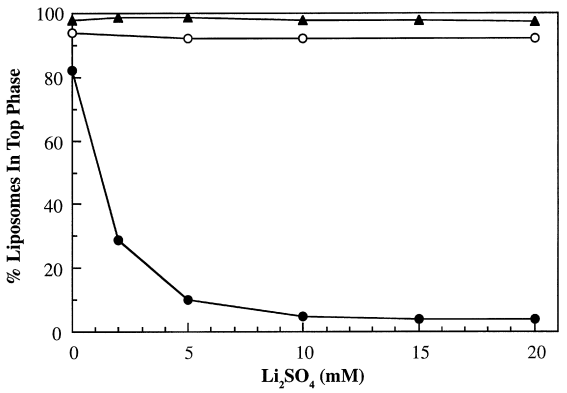

Fig. 1. Affinity partitioning of liposomes with increasing concentrations of Li_2SO_4. Each 2-g system contained 5.6% (w/w) of each phase polymer in 10 m*M* HEPES–NaOH, pH 7.5, 90 μg dextran-bound avidin (when present) and the concentration of salt indicated. Ten μl of liposome suspension was partitioned in each system. Biotinylated liposomes contained 1% (w/w) biotin-LC-DPPE. Each point represents the mean of duplicate determinations, the deviation from the mean being less than 1%. Biotinylated (●) and non-biotinylated (○) liposomes partitioned in systems containing avidin–dextran; (▲) biotinylated liposomes partitioned in systems without avidin–dextran.

dextran itself partitioned in the dextran-rich phase both in the presence and absence of Li_2SO_4.

To analyse in detail why salt had to be added to bring about affinity redistribution of the liposomes, the partitioning of both biotinylated liposomes and avidin–dextran was followed at increasing Li_2SO_4 concentrations (Fig. 2). Without added salt and at very low salt concentrations, when liposomes predominantly distributed in the top phase, slightly more avidin–dextran was found in this phase than at higher salt concentrations when both liposomes and avidin–dextran mainly partitioned in the bottom phase. The amount of avidin–dextran redistributed in this manner approximately corresponded to one avidin per liposome present in the system. This suggests that biotinylated liposomes form complexes with avidin–dextran under all conditions, which is expected from the very strong interaction between biotin and avidin, but that the liposomes caused a redistribution of the complexed fraction of avidin–dextran from the dextran-rich bottom phase to the top phase at low salt concentrations. Thus, the distribution of the adduct may be a matter of concern, at least when large structures as liposomes,

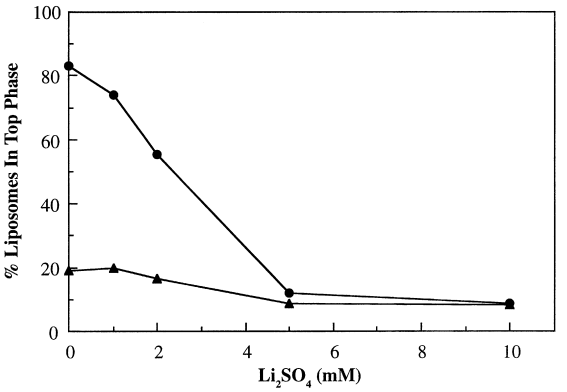

Fig. 2. Co-distribution of biotinylated liposomes and avidin–dextran at various Li_2SO_4 concentrations. Standard 2-g phase systems with 10 m*M* HEPES–NaOH buffer, pH 7.5, and 90 μg of dextran-bound avidin were used. The distribution of avidin (▲) was measured by protein analysis [12]; the phase polymers did not interfere with the analysis; (●) the distribution of liposomes.

membranes or cells are the affinity partitioned species.

As different salts may affect the partitioning of charged macromolecular species differently [14] other salts were also tested. Na_2SO_4 had the same capacity as Li_2SO_4 in bringing about an avidin–dextran dependent redistribution of biotinylated liposomes, whereas higher concentrations of LiCl and, in particular, of sodium phosphate were required (Fig. 3). NaCl had the same effect as LiCl (not shown). The liposomes remained in the top phase with all these salts when avidin–dextran was omitted from the two-phase systems (only shown for sodium phosphate in Fig. 3).

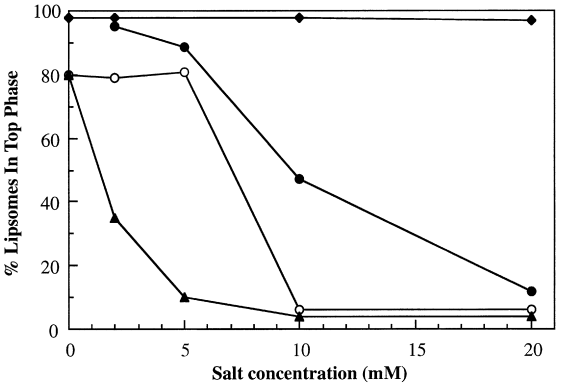

Fig. 3. Affinity partitioning of biotinylated liposomes in the presence of various salts. Conditions were as in Fig. 1. Partitionings in the presence of avidin–dextran and the indicated concentrations of Na_2SO_4 (▲), LiCl (○), or sodium phosphate (●), and sodium phosphate without avidin–dextran (♦).

Thus, all salts tested had a similar effect in bringing down the complex of liposomes and avidin–dextran to the bottom phase. This effect is likely not to be purely electrostatic, but more information on the partitioning of liposomes and phase polymer adducts in two-phase systems under different experimental conditions will be needed to explain the behaviour of the complex.

3.2. Extent of mixing

A short but thorough mixing of the liposomes with the phase system followed by immediate phase separation was not sufficient for an optimum affinity interaction between biotin and avidin. Thus, 40% of the biotinylated liposomes remained in the PEG-rich top phase under such conditions (Fig. 4). A more complete redistribution to the bottom phase, with only 5% remaining in the top phase, was observed when the mixing time was extended to 30 min before phase separation. This extended mixing time was used in the experiments to ensure optimum partitioning conditions. A tentative explanation to this behaviour is that some avidin molecules (having an average of 3.5 biotin binding sites, see Section 2.3)

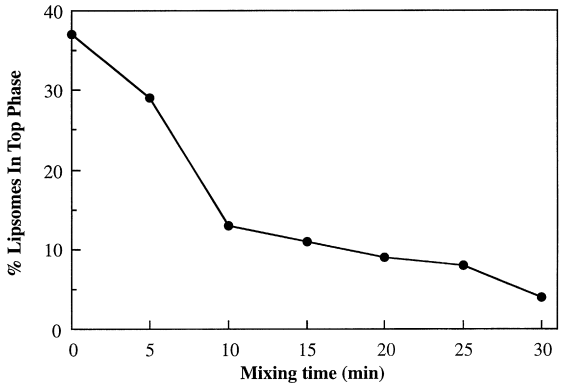

Fig. 4. The effect of mixing time on the distribution of liposomes. Affinity partitionings were performed under standard conditions (see Section 2.4) varying the extent of mixing prior to phase separation. The two-phase systems were mixed by vortexing for 20 s followed by tube inversions for the indicated times. Zero time represents vortexing followed by immediate phase separation by centrifugation.

initially might have bound more than one biotinylated liposome, and that such complexes were not stable due to steric interference by the bulky liposomes. More extended mixing times then would have allowed rearrangement to more stable stoichiometric complexes to take place (see below).

3.3. Number of binding sites required to redistribute liposomes

The effect of varying the amount of biotin in the liposomes is shown in Fig. 5. The biotin-LC-DPPE content was varied between 0.01 and 1% (w/w) of the total phospholipids, and the liposomes were partitioned in a two-phase affinity system containing 90 μg of dextran-bound avidin, i.e., a molar excess over liposomal biotin. At 0.5% biotin-LC-DPPE and above more than 95% of the liposomes were attracted to the bottom phase, whereas the amount of biotin became limiting at lower concentrations.

The results at these limiting conditions could be used to estimate the number of biotin residues required to redistribute a liposome from top to bottom phase. The number of biotin-LC-DPPE molecules per liposome can be assumed to follow a Poisson distribution. The probability, *p*, of a liposome having *k* biotins when the average number is *m* per liposome is

$$p(k) = e^{-m}m^{k}/k! \quad (1)$$

resulting in the following probabilities

$$p(0) = e^{-m} \quad (2)$$

$$p(1) = e^{-m}m \quad (3)$$

$$p(>1) = 1 - e^{-m}(1+m) \quad (4)$$

The average number of biotin residues per liposome, *m*, can be calculated at different mass percentages, *r*, of biotin-LC-DPPE

$$m = \frac{r}{100} \times \frac{Mr_{\text{liposome}}}{Mr_{\text{biotin-LC-DPPE}}} \quad (5)$$

To estimate the particle mass of the liposomes, Mr_{liposome}, their size was determined by flow field-flow fractionation [15]. Their average diameter was found to be 20 nm agreeing well with earlier determinations of the size of PC liposomes having a particle mass of $1.88 \cdot 10^6$ as calculated from hydrodynamic measurements [16]. This particle mass was used as a value for Mr_{liposome}. The molecular mass for biotin-LC-DPPE is 1133. Furthermore, PE usually prefers the outer monolayer in PC:PE vesicles containing less than 10 mol% PE [17], a tendency likely to be enhanced by the bulky biotin ligand.

The distribution of liposomes can be described by

$$T = ap(0) + bp(1) + cp(>1) \quad (6)$$

where *T* is the percentage of liposomes in the top phase and *a*, *b* and *c* are the percentages of liposomes with zero, one and more than one biotin per liposome, respectively, in the top phase. Insertion of Eqs. (2)–(4) gives

$$T = ae^{-m} + be^{-m}m + c\left[1 - e^{-m}(1+m)\right] \quad (7)$$

The set of data in Fig. 5 can be fitted to Eq. (7) giving estimations of *a*, *b* and *c*. The curve fit indicates that liposomes containing more than one biotin are maximally redistributed to the bottom phase. The estimation of the redistribution of liposomes with one biotin is less certain. The results

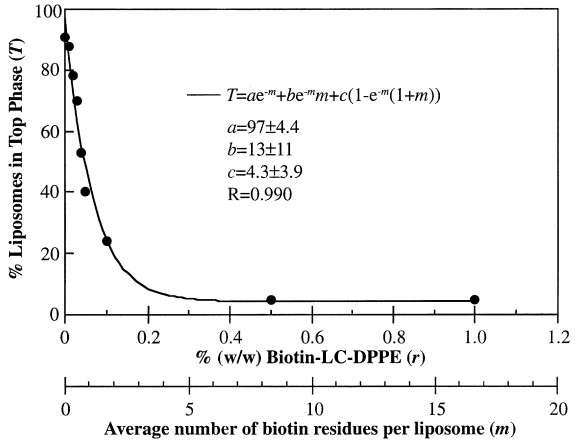

Fig. 5. Affinity partitioning of liposomes with increasing biotin content. Standard 2-g phase systems with 10 m*M* Li_2SO_4 and 90 μg of dextran-bound avidin were used. The average number of biotin per liposome (*m*) was calculated according to Eq. (5). The data set was curve fitted to Eq. (7) with KaleidaGraph by Abelbeck Software.

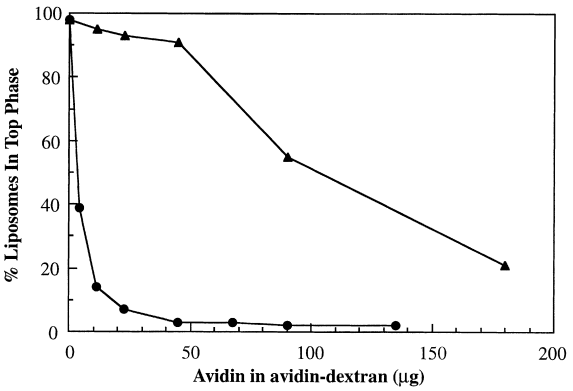

Fig. 6. Affinity partitioning of biotinylated liposomes as a function of the amount of avidin. Standard 2-g phase systems with 10 m*M* Li_2SO_4 and the indicated amounts of dextran–bound avidin were used. The liposomes contained 1% (w/w) biotin-LC-DPPE (●) or biotin-DPPE (▲).

imply, however, that a single biotin gives a significant redistribution of the liposomes.

3.4. Effect of avidin

The distribution of biotin-containing liposomes was examined at different avidin–dextran concentrations. In the presence of 45 μg avidin and above in a 2-g two-phase system most liposomes were redistributed from top to bottom phase, and already at less than 4 μg the redistribution was significant with only 40% of the biotinylated liposomes remaining in the top phase (Fig. 6). This showed that the affinity partitionings so far performed had been in the presence of a saturating amount of avidin.

The effect of a spacer was also tested by including biotin-DPPE instead of biotin-LC-DPPE in the liposomes. Biotin without a spacer was redistributed much less avidly, and more than 100 mg of avidin was required to redistribute 50% of the biotin-DPPE containing liposomes into the bottom phase (Fig. 6); in fact, a near complete redistribution was not achieved within the avidin interval examined (up to 180 μg). The requirement for a spacer was not unexpected as the biotin binding sites reside well below the surface of the avidin molecule [18].

4. Concluding remarks

The experiments described were performed to establish basic requirements for affinity partitioning of membraneous particles with the further goal of extending the technique to the purification of membranes. The model system was selected because of the strong and well-defined interaction between biotin and avidin and the possibility to reproducibly prepare small liposomes with a defined content of biotin. Furthermore, the liposomes, in contrast to membrane vesicles, do not contain proteins which may affect the partitioning process thereby complicating interpretations. The results obtained show that 1–2 strong interactions sufficed to redistribute the liposomes from top to bottom phase under favourable conditions. These conditions included the addition of certain salts to the phase system in order to avoid that the liposome–affinity adduct complex should distribute in the top phase. The next step will be to extend our studies to membrane vesicles.

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