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Journal of Chromatography A, 971 (2002) 117–127

JOURNAL OF CHROMATOGRAPHY A

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Affinity partitioning for membrane purification exploiting the biotin–NeutrAvidin interaction
Model study of mixed liposomes and membranes

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Received 10 April 2002; received in revised form 14 June 2002; accepted 17 June 2002

Abstract

Biotinylated negatively charged liposomes as well as membranes were affinity partitioned in an aqueous poly(ethylene glycol)–dextran two-phase system using NeutrAvidin conjugated to dextran as affinity ligand. Both liposomes and membranes redistributed from top to bottom phase upon addition of NeutrAvidin–dextran. The presence of 35–60 m*M* Li_2SO_4 was necessary both to force the components into the top phase without ligand and for ligand-dependent redistribution into the bottom phase. Attaching biotin via a hexanamidohexanoyl spacer and an increased density of biotin or NeutrAvidin enhanced the affinity separation. The separation conditions in these model experiments provide a basis for affinity partitioning of membranes using other affinity ligands.

Keywords: Affinity partitioning; Membranes; Aqueous two-phase systems; Liposomes; Biotin; Avidin

1. Introduction

Selectivity is an important factor when purifying biological material, as a highly selective method would tend to be both rapid and result in high purity of the material being prepared. Yet, few selective preparation methods exist for membranes, a reason perhaps being their complex structure combined with the tendency of membranes to interact unspecifically with separation matrices and also occasionally with other membranes. Affinity partitioning in aqueous polymer two-phase systems is a potentially useful separation technique for membranes [1]. This technique combines the selectivity of an affinity method with conditions where membranes are kept in a matrix-free aqueous environment during the separation process, thereby minimizing unwanted interactions.

Membranes and subfractions of membranes can be fractionated by conventional aqueous two-phase partitioning [2–4]. The membrane material distributes between the two aqueous phases and the interface, and the distribution can be modulated by the selection of separation parameters such as types and concentrations of salts and phase polymers in the system [5]. The partitioning behavior depends on rather undefined surface properties of the separated

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)00841-5

material, and a useful fractionation often requires multiple partitionings such as in a counter-current device [6]. Affinity partitioning instead aims at a selective ligand-governed distribution between the phases. Therefore, conditions are chosen to direct the bulk of membranes into one of the phases in the absence of ligand, whereas the membrane of interest selectively distributes in the other ligand-containing phase in the complete system with affinity ligand.

The possibility to prepare membranes by affinity partitioning was first explored using a ligand binding the nicotinic cholinergic receptor of electroplax membranes [7]. Further studies of these membranes [8] and of synaptic membranes using a ligand with affinity for opiate receptors [9] showed that membranes could be fractionated by affinity two-phase partitioning using the counter-current distribution technique. A further development was the purification of plasma membranes in a poly(ethylene glycol) (PEG)–dextran two-phase system using the lectin wheat germ agglutinin (WGA) conjugated to dextran as affinity ligand [10,11]. WGA interacts with abundant sialic acid and *N*-acetylglucosamine residues on the plasma membrane surface. A clear-cut separation was obtained in that more than 90% of all membranes partitioned in the PEG-rich top phase in the absence of ligand, whereas 90% of the plasma membranes selectively partitioned in the dextran phase upon the addition of WGA–dextran with little cross-contamination from other membranes [10]. This showed the possibility to separate membranes by a single affinity extraction step, perhaps followed by re-extraction to increase purity further.

Attempts to extend the affinity technique to other ligands and membranes were less successful, however. Therefore model experiments were initiated in PEG–dextran two-phase systems to define factors critical for the affinity process using biotinylated liposomes and NeutrAvidin conjugated to dextran as affinity ligand [12]. Biotinylated phosphatidylcholine (PC) liposomes were efficiently redistributed from the PEG-rich top phase to the dextran-rich bottom phase by the affinity ligand on condition that 10 m*M* Li_2SO_4 or other salts were present and that biotin was attached to the liposomes via an amidohexanoyl spacer [12]. Neither biotinylated mixed liposomes with negative charges nor membranes were pulled into the bottom phase by NeutrAvidin under these conditions, however, presumably due to charge repulsion between the liposome surface and the negatively charged rim of the deep biotin-binding pocket of NeutrAvidin [13].

We focused on the conditions required for the partitioning of mixed negatively charged liposomes and membrane fractions. The structure of the biotin-binding pocket of NeutrAvidin suggests that the introduction of a longer spacer when coupling biotin to phospholipid would avoid the close apposition between liposome and NeutrAvidin surfaces, thereby reducing their electrostatic repulsion. Apart from exploring a longer spacer, other factors affecting the affinity process have been examined to define conditions under which negatively charged liposomes as well as membrane fractions can be affinity partitioned using biotin–NeutrAvidin interactions.

2. Experimental

2.1. Chemicals

Stock solutions in water of 20% (w/w) Dextran 40 and Dextran 2000 (Pharmacia Biotech, Sweden) and 40% poly(ethylene glycol) 3350 (Carbowax 3350; Union Carbide, Danbury, CT, USA) were prepared as described [14]. The dextran was freeze–dried from aqueous solution before use [11]. PC from egg yolk, phosphatidylserine (PS) from bovine brain, phosphatidylethanolamine (PE) from sheep brain, phosphatidylinositol (PI) from soybean and synthetic dipalmitoyl phosphatidylethanolamine (DPPE) were obtained from Sigma (St. Louis, MO, USA). Immunopure NeutrAvidin, *N*-{6-[(biotinoyl)amino]-hexanoyl} dipalmitoyl - L - α - phosphatidylethanol - amine (biotin-LC-DPPE) and succinimidyl-6′-(biotinamido)-6-hexamido hexanoate (EZ-Link NHS-LC-LC-biotin) were from Pierce (Rockford, IL, USA). ^{3}H-Labeled PC was from Amersham Life Sciences (Little Chalfont, UK), and 2,2,2-trifluoroethane sulfonyl chloride (tresyl chloride) was from Synthelec (Lund, Sweden). All other reagents were of analytical grade.

2.2. Synthesis of biotin-LC-LC-DPPE

DPPE was biotinylated using a modification of a

previous protocol [15]. DPPE, 100 μmol, was dissolved in 8 ml of anhydrous methanol–chloroform (1:1, v/v), also containing 100 μmol of triethylamine, under a nitrogen atmosphere. NHS-LC-LC-biotin, 50 mg, was then dissolved in the lipid solution and the reaction was allowed to proceed until all the DPPE had been converted to biotin-LC-LC-DPPE (approx. 15 h). The conversion was followed by thin-layer chromatography (TLC) on silica gel 60-F_{254} plates (Merck, Darmstadt, Germany) with chloroform–methanol–water (65:25:4, v/v/v) as mobile phase. The disappearance of unmodified DPPE (R_F=0.45) and the appearance of biotin-LC-LC-DPPE (R_F=0.25) was followed by treating the plates with I_2 vapor and ninhydrin spray. After completion of the reaction the solvent was removed by a stream of nitrogen. Solids were redissolved in 5 ml chloroform, and water-soluble by-products extracted twice, each time by adding an equal volume of 1% (w/v) NaCl. The purity of the final product was established by TLC. The product was divided into aliquots, which were dried under a stream of nitrogen and stored at −20 °C up to several months.

2.3. Preparation of liposomes

Small unilamellar vesicles of different compositions, as indicated in each case, were prepared by sonication of phospholipid mixtures [13] with the additional admixture of 1 or 2% (w/w) of biotinylated lipid. This latter was either biotin-LC-DPPE or biotin-LC-LC-DPPE, having an amidohexanoyl or a hexanamidohexanoyl spacer between biotin and lipid, respectively. A trace of [^{3}H]PC was included to be able to follow the liposomes radiometrically in partitioning experiments.

2.4. Preparation and in vitro biotinylation of microsomal membranes

Microsomal membranes were prepared as described [16] and sonicated using a Branson Sonifier (Branson Sonic Power, Danbury, CT, USA) equipped with a microtip for 2×20 s set at output 4 and duty cycle 20% followed by 4×30 s at output 2 and duty cycle 20%. After pelleting at 100 000 *g* for 1 h, and homogenization in 10 m*M* *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES)–NaOH, pH 7.5, also containing 35 m*M* Li_2SO_4 and 0.25 *M* sucrose (HLS buffer) in a Potter-Elvehjem homogeniser, they were stored in aliquots at −80 °C until use. Biotin-LC-LC-DPPE was resuspended in 10 m*M* HEPES–NaOH, pH 7.5, after evaporation of the chloroform solvent under a stream of nitrogen. An aliquot of microsomal membranes was thawed and homogenized as above in five times its volume of HLS buffer. The membrane suspension was then added to the biotin-LC-LC-DPPE suspension in a lipid/membrane protein ratio of 1 or 2% (w/w) and vortexed for 1 min. After adjustment to 1 ml with HLS buffer the mixture was chilled and sonicated at 0 °C as described above. After incubation of the sonicated membrane suspension at 4 °C on a shaking table for 30 min, membranes were pelleted at 100 000 *g* for 1 h. The pellet was resuspended in the same volume of HLS buffer and centrifuged again. The final pellet was resuspended in HLS buffer in the smallest volume possible.

2.5. Coupling of NeutrAvidin to dextran

Freeze–dried Dextran 2000 was activated with tresyl chloride [11] and coupled to NeutrAvidin [13]. The coupling degree was 2–3 μg of NeutrAvidin per mg of dextran, as determined by protein measurement [17] using NeutrAvidin as standard.

2.6. Affinity two-phase partitioning

Affinity partitioning was performed as described earlier [13], but with a modified two-phase system. This was prepared by dissolving the required amount of NeutrAvidin–Dextran 2000 in appropriate amounts of PEG 3350, Dextran 40, Li_2SO_4 and buffer stock solutions, balancing with water. Affinity systems contained (final concentrations) 10 m*M* HEPES–NaOH, pH 7.5, 0–100 m*M* Li_2SO_4, 0–90 μg of NeutrAvidin bound to Dextran 2000, 6.45% (w/w) PEG 3350 and 8.45% (w/w) dextran (Dextran 40 plus ligand-bound Dextran 2000). In affinity partitioning experiments, 0.2 mg of liposome phospholipid was added to each 1.1-g two-phase system, or, in the case of membrane partitioning, a maximum of 130 μg of membrane protein to avoid aggregation at the interface. All operations were performed in a thermostatically controlled cold room (4 °C), as the

partitioning process is strongly dependent on temperature.

2.7. Determination of the critical point at different Li_2SO_4 concentrations

The critical point of a system with the starting composition of 8.45% dextran, 6.45% PEG and 10 m*M* HEPES–NaOH, pH 7.5 was determined at 0, 100 and 200 m*M* Li_2SO_4. Each system was titrated by adding water dropwise and weighing the system. After mixing, the system was allowed to separate into two phases. The procedure was repeated until a one-phase system was obtained, where the concentrations of the polymers were calculated giving the critical point.

2.8. Analyses

Liposomes in top and bottom phases were quantified radiometrically (liquid scintillator LS 1801, Beckman Instruments, CA, USA) as described in detail elsewhere [13]. The distribution of biotinylated membranes in the two phases was determined by protein measurement [18], the marker enzymes 5′-nucleotidase for plasma membranes [19] and arylesterase for the endoplasmic reticulum [20], and radiometrically in the same way as for liposomes [13] when [^{3}H]PC had been incorporated into the membranes. The distribution is expressed as % in the top phase of the total amount recovered in the system. This was deemed more practical rather than giving the partitioning constant as the phase volumes, and thereby concentrations, differed for different systems. Analyses were carried out at least in duplicate, and usually in triplicate.

3. Results

3.1. Effect of spacer on the partitioning of mixed PC/PS liposomes

Earlier two-phase partitioning experiments showed that an amidohexanoyl spacer did not allow an optimal interaction between biotinylated negatively charged liposomes and NeutrAvidin coupled to dextran [13]. To examine whether a longer spacer arm (longer than the depth of the biotin-binding pocket) would markedly enhance the interaction between negatively charged liposomes and NeutrAvidin–dextran, a hexanamidohexanoyl spacer was utilized. To this end, biotin was attached to PE via a hexanamidohexanoyl spacer and included in mixed PC/PS liposomes in which the PS content was 10% (w/w). The liposomes were subjected to affinity partitioning in a PEG–dextran two-phase system using NeutrAvidin coupled to dextran as ligand. Under the partitioning conditions chosen, biotinylated liposomes preferentially partitioned in the PEG-rich top phase in the absence of ligand, and preferentially in the dextran-rich bottom phase in its presence (Fig. 1A). Thus, the NeutrAvidin ligand caused a redistribution of the liposomes from approximately 90% in the top phase to 80% in the bottom phase under the most favorable conditions, i.e., in systems containing 60 μg NeutrAvidin conjugated to dextran and with a biotinylated lipid content of 2% (w/w) in the liposomes. The fraction of liposomes distributing in the bottom phase increased both with increasing concentrations of NeutrAvidin and with biotin density on the liposome surface.

As a comparison, mixed biotinylated liposomes with the shorter amidohexanoyl spacer were also partitioned (Fig. 1B). Although the preferential distribution in the bottom phase was similar to that attained with the longer spacer at 60 μg NeutrAvidin, a limited ligand-dependent redistribution (70–75% of the liposomes partitioning in the top phase) was seen at 30 μg NeutrAvidin. This indicates that biotin attached to liposomes via the longer spacer interacts more efficiently with NeutrAvidin.

3.2. Effect of Li_2SO_4 concentration

The effect of spacer arm length was tested at a set concentration of 20 m*M* Li_2SO_4 as previous experiments with biotinylated PC liposomes showed that the presence of Li_2SO_4 was essential for an efficient ligand-dependent distribution. Li_2SO_4 was selected because cations push negatively charged particles into the top phase in the order $K^+ < Na^+ < Li^+$ and anions in the order $H_2PO_4^- < SO_4^{2-}$ (for a detailed discussion on the influence of ionic composition of the phase system, see Ref. [5], pp. 118 and 119). To determine whether the effect of salt on the affinity

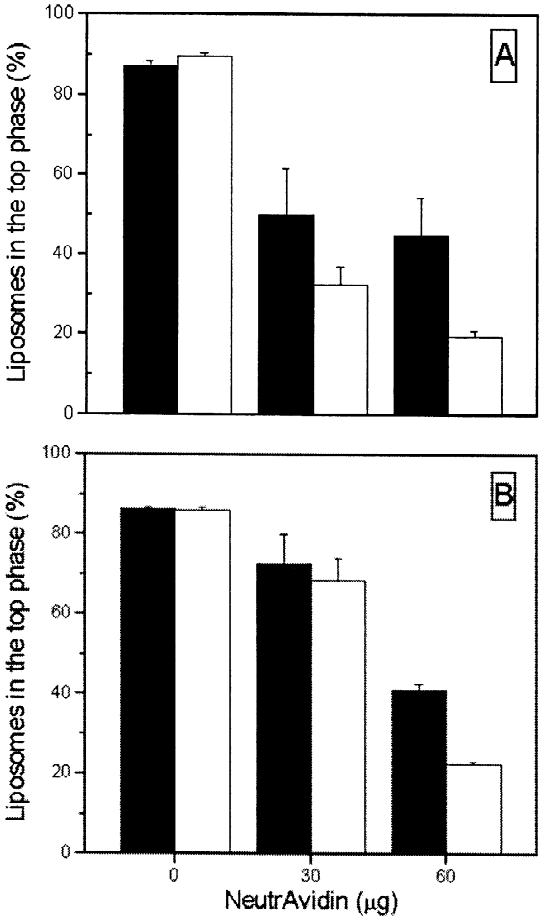

Fig. 1. Affinity partitioning of mixed PC/PS liposomes with increasing concentrations of NeutrAvidin coupled to dextran. Each 1.1-g system contained 8.45% (w/w) dextran and 6.45% (w/w) PEG 3350, 10 m*M* HEPES–NaOH, pH 7.5, and 20 m*M* Li_2SO_4. A 10-μl volume of liposome suspension was partitioned in each system. Biotinylated liposomes contained 1% (w/w) (filled bars) or 2% (w/w) (open bars) biotinylated lipid. The biotin was coupled to PE via a hexanamidohexanoyl spacer (panel A) or an amidohexanoyl spacer (panel B). The distribution of liposomes was measured radiometrically. Results are from 2 to 4 partitionings±S.E.

behavior of biotinylated mixed PC/PS liposomes was similar to that of biotinylated PC liposomes [12], the Li_2SO_4 concentration was varied (Fig. 2). An optimum concentration, where the mixed liposomes redistributed from ca. 90% in the top phase without ligand to 95% in the bottom phase in the presence of NeutrAvidin, was found in a rather narrow concentration range between 35 and 60 m*M*

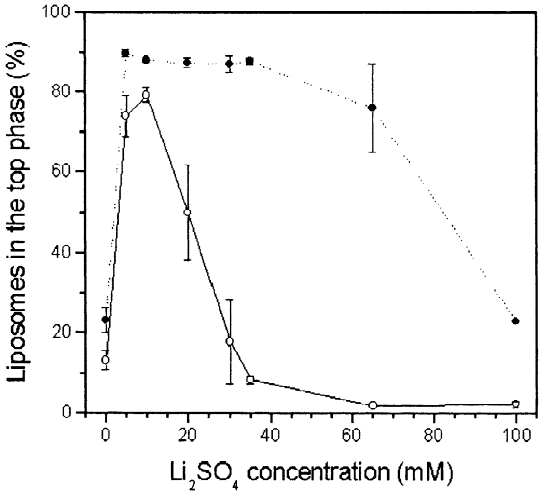

Fig. 2. Distribution of biotinylated PC/PS liposomes at various Li_2SO_4 concentrations. Standard 1.1-g two-phase systems with 10 m*M* HEPES–NaOH buffer, pH 7.5, in the presence (○) or absence (●) of 30 μg of dextran-bound NeutrAvidin. Liposomes were biotinylated by addition of 1% (w/w) biotin-LC-LC-DPPE. The distribution of liposomes was measured radiometrically. Results are from 3 partitionings±S.E. Where error bars are not shown the S.E. was smaller than the symbol.

Li_2SO_4. At salt concentrations below 35 m*M* the redistribution was less pronounced, as the biotinylated liposomes to a large extent partitioned in the top phase even in the presence of NeutrAvidin. This was particularly evident around 10 m*M* salt when 80% of the liposomes distributed in the top phase. Above 60 m*M* salt the liposomes gradually tended to distribute in the bottom phase even in the absence of affinity ligand, making these conditions less suitable for affinity partitioning. It should be noted that the liposomes preferred the bottom phase both in the presence and absence of affinity ligand when Li_2SO_4 was not added to the two-phase system. Therefore, the inclusion of an appropriate concentration of Li_2SO_4 is critical for the successful NeutrAvidin-dependent affinity partitioning of biotinylated mixed liposomes.

The partitioning of a particle is partially dependent on its interactions with the two-phase system polymers, therefore the effect of Li_2SO_4 concentration on the binodial curve, i.e., on the polymer concentration in the phases, was also investigated. Systems containing 0 or 100 m*M* salt gave two phases whereas in the presence of 200 m*M* Li_2SO_4 only one phase was

obtained. Titration with water of the systems containing 0 and 100 m*M* Li_2SO_4 showed different critical points, i.e., more water had to be added to the system containing no salt than to that with 100 m*M* Li_2SO_4. The critical points were found to be 6.21% dextran and 8.07% PEG in the absence of salt and 6.27% dextran and 8.21% PEG at 100 m*M* Li_2SO_4. Thus, there was a shift in the critical point and thereby binodial curve towards higher polymer concentrations at increasing Li_2SO_4 concentrations.

3.3. Effect of phospholipid composition

To further test the effect of negatively charged phospholipids on the affinity distribution, the proportion of PS or PI was varied (Fig. 3). With an admixture of up to 10% PS, ca. 90% of the mixed biotinylated liposomes distributed in the NeutrAvidin-containing bottom phase, while they increasingly partitioned in the top phase at higher proportions of PS. Mixed PC/PI liposomes behaved similarly, although the system tolerated up to 20% PI well.

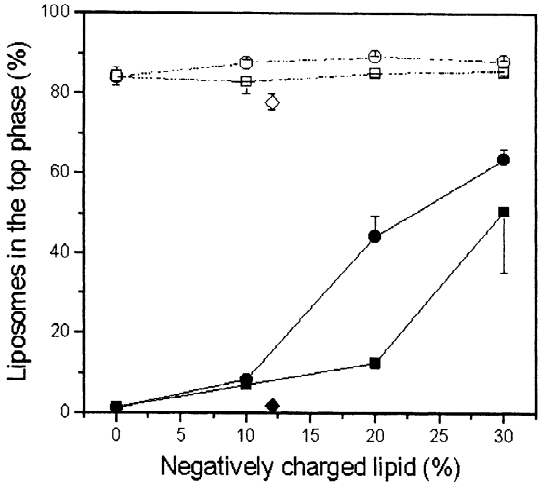

Fig. 3. Affinity partitioning of biotinylated mixed PC liposomes containing various concentrations of negatively charged phospholipids. Liposomes were biotinylated by addition of 1% biotin-LC-LC-DPPE. Standard 1.1-g two-phase systems were prepared with (closed symbols) or without (open symbols) 30 μg NeutrAvidin coupled to dextran in 10 m*M* HEPES–NaOH buffer, pH 7.5, with 35 m*M* Li_2SO_4. (●) Liposomes containing PS in addition to PC, (■) liposomes containing PI in addition to PC, (♦) liposomes with a phospholipid composition similar to that of rat liver microsomal membranes. The distribution of liposomes was measured radiometrically. Results are from 3 partitionings±S.E. Where error bars are not shown the S.E. was smaller than the symbol.

As membranes contain a more complex mixture of phospholipids, affinity partitioning of biotinylated liposomes with a composition similar to that of rat liver microsomal membranes [21] was also tested. In addition to 1% (w/w) biotin-LC-LC-DPPE, these liposomes contained a total of 11.8% of negatively charged phospholipids (total composition by mass, 58% PC, 29% PE, 3.6% PS, 1.2% PG and 7% PI). A NeutrAvidin-dependent redistribution of these liposomes was observed from more than 80% in the top phase without ligand to 98% in the bottom phase in its presence (Fig. 3). Thus, the effect of the ligand was as pronounced as that observed with mixed PC/PS or PC/PI liposomes with a similar admixture of negatively charged phospholipids.

3.4. Partitioning of membranes

Having established conditions for the affinity partitioning of mixed liposomes, the next step was to examine whether these separation conditions were also suitable for biotinylated membrane fractions. To this end, rat liver microsomal membranes were biotinylated by insertion of biotinylated DPPE containing an amidohexanoyl or a hexanamidohexanoyl spacer between the biotin moiety and the lipid headgroup, and then partitioned in two-phase systems containing 30, 60 or 90 μg NeutrAvidin coupled to dextran, or without the affinity ligand as control. The distribution of plasma membranes and endoplasmic reticulum membranes, both being enriched in microsomes, was followed by the marker enzymes 5′-nucleotidase and arylesterase, respectively, and the distribution of the total bulk of membranes by protein determination of each phase (Fig. 4).

Without NeutrAvidin ligand ca. 80–90% of the membranes partitioned in the PEG-rich top phase, while in its presence both kinds of membranes instead partitioned in the dextran-rich bottom phase; those biotinylated with the long spacer (Fig. 4A and B) more pronouncedly than those with the short one (Fig. 4C and D).

The degree of distribution in the bottom phase also depended on the biotin density on the membrane

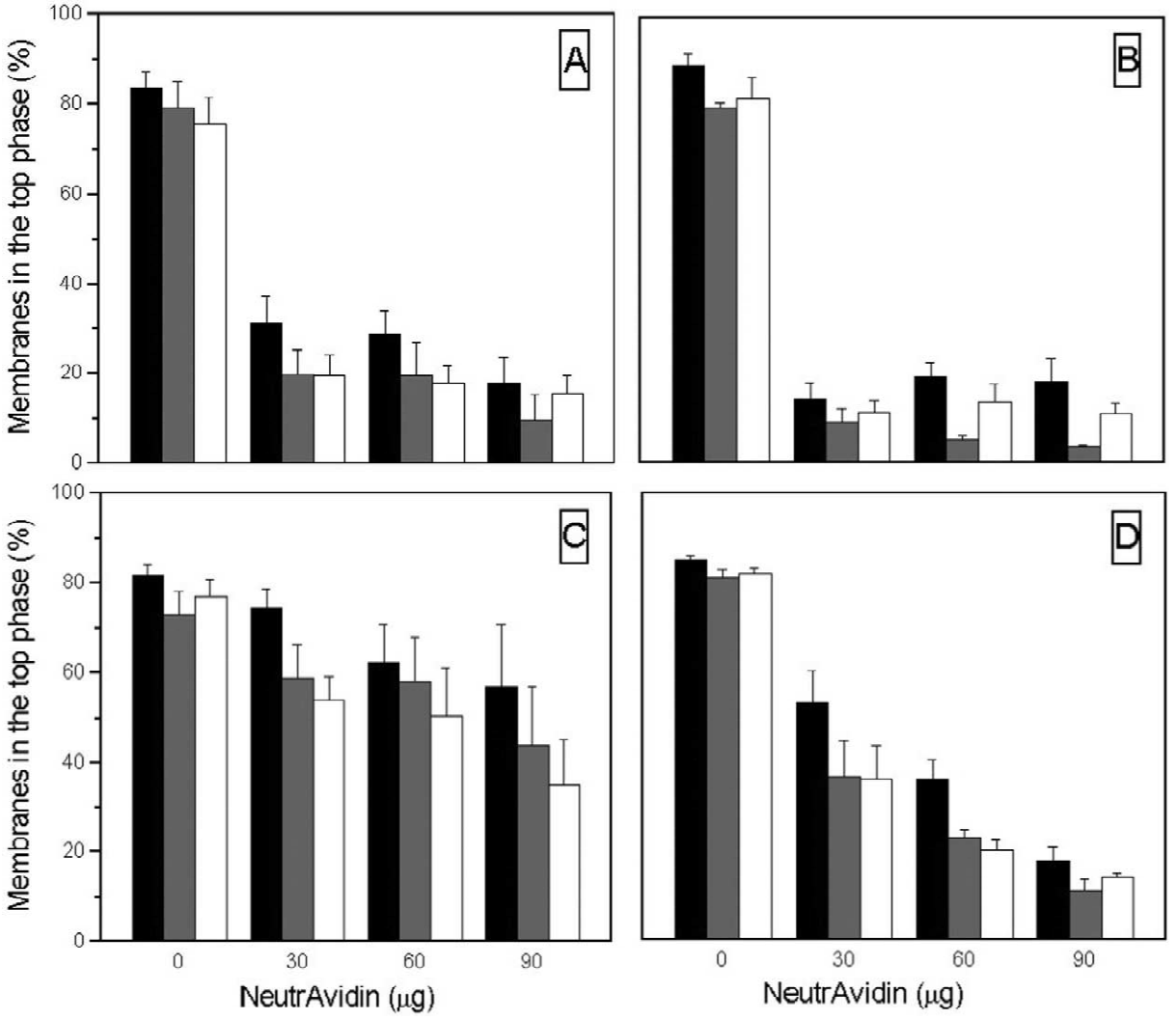

Fig. 4. Affinity partitioning of biotinylated microsomal membranes. DPPE biotinylated via the long hexanamidohexanoyl (panels A, B) or the short amidohexanoyl spacer (panels C, D) was incorporated into microsomes. The amount of biotinylated lipid was 1% (w/w) (panels A, C) or 2% (w/w) (panels B, D) of the total amount of phospholipids in the membranes. Standard 1.1-g two-phase systems were prepared with 0 to 90 μg NeutrAvidin coupled to dextran in 10 m*M* HEPES–NaOH buffer, pH 7.5 with 35 m*M* Li_2SO_4. Membrane protein, 100 to 130 μg, was added per system. The distribution of the plasma membrane marker 5′-nucleotidase (black), the endoplasmic reticulum marker arylesterase (grey), and the total membrane protein (white) is shown. Results for 4 partitionings±S.E. are shown, each analyzed in duplicate or triplicate.

surface (1 or 2%) and the concentration of affinity ligand in the system. Thus, maximum NeutrAvidin-dependent redistribution from 80 to 90% in the top phase to approximately 90% in the bottom phase already occurred at 30 μg NeutrAvidin in membranes containing 2% biotin attached via the long hexanamidohexanoyl spacer (Fig. 4B), whereas 90 μg NeutrAvidin was required to attain a similar distribution with the lower amount of biotin or with 2% biotin coupled via the short amidohexanoyl spacer (Fig. 4A and D). Less membranes distributed in the bottom phase in systems with less NeutrAvidin–dextran, particularly membranes with biotin coupled via the short spacer. As to different mem-

branes, the distribution was similar under all conditions, although the plasma membrane marker 5′-nucleotidase always partitioned slightly higher than the endoplasmic reticulum marker arylesterase. The distribution of the bulk protein followed the endoplasmic reticulum marker, which is in agreement with the fact that these membranes are by far the most abundant ones in the microsomal fraction [16]. The distribution of membranes was also confirmed by incorporating [^{3}H]PC, and this label followed the bulk protein (data not shown).

The optimal salt conditions for affinity partitioning of membranes were checked by varying the Li_2SO_4 concentration (Fig. 5). In the presence of NeutrAvidin–dextran the biotinylated membranes partitioned to more than 90% in the bottom phase at all salt concentrations tested. As with mixed liposomes (cf. Fig. 2) the membranes partitioned preferentially in the top phase in the absence of the affinity ligand at intermediate salt concentrations, and with a maximum of 80% at 35 m*M* Li_2SO_4. Below this concentration the membranes aggregated and were found at the interface, whereas the membranes distributed mainly in the bottom phase above 65 m*M*. Thus, optimum salt conditions for affinity partitioning of membranes using the biotin–NeutrAvidin interaction was between 35 and 60 m*M* Li_2SO_4, i.e., the same as for mixed PC/PS liposomes.

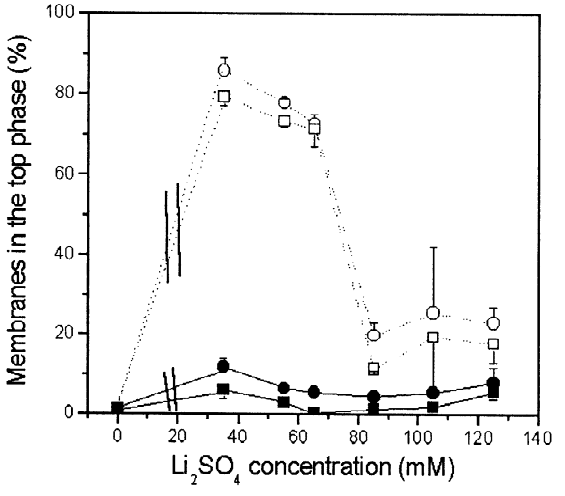

Fig. 5. Distribution of biotinylated microsomal membranes at various Li_2SO_4 concentrations. Membranes contained 2% (w/w) biotin-LC-LC-DPPE. Standard 1.1-g phase systems with 10 m*M* HEPES–NaOH buffer, pH 7.5, in the presence of 30 μg of dextran-bound NeutrAvidin (closed symbols) or in its absence (open symbols). Two membrane markers were followed: 5′-nucleotidase (●) for plasma membranes and arylesterase (■) for endoplasmic reticulum membranes. Results for 2 partitionings±S.E. are shown, each analyzed in duplicate or triplicate. Where error bars are not shown the S.E. was smaller than the symbol. Aggregation occurred below 20 m*M* Li_2SO_4 as indicated by the break in the line.

The co-distribution of the enzyme markers for plasma membranes and endoplasmic reticulum at all conditions tested indicates that both membrane fractions became biotinylated to approximately the same extent. An alternative possibility would be that one membrane fraction was biotinylated preferentially and that the other fraction co-distributed due to unspecific interactions. To test this possibility biotinylated membranes were partitioned separately and together with ^{3}H-labeled mixed PC/PS liposomes devoid of biotin. In the absence of the affinity ligand 80–90% of both liposomes and membranes, as expected, distributed in the top phase (Table 1), while in its presence the membranes partitioned in

Table 1
Specificity of the affinity purification of biotinylated membranes

Material in the two-phase system		Recovery in top phase (%)	
		NeutrAvidin (μg) bound to dextran	
		0	30
Non-biotinylated liposomes		90.5±0.5	90±0.5
Biotinylated microsomes	PM	83±3	12±2
	ER	79±2	6±2
	Protein	82±3	9±3
Mixture	Liposome	88±1	89±0
	PM	88.5±0.5	12±0
	ER	88±1	0±0
	Protein	94±5	11±2

Biotinylated microsomal membranes and non-biotinylated liposomes were partitioned separately or together in a mixture. The distribution of liposomes was followed radiometrically (^{3}H-labeled), and that of membranes by analysis of the plasma membrane marker 5′-nucleotidase (PM), endoplasmic reticulum marker arylesterase (ER) and by protein determination. Results for 2 partitionings±S.E. are shown, each analyzed in duplicate or triplicate, are expressed as % of material recovered in the top phase.

the bottom phase, leaving the non-biotinylated liposomes in the top phase, both when they were partitioned separately and when mixed with the membranes. This indicates that the distribution of both kinds of membranes was due to specific interactions with the affinity ligand.

4. Discussion

Our present work has established conditions for affinity partitioning of negatively charged liposomes as well as membranes in PEG–dextran two-phase systems based on the biotin–NeutrAvidin interaction. Crucial conditions include the presence of Li_2SO_4 in the two-phase system and the attachment of biotin to liposomes via a hexanamidohexanoyl spacer enhancing the affinity interaction.

An essential feature in affinity partitioning is that the affinity ligand should redirect the material of interest from one phase to the other ligand-containing phase away from unwanted material. The addition of Li_2SO_4 to the phase system was necessary for two reasons, to force liposomes as well as membranes to partition in the PEG-rich top phase in the absence of ligand and to enhance a ligand-dependent redistribution to the bottom phase.

The distribution of liposomes and membranes in a two-phase system under different salt conditions is governed by a combination of parameters including the uneven distribution of ions between the phases, hydrophobic interactions with phase polymers, electrostatic interactions and the effect of salt on the polymer distribution between the phases. Thus, negatively charged mixed PC/PS liposomes, and also membranes, distributed to the dextran-rich bottom phase in the absence of Li_2SO_4, presumably due to their preference for dextran molecules. In contrast, electroneutral PC liposomes preferred the PEG-rich top phase under similar conditions [12]. The addition of comparatively low concentrations of Li_2SO_4 (below 10 m*M*) redirected the negatively charged liposomes from dextran to PEG phase, an effect most likely due to the preference of lithium ions for the PEG-rich top phase and sulfate ions for the dextran-rich bottom phase (for a detailed discussion on the distribution of ions in two-phase systems, see Ref. [5], pp. 132–135). When the Li_2SO_4 concentration was increased further (above 60 m*M*) the liposomes again preferred the dextran-rich bottom phase. This finding can be interpreted on the basis of salt induced changes in the polymer concentrations of the phases. Thus, an increase in the Li_2SO_4 concentration in the two-phase system caused a shift in the phase diagram binodial in the direction of higher polymer concentrations, i.e., the two-phase system moved closer to the critical point. As a consequence, the tie-line length decreased and the two phases became more similar in phase polymer composition [22].

The partitioning coefficient, K, for particles in a two-phase system is dependent on several parameters. In the simplified relation:

$$\ln K = \ln K_0 + \ln K_{hyd} + \ln K_{el}$$

K_{hyd} represents the contribution of hydrophobic interactions between the liposomes and the phase components, K_{el} that of electrostatic interactions whereas K_0 includes other effects on the partitioning. When the phases of a system become more similar in composition, as in the case of increasing salt concentration, the hydrophobic and electrostatic contributions decrease [22]. A possible explanation for the partitioning of the liposomes to the bottom phase at increasing salt concentrations is that the electrostatic contribution decreases faster than the hydrophobic one, the latter becoming predominant at high salt concentrations. The situation then becomes similar to that prevailing in the absence of salt when mixed liposomes partition to the bottom phase (see above), i.e., the negatively charged liposomes tend to avoid the more hydrophobic PEG phase in favor of the hydrophilic dextran phase.

In the presence of affinity ligand, salt concentrations above 10 m*M* were required to pull the liposomes to the bottom phase. This might be interpreted as a screening effect: as both the mixed liposomes and the entrance to the biotin binding site of NeutrAvidin are negatively charged at neutral pH [13], sufficient electrostatic repulsion maybe at hand to obstruct binding. Salt ions attenuate this repulsion, allowing the biotin moiety on the liposomes to interact with NeutrAvidin. Once formed the binding is strong enough for the liposome to partition to the bottom phase along with NeutrAvidin–dextran. A further increase in the negative charge density of the

liposomes (increase in negatively charged phospholipid to neutral phospholipid ratio) again disturbs this interaction, more pronouncedly in mixed PC/PS liposomes, containing more ionized groups, than in PC/PI liposomes (Fig. 3).

When membranes (the microsomal fraction) were partitioned in the two-phase system a similar salt dependency as mixed liposomes was observed in the absence of affinity ligand (see Figs. 2 and 5); in its presence the membranes tended to aggregate at low salt concentrations and collect at the interface. Above 35 m*M* Li_2SO_4 however, the membranes distributed into the bottom phase, a behavior consistent with that of the liposomes. Thus, mixed liposomes was a suitable model for membranes exploiting conditions for affinity partitioning.

A crucial factor in the partitioning experiments was to enhance the affinity interaction by attaching biotin to liposomes via a spacer. Apart from ascertaining that biotin would be oriented on the outer liposome surface for steric reasons, a spacer is required for the biotin moiety to properly reach its binding site. As the carboxy group of the biotin side chain is positioned approximately 1 nm below the NeutrAvidin surface [23], the insertion of an amidohexanoyl spacer (0.9 nm in length [24]) was necessary to affinity partition electroneutral PC liposomes [12] (Fig. 6A). When subjecting negatively charged (PC/PS) liposomes to affinity partitioning at 20 m*M* Li_2SO_4 this spacer did not allow an optimum interaction, however, presumably due to electrostatic repulsion towards negatively charged amino acid side chains on the loops around the entrance of the biotin binding pocket of NeutrAvidin. An interpretation is that the concentration of salt was not sufficient to screen this repulsion. This is based on the calculated Debye length, i.e., the distance where the repulsion is attenuated [25], which for our experimental conditions was 1.2 nm. Thus, the liposomes were not allowed to get close enough to NeutrAvidin for biotin to be inserted smoothly in the binding site. Nevertheless, as the NeutrAvidin structure is quite flexible, a limited binding was attained (see Fig. 6B). The longer (1.7 nm) hexanamidohexanoyl spacer facilitated the affinity interaction, but as its length was too close to the range allowed by the Debye length, the redistribution was not optimal (Fig. 6C). When the salt concentration was increased

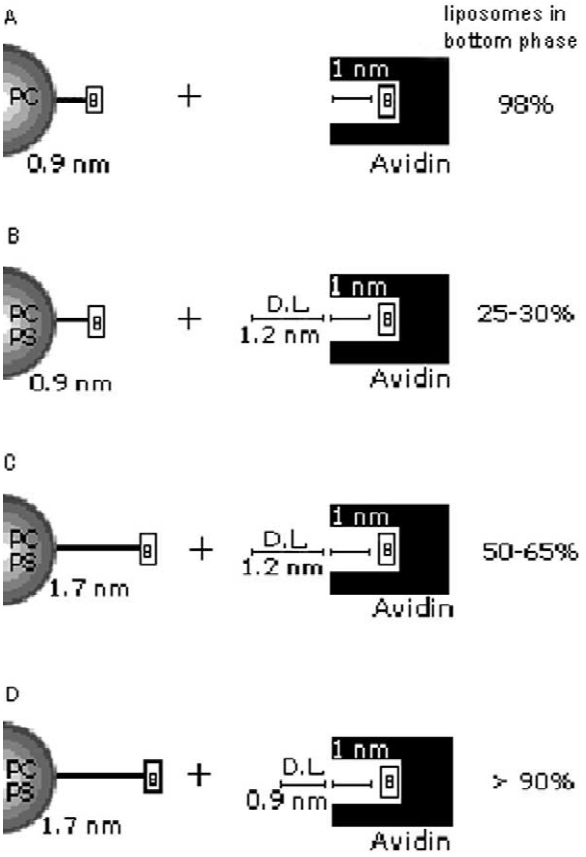

Fig. 6. Influence of the calculated Debye length (D.L.) on the interaction between biotinylated liposomes and NeutrAvidin.

to 35 m*M* the Debye length decreased to 0.9 nm allowing a more facile binding with this spacer (Fig. 6D) and therefore nicely reflecting the affinity distribution of PC/PS liposomes in this salt concentration bracket.

These studies were performed to establish minimal requirements for affinity partitioning of liposomes and membranes using the biotin–NeutrAvidin interaction. The conditions devised tolerated more negatively charged liposomes than in earlier studies [13] and also allowed the affinity partitioning of membranes, which are negatively charged as well, that has not been possible before using this affinity interaction. As shown here, limitations of the affinity interaction could be overcome by altering the ionic strength in the two-phase system and by introducing a longer spacer arm when coupling biotin to phos-

pholipid to avoid a too close apposition between the liposome and NeutrAvidin surfaces on affinity binding.

The next step will be to extend these model studies to membranes using naturally occurring affinity pairs.

Acknowledgements

We thank Professors F. Tjerneld and P.-Å. Albertsson, and Dr. H.-O. Johansson for valuable discussions. The work was supported by the Swedish Natural Science Research Council. I.B.-R.R. is grateful for a scholarship from the Basque Government: ''Beca para la formación de Investigadores del Departamento de Educación, Universidades e Investigación del Gobierno Vasco''.

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