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THE EFFECT OF THE LIPID COMPOSITION ON THE PARTITION OF LIPOSOMES IN AQUEOUS TWO-PHASE SYSTEMS

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Summary

Liposomes have been partitioned in aqueous two-phase systems consisting of water, dextran, poly(ethylene glycol), salt and buffer. Liposomes were used as a model system in order to determine the contribution of the lipids on the partition of membrane particles. The liposomes were composed of phospholipids with different polar head groups and different degrees of unsaturation. The role of cholesterol was also investigated.

The polar head group of the phospholipid plays a dominant role in determining the partition of liposomes, while the degree of unsaturation is of less importance, thereby indicating that partition in two-phase systems is a surface dependent method. Incorporation of cholesterol in liposomes reduces differences in partition between liposomes of various composition.

Introduction

Aqueous two-phase systems [1] have been widely used in the separation of membrane particles [2]. This method utilizes differences in surface properties for separation, rather than differences in size and density. These surface properties include charge and hydrophobicity.

A difference in partition has earlier been found for red blood cells from various species [3], where cells with a higher amount of phosphatidylcholine compared to sphingomyelin have a different partition than have cells with a lower amount. A correlation between the ratio poly/mono-unsaturated fatty acids and the partition of red blood cells from different species has also been reported [4]. Membrane vesicles from spinach chloroplasts with different chlorophyll *a/b* ratios have also been separated by phase partition [5].

Since the cell membrane contains a large amount of lipids, it is of interest to see the effect of each lipid on the partition behaviour and thereby determine

the contribution of the lipids on the partition of membrane particles. For this purpose liposomes were used as a model system. Liposomes were made with different polar head groups and with different degrees of unsaturation of the phospholipids. Cholesterol was also incorporated in some of the liposomes.

This investigation shows that the polar head group of the lipid is a dominant factor in determining the partition, while the degree of unsaturation and the cholesterol content are of less importance.

Materials and Methods

Dextran 40, batch No 4743, M_r = 42 000 and dextran 500, batch No 5556, M_r = 500 000 were supplied by Pharmacia, Uppsala, Sweden. Poly(ethylene glycol), M_r = 6000 was obtained from Union Carbide, New York as Carbowax. The poly(ethylene glycol) ester was synthesized by Dr. Göte Johansson [3]. L-α-Phosphatidylinositol (soya bean), purity 98%, DL-α-phosphatidylcholine-dipalmitoyl, 99% purity, L-α-phosphatidylserine (bovine brain), 98% purity, and sphingomyelin (bovine brain) were obtained from Sigma. Egg phosphatidylcholine and soya bean phosphatidylcholine were generous gifts from Dr. Björn Åkesson [6]. Cholesten-(5)-ol-(3β), purity 99% was obtained from Merck. [^{14}C]phosphatidylcholine, 50 mCi/mmol and [^{14}C]cholesterol 54 mCi/mmol, purity of both 99%, were from New England Nuclear.

Preparation of liposomes was done according to Huang and Thompson [7]. 14 μmol of lipid were dissolved in chloroform/methanol mixtures in a 5 ml flask and 4 nmol [^{14}C]phosphatidylcholine or 4 nmol [^{14}C]cholesterol were added. The solvent was evaporated under a stream of N_2. The vessel was placed in a desiccator under reduced pressure overnight. 1 ml of 0.15 M NaCl and 0.01 M sodium phosphate buffer, pH = 7, was added and the mixture was sonicated under N_2 above the transition temperature in a Bransonic Cleaning Bath 12 until clearness of the solution. The solution was fractionated on a column of Sepharose 4B, 20 × 2 cm, at 4°C, separating multilayered from the bilayer liposomes. The turbidity and the radioactivity of the eluate were measured. The same results were obtained with radioactively labelled phosphatidylcholine as with labelled cholesterol. The turbidity was measured by a ultraviolet monitor and the radioactivity by a liquid scintillation counter with toluene/Triton X-100/omnifluor, 500 ml : 250 ml : 4.25 g, as a scintillator. The large vesicles which are surrounded by many bilayers are referred to multilayered liposomes in the text, while the small liposomes which are surrounded by only one bilayer are called bilayer liposomes.

Preparation of phase systems: The phase systems used contained 4% (w/w) poly(ethylene glycol) and 8% (w/w) dextran 40 or 5% (w/w) dextran 500. Phase systems of total weight 5 g were prepared by mixing 2 g of a 20% (w/w) dextran 40 solution or 1.25 g of a 20% (w/w) dextran 500 solution with 0.5 g of a 40% (w/w) poly(ethylene glycol) solution and then adding the appropriate salt and buffer to the final concentrations. One ml of liposome suspension was included in these 5 g. The phases were mixed by 30 inversions and then allowed to separate for 25 min (with dextran 40) or 30 min (with dextran 500). All partitions were done at room temperature, 22°C. After separation, 1 ml of the upper, poly(ethylene glycol)-rich, phase and 1 ml of the lower, dextran-rich, phase were withdrawn and the radioactivity of these was measured.

Results and Discussion

Liposomes of different composition have been partitioned in phase systems composed of 5% (w/w) dextran 500 and 4% (w/w) poly(ethylene glycol) 6000 with (a) 0.15 mol/kg NaCl and 0.01 mol/kg sodium phosphate buffer, pH 7, or (b) 0.11 mol/kg sodium phosphate buffer, pH 7, and 0.03 mol/kg NaCl. Phase system a has a negative potential [8], i.e., the upper phase is more negative than the lower phase. Phase system b has a positive potential, i.e. the upper phase is more positive than the lower phase. The distribution of the different liposomes between the two phases can be seen in Table I. In this Table one can compare the partition of liposomes having different polar head groups and different degrees of unsaturation of the phospholipids in phase systems with opposite potential.

Effect of polar head group

Both multilayered and bilayer liposomes composed of phosphatidylcholine or sphingomyelin have a slightly but significantly higher partition in phase system a than in phase system b, i.e., they behave as if they carried a positive surface charge (Table I). Both multilayered and bilayer liposomes of phosphatidylserine or phosphatidylinositol have a lower partition in phase system a than in phase system b. This shows that the phase systems can detect a negative surface charge for both phosphatidylserine and phosphatidylinositol liposomes.

Incorporation of cholesterol in molar ratio 1 : 1 in liposomes of phosphatidylcholine or sphingomyelin gives larger differences in partition between phase systems of opposite potential, i.e., the positive surface charge seems to increase. The charge of the liposomes which is registered by the phase systems is thus not the same charge as the sum of the positive and the negative charges from each lipid.

The surface charge can thus be measured qualitatively by partitioning in phase systems with positive and negative potentials and comparing the results. The partition of charged liposomes can be directed by salts, as has been shown earlier for proteins and cells [1].

Phosphatidylinositol liposomes have a higher partition than phosphatidylserine liposomes (Table I). The polar head group therefore seems to be important for the partition behaviour even for liposomes with a negative surface charge.

Partition of liposomes composed of mixtures of phosphatidylcholine and phosphatidylserine shows two effects. 1, both multilayered and bilayer liposomes of phosphatidylcholine : phosphatidylserine in molar ratios 4 : 1, 1 : 1 and 1 : 4 have negative surface charge, since they have a lower partition in phase system a than in phase system b. 2, In the phase system with negative potential (phase system a) the higher the molar ratio of phosphatidylserine, the more the liposomes favour the lower phase. In contrast, in those phase systems with positive potential, liposomes with phosphatidylcholine : phosphatidylserine in molar ratio 4 : 1 favour the upper phase more than liposomes composed of only one of the phospholipids.

There thus seem to be two tendencies working in opposite direction, one depending on the surface charge and one depending on the structure of the

TABLE I

The distribution of liposomes between the two phases in 5% (w/w) dextran 500, 4% (w/w) poly(ethylene glycol) and (a) 0.15 mol/kg NaCl and 0.01 mol/kg sodium phosphate buffer or (b) 0.03 mol/kg NaCl and 0.11 mol/kg sodium phosphate buffer. These phase systems have opposite potentials. Each 5-g phase system contain 0.19 μmol phospholipid. Values in parentheses are for bilayer liposomes and values without parentheses are for multilayer liposomes.

Liposome composition	Percentage of liposomes		
	in the upper phase	at the interface	in the lower phase
Soya bean phosphatidylcholine			
a	8 (51)	76 (24)	16 (25)
b	2 (41)	64 (23)	34 (36)
Soya bean phosphatidylcholine/cholesterol: molar ratio 1 : 1			
a	(4)	(80)	(16)
b	(1)	(45)	(54)
Egg phosphatidylcholine			
a	5 (29)	74 (41)	21 (30)
b	3 (25)	81 (42)	16 (33)
Dipalmitoylphosphatidylcholine			
a	13 (5)	64 (61)	23 (34)
b	2 (4)	64 (55)	34 (41)
Dipalmitoylphosphatidylcholine/cholesterol; molar ratio 1 : 1			
a	8 (5)	79 (77)	13 (18)
b	0 (1)	52 (53)	48 (46)
Sphingomyelin			
a	1 (4)	63 (61)	36 (35)
b	1 (2)	65 (58)	34 (40)
Sphingomyelin/cholesterol; molar ratio 1 : 1			
a	11 (7)	74 (70)	15 (23)
b	1 (1)	62 (48)	37 (51)
Dipalmitoylphosphatidylcholine/phosphatidylserine; molar ratios:			
4 : 1			
a	1 (28)	41 (11)	58 (61)
b	4 (58)	77 (26)	19 (16)
1 :1			
a	1 (5)	8 (11)	91 (84)
b	2 (43)	58 (27)	40 (30)
1 : 4			
a	1 (5)	0 (5)	99 (90)
b	2 (20)	46 (31)	52 (49)
Phosphatidylserine			
a	1 (2)	0 (2)	99 (96)
b	1 (2)	28 (25)	71 (73)
Dipalmitoylphosphatidylcholine/phosphatidylinositol; molar ratio 1 : 1			
a	1	7	92
b	3	64	33
Phosphatidylinositol			
a	(20)	(8)	(72)
b	(47)	(6)	(47)

polar head group. This might cause difficulties in predicting the partition of mixed phospholipid bilayers.

Effect of degree of unsaturation

In Table I we can study also the effect of the degree of unsaturation of the phospholipids. Bilayer phosphatidylcholine liposomes with the most unsaturated lipids have a higher partition than bilayer liposomes with saturated lipids. Soya bean phosphatidylcholine has 21% saturated, 23% monounsaturated and 56%

polyunsaturated fatty acyl chains [9]. Egg phosphatidylcholine has 48% saturated, 31% monounsaturated and 20% polyunsaturated fatty acyl chains [10]. Dipalmitoylphosphatidylcholine has 100% saturated fatty acyl chains. This difference in partition between saturated and unsaturated phosphatidylcholines cannot be found for multilayered liposomes. Incorporation of cholesterol in bilayer liposomes of soya bean phosphatidylcholine or dipalmitoylphosphatidylcholine removes the differences in partition between saturated and unsaturated liposomes (Table I). Incorporation of cholesterol in liposomes of phosphatidylcholine and sphingomyelin also removes the differences in partition between these two types of liposomes.

The fact that the degree of unsaturation is reflected in partition of bilayer vesicles but not in partition of multilayered liposomes might be explained by the higher radius of curvature for bilayer liposomes compared to multilayered [11]. The hydrophobic part of the membrane will be more exposed to the surroundings in the case of bilayer vesicles. Cholesterol is supposed to reduce the membrane fluidity and this may be the explanation to the equalizing effect of cholesterol on the partition [12].

Effect of poly(ethylene glycol) palmitate

Poly(ethylene glycol) palmitate has earlier been used in experiments with erythrocytes [3]. In phase systems with 8% (w/w) dextran 40, 4% (w/w) poly(ethylene glycol) 6000, 0.15 mol/kg NaCl and 0.01 mol/kg sodium phosphate buffer, pH 7, the liposomes are at the interface and/or in the lower, dextran-rich phase. If some of the terminal hydroxylgroups of poly(ethylene glycol) are esterified with palmitic acid the liposomes will be found in the upper phase.

Neither for multilayered nor for bilayer phosphatidylcholine liposomes were there any differences in partition when the degree of unsaturation of the phospholipid was changed. Fig. 1. Incorporation of cholesterol in phosphatidylcholine liposomes in molar ratio 1 : 1 has no effect on the partition of multilayered liposomes. Bilayer vesicles with cholesterol in molar ratio 1 : 1 require somewhat less concentration of poly(ethylene glycol) palmitate to get into the upper phase than bilayer liposomes without cholesterol.

For bilayer vesicles the amount of poly(ethylene glycol) palmitate which is required to get 50% of the liposomes into the upper phase is in the order of five poly(ethylene glycol) palmitate molecules per liposome. The corresponding number for erythrocytes was $5 \cdot 10^6$ molecules per red blood cell. The amount of poly(ethylene glycol) palmitate per mole lipid that is required to get the particles into the upper phase is 10-times higher for erythrocytes than for bilayer liposomes.

Liposomes of different composition i.e. phosphatidylcholine, sphingomyelin, phosphatidylserine and phosphatidylinositol are compared in Figs. 1, 2 and 3. Multilayered phosphatidylcholine liposomes require the lowest poly(ethylene glycol) palmitate concentration to get into the upper phase. Fig. 1a. At somewhat higher concentration of poly(ethylene glycol) palmitate, bilayer vesicles will be found in the upper phase. Fig. 1b. Liposomes of sphingomyelin require higher concentrations of poly(ethylene glycol) palmitate to get into the upper phase than phosphatidylcholine liposomes (Fig. 2). The liposomes in Fig. 2

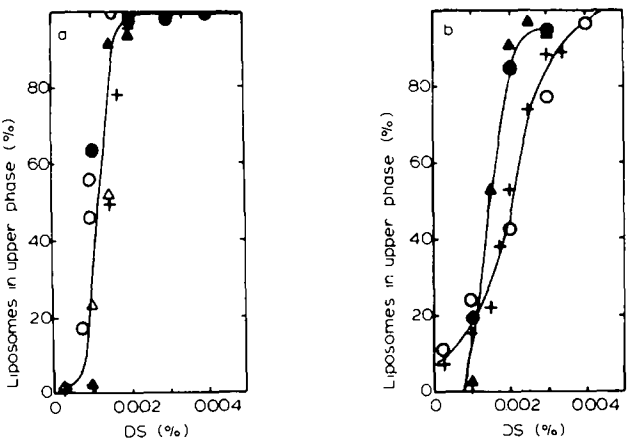

Fig. 1. Saturated and unsaturated phosphatidylcholine liposomes in phase systems containing poly(ethylene glycol) palmitate. The percentage of phosphatidylcholine liposomes in the upper phase as function of the percentage poly(ethylene glycol) end groups esterified with palmitic acid. DS, degree of substitution. The phase systems were composed of 8% (w/w) dextran 40, 4% (w/w) poly(ethylene glycol) 6000, 0.15 mol/kg NaCl and 0.01 mol/kg sodium phosphate buffer. Each 5 g phase system contains 0.35 μmol phospholipid. ○, soya bean phosphatidylcholine; +, egg phosphatidylcholine; △, dipalmitoylphosphatidylcholine. ▲, dipalmitoylphosphatidylcholine/cholesterol, molar ratio 1 : 1 and ●, soya bean phosphatidylcholine/cholesterol, molar ratio 1 : 1. Fig. 1a shows multilayered liposomes and Fig. 1b bilayer liposomes.

were sonicated until clearness of the solutions, but not separated according to size on a column, in order to be sure that no changes for the partition experiments occurred during the separation. The sphingomyelin in Fig. 2 was from bovine brain (66% saturated, 32% monounsaturated and no polyunsaturated

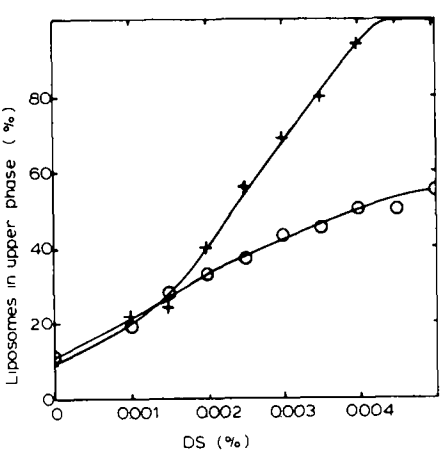

Fig. 2. Sphingomyelin and phosphatidylcholine liposomes in phase systems containing poly(ethylene glycol) palmitate. The percentage of liposomes in the upper phase as function of the percentage poly(ethylene glycol) end groups esterified with palmitic acid, DS. The phase systems were composed of 8% (w/w) dextran 40, 4% (w/w) poly(ethylene glycol) 6000, 0.15 mol/kg NaCl and 0.01 mol/kg sodium phosphate buffer. Each 5 g phase system contains 0.35 μmol phospholipid. The liposomes were not separated according to size and were thus a mixture of multilayered and bilayer liposomes. ○, sphingomyelin and +, dipalmitoylphosphatidylcholine.

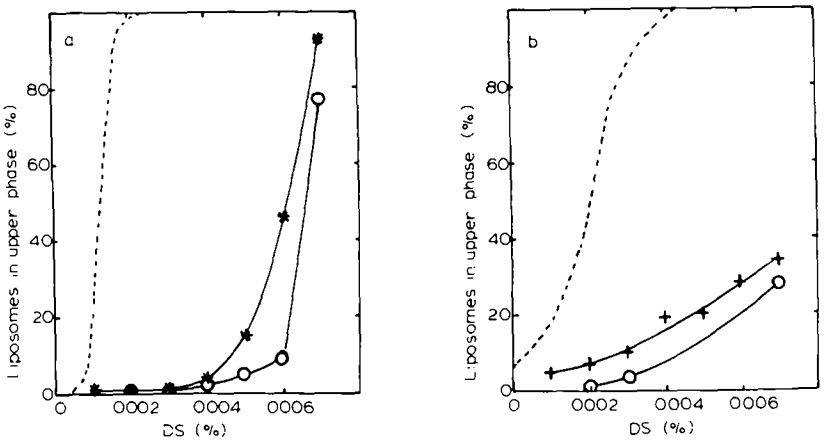

Fig. 3. Negatively-charged liposomes in phase systems containing poly(ethylene glycol) palmitate. The percentage of liposomes in the upper phase as function of the percentage poly(ethylene glycol) end groups esterified with palmitic acid, D.S. The phase systems contain 8% (w/w) dextran 40, 4% (w/w) poly(ethylene glycol) 6000, 0.15 mol/kg NaCl and 0.01 mol/kg sodium phosphate buffer. Each 5 g phase system contains 0.35 μmol phospholipid. *, phosphatidylinositol : phosphatidylcholine, molar ratio 1 : 1; +, phosphatidylinositol and ◦, phosphatidylserine. The dotted line represents the corresponding curve for phosphatidylcholine liposomes from Fig. 1. Fig. 3a shows multilayered liposomes and Fig. 3b shows bilayer liposomes.

fatty acyl chains [13]) and the phosphatidylcholine was dipalmitoylphosphatidylcholine. Incorporation of cholesterol in sphingomyelin liposomes in molar ratio 1 : 1 removes this difference in partition between phosphatidylcholine and sphingomyelin liposomes. The partition behaviour for sphingomyelin/cholesterol liposomes becomes similar to that of phospatidylcholine liposomes (not shown in figures). At even higher concentrations of poly(ethylene glycol) palmitate the negatively charged liposomes will be found in the upper phase. Fig. 3.

Differences in liposome composition can therefore be detected in phase systems containing poly(ethylene glycol) palmitate. The polar head group of the phospholipid is also here the dominant factor in determining the partition of liposomes. The degree of unsaturation of the phospholipid has negligible influence on the partition in this case. Differences in partition between liposomes of phosphoglycerides and sphingolipids with the same polar head group can be found with poly(ethylene glycol) palmitate.

It is reasonable to assume that the fatty acyl group of poly(ethylene glycol) palmitate dips down into the bilayer and in that way anchors the poly(ethylene glycol) chain. The results of this investigation shows that the strength of this binding is more dependent of the polar head group than of the fatty acyl part of the phospholipid.

Conclusions

Dextran-poly(ethylene glycol) phase systems

1. The polar head group of the phospholipid is the dominant factor in determining the partition of liposomes.

2. The charge of the liposomes can be detected. The surface charge of the liposomes is not the same charge as the sum of the charges of the phospholipids.

3. The degrees of unsaturation of the phospholipids are of less importance for the partition behaviour.

4. Cholesterol plays a minor role in determining the partition.

Dextran-poly(ethylene glycol)-palmitate phase system

1. Poly(ethylene glycol) palmitate has affinity for lipid bilayers and can change the partition of liposomes from the lower phase to the upper phase.

2. Poly(ethylene glycol) palmitate can detect differences in surface properties of liposomes. The polar head group of the phospholipid is the dominant factor in determining the partition.

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