Note

Hydrophobic affinity partition of liposomes in aqueous two-phase systems

EVA ERIKSSON

Department of Biochemistry, University of Lund, P.O. Box 740, S-220 07 Lund 7 (Sweden)

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Aqueous two-phase systems consisting of the two polymers dextran and poly-(ethylene glycol) (PEG) are useful in the separation and study of cells and membrane particles[1,2]. Both phases have a high content of water, 80–95 %, and have therefore very mild effects on biological materials. Salts and sugars may be added at suitable concentrations.

Hydrophobic affinity partition in aqueous two-phase systems with fatty acids covalently attached to PEG has been used for selective partition of proteins[3–5] and membranes[6–8]. Materials having affinity for the PEG-bound fatty acids will be partitioned from the lower dextran-rich phase to the upper PEG-rich phase. Liposomes have been partitioned in phase systems with PEG esterified with palmitic acid[9]. Differences in partition behaviour were found when the polar head groups of the phospholipids were varied, but not when the degree of unsaturation was varied. For chloroplast membranes it has been shown that the length of the PEG-bound fatty acid is of importance[6]. Intact and broken chloroplasts were separated when capric acid was attached to PEG but not when longer fatty acids were used.

This investigation was done in order to see whether the effect of PEG-bound fatty acids of various lengths was reflected in partition of liposomes of different composition. Dilauroyl phosphatidylcholine, dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine, distearoyl phosphatidylcholine and egg phosphatidylcholine as well as phosphatidylserine liposomes were partitioned in phase systems with PEG esterified with capric acid, lauric acid, myristic acid, palmitic acid and stearic acid. The longer the PEG-bound fatty acid, the lower is the amount of it required for extraction of liposomes from the lower phase.

The results show that neither differences in length nor in degree of unsaturation of the liposomal phospholipids give partition differences with PEG-bound fatty acids of various lengths. In contrast, variations in the polar part of the liposomes dominate the partition. The differences in partition of phosphatidylcholine and phosphatidylserine liposomes were more pronounced when the PEG-bound fatty acid was decreased in length from stearic acid to capric acid.

EXPERIMENTAL

Dextran 40, Batch No. 5970, $M_r = 42,000$ was supplied by Pharmacia (Uppsala, Sweden). PEG, $M_r = 6000$ was obtained from Union Carbide (New York, NY,

U.S.A.) as Carbowax. The PEG esters were synthesized by Dr. Göte Johansson[4]. Dilauroyl L-α-phosphatidylcholine, purity 98%, dimyristoyl L-α-phosphatidylcholine, purity 98%, dipalmitoyl DL-α-phosphatidylcholine, purity 99%, distearoyl L-α-phosphatidylcholine, purity 99% and L-α-phosphatidylserine (bovine brain), purity 98–99% were obtained from Sigma (St. Louis, MO, U.S.A.). Egg phosphatidylcholine was prepared according to ref. 10. [14C]Phosphatidylcholine (50 mCi/mmol), purity 99% was obtained from New England Nuclear (Boston, MA, U.S.A.).

Liposomes were prepared according to Huang and Thompson[11]: 14 μmol of lipid were dissolved in chloroform in a 5-ml flask and 4 nmol [14C]phosphatidylcholine were added. The solvent was evaporated under a stream of nitrogen, and the vessel was placed in a desiccator under reduced pressure. One ml of 0.15 M NaCl and 0.01 M sodium phosphate buffer, pH 7, was added and the mixture was sonicated under nitrogen above the transition temperature in a Bransonic cleaning bath 12 for 15 min. The resulting solution contained mostly multilayered liposomes.

Preparation of phase systems

The 5-g phase system used contained 8% (w/w) dextran 40, 4% (w/w) PEG 6000, 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7, and 0.35 μmol phospholipid. The phases were mixed by 30 inversions and allowed to separate for 25 min. All partitions were done at 22°C. After separation, 1 ml of the upper PEG-rich phase and 1 ml of the lower dextran-rich phase were withdrawn and the radioactivity was measured. The amount at the interface was calculated from the total amount in the phase system.

RESULTS

In phase systems composed of 8% (w/w) dextran 40, 4% (w/w) PEG 6000, 0.15 M NaCl and 0.01 M sodium phosphate buffer, pH 7, 93–98% of the liposomes are in the lower dextran-rich phase. If some of the terminal hydroxyl groups of PEG are esterified with fatty acids the liposomes will be found at the interface and in the upper phase.

Fig. 1 illustrates the increasing affinity of liposomes for the interface and the upper phase as the concentration of PEG-bound fatty acid increases. The longer the fatty acids of PEG, the more effective is the extraction of liposomes, *i.e.*, the lower is the concentration of PEG-bound fatty acid required for transferring the liposomes from the lower phase. Both for phosphatidylcholine and phosphatidylserine liposomes, the effectiveness of extraction follows the order: PEG caprate < PEG laurate < PEG myristate < PEG palmitate ≤ PEG stearate.

There were no differences in partition behaviour between phosphatidylcholine liposomes having different hydrophobic parts of the bilayer (Fig. 1). Thus, dilauroyl, dimyristoyl, dipalmitoyl and distearoyl phosphatidylcholines and egg phosphatidylcholine liposomes were all extracted from the lower phase at the same concentrations of PEG-bound fatty acids.

Table I shows the amount of PEG-bound fatty acid that is required to partition 50% of the liposomes at the interface and in the upper phase. It can be seen that 5–15 times more PEG-bound fatty acid is required to extract phosphatidylserine liposomes compared to phosphatidylcholine liposomes. The ratio increases when
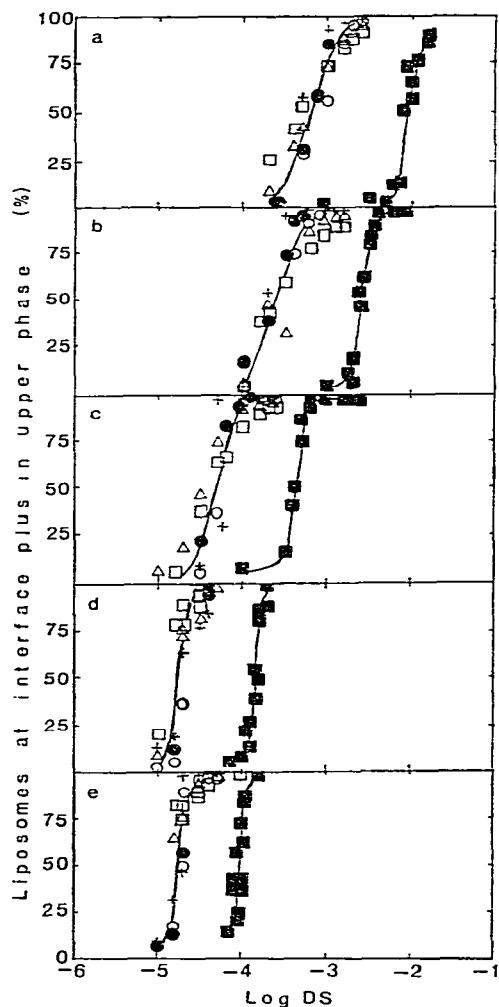
Fig. 1. The percentage of liposomes at the interface and in the upper phase as a function of the fraction of PEG end groups esterified with fatty acids, DS, *i.e.*, the degree of substitution. +, Dilauroyl phosphatidylcholine; ○, dimyristoyl phosphatidylcholine; △, dipalmitoyl phosphatidylcholine; □, distearoyl phosphatidylcholine; ●, egg phosphatidylcholine and ■, phosphatidylserine. PEG was esterified with capric acid C_{10} (a), lauric acid C_{12} (b), myristic acid C_{14} (c), palmitic acid C_{16} (d) and stearic acid C_{18} (e).
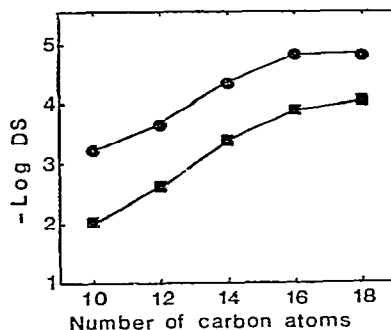
Fig. 2. The fraction of PEG end groups esterified with fatty acids, DS, for extraction of 50% of the liposomes from the lower phase as a function of the number of carbon atoms of the fatty acids. ●, Phosphatidylcholine; ■, phosphatidylserine liposomes.

shorter fatty acids are used, *i.e.*, the shorter the PEG-bound fatty acid, the higher is the concentration required to extract 50% of phosphatidylserine liposomes compared to phosphatidylcholine liposomes from the lower phase.

In Fig. 2, —log DS for extraction of 50% of the liposomes from the lower phase is plotted against the number of carbon atoms of the fatty acid attached to PEG.

TABLE I

FRACTION OF PEG END GROUPS ESTERIFIED WITH FATTY ACIDS FOR EXTRAC-
TION OF 50% OF THE LIPOSOMES FROM THE LOWER PHASE TO INTERFACE AND
UPPER PHASE

DS = degree of substitution = PEG-bound fatty acid/PEG.

| Fatty acid bound to PEG	DS		DS ratio, phosphatidyl-serine: phosphatidyl-choline liposomes
	Phosphatidylcholine liposomes	Phosphatidylserine liposomes	
Capric acid, C_{10}	$6.3 \cdot 10^{-4}$	$9.6 \cdot 10^{-3}$	15.2
Lauric acid, C_{12}	$2.4 \cdot 10^{-4}$	$2.5 \cdot 10^{-3}$	10.4
Myristic acid, C_{14}	$4.7 \cdot 10^{-5}$	$4.4 \cdot 10^{-4}$	9.4
Palmitic acid, C_{16}	$1.7 \cdot 10^{-5}$	$1.4 \cdot 10^{-4}$	8.2
Stearic acid, C_{18}	$1.7 \cdot 10^{-5}$	$9.6 \cdot 10^{-5}$	5.6

DISCUSSION

The effect of PEG-bound fatty acids on the partition of liposomes is explained by assuming an interaction of the PEG-bound fatty acid with the liposomal bilayer. Owing to a weaker interaction of the shorter fatty acids, higher concentrations of these are required for extraction of liposomes from the lower phase. The deeper the PEG-bound fatty acids dip down into the liposomal bilayer, the stronger are the hydrophobic interactions between the fatty acids and the hydrophobic part of the bilayer.

This interaction is independent of the nature of the hydrophobic part of the liposomes, since there were no differences in partition of liposomes having different lengths or different degrees of unsaturation of the phospholipid fatty acids. The phospholipids were chosen to have transition temperatures below (egg phosphatidylcholine and dilauroyl phosphatidylcholine), almost the same (dimyristoyl phosphatidylcholine) and above (dipalmitoyl and distearoyl phosphatidylcholines) the temperature during the partition. Thus the fluidity of the bilayer does not influence the partition behaviour.

The polar head group of the phospholipid plays a dominant role in determining the partition of liposomes when PEG-bound fatty acids are present. Since there were no partition differences when the hydrophobic part was varied, it is the accessibility to the hydrophobic part that is important for the interaction. Both electrostatic and steric factors may contribute to this effect. In the case of biological membranes, proteins may also influence the partition.

A simple representation of this interaction is to regard it as the binding of a PEG-bound fatty acid, F, to a binding site, B, on the liposome to give a complex, BF
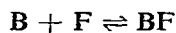
$$B + F \rightleftharpoons BF$$

with $K = [BF]/[B] [F]$. In the simplest case with only one PEG-bound fatty acid on each liposome that is removed from the lower phase and when 50% of the liposomes are extracted from the lower phase, $K = 1/[F]$. In this case [F] is directly proportional to DS. Thus $K \approx 1/DS$.

The binding of a PEG-bound fatty acid to the hydrophobic part of the bilayer may also be regarded as partition of a hydrocarbon between an aqueous phase and an organic phase. ΔG for such a process is proportional to the length, n, of the hydrocarbon, *i.e.*, in this case the length of the PEG-bound fatty acid[12]. Thus, $\Delta G = -RT \ln K \approx$ constant $\cdot n$. And with 50% of the liposomes at the interface and in the upper phase: $-RT \ln 1/DS \approx$ constant $\cdot n$, *i.e.*, log $DS \approx$ constant $\cdot n$. This is also seen in Fig. 2 where there is an almost linear relation between $-$log DS and the number of carbon atoms of the PEG-bound fatty acid until the critical number of 16 where a further increase has very little effect.

Although shorter PEG-bound fatty acids have to be used in higher concentrations they are still very useful for separation purposes. This is reflected in the greater extraction of phosphatidylcholine liposomes compared to phosphatidylserine liposomes from the lower phase as the PEG-bound fatty acid decreases in length (see ratios in Table I). Similar behaviour has been observed[6] with chloroplast membranes where intact and broken chloroplasts were separated when capric acid was attached to PEG but not when longer fatty acids were used. Thus, several different lengths of PEG-bound fatty acids should be tried in order to achieve the best separation.

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