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THE INCORPORATION OF GLYCOLIPIDS WITH DEFINED CARBOHYDRATE SEQUENCE INTO LIPOSOMES AND THE EFFECTS ON PARTITION IN AQUEOUS TWO-PHASE SYSTEMS

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A glycolipid with a defined carbohydrate sequence, derived from the glycoprotein fetuin, has been synthesised and incorporated into liposomes. The effect of the glycolipid on partition of the liposomes in aqueous two-phase systems has been investigated. Incorporation of glycolipid into liposomes changed their partition behaviour in a concentration-dependent manner. The effects on partition of the sequential removal of the terminal carbohydrates were investigated. Partition behaviour appeared to be determined by the net effect of a range of factors including the nature of the terminal carbohydrate, interactions of the lipid region of the glycolipid and possibly carbohydrate chain length. The electrostatic potential difference which can be created between the phases (by the inclusion of certain ions, notably phosphate) appeared to have no detectable effect on partition, even when *N*-acetylneuraminic acid was present as the terminal carbohydrate of the glycolipid.

Introduction

Partition between two immiscible aqueous phases is an established method of cell (and macromolecule) separation [1–3]. The basis of cell separation is the affinity of cells, dependent on their surface properties, for one of the phases. The most commonly used aqueous polymer solutions are dextran and poly(ethylene glycol). In these phase systems cells generally partition between the interface and one of the phases.

Although this is an established technique of cell separation, little is known concerning the effect of membrane surface molecules on partition behaviour. Correlations between partition behaviour and the ratio of poly/mono unsaturated fatty acid for different species of red blood cells have however, been reported in uncharged (neutral) phase systems [4,5], but the complexity of a cell membrane makes evaluation of the effects of particular surface molecules difficult. Liposomes have also been used as 'model cells' and more precise correlations between lipid and partition made, showing that in pure lipid liposomes, the lipid headgroup is the dominant factor determining partition behaviour [6]. These results however give no indication as to the influence on partition of membrane molecules other than lipid.

Although lipids therefore affect partition behaviour, it is likely that the other molecules that constitute biological membranes (eg protein, glycoprotein, glycolipid), may have a greater effect on partition since these molecules often project outwards from the membrane and are thus most accessible to the phases.

Liposomes have again been chosen for study as 'model cells' because of their relative simplicity. In this study however, the effects on partition of incorporation of a defined glycolipid molecule into the liposomes have been investigated.

Materials and Methods

Phase systems

Dextran T500 Batch FD16027 was supplied by Pharmacia, Uppsala Sweden. Poly(ethylene glycol) M_r 4000 was obtained from BDH, Poole, U.K. Each phase system was made up in 4 g batches such that on addition of 1 ml liposome suspension in water, the phase system consisted of 7% (w/w) dextran, 7% (w/w) poly(ethylene glycol). Two-phase systems of this same polymer concentration but containing different salt compositions were used: positive potential system (top phase more positive than the bottom phase), and a phase system designated 'negative' [6] which is essentially neutral but may have a top phase which is slightly more negative than the bottom phase. These were prepared as follows:

Positive: 0.03 mol/kg NaCl, 0.11 mol/kg sodium phosphate buffer (pH 7).

'Negative': 0.15 mol/kg NaCl, 0.01 mol/kg sodium phosphate buffer (pH 7).

The positive and 'negative' systems contained the same salt composition as the phase systems designated positive and negative and used for partitioning liposomes by Eriksson and Albertsson [6].

The phases (at 22°C) were mixed by 30 inversions in 5 ml graduated tubes, and then separated by spinning at $34 \times g$ for 5 min after which a narrow interfacial band of liposomes and translucent phases resulted. The volume of each phase was noted and 250 μl was removed from the centre of each phase for assay. The assays were carried out in 2 ml water in a 3 ml silica cuvette by injection of 100 μl phase (containing liposomes) followed by rapid mixing. For all concentrations of liposomes assayed, light scattering by the liposomes was a constant percentage of the total 'fluorescence' measured, and therefore no corrections were made for this. The same preparations of phases were used in all experiments.

Liposome preparation and assay

A simple, rapid fluorometric assay for liposomes was devised, based on the inclusion of a small amount of 10-(1-pyrene)-10-ketodecanoyl cholesterol into the liposomes. The fluorescence emitted at an excitation wavelength of 366 nm and an emission wavelength of 478 nm (measured on a Perkin-Elmer 3000 Fluorescence Spectrometer) was found to be linear with concentration over the range used and could therefore be used as a measure of liposome concentration.

Bilayer liposomes were prepared as follows: 25 mg dipalmitoylphosphatidylcholine 99% purity (Sigma), and 0.25 mg 10-(1-pyrene)-10-ketodecanoyl cholesterol (Sigma), were dissolved in 1 ml chloroform. Glycolipid (in methanol/water (2 : 0.8, v/v)) was added at concentrations from 2 to 20% (w/w) with respect to phospholipid. The lipid solution was freeze-dried overnight, resuspended in 3.5 ml water and sonicated under N_2 for 60 min in a Decon Sonibath [6,8]. The suspension was centrifuged at $2000 \times g$ for 5 min and the liposomes separated from the frothy surface of unsuspended lipid. 1 ml of liposome suspension was added to each phase system and the remainder assayed for 'number' of liposomes added to each phase system. All liposomes preparations were used immediately after preparation to avoid any changes induced by storage.

Bilayer liposome suspensions made in the way described contain approx. 5% (w/w) multilayer liposomes which can be removed from the suspension by fractionation on a Sepharose 4B column [8]. When bilayer liposomes were purified in this way and partitioned in the two phase systems, their partition was identical to that of unpurified bilayer liposomes (i.e., without the multilayer liposomes removed). It was concluded therefore that the number of multilayer liposomes present was too small to affect partition significantly. The gel filtration step was therefore not included in the preparation of bilayer liposomes.

Glycolipid synthesis

The synthesis of the glycolipid was carried out as described by Warren and Fowler [9]. The carbohydrate sequence of calf serum fetuin (Sigma type 3), was removed by pronase treatment and purified by gel filtration. The glycopeptide consists of three identical carbohydrate chains (Neu*N*Ac-Gal-Glc*N*Ac), linked to a single mannose which is linked to asparagine via two Glc*N*Ac residues. There are six or seven amino acid residues linked to asparagine [10]. The glycopeptide was coupled by its free amino groups to the aldehyde group of tetradecyl aldehyde using sodium cyanoborohy-

dride [9,11]. The same preparation of glycolipid was used for all assays.

Sequential removal of the terminal region carbohydrates

The terminal Neu*N*Ac residues of fetuin were removed by heating 150 mg of protein in 10 ml of 0.025 M H_2SO_4 at 80°C for 1 h, exposing galactose as the new terminal carbohydrate [12]. The terminal galactose residues were removed from desialised fetuin by incubation with 300 units of *Escherichia coli* β-galactosidase (Sigma, ONPG units) at 37°C with gentle shaking for 3 h [9]. Each modified fetuin glycopeptide was then coupled to tetradecyl aldehyde as described.

Agglutination assay

Effective removal of the terminal carbohydrates by the method described and successful incorporation of the glycolipid into liposomes was tested by agglutination as described by Warren and Fowler [9] using wheat germ lectin and peanut lectin from Boehringer Ltd.

Results

The partition of dipalmitoylphosphatidylcholine liposomes containing no glycolipid in the 7% (w/w) Dextran T500/7% (w/w) poly(ethylene glycol) 4000 phase systems described was similar to the partition obtained by Eriksson and Albertsson [6] in 5% (w/w) Dextran T500/4% (w/w) poly(ethylene glycol) 6000 phase systems (Table I). There was, however, one difference in partition between these results and those obtained by Eriksson and Albertsson [6]. The partition of liposomes into the top phase was consistantly greater in the phase system with the top phase more positive than the bottom phase, whereas Eriksson and Albertsson [6] found the partition to be essentially the same in both phase systems. Although phosphatidylcholine is electrically neutral it is possible that phosphatidylcholine liposomes have a small net positive charge since the positive charge is more exposed on the liposome surface. However, no charge is detectable on phosphatidylcholine liposomes by electrophoresis [13]. Clearly the higher partition observed in the phase system with the positive top phase cannot be accounted for by

TABLE I

PARTITION OF LIPOSOMES

Percentage distribution of dipalmitoylphosphatidylcholine (PC) liposomes, containing different amounts of glycolipid, between the top and bottom phases and the interface in two phase systems of 7% (w/w) Dextran T500/7% (w/w) poly(ethylene glycol) 4000 with positive and negative top phase potentials. Each result is the average of at least three separate experiments. Values in parentheses are the standard deviations.

Liposome composition	Phase system potential	
	+	− (neutral)
PC liposomes	14 (1.5)	7 (1.7)
(no glycolipid)	54 (2.5)	70 (0.9)
	32 (0.9)	23 (3.1)
Neu*N*Ac terminal glycolipid	16 (3.0)	14 (1.6)
(w/w) phospholipid	69 (3.1)	72 (2.3)
4%	15 (1.4)	14 (1.0)
20%	51 (2.5)	47 (3.8)
	26 (2.3)	25 (4.5)
	23 (3.9)	28 (2.0)
Galactose terminal glycolipid	17 (2.5)	10 (0.5)
(w/w) phospholipid	72 (2.5)	80 (0.9)
2%	11 (2.0)	10 (1.5)
4%	41 (2.5)	28 (2.1)
	35 (4.1)	49 (3.5)
	24 (2.0)	23 (2.9)
10%	53 (2.0)	50 (2.0)
	20 (1.0)	23 (3.6)
	27 (1.1)	27 (1.5)
20%	51 (0.9)	40 (2.5)
	26 (2.5)	37 (2.1)
	23 (3.0)	23 (0.4)
Glc*N*Ac terminal glycolipid	14 (0.7)	5 (1.1)
(w/w) phospholipid	79 (1.0)	90 (1.1)
2%	7 (0.5)	5 (1.1)
4%	31 (2.5)	17 (1.9)
	56 (2.2)	73 (3.1)
	13 (3.6)	10 (2.4)
10%	35 (2.5)	24 (0.9)
	48 (2.0)	60 (0.8)
	17 (0.9)	16 (0.5)
20%	40 (3.9)	34 (2.5)
	40 (4.2)	43 (2.3)
	20 (0.9)	23 (0.9)

the charge of the phosphatidylcholine. A possible explanation for this may be that the inclusion of pyrene cholesterol (which was not included by Eriksson), affects charge dependent partition. Cholesterol is known to affect partition of liposomes [6] and also to affect charge-associated membrane properties of erythrocytes [14].

Table I shows a summary of the partition obtained with various concentrations of glycolipid and modified glycolipid in the liposomes.

NeuNAc terminal carbohydrate glycolipid

When the 'native' Neu*N*Ac terminal glycolipid is incorporated into liposomes at a concentration of 4% (w/w) there is little change in the top phase partition but there is a decrease in bottom phase partition from 32 to 15% in the positive potential phase system. When the glycolipid concentration is increased to 20% (w/w), partition in the top phase is increased to 51% and partition in the bottom phase also increases to 23%.

When the liposomes are partitioned in the 'negative' potential phase system, there is an increase in top phase partition from 7 to 14% and a bottom phase decrease from 25 to 14% for a glycolipid concentration of 4% (w/w). At 20% (w/w) glycolipid, the top phase partition is 47% and the bottom phase partition 28%. The most striking feature of the results is that although incorporation of Neu*N*Ac terminal glycolipid into the liposomes gives them additional negative charge, this charge does not appear to have any influence on partition in the charged phase system. The top phase partition is very similar whether the top phase has a strong positive or a weak negative (or neutral) potential. Increasing the glycolipid concentration to 20% (w/w) increases partition in both phases with partition in the bottom phase being approximately the same as for the liposomes with no glycolipid.

Galactose terminal carbohydrate glycolipid

Galactose was exposed as the terminal carbohydrate by removal of terminal Neu*N*Ac as described. The efficiency of this method was at least 98% [15]. When incorporated into liposomes, the latter could be agglutinated with peanut lectin (specific for galactose), but not with wheat germ lectin which binds Neu*N*Ac and Glc*N*Ac [9].

Four concentrations of glycolipid were used (2, 4, 10 and 20% (w/w)) in the liposomes. At concentrations of 4% (w/w) and above there was increased partition in the top phase compared with liposomes containing no glycolipid. The increased top phase partition appears to be concentration dependent between 4 and 10% (w/w) and at 20 (w/w) partition is similar to that 10% (w/w).

Partition into the top phase is greater with galactose as the terminal carbohydrate than with Neu*N*Ac. At 4% (w/w), top phase partition was 41% in the positive potential system compared with 16% for the Neu*N*Ac terminal glycolipid. At 2% (w/w), the galactose terminal glycolipid produces top phase partition comparable with Neu*N*Ac glycolipid at 4%. In both phase systems the galactose terminal glycolipid has a higher top phase partition (at least 2-times), than the Neu*N*Ac terminal glycolipid at concentrations of between 4–10% (w/w).

Charge appears to have little effect on partition except that, as with liposomes without glycolipid, the top phase partition is highest in the positive potential phase system.

Partition in the bottom phase appears to follow a similar pattern to that with the Neu*N*Ac terminal glycolipid. At low concentrations the bottom phase partition is low, increases with increased concentration, but appears to remain lower than the partition of liposomes without glycolipid.

N-Acetylglucosamine terminal glycolipid

Glc*N*Ac was exposed as the terminal carbohydrate by removal of the galactose from desialised fetuin with β-galactosidase as described. The efficiency of this treatment is difficult to estimate. When liposomes were made containing the treated glycolipid they were agglutinated with wheat germ lectin which is specific for Glc*N*Ac and were not agglutinated with lectins specific for other terminal carbohydrates, suggesting that few glycolipid molecules were present with terminal carbohydrate residues other than Glc*N*Ac [9].

The same concentrations of this glycolipid were incorporated as for the galactose terminal glycolipid and the partition in the phase systems appears to follow a similar pattern.

The top phase partition is intermediate between the partition for Neu*N*Ac terminal and galactose

terminal glycolipids in all three phase systems. The top phase partition is concentration dependent but unlike the case of the galactose terminal glycolipid there is no decrease in top phase partition between 10 and 20% (w/w).

The bottom phase partition also followed a similar pattern to that of the galactose terminal glycolipid but was less at all concentrations and appeared to be less than the bottom phase partition with the Neu*N*Ac terminal glycolipid.

Tetradecyl aldehyde

In order to determine any possible effect on partition of the lipid portions of the glycolipid, tetradecyl aldehyde was incorporated into the liposomes at 1% (w/w). The effect on partition of the addition of this small amount of lipid was to greatly reduce partition in both phases for the two phase systems used to between 3 and 6% (results not shown). This represents a large decrease in bottom phase partition (i.e., from approx. 30%), and a smaller decrease in top phase partition (i.e., from approx. 15%).

Discussion

Clearly the incorporation of carbohydrate molecules, in the form of glycolipid, into liposomes, produces marked changes in partition of liposomes, in both top and bottom phases.

Top phase partition

For amounts of 4% (w/w) and above, carbohydrate molecules increase top phase partition of liposomes. Thus addition of apparently non-hydrophobic molecules to hydrophobic lipid membrane appears to increase the affinity of liposomes for the hydrophobic top phase.

Changing the terminal carbohydrate residue has a marked effect on partition. This is particularly noticeable at 4% (w/w) incorporation where there appears to be an 'order' of increasing top phase partition such that galactose > Glc*N*Ac > Neu*N*Ac. Neu*N*Ac can be considered to be the least hydrophobic of the three since it is charged and might therefore be expected to have the lowest partition into the hydrophobic phase. However, at higher glycolipid concentrations (e.g. 20% (w/w)) there is little difference in partition with the different terminal carbohydrates and it is possible that this represents a saturation effect.

Top phase partition is concentration dependent within the concentration range used, although there is a decrease in partition with the galactose terminal glycolipid at 20%(w/w).

Bottom phase partition

Incorporation of glycolipid into liposomes decreases bottom phase partition in a concentration dependent manner. The lower the concentration of glycolipid added to the liposomes, the lower the bottom phase partition. Increasing the glycolipid concentration increases the bottom phase partition, but not above the level obtained for liposomes without glycolipid. A possible explanation for this is that the tetradecyl aldehyde is having an affect on partition. Tetradecyl aldehyde alone, greatly reduces bottom phase partition at very low concentrations (i.e., 1% w/w). It is possible that at low concentrations of glycolipid the tetradecyl aldehyde has a bigger effect on partition than at higher glycolipid concentrations because it may be masked from the phases to some extent at higher concentrations by the large number of carbohydrate chains projecting from the liposomes surface.

There appears to be on order of increasing bottom phase partition with different terminal carbohydrates which is again most noticeable at 4% (w/w) glycolipid where galactose > Neu*N*Ac $\geqslant$ Glc*N*Ac. As with the top phase partition the partition at high glycolipid concentrations is very similar with each of the terminal carbohydrates.

Effect of charge

The interpretation of the partition behaviour of charged liposomes in charged phase systems is made difficult by the fact that measurements of the electrostatic potential differences between the two phases are very unreliable when determined using standard methods, e.g., agar bridge electrodes. It is, however, established that the phase system described here as 'positive' does have a significant potential difference across the phases. The system designated 'negative' probably has a small negative potential difference although this cannot be accurately measured. There does not appear to be any major effect on partition of liposomes with charged glycolipid in phase sys-

tems with a notable potential difference between the phases or with little or no potential difference. Neu*N*Ac terminal glycolipid (with three Neu*N*Ac residues per molecule of glycolipid) incorporated at 20% (w/w), does not partition any differently in the positive or 'negative' potential phase systems at the polymer concentrations used.

Eriksson and Albertsson [6] were able to detect charge-indicated partition of liposomes in the charge phase systems described which correlated with the expected charge of the liposomes. When Neu*N*Ac is removed from erythrocytes, there is a corresponding decrease in top phase partition in phase systems with a positive potential but an increase in top phase partition in uncharged (neutral) systems. However, when certain negatively charged membranes, e.g. from *Acholeplasma laidlawii* are partitioned in a positive potential phase system they partition in the bottom phase indicating that in this case charge is not a dominating factor in determining partition behaviour [17].

A possible reason why charge does not appear to affect partition of liposomes which contain a high number of negatively charged Neu*N*Ac groups on their surface is that the effect of the carbohydrate molecules, added to liposomes, on partition is so large that any smaller effects of charge are undetectable.

In all the partition experiments carried out, there was a slightly lower top phase partition (and also more liposomes remaining at the interface) in the 'negative' phase system. This was most clearly detectable, as already stated, for pure phosphatidylcholine liposomes where in the positive potential top phase partition was twice that in 'negative' phase systems. It is interesting that this trend remains for all experiments where the different glycolipids were added in different amounts. This would suggest that whatever molecule in the liposomes is causing this effect, it is detectable by the phases even when large amounts of glycolipid are incorporated. Thus it is possible that molecules both in, and projecting from the membrane can affect partition and even when large numbers of carbohydrate residues project from the membrane, the presence of molecules in the membrane can still be detected by the phases.

Conclusions

The conclusions drawn from these experiments can be summarised as follows:

(1) Incorporation of defined glycolipid molecules into liposomes produces large changes in partition in both phases in a concentration-dependent manner.

(2) The terminal carbohydrate appears to be a dominant moiety in determining partition. Different terminal carbohydrates appear to produce different partition. It is however, difficult to draw precise conclusions on the effects of a particular terminal carbohydrate since the differences are only detectable at certain (low) glycolipid concentrations.

(3) Charge appears to have no detectable affect on partition at the polymer concentrations used.

(4) The partition observed appears to result from a combination of the effects of various regions of the added glycolipid molecules. The nature of the terminal carbohydrate seems to be the major determinant of partition behaviour but changes in the carbohydrate chain length and interactions of the lipid region of the molecule probably contribute to the overall effect. Carbohydrate chain length by itself does not appear to be the main molecular feature governing partition. This is because partition behaviour does not vary with a simple relationship to chain length.

The amino acid portion of the glycolipid is constant but its effect on partition may vary as the strengths of opposing factors change.

The design of appropriate simplified model molecules should allow some dissection of the various components of partition behaviour noted above, and this approach is at present being investigated.

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