Journal of Chromatography, 544 (1991) 283–304
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 042

Review

Liposome chromatography: liposomes immobilized in gel beads as a stationary phase for aqueous column chromatography

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ABSTRACT

Liposomes have been used as a stationary phase for column chromatography with an aqueous mobile phase. They were immobilized in the pores of carrier gel beads by two methods: (A) hydrophobic ligands were coupled to the matrix of gel beads, which then were packed into a column and liposomes were applied and became associated with the ligands by hydrophobic interaction; and (B) phospholipids and detergent were dialysed in the presence of gel beads; many of the liposomes that formed in the pores of the beads were sterically immobilized by the gel matrix. Proteoliposomes containing red cell glucose transport protein in the lipid bilayers were immobilized in a column by method A. This column retained D-glucose longer than L-glucose. In contrast to L-glucose, D-glucose was transported into and out of the immobilized liposomes, causing an increased retention. Liposomes with $(\text{stearylamine})^+$ or $(\text{phosphatidylserine})^-$ in their lipid bilayers were immobilized by method B and the gel beads were packed into a column. A protein of opposite charge was applied in excess. Under suitable conditions, the protein molecules became close-packed on the liposome surfaces. Ion-exchange chromatographic experiments with proteins showed that these sterically immobilized liposomes were also stable enough to be used as a stationary phase. The loss of lipids was 5–23% in the first run at high protein load and with sodium chloride gradient elution but was lower in subsequent runs. It is proposed that water-soluble molecules can be separated and their interactions with liposome surfaces studied by chromatography on immobilized liposomes in detergent-free aqueous solution. Membrane proteins can be inserted and ligands can be anchored in the lipid bilayers for chromatographic purposes.

CONTENTS

1. INTRODUCTION

Chromatography with immobilized liposomes as a stationary phase was recently introduced [1–3] and methods for immobilization of liposomes have been developed [1–5] for this and other purposes. For brevity, the term "liposome" is here used to mean "artificial unilamellar phospholipid vesicle" ("lipid vesicle" [1–5]), although "liposome" sometimes denotes a multilamellar structure [6].

The lipid bilayer of liposomes is thought to be structurally similar to the lipid regions of biomembranes. This makes immobilized liposomes interesting as chromatographic media. Further, membrane proteins and other amphiphilic molecules can be incorporated into the lipid bilayers, and various ligands can be anchored in the bilayers by means of attached hydrophobic groups [7,8]. Immobilized liposomes can thus be used as a versatile stationary phase for mild chromatography in aqueous solution. Interactions between liposome surfaces and water-soluble molecules can be studied by chromatographic techniques under conditions similar to those prevailing in cells. The internal aqueous space of liposomes can also be utilized, for instance, in studies of transport across the lipid bilayers.

The limited stability of liposomes is a drawback to using immobilized liposomes as chromatographic media. Detergents and/or organic solvents normally cannot be employed unless the lipid bilayers are stabilized, *e.g.*, by cross-linking of the lipids. In another approach by Pidgeon and Venkataram [9], a monolayer of phospholipids was covalently coupled to silica particles. With this material a variety of solvents can be used and the restrictions caused by limited stability are less severe. However, for the purification of non-denatured proteins, for the use of membrane proteins or other amphiphilic components in lipid bilayers and for studies of interactions with liposome surfaces, the use of liposomes has a decisive advantage.

To our knowledge, liposome immobilization has not been explicitly described previously except in some recent pharmaceutical applications [10,11]; however, lipid entrapment in acrylamide gel for chromatographic purposes has been described [12] (see below), and phospholipids have been immobilized in agarose beads by hydrophobic interaction for use as phospholipase substrates [13]. We have immobilized liposomes in gel beads by two methods using (A) hydrophobic and (B) steric immobilization. Gel beads commercially available for molecular sieve chromatography were used as liposome carriers.

For method A we covalently coupled alkyl chains (C_4–C_{12}) to beads of agarose or allyldextran cross-linked with bisacrylamide and then let preformed liposomes diffuse into the gel beads. The liposomes thus became associated with some of the alkyl chains by hydrophobic interaction [1,4] within the beads and, probably, on their surfaces. This method is termed here "immobilization by hydrophobic interaction" (IHI), in analogy with the term "hydrophobic interaction chromatography" (HIC), and the liposomes are denoted "IHI-liposomes".

For method B we dialysed a mixture of gel beads and detergent-solubilized phospholipids. Liposome formation occurs both inside and outside the gel beads. Some liposomes grow too large to leave their site of formation and thus become sterically immobilized in the gel-bead matrix [2,3,5]. This method is termed here "steric immobilization" (earlier termed "entrapment" [5]) and sterically immobilized liposomes are denoted "SI-liposomes". Attempts to form a lipid bilayer around the gel beads have so far failed. The formation of 0.5–3-μm liposomes around 0.2–0.6-μm colloidal gold particles has been reported [14].

The immobilization methods and results will be described below, together with applications in chromatography of D- and L-glucose on liposomes with the glucose transport protein from human red cell membranes incorporated in the lipid bilayers [1], and ion-exchange chromatography of proteins on cationic and anionic liposomes [2,3]. Our data reveal the capacity of the carrier beads for immobilization of liposomes and proteoliposomes, the stability of IHI- and SI-liposomes, the existence of sealed aqueous compartments in these liposomes and the accessibility of the surfaces of SI-liposomes to proteins. Collectively, the results show that both IHI- and SI-liposomes can be used as stationary phases for chromatographic purposes.

In the development of liposome chromatography, experience in membrane biochemistry and separation science has been combined. The conviction that liposome immobilization and liposome chromatography would be feasible was initially derived from HIC results by Hjertén and co-workers [15–17], from the observation of Eriksson [18] that fatty acid derivatives of poly(ethylene glycol) affect the partitioning of liposomes in aqueous two-phase systems and from reports on blood-cell separations on alkyl-Sepharose gels by Halperin and Shaltiel [19,20] and Shinozuka *et al.* [21].

As mentioned briefly above, Pidgeon and Venkataram [9] recently constructed a stationary phase composed of derivatives of membrane phospholipid which are coupled to spherical silica particles via amide bonds to the terminal carbon on the C_2 fatty acid chains. A monolayer of lipid molecules is formed on the internal and external silica surfaces and is thought to cover the pore walls. Scheme 2 in ref. 9 shows a monolayer around a spherical particle and may thus be misleading. This stationary phase is very interesting and complements liposomes and their surfaces as a chromatographic separation medium. The monolayers are covalently coupled and are therefore more stable than liposomes, whereas the lipid bilayers of liposomes are more similar to the lipid regions of biomembranes and in this respect are more versatile for chromatographic purposes than are the monolayers.

Similar approaches have been reported earlier: Miyake *et al.* [22] in 1987 described non-covalent partial coating of silica gel particles with dipalmitoylphosphatidylcholine with the intent to use high-performance liquid chromatography to determine the partition coefficients of substances between octanol and water. The coating was reported to be stable in aqueous solution containing 20% acetonitrile and

phosphoric acid (pH 2.2). The mechanism of phospholipid adsorption is not known. In 1984, Uchida and Filburn [12] reported affinity chromatography on phosphatidylserine and cholesterol immobilized in polyacrylamide. The lipids were dispersed in ethanol and diluted with an aqueous solution containing acrylamide and a high proportion of N,N′-methylenebisacrylamide to make a macroporous gel that could entrap the lipids. The gel was then homogenized mechanically and packed into a column. Uchida and Filburn used the expression "trapped cholesterol–phosphatidylserine micelles", but the lipids most likely formed liposomes. The amount of entrapped lipids was low but sufficient for the purpose of enriching protein kinase C in affinity chromatographic experiments.

The present paper was originally written at the invitation of Professor Toshio Takagi and the publisher Kyoritsu Shuppan (Tokyo) and has been published in Japanese (with minor deviations from this version) in the review journal *Protein, Nucleic Acid and Enzyme* [23].

2. EXPERIMENTAL

We immobilized liposomes of average diameters in the range 30–230 nm either by IHI or by steric immobilization [1–5]. The average liposome sizes were determined by molecular sieve chromatography and by measurements of internal liposome volumes and phospholipid amounts [24,25]. As liposome carriers we used agarose gel beads (Sepharose 6B, 4B and 2B and Superose 6) and beads of allyldextran cross-linked with N,N′-methylenebisacrylamide (Sephacryl S-1000). Some pores in the latter gel were large enough to accommodate even the largest liposomes. These gels and also Sephadex G-50 M, DEAE-Sepharose 6B and a prepacked Mono S column were purchased from Pharmacia LKB (Uppsala, Sweden). Sephacryl S-1000 has recently been replaced by Sephacryl S-1000 Superfine, which has a smaller bead size.

Unless stated otherwise, the experiments were done at room temperature (about 23 ± 2°C), *i.e.*, above the transition temperature of the lipids and lipid mixtures used.

2.1. Ligands for immobilization by hydrophobic interaction (IHI)

To provide space for the polar head groups of the phospholipids in the liposomes upon IHI, alkyl ligands were covalently attached to the gel matrix via long spacer arms terminating in a thioether linkage to the hydrophobic chain (Fig. 1). These derivatives are termed "alkyl sulphide gels". The synthesis involves activation of the hydroxyl groups of the gels with 1,4-butanethiol diglycidyl ether followed by coupling of alkanethiols, as described by Maisano *et al.* [26] for agarose gels. The synthesis is simple and reliable. We found that it could also be applied to Sephacryl gels [1]. In this instance, the hydroxyl groups of the dextran moieties are the probable sites of attachment. A special merit of the alkanethiol coupling procedure is that the average ligand density can be determined simply by determination of the sulphur content of dried gel samples [1,26]. The background sulphur content of the gels must be taken into account and was surprisingly high for the Sephacryl gel [1]. The maximum number of ligands that could be attached per millilitre of packed gel was more than twice as high for Sephacryl S-1000 as for Sepharose 2B, both of which are gels with very large pores [1]. Probably the polymer strands are collected into bundles which will affect the local ligand density.

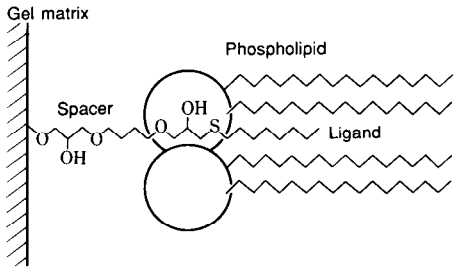

Fig. 1. Binding of phospholipids in liposomes to alkyl sulphide derivatives of gel beads: detail of immobilization of liposomes by hydrophobic interaction (IHI). The partly hydrophilic spacer arm is thought to penetrate the region of the lipid head groups, allowing hydrophobic interaction between the alkyl chain of the ligand and the fatty acid chains of lipid molecules in the outer leaflet of the lipid bilayer.

2.2. Liposomes

Egg yolk phospholipids (70% phosphatidylcholine, 21% phosphatidylethanolamine) were solubilized with cholate and liposomes for IHI (see below) were prepared in Sephadex G-50 M columns by chromatographic removal of the detergent, essentially as originally described by Brunner *et al.* [27]. We have used this procedure in several studies of the glucose transporter protein from human red cells [1,28–36]. In some liposome chromatographic experiments, integral membrane proteins and lipids from human red cell membranes were solubilized in *n*-octyl β-D-glucopyranoside (octyl glucoside) [33–36] and mixed with the cholate-solubilized lipids to form proteoliposomes [1,5]. For steric immobilization, both cholate-solubilized and octyl glucoside-solubilized egg yolk phospholipids were used in liposome preparation by dialysis [5] (see below). The preparation, characterization and preservation of liposomes have been reviewed recently by Lichtenberg and Barenholz [37].

In the chromatographic procedure, the detergent monomers migrate into the gel beads of the Sephadex G-50 M column and thereby lag behind the lipid–detergent micelles, which are (or soon become) too large to enter the beads. Detergent is released from the mixed micelles, as the amount of bound detergent depends on the concentration of free monomeric detergent in the vicinity of the micelles. The release of detergent is accompanied by lipid–lipid association through hydrophobic interaction and the sizes of the mixed micelles increase [38]. Liposomes are soon formed and are eluted at or very near the void volume of the column (Fig. 2A). The entire formation of liposomes takes place outside the Sephadex gel beads. This procedure can be used efficiently only with detergents that have a high critical micelle concentration (CMC). Octyl glucoside and cholate are such detergents.

When complexes of membrane proteins and a non-denaturing detergent of high CMC are added to the lipid–detergent mixture, most of the protein molecules become inserted into the lipid bilayers as the detergent molecules are released from the complexes and from the micelles during the chromatographic run (Fig. 2A).

For IHI, the size of the liposomes was modulated by varying the ionic strength of the buffer [1,4]. When liposomes were prepared for entrapment by dialysis the size was determined by the choice of detergent and detergent-to-lipid ratio [2,3,5]. High ionic strength promotes the formation of large liposomes when the lipids are solubilized with an ionic detergent such as cholate, but this method gave very broad size

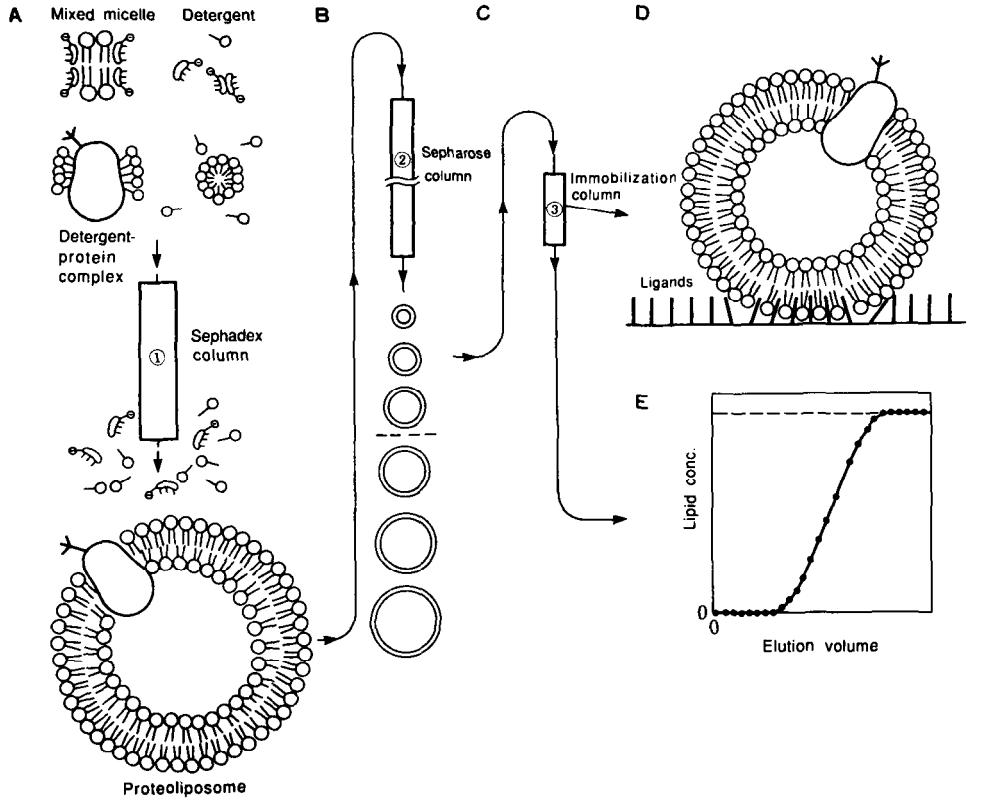

Fig. 2. (A) Preparation of proteoliposomes from phospholipids solubilized in cholate and with membrane protein(s) solubilized with a non-ionic detergent of high CMC, such as octyl glucoside. Detergent monomers are removed from the complexes by chromatography on a Sephadex G-50 M (column 1). The monomers penetrate the gel beads, but the complexes stay outside the beads, where the liposome formation takes place. The carboxyl group and the three hydroxyl groups of the cholate molecules are indicated schematically. (B) Fractionation of the prepared liposomes according to size, *e.g.*, on Sepharose 4B (column 2). (C) Immobilization of fractionated liposomes by hydrophobic interaction (IHI) with alkyl sulphide gel beads (column 3). Large-pore gels such as Sepharose 2B or Sephacryl S-1000 are used. (D) IHI probably engages several alkyl groups at each binding site. See also Fig. 1. (E) Initially all liposomes in the applied liposome suspension become immobilized if the flow-rate and the liposome concentration are not too high. The phospholipid concentration in the eluted buffer is obtained by phosphorus determination. As saturation is approached, part of the applied liposomes pass through the column. Saturation is eventually reached. The area to the left of the graph and below the dashed line corresponds to the amount of immobilized liposomes.

distributions. Liposomes of suitable size ranges were usually selected after fractionation on Sepharose 4B (Fig. 2B). The size distribution in the chosen liposome fraction was sometimes verified by chromatography on Sephacryl S-1000 [1].

In the experiments with steric immobilization of liposomes it is difficult to determine the size distribution of the immobilized liposomes. Average values can be obtained [2,5] and the size of the liposomes formed outside or escaped from the gel beads can be determined [3]. If the SI-liposomes could be revealed by electron microscopy in sections of the gel beads, one might obtain interesting data on the size distribution of the gel bead "pores" and of the liposomes.

2.3. IHI

A large volume of liposome suspension (10–120 ml) was slowly pumped into a small column (1–5 ml) of alkyl sulphide gel beads (Fig. 2C). Octyl chains were mainly used. The rate of phospholipid immobilization was up to 1.5 μmol per millilitre of packed gel per minute until half-saturation had been reached, and thereafter decreased. Usually only a fraction of the applied liposomes became immobilized, even with dilute liposome suspensions [1]. The time of immobilization was usually between 1 and 20 h. Sometimes the liposome suspension was circulated through the column overnight, whereupon the liposomes gradually became immobilized. The liposomes become attached via hydrophobic interaction, presumably with several alkyl ligands (Fig. 2D). In contrast to the situation with protein adsorption on HIC columns, IHI of liposomes does not require a high ionic strength [4]. Modest ligand densities are advantageous for increasing the rate of immobilization and reducing the leakiness of IHI-liposomes [1,4] (see below). Possibly IHI-liposomes diffuse slowly by sliding or rolling along the polymer bundles until a homogeneous distribution is achieved, as suggested in ref. 4 (see Fig. 3). Transient blocking of the entrances of the gel bead pores may contribute to a low adsorption rate, especially as saturation is approached. In addition, the diffusion of the liposomes is relatively slow compared with that of proteins, as liposomes are much larger. At a high liposome concentration in the suspension the flow-rate of application must be slow from the very beginning of the immobilization procedure to allow complete adsorption of liposomes [1].

To determine the immobilization capacity of the carrier gel beads we saturated the columns by slowly applying an excess of liposomes, and calculated the amount of adsorbed phospholipids as the difference between the applied and the eluted amounts of phospholipids (Fig. 2E). As much as about 20% of the gel bead volume could be filled with liposomes [1] (see below).

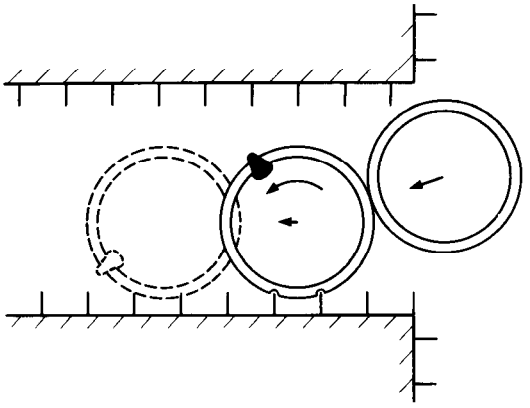

Fig. 3. Even when liposomes of sizes similar to the largest pore dimensions in the gel beads are being immobilized, the pores do not become blocked quickly. Perhaps the largest pores have many entrances. Part of the explanation for the slowly continuing immobilization may be that liposomes that are associated by hydrophobic interactions to a single site (albeit by several ligands) can move along the pores, *e.g.*, by rolling and sliding as illustrated here. In principle, the liposomes will move to regions of high ligand density or to positions where association occurs at more than one interaction site.

2.4. Steric immobilization

Steric immobilization of liposomes in gel beads [2,3,5] was described previously [5]. Gel beads were packed in a short column, a volume of detergent–phospholipid solution slightly larger than the gel bed volume was applied for mixing of the beads and lipid solution without dilution or waste of material and the gel–lipid mixture was then transferred to a thin, flat, rectangular dialysis cell. This was formed by inserting a U-shaped poly(methyl methacrylate) frame, topped by a poly(methyl methacrylate) lid, into a dialysis tube (Fig. 4A). The ends of the tube were tied off with plastic string. The thin shape of the dialysis cell increased the dialysis rate. The dialysis buffer was either changed at suitable intervals [2,5] or flowed continuously [3]. The dialysis cell can be rotated to increase the efficiency further [3,39] (Fig. 4A). Liposomes formed both inside and outside the gel beads. When the bead pore size and lipid-to-detergent ratio were chosen properly, some of the liposomes that formed inside the gel beads as the detergent monomers were removed (Fig. 4B) became sterically immobilized; they were larger than the adjacent opening in the gel matrix structure (Fig. 4C). A detergent of high CMC is necessary unless the detergent outside the dialysis cell can be efficiently adsorbed on, for instance, hydrophobic polystyrene beads. About 80% or more of the lipids formed liposomes outside the gel beads or liposomes which escape from the beads. They were removed by rinsing in a column. A small amount of liposomes was finally removed by centrifugation, but no liposomes were released on further isocratic rinsing in a column [5].

Fusion of phosphatidylserine liposomes on Ca^{2+} binding increases the amount of SI-liposomes of this type [3]. The fusion procedure was developed by Papahadjopoulos *et al.* [40] for other purposes.

3. CHARACTERISTICS OF IMMOBILIZED LIPOSOMES

3.1. Surface area

The amount of liposomes in the gel beads can be expressed in terms of the amount of lipids or of the liposome surface area. The highest values were found on IHI of "small" liposomes [1] (average diameter 40 nm). About half of the gel bead volume of Sepharose 2B and Sephacryl S-1000 is accessible to these liposomes, and the final liposome binding levels were very high, as illustrated in Fig. 5 for Sephacryl S-1000. The saturation levels, about 110 μmol/ml gel, correspond to an outer liposome surface area of 28 m^2 per millilitre of packed gel. We have assumed that the surface area is 70 $Å^2$ per phospholipid molecule in the outer and inner lipid bilayers and that the bilayer thickness is 4 nm. For liposomes of average diameter 200 nm, the corresponding value is 4 m^2/ml, as calculated from data in ref. 1.

Fig. 4. Steric immobilization of liposomes by dialysis. (A) A mixture of large-pore gel beads, phospholipids and detergent of high CMC is dialyzed in a thin, rotating dialysis cell surrounded by flowing buffer. (B) The phospholipid–detergent complexes penetrate the gel bead structure (connected bars). Detergent monomers (cholate in this illustration) leave the dialysis cell through the pores of the dialysis membrane (in the lower half of the figure). The molar ratio between phospholipids and detergent thus increases in the gel beads and large aggregates of phospholipid molecules are formed, eventually leading to the formation of liposomes inside the gel beads as well as outside. (C) Some of the liposomes formed in the beads are too large to pass through the exits to the space in which they were formed and are thus sterically immobilized in the gel matrix.

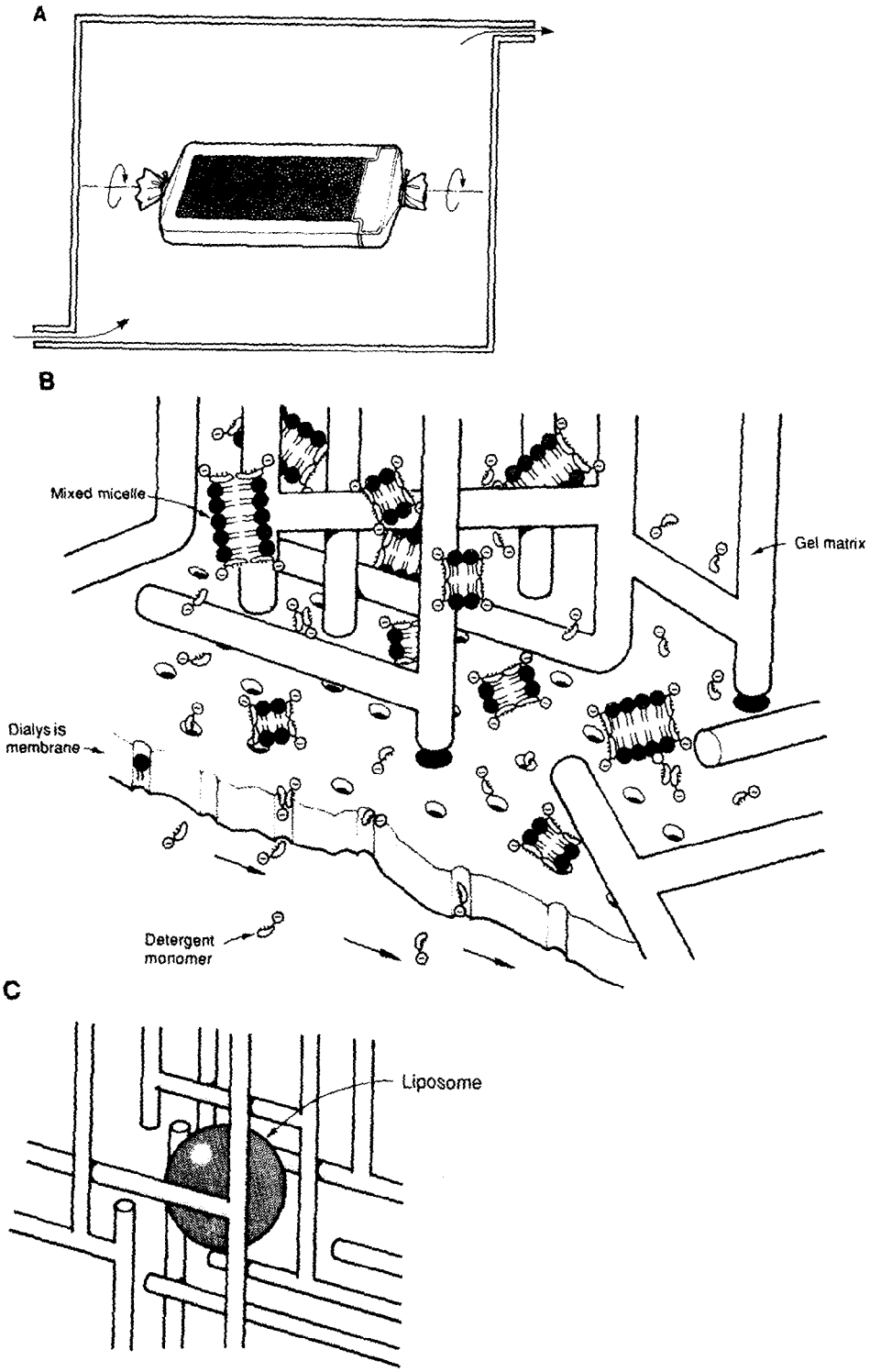
A
B
Mixed micelle
Gel matrix
Dialysis membrane
Detergent monomer
C
Liposome

Fig. 4.

The amounts of SI-liposomes were lower and did not depend on the liposome size in any simple way. Under various conditions of steric immobilization in Sepharose 6B and Sephacryl S-1000 beads, the amount of lipids in the SI-liposomes was 5 ± 4 μmol/ml (n = 19) (data from refs. 2, 3 and 5), which corresponds to about 1.0 ± 0.8 m^2/ml. This is sufficient for many chromatographic applications. The actual surface area is not very important. However, the possible interaction between the substance of interest and the carrier gel bead material has to be determined in separate control experiments without liposomes and such an interaction becomes increasingly more important with decreasing liposome surface area, especially when the interactions with the liposome surface are weak.

The surface area available for gases in and on the silica spheres, Nucleosil-300 ($7NH_2$) (Machery, Nagel & Co., Düren, Germany), which were used in "immobilized artificial membrane chromatography" [9] is 100 m^2/g, according to data from the manufacturer cited in ref. 9, whereas the surface area accessible to propylamine, and thus possibly to phospholipids, was about 20–25 m^2/g (interpretation of estimates in ref. 9). The usefulness of this large surface area is limited by the fact that the surfaces of the lipid monolayers are partially hydrophobic ("similar to a C_2 or C_3 reversed-phase column" [9]), presumably owing to imperfect packing of the lipid molecules. In our IHI-liposome columns many hydrophobic ligands are free and accessible. In contrast, the SI-liposomes in agarose gel beads have purely hydrophilic surfaces, although of moderate area, and the carrier gel beads used are known to exhibit minimum adsorption and interaction with most biomolecules.

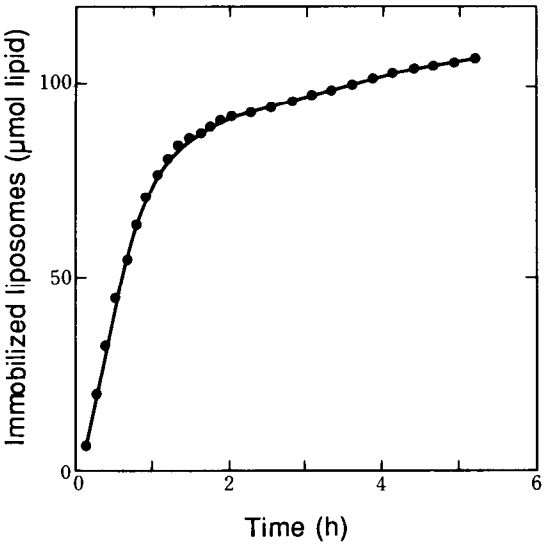

Fig. 5. Example of immobilization by hydrophobic interaction (IHI). Egg yolk phospholipid liposomes of average diameter 40 nm were applied on octyl sulphide Sephacryl S-1000. Ligand density, 14 μmol/ml packed gel; flow-rate, 21 ml/h; column volume, 1.0 ml; phospholipid (phosphorus) concentration in the applied liposome suspension, 4.5 mM; total amount of phospholipids applied, 520 μmol; amount of phospholipids immobilized, 110 μmol; corresponding values after 1 h of immobilization, 95 μmol applied, 78 μmol immobilized (82%). Data from ref. 1.

3.2. Stability of immobilized liposomes

The release of phospholipids or liposomes from the columns was determined by phosphorus assay of all fractions of eluent, according to the method of Bartlett [25]. The leakage of phospholipids from IHI-liposomes was very low (no attempt was made to measure it accurately) and may correspond approximately to the solubility (CMC) of phospholipids in water, which is usually in the nanomolar range, *e.g.*, $4.7 \cdot 10^{-10}$ *M* for dipalmitoylphosphatidylcholine [41,42]. No release of liposomes was observed.

For ionic SI-liposomes we observed liposome losses in the range 5–23% in the first chromatographic experiment on each column in instances where a large amount of protein(s) was bound to the liposomes and then released by a sodium chloride gradient [2,3]. These losses were higher with than without protein binding. Possibly the protein binding to the liposomes is accompanied by formation of "microdomains" which cause increased shrinkage by the osmotic effect of the sodium chloride gradient [3,43]. The increase in osmotic pressure on elution of proteins with sodium chloride was the main cause of liposome leakage from the column. Elution by a decrease in pH gave lower losses. The losses were lower in subsequent experiments performed on the same column, as illustrated in Fig. 6. The use of buffer solutions of osmolarity below that of the solution used for the steric immobilization promoted the stability of the SI-liposomes. Some instability is the inevitable consequence of the use of a stationary phase that is closely similar to the thin and flexible lipid regions of biomembranes and which is not bound covalently or associated via ligands to the carrier. Nevertheless, up to eight chromatographic ion-exchange experiments over a period of 3 days done without problems on a single column. The release of phospholipids on storage of a Sepharose 6B column with sterically immobilized egg yolk phospholipid liposomes for 9 days at 4°C was 9%.

Further long-term stability tests should be done under various conditions. The present results allow fairly safe predictions about the outcome of such tests. The main point is that osmotic and charge-related shrinkage of entrapped liposomes should be kept small.

Liposome phospholipids can be cross-linked [44–46], which would increase liposome stability, but this obviously leads also to changes in the properties of the lipid bilayers.

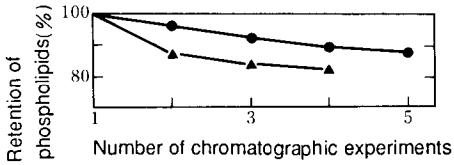

Fig. 6. Stability of sterically immobilized cationic liposomes on ion-exchange chromatography. Liposomes composed of egg yolk phospholipids and stearylamine (molar ratio 4:1) were immobilized in the presence of 0.2 *M* NaCl. Bovine serum albumin was adsorbed at low ionic strength and then released by an increase in NaCl concentration from 0 *M* to (●) 0.2 *M* or (▲) 0.5 *M*. The low ionic strength was restored and the experiment was repeated two or three times. Each curve corresponds to experiments with a single column and shows the amount of phospholipids (liposomes) retained in the column before each experiment. Some stearylamine was probably released from the liposomes remaining in the gel beads in each chromatographic experiment, which presumably contributed to shrinkage of liposomes with attendant release. The losses in experiments 4 and 5 were small. (Redrawn from Fig. 1 in ref. 2, with permission).

The covalently coupled phospholipid monolayers synthesized by Pidgeon and Venkataram [9] are, like immobilized liposomes, stable in aqueous solution at neutral pH but lose material in some organic solvents and some aqueous solutions owing to hydrolysis of the ester bonds of the fatty acid chains. This can be monitored ingeniously by Fourier transform infrared assay [47]. The losses ranged from negligible to large, depending on the solvent [47].

3.3. Liposome volumes

The sum of the volumes of all internal aqueous compartments of unilamellar liposomes composed of a given amount of lipids increases linearly with increasing liposome radius [24]. The reason is that the number of liposomes decreases linearly with the square of the increasing radius, whereas the volume of each liposome increases linearly with the cube of the increasing radius. To achieve a large internal volume of IHI-liposomes, liposomes of large size should therefore be used. However, for IHI in a given type of gel beads, the bead space accessible to the liposomes decreases with increasing liposome radius. We thus found that the internal compartments of IHI-liposomes of average diameters 40, 100 and 200 nm, on saturating alkyl sulphide Sephacryl S-1000, all occupied about 12% of the gel-bed volume, or about 17% of the volume of the beads, as illustrated schematically in Fig. 7. SI-liposomes occupied only 1.0–2.2% of the gel-bead volume, according to data from ref. 5.

The total volume of IHI-liposomes (aqueous and bilayer volumes) could be observed directly as the difference in the elution volume of D-[^{14}C]glucose on an octyl sulphide gel column before and after immobilization [1]. In the absence of glucose transporter protein, D-glucose passes essentially outside the IHI-liposomes.

In cases with little leakage (low ligand density), the internal volumes of IHI-liposomes were determined with the use of calcein, a fluorescent compound resembling a conjugate of fluorescein and EDTA. Calcein was encapsulated in the liposomes during preparation and external calcein was removed by molecular sieve chromatography before IHI. The calcein in the immobilized liposomes was determined by fluorimetry after destruction of the liposomes with cholate [1]. The internal volumes of SI-liposomes were determined similarly in some experiments. These measurements

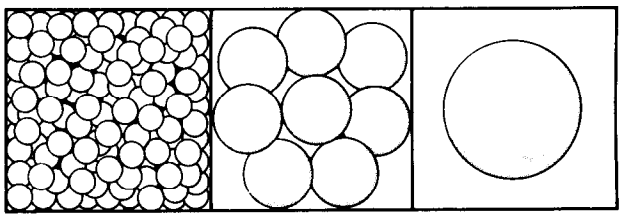

Fig. 7. Saturation of Sephacryl S-1000 with liposomes immobilized by hydrophobic interaction (IHI). The figure shows schematically the liposomes immobilized in a given volume element, except that the left panel should contain 50% more liposomes than are drawn. The point is that the summed internal liposome volumes constitute *ca.* 17% of the gel beads volumes when the beads are saturated with liposomes, in all three panels, although the amount of phospholipids and thus the surface area of the IHI-liposomes is much higher in the left than in the middle panel, and higher in the middle than in the right panel. Average liposome diameters, 40, 100 and 200 nm, from left to right. Data from ref. 1.

prove that the lipid material in the gel beads is in the form of liposomes with a sealed internal aqueous compartment; the liposomes are not destroyed on immobilization. The volumes of IHI-liposomes were consistent with the amounts of phospholipids and the liposome diameters determined by molecular sieve chromatography of the liposomes before immobilization.

3.4. Bilayer permeability

The outleakage of calcein during IHI of calcein-containing liposomes depended dramatically on the ligand density. At an octyl chain density of 14 μmol per millilitre of packed gel the initial leakage during the immobilization procedure was very high (75% of the encapsulated calcein for liposomes of diameter 40 nm), whereas it was only 2–3% at 6 μmol/ml [1]. In the final immobilized state the leakage became very much lower (less than 1%/h), although it was still highest at the higher ligand density [1]. It seems that penetration of a certain number of adjacent octyl chains into the lipid bilayer causes a temporary high leakage, but that the bilayer structure is then largely restored.

The calcein leakage from SI-liposomes in Sepharose or Sephacryl gels was below the detection limit. These liposomes probably retain their normal structure. However, in other experiments, contact between large freeze–thawed liposomes and the surfaces of Sephadex G-50 M gel beads seemed to cause some glucose leakage from the liposomes [36]. Further investigations of interactions between liposomes and various polymers and surfaces may be useful. The adsorption of liposomes on microporous filters can cause a high leakage [36]. Leakage on adsorption of liposomes on glass has been reported [48]. The steric inclusion of liposomes in agarose or bisacrylamide–allyldextran gels seems to have extremely small effects on the bilayers.

4. IMMOBILIZATION OF PROTEOLIPOSOMES

4.1. IHI

Integral membrane proteins and membrane lipids from human red cells were prepared by hypotonic lysis of the red cells followed by molecular sieve chromatography at pH 8 and 10.5 and centrifugations at pH 10.5, 12 and finally 6.8 [1,33]. The proteins were partially solubilized with octyl glucoside [1]. The solution contained mainly the anion transporter, the glucose transporter, glycophorin A and membrane lipids [33]. The solubilization was partly selective for the glucose transporter [35,49] and essentially all of the solubilized proteins were reconstituted into proteoliposomes (unpublished data). The proteoliposomes were prepared by molecular sieve chromatography on Sephadex G-50 M as described above (Fig. 2) and were fractionated on Sepharose 4B. A fraction corresponding to a diameter of about 100 nm was chosen. Octyl sulphide Sephacryl S-1000 was used with a ligand concentration of 6 μmol/ml. About 30 μmol of phospholipids were immobilized per millilitre of gel, with a total specific liposome volume (*cf.*, ref. 24) of 2 μl/μmol lipid. The IHI-proteoliposome column was used for "transport retention chromatography" [1] (see below).

4.2. Steric immobilization of proteoliposomes

Integral red cell membrane proteins, added in octyl glucoside solution to the detergent–lipid mixtures, could also be incorporated into the lipid bilayers on steric

immobilization of liposomes (Fig. 4), as judged by electrophoretic analyses [5]. The amount of phospholipid in SI-proteoliposomes per millilitre of gel, under various conditions, was 70–115% of the corresponding amount of phospholipid for protein-free liposomes sterically immobilized under similar conditions (data from ref. 5). Relatively little anion transport protein was found in SI-liposomes in Sepharose 6B gel beads. Monomers of this protein rapidly associate to form oligomers of high molecular weight in octyl glucoside [34]. These oligomers can only enter a small fraction of the Sepharose 6B pores during the immobilization procedure, which probably is the cause of the decreased yield of the anion transporter in the SI-liposomes. The tendency of some membrane proteins to form oligomers in non-ionic detergents must be considered in reconstitution and immobilization procedures.

In some instances, integral membrane proteins can be incorporated into the lipid bilayers by mixing the proteins with preformed liposomes [50].

5. LIPOSOME CHROMATOGRAPHY

5.1. Transport retention chromatography

A chromatographic effect of transport across the lipid bilayers of the liposomes, mediated by a transporter protein, has been demonstrated [1] for the glucose transporter from human red cells, which is a stereospecific gate for facilitated diffusion of glucose. This protein belongs to a family of tissue-specific but partly sequence-homologous glucose transporters [51,52] which are abundant in, for instance, the blood–brain [53] and the blood–eye barriers [54]. In transport retention chromatography, the internal volume of the immobilized liposomes is used.

The elution volume, V_A, for D-[^{14}C]glucose on a small column of octyl sulphide Sephacryl S-1000 was determined accurately. Proteoliposomes with integral red cell membrane proteins in the bilayers were then immobilized on the column. The glucose transporter constitutes about 20% of the protein by weight. D-[^{14}C]Glucose, which is transported, and L-[^{14}C]glucose, which is not, were applied in separate runs, and the elution volumes, V_D and V_L, were determined. The D-glucose, which has access to the internal compartments of the proteoliposomes, was eluted later than the L-glucose (Figs. 8 and 9). The chromatographic set-up was identical in all three runs. $V_A - V_L$, together with the liposome radius (*ca.* 50 nm) and the bilayer thickness (*ca.* 4 nm), give the internal volume of the IHI-liposomes, V_i, which was about 200 μl. The transport retention, $V_D - V_L$, was 90–95 μl, or about half of V_i, as shown by two similar experiments [1], one of which is illustrated in Fig. 9. $V_D - V_L$ is small, but significant, and consistent with D-glucose transport, being about half the maximum value, V_i. Higher flow-rates gave lower transport retention, probably because of incomplete equilibration of D-glucose between the external medium and the internal volume of the liposomes. Protein-free IHI-liposomes and IHI-proteoliposomes containing inactive glucose transporter showed the same elution volume for D-glucose as for L-glucose.

Smaller carrier gel beads of more homogeneous size and with larger pores may be required to reduce the zone widths and to accommodate more and larger liposomes in order to increase $V_D - V_L$ greatly. The amount of immobilized proteoliposomes can be increased by improvements of the immobilization procedures and the leakage of L-glucose into the proteoliposomes can be reduced by reconstitution of purified glucose transporter instead of the mixture of integral membrane proteins [36].

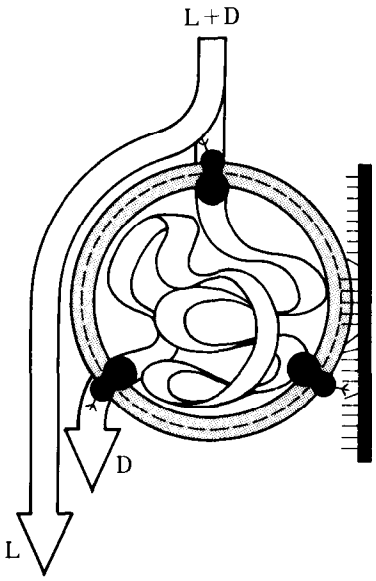

Fig. 8. Retardation of D-glucose (D) in relation to L-glucose (L) on a column of glucose-transporter IHI-liposomes. D-Glucose enters the internal compartments of the liposomes but L-glucose does not. Therefore, D-glucose passes a larger volume than does L-glucose and becomes retarded as illustrated schematically ("transport retention chromatography").

Similar glucose chromatographic experiments, although done with red cell membrane fragments instead of proteoliposomes, were reported in 1966 by Bobinski and Stein [55] and Bonsall and Hunt [56]. The chromatographic effects were extremely small and were attributed to D-glucose binding rather than transport. It was not recognized at that time that the membrane fragments presumably formed more or less sealed vesicles.

An entirely different use of glucose transport for a separation purpose,

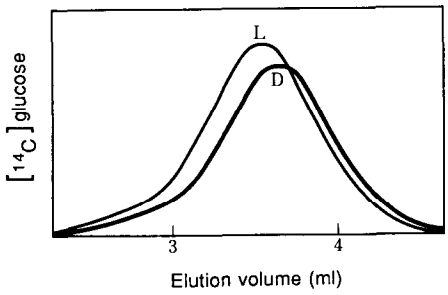

Fig. 9. Example of transport retention chromatography. L-Glucose was eluted earlier than D-glucose from a column of IHI-proteoliposomes in octyl sulphide Sephacryl S-1000, containing human red cell glucose transporter among the proteins in the lipid bilayer. Column volume, 3.8 ml; column size, 4.8 cm × 1.0 cm I.D.; sample volume, 100 μl; flow-rate, 6 ml/h. A longer column would increase the resolution, in a similar manner as in molecular sieve chromatography. Data from ref. 1.

"transport-specificity fractionation", was described by Goldin and Rhoden [57]. Liposomes containing a glucose transporter protein in their bilayer were separated by centrifugation from liposomes containing other single membrane proteins. D-Glucose was preloaded into the liposomes. The separation utilized the decrease in density of the glucose transporter liposomes that occurred as D-glucose was released. An early and correct identification of the glucose transport protein was made. Separations according to the same principle have been done recently with ATP-dependent Ca^{2+} transport proteins and other transporters [58].

5.2. Liposome-surface chromatography

In liposome-surface chromatography, binding to the surfaces of the lipid bilayer is utilized. Our chromatographic experiments of this type, which were made with SI-liposomes, are presented in two papers [2,3], both of which provide data on the chromatographic stability of immobilization, as discussed above. The question of whether the surfaces of SI-liposomes are easily accessible to macromolecules was addressed and ion-exchange chromatographic experiments were made [2]. We showed that the protein-binding capacity of sterically immobilized ionic liposomes increases with increasing surface charge in ion-exchange experiments and gave an example of the use of a small-beaded and highly cross-linked agarose gel as liposome carrier [3].

The charge-density experiments were partly motivated by indications that the surface charge of liposomes with 20% stearylamine was too low for close packing of ferritin molecules on the surfaces [2].

Liposome surfaces with charged ligands show interesting fundamental differences from conventional ion exchangers for proteins. The charge density can be varied easily within limits, but is relatively low. The charges are located on a fairly smooth surface and are probably on average distributed evenly in the absence of a binding molecule, but are mobile in the plane of the liposome surface. We found that nearly all of the surface charges on SI-liposomes were accessible to proteins and that the gel matrix did not significantly block the surface area.

5.2.1. Accessibility of the surfaces of SI-liposomes. At pH 7, negatively charged ferritin molecules (M_r 440 000, diameter 12 nm) became extensively bound to SI-liposomes composed of positively charged stearylamine molecules and neutral egg yolk phospholipids (EYP) in the molar ratio 1:4. The column became dark brown. The carrier gel beads (Sepharose 6B) with and without neutral EYP liposomes showed 1% of the ferritin binding obtained with the cationic liposomes; 30% of the surface area of the positively charged liposomes became covered with ferritin (Fig. 10). A large part of the surface area of SI-liposomes is thus accessible to large proteins, even with small-pore carrier gel beads such as Sepharose 6B. This is consistent with the facts that those pore spaces in which liposomes can be accommodated are much larger than proteins and that the agarose polysaccharides, and probably also most of the Sephacryl polymers, are collected in bundles (Fig. 5 in ref. 5 and Figs. 4C and 10 here). The minimum diameter of a liposome is 20–25 nm, about twice the diameter of ferritin. The liposome in Fig. 10 is probably atypically small but, unfortunately, we do not know the size of the stearylamine–EYP liposomes used in the ferritin binding experiments. Liposomes prepared similarly, but without stearylamine, had an average diameter of about 60 nm [5].

The reason why not more than 30% coverage of the surface was realized is

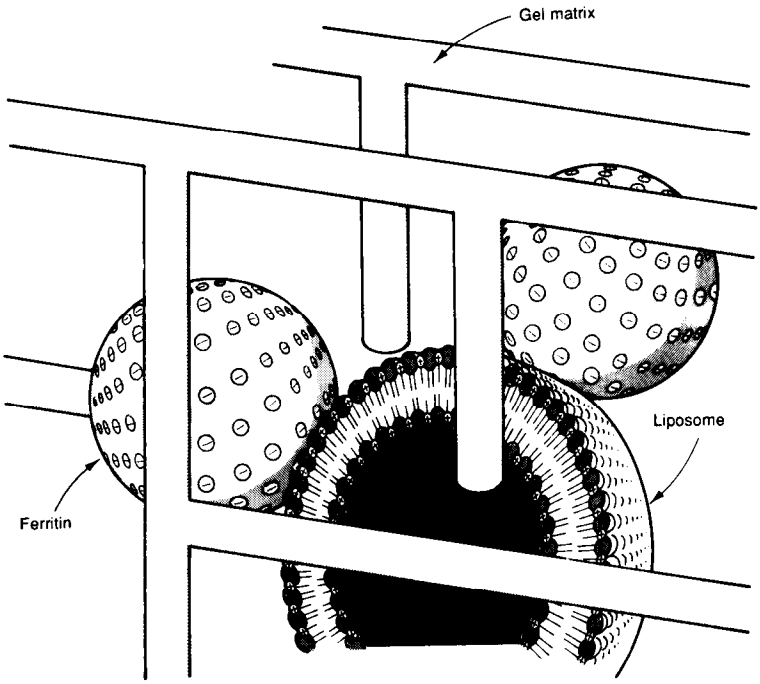

Fig. 10. The surfaces of sterically immobilized liposomes are readily accessible even to proteins as large as ferritin, as illustrated here. Egg yolk phospholipid–stearylamine liposomes were prepared by dialysis entrapment. Up to 0.36 mg of ferritin could be bound per micromole of phospholipid, which corresponds to a coverage of 30% of the liposome surfaces. The main factor limiting the binding is presumably the lack of charges on the surfaces of the liposomes. The relative size of the liposomes used in these experiments is probably, on average, larger than illustrated. Data from ref. 2.

probably that the number of stearylamine charges in the liposome surfaces was too low in relation to the ferritin charge. A coverage of 30% corresponds approximately to electroneutrality. More than 30% of the surface area may thus be sterically accessible to ferritin.

Lysozyme (M_r 14 600) covered about 100% of the surface area of sterically immobilized negatively charged phosphatidylserine liposomes [3] (Fig. 11), whereas citraconylated myoglobin, which, at least at high ionic strength, is only slightly larger than lysozyme, covered only 10% of the stearylamine–EYP liposomes (as calculated by use of data from ref. 2), presumably again mainly due to insufficient surface charge density. We assume that protein–protein interactions can be neglected in the above experiments, as the protein molecules are charged and the ionic strength is low.

The protein-binding capacities are exemplified by the facts that the stearylamine–EYP (1:4) liposomes bound 4 mg of ferritin or 0.5 mg of citraconylated myoglobin per millilitre of gel and phosphatidylserine liposomes bound 1.6 mg of lysozyme per millilitre of gel.

5.2.2. Charge dependence of protein binding to ionic SI-liposomes. When sterically immobilized stearylamine–EYP (1:4) liposomes were saturated with ferritin

or with citraconylated myoglobin, and when phosphatidylserine- or phosphatidylserine–phosphatidylcholine (1:4) liposomes were likewise saturated with lysozyme, the ratio between the charges of the bound proteins and the charges of the liposome surfaces was 0.85 ± 0.16 (data from refs. 2 and 3; with the charge of citraconylated myoglobin estimated at −30 unit charges per polypeptide). About 70–100% of the liposome surface charges were thus utilized for binding of the various proteins, and the surfaces were covered with protein until near electroneutrality, in some instances equivalent to close packing (Fig. 11).

The protein-binding capacity increased for sterically immobilized phosphatidylserine–phosphatidylcholine liposomes with increasing phosphatidylserine content, in accordance with the above rule of electroneutrality, except that the binding at 45% phosphatidylserine was very low. We do not known the reason. Tentative suggestions are that the phosphatidylserine forms microdomains at this particular composition, or that the orientation of bound lysozyme molecules changes at the corresponding charge density.

5.2.3. Ion-exchange chromatography on SI-liposomes. Bovine serum albumin monomers and dimers separated well on cationic SI-liposomes [2] (Fig. 12A). The resolution was better than that on DEAE-Sepharose (Fig. 12B) and required a lower ionic strength. On a Sepharose 6B column in the absence of liposomes, the monomers and dimers were eluted together at 2–3 ml in an experiment corresponding to that in Fig. 12A (not shown). Separations of several plasma proteins have also been achieved on cationic liposomes [2], in addition to separations by "high-performance" ion-exchange liposome-surface chromatography on phosphatidylserine liposomes in 13-μm rigid agarose gel beads (Superose 6) [3]. In the latter instance, ribonuclease A separated from lysozyme and cytochrome *c*, which were fairly well resolved (Fig. 13A). Some protein retardation was observed even in the absence of the anionic liposomes, probably because the Superose matrix is slightly negatively charged and thus not ideal for the present purpose (see Fig. 3B in ref. 3). The ionic strength had to be kept low (0.03–0.15 *M*) with the liposomes for protein binding. This is favourable for the

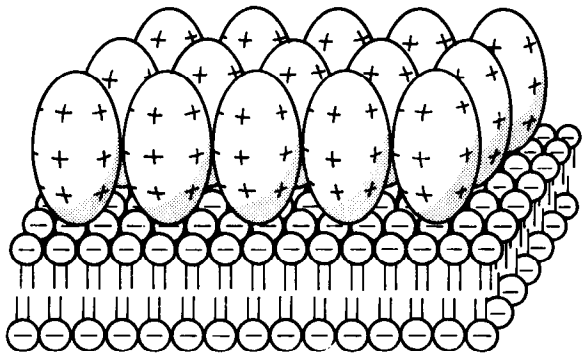

Fig. 11. The surface charge of sterically immobilized phosphatidylserine liposomes was sufficient to afford close packing of lysozyme on the outer liposome surface at pH 7, as illustrated schematically here. Each lysozyme molecule corresponds to about twelve phosphatidylserine molecules in the outer leaflet of the bilayer of the liposome. This could be determined conveniently by chromatography of lysozyme on the immobilized liposomes. The net charge of a lysozyme molecule is about +10 at pH 7. Data from ref. 3.

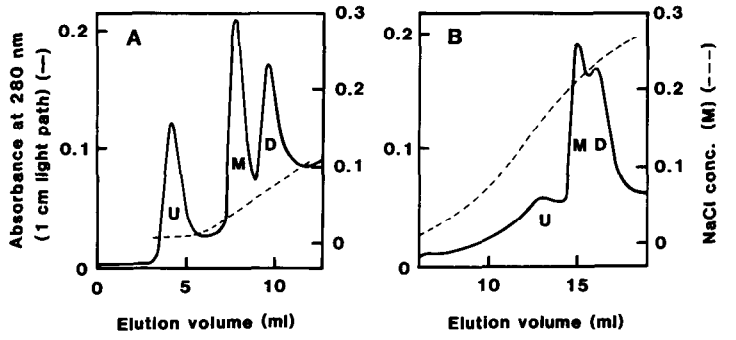

Fig. 12. (A) Ion-exchange chromatography of bovine serum albumin monomers (M) and dimers (D) on sterically immobilized cationic liposomes composed of egg yolk phospholipids and stearylamine. The carrier gel beads were Sepharose 6B. Column, 2.9 cm × 1.0 cm I.D.; sample volume, 200 μl; sample concentration, 1 mg/ml each of M and D. U, unidentified material (not protein). (B) Comparative experiment on the anion exchanger DEAE-Sepharose 6B. The experiment was done as in (A). Redrawn from Figs. 4 and 5 in ref. 2, with permission.

stability of steric immobilization, as the osmolarity used during the immobilization was higher and the liposomes thus swell rather than shrink. The highly substituted strong cation exchanger Mono S (Pharmacia LKB), which has 10-μm beads, was used for comparison (Fig. 13B). The separation of ribonuclease A from lysozyme was poor in this instance, but lysozyme and cytochrome *c* separated slightly better than on the liposome column [3]. The ionic strength required with the Mono S ion exchanger was five times higher than that used with the liposomes. The same flow-rate was used except during sample application [3]. Further improvements in the resolution in

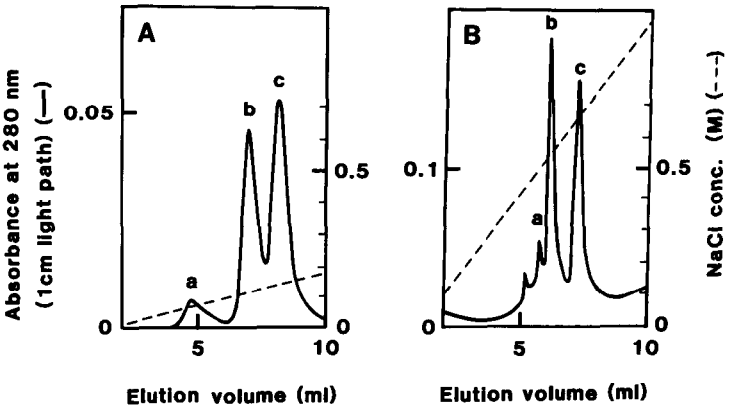

Fig. 13. (A) Ion-exchange chromatography on phosphatidylserine liposomes sterically immobilized in a small-bead (13-μm) gel, Superose 6. (a) Ribonuclease A, (b) lysozyme and (c) cytochrome *c* were eluted at much lower ionic strength than is required with ion exchangers such as Mono S. Experiments of this type confirm that sterically immobilized liposomes are stable enough for chromatographic use. The dashed line shows the NaCl gradient during the elution. Column, 4 cm × 0.5 cm I.D.; flow-rate, 0.4 ml/min; sample volume, 25 μl; protein concentrations, (a) 0.5 and (b and c) 1.0 mg/ml. (B) Comparative experiment on the cation exchanger Mono S (10-μm beads). Column, 5.5 cm × 0.5 cm I.D. The experiment was done essentially as in (A). Redrawn from Fig. 3A and C in ref. 3, with permission.

chromatography on liposomes can presumably be achieved by use of still smaller carrier beads. Obviously, this applies to all types of liposome chromatography and not only to ion-exchange chromatography.

The above ion-exchange chromatographic experiments indicate that proteins cannot bind by ionic interactions to charged lipid bilayers at the relatively high ionic strengths encountered in living cells and at cell surfaces under physiological conditions. This is consistent with earlier results on protein binding to biomembranes [59].

The chromatographic experiments were made partly to gain experience on the stability of the SI-liposomes as a stationary phase for aqueous column chromatography. Several experiments were done on each liposome column, with only moderate losses of binding capacity, before elution and solubilization of the liposomes for determination of the amount of retained phospholipids.

6. CONCLUSIONS

Liposomes can be sterically immobilized or immobilized by hydrophobic interaction in carrier gel beads. Immobilized liposomes offer sufficient stability and accessibility to be usable as stationary phases for a variety of chromatographic applications on a small laboratory scale in detergent-free aqueous solution. Some SI-liposomes will usually be lost in each chromatographic experiment. Large increases in osmolarity should be avoided when the liposomes are sterically immobilized. When the presence of exposed hydrophobic ligands poses no problem, liposomes or proteoliposomes can be immobilized by the use of hydrophobic ligands coupled to carrier gel beads, affording high capacity and high immobilization stability and providing a high internal liposome volume per unit gel volume. This is important for encapsulation and for transport retention chromatography in which the internal volume is used. A variety of ligands can be anchored in the bilayers of immobilized liposomes for a variety of chromatographic purposes.

7. ACKNOWLEDGEMENTS

We thank Dr. Maria Wallstén and Mrs. Eva Greijer for fruitful cooperation during the development of liposome immobilization and chromatography, Dr. Makonnen Belew for kindly introducing us to alkyl sulphide ligands and Mr. Lars Andersson, Mrs. Ann-Kristin Englund and Dr. Erik Mascher for valuable discussions, encouragement and related research giving important information. One of the authors (P.L.) appreciates the possibility of working on this review during part of a 6-month stay at the Institute for Protein Research, Osaka University, and is grateful to the Director of the Institute, Professor Yukiteru Katsube, and to the staff of the Research Centre for Protein Engineering and the Division of Physical Chemistry for their encouragement and help. Valuable suggestions on the manuscript were provided by Mr. Hideki Hayashi, Professor Yutaro Hayashi, Mr. Keiichi Kameyama, Dr. Jun Miyake, Professor Hiroshi Terada and Professor Tetsuro Yoshimura. We thank Dr. David Eaker for linguistic revision. The work was supported by Osaka University, the Swedish National Board for Technical Development (STUF), the Swedish Natural Science Research Council and the O.E. and Edla Johansson Science Foundation.

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