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Properties of immobilized-liposome–chromatographic supports for interaction analysis

Eggert Brekkan, Lili Lu, Per Lundahl*

Department of Biochemistry, Biomedical Center, Uppsala University, Box 576, S-751 23, Uppsala, Sweden

Abstract

Liposomes, composed of egg yolk phospholipids or phosphatidylcholine, were sterically immobilized in Superdex 200 PG and TSK 6000 PW gel beads by freeze–thawing. The presence of Percoll improved and speeded up the separation of non-immobilized liposomes from gel beads during centrifugal washes. Depending on the concentration of the added liposome suspension and the type of gel beads and lipids used, the amount of lipids immobilized in the gel beads was 20–77 μmol/ml packed gel, with corresponding internal volumes of 20–150 μl/ml. The specific internal volumes were 0.5–2.5 μl/μmol. The external surface area of the immobilized liposomes was dependent on the amount of lipids immobilized and increased during one week of storage, which indicates structural changes. Immobilization of liposomes in Superdex beads did not significantly affect the back-pressure over the columns (0.5 cm I.D.), which could be run at flow-rates up to 2 ml/min. For drug interaction studies the liposome columns were used for a period of two weeks, at flow-rates between 0.1 and 1 ml/min, for more than 100 runs with good reproducibility and less than 3% loss of lipids. The liposome–chromatographic supports are stable and can be used for the analysis of interactions with membranes and membrane proteins.

1. Introduction

The lipid bilayers of liposomes mimic properties of cell membranes. Lipids [1–9] or vesicles [10] have earlier been immobilized by various methods in gel matrices to study their interactions with, for example, proteins, peptides or drugs by chromatography.

High-performance liquid chromatography (HPLC) has been used to study the partitioning of solutes or drugs into hydrophobic stationary phases [11]. However, the organic phases are not sufficiently similar to biological membranes to reveal specific interactions between compounds and phospholipid bilayers [12]. Reversed-phase HPLC columns with hydrophobic coatings, such as dimyristoyl phosphatidylcholine (DMPC) or phosphatidylcholine (PC), have been used for studies of partitioning coefficients of drugs [6,11], binding constant of biomolecules to phospholipid membranes [13,14] and prediction of drug transport across biological membranes [8]. However, since fluid lipid bilayers of liposomes are structurally more similar to biological membranes than the covalently attached coatings, chromatographic studies of drug interactions on columns containing immobilized liposomes may be more informative and may become applicable in, for example, the screening of drug prototypes.

* Corresponding author.

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In our laboratory we have immobilized liposomes or proteoliposomes in gel beads either by hydrophobic interactions [15–20] or sterically by dialysis [21–23], reversed-phase evaporation, freeze-drying and rehydration or freeze-thawing [24]. Gel beads with hydrophobically immobilized liposomes have been used for ion-exchange chromatography under mild conditions [22,23] and for hydrogen-bonding "transport retention chromatography", where the human red cell glucose transporter (Glut1) was incorporated into the liposomes and D-glucose and L-glucose could be separated [19]. The above immobilization techniques have been used mostly with relatively large agarose (Sepharose) or allyldextran–bisacrylamide (Sephacryl) gel beads that have a broad size distribution and large pores and limited resolution and flow-rate. To achieve improved chromatographic properties of the liposome columns we have immobilized liposomes in Superdex 200 PG and TSK 6000 PW (also denoted as Superdex and TSK), small rigid gel beads of relatively homogeneous size distribution, by the freeze–thawing method. Stable immobilization and relatively large surface area was achieved. Immobilizing liposomes in Superdex beads caused only minor changes in the back-pressure over the gel bed. Because of the rigidity of these gel beads, the columns could be run at high flow-rates. We have also studied the interaction of three model drugs with immobilized liposomes, and reproducible retentions were obtained even at high flow-rates.

The liposome–Superdex columns can be used to study peptide interactions with lipid bilayers (Y. Zhang et al., unpublished results) and on-line analytical proteoliposome affinity chromatography has been used to determine the association of the specific inhibitor, cytochalasin B, to Glut1 (Q. Yang and P. Lundahl [30]).

2. Experimental

2.1. Materials

Superdex 200 prep grade (PG) gel beads and Percoll were obtained from Pharmacia (Uppsala, Sweden) and TSK 6000 PW gel beads from Tosoh (Tokyo, Japan). [^{3}H]Percoll was a gift from Dr. Håkan Pertoft and [^{125}I]Percoll was prepared essentially as described by Hjorth and Pertoft [25]. Calcein {4′,5′-bis[N,N-bis(carboxymethyl)aminomethyl]-fluorescein} and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma (St. Louis, MO, USA). Cholic acid (>99%) was bought from Fluka (Buchs, Switzerland). PC from hen's egg yolk (95% pure) and phosphatidylethanolamine (PE, 95% pure) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Buffer A was 10 m*M* Tris–HCl (pH 7.4 at 22°C), 150 m*M* NaCl, 1 m*M* Na_2EDTA. All chemicals were of analytical grade. Solutions were filtered through 0.2-μm filters.

2.2. Lipids and lipid solutions

Egg yolk phospholipids (EYP), containing approximately 70% PC, 21% PE and 9% other phospholipids, were prepared from fresh egg yolk essentially as described in detail earlier [26]. EYP solutions were prepared by dispersing the lipids in a cholate-containing aqueous medium by vigorous stirring overnight at pH 7–8 and 6°C. The pH was adjusted to 8.4, the mixture was centrifuged at 160 000 *g* at 2°C and the floating material was discarded [26]. The final phospholipid solution contained 200 m*M* phospholipids, 250 m*M* cholate, 200 m*M* NaCl and 1 m*M* Na_2EDTA (pH 8.4). PC or PC–PE (95:5, w/w) solutions were prepared as described earlier [24]. The lipids were dissolved in chloroform and the chloroform was removed by rotary evaporator upon which a thin lipid film was obtained. The dissolution and evaporation was repeated twice with diethyl ether to remove residual chloroform. Finally, the lipids were dissolved in 200 m*M* cholate in buffer A by vigorous vortex-mixing followed by rotation under high vacuum for 30 min. The final lipid concentration was 100 m*M*.

2.3. Preparation of liposomes

Liposomes composed of EYP, PC or PC–PE (95:5) were prepared from the above cholate-

solubilized lipids by chromatographic removal of the cholate on a Sephadex G-50 M gel bed. The liposomes eluting in the void volume were pooled and concentrated in a Minicon B-15 membrane concentrator with water-absorbing material (Amicon, Beverly, MA, USA). The procedure was essentially as described earlier [24].

2.4. Steric immobilization of liposomes in gel beads by freeze–thawing

The gel beads were either suction-dried on a funnel with a water aspirator or dried by washing with increasing concentration of ethanol (20, 50 and 95%) before they were transferred to a desiccator containing anhydrous $CaCl_2$ where they were stored under vacuum. One part (weight, g) dry beads was mixed with 1.0–1.5 parts (volume, ml) of liposome concentrate in a conically bottomed plastic tube (for internal volume determinations the concentrate contained 10 m*M* calcein). The gel–liposome mixture was thoroughly mixed by vortexing, followed by degassing, and was allowed to swell for at least 3 h at room temperature. The mixture was frozen for 15 min at −70°C in ethanol–CO_2 (s), thawed and kept at room temperature for at least 1 h. The gel–liposome suspension was transferred to a 14-ml centrifugation tube and mixed with buffer A and isotonic 90% Percoll solution in buffer A to a final concentration of 15% Percoll and a total volume of 12 ml. The mixture was centrifuged at 350 *g* for 15 min and 20°C. The supernatant containing the non-immobilized liposomes was removed and replaced with buffer A. The gel–liposome mixture was resuspended in buffer A and centrifuged at 350 g for 5 min at 20°C. The supernatant was removed and replaced with buffer A as described above. This washing procedure was repeated three times (or more if Percoll was not used) in order to further remove loosely bound and non-immobilized liposomes and Percoll. The gel was further washed in a column with at least 15 column volumes of buffer A. The procedure is essentially as described by Yang and Lundahl [24] except that Percoll was used during centrifugal washes.

2.5. Immobilized amount and internal volume

The amount of immobilized liposomes was expressed as the amount of immobilized phospholipids per millilitre of packed gel. Aliquots were taken out from the centrifugation tubes after the final centrifugal wash to determine the initial capacity. When the experiment was finished the final capacity was determined by eluting the liposomes from the gel with 100 m*M* cholate and determining the amount of phospholipids in the eluate. The phospholipid amount was determined as phosphorus by the method of Bartlett [27], with minor modifications [15]. The internal volume of the liposomes (μl/ml packed gel) was determined by mixing calcein with the liposome concentrate to a final calcein concentration of 10 m*M*, and this solution was added to the dry gel beads. The immobilization procedure was otherwise as described above. Non-entrapped calcein was eluted from the column, whereafter the entrapped calcein was released by solubilization of the liposomes with 100 m*M* cholate and the amount of entrapped calcein determined fluorometrically.

2.6. External surface area of immobilized and non-immobilized liposomes

The external surface area of free liposomes was determined by reacting the primary amino groups of PE in EYP or PC–PE (95:5) liposomes with TNBS in the absence and presence of detergent. Assuming an even inside–outside distribution of PE in the liposomes, the area ratio, in percent, of the external lipid surface to the total lipid surface could be determined photometrically using a standard curve. The procedure was essentially as described by Barenholz et al. [28] with minor modifications. As the lipid bilayers are slightly permeable to the TNBS reagent, an overestimate of the external surface PE can be expected [29]. To correct for the leakage of the reagent through the bilayer, three incubation times were used: 10, 30 and 60 min. The obtained external surface area at each incubation time was plotted against the incubation time, and the graph was extrapolated to

time zero. For immobilized liposomes, the gel beads were spun down and aliquots of the supernatant were taken for absorbance measurements. The procedure was otherwise as for free liposomes.

2.7. Column back-pressure

An HPLC pump (HPLC pump 2248, Pharmacia) with an internal manometer was used together with a detector, a fraction collector and a recorder. The manometer reading was recorded to obtain the column back-pressure.

2.8 Residual Percoll in Superdex with immobilized liposomes

PC liposomes were immobilized in Superdex gel beads and free liposomes were removed by centrifugal washes as described above, except that the cold Percoll was supplemented with [^{3}H]Percoll or [^{125}I]Percoll. Aliquots were taken out from the centrifugation tubes for scintillation counting after each wash. The gel containing the immobilized liposomes was packed in a 1-ml column and equilibrated with 15 column volumes. The gel was finally removed and the amount of residual Percoll in the gel with the immobilized liposomes was determined. The Percoll was efficiently removed with the washing procedures (Fig. 1). After one centrifugal wash 7% remained and the amount further decreased to 0.80% and 0.15% after two and three washes, respectively. When the gel bed with the immobilized liposomes had been equilibrated with 15 column volumes of buffer A, only 0.02% of the Percoll remained, as determined with ^{125}I-labelled Percoll. With [^{3}H]Percoll no remaining Percoll could be detected. Percoll did not have any detectable effect on the amount of liposomes immobilized as the same amount of liposomes was immobilized with or without Percoll in the washing steps. With Percoll the removal of non-immobilized liposomes was convenient as most of the liposomes were concentrated at the top of the centrifugation tube, while the gel beads with the immobilized liposomes remained at the bottom. The use of Percoll was optional, but fewer

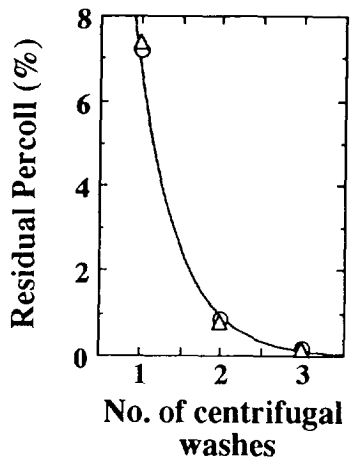

Fig. 1. Residual Percoll in Superdex 200 PG gel beads with immobilized PC liposomes after removal of free and loosely immobilized liposomes by one, two and three centrifugal washes. The percentage of Percoll, either [^{3}H]Percoll (open circles) or [^{125}I]Percoll (open triangles), originally added is plotted against the number of centrifugal washes.

centrifugal washes were needed if Percoll was used.

2.9. Chromatography of drugs

Superdex gel beads with immobilized PC at different concentration were packed in 1-ml columns (5 cm × 0.5 cm). The drugs studied were atenolol, metoprolol and hydrocortisone. Mannitol was applied before and after elution of the liposomes to estimate the volume of the immobilized liposomes. The drugs were dissolved in ethanol and diluted 500 times with buffer A (final concentration, 20 μg/ml). Mannitol was dissolved directly in buffer A (10 mg/ml). Samples of 25 μl were applied at room temperature at flow-rates of 0.1, 0.2, 0.5 and 1.0 ml/min. Detection was done at 210 nm (Waters 486 detector, Millipore, Milford, MA, USA). The immobilized liposomes were finally removed by elution with 100 m*M* cholate and the drugs were run on the liposome-free column to correct for the contribution of the gel matrix to retardation on the liposome–Superdex bed. The columns were used for a period up to two weeks to study the effect of storage on the performance of

the columns. The stability of the immobilized liposomes was determined by measuring the amount of phospholipids immobilized on the column at the beginning and at the end of the experiment.

3. Results

3.1. Immobilized amount and internal volume

Liposomes, composed of EYP or PC, were immobilized in Superdex 200 PG or TSK 6000 PW gel beads as described above. For Superdex beads, 20 μmol EYP were immobilized per milliliter packed gel by freeze-thawing when the concentration of the added liposome suspension was 46 m*M* lipid, and the amount increased to 65 μmol/ml as the concentration was increased to 233 m*M* (Fig. 2A). The amount of PC liposomes immobilized in Superdex beads was increased similarly, but slightly more was immobilized (Fig. 2A). The amount of EYP liposomes immobilized in TSK beads (Fig. 2B) was comparable to the amount of EYP liposomes immobilized in Superdex beads. When PC or EYP liposomes were immobilized in Superdex beads by vortex-mixing and degassing, without freeze-thawing, about 20 μmol/ml were immobilized, independent of the concentration of the added suspension (Fig. 2A). The internal volume of EYP or PC liposomes immobilized by freeze-

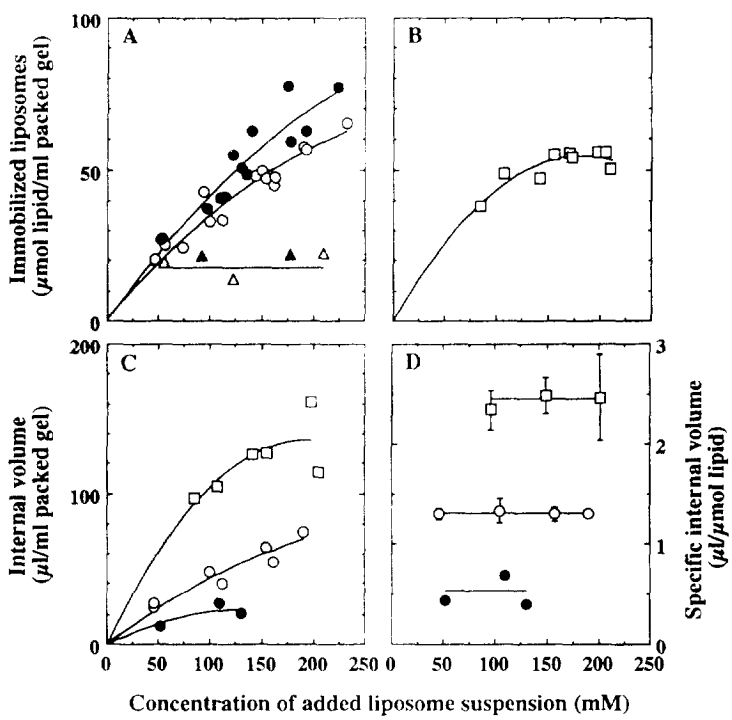

Fig. 2. Steric immobilization of liposomes in Superdex 200 PG or TSK 6000 PW gel beads with freeze–thawing (circles and squares) or without freeze–thawing (triangles). The amount of immobilized liposomes, the internal volume and the specific internal volume of the immobilized liposomes is plotted against the lipid concentration of the liposome suspension that was added to the dry gel beads. (A) The amount of EYP liposomes (open circles) and PC liposomes (closed circles) immobilized in Superdex beads by freeze–thawing and the amount of EYP (open triangles) or PC (closed triangles) immobilized in Superdex beads without freeze–thawing. (B) The amount of EYP liposomes immobilized in TSK beads by freeze-thawing. (C) Internal volume of EYP liposomes (open circles) or PC liposomes (closed circles) immobilized by freeze–thawing in Superdex beads, or of EYP liposomes immobilized in TSK beads. (D) The specific internal volume (μl/μmol lipid) of liposomes immobilized by freeze–thawing in Superdex or TSK gel beads. The same symbols were used as in C.

thawing in the gel beads (Fig. 2C) increased as the concentration of added liposomes was increased. For example, for EYP liposomes in Superdex beads, the internal volume increased from 25 to 75 μl/ml packed gel as the concentration of the added suspension was increased from 46 to 205 m*M*. The internal volume of EYP liposomes immobilized in TSK beads was much larger than for those immobilized in Superdex beads. The specific internal volume (μl/μmol immobilized lipid) of EYP liposomes in TSK and Superdex beads and PC liposomes in Superdex beads was essentially constant in the concentration interval studied, about 2.5 μl/μmol for EYP liposomes in TSK beads, about 1.3 μl/μmol for EYP liposomes in Superdex beads and about 0.5 μl/μmol for PC liposomes in Superdex beads (Fig. 2D). The freezing and thawing procedure did only increase the amount of sterically immobilized liposomes above lipid concentrations of 45 m*M*. The immobilization obtained with non-freeze–thawed material may have been induced by the vigorous liposome–gel bead mixing procedure. There was little adsorption of lipids to the gel matrix, as only about 0.3 μmol lipid/ml had been adsorbed/immobilized during overnight recirculation of the liposomes on a Superdex column.

3.2. External surface area of immobilized and non-immobilized liposomes

The external liposome surface area was determined as described in the Experimental section. Non-immobilized EYP liposomes were taken directly from the preparation of liposomes by gel filtration. The surface area of non-immobilized liposomes after freezing and thawing at concentrations between 5 and 280 m*M* varied from 53% down to about 13% (Fig. 3A). Liposomes freeze–thawed at 5 m*M* lipid concentration gave the same surface area of liposomes that had not been frozen and thawed (dashed line in Fig. 3A), which suggests that no fusion of liposomes or change in the liposome structure had occurred during freeze–thawing at 5 m*M* lipid concentration. For non-immobilized liposomes, the critical concentration interval for

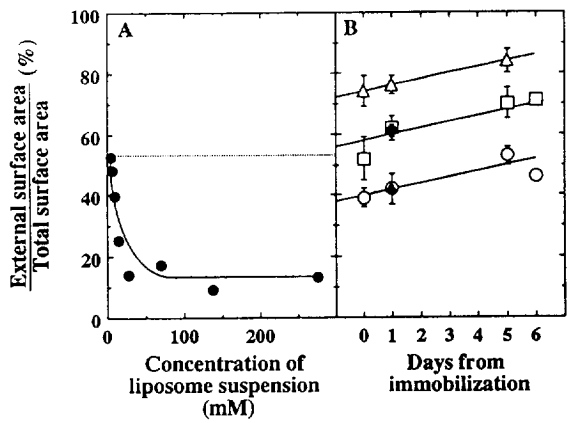

Fig. 3. Ratio between the external liposome surface area and the total surface area in percent. (A) The external surface area of non-immobilized liposomes plotted against the concentration at which they were freeze–thawed. Dotted line indicates the external surface area of liposomes that had not been freeze–thawed. (B) External surface area of EYP or PC–PE (95:5) immobilized in Superdex 200 PG gel beads. EYP liposomes immobilized at 28 m*M* (open triangles), 138 m*M* (open squares) and 280 m*M* (open circles) liposome concentrations, and PC–PE liposomes immobilized at 54 m*M* (closed circle) and 136 m*M* (closed triangle) liposome concentrations.

freeze–thawing-induced fusion and formation of large multilameller vesicles was between 5 and 60 m*M*. At concentrations above 60 m*M* the surface area was constant at ≈13% (Fig. 3A).

The external surface area of liposomes immobilized by freeze–thawing in Superdex beads, estimated as described in the Experimental section, was dependent on the concentration of added liposome suspension and on storage time. The external surface area was measured over a period of seven days, day 0 being the day of immobilization, on which EYP liposomes immobilized at concentrations of 28, 138 and 280 m*M* had a surface area of 74, 52 and 39% of the total lipid area, respectively (Fig. 3B). Large external surface area for liposomes immobilized at low concentration indicates that the immobilized liposomes are small, for example 74% external surface area corresponds to unilamellar vesicles with a diameter of 390 Å and a wall thickness of 40 Å. As the concentration of the liposomes is increased the external surface area of the immobilized liposomes decreased, sug-

gesting that mostly large liposomes, with some degree of multilamellarity, are formed. The measured surface area increased during seven days of storage, which shows that the liposomes undergo structural changes. Small liposomes probably fuse and form larger liposomes, since the strains caused by the curvature of the lipid bilayers make them energetically unstable. Larger external surface areas of immobilized liposomes than of non-immobilized liposomes are presumably due to the presence of the gel beads that affects the freeze–thawing and minimizes the degree of multilamellarity. Only little adsorption of lipids/liposomes to the matrix was observed (see previous section), and the large external surface areas of immobilized liposomes are therefore not due to such adsorption. The surface areas of PC–PE (95:5) liposomes immobilized in Superdex beads, measured the day after immobilization (day 1), were 61 and 42% at concentrations of 54 and 136 m*M*, respectively. A higher degree of multilamellarity was thus obtained with PC–PE liposomes than with EYP liposomes (Fig. 3B). The concentration dependency and the effect of storage on the measured surface area of immobilized liposomes is evident and the presence of the gel beads during freeze–thawing markedly affects the structure of the liposomes formed after freeze–thawing.

3.3. Effect of immobilized liposomes on column back-pressure (Fig. 4)

The flow-rate was changed stepwise and the pressure was continuously monitored as described in the Experimental section. Two 1-ml Superdex columns (5 cm × 0.5 cm) containing 24 and 61 μmol immobilized liposomes, respectively, were studied. The column back-pressure was almost the same for both columns. It increased linearly from 0 to 0.10 MPa/cm bed height as the flow-rate was increased from 0 to 2 ml/min. The pressure over the liposome columns was slightly higher than the pressure over the columns after elution of the immobilized liposomes with detergent. The gel beads probably swell more in the presence of liposomes, which results in larger beads and lower back-pressure over the column.

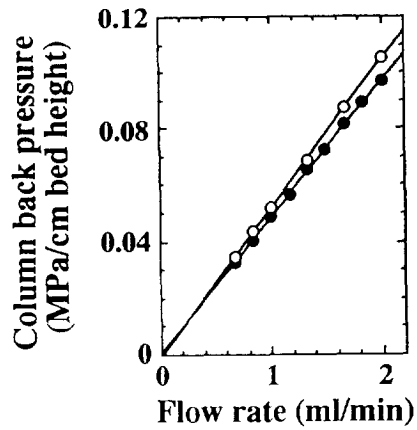

Fig. 4. Effect of PC liposomes immobilized in Superdex 200 HR gel beads on the back-pressure on the Superdex gel bed. The back-pressure (MPa/cm bed height) is plotted against the flow-rate (ml/min). The average of the column back-pressure on two 1-ml Superdex columns (5 cm × 0.5 cm) with 24 and 61 μmol PC liposomes immobilized, before (closed circles) and after (open circles) elution of the immobilized liposomes with cholate. Average values shown for clarity (error limits, less than 0.002 MPa/cm, ommited).

A slight compression of the gel bed was observed after the elution of the immobilized liposomes, which supports these findings. Care must be taken not to compress the gel bed with the immobilized liposomes upon packing of the gel, since in that case a high back-pressure may build up, due to deformation of the beads, followed by release and aggregation of the liposomes. The immobilized liposomes were stably immobilized with respect to changes in flow-rate (see section below).

3.4. Chromatography of drugs

Beigi et al. [31] have studied the interactions of various drugs and PC liposomes immobilized in Superdex gel beds by chromatography. To obtain a measure of the specific interaction of the drugs with the liposome bilayers, they introduced the specific capacity factor, SCF, defined as

$$SCF = [V_R - (V_0 - V_L)]/[V_0 - V_L)B]$$

where V_R is the retention volume on the lipo-

some column, V_0 is the retention volume after elution of liposomes with detergent, V_L is the liposome volume and B is the concentration of immobilized phospholipids (in mmol/ml packed gel). The flow-rate used was relatively low, only 0.1 ml/min (0.5-cm-I.D. column). We have studied the effect of flow-rate, storage and amount of immobilized liposomes on the SCF for atenolol, metoprolol and hydrocortisone. PC liposomes were immobilized in Superdex gel beads by freeze–thawing as described in the Experimental section and drugs were applied and the SCF calculated from the retention volumes. The same or similar SCF was obtained with flow-rates from 0.1 to 1.0 ml/min (Fig. 5A). The hydrophobic nature of the drugs was reflected in the SCF as hydrocortisone was the most retarded and atenolol the least. The relatively hydrophilic drug, atenolol, was only slightly retarded by the liposomes and that in turn led to more uncertain values of the SCF, as the difference in retention with or without liposomes immobilized was small. For metoprolol and hydrocortisone the reproducibility was better. The columns were stable and could be run with

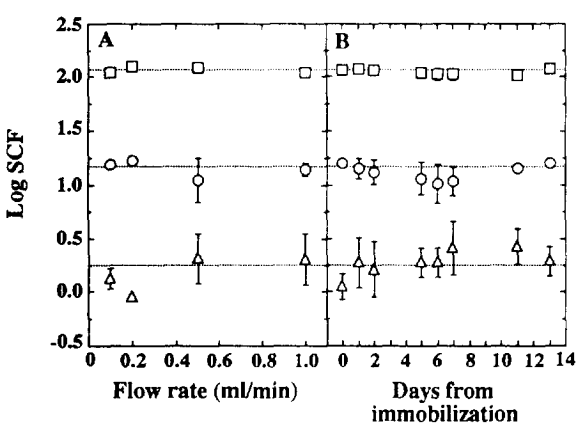

Fig. 5. Interaction of three drugs of different hydrophobicities with PC liposomes immobilized in Superdex gel beads studied by immobilized-liposome chromatography. The logarithm of the specific capacity factor (SCF, calculated by use of the retention volume for the drugs and the concentration of lipids in the column, see Results, *Chromatography of drugs*) for atenolol (open triangles), metoprolol (open circles) and hydrocortisone (open squares) are plotted against the flow-rate (A) or against the number of days from immobilization (B).

over 100 sample applications during two weeks, without any significant change in SCF (Fig. 5B) and less than 3% of the lipids were lost during this time. The retentions were influenced by the amount of lipids immobilized. For example, at a flow-rate of 1 ml/min the elution volume of hydrocortisone was 4.9 ml when the column contained 33 μmol lipid/ml packed gel, but was 8.4 ml on a gel bed of the same dimension, but with 60 μmol lipid/ml. The retardation of the drug was thus 0.14 column volumes/μmol immobilized lipids.

4. Discussion

In our laboratory, liposomes have previously been immobilized in gel beads by hydrophobic interactions or sterically by various methods (see the Introduction). The use of derivatized gel beads to immobilize liposomes hydrophobically has its drawbacks as the free hydrophobic ligands tend to limit the applicability of the columns for immobilized-liposome chromatography. Of the sterical methods, freeze–thawing seems to be the most promising for preparation of liposome and proteoliposome columns. Large amounts of liposomes could be immobilized. The size, size distribution and the relative softness of previously tested gel beads, such as Sephacryl and Sepharose, limits their practical use for immobilized-liposome chromatography. We have therefore used small rigid gel beads with relatively narrow size distribution. Less reproducible results were obtained with TSK 6000 PW beads (17 μm average diameter) than with Superdex 200 PG beads (34 μm average diameter). Superdex 200 HR beads were therefore used in most experiments. The amount of liposomes immobilized in Superdex or TSK beads was relatively high compared to previously test types of gel beads, such as Sephacryl and Sepharose gel beads. The composition of the liposomes used affected the amount immobilized, and slightly more PC liposomes than EYP liposomes were immobilized in Superdex beads. PC or EYP liposomes were also immobilized without freezing–thawing. By comparing the amounts immobilized with the two

immobilization methods the contribution of the freezing and thawing was clearly illustrated (Fig. 2A). The induced fusion and formation of large liposomes within the gel beads by freeze–thawing required high liposome concentrations, above 50 m*M*. The internal volumes of the immobilized liposomes were notably affected by the type of liposomes and the type of gel beads used. The structure of the gel beads affects the disruption and resealing of the liposomes by freeze–thawing. The structure of the polymer-based TSK beads might differ from the structure of the Superdex beads, which is made of a cross-linked sugar polymer (agarose) with dextran grafted to the matrix, and it is therefore not surprising that the different internal volumes can be obtained. The PC liposomes immobilized in Superdex beads are more multilamellar than EYP liposomes in Superdex as indicated by lower specific internal volume (Fig. 2D) and less external surface area (Fig. 3B). The nearly constant specific internal volume of EYP or PC liposomes immobilized in Superdex beads, independent of the concentration of the added liposome suspension, and decreased external surface area with increasing concentration, suggests that liposomes immobilized at low concentrations are small, but their size and degree of multilamellarity increases with increasing concentration of added liposome suspension.

The column back-pressure over Superdex columns with immobilized PC liposomes was almost independent of the amount of liposomes immobilized and was only marginally different from the pressure over a Superdex bed without liposomes. With Sephacryl or Sepharose columns with immobilized liposomes a gradual increase in column back-pressure was observed unless low flow-rates were used, and due to this the column had sometimes to be repacked, whereafter 20–30% of the immobilized liposomes were released [24]. This problem was completely eliminated by using Superdex gel beads for immobilization and high flow-rates could be used.

The interactions of drugs with lipid bilayers were studied by using Superdex columns with immobilized liposomes. Three drugs of different hydrophobicities were applied on Superdex columns with immobilized PC liposomes and the effect of flow-rate, amount of liposomes immobilized and storage of the columns on the specific capacity factor, SCF (see Results, *Chromatography of drugs*) was investigated. Superdex columns with 17, 19, 28 and 50 μmol PC liposomes immobilized gave the same specific capacity factor for the three drugs, atenolol, metoprolol and hydrocortisone. For more hydrophilic drugs like atenolol, the determination became more uncertain as the difference in elution volumes of the drug on the column with or without liposomes was small. The external surface area of the immobilized liposomes increased during one week of storage (Fig. 3B), but this did not affect the specific capacity factor of the drugs, probably because the drugs are able to penetrate the liposome bilayers.

The immobilized-liposome–chromatographic supports are stable and easily prepared. They can be used to study interactions of various biologically interesting substances with the bilayers of the immobilized liposomes or with membrane proteins reconstituted into the immobilized liposomes. Future experiments should include visualization of the immobilized liposomes by electron microscopy and further exploration of the application possibilities of these chromatographic supports.

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