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Avidin–biotin immobilization of unilamellar liposomes in gel beads for chromatographic analysis of drug–membrane partitioning

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Abstract

To construct a homogeneous lipid membrane chromatographic phase, biotinylated unilamellar liposomes of small and large sizes (SUVs and LUVs, respectively) were immobilized in avidin- or streptavidin-derived gel beads in amounts up to 55 μmol phospholipid/ml gel bed at yields above 50%. The immobilized liposomes exhibited excellent stability due to avidin–biotin multiple-site binding. The trapped volume and size distribution of the immobilized liposomes (0.33–0.42 μl/μmol lipid and 20–30 nm diameter for SUVs, 1.7–1.9 μl/μmol lipid and 80–120 nm for LUVs) indicated the unilamellarity and integrity of the immobilized liposomes. Partitioning of 15 pharmaceutical drugs into the bilayers of LUVs immobilized in different gel matrices correlated very well, as shown by chromatographic drug retention analysis. The partitioning of several β-blockers into the immobilized LUVs showed a close correlation with their partitioning, reported in the literature, into free liposomes. The avidin–biotin-immobilized unilamellar liposomes can thus be used for chromatographic analysis and screening of solute–membrane interactions. © 1998 Elsevier Science B.V.

Keywords: Avidin–biotin immobilization; Unilamellar liposomes; Drug–membrane partitioning

1. Introduction

Immobilized liposomes and proteoliposomes in gel beads have been used as a lipid membrane stationary phase for column-chromatography studies of solute interactions with membranes and membrane proteins [1–8]. The retention volume of a solute on a gel bed with immobilized liposomes or proteoliposomes reflects the extent of the solute interaction with the liposomal membranes and membrane components, and an equilibrium constant or partition coefficient for the interaction may be obtained. The chromatographic approach generally provides a rapid and accurate analysis of solute–membrane interaction and can be automatically performed by use of modern experimental instruments.

Liposomes, proteoliposomes and red cell mem-

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brane vesicles have been immobilized in gel beads in several ways by binding to hydrophobic ligands coupled to the gel matrix or by entrapment as reviewed and described in Refs. [2,9–11]. Hydrophobic ligands offer high immobilized amounts and yields [6,12], but perturb the liposomal membranes, causing them to leak. An excess of the ligands may affect the solute–membrane interaction, although low ligand density diminishes the effect [6,13]. Entrapment of (proteo)liposomes in gel bead pores occurs upon formation of sufficiently large vesicles in the beads [14–16], e.g., by freeze–thawing [15,16], which is a simple method. However, the yield is below 40% [17] and multilamellar liposomes (MLVs) are likely to form at the high lipid concentrations used. The aim of the present work was to achieve a very stable high-yield immobilization of unilamellar liposomes with narrow size distribution, thereby creating a simple and homogeneous membrane stationary phase for chromatographic analysis of solute–membrane partitioning, avoiding liposome losses and equilibration of analytes between the multiple lamellas. It has been suggested that unilamellar liposomes with defined sizes is the most attractive membrane system for determination of drug–liposome partitioning [18].

Avidin–biotin or streptavidin–biotin binding ($K_d = 10^{-15}$–10^{-4} *M* [19,20]) has been used to create supramolecular aggregates by a self-assembly process [21,22] to study surface adhesion of liposomes [23] or to immobilize biotinylated membrane vesicles on avidin-coupled solid surfaces [24]. We have here utilized avidin–biotin technology to immobilize unilamellar liposomes, as they were prepared originally, in gel beads. The chromatographic retention of several β-blockers in the avidin–biotin-immobilized liposomes was related to literature values of their partitioning in the free liposomes.

2. Experimental

2.1. Materials

Sephacryl S-1000 Superfine, Superdex 200 prep. grad. and CNBr-activated Sepharose 4B were purchased from Pharmacia (Uppsala, Sweden), and TSK G6000PW from Tosoh (Tokyo, Japan). Egg-white avidin [relative molecular mass (M_r) 66 000] and streptavidin (M_r 60 000) were bought from Calbiochem (La Jolla, CA, USA), and Bradford dye reagent (No. 500-0006) from Bio-Rad (Hercules, CA, USA). Egg yolk phosphatidylcholine (EPC) (95%), egg yolk phosphatidylethanolamine (EPE) (99%), phosphatidylserine from brain extract (BPS) (99%), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), 1,2-dioleoyl-phosphatidylethanolamine-N-(cap biotinyl) (biotin-cPE) and 1,2-dioleoyl-phosphatidylethanolamine-N-biotinyl (biotin-PE) were purchased from Avanti (Alabaster, AL, USA). 1,2-Palmitoyl-phosphatidylcholine (DPPC), (−)-isoproterenol, (±)-isoproterenol, salbutamol, DL-octopamine, (±)-synephrine, bupivacaine, disopyramide, clenbuterol, (±)-metoprolol, atenolol, alprenolol, oxprenolol, pindolol, acebutolol, DL-propranolol, and Fiske and Subbarow reducer were purchased from Sigma (St. Louis, MO, USA). *n*-Octyl-β-D-glucopyranoside (OG), 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) and 3,3-bis(N,N-di(carboxymethyl)aminomethyl)-fluorescein (calcein) were from Dojindo (Kumamoto, Japan), 4-nitrophenyl chloroformate and 4-dimethylaminopyridine from Aldrich (Milwaukee, WI, USA), stearylamine (STA) and (+)-biotin from Wako (Osaka, Japan), and cholesterol (Chol) from Nacalai Tesque (Kyoto, Japan). Other chemicals were of analytical grade.

2.2. Activation of gels with chloroformate and coupling of avidin to the gels

Sephacryl S-1000, Superdex 200 prep. grad. and TSK G6000PW gels (denoted Sephacryl, Superdex or TSK, respectively, below) were activated essentially as described in Ref. [25]. A gel slurry (10–25 ml) was washed in a glass filtering funnel (type 17G3; Sibata, Tokyo, Japan) with distilled water and increasing concentrations of acetone (25%, 50%, 75%, 99.5%, and 100%, about 500 ml each) and was mixed with 4-nitrophenyl chloroformate (0.05–0.1 g to 1 g moist gel) in 100% acetone. 4-Dimethylaminopyridine (0.05–0.1 g to 1 g moist gel) in the same solvent was added dropwise with gentle stirring. The mixture was kept at 23°C for 2–3 h or at 4°C overnight, washed extensively with acetone, acetone–2-propanol (1:1), 2-propanol, 2-propanol–water (1:1) and water (about 1 l for each washing),

and was finally suspended in a coupling buffer (0.1 *M* $NaHCO_3$, pH 8.1). The ligand density of the activated gels was determined [25] to be 5–7 μmol/g for the Sephacryl, 31–40 μmol/g for the TSK, and 43–60 μmol/g for the Superdex. Avidin dissolved in the coupling buffer (3–4 mg/ml) was then mixed with the activated gel overnight at 4°C (4 mg protein to 1 g moist gel), followed by washing successively with water, 0.2 *M* acetic acid and 10 m*M* NaOH (200–400 ml for each washing), as described in Ref. [26]. Excess chloroformate in the TSK and the Superdex gels was blocked by further mixing the gels with 1 *M* ethanolamine (pH 8.5) overnight at 4°C or 2 h at 23°C. Alternatively, avidin or streptavidin was coupled to CNBr–Sepharose 4B (denoted Sepharose) according to the procedures described in Ref. [27]. The gels contained 3.0–3.5 mg (strept)avidin/ml gel bed as determined by use of the modified Bradford assays [28], and were stored at 4°C in buffer H (10 m*M* HEPES, 150 m*M* NaCl, 0.1 m*M* Na_2-EDTA, pH 7.5) supplemented with 3 m*M* NaN_3.

2.3. Preparation of biotinylated liposomes

EPC supplemented with biotin-cPE (molar ratio 1–4%) in chloroform was dried in a round-bottomed flask by rotary evaporation and redissolved in diethyl ether, whereafter the ether was evaporated to form a lipid film. The film was flushed with nitrogen, kept under vacuum for at least 3 h, and hydrated by dispersal in 4 ml of buffer H or, for preparation of calcein-entrapped liposomes, in 100 m*M* calcein in deionized water (pH 7.5), to form MLVs. Small unilamellar liposomes (SUVs) were prepared by probe sonication (Sonifier 450; Branson, Danbury, CT, USA) of a MLV suspension in a 10-ml plastic tube on an ice-bath at 20–40 W for 4×5 min under a continuous flow of nitrogen, followed by sedimentation of titanium particles and residual MLVs at 100 000 *g* for 90 min at 4°C. Large unilamellar liposomes (LUVs) were prepared by extrusion [29] of a MLV suspension, that was frozen in liquid nitrogen, thawed in a water-bath at 25°C (or 50°C for DPPC MLVs) for five cycles, and passed ten times through two stacked polycarbonate filters of 100-nm pore size (Nuclepore®; Costar, Cambridge, MA, USA) at 23°C (or 50°C for DPPC LUVs) in a high-pressure vesicle extruder with thermobarrel (Lipex Biomembranes, Vancouver, BC, Canada).

2.4. Immobilization of biotinylated liposomes

A suspension of biotinylated SUVs or LUVs (30–55 μmol phospholipid in 1–3 ml of buffer H) was gently mixed with moist (strept)avidin–gel (0.7 g TSK or 0.6 g Sepharose, Superdex and Sephacryl, which gave an 0.5-ml gel bed) by rotation for 2–3 h at 23°C or overnight at 4°C under nitrogen, with the exception of DPPC liposomes which were mixed with the gel at 45°C for 3 h. Free liposomes were then removed by washing with buffer H on the filtering funnel (17G3). Alternatively, the suspension was circulated at a flow-rate of 0.2 ml/min through a (strept)avidin–gel bed for 2–3 h at 23°C or overnight at 4°C. Free liposomes were eluted with buffer H.

2.5. Dynamic light scattering analysis

The size and size distribution of liposomes were determined on a dynamic light scattering (DLS) analyzer equipped with an argon laser (488 nm) (DLS-6000AS; Otsuka, Osaka, Japan). Best fits [30] were obtained and mean size and size distribution of the liposomes were analyzed by the histogram method [31]. Intensity-weighted parameters were used. The buffer was filtered twice through a 0.22-μm filter (Millipore, Bedford, MA, USA). A 30–60 μl aliquot of SUVs or LUVs in buffer H (9 ml) in a 10-ml dust-free scattering cell was placed in a vat of refraction-index-matching fluid. DLS measurements were done at 25°C at a scattering angle of 90°.

2.6. Determination of trapped volume of immobilized liposomes

The calcein-entrapped SUVs or LUVs in the presence of 100 m*M* calcein were mixed with avidin–gel overnight at 4°C and then washed with buffer H on the filtering funnel to remove nonimmobilized liposomes together with nonentrapped calcein. The liposome-entrapped calcein was released by addition of a 1 *M* OG solution to a final OG concentration of 100 m*M* and determined in a fluorescence spectrophotometer (F-4500; Hitachi, Tokyo, Japan) at excitation and emission wave-

lengths of 492 and 517 nm, respectively. The specific internal (trapped) volume of the liposomes was calculated from the amount of trapped calcein, the concentration of calcein in the initial medium before washing, and the amount of immobilized liposomal phospholipids. Alternatively, nonentrapped calcein was removed from calcein-entrapped liposomes by size-exclusion chromatography on Sephacryl S-300 (column dimensions, 2.0 cm I.D.×35 cm), and the calcein-entrapped liposomes were then mixed with the avidin–gels for immobilization. The two procedures gave very similar values.

2.7. Phosphorus determination

The phospholipids amounts in aliquots of gels containing immobilized liposomes or in eluates with 200 m*M* OG from used gel beds were determined as phosphorus, as described by Bartlett [32].

2.8. Chromatography of drugs on the immobilized liposomes

The gel beads containing avidin–biotin-immobilized liposomes were packed into a 5 mm I.D.×5–5.5 cm gel bed in a glass column (HR 5/5; Pharmacia). Pharmaceutical basic drugs (1 μg/μl, 5 μl) were applied to the immobilized liposome gel bed and were eluted with buffer H at a flow-rate of 0.3 ml/min (Sephacryl gel bed) or 1 ml/min (Superdex or TSK gel bed). The chromatographic runs were performed by using a Tosoh high-performance liquid chromatography system with a pump, a detector (UV-8010) set at 220 nm, and a recorder interfaced with an IBM computer. A column was placed in a column oven (CO-8020; Tosoh) equipped with a sample injector. The temperature for the chromatographic runs was 23°C, except for runs on immobilized DPPC liposomes which were done at 30–50°C.

The solute retention on immobilized liposome–gel beds of different dimensions and different lipid amounts can be normalized, as described in Ref. [33], by defining a specific retention factor, K_s, as $(V_R - V_0)/M$, where V_R is the retention volume (ml) of solute on a liposome–gel bed, V_0 is the elution volume (ml) of an unretained solute (NaN_3) on the same bed, and M is the amount of immobilized liposomes (mmol phospholipid).

3. Results

3.1. Immobilization of liposomes by avidin–biotin binding in gel beads

Biotinylated liposomes composed of different lipid compositions were immobilized in high amounts and yields in (strept)avidin-derived gels of various types, as summarized in Table 1. High immobilized amounts were obtained for SUVs in the avidin–gels of all types and for LUVs in avidin–Sephacryl S-1000 or avidin–TSK 6000PW (pore sizes up to 400 nm and 500 nm, respectively [34]). A small amount of LUVs was immobilized at a low yield in avidin–Sepharose 4B or avidin–Superdex 200. As expected, the binding immobilization is affected by the pore-size of the gel beads used. Probably owing to ion-exclusion effects, negatively or positively charged liposomes were immobilized in a relatively low amount and yield (Table 1, rows 5 and 6, respectively). By increasing the amount of SUVs mixed with 1 ml gel beads from 60 to 100 μmol lipid, the immobilized amount was increased from 39 to 55 μmol lipid/ml gel and the immobilization yield was decreased from 76% to 52%. Similar results were found when SUVs were immobilized in streptavidin–Sepharose gel or when LUVs were immobilized in avidin–Sephacryl gel. The average values of the corresponding immobilized amounts and yields are shown in Table 1, rows 1–3.

The amount of SUVs bound in avidin–Sepharose gel gradually increased as the molar ratio of biotin-cPE to EPC in the liposomes was increased (Fig. 1). The immobilization was essentially due to specific binding of the incorporated biotinyl lipid to the coupled (strept)avidin, since blocking the avidin–gels with biotin diminished the amount of bound liposomal lipid to only 0.6–3 μmol/ml gel.

When SUVs and LUVs containing biotin-PE without a spacer arm were mixed with the avidin–Sephacryl gel (mixing cycle 1, Fig. 2), the amount of liposomes immobilized became half of that obtained with liposomes containing biotin-cPE with a spacer arm between the primary amine and the biotin (Table 1, row 3). The effect of spacer arms of biotinyl phospholipids on avidin-mediated liposome precipitation has been reported by Hashimoto et al. [35]. Additional biotinylated liposomes could be immobil-

Table 1
Amounts of biotinylated liposomes immobilized in (strept)avidin–gels of various types

Lipid composition[a]	Gels coupled with avidin or streptavidin	Immobilized SUVs		Immobilized LUVs	
		Amount (μmol lipid/ml gel bed)	Yield[b] (%)	Amount (μmol lipid/ml gel bed)	Yield (%)
EPC	Sepharose	47±8	64±12 (n=4)	4	7
EPC	Sepharose[c]	47±9	60±6 (n=3)	n.d.[d]	n.d.
EPC	Sephacryl	43±2	66±14 (n=3)	43±4	64±8 (n=3)
EPC:EPE:Chol. (7:2:3)	Sephacryl	n.d.	n.d.	40	51
EPC:BPS (1:1)	Sephacryl	n.d.	n.d.	24	38
EPC:STA (3:1)	Sephacryl	n.d.	n.d.	37	37
EPC	Superdex	37±2	53±3 (n=3)	4±1	6±1 (n=2)
EPC	TSK	n.d.	n.d.	42	57
POPC	TSK	35±0	47±0 (n=2)	n.d.	n.d.
DPPC	TSK	n.d.	n.d.	35	43

[a] The liposomes were composed of different lipid compositions supplemented with 2 mol% biotin-cPE with an exception of POPC SUVs, which contained 1 mol% biotin-cPE.
[b] Yield was expressed as the immobilized amount in percent of the added amount of biotinylated liposomes.
[c] Streptavidin-coupled gel.
[d] n.d., not determined.

ized on the bound and avidin-saturated biotinylated liposomes (Fig. 2). As a result, the immobilized amounts of the SUVs and LUVs could be considerably increased, probably by forming liposome assemblies in the gel beads.

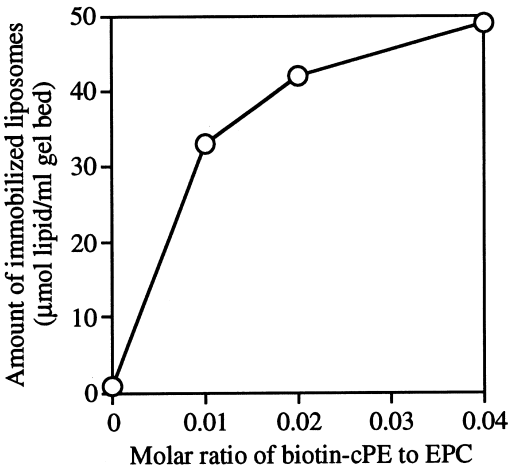

Fig. 1. Amount of biotinylated SUVs immobilized in avidin–Sepharose, versus the molar ratio of biotin-cPE to EPC in the lipid mixture used for liposome preparation. The immobilization was achieved by circulating a liposome suspension (6 ml, 130 μmol lipid, single experiment) through a 1-ml gel bed at 4°C (see Section 2.4).

3.2. Size and size distribution of the immobilized liposomes

The size distribution of the bound liposomes was estimated indirectly by DLS analysis of the mean size and the apparent size distribution of the originally prepared liposomes and of those remaining unbound after mixing with the gels for the binding immobilization, as shown in Table 2 and Fig. 3. After mixing biotinylated SUVs with avidin–Superdex and avidin–Sepharose, the nonbound liposomes showed a narrow apparent size distribution that had shifted to larger diameters than in the original preparation (Fig. 3 and Table 2), whereas little or no changes in size (Table 2) and the size distribution (data not shown) for both the SUVs and the LUVs were observed after the mixing with avidin–TSK gel, indicating that the TSK-bound liposomes had the same size population as originally prepared (80–120 nm, Table 2). From the data in Table 2, and by assuming an immobilization yield of 50%, the diameters of the SUVs immobilized in avidin–Superdex or avidin–Sepharose and the LUVs immobilized

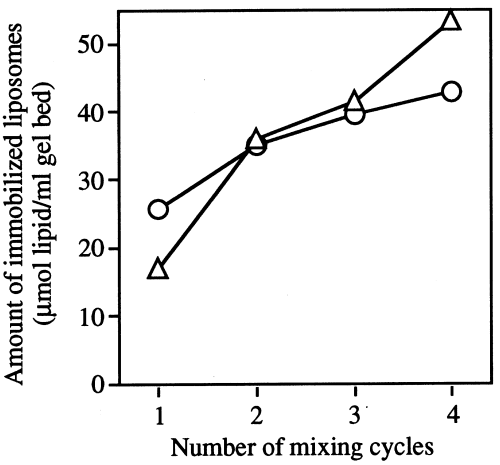

Fig. 2. Immobilization of biotinylated liposome assemblies of SUVs (△) and LUVs (○). An excess of liposomes (130 μmol lipid) were gently mixed with moist avidin–Sephacryl gel (1 g, 15 h, 4°C) and the free liposomes were collected for another mixing cycle by washing on a filter (mixing cycle 1). Avidin (5 mg in 5 ml of buffer H) was added to the gel containing immobilized biotinylated liposomes. The mixture was incubated (30 min, 23°C) and washed to remove free avidin. The gel was then mixed with the remaining free biotinylated liposomes (30 min, 23°C), after which free liposomes were collected as above (mixing cycle 2). The mixing cycle 2 was repeated twice. The amount of immobilized liposomes (phospholipids) in a gel aliquot was determined after each mixing cycle (single experiment). The initial immobilized amounts were relatively low, since biotinylated lipids without spacer arm were used.

in avidin–Sephacryl were estimated to be in the range of 20–30 nm and 80–100 nm in diameter, respectively, consistent with the size-exclusion properties of Sepharose 4B [36] and Sephacryl S-1000 gels [34].

3.3. Internal (trapped) volume of immobilized liposomes

The trapped specific volume of the immobilized SUVs and LUVs (Table 3) was consistent with the literature data of 0.2–0.5 μl/μmol lipid (SUVs [37]) and 1.5 μl/μmol lipid (extruded LUVs [29,37]). From the trapped volumes and the mean diameters (Table 2) of the immobilized liposomes, the avidin–biotin-immobilized liposomes were estimated to retain their unilamellarity [37]. Only 3–4% of the initial calcein trapped in the immobilized SUVs or LUVs leaked out during storage for 2 weeks at 4°C. The retention of calcein in the internal volume of the immobilized liposomes indicates clearly the integrity of their bilayers.

3.4. Stability of avidin–biotin-immobilized liposomes

The (strept)avidin–biotin-immobilized liposomes exhibited excellent stability with no detectable loss during short-term storage, and only about 10% of the liposomes had been lost after storage for more than 10 months (Fig. 4). This loss may be attributed to oxidation and hydrolysis of the liposomal phospholipids and the biotinylated PE. Consistent with the storage stability of the liposomes, almost identical specific retention factors were obtained between two series of chromatographic runs with 15 kinds of drugs eluted with buffer H on a gel bed containing immobilized LUVs or SUVs. The SUV- or LUV-column was stored at 4°C for one month between the two series of runs. The thermostability of the avidin–

Table 2
Mean diameters (nm) of biotinylated liposomes before mixing with avidin–gels and of unbound biotinylated liposomes after mixing with avidin–gels for immobilization

Biotinylated liposomes	Before mixing	After mixing, unbound liposomes			
		Sepharose	Superdex	Sephacryl	TSK
SUVs[a]	30±11 (n=2)	40±10 (n=1)	43±11 (n=2)	n.d.[b]	30±10 (n=1)
LUVs[c]	102±20 (n=5)	n.d.	n.d.	121±25 (n=3)[d]	97±22 (n=2)[e]

[a] The SUVs are composed of EPC.
[b] n.d., not done.
[c] The LUVs are composed of EPC, DPPC, EPC:BPS (molar ratio 1:1) or EPC:EPE:Chol (7:2:3).
[d] The LUVs are composed of EPC:EPE:Chol (molar ratio, 7:2:3), EPC:BPS (1:1) or EPC alone.
[e] The LUVs are composed of EPC or DPPC.

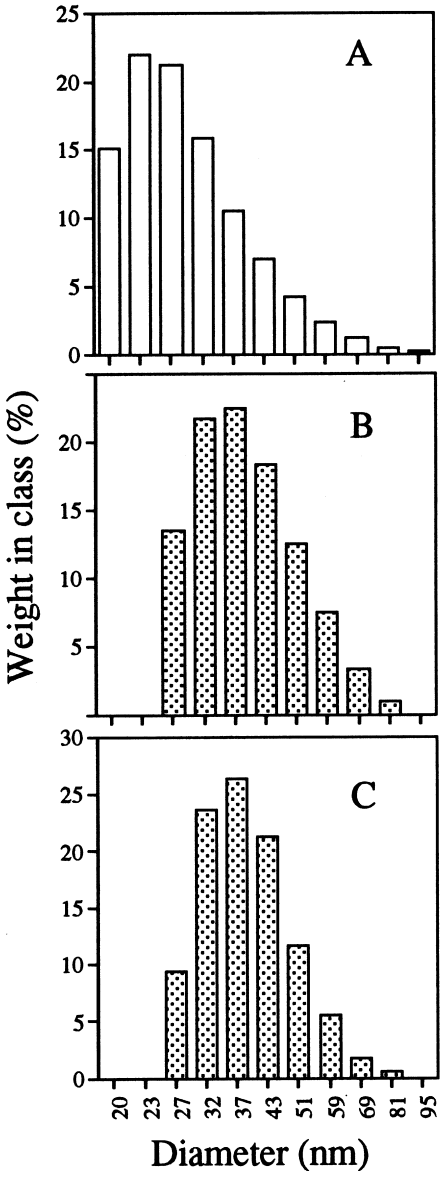

Fig. 3. DLS analysis of apparent size distribution (relative weight) for SUVs composed of EPC suspended in buffer H before (A) and after (B and C) mixing with avidin–Superdex (B) and with avidin–Sepharose (C) for 3 h at 23°C. The analysis in panel A was done on an aliquot of the originally prepared SUVs kept under nitrogen for 3 h at 23°C while the immobilization of the other samples took place.

biotin-immobilized liposomes was demonstrated by the fact that only 7% of the immobilized DPPC LUVs in TSK gel was lost after series of chromatographic runs with the drugs at 50°C, 45°C, 37°C, 30°C and again at 45°C with a total of 75 runs.

3.5. Partitioning of drugs in the avidin–biotin-immobilized liposomes

In our previous work [15,16] and in this work, several kinds of carrier-gels have been used to immobilize liposomes. The effect of the gel bead matrices on drug interactions with the gel immobilized membrane stationary phase was thus evaluated by chromatography of 15 pharmaceutical basic drugs on the immobilized liposomes. The drug partitioning into the bilayers of SUVs or LUVs immobilized in avidin–gels of Superdex (crosslinked agarose grafted with dextran), TSK 6000PW (polymer-based) and Sephacryl (alkyldextran–bisacrylamide) showed linear and high correlation between the series of log K_s values obtained with the different matrices (Fig. 5). The correlation between the drug partitioning into the bilayers of the immobilized SUVs and LUVs was very good (Fig. 5A, △) with slightly high log K_s values for the SUVs. It should be noted that the drug partitioning into the TSK-immobilized LUVs analyzed at a flow-rate of 1 ml/min correlated well with that in Sephacryl-immobilized LUVs analyzed at 0.3 ml/min (Fig. 5B).

Fig. 6A shows that a good correlation was obtained between partitioning of several β-blockers into the avidin–biotin-immobilized liposomes and the corresponding partitioning reported by Betageri and Rogers [38] for free liposomes. An even better correlation was obtained between log K_s values on immobilized and free liposomes of the same lipid composition (DPPC) (Fig. 6A, △). The correlation between the partitioning into immobilized liposomes and the partitioning in a *n*-octanol–buffer system was lower (Fig. 6B), probably owing to the fundamental differences between the membrane and the bulk oil phases [39–41].

4. Discussion

Avidin–biotin and streptavidin–biotin binding are characterized by their exceptionally high affinity, stability and site-specificity [20]. Accordingly, liposomes and liposome assemblies were immobilized in

Table 3
Trapped volume of biotinylated EPC liposomes immobilized in avidin–gels

Type of immobilized liposomes	Type of gels coupled with avidin	Amount of immobilized liposomes (μmol lipid/ml gel bed)	Trapped volume of immobilized liposomes (μl/μmol lipid)
LUVs	TSK	36.5±0.5 (n=3)	1.9±0.2 (n=3)
LUVs	Sephacryl	34	1.7
LUVs	Superdex	3.1	1.8
SUVs	Superdex	36.7±1.4 (n=2)	0.33±0.01 (n=2)
SUVs	Sepharsoe	46	0.42

gel beads with a high yield and excellent stability (Table 1 and Fig. 4) by strong binding between avidin coupled to gel matrices and the biotinylated phospholipids incorporated in the liposomal bilayers, as was verified by control experiments (Section 3.1). However, the binding of (strept)avidin to biotin-PE in liposome membranes is significantly weaker than that of (strept)avidin to nonmodified biotin molecules

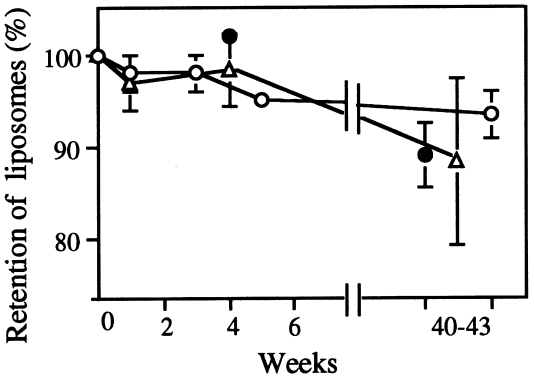

Fig. 4. Stability of (strept)avidin–biotin-immobilized liposomes upon storage: SUVs in avidin–Sepharose (initial amount 41–52 μmol lipid/ml gel) (○), SUVs in streptavidin–Sepharose (44–53 μmol/ml) (●), and LUVs in avidin–Sephacryl (31–48 μmol/ml) (△). The liposomes were composed of EPC supplemented with 2 mol% of biotin-cPE. The gels with the immobilized liposomes were stored at 4°C under nitrogen. After the chosen period of storage the liposome–gel suspension was washed on the filtering funnel with buffer H and resuspended in the buffer (about 5 ml). Aliquots (10–20 μl) were taken before and after the washing for phosphorus determination and the difference in phospholipid amounts was expressed in percentage of the initial amount of the lipids. Average values from two or three batches of immobilized liposome gels are given.

[21,24]. It is very likely that several (strept)avidin molecules coupled to the gel offer multiple-site binding of each biotinylated liposome, as illustrated in Fig. 7. This probably greatly contributed to the remarkable stability of immobilization. In the system used, 80 gel-coupled avidin molecules (M_r 66 000) were available for binding to each liposome, which exposed 70–800 biotinylated lipid molecules on the outer surface (liposome diameter 30–100 nm, 0.7–8×10^4 phospholipid molecules [42]). Stable multiple-site immobilization of liposomes may also be achieved by use of other types of affinity binding systems, e.g., lectin–glycolipid or antibody–antigen.

Both the sonicated SUVs and extruded LUVs have been well characterized as regards their homogeneous size distribution and unilamellar structure [29,36,43–45] and they can be prepared in a simple and reproducible manner. Immobilization of these liposomes in unchanged form was achieved (see Tables 2 and 3, and the corresponding sections) by (strept)avidin–biotin site-specific binding, since the binding did not perturb the liposomal membranes, as inferred from the result shown by Chiruvolu et al. [21] that biotinylated liposomes in streptavidin-mediated assemblies retained their spherical shapes and unstressed membrane surfaces.

For immobilized liposomes chromatography (ILC), solute interactions with the gel matrices and the coupling-(strept)avidin molecules should preferably be minimal. In fact, the electrostatic interaction between the basic drugs and the (strept)avidin–gels were negligible, since the elution volumes of the most hydrophilic drugs (log K_s values below 1.5) were very similar to the elution volume of NaN_3 (data not shown), and since the most hydrophobic

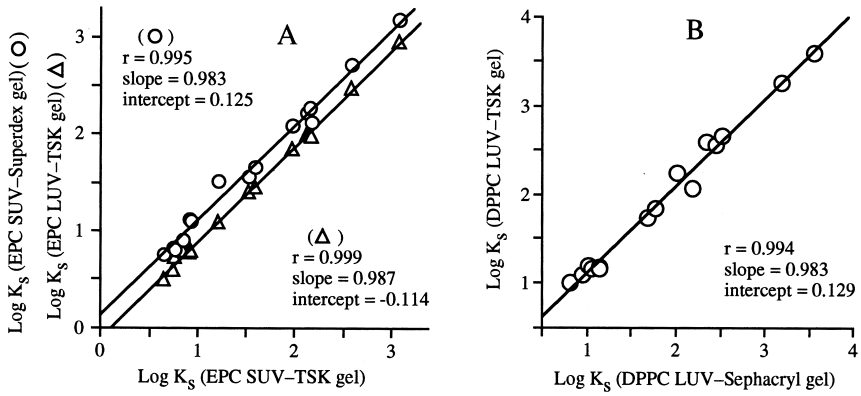

Fig. 5. Correlations of drug partitioning into the bilayers of immobilized liposomes in avidin–gels of different types. Fifteen drugs (see Section 2.1) were applied to the liposome–gel beds and were eluted with buffer H as described in Section 2.8. The K_s values of (−)-isoproterenol and (±)-isoproterenol were overlaped due to the same retention volume obtained for these two drugs. The chromatographic runs on immobilized LUVs composed of DPPC (Panel B) were done at 37°C.

drugs (log K_s values >1.9) showed only slight retardation on the liposome gel beds (data not shown) upon chromatographic elution with the buffer of an intermediate ionic strength (≈0.15) and neutral pH. The close correlation of drug partitioning into the bilayers of the immobilized liposomes in different gel matrices (Fig. 5) was expected, since the gel matrices used as liposome carriers are highly hydrophilic, designed for size-exclusion chromatography in aqueous solution, and since the (strept)avidin molecules coupled to the gel matrices are also hydrophilic. The (strept)avidin–gels were therefore suitable for the binding immobilization of liposomes, provided that a substantial fraction of their pores are sufficiently large to allow liposomes of a given size range to enter the beads. TSK G6000PW and Sephacryl S-1000 gels coupled with avidin accommodated both SUVs and LUVs in large amounts and high yield (Table 1). Moreover, the rigid 17-μm TSK gel beads allowed high-resolution analysis at

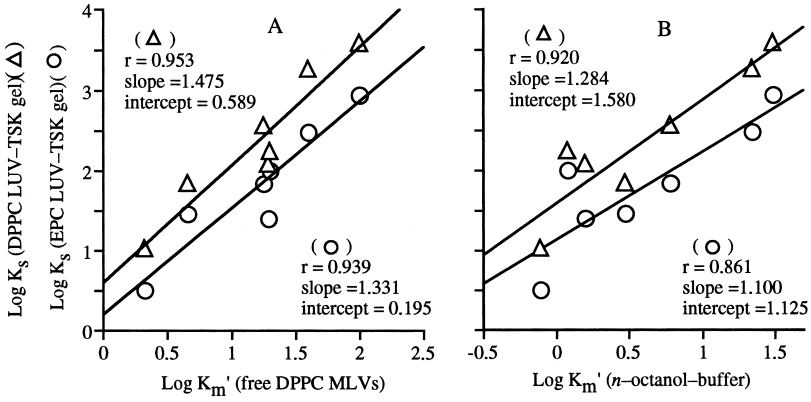

Fig. 6. Correlation of the partitioning of several β-blockers into the bilayers of immobilized liposomes (log K_s) and of free DPPC liposomes (log K'_m) (Panel A), and in an *n*-octanol–buffer system (Panel B). The β-blockers used are propranolol, alprenolol, oxprenolol, pindolol, metoprolol, acebutolol and atenolol. The apparent partition coefficients (K'_m) into liposome and in the *n*-octanol–buffer systems were measured by Betageri and Rogers in phosphate buffer (pH 7.4) [38]. The chromatographic runs on the DPPC LUV TSK gel bed were done at 37°C.

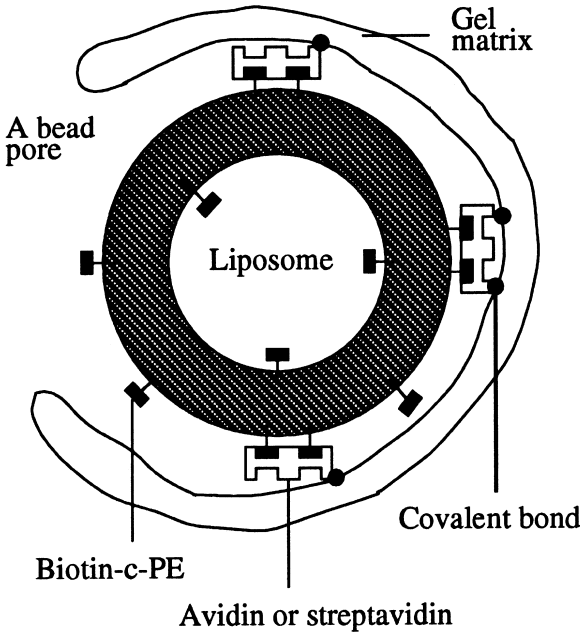

Fig. 7. Schematic illustration of a biotinylated liposome immobilized by multiple-site (strept)avidin–biotin binding in a gel matrix.

high flow-rate (Fig. 5B) of drug–membrane partitioning into immobilized liposomes. The avidin–biotin liposome binding thus constitutes another versatile biomembrane immobilization method that has stability and yield advantages which in many cases may prove useful.

Correlation of the chromatographic retention volumes, normalized as K_s, for several β-blockers on the binding-immobilized LUVs (Fig. 6A) with their partitioning into free liposomes (literature values), demonstrated the chromatographic analysis of drug partitioning into the liposomes, as has been shown with sterically immobilized MLVs and other membrane systems [3,5,33]. The partitioning of the drugs seemed to be higher into the LUVs (Fig. 6A) than that into the MLVs, as shown by comparison with data of Beigi et al. (Fig. 2A in Ref. [33]), which may be explained by slow or nonexisting equilibration of hydrophilic analytes between the multiple lamellas. Interestingly, electrostatically adsorbed red cell ghosts with their unilamellar membranes (Fig. 2D in Ref. [33]) also showed higher log K_s values for hydrophilic drugs than did the presumably multilamellar liposomes and vesicles (Fig. 2(A–C) in Ref. [33]). The amount of lipid interacting with the most hydrophilic drugs was possibly only that contained in the outer bilayer in the latter cases, whereas the total amount of lipids was used to calculate the log K_s values. In the present work it was also noted that the log K_s values were significantly higher with the SUVs than with the LUVs (Fig. 5A), which may be attributed to the slightly larger surface area and corresponding lower membrane lipid surface density [46] resulting from the asymmetric phospholipid bilayer packing and extreme membrane curvature of SUVs of small radius (less than 15 nm) [47]. Solute partitioning into a lipid bilayer interfacial phase has been shown to be affected by liposome types [48] and the membrane surface density [39–41]. Further studies on the difference in drug partitioning into the immobilized SUVs, LUVs and MLVs, and the effect of the partitioning by the chain ordering in the liposomal lipid bilayers upon ILC are being carried out in our laboratory.

5. Conclusions

The biotinylated SUVs and LUVs were prepared in a reproducible manner and could readily bind to the (strept)avidin–gel beads upon mixing of them. Stable and high-yield immobilization of the liposomes in the gel beads was achieved by avidin–biotin multiple-site binding. The immobilized unilamellar liposomes of defined size distribution constitute a relatively homogeneous stationary phase for chromatographic analysis or screening of interactions of solutes (e.g. synthetic drugs or peptides) with the lipid bilayers. The high immobilization yield is useful for immobilization of liposomes composed of expensive phospholipids.

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