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Review

Chromatographic approaches to liposomes, proteoliposomes and biomembrane vesicles

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Abstract

Size-exclusion chromatography has been used for fractionation of liposomes, proteoliposomes and biomembrane vesicles of up to approximately 500 nm in size and for separation of these entities from smaller components. Liposome sizes, encapsulation stability, and solute affinities for membrane proteins have been determined. Counter-current distribution in aqueous two-phase systems has widened the range of applications to larger structures. Immobilized biomembrane vesicles and (proteo)liposomes provide stationary phases for chromatographic analysis of specific or nonspecific membrane–solute interactions.

Keywords: Reviews; Membrane vesicles; Liposomes; Proteoliposomes; Proteins

Contents

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1. Introduction

The purpose of the present review is to focus attention on the use of chromatographic techniques, mainly size-exclusion chromatography (SEC), for fractionation and analysis of liposomes, proteoliposomes and membrane vesicles. Ion-exchange chromatography of proteoliposomes, Hummel and Dreyer analysis of membrane protein affinities for solutes, and counter-current distribution of membrane structures in aqueous polymer phases are exemplified. The recently reviewed [1] use of cells, vesicles, proteoliposomes and liposomes as stationary phases for chromatographic analysis and separation is briefly described.

Liposomes (lipid vesicles) are formed by self-assembly in water of phospholipids or other amphiphiles with related properties [2–5]. The liposome size and the number of lipid bilayers (lamellae) are regulated by the methods used to disperse or solubilize the lipids and the conditions for re-association. Bangham and Horne were the first to prepare liposomes and study their structures by electron microscopy [6,7]. The interest in liposomes and in proteoliposomes, composed of lipids and reconstituted membrane proteins [8–11], is founded on their use as models for biomembrane structures. Liposomes are also used as drug carriers [2] for facilitation of drug uptake, protection of drugs and drug targeting, which makes the analysis of liposome encapsulation important. Biological membranes and membrane vesicles are prepared by disintegration of cells and fractionation by centrifugal or other techniques [12]. The application of various fractionation techniques to separation problems involving liposomes, proteoliposomes and membrane vesicles is mostly governed by the large size of these structures and by their tendency to adsorb to gel matrices.

2. Chromatography of liposomes, proteoliposomes and biomembrane vesicles

2.1. Size-exclusion chromatography

2.1.1. Brief history and pioneering liposome fractionation on agarose gels

The SEC separation principle was understood already in 1952 by Deuel and Neukom [13], as noted in [14]. Hjertén fortuitously observed size separation on dextran gel particles, as described later [15], whereafter the development gained momentum. The primary report on size fractionation of small solutes and desalting of proteins on the easily handled dextran gels was published in 1959 [16]. Granulated agar [17], granulated cross-linked polyacrylamide polymer [18] and other granulated materials [19] were introduced. The first protein separations were performed on beds of cross-linked polyacrylamide; see the review by Tiselius [20]. As early as 1962, Hjertén fractionated subcellular particles such as viruses, ribosomes and proteins in agarose gel suspensions [21]. The further development of SEC matrices [22,23] and of various liposomal structures [2] took place in parallel to mutual benefit.

Beaded agarose gel [22] was ingeniously prepared in 1963 [24] and was first applied to liposome fractionation by Huang in 1969 [25] (Fig. 1). Large multilamellar structures and small unilamellar liposomes in sonicated lipid dispersions [26] were

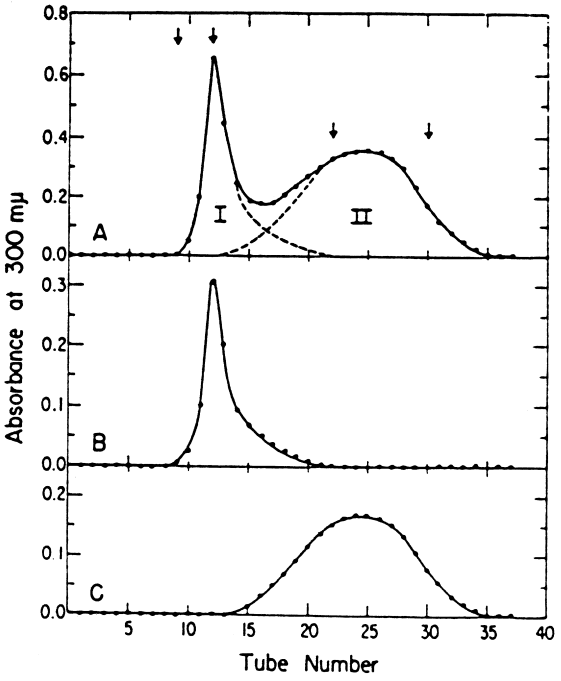

Fig. 1. Elution profiles for phosphatidylcholine (PC) liposomes on Sepharose 4B (Amersham Pharmacia Biotech, Uppsala) at 4°C. (A) Dispersion obtained by ultrasonic treatment, (B) fraction I and (C) concentrated small unilamellar liposomes, average diameter ≈25 nm, from fraction II of panel A. The fractions were collected between the arrows. Bead size range: 45–165 μm. Bed dimensions: 50×2.5 cm (diam.). (Reprinted from [25], with kind permission from the American Chemical Society).

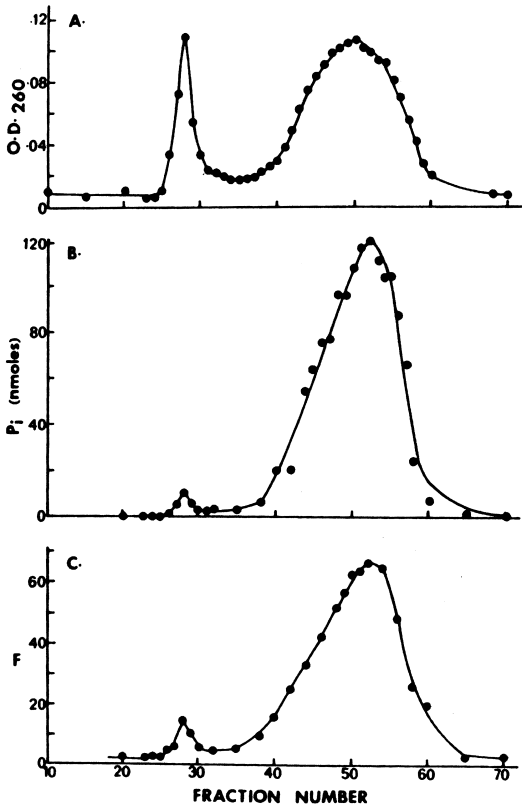

Fig. 2. Elution profiles on Sepharose 4B for dimyristoyl PC liposomes prepared by sonication. The fractions were monitored by (A) optical density at 260 mn, (B) phosphorus analysis, and (C) diphenylhexatriene fluorescence enhancement by association with phospholipid bilayers. Bed dimensions: 40×1.5 cm (diam.). (Reprinted from [27], with kind permission from Academic Press).

separated. Similar experiments done by London and Feigenson [27], using different detection methods, confirmed and extended the results (Fig. 2). Diffusion analysis, electron microscopy and column calibration with viruses showed that size distributions of the small liposomes were broad.

In the further development of liposome preparation methods SEC has frequently been used as a simple and informative method. Sepharose 4B has been applied even when the liposomes were largely excluded from the gel, e.g., for unilamellar 60-nm and 100-nm liposomes prepared by dialysis of cholate–phospholipid mixed micelles [28] and by SEC on Sephadex G-25 (Amersham Pharmacia Biotech) of deoxycholate–phospholipid mixed micelles [29], respectively. Sephadex G-25 retards and adsorbs deoxycholate [30]. Sepharose 2B allows separation of liposomes of up to 100 nm in size [31–34].

2.1.2. Extended fractionation range on allyl dextran–bisacrylamide copolymer gels

The design of the rigid allyl dextran–methylene bisacrylamide copolymer gel Sephacryl S–1000 (Amersham Pharmacia Biotech) extended the separation range to large liposomes and allowed high flow-rates. Liposomes of sizes in the range of 30–250 nm obtained by dialysis of lipids solubilized with different detergents were eluted at different elution volumes on this gel [35,36] (Fig. 3). The

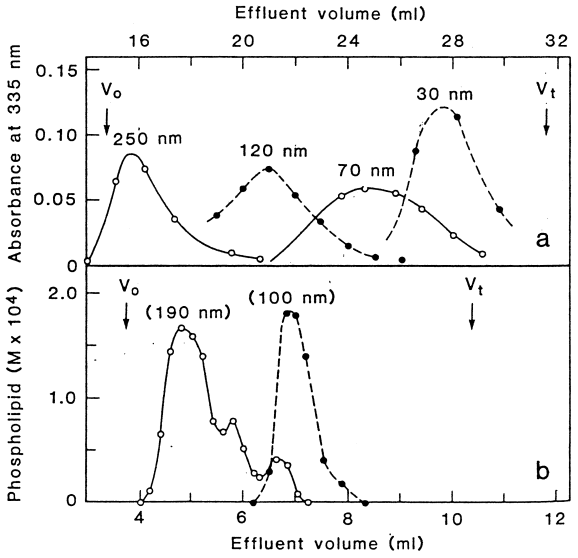

Fig. 3. Elution profiles on Sephacryl S-1000 for liposomes prepared by dialysis of lipids solubilized with (a), from left to right, octyl glucoside, dodecyl octa(ethylene glycol) monoether ($C_{12}E_8$), dodecyl nona(ethylene glycol) monoether ($C_{12}E_9$), and sodium cholate, and (b) octyl glucoside, with two different dialysis procedures. Bead size range: 40–105 μm. Bed dimensions: (a) 50×0.9 cm (diam.), (b) 28×0.7 cm (diam.). (Reprinted from [35], with kind permission from the American Association for the Advancement of Science).

peak position expressed as erf^{-1} $(1-K_D)$ showed a rectilinear dependence on the liposome diameter[1], as for Ficoll (Sigma, St. Louis, MO, USA), proteins, oligosaccharides and poly(ethylene glycols) (PEGs) on other gels [38].

The internal volume of proteoliposomes that are eluted near to the void volume of Sephacryl S-1000 is sufficient for determinations of the initial influx rate of substrates into the proteoliposomes, whereas proteoliposomes of low internal volume will be eluted later due to the large matrix pore sizes. For example, large red cell membrane protein proteoliposomes [39] were separated from smaller proteoliposomes on Sephacryl S-1000 (Fig. 4A) and used for transport measurements. Owing to the fast D-glucose transport by the abundant glucose transporter, Glut1, D-glucose approached equilibrium rapidly, whereas the initial rates of L-glucose and tyrosine uptake were lower (Fig. 4B). The discarded small proteoliposomes would not have contributed to the uptake values in proportion to their protein amount.

The Sephacryl gel S-500 HR is suitable for fractionation of liposomes in the size range up to ≈100 nm. In studies of asymmetric reconstitution of the human red cell anion transporter in the bilayers of spherical liposomes [42], 70-nm proteoliposomes were French-pressed to obtain smaller vesicles, which were fractionated on this gel into 50-nm bilamellar anion-transporter proteoliposomes with right-side-out protein that transported sulfate, 30-nm unilamellar proteoliposomes, and even smaller liposomes that carried no protein [43,44].

Sephacryl gels of even smaller pore sizes are available. In analyses of incorporation of PEG derivatives into the bilayers of extruded 160-nm liposomes, chromatography on Sephacryl S-400 HR excluded the liposomes [45]. Furthermore, association of the 133-residue polypeptide interleukin-2 with the bilayers of PC liposomes at pH 3 was demonstrated by several methods including chromatography on Sephacryl S-300 HR. The 60-nm liposomes were eluted at the void volume. The polypeptide decreased the size of the liposomes prepared by sonication and prevented fusion [46]. Polypeptide binding to the internal bilayer surfaces may have increased the degree of encapsulation, a possibility that was not discussed.

2.1.3. HPLC fractionation on ethylene glycol–methacrylate copolymer gels

Size analysis of liposomes on the ethylene glycol–methacrylate gel TSKgel G6000PW (Tosoh, Tokyo; TosoHaas, Montgomeryville, PA, USA) in HPLC columns with 17-μm beads has been extensively studied [47–49]. Semipreparative columns with 25-μm beads are available. This 'hydroxylated polyether-based' gel shows a larger exclusion limit than do other gels (Section 2.1.4.2). Low recoveries were observed for 0.5–1-μm structures [49]. Liposome elution profiles on TSKgel G6000PW are shown in Fig. 5. Liposomes of ≈300-nm size were eluted earlier than were 145-nm liposomes, but with an overlap presumably due to both size heterogeneity and retardation by weak association with the matrix. The differences in peak elution volumes of 145-nm, 80-nm and 45-nm liposomes were smaller. Several

[1]The erf function is defined in [37] under the heading 'The Error Function'.

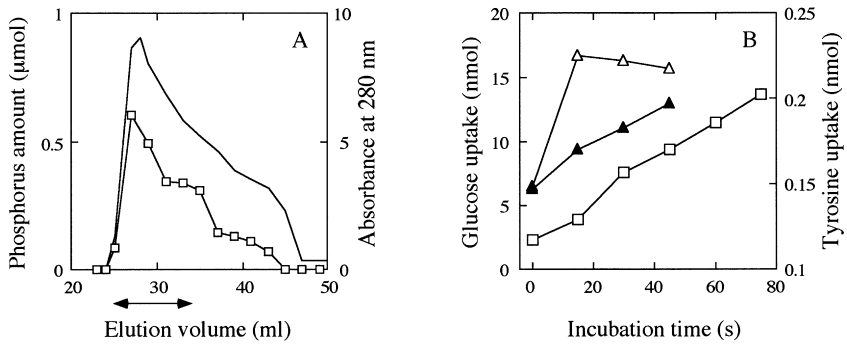

Fig. 4. (A) Elution profile on Sephacryl S-1000 of freeze–thawed proteoliposomes prepared from red cell membrane proteins and egg yolk phospholipids (squares: phosphorus amount). A fraction (double arrow) was concentrated twofold for transport measurements. (B) Uptake of D-glucose (open triangles), L-glucose (filled triangles) and L-tyrosine (squares). The influx rates per amount of protein, in pmol s^{-1} $(\mu g)^{-1}$, were >6.5, 2.8 and 0.018, respectively, at 1.6 m*M* D- and L-glucose and 30 μ*M* tyrosine, i.e., concentrations equal to the estimated K_m values for zero-trans-entry in native systems of D-glucose [40] and tyrosine [41], respectively. The protein concentration in the incubation mixture was 0.24 mg/ml, of which Glut1 comprised 0.043 mg/ml. Incubation buffer and eluent: 15 m*M* Tris–HCl, 150 m*M* NaCI, pH 7.4; Temperature: 23°C. Bed dimensions: 36×1.4 cm (diam.). Studies in cooperation with J. Fischbarg et al. (Unpublished data.)

other liposome elution profiles on this TSKgel are illustrated in [47]. The relatively small and homogeneous bead size of the TSKgel G6000PW provides increased resolution, although the slow diffusion of large vesicles probably limits the resolution at high flow-rate, whereas low flow-rate promotes adsorption of the material to the beads (Section 2.1.4). High-performance (XL) columns with 13-μm beads have been reported to give low recovery of liposomes [49]. The TSKgel G5000PW is suitable for small liposomes [49].

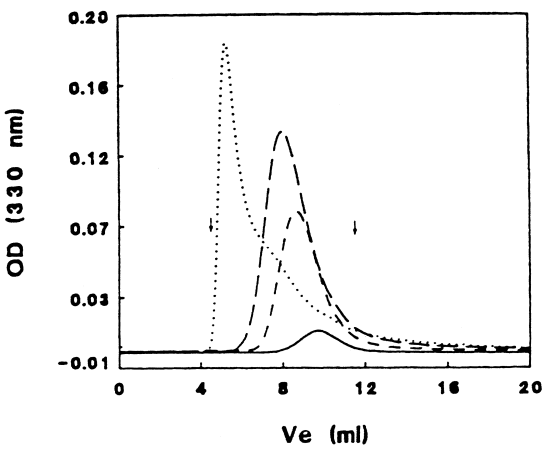

Fig. 5. Elution profiles of liposomes of different sizes on TSKgel G6000PW. The samples were: (· · ·) and (– – –), 300-nm and 145-nm liposomes, respectively, prepared by extrusion, (- - -) 80-nm liposomes prepared by spontaneous fusion of small unilamellar vesicles, (———) 25-nm unilamellar vesicles prepared by sonication. The 80-nm liposomes were composed of dipalmitoyl PC, the others of egg PC–egg phosphatidic acid (9:1, w/w). The liposome diameters were determined by dynamic light scattering. The arrows indicate V_0 and V_t; bead size: 17 μm; gel bed dimensions: 30×0.7 cm (diam.). (Reprinted from [49], with kind permission from CRC Press).

2.1.4. Methodology

2.1.4.1. *Aspects on the separation principle*. SEC of liposomes and similar structures is affected by the size and shape of the structures, and possibly also by their flexibility. Liposomes may be deformed and thereby either pass through relatively narrow pores or be excluded from relatively large pores, dependent on shape and orientation relative to the pores. The net effect is difficult to predict. Furthermore, as touched upon above, affinity interactions with the gel matrix tend to retard liposomal structures in addition to retardation by the size exclusion effect. Gel matrices provide a multitude of interaction sites of a wide range of affinities for liposomes. The strength of interaction will depend on the lipid bilayer composition, and the number of possible interaction sites will be affected by the size and flexibility or rigidity of the liposomal structures. A further factor

is that the relation between surface area and internal volume limits the deformability of small liposomes.

2.1.4.2. *Choice of gel and column*. Ref. [47] provides a valuable and thorough overview of the methodology for chromatographic size separation of liposomes in general, and for size analyses on TSKgel G6000PW in particular, and gives references to many interesting applications. Several matrices are compared. However, reliable comparisons between factory-produced and laboratory-packed columns differing in matrix material as well as in the size and size distribution of both beads and pores require that identical samples, preferably liposomes of different but homogeneous sizes, are analyzed on columns of identical dimensions. Such experiments have not been reported, to our knowledge, and trial experiment in novel cases may be recommendable. Table 1 in [49] and Table 1 in the present review give preliminary guidance. For example, liposomes of sizes up to ≈500 nm can be fractionated on TSKgel G6000PW, those up to 200–300 nm on Sephacryl S-1000 and smaller liposomes on, e.g, Sepharose 4B or CL-4B, Sephacryl S-500 HR or S-400 HR, or TSKgel G5000PW. As pointed out by Walter et al. [49], no SEC beads are available for fractionation of very large vesicles, e.g., >0.8 μm, which is a drawback also in transport retention chromatography (TRC) (Section 4.3). Information on SEC of liposomes is given also in Section VI of [51].

Both the size distribution of the bead pores and the space between the beads in a column affect the passage of large vesicles [47]. The inter-bead spaces will be decreased when the beads are compressed and deformed upon packing of the bed or during runs at high flow-rate and also if the beads swell in a closed column upon a change of eluent.

The column design and dimensions must be chosen according to the type of application (preparative or analytical), the resolution needed, and the scale of operation. Empty columns are available from several manufacturers, e.g., Amersham Pharmacia Biotech and Bio-Rad (Hercules, CA, USA). Some gels, such as the TSKgel G6000PW, are commercially available only in prepacked columns. For analytical applications an HPLC pump that provides constant flow-rate, in combination with an HPLC injector, is advantageous. Small volumes of expensive eluents can be supplied by use of a Superloop (Amersham Pharmacia Biotech), as for application of large-volume samples in frontal chromatography (Section 4.4) [53], and, in combination with the use of two injectors, as for Hummel and Dreyer analyses (Section 2.1.6.4).

2.1.4.3. *Presaturation and recovery*. Adsorption of liposomes, proteoliposomes and membrane vesicles to gel matrices lowers the chromatographic yield, e.g., to 30–40% for certain phospholipid liposomes on Sepharose 2B [31]. Presaturation of the gel bed with a suitable liposome preparation improves the lipid recovery, e.g., to ≈95% on Sephacryl S-1000 [36] and Sepharose 4B [50], and all SEC gel beds, including TSK gel G6000PW, are usually pretreated with (proteo)liposomes. The liposomal lipid composition affects the recovery. This may be low even after presaturation [47]. A delicate matter is to evaluate whether the adsorbed material is displaced by or exchanged for suspended liposomes passing by, and whether lipids are selectively adsorbed or exchanged, which, in principle, would have to be tested for each individual lipid composition and gel type [47]. In a particular case, no exchange of adsorbed radioactive PC with lipid in the applied

Table 1
Approximate diameter, in nm, of liposomes eluted upon SEC on commercial gels at a volume V_e corresponding to the given values of $K_D=(V_e-V_0)/(V_t-V_0)$ [23][a]

Gel	$K_D=0$	$K_D=0.1$	$K_D=0.5$	References
Sepharose 4B	>60	50	20	[50]
Sepharose 2B	>100	70		[32]
Sephacryl S-1000	>200–300[b]	200	80	[22,35,36,50–52]
TSKgel G6000PW	>500	400	130	[47,48]

[a] Values either given in the cited reference(s) or estimated from data given therein.
[b] Refs. [51,52] give the value of 200 nm.

liposome suspension was observed [36]. Interestingly, liposomes prepared by a polyol dilution method that left up to 3% of glycerol or propylene glycol in the final aqueous liposome suspension showed high stability and 100% recovery from Sepharose CL-4B [54]. The recovery of (proteo)liposomes or membrane vesicles larger than 1 μm is highest at high flow-rate, probably since the hydrodynamic lifting force [55–57] keeps them away from the gel bead surfaces.

2.1.4.4. *Calibration*. Calibration of gel beds with latex beads, viruses and large proteins may lead to underestimation of the liposome size upon SEC. Lesieur et al. suggested that unilamellar liposomes of sizes up to 100 nm 'penetrate the pores according to their equivalent hydrodynamic volume without any deformation', whereas the elution of larger flexible liposomes is delayed compared to rigid liposomes (or other particles) of the same size [47]. When the retardation of small liposomes by matrix association on presaturated Sepharose 4B was suppressed by inclusion of liposomes in the eluent (Fig. 6A) or by applying samples containing large amounts of liposomes (Fig. 6B), calibration with viruses and proteins indicated a liposome diameter considerably below the value obtained by inner-volume analysis (unpublished data) [1]. This indicates residual matrix-association, possibly in combination with increased elution volume of small liposome owing to deformability. A similar discrepancy was previously observed [58].

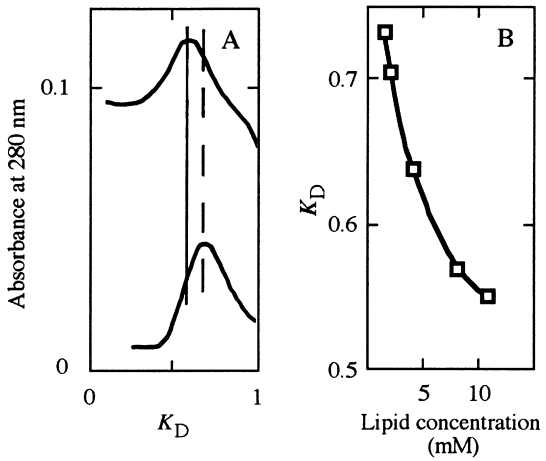

Fig. 6. Fractionation of egg yolk phospholipid liposomes on Sepharose 4B at 23°C. (A) Elution in the absence (lower peak) and presence (upper peak) of liposomes (1 m*M* phospholipid) in the eluent. (B) K_D values for 0.9-ml liposome samples of the phospholipid concentrations indicated. Gel bed dimensions: 42× 1.0 cm (diam.). (Unpublished data.)

2.1.4.5. *Detection*. Since large liposomes scatter light, their amount tends to be overestimated by ultraviolet light absorbance detection (Fig. 1), whereas the elution profiles obtained by phosphorus analysis [59], fluorescence detection [27] or refractivity detection [49] correctly reflect the amount of lipid (Fig. 2). The latter applies also to scintillation counting with radioactively labeled lipids. The protein amount in proteoliposome or membrane vesicle fractions can be determined by automated amino acid analysis [39,57]. Other quantitative methods for protein determination [60] are subject to disturbances caused by other substances present. SDS-PAGE analysis allows analysis of the protein composition of the fractions.

2.1.5. Preparative applications: vesicles

2.1.5.1. *General*. Membrane vesicles of natural origin can be fractionated similarly to liposomes: more commonly by centrifugation techniques, two-phase aqueous partitioning and other free-solution methods than by SEC [12,61,62]. However, SEC is simple and proteins are often efficiently separated from the vesicles, as pointed out in a study on removal of Percoll (Amersham Pharmacia Biotech) from rat liver vesicles [63].

Sephacryl S-1000 and porous glass beads (CPG-3000; CPG, Fairfield, NJ, USA) have been applied for isolation of synaptic membrane vesicles from rat brain homogenate [64,65], and CPG-3000 derivatized with glycidoxypropyltrimetoxysilane to prevent protein adsorption was used for purification of skim-milk membrane vesicles [66]. Sepharose CL-6B was applied for the purification of extracellular membrane vesicles from rabbit aorta [67], and Sephacryl S-1000 as a final preparation step for purification of small vesicles from dog spleen [68]. The combination of centrifugation procedures with SEC on Sephacryl S-1000 allowed the efficient preparation of clathrin-coated vesicles from brain [69] and of the

'major vault protein' of stingray cytoplasmic ribonucleoprotein particles [70]. The vault protein and the synaptic membrane vesicles were co-sedimented upon discontinuous sucrose density centrifugation and were then separated on the Sephacryl gel (Fig. 7).

2.1.5.2. *Red cell membrane vesicles*. We have routinely prepared cytoskeleton-depleted red cell membrane vesicles by chromatography of lysed cells on Sepharose CL-4B and CL-6B columns in tandem at a linear flow-rate of 10 cm/h, followed by centrifugation steps [71,72]. Proteins other than integral membrane proteins were removed. Any decrease in the flow-rate decreased the vesicle yield, consistent with the hydrodynamic effect mentioned in Section 2.1.4.2. The use of a membrane pump eliminated this problem [72]. In each series of preparations, the first one on washed and stored gel beds yielded, on the average, 15% less membrane vesicles than did following ones, probably due to adsorption.

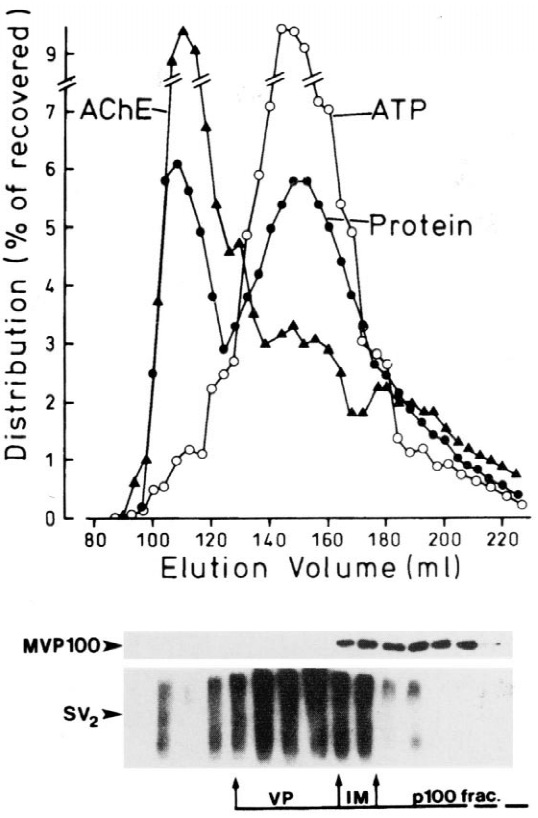

Fig. 7. Separation of the major vault protein (MVP100) from synaptic membrane vesicles by chromatography on Sephacryl S-1000. The vesicles, identified by their ATP contents, were separated from cell membrane fragments, enriched in acetylcholinesterase (AChE). The protein curve represents mainly membrane proteins of the vesicles and the fragments. The eluted MVP100, as analyzed by SDS-PAGE with immunoblotting, only partly overlaps with the eluted synaptic vesicle protein (SV_2). (Reprinted from [70], with kind permission from the American Society for Biochemistry and Molecular Biology.)

2.1.6. Analytical applications

2.1.6.1. *Size analysis*. Three methods for liposome size analysis by SEC have been described in detail by Lesieur et al. [47]: dynamic light-scattering analysis of SEC fractions; rechromatography of SEC fractions on a calibrated column with turbidity measurements; and SEC with on-line turbidity and refractive index detection. The rechromatography method was judged to be the most reliable, although the sensitivity suffered from the dilution in the two chromatographic steps. Interestingly, the SEC elution volume seems to reflect the size of individual vesicles, even in cases where analyses by dynamic light-scattering and turbidimetry show much larger sizes, presumably indicating vesicle aggregation [47]. The rationale may be that when a single vesicle of an aggregate just enters a gel bead pore upon SEC, shearing forces instantly rip off the entering vesicle. Repetition of the process will rapidly dissociate the aggregate.

In addition to the studies mentioned above and in preceding sections, there are several examples of the monitoring of liposome preparation procedures and characterization of liposomes by SEC on, e.g., Sepharose 4B [50], Agarose A-150m (Bio-Rad) [73] and Sephacryl S-1000 [52,74]. Brunner et al. [58] prepared 30-nm unilamellar egg PC liposomes by detergent depletion of cholate-solubilized lipids on Sephadex G-50 M, and showed by SEC on Sepharose 4B (Fig. 8) and other methods that these liposomes had a more narrow size distribution than those produced by sonication or dilution in water (Figs. 1 and 2). When detergent-solubilized membrane proteins are added they can be reconstituted concomitantly with the liposome preparation by this method [77–80].

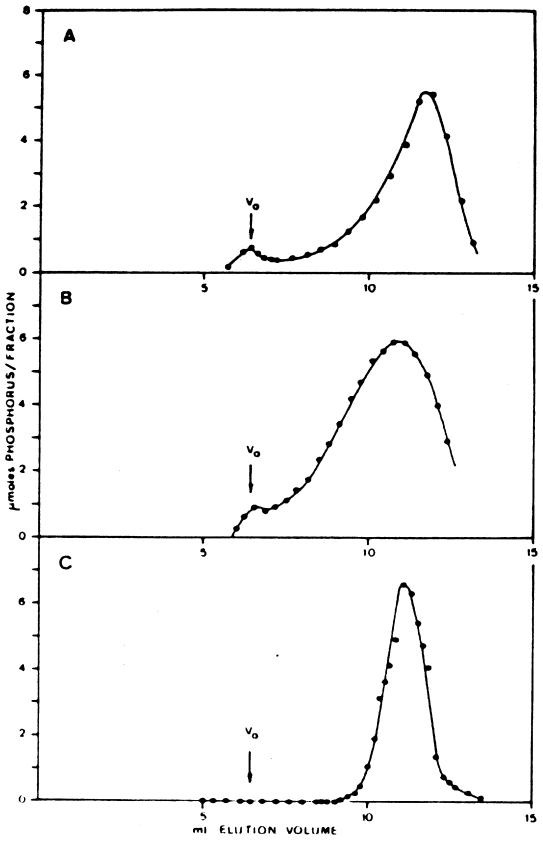

Fig. 8. Elution profiles of egg lecithin vesicles on Sepharose 4B. The column was calibrated by the use of proteins and a virus. (A) Liposomes prepared by sonication as described in [75], (B) vesicles prepared according to the method of Batzri and Korn [76], (C) small unilamellar liposomes of homogeneous size prepared by SEC on Sephadex G-50. (Reprinted from [58], with kind permission).

Qui and MacDonald fractionated sonicated liposomes on Sepharose 4B [81]. The liposomes were claimed to be metastable. Liposome coating improves the stability [2], as shown by Ishiwata et al., who prepared PEG-coated liposomes by inclusion of cholesteryl-PEG in the bilayers and separated the coated liposomes from free cholesteryl-PEG on Sepharose CL-4B [82]. The coating of liposomes with cholesteryl-pullulan was studied by Kang et al. who performed extensive quantitative analyses of the insertion of the cholesteryl moiety of the derivative into the liposome bilayers by chromatography on Sepharose 4B and by other means [83].

A delicate membrane vesicle size analysis was presented by Sturgis and Niederman [84], who French-pressed *Rhodobacter spheroides* cells to release intracytoplasmic membrane vesicles. Cells grown in strong light gave slightly smaller vesicles than did cells grown in weaker light, as indicated by Sepharose 2B chromatography, zonal centrifugation and electron microscopy. This was proposed to reveal a role of the peripheral light-harvesting complex in vesicularization of the intracytoplasmic membrane.

A puzzling result was obtained when TSKgel G6000PW was applied for size analysis of 'supramolecular biovectors' prepared by mixing octyl glucoside and phospholipids with 20-nm starch particles grafted with fatty acids, diluting with water and removing the detergent by dialysis [85]. Lipid-coated particles were obtained, whereas 20-nm liposomes were formed in the absence of the starch particles. Although dynamic light-scattering data indicated that the lipid-coated particles were of the same size as the liposomes the coated particles were eluted much earlier on the TSK gel [85], perhaps since the 'biovectors' were rigid particles, whereas the liposomes were flexible. As discussed in Section 2.1.4.1 and 2.1.4.4, deformability may confer a stronger affinity for the matrix.

2.1.6.2. *Solute encapsulation analysis*. To counteract or regulate leakage of entrapped drugs it is important to be able to analyze and control the liposome stability and permeability. HPLC analyses of drug encapsulation on the basis of SEC of liposomes have been done on TSKgel G5000PW [86] and on TSKgel G6000PW in tandem with TSKgel G4000PW [87]. These systems were claimed to have major advantages regarding run times, sample recovery and economy with sample amounts. However, SEC or ion-exchange chromatography in minicolumns has been recommended for the separation of free drugs from liposomes, since this methodology is fast, requires only small sample volumes and does not affect the liposomes [51]. Commercially available minicolumns can be run in a centrifuge, which shortens the time required for separation. Comparison of several methods for separation of non-encapsulated drug from unilamellar liposomes [88] showed that centrifugation of dilute liposome suspensions on a filter in Centrifree tubes (Amicon,

Beverly, MA, USA) and centrifugation in a Ficoll density gradient are more rapid than other methods.

Conventional SEC is often used to remove free drug. For example, Lidgate et al. [89] prepared liposomes by rehydrating thin lipid films, encapsulating mannitol and fractionating the liposomes on Sepharose 6B, yielding a baseline separation of free mannitol. Chromatography on Sephadex G-50 revealed that large oligolamellar liposomes contained much more doxorubicin than did smaller unilamellar liposomes [90]. At least 95% of the drug was membrane-associated. Lichtenstein and Margalit reported that upon Sephadex G-50 chromatography of liposomes encapsulating silver sulfadiazine a fraction of the drug, possibly stable drug aggregates, surprisingly preceded the liposomes [91]. However, this would rather indicate retardation of the liposomes. Another case, encapsulation of interleukin-2, is described in Section 2.1.2.

A special case is Ca^{2+}-alginate-entrapped poly(L-lysine)-coated liposomes, which release their contents in bursts [92]. SEC on Ultrahydrogel 2000 (Waters, Milford, MA, USA) gave a peak of alginate–liposomes that appeared earlier than the liposomes alone and indicated the expected interaction between the alginate and the liposomes. A more general effect is the destabilization of liposomes in blood by phospholipid transfer to lipoproteins. Human serum albumin may give a similar effect, as indicated by the loss of liposomal material after incubation with albumin revealed by SEC on Sepharose CL-2B [93].

Effects of contact surfaces on liposome stability have been studied. Inhibited Glut1 proteoliposomes released entrapped glucose when the liposomes were washed on cellulose nitrate and cellulose acetate filters [57] and, to a much lower degree, upon SEC on presaturated Sephadex G-50 M at high flow-rate.

2.1.6.3. *Liposome–protein interaction analysis*. Sepharose and Sephacryl gels allow separation of liposomes from proteins (see also Section 2.1.5), as upon analysis on Sepharose CL-4B of apolipoprotein-B conjugation with liposomes as a means to increase the specificity of cellular uptake of the liposomes [94]. Another example is separation on Sepharose 2B of liposomes incubated with plasma from the non-adsorbed plasma components, which was studied because adsorption of plasma proteins on intravenously injected liposomes is important for the fate of liposomes used as drug carriers. The adsorbed proteins were identified by two-dimensional electrophoresis [95]. Recently, CTP–phosphocholine cytidylyltransferase association with PC–oleic acid vesicles was demonstrated by SEC analysis on Sephacryl S-400 in the presence of a low concentration of Triton X-100 micelles. An amphipathic α-helix of the transferase is the principal membrane-embedded region as revealed by photolabelling and proteolytic dissection [96].

2.1.6.4. *Hummel–Dreyer analysis of solute–membrane protein interaction*. The Hummel and Dreyer method [97] has been widely used for measuring small-molecule–protein interactions [98]. As an example of adequate quantitative analysis of solute binding to a membrane protein both in proteoliposomes and membrane vesicles, we have studied [99] interactions between the transport inhibitor cytochalasin B (CB) and Glut1 in cytoskeleton-depleted human red cell membrane vesicles [71] and in egg yolk phospholipid proteoliposomes [57]. The first application to Glut1 proteoliposomes gave unsatisfactory results [100] and perhaps discouraged further use of the method until now.

For the present experiments the proteoliposomes and membrane vesicles were stored at −70°C. Thawed and refrozen material was kept in aliquots at −20°C for up to a week during each series of experiments and was applied within 30 min after the final thawing. CB was included in the mobile phase. Before the first experiment in each series the gel bed was treated with liposomes.

The chromatogram for CB binding to the membrane vesicles (Fig. 9) shows the positive peak of CB–Glutl and CB partitioned into the lipid bilayers at the void volume of the column.

The negative peak, representing the deficiency of free CB in the eluent, was chosen for calculation of the amount of bound CB, since its area showed better reproducibility, e.g., ±3% at 40 n*M* CB ($n=3$) than did the area of the positive peak, possibly due to partial adsorption of the material. Analyses over a range of flow-rates gave constant peak area, which indicates that equilibrium prevailed. The binding constants and numbers of binding sites were calcu-

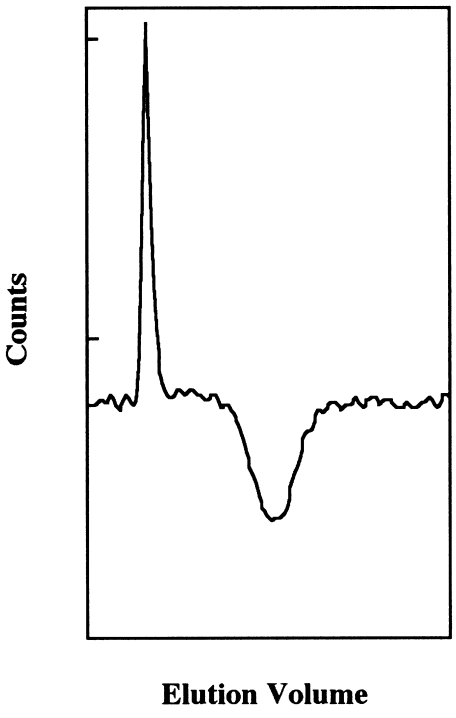

Fig. 9. Example of Hummel and Dreyer flow-scintillation chromatogram on Superdex 75 (Amersham Pharmacia Biotech) for cytoskeleton-depleted red cell membrane vesicles suspended in the eluent, which contained 30 nM nonlabelled CB. The chromatograms for Glut1-proteoliposome samples were similar to that illustrated here. (Modified from [99].)

lated by use of multiple equilibria theory, according to which the reversible binding of the ligand CB to Glut1 in vesicles or proteoliposomes can be described by the following equation [98]:

$$B = \sum_{i=1}^{m} \frac{N_i K_i [\mathrm{CB}]}{1 + K_i [\mathrm{CB}]} \quad (1)$$

where B represents the amount of CB specifically bound to Glut1; m is the number of classes of independent binding sites, where each class, i, has a total amount of binding sites, N_i, with a binding affinity K_i, and [CB] is the concentration of the unbound CB and [^{3}H]CB in the eluent. The binding parameters, N and K (the inverse $1/K = K_d$, the dissociation constant) were calculated by fitting Eq. (1) to the experimental data [99].

The apparent dissociation constant $K_{d,app} = IC_{50}/(1 + [CB]/K_d$, CB) [101] for competitive D-glucose inhibition of CB interaction with Glut1 in proteoliposomes was ≈80 m*M*, about twice as high as the average value obtained by frontal immobilized biomembrane affinity chromatography [53,72,102]. The amount of nonspecifically bound CB was determined by including D-glucose in the eluent at 300 m*M* or higher concentration and was subtracted from the specific binding.

The binding parameters and the ratio between N and the number of protein monomers for vesicles and proteoliposomes are given in Table 2. CB showed a higher affinity for Glut1 in membrane vesicles than for reconstituted Glut1, consistent with earlier analyses [72]. The K_d value for membrane vesicles was identical to that obtained by immobilized vesicle affinity chromatography, whereas, probably owing to residual detergents in the proteoliposomes, the K_d value for proteoliposomes was higher than the corresponding immobilized proteoliposome affinity chromatographic value (Table 2). The number of

Table 2
CB-Glut1 binding parameters determined by the Hummel and Dreyer method[a] and by immobilized biomembrane affinity chromatography (values within parenthesis[b])

Sample	K_d (n*M*)	Binding sites per Glut1 monomer
Red cell membrane vesicles[c]	61±6 (60±2)	0.43[d]±0.03 (0.4[e]±0.05)
Glut1-proteoliposomes[f]	115±18 (80±6)	0.39±0.04 (0.33[g]±0.02)

[a] *I* 0.08, pH 7.4, 23°C.
[b] Average values from [133] adjusted to $I = 0.08$ according to Fig. 2 of [102].
[c] Sample (20 μl) contained 128 μg of protein and $N = 117$ pmol of binding sites (estimated error±5 pmol).
[d] Based on the estimate that 11.8% of the vesicle protein is Glut1 [39].
[e] Unpublished value.
[f] Sample (50 μl) contained 6.3 μg of protein (116 pmol of Glut1 monomers) and $N = 46$ pmol of binding sites (estimated error±3 pmol).
[g] From [53,72,102].

CB-binding sites per Glut1 monomer was ≈0.4 for both proteoliposomes and membrane vesicles, in contrast to the higher ratios in the range of 0.66–0.92 previously reported for Glut1 in proteoliposomes [102–107], also when Glut1 was prepared in the presence of 5–10 m*M* dithiothreitol [107]. However, the lower ratio (0.44) was obtained for Glut1 prepared in the absence of reducing agent [107]. Because CB does not bind to the outward-facing glucose binding site, the number of CB binding sites per Glut1 monomer (Table 2) may reflect the probability that the cytoplasmic face of the transporter shows the conformation open to CB and glucose. This probability will vary with the detailed state of Glut1 which may depend on the preparation procedure.

2.2. Other chromatographic methods

2.2.1. Ion-exchange chromatography

Ion-exchange chromatography has been applied for fractionation of small proteoliposomes which had been prepared to contain a single or at most a few red cell membrane proteins per proteoliposome [80] (Fig. 10). The recovery was ≈70%. Liposomes without protein showed a higher amount of non-adsorbed material and were eluted slightly earlier (not shown). This indicates that negative charges on the membrane proteins and on their oligosaccharide chains contributed to an increased affinity of the proteoliposomes for the ion exchanger. Since the orientation of the proteins upon reconstitution is essentially random, and since each proteoliposome contained few protein molecules, the peaks obtained may reflect the number of exposed internal and external protein faces. However, repetition of the experiment with purified Glut1 would simplify the interpretation.

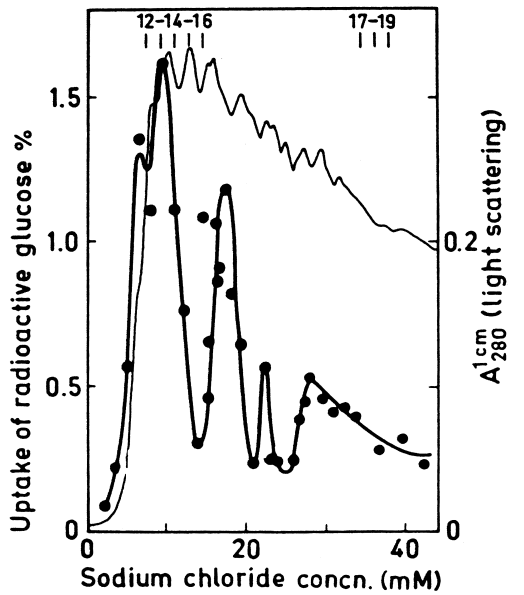

Fig. 10. Ion-exchange chromatography of proteoliposomes on microcrystalline DEAE-cellulose (DE-52, Waters). Egg yolk phospholipids proteoliposomes with human red cell membrane proteins were desalted on Sephadex G-50 and applied on the ion-exchange column. The material was eluted in a shallow NaCl gradient and the D-glucose transport activity was determined. Bed dimensions: 52×1.9 cm (diam.). (Reprinted from [80], with kind permission).

2.2.2. Hydroxyapatite chromatography

Liposome interaction with hydroxyapatite has been investigated by Hirsh et al. [108], who mixed liposomes with hydroxyapatite seed crystals as a model for the calcification of atherosclerotic plaques and discovered that hydroxyapatite induced aggregation of liposomes. It thus seems that hydroxyapatite interaction with liposomes may have implications for chromatographic purposes. This view is supported by the fact that lipoproteins can be separated on hydroxyapatite as recently reviewed by Shibusawa [109]. This method is a complement to previously used methods such as SEC on various gels. Three classes of lipoproteins from human serum, high-, low- and very-low-density lipoproteins (HDL, LDL and VLDL), were eluted stepwise with 0.1, 0.3 and 0.5 *M* potassium phosphate buffer at pH 7.4 from a Bio-Gel HTP DNA grade (Bio-Rad) bed: 85–90% of the loaded lipoprotein fraction was recovered. Shibusawa also showed that LDL and VLDL can be separated from HDL and serum proteins on a hydroxyapatite column without any prior fractionation of the serum. For further separation of HDL from serum proteins, a cross-axis coil planet centrifuge was used for counter-current chromatography [109].

3. Aqueous two-phase partitioning and counter-current distribution

Aqueous solutions of two polymers, such as PEG and dextran, form two-phase systems at appropriate concentrations. These can be used to fractionate (proteo)liposomes or membrane vesicles of different size or different surface properties in a single step or in a multiple-step counter-current distribution mode. Liposome partitioning between aqueous phases has been known for two decades [110–112]. Recent examples were presented by Tilcock and Fisher, who observed that an increasing content of negatively charged phospholipids in the liposomes transferred them from the lower dextran-rich phase to the upper PEG-rich phase [113], and by Van Alstine et al., who observed that PEG-amphiphile adsorption at hydrophobic and phospholipid surfaces correlated with changes in the partition of PC liposomes in aqueous PEG–dextran systems [114]. An efficient two-phase system where 90% of the negatively charged liposomes accumulated in the PEG phase was developed by chemometric evaluation of the influence of five parameters on liposome partitioning in a PEG–dextran two-phase system [115]. Furthermore, Senior et al. have shown by the use of a two-phase method that liposomes coated with monomethoxyPEG appear to adsorb plasma components more slowly than do liposomes without the polymer [116]. From soybean cells grown in culture, Dehahn et al. [117] prepared plasma membrane vesicles with either right-side-out or inside-out orientation by the use of two-phase partitioning and saw that NADH oxidase activity was present on both the external and internal surfaces of the plasma membrane. Johansson et al. used ten two-phase systems and did manual transfers. The inclusion of PEG-coupled ligands in the system affected the distribution behavior of various fractions of a synaptic membrane preparation [118].

The counter-current distribution mode has been used extensively for studies of chloroplasts and other plant cell structures. Peltier and Rossignol used counter-current distribution to fractionate the plasma membrane from tobacco cells when they studied auxin-induced differential sensitivity of the H^+-ATPase [119]. Stefánsson et al. used a centrifugal countercurrent distribution apparatus for fractionation of membrane fragments obtained by sonication of spinach chloroplast and *Dunaliella salina* thylakoids and derived from different structural domains [120,121]. Askerlund separated endoplasmatic reticulum from cauliflower from vacuolar membranes co-fractionated with a Ca^{2+}-ATPase of molecular mass 111 000 [122]. Hensley and Mircheff were able to separate 21 physically and biochemically distinct membrane populations from rabbit cortex by use of a 'three-dimensional' separation procedure based on differential sedimentation, density gradient centrifugation and counter-current distribution, in studies of sodium-dependent amino acid transport systems [123].

The counter-current technique is analogous to chromatographic fractionation and has the advantages that the particle-size is in principle unlimited, that there are no problems with adsorption to a stationary phase, and, if transfers are done manually, that no special equipment is needed.

4. Immobilized liposomes, proteoliposomes and biomembranes for chromatographic analysis

Ever since 1986 [124] our research group has been pursuing the use of immobilized liposomes and proteoliposomes as stationary phases for chromatography. All gel beads with sufficiently large pores or cavities, on which the (proteo)liposomes are retarded upon SEC, can be used for this purpose, and allow immobilization of relatively large amounts of liposomes. If only a very small amount of immobilized material is required, as for drug–bilayer partitioning analysis with strongly lipophilic drugs, coupling of the liposomes to the surfaces of small gel beads may be used. Also membrane vesicles and red cells were recently immobilized for chromatographic analyses [1,101,125]. Immobilized liposomes have been used for ion-exchange chromatography [126], prediction of drug uptake through cell membranes [127–130], and analysis of peptide–liposome interactions [131]. Immobilized proteoliposomes and red cells allow TRC [125,126,132], and quantitative affinity chromatography on immobilized proteoliposomes, membrane vesicles and red cells has provided data on specific solute interactions with membrane proteins,

including equilibrium constants and binding ratios [53,102,133,134].

The immobilization of (proteo)liposomes and membrane vesicles has been done in several ways [134]. Swelling of dried gel beads in a vesicle suspension followed by freeze–thawing is perhaps the most convenient procedure [60,135]. A large fraction of the gel bead volume can be occupied by liposomes, e.g., at least 20% for Sephacryl S–1000 with an internal liposomal volume up to 4 l/mol of phospholipid (Fig. 2B in [136]), corresponding to a diameter of homogeneously sized liposomes of 130 nm [29], and 16% for TSKgel G6000PW, with corresponding values of 2.4 l/mol (Fig. 2D, squares, in [137]) and 80 nm. The cross-linked agarose gel Superdex 200 (Amersham Pharmacia Biotech) onto which dextran is grafted, has allowed extensive filling of the beads as determined by confocal laser scanning microscopy [135] and, in analogy with the stabilization of liposomes by coating with a hydrophilic polymer [2], seems to protect the immobilized vesicles particularly well [1,53,102].

4.1. Immobilized-liposome ion-exchange chromatography

The surface of charged liposomes can be used as an ion-exchange matrix. The negative surface charge of sterically immobilized phosphatidylserine liposomes was sufficient to achieve tight packing of lysozyme on the liposome surfaces at pH 7 [138]. Upon saturation with small counter-charged proteins, ≈70–100% of the liposome surface charges were utilized for protein binding and the surfaces became covered to nearly achieve electroneutrality [126].

The ion-exchange chromatographic resolution on the above liposomes of ribonuclease A, lysozyme and cytochrome *c* was higher and the proteins were eluted at much lower ionic strength than on the ion-exchange matrix Mono S (Amersham Pharmacia Biotech). Cationic liposomes composed of egg yolk phospholipids and sterylamine separated monomers and dimers of bovine serum albumin better than did DEAE-Sepharose 6B (Amersham Pharmacia Biotech) and required lower ionic strengths for adsorption and desorption [139].

4.2. Immobilized-liposome chromatography for prediction of drug uptake

The capability of drugs for passive diffusion over cell membranes is of major interest in pharmaceutic sciences, since diffusion over the epithelium of the small intestine is the crucial step for the absorption of 95% of all commercially available drugs. In 1995, Beigi et al. [127] were the first to use immobilized liposomes as an epithelium model for the prediction of the absorption of drugs in humans. Recently, the method was extended to the use of charged liposomes and different membranes prepared from red cells or red cell membrane components, and the influences of chromatographic parameters, temperature and pH were studied [129]. It appeared that the drugs which were known to have the highest level of absorption in humans had intermediate retention values. The method was also applied in a continuous-bed capillary chromatographic system [130], and liposomes were used as a pseudostationary phase for analogous capillary electrophoretic studies [140].

The immobilized liposome or biomembrane models seem to be more similar to natural membranes than are other more simplified models, such as the octanol–water and immobilized artificial (monolayer) membrane (IAM) systems [141]. Furthermore, the composition of the immobilized (bilayer) membranes can be chosen to closely resemble that of natural membranes, except for the biological membrane asymmetry.

4.3. Transport retention chromatography

Transport membrane proteins reconstituted in proteoliposomes immobilized in a gel matrix can be used as stationary phase to partially separate transported molecules from non-transported ones. This type of separation on red cell ghosts or protein extracts from the same material was first shown in 1966, although the separation mechanism was incorrectly attributed to binding in the initial work [142,143]. Recent examples are the slightly differing elution volumes of D-glucose and L-glucose on Glut1 proteoliposomes immobilized in Sephacryl S-1000 [126,132] and the partial separation of these enantiomers on red cells or ghosts electrostatically adsorbed

on the wrinkled and channeled surface of particles of continuous-bed polymer [125]. The general applicability of TRC on proteoliposomes and membrane vesicles would be greatly improved if gel beads with pores or cavities in the micrometer range became available, since a large inner volume is favorable and requires a large proteoliposome or vesicle size.

4.4. Quantitative affinity chromatography on immobilized proteoliposomes or biomembranes

Quantitative affinity chromatography was introduced long ago [144,145] and has mostly been used to study ligand interactions with water-soluble macromolecules. Recently, the method was applied to determine biospecific interactions between membrane proteins and substrates and inhibitors. Proteoliposomes or vesicles were immobilized in gel beads and the interactant was applied either in a narrow zone [146] or as a large-volume front [101]. Examples are studies on the glucose affinity for the glucose transporter Glut1 in native or reconstituted lipid bilayers [134], the temperature-dependence of affinity constants in these systems [147], and the affinity of a nucleoside transport inhibitor for the red cell nucleoside transport protein [133]. Glut1 was remarkably stable in the immobilized state, allowing analyses over periods of up to 3 months at room temperature [53,72,102,133,134]. This indicates that proteolytic activity is absent, that the protein is protected by the lipid environment, that Glut1 is intrinsically stable, and that the membrane structures as such are stabilized by the entrapment.

5. List of abbreviations

CB	Cytochalasin B
Glut1	Human red cell glucose transporter
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
PC	Phosphatidylcholine
PEG	Poly(ethylene glycol)
SEC	Size-exclusion chromatography
VLDL	Very-low-density lipoprotein
TRC	Transport retention chromatography

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