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Avidin–biotin-immobilized liposome column for chromatographic fluorescence on-line analysis of solute–membrane interactions

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Abstract

Unilamellar liposomes with entrapped fluorescent dye calcein were stably immobilized in gel beads by avidin–biotin-binding. The immobilized liposomes remained extremely stable upon storage and chromatographic runs. The immobilized calcein-entrapped liposomes were utilized for fluorescent analysis of solute–membrane interactions, which in some cases are too weak to be detected by chromatographic retardation. A liposome column was used as a sensitive probe to detect the interactions of membranes with pharmaceutical drugs, peptides and proteins. Retardation of the solutes was monitored using a UV detector. Perturbation of the membranes, reflected as leakage of the entrapped calcein by some of the solutes, can thus be detected on-line using a flow-fluorescent detector. For the amphiphilic drugs or synthetic peptides, perturbation of membranes became more pronounced when the retardation (hydrophobicity) of the molecules increased. On the other hand, in the case of positively-charged peptides, polylysine, or partially denatured bovine carbonic anhydrase, significant dye leakage from the liposomes was observed although the retardation was hardly to be measured. Weak protein–membrane interactions can thus be assumed from the large leakage of calcein from the liposomes. This provides additional useful information for solute–membrane interactions, as perturbation of the membranes was also indicated by avidin–biotin-immobilized liposome chromatography (ILC).

Keywords: Avidin–biotin-immobilized liposome

1. Introduction

Liposomes possess a lipid bilayer structure which simulates biological membranes, and therefore they are frequently used as models to study interactions between membranes and biological molecules such as proteins, peptides and drugs. For chromatographic analysis, liposomes and biomembranes have been immobilized in gel beads by steric entrapment [1], hydrophobic binding [2], avidin–biotin affinity binding [3] or covalent attachment [4]. The interaction of solutes (e.g., drugs, peptides or proteins) with liposomes or proteoliposomes have been analyzed by immobilized liposome chromatography (ILC) [5–10]. Variations in retention volume depend on the extent of solute–membrane interaction and can be precisely measured. However, it is often difficult to

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detect weak interactions of proteins and peptides with membranes from retention volume using ILC analysis. Liposomes containing entrapped fluorescent labels within the aqueous space can be used to investigate the changes in membrane permeability [11]. This implies that fluorescent dye-entrapped liposome chromatography may provide more information about solute–membrane interactions. From this point of view, a relatively homogeneous membrane stationary phase and a stable immobilization method are important for chromatographic analysis or screening of solute–membrane interactions. Compared to other ILC methods of low-yield immobilization [12], hydrophobic ligand effect [2] or membrane permeability [4], small or large unilamellar liposomes can be immobilized by avidin–biotin binding [3,8] with high-yield immobilization and excellent stability. The avidin–biotin technology [13], which is derived from the extremely tight binding between the vitamin biotin and the egg-white glycoprotein avidin [14], has been widely used in (immuno)affinity chromatography [15,16] and molecular assemblies [17].

In the present study, unilamellar liposomes with entrapped fluorescent dye calcein were immobilized by avidin–biotin binding and used as a sensitive probe to detect solute–membrane interactions. In this system, the retention of the solutes was detected by a UV monitor and the perturbation of membranes resulting from leakage of calcein was monitored on a flow-fluorescent detector. The interactions of drugs, peptides and proteins with liposomal membranes were investigated by this ILC-fluorescent on-line system. Further, the avidin–biotin-bound fluorescent liposomes were evaluated for their stability and membrane integrity.

2. Materials and methods

2.1. Materials

Sephacryl® S-1000 Superfine, Superdex 200 prep grade and CNBr-activated Sepharose® 4B were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). TSK G6000PW was from Tosoh (Tokyo, Japan). Egg-white avidin was purchased from Calbiochem (La Jolla, CA, USA). Egg yolk phosphatidylcholine (EPC, >99%) and 1,2-dioleoyl-phosphatidylethanolamine-*N*-(cap biotinyl) (biotin-cPE) were purchased from Avanti Polar Lipids (Alabaster, AL, 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) came from Dojindo Laboratories (Kumamoto, Japan).

(±)-Metoprolol, atenolol, alprenolol, oxprenolol, pindolol, acebutolol, DL-propranolol and bovine carbonic anhydrase (CAB) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

Amphiphilic peptides were synthesized by a 9050 PepSynthesizer (PerSeptive Biosystems) using the FMOC (9-Fluorenylmethoxycarbonyl) solid-phase method [18]. Crude peptides were purified by reversed-phase chromatography (Shimadzu, Japan) as described previously [19]. Three different amphiphilic peptides were used, AC-$(KLLELK)_2$-(KLLE)-NH_2, AC-$(KALELK)_2$-(KLAE)-NH_2 and AC-$(KLLEELK)_4$-(KLLE)-NH_2 (denoted P18L, P18LA and P32L, respectively). Polylysines of 10 residues (purity, 93%) and 30 residues (purity, 83%) (denoted p-Lys10 and p-Lys30, respectively) were obtained from Millipore Co., Ltd. (Japan).

2.2. Coupling of avidin to the activated gels

Sephacryl S-1000 gel, TSK G6000PW gel or Superdex 200 gel (denoted Sephacryl, TSK or Superdex, respectively, below) was activated by 4-nitrophenyl chloroformate to a chloroformate density of 20–30 μmol/ml gel [3]. Avidin was coupled to the activated gel at a concentration of about 3 mg/ml gel as described in Ref. [3]. The gels were washed using a 10 μm filter (Millipore, Bedford, MA, USA) fixed in a glass funnel. Alternatively, avidin was coupled to CNBr-activated Sepharose 4B (denoted Sepharose) to 3.0–3.5 mg/ml of gel bed according to the manufacturer's specifications. The avidin-gels were stored at 4°C in buffer H (10 m*M* HEPES, 150 m*M* NaCl, pH 7.5) supplemented with 3 m*M* NaN_3.

2.3. Immobilization of biotinylated liposomes

EPC supplemented with 2 mol% biotin-cPE was evaporated on a rotary evaporator to form a dry film. The film was flashed with nitrogen, kept under a high vacuum for at least 3 h and then dispersed in

100 m*M* calcein solution (pH 7.5) to form multilamellar vesicles (MLVs). Small or large unilamellar liposomes (denoted SUVs or LUVs, respectively) were prepared by probe sonication or extrusion to mean diameters of 29±5 nm and 105±20 nm, respectively [3]. The calcein-entrapped biotinylated liposomes were mixed with avidin gels by gentle rotation for 2–3 h at 23°C or overnight at 4°C under nitrogen for immobilization. Non-immobilized liposomes together with non-entrapped calcein were then removed by washing with buffer H using the 10 μm filter.

2.4. Covalent attachment of liposomes to activated gels

The covalently immobilized liposomes were prepared as described in Ref. [4]. Briefly, the chloroformate activated Sephacryl, TSK or Superdex gel and CNBr-activated Sepharose gel prepared as previous [3]. An aliquot of the gel was mixed with a liposome suspension, prepared as described in Section 2.3. Excess active groups were blocked by 0.5 *M* ethanolamine (pH 8.2) incubation overnight at 4°C.

2.5. Phosphorus and fluorescence determination

Using the Bartlett method, phospholipids of the immobilized liposomes were examined in order to determine the phosphorus content of the gel beads [20]. The direct phosphorus assay without solubilizing liposomes from the gel beads has previously been described in detail [4]. After solubilization with 100 m*M* β-OG, the liposome-entrapped calcein was measured using a fluorescent spectrophotometer (F-4500, Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 490 nm and 520 nm, respectively. The specific internal volume of the liposome was calculated as described before ([3]and references therein).

2.6. Immobilized liposome chromatographic (ILC) fluorescent analysis

The LUVs or SUVs immobilized in gel beads were packed in a 5 mm I.D. glass column (HR 5/5, Pharmacia Biotech) to form a 0.5 ml or 1 ml gel bed. The liposome column was placed in a column oven (CO-8020, Tosoh) equipped with a sample injector and connected to an HPLC pump (CCPM-II, Tosoh). The chromatographic runs were monitored using a UV-detector (UV-8010, Tosoh) set at 220 nm for drugs and peptides, and 267 nm for CAB, respectively; a fluorescence detector (FS-8020, Tosoh) at excitation and emission wavelength of 490 and 520 nm, respectively, and a recorder interfaced with an IBM computer. The chromatograms were analyzed by the Tosoh HPLC SYSTEM 1. Pharmaceutical drugs (1 μg/μl, 15 μl), peptides (200 μ*M*, 100 μl) and CAB (25 μ*M*, 10 μl) were applied to the liposome column and eluted with buffer H at a flow rate of 0.15–0.3 ml/min at 25°C.

Retention of the solute was expressed as a specific retention factor, K_s [6,3]. Further, perturbation of the liposomal membranes by interactions resulting in increased permeability can be observed by the calcein released from the immobilized liposomes. The amount of released calcein can be estimated from the peak area of eluted calcein.

3. Results and discussion

3.1. Membrane integrity of immobilized calcein-entrapped liposomes

Calcein-entrapped SUVs and LUVs were immobilized in the avidin-gel beads at amounts of 30–44 μmol lipid/ml gel bed (Table 1). LUVs were immobilized only in avidin-coupled Sephacryl and TSK gel beads, because the pore sizes of Sephacryl and TSK gels are up to 400 and 500 nm, respectively, favoring the immobilization of the large unilamellar liposomes ([3] and references therein). The specific trapped volumes of the avidin–biotin immobilized SUVs and LUVs were similar to the literature data [21]. These values together with the mean size of SUVs (29±5 nm) or LUVs (105±20 nm) indicated the unilamellarity of the avidin–biotin-immobilized liposomes. Only 0.4–1.1% of the initially entrapped calcein was released from the immobilized SUVs and LUVs during storage for 1 week at 4°C (Table 1), the avidin–biotin-immobilized liposomes retained their integrity well. The avidin–biotin site binding did not perturb the liposomal membrane, as it has been shown that biotinylated liposomes in streptavidin-mediated aggregation are

Table 1
Trapped volume of biotinylated EPC liposomes in avidin-gels and leakage of the liposome-entrapped calcein after storage for 1 week at 4°C

Type of liposome	Type of avidin gel	Amount (μmol lipid/ml gel)	Trapped volume (μl H_2O/μmol lipid)	Leakage (%)
SUVs	Sepharose	37.6	0.54	0.53
	Sephacryl	41.5	0.68	0.77
	Superdex	30.2	0.66	1.08
	TSK	30.3	0.60	0.74
LUVs	Sephacryl	44.2	2.30	0.43
	TSK	34.3	1.90	0.47
LUVs[a]	Sephacryl	42.5	1.95	1.10

[a] LUVs were prepared under the experimental conditions described in Section 2.3. Alternatively, nonentrapped calcein was removed by gel filtration on a Sephacryl S-300 column (2.0 cm I.D.×35 cm) that had been equilibrated with buffer H, and the calcein-entrapped liposomes were then mixed with the avidin-Sephacryl for immobilization.

likely to be spherical and unstressed [17]. It should be noted that similar results were obtained when calcein-entrapped liposomes or calcein-entrapped liposomes in the presence of free calcein were used in immobilization procedures (rows 5 and 7, Table 1). This meant that, first, only biotinylated liposomes were immobilized in avidin-gel beads but not free calcein, and second, the immobilization method of Section 2.3 was simpler because the gel filtration in the footnote of Table 1 was avoided.

3.2. Long-term stability of immobilized calcein-entrapped liposome

Both the avidin–biotin-immobilized SUVs and LUVs showed excellent stability with a small leakage of calcein during long-term storage compared with the large amount of entrapped calcein that was released from the covalent-immobilized liposomes (Fig. 1). Only 3–4% of the entrapped dye leaked out of the avidin–biotin-immobilized liposomes after storage at 4°C for 7 months. By contrast, the liposomes immobilized by covalent attachment had poor their membrane integrity with 30–50% of the entrapped calcein leaking out after storage for 7 months (Fig. 1), consistent with the results of Yang et al. [4]. This loss may be explained by the change in packing geometry of the coupled lipid in the membranes. A certain disordering of lipid packing in the membranes occurs when the crosslinking of phospholipid molecules with the gel matrix changes the shape-balance of the coupled lipid molecules [22]. The avidin–biotin bound liposomes were less permeable to the entrapped hydrophilic molecules than covalently bound liposomes, thus have long provided a fluorescent probe for chromatographic applications.

Fig. 1 further shows that the leakage of entrapped calcein from immobilized SUVs was more pronounced than that of LUVs during storage. It has been reported that small, sonicated vesicles (approx. 25 nm in diameter) retained only 40% of trapped

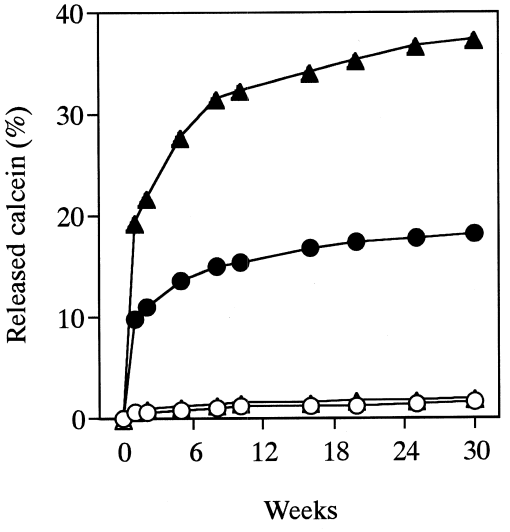

Fig. 1. Stability of avidin–biotin (open symbols) and covalently (solid symbols) immobilized SUVs (triangles) and LUVs (circles) upon storage. The liposomes immobilized gels were stored at 4°C under nitrogen. After the chosen period of storage, the liposome-gel supernatant was removed to allow fluorescence determination. The gels were then washed by centrifugation at 700 *g* for 5 min with buffer H and resuspended in buffer H (about 4 ml). The released calcein is expressed as a percentage of the initial amount of entrapped calcein.

fluorescent dye compared with almost 100% of large, extruded vesicles (50–100 nm in diameter) during freeze–drying [23]. The permeability of SUVs is due to their high radius of curvature. In the case of covalent-immobilization, the calcein leakage from SUVs was 15% higher than that from LUVs. However, for the avidin–biotin-immobilization, the calcein leakage from SUVs was only 0.5% higher than that from LUVs, indicating that liposome size has little effect on the entrapped calcein leakage from the avidin–biotin-immobilized liposomes.

In contrast to free-form liposomes, which were largely aggregated and precipitated upon storage, the liposomes immobilized in gel beads are protected from such aggregation by the gel matrix. Stability of the avidin–biotin-immobilized liposomes was excellent with a loss of only 3–4% of the entrapped calcein and 5% of the lipid after 5 months storage at 4°C under nitrogen. It should be noted that the retardation and peak area of released calcein caused by all β-blockers show almost no difference for two chromatographic runs done using each β-blockers on the same calcein-entrapped liposome column at 25°C. On the other hand, the amount of calcein and immobilized liposomes lost was only about 0.1% within the experimental error after chromatographic runs by the drugs at 25°C with a total of 14 runs. These results suggest that the avidin–biotin-binding method in liposome chromatography is especially suitable for fluorescent dye-entrapped liposomes as a sensitive long-term probe.

3.3. ILC-fluorescent analysis of solute–membrane interactions

3.3.1. Interaction of pharmaceutical drug and membranes

Interactions of drugs with membranes have been studied by immobilized liposome and biomembrane chromatography [3,5]. The chromatographic retention volume, normalized as K_s for several β-blockers on the avidin–biotin-immobilized liposomes showed good correlation with their partitioning into free liposomes (Fig. 6A in Ref. [3]), indicating the potential of the ILC column for prediction of drug diffusion. Fig. 2 shows that an excellent correlation and similar values of K_s were obtained by performing the ILC runs on two kinds of gel bed containing

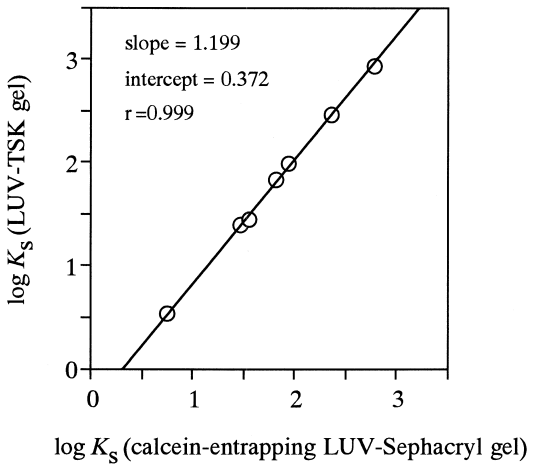

Fig. 2. Correlation of the Log K_s values for β-blockers ((±)-metoprolol, atenolol, alprenolol, oxprenolol, pindolol, acebutolol, DL-propranolol) by ILC on the calcein-entrapping LUV-Sephacryl column of 0.5-ml gel bed and the LUV-TSK column of 1-ml gel bed (data from Ref. [3]).

different amounts of liposome. This is important from the practical point of view in the use of the ILC method for quantitative analysis of solute–membrane partitioning in the laboratory, since it is hardly possible to prepare a constant amount of immobilized liposomes in gel beds of the same dimension by batchwise procedures. It also meant that the entrapped calcein molecule has no effect on drug partitioning into membranes. Nevertheless calcein leakage caused by drug partitioning can be observed. A linear relationship ($r=0.96$) between the chromatographic retardation of drugs and the calcein leakage of the liposomal membrane by the partitioning of drugs was obtained (Fig. 3). Drugs of greater hydrophobicity (higher retention volume) caused pronounced leakage of the entrapped calcein from the liposomes, indicating that the drugs penetrate into the immobilized liposomal membranes. A similar relationship was found in the interactions between lipophilic cations and liposomes by ILC [8].

3.3.2. Interactions between peptides and membranes

P18L with a short chain length (18 Lys residues) and 8 Leu residues showed significant binding and had a pronounced effect on liposome permeability (curve 1, Fig. 4a and b), but neither P18LA with Leu

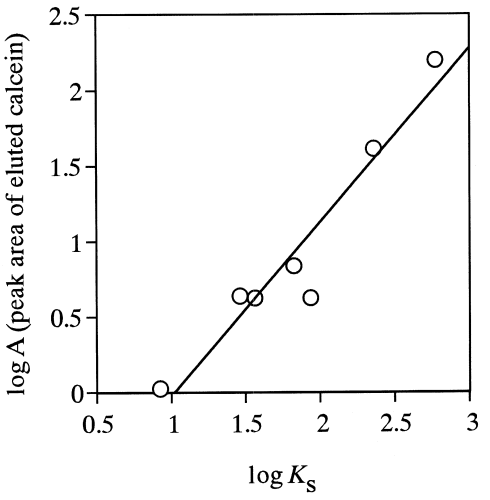

Fig. 3. Log K_s values of β-blockers obtained on the calcein-entrapping LUV-Sephacryl column versus the logarithms of the peak area of calcein released from the same liposome column (5 mm I.D.×20 mm). The amount of immobilized liposomes and entrapped calcein were 10.7 μmol of phospholipid and 2.1 μmol, respectively. The linear correlation is $r=0.95$.

residues of 5 and hydrophilic Ala residues of 3 nor P32L with long chain length (32 Lys residues), as shown in Fig. 4a and 4b (curves 2 and 3) did. It has been determined that P32L has a α-helical content of 75% [19], P18L has 50% α-helical content and P18LA shows a random structure in aqueous buffer by CD measurement (unpublished data). It seems that P18L of short chain length (compared to P32L) and more hydrophobic peptides with α-helical structure (compared to P18LA) can penetrate into the immobilized liposomal membranes and cause leakage of the membrane. Recent studies have shown that certain chain lengths (15–20 residues) and amino acid compositions (Leu and Lys) as well as amphiphilic structure of the peptides are needed for hemolytic and antimicrobial activity, which means perturbing the barrier function of membranes [24,25]. In addition, peptide Hel11-7 [26], which has a structure similar to P18L, can bind to calcein-entrapped liposomes resulting in leakage of calcein. Peptide-induced calcein leakage from immobilized liposomes coincided with the results obtained in free-form liposomes (data not shown). In addition, it should be noted that from the chromatographic retardation (curve 1, Fig. 4a), P18L was not eluted out from the liposome column, indicating that P18L probably bound strongly to the lipid bilayer. On the other hand, significant calcein leakage (curve 1, Fig. 4b) from the liposome clearly confirmed that P18L penetrated into the lipid bilayer and caused membrane leakage.

In contrast to the amphiphilic peptides, hydrophilic peptides, such as p-Lys30, caused more leakage of calcein from the immobilized liposomes than p-Lys10 (Fig. 4d) because the amino acid concentration of p-Lys30 solution was higher than that of p-Lys10. This can probably be explained by electrostatic binding of the positively charged lysine on the liposomal surface which is slightly negatively charged (2% biotin c-PE). Although the elution volume (Fig. 4c) showed no difference, which means the same retardation on the immobilized liposome column, the difference of membrane permeability was detectable on ILC-fluorescent analysis.

It seems that the penetration of the α-helical hydrophobic peptide into lipid bilayer caused pronounced membrane perturbation than the binding of positively charged hydrophilic peptide on the liposome surface. Both the hydrophobic interaction between lipid acyl chain and hydrophobic residues of the peptide and the electrostatic interactions between the polar residues of the peptide and the phospholipid head groups can be revealed by the membrane leakage on the immobilized fluorescent liposome column, although the interactions were difficult to detect by their retardations.

3.3.3. Interaction between protein or partly denatured protein and membranes

Several proteins can be denatured from a native to an unfolded state through to a molten globule state [27,28] by increasing the concentration of GuHCl. A protein in a molten globule state exposes a greater-than-native fraction of nonpolar residues to the solvent. The hydrophobicity of proteins in a molten globule state, which is likely to occur at the concentration of 2 *M* GuHCl, has been observed to be very high [27,29]. Therefore, the interactions between partially denatured proteins and neutral immobilized liposomal membranes are presumably dominated by hydrophobic binding. Fig. 5 shows the interaction of immobilized liposomes with the water

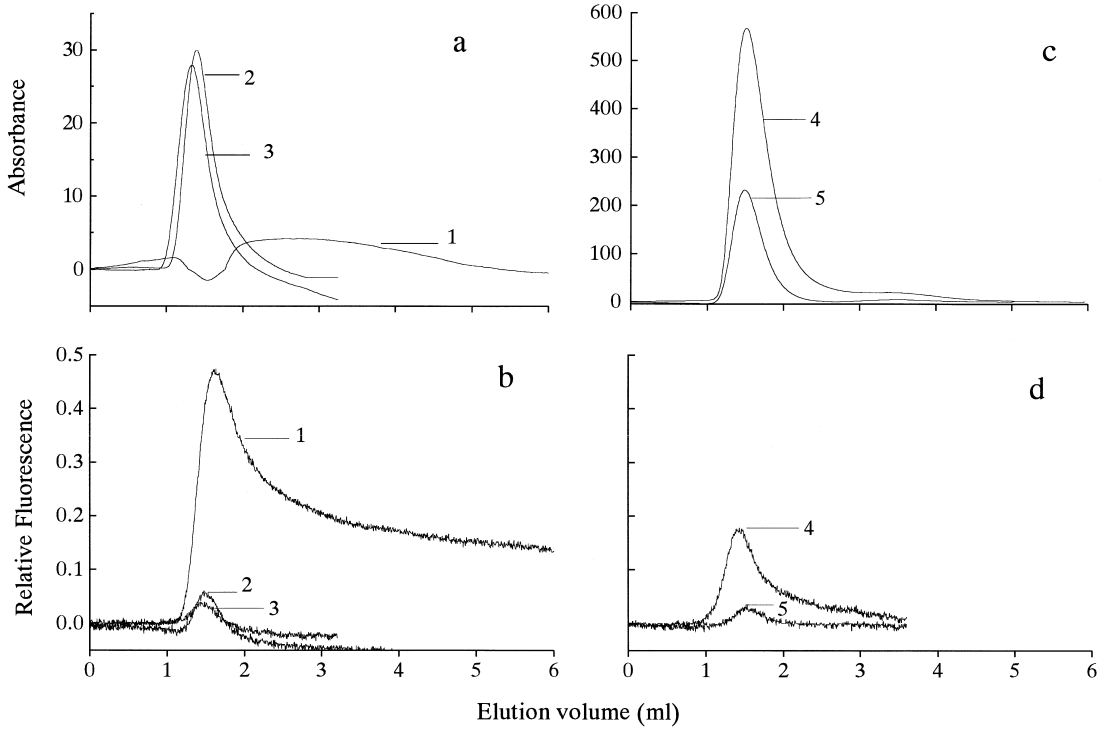

Fig. 4. Elution profiles for synthetic amphiphilic peptides (panels a and b) and polylysine (panels c and d) on the calcein-entrapping SUV-Sepharose column (5 mm I.D.×50 mm). The amounts of entrapped calcein and immobilized liposomes were 2.0 μmol and 42 μmol, respectively. Curves 1–5 correspond to the elution profiles of P18L, P18LA, P32L, p-Lys30 and p-Lys10, respectively.

soluble proteins CAB (panels a and b), which had been partly denatured by 0.5–2.5 *M* guanidinium hydrochloride (GuHCl). With an increase in GuHCl concentration from 0 to 2.5 *M*, slight retardation of denatured CAB was observed on the immobilized liposome column (Fig. 5a); meanwhile the calcein leakage of liposomal membranes was significantly increased (Fig. 5b). Thus, more denatured CAB binds more strongly to liposomes and causes more perturbation of the lipid bilayers. Protein folding has been extensively studied using size-exclusion chromatography (SEC) in terms of changes in retention time [27,30]. Generally, unmodified gel beads for SEC should be more hydrophilic in order to minimize the hydrophobic interactions between proteins and the stationary phase. Also, in ILC analysis proteins should not interact with the gel matrix but only with the liposomal membrane phase. The nonspecific binding of solutes on Sepharose gel is lower than other matrix [8]. Indistinguishable retardation of CAB at various concentrations of GuHCl on blank Sepharose gel (not shown) was obtained in contrast to the significant retardation on the liposome column, indicating clearly that the retardation on the ILC was caused by the binding of CAB to the immobilized liposomes and not to the gel matrix. The denatured CAB retards more than native CAB, indicating that CAB refolding is likely to have occurred, which agrees with the results of Yoshimoto et al. [9]. Although the retention volume of CAB at various concentrations of GuHCl is very similar (±0.07 ml) due to weak interaction, the calcein release showed a more pronounced difference (Fig. 5a and b). The peak area of the released calcein caused by denatured CAB was increased from 25, 40, 121 to 494 mv sec as the GuHCl concentration increased from 0, 0.5, 1.5 to 2.5 *M*. It should be noted that the peak area of the released calcein with only a 2.5 *M* GuHCl solution was 30 mv sec. Therefore, the membrane perturbation by CAB and retardation of CAB on ILC

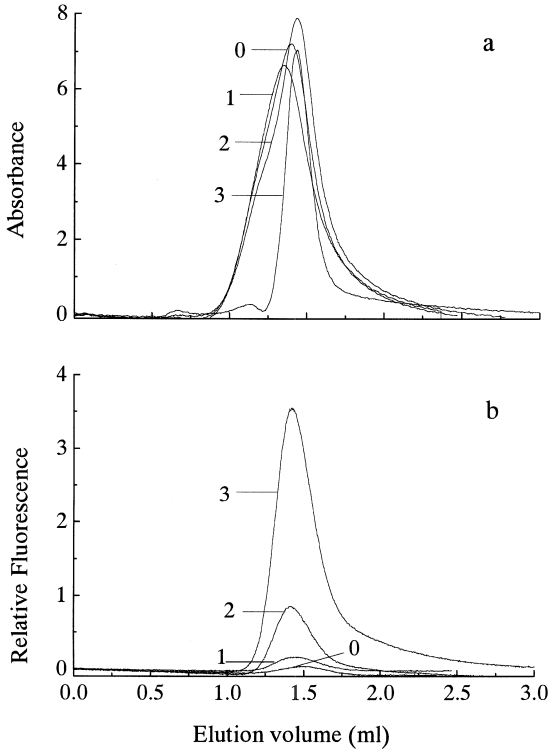

Fig. 5. Elution profiles for CAB (panels a and b) on the calcein-entrapping SUV-Sepharose column (5 mm I.D.×50 mm). The proteins were preequilibrated in the absence (curve 0) and in the presence of 0.5, 1.5, 2.5 *M* GuHCl (curves 1–3, respectively) for 2 h at room temperature before injection. The samples were eluted with buffer H (flow-rate: 0.15 ml/min). The amounts of entrapped calcein and immobilized liposomes were 1.9 and 32 μmol, respectively.

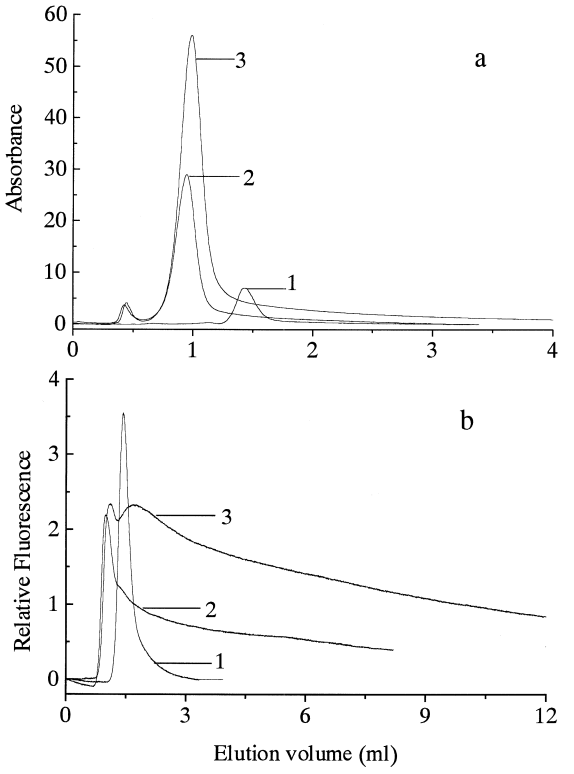

Fig. 6. The effect of protein concentration on chromatographic elution of 2.5 *M* GuHCl denatured CAB on the calcein-entrapping SUV-Sepharose column. Curve 1–3 correspond to the CAB concentration of 0.025, 0.25 and 0.5 m*M*, respectively. The column was the same as that used in Fig. 5.

are thought to be due to protein–liposome interactions during the refolding process.

The effect of protein concentration on the immobilized liposome column is shown in Fig. 6. First, from the retardation of CAB in 2.5 *M* GuHCl (Fig. 6a) on ILC, it was found that the retention volume decreased with increasing protein concentration. This can be explained by the protein aggregation at high concentrations. It has been reported that large aggregated forms of CAB were observed at a protein concentration >1 mg/ml (0.035 m*M*) in the presence of 1 *M* GuHCl [31]. Therefore, the aggregated CAB eluted out early. Secondly, from the calcein leakage of liposomes, it was found that the peak area of released calcein by 2.5 *M* GuHCl-denatured CAB (Fig. 6b) increased with the increasing CAB concentration, but the elution profile was quite different in Fig. 6b. At higher protein concentrations of 0.25 m*M* and 0.5 m*M*, the fluorescent signal can not return to the baseline, and the peak height of released calcein by denatured CAB was lower than that at a lower protein concentration of 0.025 m*M*, although the elution peak broadens significantly. By contrast, at lower protein concentration, the calcein elution peak is almost symmetrical. It implies that after interaction with liposomal membrane, the monomer protein was eluted out, and the membrane integrity was the same as the previous state. At higher concentrations of protein, self-association of the first intermediate during protein refolding produces dimers and trimers, which in turn can irreversibly form aggregates [31,32]. When the aggregated protein

binds to the lipid bilayer, the process is irreversible, resulting in continued leakage. Besides the retardation of protein, the dye-entrapped liposome column provides more information of membrane leakage by the proteins with quaternary structure (monomer and aggregates) function of protein concentration during protein refolding.

Considering of the size of molecules, it was found that the perturbation of membranes by the solutes increased, while the retardation of the solutes decreased, when the molecule size was changed from chemical molecular, supermolecular to protein molecular. For example, the order of retardation is propranolol (MW 259)>P18L (MW 2108)>partly denatured CAB (MW 28800); however, the order of calcein leakage of the membranes was partly denatured CAB>P18L>propranolol. It is likely that propranolol penetrated into the liposomes, P18L peptides and partly denatured CAB penetrated in the outer layer of liposomes or bound on the hydrophobic region of the lipid bilayers. Different mechanisms of solute–membrane interactions were revealed by ILC-fluorescent on-line analysis of both the retardation of solutes and leakage of membranes.

4. Conclusion

Using immobilized liposome chromatographic fluorescence analysis, the interaction of solutes (e.g., drugs, peptides and proteins) with membranes can be analyzed not only from their retardation on the column but also from the leakage of the liposomal membranes, which is caused by solute binding and/or penetration on/into membranes. Thus the fluorescent dye-entrapped liposomes immobilized in gel beads by avidin–biotin binding with a dual-detection system provides a useful and sensitive probe to detect weak solute–membrane interaction. Moreover, most of the entrapped calcein was retained in the immobilized liposomes, and the immobilized liposomes remain stable during the chromatographic runs and long-term storage. It was again demonstrated that the avidin–biotin immobilized liposomes have excellent stability [3,8] and membrane integrity when used as a membrane stationary phase for analysis of solute–membrane interaction.

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