Binding of lysozyme on the surface of entrapped phosphatidylserine–phosphatidylcholine vesicles and an example of high-performance lipid vesicle surface chromatography

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ABSTRACT

Phospholipid vesicles (liposomes) composed of phosphatidylserine (PS) and phosphatidylcholine (PC) in five different molar ratios were formed and entrapped in agarose gel beads (Sepharose 6B and Superose 6). For this purpose a previous dialysis–entrapment procedure was improved by use of an apparatus with continuous buffer flow and rotating dialysis cells. By rotary dialysis combined with a method of vesicles fusion with the aid of Ca^{2+} ions, the capacity of entrapped vesicles composed of 80–85% phosphatidylserine from bovine brain [denoted "PS (brain extract)"] was increased. The entrapment of PS–PC vesicles increased as the content of PS was increased. Lysozyme was applied to a PS–PC vesicle–Sepharose 6B column. The amount of bound lysozyme was about 9 nmol per μmole of lipids for entrapped PS–PC vesicles with 20 and 45 mol-% PS contents. The amounts of bound lysozyme increased to 29, 30 and 40 nmol per μmole of lipids as the fraction of PS in the vesicles was increased to 65, 80–85 and 100 mol-% PS, respectively. The lysozyme molecules thus closely packed on the surface of the vesicles with the higher PS contents. The losses of the entrapped vesicles in the first two chromatographic lysozyme-binding experiments were 5% for 80–85% PS (brain extract) vesicles, 10–12% for vesicles with 20 to 65 mol-% PS content and 23% for pure PS vesicles. PS (brain extract) vesicles were also formed and entrapped in Superose 6 gel beads (diameter 13 μm). Ribonuclease A, lysozyme and cytochrome *c* were well separated on this PS-vesicle–Superose column at an ionic strength of 0.05–0.15 *M*.

INTRODUCTION

Dialysis entrapment of lipid vesicles and protein lipid vesicles in gel beads without the use of hydrophobic ligands, *i.e.*, entrapment of vesicles (liposomes) in gel bead pores as the vesicles are formed on dialysis, has recently been developed in our

laboratory[1]. Separation of bovine albumin monomers and dimers, for instance, can be done by anion-exchange chromatography on 20% stearylamine–egg yolk phospholipid vesicles entrapped in Sepharose 6B[2]. For chromatographic purposes it is of interest to know the effects of the ligand density of charged lipids in the entrapped vesicle bilayer on the capacity of binding of protein to the vesicles and on the reproducibility of the capacity values. For the separation of bound and unbound proteins and for the release of the bound proteins from the vesicle surfaces, chromatography of the proteins on the entrapped vesicles should be simpler than the ultracentrifugation method, which has been employed for binding studies heretofore[3-6]. Entrapped vesicles might provide a convenient and rapid method for investigating the binding of proteins and peptides to lipid vesicle surfaces, especially for testing the reversibility of the binding.

By the use of pure phosphatidylserine (PS) and pure phosphatidylcholine (PC) we studied the effects of the ligand density in the entrapped vesicle surfaces on the binding of lysozyme. Lysozyme, a basic protein, has been widely used in studies of interactions of protein with negatively charged phospholipid vesicles[4,7-9]. We have also carried out the high-performance ion-exchange chromatography of lysozyme, ribonuclease A and cytochrome c on anionic vesicles entrapped in a small-bead agarose gel (Superose 6). In this work we used a dialysis apparatus with a rotating cell surrounded by slowly flowing buffer in combination with a Ca^{2+}–EDTA chelation procedure for vesicle fusion in order to increase the entrapment capacity of the vesicles composed of PS. The Ca^{2+}–EDTA chelation method, previously reported by Papahadjopoulos et al.[10], was improved by Gould-Fogerite and Mannino[11], who employed an apparatus for rotary dialysis.

EXPERIMENTAL

Materials

Sepharose 6B and Sephacryl S-1000 gels as well as prepacked Mono S and Superose 6 columns were obtained from Pharmacia-LKB Biotechnology (Uppsala, Sweden). Lysozyme (chicken egg white, L-6876), ribonuclease A (bovine pancreas, type III-A, R-5125) and cytochrome c (horse heart type III, C-2506) were purchased from Sigma (St. Louis, MO, U.S.A.). Spectrapor dialysis membrane tubing with a nominal molecular weight cut-off of 6000–8000 and a diameter of 20.4 mm was bought from Spectrum Medical Industries (Los Angeles, CA, U.S.A.). Phosphatidylcholine (PC) from egg yolk and purified phosphatidylserine (PS) from bovine brain were purchased in the form of chloroform solutions in sealed ampoules from Avanti Polar Lipids (Birmingham, AL, U.S.A.) and were stored at $-70°C$. Brain extract containing 80–85% PS and other brain lipids [here denoted "PS (brain extract)"] (bovine brain type III, B-1627) and Tris (Trizma base) were bought from Sigma, $CaCl_2$, NaCl and EDTA were obtained from Merck (Darmstadt, F.R.G.) and 2-mercaptoethanol and cholic acid from Fluka (Buchs, Switzerland).

Rotary dialysis–entrapment

An appropriate amount of PC and PS in chloroform was transferred to a 5-ml glass vial fitted with a screw-cap lined with aluminium foil. The mixture was dried to a thin film under a stream of nitrogen, and nitrogen was passed over the lipid film for

10 min in order to remove the residual traces of chloroform. The lipid film was dissolved in 1.5 ml of dialysate solution (200 mM NaCl–1 mM 2-mercaptoethanol–5 mM Tris–HCl, pH 7.1) supplemented with 100 mM cholate under nitrogen at room temperature. The sum of the concentrations of PS and PC was 40 mM. A 1.2-ml volume of the lipid solution was pumped into a Sepharose 6B column (12 mm × 10 mm I.D.) or a Superose 6 column (50 mm × 5 mm I.D.) at a flow-rate of 15 ml/h. The gel bead diameters were 45–165 μm for Sepharose 6B and 13 ± 2 μm for Superose 6. The mixture of the gel and the lipid solution was then transferred from the column into a dialysis cell (see Fig. 1 in ref. 1) by use of the excess 0.3 ml of the lipid solution. To control the rate of detergent removal on vesicle formation and provide thorough mixing of the gel beads with the lipid–cholate solution during the dialysis–entrapment process, a rotary dialysis apparatus consisting of a magnetically driven Perspex rotor inside a 300-ml dialysate container was constructed. Two dialysis cells were fixed at opposite sides of the dialysis rotor and buffer was pumped slowly through the container, as detailed below. The cells containing the mixture of the gel and lipid solution (see above) were dialyzed with rotation, first for 12 h against dialysate solution in order to form vesicles (rotor speed 15 rpm), second against dialysate solution containing 5 or 50 mM CaCl$_2$ for 8 h to induce fusion of the vesicles and third against dialysate containing 10 mM EDTA (buffer E) for 15 h at a rotor speed of 40 rpm. The rate of buffer flow through the container was 120 ml/h. After dialysis the gel beads were washed three times with buffer E by centrifugation at 150 g for 5 min. The suspensions containing non-entrapped vesicles were collected and 200 μl of the suspension were applied to a Sephacryl S-1000 column for determination of the K_d value (for details see the legend to Fig. 1). The gel beads containing entrapped anionic vesicles were repacked into columns (sizes as above) and washed further by elution with 3–5 column volumes of buffer E before equilibration with starting buffer (see below).

Determination of lysozyme binding capacities of entrapped vesicles containing five different amounts of phosphatidylserine

A 5-mg amount of lysozyme in 2.5 ml of starting buffer was applied to an anionic vesicle–Sepharose 6B column (12 mm × 10 mm I.D.). This column was pre-equilibrated with starting buffer (50 mM NaCl–0.1 mM EDTA–5 mM Tris–HCl, pH 7.1) and connected to a UV monitor (UV-2; Pharmacia-LKB Biotechnology) and a recorder for monitoring the course of saturation of the entrapped vesicles. Following sample application the column was washed with ten bed volumes of the above buffer. The bound lysozyme was then released by elution with 0.3 M NaCl containing 0.1 mM EDTA and 5 mM Tris–HCl (pH 7.1). Two chromatographic experiments were done on each column. All of the effluent fractions from the rinsing, elution and equilibration were collected in tared tubes for weight determination and the amount of released phospholipids present in each was determined by phosphorus analyses according to the method of Bartlett[12]. The initial amount of entrapped phospholipids was calculated as the sum of the amount of lipids released during the chromatographic experiments and the amount of lipids solubilized with 100 mM cholate.

The amounts of desorbed lysozyme were determined by automated amino acid analysis following hydrolysis for 24 h in 6 M HCl. Owing to the presence of trace amounts of serine from PS, the amount of protein was calculated from the known

amino acid composition of lysozyme using the analysis values for a few stable amino acids.

High-performance ion-exchange chromatography

Superose 6 (cross-linked agarose gel beads of diameter 13 ± 2 μm) with entrapped PS (brain extract) vesicles was packed into a column (40 or 50 mm $\times$ 5 mm I.D.). This column, or a 55 mm $\times$ 5 mm I.D. column of Mono S (a strong cation exchanger with a gel bead diameter of 10 μm) used as a control, was connected to two precision pumps (P-500), a mixer, a sample injection valve (V-7) and UV monitor set at 280 nm (UV-1). This system was controlled by a liquid chromatography controller (LCC-500 Plus). All of these components were provided by Pharmacia-LKB Biotechnology.

RESULTS

Entrapment of negatively charged phospholipid vesicles

The capacity for entrapment of PS (brain extract) vesicles in Sepharose 6B was 2.0 μmol lipid/ml packed gel on rotary dialysis without addition of $CaCl_2$. On addition of 5 mM Ca^{2+} to a dialysate solution (see Experimental) to induce fusion of the vesicles, the capacity increased to 4.4 μmol lipid/ml packed gel, $i.e.$, by a factor of about two. The K_d value of the corresponding non-entrapped vesicles on Sephacryl S-1000 (see legend to Fig. 1) was 0 for the fused vesicles and 0.39 for the non-fused vesicles. The vesicles thus grow considerably in the fusion process. The effect of Ca^{2+} on the entrapment capacity may be due to this enlarged size of the vesicles on fusion. Under the same conditions of fusion and entrapment for PS–PC (molar ratio 82.5:17.5) vesicles the capacity was 2.2 μmol/ml packed gel and the K_d value was 0.41. Fusion of
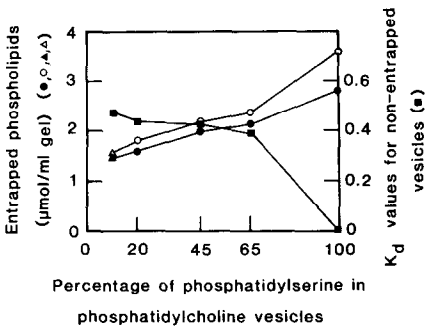

Fig. 1. Capacities for entrapment of PS–PC vesicles in Sepharose 6B gel beads. Formation of vesicles and simultaneous entrapment were done by dialysis of cholate-solubilized phospholipids in the presence of gel beads (see Experimental). ($\bullet$, $\bigcirc$) Dialysis against dialysate solution in the presence of 50 mM Ca^{2+} (see Experimental); ($\blacktriangle$, $\triangle$) dialysis against dialysate solution without addition of Ca^{2+} ions; ($\bigcirc$, $\triangle$) calculated initial amounts of entrapped phospholipids before chromatography of lysozyme (see Experimental); ($\bullet$, $\blacktriangle$) final amounts of entrapped phospholipids, determined after solubilization with 100 mM cholate following chromatographic experiments. K_d values ($\blacksquare$) were obtained by chromatography on non-entrappd vesicles on Sephacryl S-1000 [column 60 cm $\times$ 1 cm I.D., flow-rate 6 ml/h (determined by weighing) and sample volume 0.2 ml]. The K_d value is defined as $(V_e - V_0)/(V_t - V_0)$, where V_0 and V_t are the void volume and total volume of the packed column, respectively, and V_e is the elution volume of non-entrapped vesicles (see Experimental).

the PS–PC vesicles at room temperature, which is above the phase transition temperature, was very limited compared with that observed with the PS (brain extract) vesicles, where fusion was enhanced by contamination with phosphatidylethanol amine[13–15]. It has been reported that for phosphatidylserine–dipalmitoylphosphatidylcholine (1:1) vesicle fusion was completely inhibited at 30°C and at 10 mM Ca^{2+} concentration (*cf.*, Fig 4 in ref. 16). Entrapped capacities of vesicles with five different molar ratios of PS to PC at a Ca^{2+} concentration of 50 mM are illustrated in Fig. 1 (○, ●). The amount of entrapped lipids increased with increasing content of PS. This may be consistent with an increase in vesicle size to different extents, which is reflected by the decreases in the K_d values of the corresponding non-entrapped vesicles (Fig. 1, ■).

The initial amount of entrapped phospholipid (Fig. 1, △, ○; see also Experimental) decreased by 23% for the pure PS vesicles and by 10–12% for entrapped PS–PC vesicles with lower PS contents (Fig. 1, ▲, ●) during the first two chromatographic lysozyme-binding experiments (see below) done after the entrapment. These losses seem to be related to protein effects on the lipid bilayers (see Discussion).

Effect of phosphatidylserine density

A basic protein, lysozyme (pI = 11), was used as a model protein in order to investigate the effect of ligand density on protein binding to vesicle surfaces. We found that the capacity of lysozyme binding to the PS–PC vesicles increased with increasing charge density of PS from 45 to 100 mol-%. Unexpectedly, the capacity was lower at 45
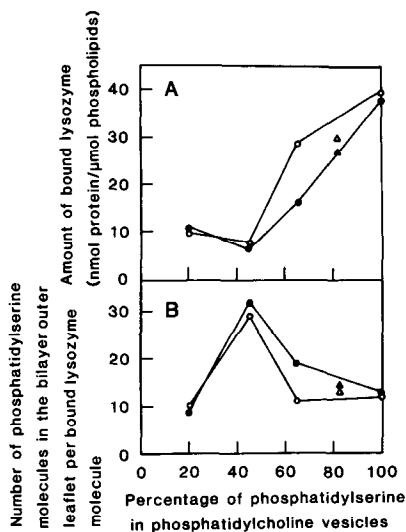

Fig. 2. (A) Binding of lysozyme to entrapped PS–PC vesicles (●, ○) and PS (brain extract) vesicles (▲, △) (see Experimental). An excess amount of lysozyme was applied to an entrapped vesicle–Sephadex 6B column for chromatographic binding experiments (see Experimental). (○, △) Amount of bound protein eluted during the first chromatographic run; (●, ▲) amount of protein eluted in the second chromatographic run. (B) Number of PS molecules in the outer lipid leaflet of the vesicles corresponding to one bound lysozyme molecule. The estimates were made by use of the data from (A) and the percentages of PS in PC vesicles. It is assumed that half of the total number of phospholipid molecules are in the outer leaflet of the bilayers. Symbols as in (A).

mol-% PS content than at 20 mol-% (Fig. 2A). Both of them showed lower protein binding capacities with respect to the capacities for the vesicles with higher PS contents (Fig. 2A). From these data we calculated the number of PS molecules in the outer phospholipid monolayer per bound lysozyme molecule (Fig. 2B). After each dialysis–entrapment the first chromatographic run showed 10–13 PS molecules per bound lysozyme molecule, except for the vesicles with 45 mol-% PS (Fig. 2B, △, ○). Not more than ten charges were therefore needed to bind each molecule of lysozyme on the vesicle surfaces on chromatography at an ionic strength about 50 mM.

The capacity for protein binding per μmole of lipids decreased by 41% from the first to the second chromatographic run on the PS–PC (65:35) vesicles (Fig. 2A). This decrease in protein capacity was accompanied by a phospholipid loss of only 7%. A possible explanation is that the released phospholipids in the eluted fractions may be enriched in PS. The charge density on the vesicle surfaces may therefore become decreased. For PS–PC (45:55) and PS–PC (20:80) vesicles the protein binding capacities were very similar in the first and second chromatographic runs (Fig. 2A). It seems that the charge density on the surface of the vesicles with lower PS contents was not changed much despite small losses of the entrapped vesicles (8% and 10%, respectively). The 80–85% PS vesicles entrapped in Sepharose 6B gel beads showed a high and nearly constant protein binding capacity (Fig. 2A) with a small loss of entrapped vesicles (about 5%) during two chromatographic experiments. For pure PS vesicles the loss of phospholipids on chromatography (Fig. 1) was higher (23%) compared with other PS–PC vesicles. This may be due to the extreme close-packing of protein molecules on the vesicle outer leaflet (see Discussion).

High-performance ion-exchange chromatography on entrapped-vesicle surfaces

PS (brain extract) vesicles were entrapped in Superose 6 gel beads. A mixture of ribonuclease A, lysozyme and cytochrome c was separated at an ionic strength below 0.15 M on the PS vesicle–Superose 6 column (Fig. 3A). A five-fold higher ionic strength was required for elution of the proteins from the commercial cation exchanger Mono S (Fig. 3C). For a control experiment the entrapped vesicles were solubilized and eluted from the Superose 6 column with 100 mM cholate. Chromatography of the protein mixture on the lipid-free Superose 6 column following equilibration of the column with starting buffer (see legend to Fig. 3) showed that most of the cytochrome c and ribonuclease A passed through the column on rinsing with starting buffer, whereas lysozyme was retarded in the column and was eluted as a very broad peak with increasing salt concentration (Fig. 3B). This may be due to both ionic and hydrophobic interactions of the protein(s) with the gel matrix. High-performance chromatography on the vesicle–Superose 6 column utilizing the entrapped vesicles was performed several times at room temperature over a period of 24 h in order to optimize the experimental conditions for separation. The total loss of lipids during the 24-h series was *ca.* 15%. Only the first elution profile was disturbed owing to the release of a small amount of entrapped vesicles from the column. To remove these unstable vesicles entrapped in Superose 6 gel beads one or two prechromatographic runs without application of proteins are recommended. Such a prechromatographic run caused a loss of entrapped vesicles of about 4% in a separate experiment. The amounts of entrapped lipids in Superose 6 gel beads were 2.5–3 μmol/ml packed gel.

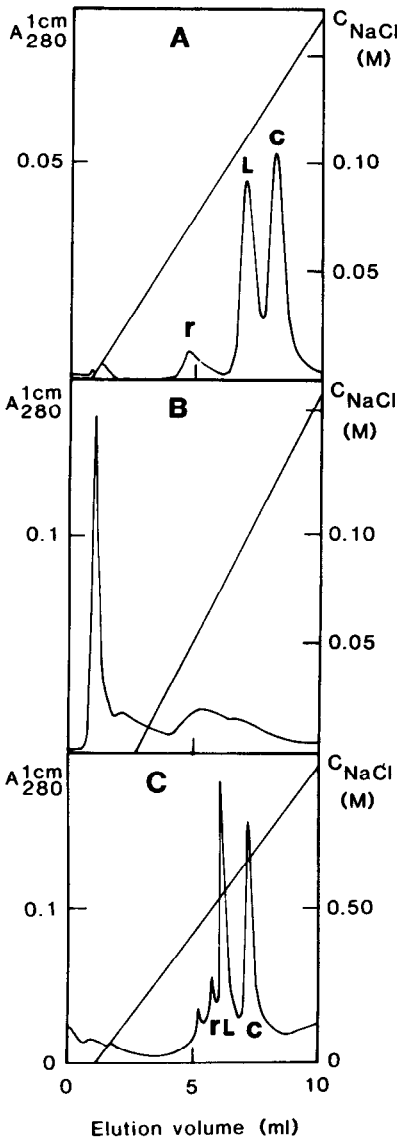We repeated the separation of the protein mixture on another PS vesicle–

Fig. 3. High-performance ion-exchange chromatography of ribonuclease A (r), lysozyme (L) and cytochrome c (C). Sample: 25 μl of the protein mixture [protein concentrations are each 0.5 mg/ml for (A) and 1 mg/ml for (B) and (C)]. (A) Chromatography on PS vesicles entrapped in Superose 6 gel beads (column 40 mm × 5 mm I.D.). Flow-rate, 0.4 ml/min, except during sample application, when it was 0.1 ml/min. Starting buffer, 25 mM NaCl–0.1 mM EDTA–5 mM Tris–HCl (pH 7.0); end buffer, 0.2 M NaCl–0.1 mM EDTA–5 mM Tris–HCl (pH 7.0). (B) Chromatography on the lipid-free Superose 6 column. The entrapped vesicles were solubilized and eluted from the column in (A) with 100 mM cholate solution. This column was then equilibrated overnight with at least 100 column volumes of starting buffer to ensure complete removal of cholate, which may bind to the gel matrix. Flow-rate, 0.2 ml/min. Starting and end buffers as in (A). (C) Comparative experiment: chromatography on a Mono S column (55 mm × 5 mm I.D.). Flow-rate, 0.4 ml/min. 0.05 M NaCl for starting buffer and 1 M NaCl for end buffer in 5mM Tris–HCl (pH 7.1) containing 0.1 mM EDTA. Note the difference in ionic strength between (A) and (C).

Superose 6 column and obtained the same result as in Fig. 3A (not shown). The same result was also obtained after storage of this latter column at 7°C for about 72 h. When the entrapped vesicles were saturated with lysozyme and the protein was released by elution, 23% of the lipids were lost from the PS vesicle–Superose 6 column, much more than the corresponding loss of the same type of vesicles entrapped in Sepharose 6B gel beads. In other words, entrapped PS (brain extract) vesicles are more stable in Sepharose 6B than in Superose 6 gel beads. The reason for this is not clear.

DISCUSSION

Lysozyme is a basic protein with a pI value of 11 and the dimensions $45 \times 30 \times 30$ Å. By use of the estimated cross-sectional areas of lysozyme ($30 \times 30 = 900$ Å^2; cf., Fig. 3 in ref. 7 and Fig. 5 in ref. 8) and of egg yolk phospholipid molecules ($ca.$ 70 Å^2, ref. 17) we can estimate from the results shown in Fig. 2A that about 30% of the outer leaflet of the bilayer became occupied by the protein for PS–PC (20:80) vesicles and about 20% for PS–PC (45:55) vesicles. These values are consistent with the value reported previously[2] for ferritin binding: the area covered by ferritin was 30% on egg yolk phospholipid vesicles containing 20% stearylamine. These estimates of the protein-covered area of the vesicle surface imply that for charge densities corresponding to 20–50% PS, the area that may be covered by proteins on vesicle surfaces is limited to $ca.$ 20–30%, presumably owing to spatial limitations, electrostatic repulsion between bound protein molecules and weak attraction between protein and the vesicle surface. The result shown in Fig. 2B for PS–PC (45:55) vesicles may therefore indicate that more than half of the PS molecules were not involved in binding of protein molecules. We propose that a 50% PS ligand density is a sort of threshold value for close packing of lysozyme onto the vesicle surfaces. Above this density the attraction between the protein and the vesicle surfaces may become the dominant force. Using the cross-sectional area of 900 Å^2 per lysozyme molecule, we obtained values of about 75%, 79% and 100% protein-covered area of the vesicle surface for 65% PS, 80–85% PS (brain extract) and 100% PS vesicles (data from Fig. 2A, $\bigcirc$, $\triangle$), respectively. These values indicate that the packing of the protein molecules on the vesicle surface containing high PS contents is extremely compact.

It is interesting that the packing of cytochrome c reported in Table I in ref. 18 corresponds to about ten lipid molecules per bound protein molecule, similar to the values we have found for lysozyme binding on PS vesicles. As about ten PS molecules were available per bound lysozyme molecule (except at 45% PS) and as the net charge of lysozyme is about $+10$ at pH 7, we can possibly regard the protein as a type of multivalent counter ion to the vesicle surface charges. As the vesicle surface is flexible and the charged lipid molecules can diffuse laterally, optimum binding can always be attained, either between a few charges on the protein (at close packing) and many charges on the vesicle surface (at high charge density), or between several charges on the protein (at optimum protein orientation) and a limited number of vesicle surface charges (at low charge density). In most instances the total net charge of all bound protein molecules at saturation seems to correspond well with the total net charge of the vesicle surfaces.

The losses of entrapped phospholipid vesicles on saturation of the vesicles with protein followed by elution with increasing ionic strength were always higher than

those observed with gradient elution alone without application of protein (see Results). The same phenomenon was also observed in previous experiments (cf., Fig.1 in ref. 2). The fact that the loss of lipid vesicles was affected not only by increasing osmotic pressure but also by the binding and release of protein may be explained as follows: binding of lysozyme may form microdomains in the membrane bilayers with different "microviscosity"[18]. The bilayer may therefore have become pertubed by the surface-bound protein molecules[18,19]. Further, hydrophobic interaction between bound lysozyme and the lipid membrane may occur to some extent[4,7,8]. The entrapped vesicles containing microdomains on their surfaces may shrink more in response to increasing salt concentration than do those lacking the microdomains. Some of the shrunken vesicles became smaller than pore size of the gel beads in which they were formed and were thus released from the vesicle–gel column.

Pidgeon and Venkataram[20] reported the separation of proteins and peptides on a high-performance liquid chromatographic column of silica beads (7 μm in diameter) covered by phosphatidylcholine. Using entrapped PS vesicles in Superose 6 gel beads (13 μm in diameter), we succeeded in demonstrating the separation of model proteins on the vesicle surface by high-performance ion-exchange chromatography. The entrapped vesicles are relatively stable toward changes in osmotic pressure and on storage and, as expected, against higher pressures over the column. For the vesicle–Superose 6 column the ionic strength required for elution of the proteins was much lower than that required for the commercial Mono S column (see Fig. 3A and C). This is due to the low charge density and the uniform charge distribution on the vesicle surfaces and indicates that vesicle surface ion-exchange chromatography is a very mild method.

CONCLUSIONS

An increased capacity for the entrapment of negatively charged vesicles in gel beads can be obtained by a vesicle fusion procedure. Lipid vesicles entrapped in gel beads can be used for chromatographic studies of lipid bilayer–protein interactions. Close packing of lysozyme on PS–PC vesicles was observed at PS contents above 45%. Further, high-performance vesicle surface chromatography can be done under very mild experimental conditions, as exemplified by the ion-exchange chromatography of basic proteins on entrapped PS vesicles.

ACKNOWLEDGEMENTS

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