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Partitioning of triphenylalkylphosphonium homologues in gel bead-immobilized liposomes: chromatographic measurement of their membrane partition coefficients

Qing Yang [a,*], Xue-Ying Liu [a], Koujirou Umetani [b], Naoki Kamo [b], Jun Miyake [a]

[a] *National Institute for Advanced Interdisciplinary Research, Agency of Industrial Science and Technology, 1-1-4 Higashi, Tsukuba, Ibaraki 305, Japan*

[b] *Laboratory of Biophysical Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan*

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Abstract

Unilamellar liposomes of small or large size, SUVs and LUVs, respectively, were stably immobilized in the highly hydrophilic Sepharose 4B or Sephacryl S-1000 gel beads as a membrane stationary phase for immobilized liposome chromatography (ILC). Lipophilic cations of triphenylmethylphosphonium and tetraphenylphosphonium (TPP^+) have been used as probes of the membrane potential of cells. Interaction of TPP^+ and triphenylalkylphosphonium homologues with the immobilized liposomal membranes was shown by their elution profiles on both zonal and frontal ILC. Retardation of the lipophilic cations on the liposome gel bed was increased as the hydrophobicity of the cations increased, indicating the partitioning of lipophilic cations into the hydrocarbon region of the membranes. The cations did not retard on the Sepharose or Sephacryl gel bed without liposomes, confirming that the cations only interact with the immobilized liposomes. Effects of the solute concentration, flow rate, and gel-matrix substance on the ILC were studied. The stationary phase volume of the liposomal membranes was calculated from the volume of a phospholipid molecule and the amount of the immobilized phospholipid, which allowed us to determine the membrane partition coefficient (K_{LM}) for the lipophilic cations distributed between the aqueous mobile and membrane stationary phases. The values of K_{LM} were generally increased with the hydrophobicity of the solutes increased, and were higher for the SUVs than for the LUVs. The ILC method described here can be applied to measure membrane partition coefficients for other lipophilic solutes (e.g., drugs).

Keywords: Lipophilic cation; Membrane partition coefficient; Immobilized liposome chromatography (ILC)

1. Introduction

Ions generally cannot penetrate through pure lipid bilayer membranes unless the charge is surrounded by hydrophobic groups to form lipophilic (hydrophobic) ions [1,2]. Partitioning of lipophilic ions into the membrane hydrocarbon region is an essential property for their membrane transport. Tetraphenylphosphonium (TPP^+) and triphenylmethylphosphonium ($TPMP^+$) are two examples of lipophilic cations, which are able to pass through the middle part of energized membranes [1–3]. Thus, they have been used as probes to measure

* Corresponding author. Tel.: +81-298 542558; Fax: +81-298 543009; E-mail: qing@nair.go.jp

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the membrane potentials of bacteria [4], vesicles [5], and liposomes [6], which are too small to be measured by use of microelectrodes. Binding of lipophilic cations to liposomal membranes has been determined using equilibrium dialysis [2], an electrode selective for lipophilic ions [7], and the EPR spin label method [2,7]. In the case of the lipophilic cations of lower hydrophobicity and consequently relatively weak interaction with the membranes, the precise and reproducible measurements of the membrane-bound and free cations were found to be experimentally difficult [7]. Furthermore, the binding parameters determined by conventional methods need to be converted to a dimensionless binding constant [2,3] for thermodynamic analysis.

Interaction of solutes (e.g., drugs and peptides) with membranes has been analyzed by immobilized liposome chromatography (ILC) as reviewed in [8]. Variations of retention volume depend on the extent of solute-membrane interaction and can be precisely measured. The specific capacity factor has been used to normalize the retention volumes measured on liposome columns of different sizes and with different amounts of phospholipid, and was found to correlate well with membrane partition coefficients determined in liposomes by sedimentation or equilibrium dialysis ([8–10], and Refs. cited therein). Determination of the partition coefficients in liposomes (usually, multilamellar liposomes) by conventional methods is generally time consuming and tedious, and it will be troublesome when weak interaction between the solute and membranes is encountered. A rapid and convenient method has been introduced by Pidgeon et al. to estimate membrane partition coefficients by chromatography using the covalently and densely bound lipids in silica gel beads, named immobilized artificial membranes (IAM) [11,12]. In contrast to the IAM method, the ILC method presented in this work provides a chromatographic measure of membrane partition coefficients in unilamellar liposomes. Furthermore, the stationary phase volume of the immobilized liposomal membranes can be calculated from the known volume of a single phospholipid molecule in membrane [13], and this combined with the retention volume allow us to use the chromatographic equation [14] (Eq. 1, below) to estimate the liposome membrane partition coefficients by ILC.

Here we report on ILC measurement of liposome membrane partition coefficients for the lipophilic cations of TPP^+ and triphenylalkylphosphonium cations formulated as $(Phe)_3\text{-}P^+\text{-}(CH_2)_n\text{-}CH_3$ ($n = 0$–6). Small or large unilamellar liposomes were immobilized by avidin-biotin binding [10] to construct the membrane stationary phase. Chromatographic retardation of the lipophilic cations on the liposome membrane phase was demonstrated by both zonal and frontal ILC runs and was used for quantitative analysis of cation-membrane interactions. The effects of liposome size, cation concentration, chromatographic flow rate, and gel matrix on the cation-membrane partitioning reported in this paper will provide a greater insight into ILC analysis of cation-membrane interaction. The present ILC method can be applied to measure the membrane partition coefficient for other solutes (e.g., drugs) interacted with the liposome membranes.

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), and TSK G6000PW was from Tosoh (Tokyo, Japan). Triphenylmethylphosphonium ($TPMP^+$), triphenylethylphosphonium ($TPEP^+$), triphenylpropylphosphonium ($TPPP^+$), triphenylbutylphosphonium ($TPBP^+$), triphenylamylphosphonium ($TPAP^+$), triphenylhexylphosphonium ($TPHP^+$), triphenylheptylphosphonium ($TPHPP^+$), tetraphenylphosphonium (TPP^+) were from Tokyo Kasei (Tokyo, Japan). Egg white avidin was purchased from Calbiochem (La Jolla, CA, USA). Egg yolk phosphatidylcholine (EPC, $>99\%$) and 1,2-dioleoylphosphatidylethanolamine-*N*-(cap biotinyl) (biotin-cPE) were from Avanti Polar Lipids (Alabaster, AL, USA). (2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) and 3,3-bis(*N*,*N*-di(carboxymethyl)aminomethyl)fluorescein (calcein) were from Dojindo Laboratories (Kumamoto, Japan). 4-Nitrophenyl chloroformate and 4-dimethylaminopyridine were from Aldrich (Milwaukee, WI, USA). Other chemicals were of analytical grade.

2.2. Synthesis of avidin adsorbent

Sephacryl S-1000 gel or TSK G6000PW gel was activated by 4-nitrophenyl chloroformate to the chloroformate density of 20–30 μmol/ml gel. Avidin was coupled to the activated gel to about 3 mg/ml gel as described in [10]. The gels were washed on a 10 μm filter (Millipore, Bedford, MA, USA) fixed in a glass funnel. Alternatively, avidin was coupled to CNBr-activated Sepharose 4B 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 mM HEPES, 150 mM NaCl, pH 7.4) supplemented with 3 mM NaN_3.

2.3. Preparation and immobilization of biotinylated liposomes

Small or large unilamellar liposomes (denoted SUVs or LUVs, respectively) supplemented with 2 mol% of biotin-cPE were prepared by probe sonication or extrusion to mean diameters of 30 ± 10 nm and 100 ± 20 nm, respectively [10]. To prepare calcein-entrapped liposomes, a 100 mM calcein solution (pH 7.5) was used instead of buffer H. The biotinylated liposomes were mixed with avidin-Sepharose 4B (for SUVs) or avidin-Sephacryl S-1000 (for LUVs) under nitrogen for 2–3 h at 23°C or overnight at 4°C for immobilization [10]. Non-immobilized liposomes or non-entrapped calcein was then removed by washing with buffer H in the 10 μm filter. Phospholipids of the immobilized liposomes in the gel beads were determined as phosphorus by the method of Bartlett [15].

2.4. Immobilized liposome chromatography (ILC)

The immobilized LUVs or SUVs were packed in a 5 mm i.d. glass column (HR 5/5, Pharmacia Biotech) to form a 0.7 or 1 ml gel bed. The liposome column was placed in a column oven (CO-8020, Tosoh) equipped with an injector, connected to a HPLC pump (CCPM-II, Tosoh) and a UV detector (UV-8010, Tosoh) set at 267 nm that was interfaced with an IBM computer. The chromatograms were analyzed by the Tosoh HPLC SYSTEM 1. Lipophilic phosphonium cations (1–2.5 mM, 10–15 μl) were applied to the liposome column and eluted with buffer H at a flow rate of 0.3 or 0.5 ml/min at 25°C. Frontal chromatographic runs were performed under the same experimental conditions, except that the lipophilic cations were applied in a low concentration (15 μM) in a large buffer volume (6–40 ml) to the liposome column using a 50 ml Superloop (Amersham Pharmacia Biotech).

Zonal runs were performed on liposome-free avidin-gel beds as controls. Retention of the solute was expressed as a capacity factor, $k = (V_r - V_0)/V_0$, where V_r is elution volume of a solute on the gel bed and V_0 is liquid volume of the gel bed obtained by chromatography of NaN_3.

3. Results and discussion

3.1. Partitioning chromatography of lipophilic cations on immobilized liposomes

Binding of lipophilic phosphonium cations to the immobilized liposomes was revealed by the chromatographic retardation on ILC in both zonal and frontal runs (Figs. 1 and 2). The retardation was increased as the alkyl chain length of the lipophilic cations was increased, indicating that the interaction of the solutes with the membranes was mostly by hydrophobic interaction with the hydrocarbon region

Table 1
Membrane partition coefficients (K_{LM})[a] of lipophilic phosphonium cations[b] obtained by the ILC method with the immobilized unilamellar liposomes

Immobilized liposomes	$TPMP^+$ ($n=0$)	$TPEP^+$ ($n=1$)	$TPPP^+$ ($n=2$)	$TPBP^+$ ($n=3$)	TPP^+	$TPAP^+$ ($n=4$)	$TPHP^+$ ($n=5$)	$TPHPP^+$ ($n=6$)
SUVs	23 ± 1	21 ± 1	22 ± 1	36 ± 4	42 ± 2	82 ± 6	222 ± 3	686 ± 70
LUVs	20 ± 1	17 ± 0	17 ± 1	25 ± 1	37 ± 4	52 ± 1	148 ± 9	477 ± 43

[a]Mean values obtained from the retention volumes of zonal and frontal runs (Figs. 1 and 2).
[b]The numbers n indicate the number of methylene groups in the cations.

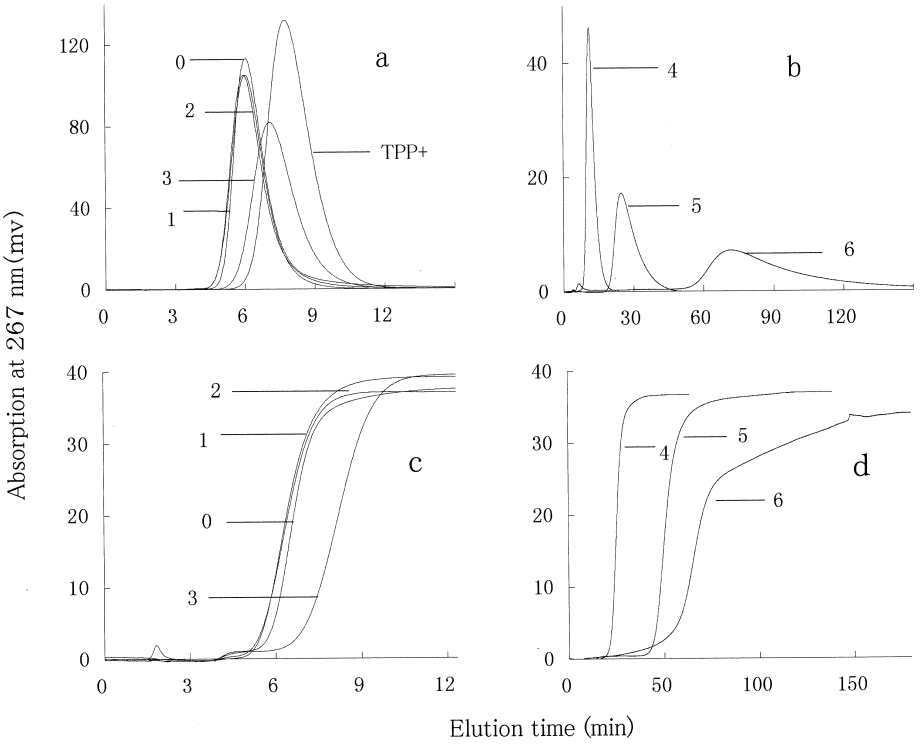

Fig. 1. ILC of zonal (a,b) and frontal (c,d) runs on a SUV-Sepharose 4B column (5 mm i.d.×50 mm). The amount of immobilized liposomes was 39 μmol of phospholipid. The flow rate was 0.3 ml/min. Curves 0–6 correspond to the elution profiles of $TPMP^+$, $TPEP^+$, $TPPP^+$, $TPBP^+$, $TPAP^+$, $TPHP^+$, and $TPHPP^+$, respectively, with number of the CH_2 groups ranging from 0 to 6.

of the membranes, since cation-membrane binding is an entropy-driven process [2,3]. Probably the lipophilic cations penetrate into a narrow region near the membrane surfaces [1,2]. Higher retardation was found on the SUVs (Fig. 1) than on the LUVs (Fig. 2) as will be discussed later. According to the extent of retardation by zonal and frontal runs on both the SUVs and LUVs, the lipophilic cations may be divided into three groups: group 1 ($TPMP^+$, $TPEP^+$, and $TPPP^+$) with weak retardation, group 2 ($TPBP^+$ and TPP^+) with intermediate retardation, and group 3 ($TPAP^+$, $TPHP^+$ and $TPHPP^+$) with strong retardation. Small differences in the retention volumes for the weakly bound cations in group 1 could be distinguished by the ILC runs (curves 0–2, Figs. 1 and 2); $TPMP^+$, which has the lowest hydrophobicity, gave a slightly higher retardation volume than other two cations in group 1. We measured limiting molar conductivity and limiting partial molar volume of these lipophilic phosphonium cations; the limiting molar conductivity of the cations decreased as their hydrophobicity (the number of CH_2 group) increased except for $TPMP^+$, which showed significantly lower conductivity than $TPEP^+$ and $TPPP^+$ in group 1 (unpublished data). The relatively large retention volume of $TPMP^+$ might be related to its physical properties but exact reasons are not known.

In the frontal runs (Figs. 1 and 2) lipophilic cations at a low concentration and in a large volume were applied to obtain an elution profile with a plateau, where the concentration of the cation is equal to the initial concentration in the sample. In contrast, the zonal run samples were applied in a narrow zone at a relatively high initial concentration. The frontal and zonal modes gave identical elution volumes within the experimental errors (Figs. 1 and 2), which shows that both ILC modes can be used for studies of solute-membrane partitioning. Zonal experiments have advantages over the frontal ones in their convenience and shorter elution time. By use of the frontal run, a constant sample concentration at equilibrium is obtained that can be used to study the effect of concentration on solute-membrane binding.

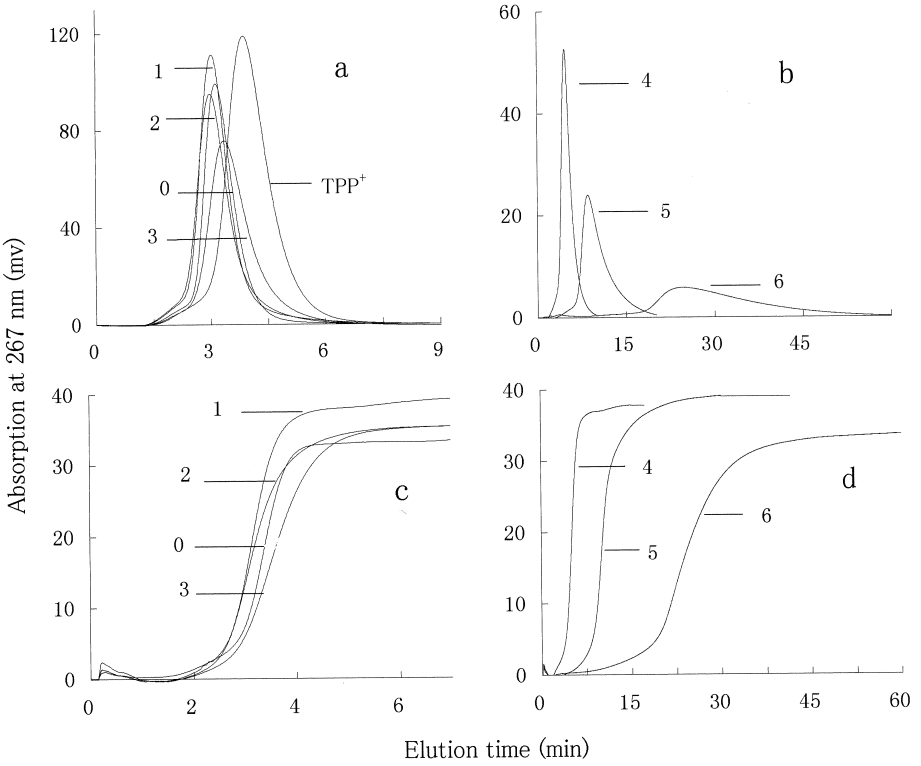

Fig. 2. ILC of zonal (a,b) and frontal (c,d) ILC runs on a LUV-Sephacryl S-1000 column (5 mm i.d.×50 mm). The amount of immobilized liposomes was 33 μmol of phospholipid. Flow rate was 0.5 ml/min. Curves 0–6 correspond to the elution profiles of the cations as in Fig. 1.

Perturbation of the liposomal membranes by the binding of the lipophilic cations was observed from the leakage of calcein trapped in the immobilized liposomes (Fig. 3). The perturbation became more pronounced when the hydrophobicity of the solutes (i.e., the chromatographic retardation) was increased. The linear relationship between the solute hydrophobicity and the calcein leakage indicates that the lipophilic cations penetrate into the immobilized liposomal membranes.

3.2. *Factors affecting chromatographic elution of lipophilic cations*

When chromatography of lipophilic cations was performed on an avidin-Sepharose 4B or Sephacryl S-1000 gel bed in the absence of liposomes, no retardation of the cations was observed. The mean capacity factors for the control runs were 0.11 ± 0.04 ($n = 8$) and 0.25 ± 0.05 ($n = 8$), respectively, which means that the non-specific binding of the lipophilic cations to these gel matrices was negligible. In other words, the retardation of the cations on the liposome gel beds (Figs. 1 and 2) does only come from their interaction with the liposomal membranes. However, lipophilic cations bound strongly to the TSK 6000 gel matrix. The capacity factor for the cations retarded on a TSK gel bed ranged from 2.5 to 35, increasing as the number of CH_2 groups of each cation was increased from 0 to 6. This clearly shows that the non-specific binding was due to hydrophobic adsorption to the TSK gel matrix. As a result, the partitioning of lipophilic cations into the immobilized liposome membranes was overestimated (data not shown). Consideration should be taken in the use of gel matrices as support materials for ILC in relation to the compounds to be studied. Ideally, in ILC analysis lipophilic solutes should not interact with the gel matrix but only with the liposomal membranes. Sepharose gel with a very hydrophilic network composed of polysaccharide agarose [16] makes a good matrix as a liposome carrier, although it cannot be used for high flow ILC runs. TSK 6000 gel beads, which are rigid, can be run at a flow rate of

up to 2 ml/min but in the ILC study the non-specific binding of lipophilic cations to the gel matrix has to be subtracted from the total retention volume. The order of the hydrophilicities of the gel matrices tested by chromatography of lipophilic cations was Sepharose 4B > Sephacryl S-1000 > Superdex 200 > TSK G6000PW.

The concentration of lipophilic phosphonium cations used for frontal runs was typically 15 μM, which was only 0.05% of the liposomal lipid concentration in the gel bed. The elution volumes of the cations did not change (variation less than 0.01 ml) in the concentration range from 0.5 to 30 μM, as examined by the frontal runs. This is consistent with the concentration-independent binding of several β-blockers studied by ILC in the wide concentration range of 0.002–40 μg/ml [9]. However, when the concentration was increased to 50 μM, the cation elution volumes decreased slightly. This may be explained by the saturation binding property of the phosphonium cations in the region near the membrane surface [1,2]. The saturation binding of solutes may be studied by ILC, similarly as solute-membrane protein affinity binding studied by quantitative affinity chromatography on immobilized proteoliposomes [17,18].

The effect of the flow rate on the ILC elution volumes of lipophilic cations was examined by ILC of three lipophilic cations of weak, intermediate, and strong hydrophobicity belonging to group 1, 2 and 3, respectively. Within the range of experimental error no differences in elution volumes were found (Fig. 4A), consistent with the results by Beigi et al. [9]. The cation peaks eluted on the liposome column broadened significantly in response to an increase in flow rate, while only a slight increase in the peak width of the cation on a liposome-free gel bed was observed (Fig. 4B). These results can be explained by the non-equilibrium theory of chromatography ([19] and Refs. cited therein). The widening of elution peaks is increased by the degree of non-equi-

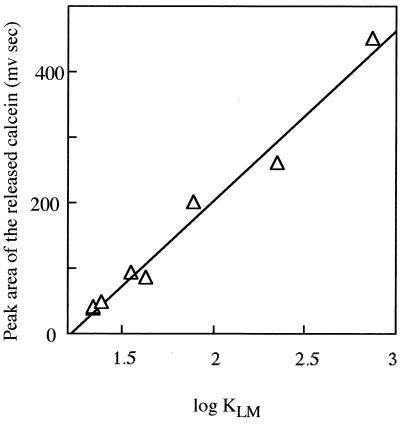

Fig. 3. The peak area of calcein released from the calcein-entrapping SUV-Sepharose column (5 mm i.d.×35 mm) was versus the membrane partition coefficients (log K_{LM}) (see Section 3.3). The amount of immobilized liposomes was 27 μmol of phospholipid. The column was stored at 4°C for 1 year. Zonal ILC runs were performed under the same experimental conditions as described in Section 2, except that the released calcein was measured on-line by a fluorescence detector (FS-8020, Tosoh) at excitation and emission wavelengths of 492 nm and 517 nm, respectively.

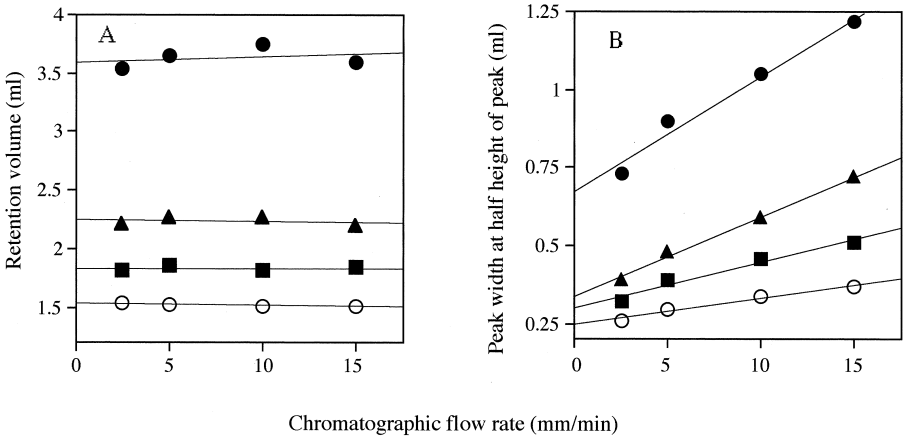

Fig. 4. The effect of flow rate on chromatographic elutions of lipophilic cations of $TPEP^+$ (squares), $TPBP^+$ (triangles), and $TPAP^+$ (circles) on a SUV-Sepharose gel bed (solid symbols) and on a 1 ml avidin-Sepharose gel bed with $TPAP^+$ (open symbols) for a control.

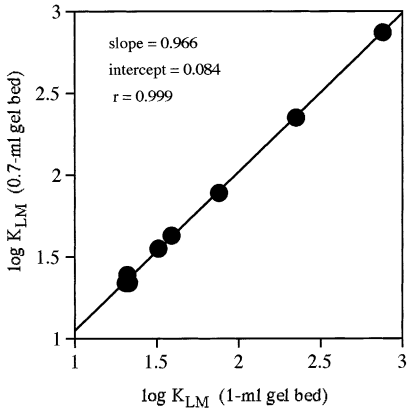

Fig. 5. Correlation of the K_{LM} values obtained by ILC on the SUV-Sepharose column in Fig. 1 (bed volume 1 ml) and the calcein-entrapping SUV-Sepharose column in Fig. 3 (bed volume 0.7 ml).

librium, or, equivalently, the rate of mass transfer of solutes between the chromatographic phases. If the exchange of solute between the mobile and stationary phases is large, or if the zone migrates slowly, then degree of zone spreading will decrease. However, at the center of the migration zone, which was used for the measurement, equilibrium of solutes partitioning between the mobile phase and the stationary phase is attained [19]. In the case of ILC, the rate of mass transfer is determined by the rate of lipophilic solute binding to and release from the membrane stationary phase inside the gel matrix, and the rate of the solute diffusing in and out of the gel beads. As a result, broader peaks were observed for the cations eluted from the liposome column than from the liposome-free column (Fig. 4B). As also shown in the figure, the peak width at a given flow rate was increased as the hydrophobicity of the cations was increased in relation to the increase in their membrane-binding rate. This intrinsic relationship between the binding rate and the peak width may be used to calculate the rate constant, as has been described for the quantitative zonal affinity chromatography [20,21].

The retardation of the solutes by ILC will be governed by variation in the amount of the immobilized liposomes. However, this does not affect calculation of membrane partition coefficients by ILC, since the immobilized lipid amount related to the retention volume is taken for the calculation as described below, as in previous work on ILC of drugs [9,10].

3.3. Membrane partition coefficients estimated by ILC

For column partitioning chromatography a solute distributed between an aqueous mobile phase and a gel stationary phase can be expressed as distribution coefficient [14],

$$K = (V_e - V_0)/V_s \quad (1)$$

where V_e is the elution volume of the solute, and V_0 and V_s refer to the mobile and stationary phase volumes, respectively. Thus, in ILC, the partition coefficient (K_{LM}) for a solute partitioning between the aqueous phase and the liposome membrane stationary phase may be obtained by

$$K_{LM} = (V_r - V_0)/V_{LM} \quad (2)$$

where V_r is the retention volume of the solute and V_{LM} is the volume of the liposomal membranes immobilized. Since the volume per phospholipid molecule packed in the liposome membrane has been well documented in the literature [2,13], V_{LM} can be calculated from the EPC volume, 1253 Å^3 in anhydrous membranes [13] and the immobilized liposome amount (A, mmole phospholipid), provided that the unilamellar liposomal membranes are accessible for the binding of the solutes. Thus, Eq. 2 can be written as

$$K_{LM} = (V_r - V_0)/0.755A \quad (3)$$

Using Eq. 3, K_{LM} can be calculated from the retention volume of the lipophilic cations measured by ILC in both zonal and frontal modes (Figs. 1 and 2). The K_{LM} values for the lipophilic cation-membrane partitioning are summarized in Table 1. The partition coefficients derived from the retention volumes varied by the differences in hydrophobicity of the cations and the size of the liposomes. The K_{LM} values were higher in the SUVs than in the LUVs (Table 1). Since the lipophilic cations bound mostly to the outer membrane surfaces of the liposomes as further discussed below, this result may be attributed to the larger surface area of the outer membranes of SUVs compared to LUVs owing to the asymmetric bilayer lipid packing of SUVs of small radius. The lower membrane surface density of SUVs caused by their extreme membrane curvature may also favor the solute partitioning (cf. [22]).

It has been reported that the translocation rate of

lipophilic cations was as low as that determined for TPP^+, 1–10 Å/s, or 10^{-2}–10^{-3}/s [2], due to the existence of high potential barriers in the bilayer interior and repulsive energy derived from the membrane dipole potential (positive inside) [2,23]. Probably almost all the cations applied to the liposome column could interact only with the outer membrane surfaces of the liposomes. Consequently, the ILC elution profiles obtained very likely reflect the process of the cation association with and dissociation from the outer leaflets of the membranes. A very small percentage of the population of the cations applied might have time to pass through the membrane and then transport out, but will be largely diluted by elution at the linear flow rate of 2.5×10^{-2} cm/s to a too low concentration to be experimentally detected. Only a single peak was observed in the elution pattern of each lipophilic cation (Figs. 1 and 2) and the retention time was independent of the flow rate (Fig. 4). The partition coefficient, K_{LM}, obtained by the ILC method, may correspond to the thermodynamically defined binding constant (K), a ratio of membrane-bound and free cation concentrations [2]. For partitioning of TPP^+ in SUVs, the K value was 100 [2] compared to the K_{LM} of 42 (Table 1). Part of the membrane thickness (0.4 nm) was used to calculate the K value compared to the entire membrane volume used in the ILC method, which may account for the discrepancy between the two values, in addition to the differences in the methods and experimental conditions (e.g., salt concentration). When only the outer lipid monolayer of the bilayer was taken as the membrane stationary phase volume for the ILC calculation, the K_{LM} value became similar to the K value.

The membrane partition coefficient calculated by Eq. 3 was independent of the dimensions of the gel beds and amounts of immobilized liposomes, as was the specific capacity factor (K_s) used previously to normalize the ILC results [9,10]. Fig. 5 shows a very good correlation and similar values of K_{LM} were obtained by performing the ILC runs on two gel beds of different sizes containing different liposome amounts. 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.

3.4. Concluding remarks

The present paper shows that the ILC method is easy, sensitive, and reproducible to estimate the membrane partition coefficient (K_{LM}) of lipophilic phosphonium cations in liposomes. This chromatographic method is applicable to a variety of solutes (e.g., drugs, peptides and proteins), which can penetrate into lipid bilayer membranes to some extent. Both weak and relatively strong interactions between solutes and lipid membranes can be determined by ILC. The adsorbent concentration of 30–40 μmol liposomal phospholipid/ml gel bed is sufficient for the ILC analysis (Figs. 1 and 2). Furthermore, the ILC can be performed at various temperatures, which allows us to analyze thermodynamic binding of the lipophilic cations to liposome membranes by linear van't Hoff plots (unpublished results). It should also be noted that one of the liposome columns with entrapped calcein had been stored at 4°C for 1 year with little effect on the ILC measurement of solute-membrane partitioning (Fig. 5). The amounts of immobilized liposomes before and after the storage were the same. Moreover, the entrapped calcein was mostly retained in the immobilized SUVs and could be used for the chromatographic detection of its leakage. It was again proved that the avidin-biotin immobilized liposomes have excellent stability and membrane integrity [10,24] upon use as a membrane phase for analysis of solute-membrane interaction.

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References

[1] B. Ketterer, B. Neumcke, P. Läuger, J. Membr. Biol. 5 (1971) 225–245.
[2] R.F. Flewelling, W.L. Hubbell, Biophys. J. 49 (1986) 531–540.
[3] A. Ono, S. Miyauchi, M. Demura, T. Asakura, N. Kamo, Biochemistry 33 (1994) 4312–4318.
[4] P.D. Hockings, P.J. Rogers, Biochim. Biophys. Acta 1282 (1996) 101–106.
[5] C.G. Davis, S. Hestrin, H. Landahl, A.S. Gordon, I. Diamond, J.I. Korenbrot, Nature 302 (1983) 525–528.
[6] T. Shinbo, M. Sugiura, N. Kamo, Y. Kobatake, Nihon Kagaku Zashi 6 (1983) 917–923.
[7] M. Demura, N. Kamo, Y. Kobatake, Biochim. Biophys. Acta 903 (1987) 303–308.
[8] P. Lundahl, F. Beigi, Adv. Drug Deliv. Rev. 23 (1997) 221–227.
[9] F. Beigi, I. Gottschalk, C. Lagerquist Hägglund, L. Haneskog, E. Brekkan, Y. Zhang, T. Österberg, P. Lundahl, Int. J. Pharm. 164 (1998) 129–137.
[10] Q. Yang, X.-Y. Liu, S.-i. Ajiki, M. Hara, P. Lundahl, J. Miyake, J. Chromatogr. B 707 (1998) 131–141.
[11] S. Ong, H. Liu, X. Qiu, G. Bhat, C. Pidgeon, Anal. Chem. 67 (1995) 755–762.
[12] S. Ong, C. Pidgeon, Anal. Chem. 67 (1995) 2119–2128.
[13] C. Huang, J.T. Mason, Proc. Natl. Acad. Sci. USA 75 (1978) 308–310.
[14] D.H. Freeman, Anal. Chem. 44 (1972) 117–120.
[15] G.R. Bartlett, J. Biol. Chem. 234 (1959) 466–468.
[16] P.-O. Larsson, in: T. Kline (Ed.), Handbook of Affinity Chromatography, Marcel Dekker, New York, 1993, pp. 61–75.
[17] Q. Yang, P. Lundahl, Biochemistry 34 (1995) 7289–7294.
[18] E. Brekkan, A. Lundqvist, P. Lundahl, Biochemistry 35 (1996) 12141–12145.
[19] J.C. Giddings, Dynamics of Chromatography. Part I. Principle and Theory, Marcel Dekker, New York, 1965, Ch. 3, pp. 95–118.
[20] C. DeLisi, H.W. Hethcote, in: I.M. Chaiken (Ed.), Analytical Affinity Chromatography, CRC Press, Boca Raton, FL, 1987, Ch. 1, pp. 1–63.
[21] R.R. Walters, in: I.M. Chaiken (Ed.), Analytical Affinity Chromatography, CRC Press, Boca Raton, FL, 1987, Ch. 3, pp. 117–156.
[22] L.R. De Young, K.A. Dill, Biochemistry 27 (1988) 5281–5289.
[23] R.F. Flewelling, W.L. Hubbell, Biophys. J. 49 (1986) 541–552.
[24] S. Chiruvolu, S. Walker, J. Israelachvili, F.-J. Schmitt, D. Leckband, J.A. Zasadzinski, Science 264 (1994) 1753–1756.