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Journal of Pharmaceutical and Biomedical Analysis
30 (2003) 1869–1877

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Role of phospholipids in drug–LDL bindings as studied by high-performance frontal analysis/capillary electrophoresis

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Received 26 April 2002; received in revised form 3 August 2002; accepted 8 August 2002

Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

The binding study between basic drugs ((*S*)-verapamil (VER) and (*S*)-propranolol (PRO)) and phospholipid liposomes was performed by using high-performance frontal analysis/capillary electrophoresis (HPFA/CE) in order to investigate the effect of oxidative modification of low-density lipoprotein (LDL) upon drug-binding affinity from molecule-based viewpoint. 1-Palmitoyl-2-oleoyl-phosphatidylcholine (POPC, 16:0, 18:1), 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC, 16:0, 18:2), dilauloyl-phosphatidylcholine (DLaPC, 12:0, 12:0), 1-palmitoyl-2-oleoyl-phosphatidyl-glycerol (POPG, 16:0, 18:1), and 1-palmitoyl-sn-glycero-3-phosphocholine (monoPPC, 16:0) were used to prepare the model liposomes. At physiological pH (pH 7.4), the model liposome prepared from POPG+POPC had negative net charges, while the total net charge of the other model liposomes (POPC liposome, PLPC liposome, DLaPC liposome, and monoPPC+POPC liposome) was zero. The drug and the model liposome mixed solutions were subjected to HPFA/CE, and the total binding affinities (nK) were calculated. The nK values of VER and PRO to POPG+POPC liposome were more than six and 10 times higher than those of other liposomes, respectively. On the other hand, the nK values of the model drugs to POPC liposome, PLPC liposome, DLaPC liposome and monoPPC+POPC liposome showed small differences less than twice. These results indicate that the electrostatic interaction plays an important effect on drug–liposome binding, and suggest that the increase in the negative charge of LDL phospholipids gives more significant effect on the drug-binding affinity of the basic drugs than the acyl-chain structure.

Keywords: Low-density lipoprotein; Liposome; Capillary electrophoresis; Verapamil; Propranolol

Abbreviations: LDL, low-density lipoprotein; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; PLPC, 1-palmitoyl-2-linoleoyl-phosphatidylcholine; DLaPC, dilauloyl-phosphatidylcholine; monoPPC, 1-palmitoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-phosphatidyl-glycerol; VER, (*S*)-verapamil; PRO, (*S*)-propranolol; SUV, small unilamellar vesicle.

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0731-7085/02/$ - see front matter
PII: S0731-7085(02)00530-7

1. Introduction

It has been well known that drug–plasma protein binding affects pharmacological effect and pharmacokinetics [1–3]. Low-density lipoprotein (LDL) is the most abundant subclass of human plasma lipoproteins, and concerned to the plasma protein binding of hydrophobic and/or basic drugs [4,5]. Under disease state such as hyperlipidemia and atherosclerosis, LDL suffers from oxidative modification by arterial smooth muscle cells [6], macrophages [7], and endothelial cells [6] as well as the increase in its plasma concentration, resulting in the change in drug-binding property [8–10]. The oxidized LDL (oxLDL) is taken up by macrophages via the scavenger receptor, while normal LDL is taken up via LDL receptor. The macrophages significantly accumulate lipids into themselves in unregulated manner, resulting in the formation of foam cells [11]. In this way, the behavior of oxLDL differs from that of normal LDL, which may give significant effect on pharmacokinetics and pharmacological action. Therefore, the drug-binding study of oxLDL is indispensable to the safe and rational use of drugs.

In general, hydrophobic drugs are bound to LDL with high affinity, and the conventional methods such as ultrafiltration and equilibrium dialysis are very often difficult to be applied to the binding analysis of hydrophobic drugs, because of technical difficulty such as undesirable drug adsorption onto the membrane. For this reason, ultracentrifugation method has been used in drug–lipoprotein-binding analysis. However, this technique has difficulty in keeping the binding equilibrium constant during separation process. On the other hand, immobilized liposome chromatography (ILC) has been used so as to estimate interaction between drug and lipid bilayer [12]. For example, Yang and Lundahl's [13] group expanded this technique to proteoliposome and estimated binding affinity between glucose transporter Glt1 and its substrate/inhibitor. Besides, liposome capillary electrophoresis (LCE) is new developing method proposed by Hjerten and coworkers [14], which affords effective separation of several pharmaceuticals. By using LCE, they successfully estimate the differences in free energy of interaction between drugs or peptides and liposomes. These methodologies are excellent techniques in prediction of interaction between drug and living cells of which membrane is composed by lipid bilayer. However, several drawbacks are still remaining in these methods. For example, ILC needs laborious preparation of solid phase appropriate for each analysis, and in LCE, the electrophoretic migration of the charged liposome causes complicated theoretical equation, because the estimation of interaction depends on migration time of the analytes. On the other hand, high-performance frontal analysis/capillary electrophoresis (HPFA/CE) enables determination of unbound drug concentration in drug–protein mixed solution [15,16]. This technique consumes very small sample volume (<100 nl), does not suffer from drug adsorption onto the membrane, and requires no special pre-treatment of the analytical system. These advantages are convenient for binding analysis of hydrophobic drugs.

Drug–LDL binding is a reversible and kinetically rapid process, where the concentrations of unbound drug, unbound LDL and drug–LDL complex easily reach equilibrium state. In addition, drugs are bound to LDL in non-specific and partition-like manner [8–10], and enantioselectivity is not observed [8–10]. These binding characters strongly suggest that drugs are not 'dissolved' into the core lipids (such as cholesterol, cholesteryl ester and triglycerides) of LDL, but are 'adsorbed' onto the surface phospholipids of LDL. Our previous studies using HPFA/CE revealed that the drug-binding affinity of LDL was enhanced by LDL oxidation [8–10]. LDL oxidation involves several kinds of surface lipid modifications such as increase in conjugated diene [17], decrease in acyl-chain length [18], formation of Schiff base [19], hydroperoxides [20] and lysophosphatidylcholine [21], and increase in negative electric charge [16]. However, the contribution of each lipid modification to the enhancement of the drug-binding affinity has not been investigated. In this paper, the binding study using model liposomes prepared from various phospholipids was carried out in order to investigate the contribution of the surface (lyso)phospholipids in drug–LDL

binding. 1-Palmitoyl-2-oleoyl-phosphatidylcholine (POPC, 16:0, 18:1), 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC, 16:0, 18:2), dilauloyl-phosphatidylcholine (DLaPC, 12:0, 12:0), 1-palmitoyl-sn-glycero-3-phosphocholine (monoPPC, 16:0) or 1-palmitoyl-2-oleoyl-phosphatidyl-glycerol (POPG, 16:0, 18:1) were used to prepare the model liposome, and subjected to binding analyses with (*S*)-verapamil (VER) and (*S*)-propranolol (PRO), both of which are antiarrhythmic drugs (Fig. 1). Because these drugs did not show enantioselective binding to LDL, the (*S*)-isomers, which are the pharmacologically active form, were used in this study.

2. Material and methods

2.1. Material

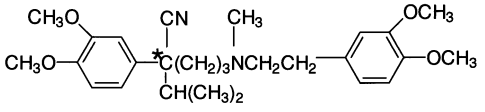
POPC, PLPC, DLaPC, POPG (Fig. 2) and PRO were obtained from Sigma (St. Louis, MO). MonoPPC was purchased from Bachem (Bubendorf, Switzerland). VER was from Research Biochemicals Int. (Natick, MA). Other chemicals of the highest grade were from local suppliers.

2.2. Preparation of liposome solution

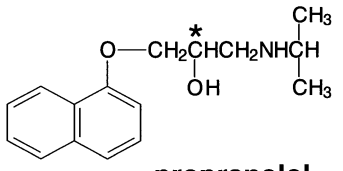
Model liposome was prepared by ultrasonication method according to Sunamoto et al. [22]. About 50 mg of phospholipid was dissolved in 4 ml of chloroform in a round-bottom flask, and chloroform was evaporated using a rotary evaporator to form a thin layer of phospholipids in the inner surface of the flask. Then, the trace residue of chloroform was evaporated under vacuum overnight. Four milliliters of sodium phosphate buffer (pH 7.4, ionic strength 0.17) was added to the dry lipid layer, and the flask was strongly shaken for 20 min to make multilamellar vesicle. Then, the multilamellar vesicle solution was subjected to ultrasonication under nitrogen for 10–30 min at 25 °C to make small unilamellar vesicle (SUV). The SUV solution was filtrated twice to five times through Millex-VV membrane filter (Millipore, pore size, 100 nm) to give a diameter of ca. 100 nm except for monoPPC+POPC liposome, as measured by dynamic light scattering (DLS) method (LPA-3000/3100, Otsuka Electronics, Osaka, Japan). The concentration of phosphatidylcholine monomer was determined by choline oxidase method, and the liposome solution was diluted to final monomer concentration of 455 μM. Then, the particle concentration of the SUV solution was calculated by the following equation:

$$\text{Particle concentration} = \frac{C}{4\pi(d/2)^2 \times 2/S},$$

where C, d, S represent monomer concentration (455 μM), diameter of liposome, shared area of one phospholipid monomer (0.6 nm^2 [23]), respectively. Because concentration of POPG could not be determined by choline oxidase method, POPC was mixed at the molar ratio of POPG/POPC = 4/1. In the case of monoPPC+POPC liposome, POPC was mixed at the molar ratio of monoPPC/POPC = 1/9, because monoPPC does not form liposome without phospholipids. There was no difference in UV adsorption intensity at 234 nm, and in fluorescent emission intensity at 430 nm (excitation 360 nm), which means that oxidation of liposome did not occur.



verapamil



propranolol

Fig. 1. Chemical structures of VER and PRO.

2.3. Determination of the ratio between monoPPC and POPC

MonoPPC micelle as a side product of monoPPC+POPC liposome was removed by two-step

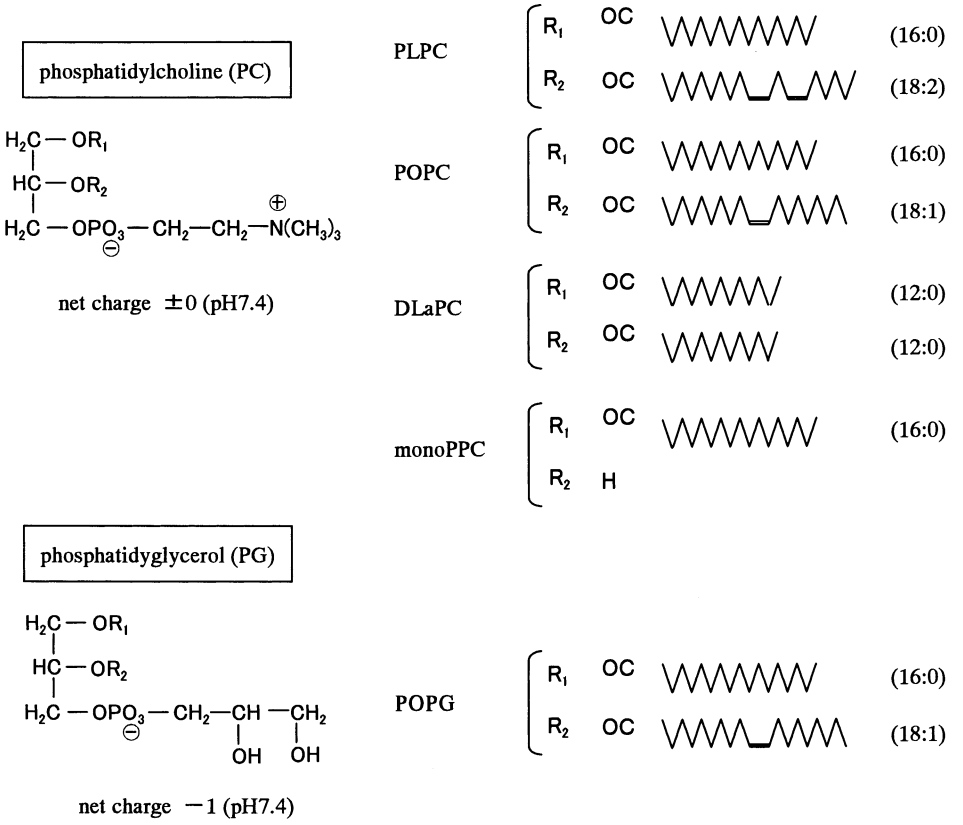


Fig. 2. Chemical structures of model phospholipids. POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; PLPC, 1-palmitoyl-2-linoleoyl-phosphatidylcholine; DLaPC, dilauloyl-phosphatidylcholine; monoPPC, 1-palmitoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol.

ultracentrifugation [24]. Just after the ultrasonication, the monoPPC and POPC mixed solution was centrifuged at 4 °C, 33 000 rpm for 10 min, and then the supernatant was centrifuged again at 4 °C, 42 000 rpm for 195 min to give monoPPC precipitant. The monoPPC and POPC concentrations in the supernatant liposome solution were determined by the following method. POPC concentration was determined by HPLC [25], of which conditions were as followed: mobile phase, MeOH:CH_3CN:H_2O = 475:475:50; flow rate, 1.2 ml/min; detection, UV 205 nm. Total PC (monoPPC + POPC) concentration was determined by choline oxidase method. Finally, monoPPC concentration was obtained by subtraction of POPC concentration from total PC concentration. From this, the ratio in the mixed liposome solution was estimated as monoPPC:POPC = 15.8:84.2. The mean diameter of the mixed liposome was measured by DLS method to give 56 nm.

2.4. Determination of unbound drug concentration in drug liposome mixed solution by HPFA/CE

The unbound concentration of VER or PRO was determined by HPFA/CE as reported elsewhere [8–10]. Briefly, VER or PRO (70–100 μM) and liposome (455 μM as monomer concentration) mixed solutions were dissolved in 66.7 mM sodium phosphate buffer (pH 7.4, ionic strength 0.17), and the solution was kept at room temperature for 1 h to reach equilibrium state. CAPI-3000 (Otsuka electronics, Osaka, Japan) capillary electrophoresis system was equipped with bare fused silica

capillary (total length 42 cm, effective length 30 cm, inner diameter 75 μm, GL science, Tokyo). The sodium phosphate buffer (pH 7.4, ionic strength 0.17) was used as background electrolyte. A 200 μl portion of sample solution was put in a cuvette, and was subjected to the hydrodynamic sample introduction for 3 s. A positive voltage (+7 kV) was applied to perform electrophoresis. Because both VER and PRO are basic drugs, whereas model liposomes are neutral (in the case of PC liposomes) or acidic (in the case of PG liposome), the drugs migrate faster than liposomes while keeping the binding equilibrium. The electropherograms were obtained by the measurement of UV adsorption intensity at 205 nm for VER and 215 nm for PRO. The drugs were detected as the zonal peak with plateau region, and the unbound drug concentration was measured from the plateau height. Before each measurement, the capillary was rinsed for 5 min with 30 mM sodium dodecyl sulphate dissolved in the background electrolyte, and 2 min with 0.1 M sodium hydroxide solution. A series of the standard drug solutions (10–100 μM of VER and 10–70 μM of PRO in sodium phosphate buffer, pH 7.4, ionic strength 0.17) were used to prepare calibration lines, and good linearity was obtained ($R^2 > 0.993$).

3. Results and discussion

HPFA/CE is applicable to binding analyses when the binding equilibrium is rapidly established. In order to foresee that the drug–liposome binding is kinetically rapid enough for HPFA/CE analysis, a model sample (50 μM VER and DLaPC liposome mixed solution) was analyzed at two different applied voltages. If the binding equilibrium is established rapidly, the plateau height of trapezoidal drug peak keeps constant even when the drug–liposome separation time is varied. Otherwise, the plateau height does not reflect the equilibrium concentration. The model sample solution was subjected to HPFA/CE analysis at +7 and +3 kV. Although, the lower applied voltage causes more than twofold longer separation time, no significant difference was observed in plateau heights. This result verifies that the HPFA/CE is applicable to analysis of drug–liposome binding. Our previous studies indicated that drug–LDL bindings were also kinetically rapid enough to be applied to HPFA/CE analysis [8–10].

In our preliminary study, it was found that when the liposome concentration in the drug–liposome mixed solution was the same as the plasma LDL concentration in healthy state (2 μM), the unbound drug concentration was too low to be detected. Therefore, all the following binding analyses were carried out at the liposome concentration of 4.3 nM (monomer concentration, 455 μM). A typical electropherogram of VER and PLPC liposome mixed solution subjected to HPFA/CE was shown in Fig. 3. VER was separated from the liposome and detected as trapezoidal peak with plateau region. This plateau height corresponds to the unbound drug concentration in the sample solution.

3.1. The unsaturated binding between drug–liposome

It was previously reported that drugs were bound to LDL in non-specific and partition-like manner [8–10]. Before use of liposome as a model of LDL, it was necessary to confirm that drug–liposome binding also showed the partition-like

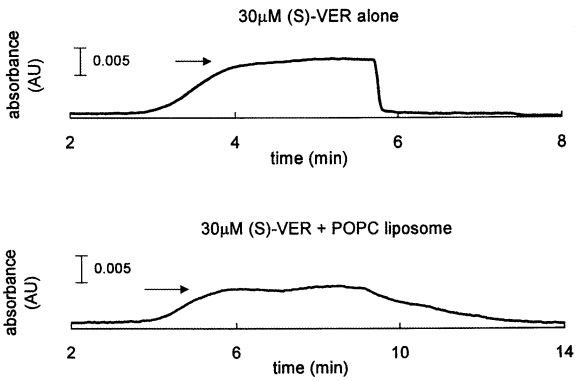


Fig. 3. Electropherograms of 30 μM VER solution (upper) and 30 μM VER+POPC liposome solution (lower). Liposome concentration was 4.35 nM, which correspond to 455 μM of the phospholipids monomer. Capillary: 42 cm (effective length, 30 cm), 75 μm i.d.; buffer: sodium phosphate buffer (pH 7.4, ionic strength 0.17); applied voltage: +7 kV; temperature: 25 °C; detection: 205 nm.

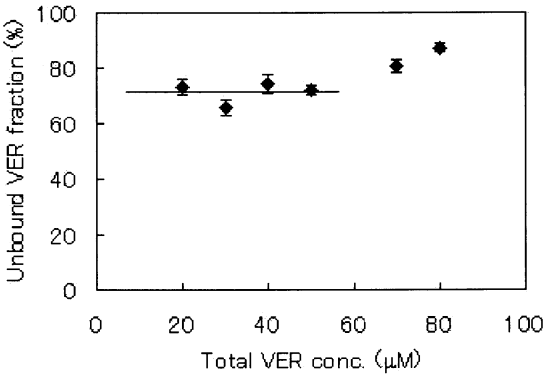


Fig. 4. Unbound VER fraction in DLaPC liposome solution. The concentration of DLaPC liposome was 4.36 nM. Measured by HPFA/CE as described in the text.

binding character. Fig. 4 indicates the unbound VER fraction in VER and DLaPC liposome mixed solutions of which total drug concentration was varied from 20 to 80 μM. The unbound fraction was constant when the total drug concentration was in the range 20–50 μM, though the unbound fraction was increased when the total drug concentration was more than 70 μM. This result means that the binding between VER and POPC liposome was non-specific and unsaturated under 50 μM of total drug concentration, whereas the saturation was observed in the higher total drug concentration. Therefore in the following binding study, the total VER concentration was fixed at 50 μM for calculation of total binding affinity (nK). Similarly, the relationship between unbound drug fraction and total drug (VER and PRO) concentration was also investigated in the other liposome solutions (data not shown). From these results, in the following study, the total drug concentration was fixed at 30 μM for PLPC, POPC or monoPPC+POPC liposome, and 50 μM for DLaPC or POPG+POPC liposome.

3.2. The effect of liposome diameter on the drug binding

Table 1 lists the particle concentrations of the model liposomes and their mean diameters measured by DLS. Although the diameter of LDL is about 20–30 nm, the liposome diameters listed were much larger than that of LDL. As measured by DLS, two major peaks in the distribution of diameter were observed in each liposome solution: one was around 20–30 nm and the other was over 200 nm, to give "mean" diameter of ca. 100 or 56 nm as listed in Table 1. However, the abundance of the smaller liposome (20–30 nm) was extremely larger than that of the larger liposome (>200 nm) because of the filtration through Millex-VV membrane (pore size 100 nm). Therefore, it could be considered that the liposome was appropriate as a model of LDL.

It was difficult to prepare monoPPC+POPC liposome of 100 nm diameter, and the diameter of monoPPC+POPC liposome (56 nm) was smaller than those of the other liposomes (100 nm). Therefore, the effect of liposome diameter on the drug bindability was investigated. Table 2 lists the unbound VER fraction in the POPG+POPC liposome solution of which diameters were varied from 56 to 100 nm. POPG+POPC was easiest to control liposome diameter. There was no significant difference in the unbound VER fraction. This result indicates that the variation in liposome diameter within this range does not give any significant effect on the drug-binding affinity.

3.3. Comparison of drug-binding affinity between the model liposomes

Because the binding between model drugs and liposomes were unsaturated and non-specific as mentioned above, the total binding affinity (nK) was calculated by the following equation:

$$\mathrm{nK} = \frac{C_\mathrm{t} - C_\mathrm{u}}{L_\mathrm{t} C_\mathrm{u}},$$

where C_t, C_u and L_t represent total drug concentration, unbound drug concentration and total liposome (as particle) concentration, respectively. Because the diameter of monoPPC+POPC liposome was smaller than those of the others, the liposome (as particle) concentration was calculated to be higher value. However, as mentioned above, the particle diameter does not give any significant effect on drug binding. Therefore, the total binding affinity of monoPPC was calculated on the

Table 1
Particle concentrations and diameters of the model liposome

Phospholipids	Monomer concentration (μM)	Diameter (nm)	L_t (nM)
PLPC	455	101	4.26
POPC	455	100	4.35
DLaPC	455	100	4.36
monoPPC+POPC	455	56	14.1
POPG+POPC	455	101	4.29

assumption that the unbound drug concentration was constant between 56 and 100 nm of monoPPC+POPC liposome diameters. The effect of the number of carbon=carbon double bond on total binding affinity becomes clear by comparison of total binding affinity between PLPC and POPC. In the same way, the effect of carbon chain length comes clear by comparison between PLPC and DLaPC. The effect of electrical charge on polar head group is known from comparison between POPC and POPG+POPC, and the effect of lysophosphatidylcholine is known from comparison between POPC and monoPPC+POPC liposomes. Table 3 lists the total binding affinity (nK) of VER and PRO to these model liposomes. As mentioned above, the liposome concentration was lower than physiological plasma LDL level. In the case of PRO, the nK value in POPG+POPC liposome was about 13-fold higher than the others, while there was no remarkable difference in PLPC, POPC, DLaPC and monoPPC+POPC liposome solutions. This result suggests that the negative charge on the surface phospholipids gives significant effect on binding of PRO to liposome, which is in accordance with a previous report [26]. Therefore, the formation of hydroperoxides or Schiff base, which gives negative charge to oxLDL, would be a main factor of increase in drug-binding affinity. On the other hand, the change in the number of double bonds and in carbon chain length, and formation of lysophosphatidylcholine do not give dominant effect on increase in binding affinity of PRO to oxLDL. This suggests a possibility that PRO is bound to the polar head groups of phospholipids on LDL surface, and that hydrophobic interior of lipid bilayer, i.e. acyl-chain moiety, does not contribute to the binding so much as the ionic interaction.

In the case of VER, the nK value in POPG+POPC liposome was sixfold or much higher than the other liposomes. The electrophoretic mobility of the POPG+POPC liposome was estimated as -4.74×10^{-4} cm^2/(V s), while those of the other liposomes were almost zero because of their net charge (± 0 at pH 7.4). On the other hand, those of 2 h copper (II) oxLDL were -2.63×10^{-4} cm^2/(V s) [13], which was 55.5% of POPG+POPC liposome. Assuming that the enhancement in VER bindability was solely due to the electrostatic interaction, the total binding affinity of POPG+POPC liposome of which electrophoretic mobility is equivalent to that of 2 h oxLDL ($nK^*_{POPG+POPC}$) could be predicted from the total binding affinity of POPG+POPC liposome ($nK_{POPG+POPC}$) and of POPC liposome (nK_{POPC}):

$$nK^*_{POPG+POPC} = (nK_{POPG+POPC} - nK_{POPC}) \times 0.555 + nK_{POPC}$$

to give 6.46×10^8 M^{-1}, which is significantly

Table 2
Unbound VER fraction in the POPG+POPC liposome solution

Diameter (nm)	Monomer concentration (μM)	Total drug concentration (μM)	Unbound drug fraction (%)
56	455	50	18.3±0.3
100	455	50	18.5±0.8

Table 3
Total binding affinity of VER and PRO to liposome

Phospholipids	VER			PRO		
	L_t (nM)	C_t (μM)	nK ($\times 10^7$ M^{-1})	L_t (nM)	C_t (μM)	nK ($\times 10^7$ M^{-1})
PLPC	4.26	30	8.45±0.43	4.46	30	15.3±0.47
POPC	4.35	30	16.8±0.72	4.15	30	13.0±0.97
DLaPC	4.36	50	8.80±0.88	4.17	30	12.3±0.97
monoPPC+POPC	4.35[a]	30	10.4±0.05	4.35[a]	30	15.4±0.30
POPG+POPC	4.29	50	103±5.42	4.29	30	178±2.27

[a] Calculated values on the assumption that the liposome diameter was 100 nm.

higher than the nK values of the other liposomes. Therefore, it follows that the negative charge of phospholipid gives significant effect on binding of VER to LDL, as observed in the case of PRO. In addition, nK value in POPC liposome solution was about twofold higher than those in PLPC and DLaPC liposome solutions. Besides, nK value in POPC was decreased by the addition of monoPPC (which was less hydrophobic than POPC due to defect of an acyl chain). These observations were not found in the case of PRO. Although the molecular mechanism of these phenomena cannot be explained in detail only from these results, the smaller nK value of monoPPC+POPC liposomes than POPC liposome implied the contribution of hydrophobic interaction to the total binding affinity between VER and phospholipids. These results indicate that not only polar headgroup but also acyl-chain moiety would affect the increase in the binding affinity of VER by oxidation of LDL, and that the contribution of hydrophobic interaction to drug-binding affinity varies between (basic) drugs.

4. Concluding remarks

The role of various phospholipids in the drug binding to model liposome was investigated by means of HPFA/CE. The drug–liposome binding showed unsaturable and non-specific character as in the case of drug–LDL binding. The total binding affinity of VER and PRO was significantly higher in POPG+POPC liposome than in PLPC, POPC, DLaPC and monoPPC+POPC liposomes. This result suggests that the electrostatic interaction is the dominant contribution to the enhancement in drug-binding affinity along with oxidation of LDL. In addition, in the case of VER, the total binding affinity of POPC was significantly higher than those of PLPC, DLaPC and monoPPC+POPC liposomes, while the nK values of PRO to these liposomes were almost the same. This suggests that the contribution of hydrophobic interaction to drug-binding affinity varies among (basic) drugs. HPFA/CE is favorable for drug–LDL or liposome binding because of the following two reasons. First, this technique requires very small sample volume. Because it is difficult to obtain LDL or liposome and to preserve them for a long term, the small consumption is convenient for sample preparation. Second, this technique is free from disturbance of drug adsorption on membrane, which is accompanied with the conventional methods such as equilibrium dialysis and ultrafiltration.

Acknowledgements

The authors thank Prof. Tetsuro Handa of Graduate School of Pharmaceutical Sciences, Kyoto University, for his kind advice. This work was supported by Grant-in-Aid for the Scientific Research (No. 11672139) from the Ministry of Education, Science and Culture, Japan.

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