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Characteristics of the binding of asialofetuin-labeled liposomes to isolated rat hepatocytes

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Summary

The binding of asialofetuin(AF)-labeled liposomes (AF-liposomes) to isolated rat hepatocytes was examined at 4°C. AF-liposomes consisting of phosphatidylcholine, phosphatidic acid (PA) and cholesterol (molar ratio 6 : 1 : 3) rapidly bound to hepatocytes. A notable contribution of AF was demonstrated by the fact that the binding was inhibited by AF and dissociated by EDTA. An increase in the PA content of AF-liposomes enhanced the extent of their binding, whereas the extent of AF-mediated binding remained the same irrespective of the PA content. There were two classes of binding sites at which AF-liposomes bound to hepatocytes: a high-affinity site with an association constant of 6.45×10^9 M^{-1} and a low-affinity site with an association constant of 4.50×10^7 M^{-1}, this value being compatible with that of control liposomes. Phosphatidylserine- or phosphatidylglycerol-containing liposomes bound to hepatocytes as did liposomes containing PA. The binding of stearylamine-containing liposomes or liposomes without charged lipid, however, was quite low. Thus, it is evident that AF-liposomes bind to hepatocytes through the high-affinity interaction between AF and asialoglycoprotein receptors and the low-affinity interaction that occurs as a result of liposomal negative charge.

Introduction

The use of liposomes as a carrier of drugs is a subject about which extensive research has been made (Ostro, 1987). For instance, they have been used for the site-specific delivery of drugs, and their modification using antibodies, glycolipids and glycoproteins has been conducted successfully (Gregoriadis and Senior, 1984; Das et al., 1985; Connor and Huang, 1986).

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Fenestrations of endothelial lining of liver capillary (Wisse et al., 1982) are advantageous for targeting particulate carriers, and parenchymal cells (hepatocytes) have asialoglycoprotein receptors capable of recognizing a sugar with a galactose configuration (Van Lenten and Ashwell, 1972). Asialofetuin (AF) is a glycoprotein molecule possessing several triantennary galactose terminated sugar chains (Spiro, 1973). Our previous study indicated a preferential uptake of AF-labeled liposomes (AF-liposomes) by hepatocytes (Hara et al., 1987, 1988). Since internalization follows binding, an initial interaction is an indispensible step for the cellular uptake of AF-lipo-

somes. The present study was conducted to characterize the binding between AF-liposomes and isolated rat hepatocytes.

Materials and Methods

Materials

Fetuin (type III), L-α-phosphatidic acid (PA) from egg yolk, L-α-phosphatidyl-L-serine (PS) from bovine brain, L-α-phosphatidyl-DL-glycerol (PG), dicetyl phosphate (DCP) and stearylamine (SA) were purchased from Sigma. Asialofetuin (AF) was prepared by splitting the terminal *N*-acetylneuraminic acid (NANA) of fetuin by the method of Spiro (1960), so that more than 90% of the NANA was removed (Aramaki et al., 1985). Cholesterol (Chol), egg yolk phosphatidylcholine (PC) and collagenase were from Wako Pure Chemicals. Bovine serum albumin (BSA, fraction V), 5,6-carboxyfluorescein (6-CF) and [^{3}H]inulin were obtained from Boehringer, Eastman Kodak and American Radiolabeled Chemicals, respectively. All other reagents were of reagent grade.

Preparation of liposomes

Unilamellar AF-liposomes consisting of PC, PA and Chol at molar ratios of 7 : 0 : 3, 6 : 1 : 3 and 4 : 3 : 3 were prepared by the detergent dialysis method using 42 μmol of total lipids and 5 mg of palmitoyl-AF (Tsuchiya et al., 1986). Control liposomes (N-liposomes) consisting of the same lipids or containing PS, PG, DCP or SA, instead of PA, were also prepared. As an aqueous marker, 6-CF was encapsulated in these liposomes except for SA-containing liposomes. In the aqueous space of SA-containing liposomes, [^{3}H]inulin was encapsulated. They were successively extruded through polycarbonate membranes (Nuclepore Corp., 0.4, 0.2 and 0.1 μm). The markers not encapsulated and palmitoyl-AF not incorporated into the liposomal bilayer were separated from liposomes by a Sepharose CL-4B column (2.5 × 40 cm) equilibrated with 10 mM Tris-HCl buffered saline (THBS, pH 7.4). Usually, about 4% of the markers and 30% of palmitoyl-AF were eluted in the liposomal fractions. The amount of AF attached to the outer surface of liposomes remained virtually the same (140–165 μg/μmol phospholipid (PL)) irrespective of the lipid composition, and was calculated to be about 500 AF molecules per vesicle according to the method of Enoch and Strittmatter (1979). The average of liposomal mean diameter was 0.130 μm (range 0.122–0.143 μm) irrespective of AF-labeling and the lipid composition.

Isolation of hepatocytes

Hepatocytes were isolated from male Wistar rats (200–250 g) by the in situ collagenase perfusion method (Tanaka et al., 1978). Isolated cells were suspended in Krebs-Henseleit buffer (KHBB, pH 7.4) containing BSA (2%) and were routinely 60–70% single and 90–95% viable.

Binding assay

Liposome suspensions (0.4 ml) at various concentrations were added to the freshly isolated hepatocyte suspension (2×10^7 cells, 4.6 ml) in a vial (2.5 × 4.5 cm) followed by incubation at 4°C for a specified period. In some experiments, AF (6 mg) and/or N-liposomes without a marker (empty N-liposomes, 7.44 μmol PL) were added to the hepatocyte suspension 30 min before incubation. The mixture was gently swirled every 10 min. Triplicate samples (1 ml, 4×10^6 cells) were withdrawn and diluted with 5 ml of ice-cold KHBB or Ca^{2+}-free KHBB containing 5 mM EDTA. After centrifugation (100 × *g*, 2 min), the cell pellets were washed twice with 5 ml of ice-cold KHBB. The liposomes associated with the cell surface were solubilized by 1% Triton X-100 (3 ml) and the fluorescence intensity of 6-CF or the radioactivity of [^{3}H]inulin was measured. Binding was calculated from the markers using the ratio of 6-CF/PL (μg/μmol) or [^{3}H]inulin/PL (dpm/μmol) of liposomes, and expressed as pmol PL/10^6 cells.

Physical analysis

Liposomal diameter and zeta (ζ)-potential were measured by a submicron particle sizer (Nicomp Model 370, Pacific Scientific) and a microelectrophoresis apparatus (Laser Zee Model 501, PEN KEM Inc.), respectively.

Analytical method

6-CF was measured by a fluorescence spectrophotometer (MFP-4, Hitachi) at excitation and emission wavelengths of 494 and 515 nm, respectively. The radioactivity of [^{3}H]inulin was measured by a liquid scintillation counter (Aloka 903, Aloka). The concentration of PL was determined from inorganic phosphorus (Chen et al., 1956) following perchloric acid ashing. The amount of AF on the outer surface of AF-liposomes was determined by a hemoagglutination inhibition assay (Tsuchiya et al., 1986). Isolated hepatocyte viability was estimated by a trypan-blue exclusion test using a hemocytometer (Erma).

Results

Binding of AF-liposomes to hepatocytes

The binding of AF- and N-liposomes consisting of PC : PA : Chol (6 : 1 : 3) to hepatocytes was examined as a function of time. As shown in Fig. 1, both types of liposomes rapidly bound to hepatocytes, reaching a constant level within 30 min. The binding of AF-liposomes, however, was about 2 times greater than that of N-liposomes throughout this experiment. In the presence of free AF (6 mg), 50-fold excess as against the amount of AF on the surface of AF-liposomes, AF-liposome binding was inhibited by about 50%, and the values were the same level as those of N-liposomes. AF, however, failed to have any effect on the binding of N-liposomes.

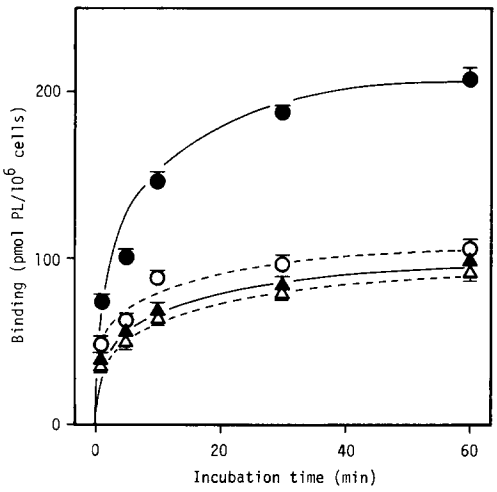

Fig. 1. Binding of AF- and N-(control) liposomes to hepatocytes. Freshly isolated hepatocytes (2×10^7 cells) were incubated with liposomes consisting of PC : PA : Chol (6 : 1 : 3) in 5 ml of KHBB (pH 7.4) containing BSA (2%) at 4°C for 30 min (circles; AF-liposomes [0.78–1.09 μmol PL, 8.54–8.78 μg 6-CF]: triangles; N-liposomes [0.84–0.92 μmol PL, 7.38–8.21 μg 6-CF]). The cells were preincubated without (closed symbols) or with 6 mg of AF (open symbols) at 4°C for 30 min. Triplicate samples (1 ml, 4×10^6 cells) were withdrawn, and binding of liposomes was determined as described in Materials and Methods. Each point represents the mean from three individual experiments ± S.D.

Inhibition and dissociation of liposomal binding

The effects of various treatments on the binding of liposomes to hepatocytes were examined (Fig. 2). Addition of free AF or empty N-liposomes inhibited the binding of AF-liposomes (187 pmol PL/10^6 cells) by about 50%. The binding of N-liposomes (84 pmol PL/10^6 cells) was not affected by AF, though about 70% inhibition was observed with empty N-liposomes. Addition of AF and empty N-liposomes together caused quite extensive inhibition (81%) of the AF-liposome binding. While the cells were being washed with Ca^{2+}-free KHBB containing EDTA, the binding of AF-liposomes was reduced to about 50%, whereas that of N-liposomes showed virtually no change.

Effect of PA content

The effect of PA content on the binding of liposomes to hepatocytes was examined (Fig. 3). The binding was found to increase with PA content in the bilayer. Following addition of free AF, the binding of AF-liposomes decreased by an almost constant value (86-114 pmol PL/10^6 cells) irrespective of the PA content. The residual binding was always the same as that of N-liposomes, whose binding capacity was not affected by addition of AF. In the case of PA-free liposomes, the binding of AF-liposomes was almost completely inhibited by free AF, and that of N-liposomes was extremely slight.

Estimation of binding parameters

The binding of AF- or N-liposomes to hepatocytes increased with their concentrations, and the

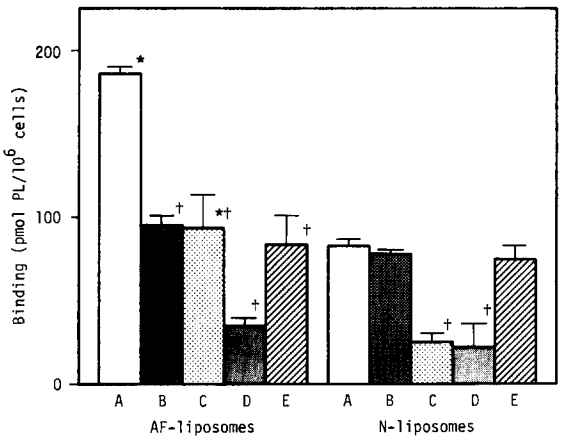

Fig. 2. Effects of AF, empty N-liposomes and EDTA on the binding of AF- and N-(control) liposomes. Hepatocytes (2×10^7 cells) were incubated with AF-liposomes (0.78–1.09 μmol PL, 8.54–8.78 μg 6-CF) or N-liposomes (0.84–0.92 μmol PL, 7.38–8.21 μg 6-CF) consisting of PC:PA:Chol (6:1:3) in 5 ml of KHBB (pH 7.4) containing BSA (2%) at 4°C for 30 min (A). Free AF (6 mg) were added (B), empty N-liposomes (7.44 μmol PL, without 6-CF) were added (C) and both free AF and empty N-liposomes were added (D). Triplicate samples were withdrawn and diluted with 5 ml of ice-cold KHBB (A–D) or Ca^{2+}-free KHBB containing 5 mM EDTA (E). Following centrifugation, the cells were washed twice with 5 ml of ice-cold KHBB. Binding of liposomes was determined as described in Materials and Methods. Each column presents the mean from three individual experiments ± S.D. Statistically significant differences: from N-liposomes, $P < 0.001$ (*); from treatment A, $P < 0.001$ (†).

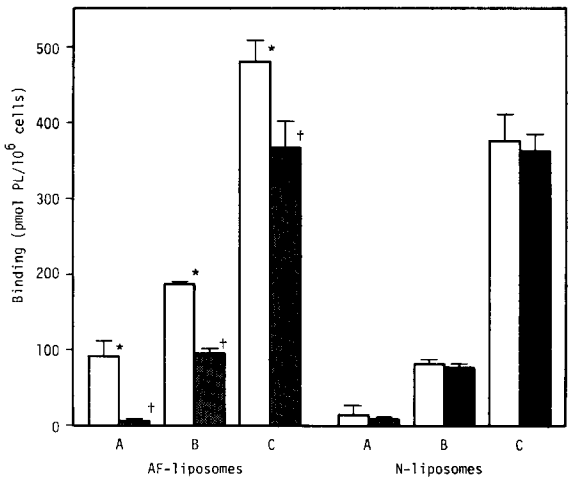

Fig. 3. Effect of PA content on the binding of AF- and N-(control) liposomes. Hepatocytes (2×10^7 cells) were incubated with AF-liposomes (0.77–1.09 μmol PL, 7.39–8.78 μg 6-CF) or N-liposomes (0.70–0.92 μmol PL, 7.27–8.21 μg 6-CF) consisting of PC:PA:Chol (A) 7:0:3; (B) 6:1:3; (C) 4:3:3 in 5 ml of KHBB (pH 7.4) containing BSA (2%) at 4°C for 30 min without AF (open column) and with 6 mg of AF (dotted column). Binding of liposomes was determined as described in Materials and Methods. Each column indicates the mean from three individual experiments ± S.D. Statistically significant differences: between AF-liposomes and N-liposomes, $P < 0.001$ (*); between without AF and with AF, $P < 0.001$ (†).

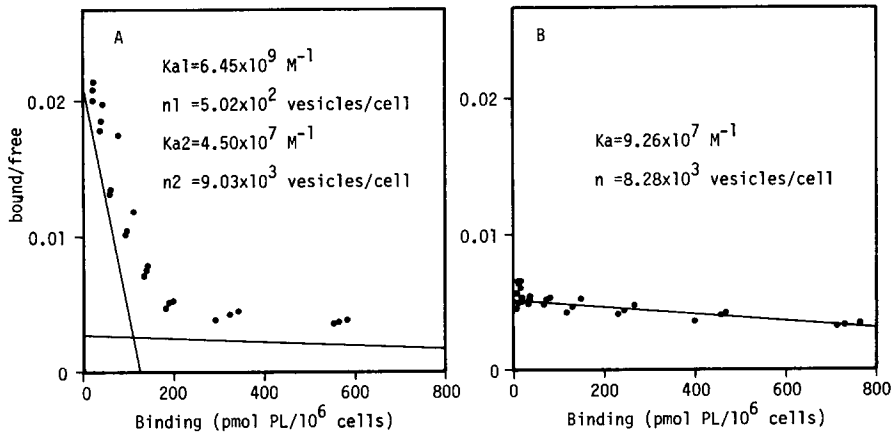

Fig. 4. Scatchard plots of AF- and N-(control) liposomes. Hepatocytes (2×10^7 cells) were incubated with various amounts of AF-liposomes (A, 23.81–3048 nmol PL, 0.245–31.34 μg 6-CF) or N-liposomes (B, 17.42–4460 nmol PL, 0.137–35.04 μg 6-CF) in 5 ml of KHBB (pH 7.4) containing BSA (2%) at 4°C for 30 min. Binding of liposomes was determined as described in Materials and Methods. Each point is the result of individual experiment. Solid lines were obtained from binding parameters estimated by non-linear least square programs, MULTI.

association constant and the number of sites were determined from the Langmuir equation using the non-linear least-squares program, MULTI (Yamaoka et al., 1981), assuming the molecular weight of liposomes with a diameter of 0.13 μm to be $800 \times 1.5 \times 10^5$ (Enoch and Strittmatter, 1979). The values are shown in Fig. 4 along with Scatchard plots. For AF-liposomes, the best curve fitting was obtained when the existence of two classes of binding site was assumed. The association constant of the high-affinity site (K_{a_1}) was about 140 times that of the low-affinity site (K_{a_2}, Fig. 4A). N-liposome binding could be best fitted assuming one class of binding site (Fig. 4B), and its association constant and number of binding sites closely resembled those of the low-affinity site for AF-liposomes.

Effect of liposomal charge

The relationships between the ζ-potential of liposomes and their binding to hepatocytes were sought using N-liposomes consisting of different kinds of lipids. As shown in Table 1, negatively charged PA-containing liposomes bound to hepatocytes, while this was not the case with neutral liposomes (PC : Chol = 7 : 3) or positively charged SA-containing liposomes. With increasing PA content in the bilayer, both the absolute value of the ζ-potential and the extent of the binding increased. The ζ-potential and the binding to hepatocytes of PS- or PG-containing liposomes were the same as those of PA-containing liposomes (6 : 1 : 3). In contrast, DCP-containing liposomes hardly bound to hepatocytes in spite of the high ζ-potential. AF-labeling or encapsulation of 6-CF or [^{3}H]inulin resulted in no change in the ζ-potential of the original liposomes (data not shown).

TABLE 1

Effect of liposomal surface charge on the binding of N-liposomes to hepatocytes

Composition	Molar ratio	ζ-potential [a] (mV)	Binding [b] (pmol PL/10^6 cells)
PC : Chol	7 : 3	−2.2	14.1 ± 12.9
PC : PA : Chol	6 : 1 : 3	−14.3	83.5 ± 3.5
PC : PA : Chol	4 : 3 : 3	−29.0	376.3 ± 34.5
PC : PS : Chol	6 : 1 : 3	−19.3	89.9 ± 13.5
PC : PG : Chol	6 : 1 : 3	–	94.1 ± 10.3
PC : DCP : Chol	6 : 1 : 3	−19.5	16.2 ± 7.2
PC : SA : Chol	6 : 1 : 3	+13.0	7.8 ± 2.8 [c]

[a] ζ-Potential of N-liposomes suspended in KHBB (pH 7.4) containing BSA (2%) was measured by a microelectrophoresis apparatus (Laser Zee Model 501) at 25 °C. Each value represents the mean from three samples.

[b] Hepatocytes (2×10^7 cells) were incubated with N-liposomes (0.84–0.92 μmol PL, 7.38–8.21 μg 6-CF) in KHBB (pH 7.4, 5 ml) containing BSA (2%) at 4 °C for 30 min. Binding of liposomes was determined as described in Materials and Methods. Each value represents the mean from three individual experiments ± S.D.

[c] Binding of SA-containing N-liposomes was determined by measuring the radioactivity of [^{3}H]inulin encapsulated in the aqueous space.

Discussion

Clarification of the cellular uptake mechanism of AF-liposomes is absolutely necessary for estimating their validity as a drug carrier. In our previous study, AF-liposomes consisting of PC, PA and Chol (6 : 1 : 3) were taken up by hepatocytes by means of specific recognition by asialoglycoprotein receptors, and this process was accelerated by an increase in the Chol content in the bilayer. However, when asialoglycoprotein receptors were saturated by free AF, the uptake of AF-liposomes could not be inhibited completely (Hara et al., 1988). This fact indicates that another mechanism in which AF does not take part is also concerned with the cellular uptake of AF-liposomes. Binding of liposomes to macrophages has been affected by the incorporation of acidic or basic lipids into their bilayer (Schwendener et al., 1984; Dijkstra et al., 1985). These findings suggest that PA plays some roles in cellular uptake of liposomes and that Chol participates in the internalization process. Since binding is the first step of the uptake process, examinations were focused on the characteristics of binding of AF-liposomes to hepatocytes, and the effect of PA was elucidated using AF-liposomes with a constant Chol content.

For a reliable measurement of the liposomal binding, we chose 6-CF or [^{3}H]inulin as an aqueous phase marker. In our previous experiment of

liposomal uptake, [^{14}C]Chol was used as a membranous marker (Hara et al., 1988), but an exchange of Chol takes place between liposomal and cellular membranes (Bruckdorfer et al., 1968). 6-CF or [^{3}H]inulin encapsulated in the internal aqueous space of liposomes can not interact with cellular membranes, and the leakage was negligible during the binding experiments (data not shown).

Incubation at 4°C makes it impossible for cells to undergo endocytosis (Zeitlin and Hubbard, 1982), thus permitting an examination of the binding of liposomes as a process separate and distinct from their internalization. Actually, the uptake of AF-liposomes by hepatocytes estimated at 37°C continued for 90 min (Hara et al., 1988), while the binding at 4°C quickly attained the steady-state level (Fig. 1) with the value of one third that of the uptake at 37°C.

AF-liposomes consisting of PC, PA and Chol bind to hepatocytes in two ways; AF-mediated binding and PA-mediated binding. The former occurs through interaction between the galactose residues of AF and asialoglycoprotein receptors of the cell, and is inhibited equally by AF and EDTA (Fig. 2). Since an asialoglycoprotein receptor requires Ca^{2+} for its binding (Hudgin et al., 1974), a divalent cation chelator may possibly cause AF-liposomes to be dissociated from their receptors. The facts that binding of AF-liposomes without PA was completely inhibited by AF and that binding of N-liposomes increased with PA content (Fig. 3) are evidence in favor of PA-mediated binding. In the case of AF-liposomes of PC, PA and Chol (6 : 1 : 3), both types of binding were found to contribute equally under these experimental conditions as described in Fig. 3. This is consistent with the results of our previous uptake experiment (Hara et al., 1988), but whether AF-liposomes bound to hepatocytes through PA-mediated binding are internalized remains to be investigated.

The Scatchard plot for the binding of AF-liposomes to hepatocytes was curvilinear, and two classes of binding site were determined (Fig. 4A). For N-liposomes, however, there was only one class of binding site with an association constant corresponding to the lower affinity site for AF-liposomes (Fig. 4B). The high- and low-affinity sites for AF-liposomes may thus be attributed to AF-mediated and PA-mediated binding, respectively. It should be emphasized that the affinity of AF-mediated binding is 140 times that of PA-mediated binding. The number of asialoglycoprotein receptors on a hepatocyte has been reported as 7×10^4 (Steer and Ashwell, 1980; Weigel, 1980) while the number of high-affinity sites for AF-liposomes on a hepatocyte was found to be about 5×10^2 (Fig. 4A). Since an AF-liposome has about 500 molecules of AF on its outer surface, and when 28% of the AF (140 molecules) are assumed to be involved in the binding, the receptors on a hepatocyte should be saturated by 500 vesicles of AF-liposomes. The binding parameters obtained at 4°C are considered to be not always equivalent to those in vivo. The binding of AF or asialotransferrin to plasma membrane of rat liver is reduced at low temperature (Debanne et al., 1980, 1981). It is likely that AF-liposomes bind more strongly to hepatocytes in vivo. Only a small fraction ($< 1\%$) of liposomes was found to bind to hepatocytes (Figs 1–3 and Table 1), but it was because the number of liposomes far exceeded (1.7×10^5 fold) that of hepatocytes. Under these conditions, the high-affinity sites of the cells were almost saturated by 200 pmol PL of AF-liposomes (Fig. 4A). Since the percentage of binding varies according to the ratio of liposomes to cells, it increased up to 2% when a lower number of liposomes was used (Fig. 4A). When these experiments were carried out at 37°C, the binding and internalization of liposomes occurred repetitively, and the uptake reached 7% of the added liposomes within 30 min (Hara et al., 1988). Thus, the results obtained in this experiment are considered to reflect the characteristics of the binding of the liposomal population.

The PA-mediated binding of AF-liposomes is considered to be due to negative charge. The binding of PS- or PG-containing N-liposomes to hepatocytes was also found to occur as well as that of PA-containing (10 mol%) N-liposomes (Table 1). The binding mechanism may involve interactions other than those of electrostatic nature. Liver macrophages (Kupffer cells) have been shown to take up preferentially negatively charged

liposomes through proteinaceous receptors (Dijkstra et al., 1985).

A very low level of binding was noted in DCP-containing N-liposomes in spite of their negative charge (Table 1). The negatively charged PLs examined were glycerophospholipids except for DCP. We cannot rule out the possibility of the existence of receptors for negatively charged glycerophospholipid. It has been proposed that solidifying liposomal membrane hinders the penetration of the cell surface protein into the liposomal membrane (Spanjer et al., 1986). Thus, the effect of lipid packing on the binding may also be considered as another possibility. The reason for the low binding of DCP-containing N-liposomes to hepatocytes is still unclear.

In conclusion, AF-liposomes consisting of PC, PA and Chol bind to hepatocytes in two different ways; AF-mediated high-affinity interaction in which asialoglycoprotein receptors participate and low-affinity interaction in which negative charges are involved.

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