Molecular Interactions between Surface-Modified Lipid Bilayers and a Water-Soluble Polymer

Ayumi YOSHIDA*1, Kaname HASHIZAKI*1, Mona El-MAHDY*1, Hitoshi YAMAUCHI*1, Hideki SAKAI*1,•2, Shoko YOKOYAMA*3, and Masahiko ABE*1,•2

*1 Faculty of Science and Technology, Science University of Tokyo
(2641, Yamazaki, Noda-shi, Chiba-ken 278-8510)
*2 Institute of Colloid and Interface Science, Science University of Tokyo
(1-3, Kagurazaka, Shinjuku-ku, Tokyo 162-0825)
*3 Kyoritsu College of Pharmacy
(1-5-30, Shibakoen, Minato-ku, Tokyo 105-8512)

Abstract: Molecular interactions between liposomes, the surface of which was modified by adding 6 mol% of poly(ethylene glycol)-lipid (termed PEG-liposomes), and chondroitin sulfate C (CS) as a water-soluble polymer have been investigated in terms of the particle size, zeta-potential, microviscosity, micropolarity and permeability of PEG-liposomes. The particle size and the absolute value of the zeta-potential of PEG-liposomes were found to increase with increasing concentration of CS added. The permeability of PEG-liposomes was decreased by the addition of CS. These were considered to be caused by the complexation between CS and liposomal PEG-chains and by the further adsorption of CS onto CS-PEG-liposomes. The effect of CS on the microviscosity of PEG-liposomes was dependent on the concentration of CS. The packing state of PEG-liposomal lipid bilayer became most rigid by the addition of ca. 4 mg mL⁻¹ of CS.

Key words: PEG-liposome, chondroitin sulfate, particle size, microviscosity, permeability

1 Introduction

Liposomal drug delivery systems have widely been researched as a sustained release system, as well as a targeted drug delivery system¹, however, there are important drawbacks to their use in vivo. Liposomes are rapidly removed from circulation following their intravenous administration primarily by Kupffer cells of the liver and fixed macrophages of the spleen. A prolonged residence of drug-entrapped liposomes in the circulation is important for sustained drug release. Thus, it is important to develop modified liposomes which can avoid uptake by the reticuloendothelial system (RES) and extend their circulation half-lives in vivo. Klibanov et al.²) showed that the conjugation of amphiphatic polyethylene glycol (PEG) with liposomes significantly increased the blood circulation half-life of the liposomes over that of those without PEG. Maruyama et al.³) reported that PEG-liposomes with a 2000 molecular weight of PEG displayed a high concentration in the blood, approximately 40% of the dose, 6 h after the injection. The mechanisms of recognition-evasion and liposome blood clearance are not fully understood yet. Many studies have been carried out from the viewpoint of biochemistry. It is necessary to clarify the surface state of PEG–liposomes to discuss the mechanisms of prolonged circulation time of PEG–liposomes in blood. We have reported in the previous paper⁴) that the packing state of liposomal lipid bilayers becomes most rigid by the addition of 6 mol% of PEG–lipid and the lipid bilayers are microscopically stabilized.

The dynamic behavior of PEG–liposomes in vivo must be dependent on the interaction be-

Corresponding author: Masahiko ABE (at the Faculty of Science and Technology)
between the cell and PEG-liposomes. In this study, chondroitin sulfate (CS), a water-soluble polymer, was used as a typical example of the acidic mucopolysaccharides of the biological membranes. In order to investigate the molecular interactions between PEG-liposomes and CS as a model interaction of the biological membrane, the particle size, zeta-potential, permeability, microviscosity and micropolarity of the liposomal bilayer membranes were measured, and the effects of CS on the PEG-liposomes were discussed from a physicochemical point of view.

2 Experimental Section

2-1 Materials

Phospholipids: Poly(ethylene glycol)-succinyl-distearoyl-phosphatidylethanolamine (PEG-lipid) was purchased from Funakoshi Co. and was used without further purification. Its weight-average molecular weight was approximately 1900 (as the molecular weight of poly(ethylene glycol)). All of the following materials other than pyrene were used as received from the respective companies whose names are indicated inside the brackets. Phospholipid: L-α-Dipalmitoylphosphatidylcholine (DPPC, 99.6% pure) [Nippon Oil and Fats Co., Ltd.]. Steroid: Cholesterol (Chol, 99.9% pure) [Sigma Chemical Co.]. Charged Material: Dicetyl phosphate (DCP, 99.6% pure) [Sigma Chemical Co.]. Water-Soluble Polymer: Chondroitin sulfate C (sodium salt, CS, 90% pure) [Sigma Chemical Co.]. Water-Soluble Polymer: Chondroitin sulfate C (sodium salt, CS, 90% pure) [Sigma Chemical Co.]. Water-Soluble Polymer: Chondroitin sulfate C (sodium salt, CS, 90% pure) [Sigma Chemical Co.]. Water-Soluble Polymer: Chondroitin sulfate C (sodium salt, CS, 90% pure) [Sigma Chemical Co.]. Water-Soluble Polymer: Chondroitin sulfate C (sodium salt, CS, 90% pure) [Sigma Chemical Co.]. Pyrene (98.0% pure) from Sigma Chemical Co. was recrystallized from ethanol several times and purified by column chromatography on silica gel. Water-Soluble Marker: Calcein (2’,7’-[bis(carboxymethyl)-amino]-methyl) fluorescein, 98.0% pure) [Sigma Chemical Co.].

In this experiment, water for injection [Otsuka Pharmaceutical Co.] was used. Phosphate buffered saline (PBS) whose pH is 7.4 was purchased from Nissui Pharmaceutical Co., Ltd.. All other chemicals were commercial products of reagent grade.

2-2 Method

2-2-1 Composition of PEG-Liposomal Membrane

Since we have found⁴ that the optimum amount of PEG-lipid in the liposomes is 6 mol%, the PEG-liposomes, the mole fraction of PEG-lipid of which is 6 mol%, were used in this study. The composition of PEG-liposomes was as follows: PEG-lipid : DPPC : Chol : DCP = 0.68 : 7 : 3 : 0.7 (molar ratio).

2-2-2 Preparation of PEG-Liposomes

PEG-liposomes were prepared using a conventional technique similar to Bangham’s method⁵. Briefly, PEG-lipid, DPPC, Chol and DCP were dissolved in chloroform in a test tube. The solvent was then removed by blowing nitrogen gas into the test tube, and the residual solvent was further dried overnight at room temperature in a desiccator under vacuum. A 4 ml portion of PBS (pH 7.4) was added to this lipid film and warmed (55–60°C) above the phase transition temperature of DPPC (Tc = 41°C) for 30 min. The test tube was then shaken vigorously on a vortex mixer and a homogeneous liposomal suspension was obtained after 1 h of sonication (120 W, bath type; Branson B–220). The final concentration of DPPC forming liposomes was 1×10⁻³ mol L⁻¹.

2-2-3 Determination of DPPC Concentration

The concentration of DPPC in PEG-liposomes was determined by the choline oxidase–phenol method⁶ with a spectrophotometer (type MPS-2000, Shimadzu Co.).

2-2-4 Addition of a Water–Soluble Polymer to PEG–Liposomes

Given amounts of CS as a water-soluble polymer were dissolved in PBS solution, and each of the resultant solutions was added to the PEG-liposomal dispersion. The mixtures were incubated at 37°C for 1 h and submitted for measurements.
2.2.5 Measurement of Particle Size of PEG–Liposomes
The particle size of PEG–liposomes was measured using a 4700–type submicrometer particle analyzer (Malvern Instrument Ltd., UK), with a multibit 8 Malvern correlator with delayed channels. The light source was an argon laser (Coherent Co., Innova 90) with a wavelength of 488 nm and a power of 5 W or less, and the time–dependence correlation function on the scattered light intensity was measured at a scattering angle of 90°.

2.2.6 Measurement of Zeta–Potential of PEG–Liposomes
The zeta–potential of PEG–liposomes was measured using a zetasizer II c (Malvern Instrument Ltd., UK), which is a laser Doppler electrophoresis apparatus. This apparatus is equipped with a multibit 8 Malvern correlator with delayed channel and 15 mV He–Ne Laser (λ=633 nm).

2.2.7 Measurement of Entrapped Efficiency of Calcein in PEG–Liposomes
The entrapped efficiency of calcein as a marker in PEG–liposomes was determined by a fluorescence method using a spectrofluorophotometer (type RF-5000, Shimadzu Co.). The calcein concentration entrapped in the PEG–liposomes was 1×10⁻⁴ mol L⁻¹ under consideration of osmotic pressure. The liposomal dispersion was diluted 100 times with PBS, when the total fluorescence intensity was measured (I_{Total}). Calcein existing in an outer aqueous phase was then quenched by the complexation with cobalt ions (using cobalt chloride, anhydrous from Wako Pure Chemical Industries Ltd.), when the fluorescence intensity was measured (I_{In}). Finally, PEG–liposomes were destroyed with Triton X-100 (t-octyl-phenoxy-polyethoxy-ethanol, from Sigma Chemical Co.), when the fluorescence intensity was measured again (I_{TX}). The entrapped efficiency of calcein was calculated according to the following expression:

\[
\text{Entrapped efficiency/\%} = \frac{I_{In} - I_{TX} \times r}{I_{Total} - I_{TX} \times r} \times 100
\]

where r is the volume correction factor\(^7\).

2.2.8 Measurement of Microviscosity of Bilayer Membranes
PEG–Liposomes containing fluorescent fatty acid probes, either 2AP or 12-AS, in their membrane and enclosing PBS in their internal water phase, were prepared. The molar ratio of lipids to probe was 300 : 1. The microviscosity of PEG–liposomes was estimated from the fluorescence polarization (P), which can be calculated according to the following equation:

\[
P = \frac{I_p - G \cdot I_v}{I_p + G \cdot I_v}
\]

where \(I_p\) and \(I_v\) are the fluorescence intensities of the emitted light polarized parallel and vertically to the exciting light, respectively, and \(G\) is the grating correction factor\(^8\). \(I_p\) and \(I_v\) were measured with a spectrofluorophotometer, and excitation and emission wavelengths were 365 and 440 nm, respectively.

2.2.9 Measurement of Micropolarity of Bilayer Membranes
PEG–Liposomes with 5.0×10⁻⁶ mol L⁻¹ of pyrene solubilized in their membranes were prepared. The micropolarity of PEG–liposomes was determined by measuring the fluorescence intensity of pyrene using a spectrofluorophotometer at excitation and emission wavelengths of 335 and 350–550 nm, respectively\(^9\).

All experiments were carried out at 37°C.

3 Results and Discussion

3.1 Effects of CS on Macroscopic–State of PEG–Liposomes
The particle size and the zeta–potential of PEG–liposomes in the presence of CS were measured. The results are shown in Figs. 1 and 2 against the amounts of CS added.

The particle size of PEG–liposomes changed little in the lower concentration range of CS, while it increased gradually and was likely to reach a certain limited size above 4 mg mL⁻¹.
Fig. 1 Effect of CS on the Particle Size of PEG-liposomes at 37°C.

Fig. 2 Effect of CS on the zeta-potential of PEG-liposomes at 37°C.

In parallel with this, the absolute value of the negative zeta-potential of PEG-liposomes increased only slightly up to 8 mg mL\(^{-1}\) of CS, above which it showed an increasing trend and approached a certain limiting value as the CS concentration increased, as seen in Fig. 2. These changes in particle size and zeta-potential of PEG-liposomes are considered to be due to the interactions between CS and the PEG-chains on the PEG-liposomal surface. The mode of the interaction between CS and PEG-liposome in the lower concentration range of CS may be different from that in the higher concentration range of CS. It is reported\(^{10,11}\) that PEG forms hydrogen-bonded complexes with polysaccharides such as dextrans or insulins in 1 : 1 molar ratio. Thus, PEG chains are likely to form hydrogen-bonded complexes with CS on the liposomal surface at low CS concentrations. At high CS concentrations, PEG-liposomes forming complexes with CS would allow CS molecules to adsorb more on their surface. Sugar layers of CS would then be formed surrounding the CS-PEG-liposomal surface by the further adsorption of CS, thereby increasing the apparent liposomal size. The number of adsorption site, however, is limited, and hence, the particle size would approach a certain limiting value. The increase in the absolute value of the negative zeta-potential of PEG-liposomes is probably caused by the negative charge of CS.

A cellular aggregation by the complexation between PEG and sugar chains exposed on the cellular surface has been found\(^{12}\). This suggests that PEG-liposomes may aggregate by the addition of CS. In this study, however, no aggregation of PEG-liposomes with CS was found, indicating no significant changes in their dispersibility. This is quite likely to be due to the electrostatic repulsion among the negatively charged CS chains, thereby preventing PEG-liposomes from aggregation with CS.

**3.2 Effect of CS on Permeability of PEG-Liposomes to Calcein**

Fig. 3 shows the time course of the entrapped efficiency of calcein as a marker at 37°C, in which the ordinate represents the ratio of the entrapped efficiency after incubation at 37°C for \(t\) hours \((E_t)\) to that before incubation \((E_0)\). Decrease in this ratio with time means that calcein leakage is promoted.

As is seen in Fig. 3, calcein leakage from PEG-liposomes was depressed by the addition of CS. The depression caused by CS would be due to changes in the packing state of the liposomal bilayer membranes through the complexation between CS and the liposomal PEG-chains.
3.3 Effects of CS on Microscopic State of PEG-Liposomes

Fluorescent fatty acid probes were used in this study to interpret the microviscosity at different depths in the liposomal bilayer membranes. It is generally known that 2-AP can probe the region close to the membrane surface, while 12-AS can probe the region near the bilayer center. The microviscosity related to fluorescence polarization can be calculated using the Perrin–Weber’s equation. It is also known that fluorescence polarization increases with increasing microviscosity. Fig. 4 (a) (2-AP probe) and (b) (12-AS probe) show the relationship between fluorescence polarization and the amount of CS added.

Both the microviscosities in the region close to the membrane surface of the bilayer membranes and in the region near the bilayer center are increased by the addition of CS. The rapid increase in the microviscosity is caused by the tightened packing of the lipid bilayers which is dependent on the fluid depression of the acyl chains. When CS molecules are adsorbed on the liposome surfaces to form complexes with the liposomal PEG-chains, the flu-
idity is depressed not only in the head group region but also in the acyl chain region. As a result, the microviscosities are likely to increase both at the regions close to the membrane surface and near the bilayer center. The microviscosities in both of the regions show, however, a maximum value at 4 mg mL\(^{-1}\) of CS, and they decrease at concentrations above 4 mg mL\(^{-1}\) of CS. CS is a straight-chain molecule with a length of approximately 1200 Å\(^{14}_{15}\). Then, the following arguments may be made: CS molecules form complexes with the liposomal PEG-chains accompanied by a tightening of the packing of the lipid bilayer membranes, while further adsorption of CS molecules on the surface reduces the microviscosity by the steric and/or electrostatic repulsion among the negatively charged CS molecules, thereby increasing the fluidity in the lipid bilayer membrane and decreasing the microviscosity at concentrations above 4 mg mL\(^{-1}\) of CS.

The micropolarity of PEG–liposomes was then measured using pyrene, as the marker in the region near the bilayer center. The results are shown in Fig. 5 where the \(I_1/I_3\) ratio\(^{16}_{18}\) of pyrene monomer fluorescence is plotted against the amount of CS added. Larger \(I_1/I_3\) ratio means greater polarity.

![Fig. 5 Effect of CS on the Micropolarity of the PEG–liposomal Bilayer Membrane; plots of \(I_1/I_3\) ratio of pyrene monomer fluorescence vs. concentration of CS.](image)

The micropolarity shows a minimum value at 4 mg mL\(^{-1}\) of CS. The decrease in the micropolarity at concentrations below 4 mg mL\(^{-1}\) of CS means an increase in the hydrophobicity of lipid bilayer. This is caused by a tightening of the Packing of the lipid bilayer, resulting in a less influence of polar substances upon the hydrophobic region in the membrane bilayer. The increase in the micropolarity above 4 mg mL\(^{-1}\) of CS corresponds to an increase in the fluidity of acyl chain in the lipid bilayer. This would considered to be caused by the steric and/or electrostatic repulsion of high concentration of CS molecules, resulting in a looser packing state of the lipid bilayers above 4 mg mL\(^{-1}\) of CS. This figure also implies the complexation of CS with PEG–liposomes at lower CS concentrations and a further adsorption of CS onto the complexed liposomal surfaces at higher CS concentrations.

### 4 Conclusion

Addition of CS brought about an increase in the apparent particle size and a decrease in the permeability of PEG–liposomes. These were likely to be caused by the complexation between CS and the liposomal PEG–chains and by further adsorption of CS onto the complexed
liposomal surface. The effect of CS on the microscopic-state of PEG-liposomes was dependent on the concentration of CS. The packing state of the PEG-liposomal lipid bilayer became highly rigid by the addition of ca. 4 mg mL\(^{-1}\) (at least 4\(~\sim\) 8 mg mL\(^{-1}\)) of CS.

These results suggest that PEG-liposomes that can form a complex with CS have a high affinity to the biological membrane and that the liposomes might be used as a carrier for sustained drug release.

(Received Jul. 16, 1998; Accepted Sept. 21, 1998)

References

4) A. Yoshida, K. Hashizaki, H. Yamauchi, H. Sakai, S. Yokoyama, M. Abe, to be submitted.
12) J. D. Robertson, Protoplasma, 63, 218 (1967).
[報文] 表面修飾した脂質二分子膜と水溶性高分子と の 相 互 作 用

吉田あゆみ*1・橋崎 要*1・モナ・エルマハディー*1
山内仁史*1・酒井 秀樹*1,2・横山 祥子*3
阿部 正彦*1,2
*1 東京理科大学理工学部 (〒278-8510 千葉県野田市山崎 2641)
*2 東京理科大学界面科研 (〒162-0825 東京都新宿区神楽坂 1-3)
*3 共立薬科大学 (〒105-8512 東京都港区芝公園 1-5-30)

ポリエチレングリコール誘導脂質を 6 mol% 添加して表面修飾したリポソーム (PEG リポソーム) と水溶性高分子であるコンドロイチン硫酸 (CS) との相互作用を、PEG リポソームの粒子径、ゼータ電位、微視的粘性、微視的極性、膜透過性から検討した。その結果、PEG リポソームの粒子径およびゼータ電位の絶対値は CS を添加することにより増加した。また、PEG リポソームの膜透過性は CS の添加により抑制された。これらの結果は、リポソーム表面での PEG 鎖と CS との分子間錯体の形成、およびその表面への CS 分子の更なる吸着に起因するものと考えられた。PEG リポソームの微視的粘性に及ぼす CS の影響は CS 濃度に依存した。PEG リポソーム二分子膜のパッキングは CS 濃度が約 4 mg mL⁻¹ の時に最もリジェットになった。

（連絡者：阿部正彦）Vol. 47, No. 12, 1323 (1998)

[報文] 油／水界面で形成する液晶を利用した
W/O 型液晶エマーショ－ン
—高水相 W/O 型エマーショ－ンの調製法とその乳化機構—

中間 康成・塩島 義浩・春澤 文則
(株)資生堂 基盤技術研究所 (〒223-0057 横浜市港北区新羽町 1050)

油/水界面で形成する液晶を用いた W/O 型液晶エマーショ－ンの調製法とその乳化機構について報告した。その調製法は、オレイン酸 (OA) を溶解した油相に、攪拌下でラウリルメチルアミノ酢酸ペタイン (LB) 水溶液を添加して W/O 型エマーショ－ンを調製する方法である。この方法を用いることによって、OA と LB の静電的結合により油/水界面で、OA, LB, 油, 水の 4 成分からなる液晶が効率よく生成し、水滴の回りを液晶が取り囲んだ W/O 型の液晶エマーショ－ンが調製できる。そして、90% 以上もの水を包含した乳化粒子の合一、クリーミングに対すて安定な高水相の W/O 型液晶エマーショ－ンを容易に調製することができた。相平衡図の解析から、これは、水の一部及び油をそれぞれ親水基部分、親油基部分に取り囲んだ硬い逆ヘキサゴナル液晶が、水滴を不動化したためであった。

（連絡者：中間康成）Vol. 47, No. 12, 1331 (1998)