Effects of the PEG Molecular Weight of a PEG-Lipid and Cholesterol on PEG Chain Flexibility on Liposome Surfaces

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ABSTRACT

A variable-temperature ^{1}H NMR study was performed to investigate the effects of the molecular weight of poly(ethylene glycol) (PEG) in PEG–lipids and cholesterol addition to the lipid bilayer on PEG chain flexibility at the liposomal surface. PEG–lipids, i.e., L-α-distearoylphosphatidylethanolamine (DSPE)–PEG, with PEG molecular weights of 750, 2000, and 5000 were modified to liposomes of ca. 100 nm. The ^{1}H NMR peak of PEG in DSPE–PEG was overlapped by broad and sharp peaks, corresponding to rigid and flexible PEG components, respectively. When the PEG molecular weight was increased, the PEG peak became sharp, indicating that long-chain PEGs were more flexible on the liposome surface. The proportion of flexible components projecting into the water phase increased as the PEG chain length increased. Peak sharpening also occurred when the cholesterol content was increased from 0 to 30 mol%, demonstrating that cholesterol incorporation into the lipid bilayer enhanced the PEG chain flexibility. In addition, the PEG chain flexibility significantly increased when cholesterol was delocalized in the lipid bilayer at concentrations above 20 mol%. Lateral diffusion of the lipid with the presence of cholesterol in the lipid bilayer significantly affected the PEG chain flexibility.

Key Words: liposome; NMR; flexibility; cholesterol; PEG molecular weight

1. Introduction

Liposomes, which are used in drug delivery systems, are small vesicles composed of phospholipid bilayers. However, short circulation lifetimes in blood are a major problem for liposome formulations. To decrease the clearance of liposomes from blood and deliver liposomes efficiently to target areas in the body, it is necessary to control the fluidity of the lipid bilayer, the surface modification, and the liposome size.[1-4] A lipid bilayer has an inherent transition temperature (T_c).[5] Below T_c, the bilayer forms a gel phase, in which acyl chains are densely packed in an ordered array. A liquid crystalline phase with disordered acyl chains and lateral lipid diffusion are observed above T_c. The effect of the incorporation of cholesterol into the lipid bilayer on the behavior of the lipid bilayer depends on the phase.[6, 7] In a lipid bilayer, cholesterol fills the gaps between the acyl chains of the lipid in the liquid crystalline phase and orders the acyl chains (condensing effect); this decreases the permeability of substances through the lipid bilayer.[8-10] The lateral diffusion or fluidity of the lipid bilayer is enhanced by the incorporation of cholesterol into the gel phase (fluidizing effect).[11, 12] Both effects of cholesterol incorporation into the lipid bilayer contribute to the improvement of the physical stability of the liposome and enhance its circulation lifetime.[4, 13]

Modification of the liposome surface with poly(ethylene glycol) (PEG)–lipids is generally accepted to improve the liposome circulation lifetime in blood.[14, 15] PEG on the liposome surface forms a hydration shell, where water molecules associate with hydrophilic PEG chains.[16] This hydration shell prevents the liposome

from interacting with blood components and fusing with other liposomes through steric hindrance. It suppresses the activation of complement systems and recognition by mononuclear phagocyte systems (stealth effect), resulting in an extended liposome circulation lifetime.[2, 14, 17] The biological distribution and circulation time of a liposome are also affected by its size and uniformity.[4, 18] Liposomes are roughly classified into multilamellar vesicles (MLV) that are 0.5–10 μm, small unilamellar vesicles (SLV) that are less than 100 nm, and large unilamellar vesicles (LUV) that are 0.1–1 μm. Liposomes with sizes controlled to approximately 100 nm efficiently accumulate in tumor tissue and inflammatory sites because of their enhanced permeability and retention effects.[19, 20] Liposomes that are ca. 100 nm in size and that have cholesterol incorporation and PEG modification of the surface are therefore useful as drug carriers targeting tumor tissue (passive targeting). Doxil®, a PEG–liposome formulation of doxorubicin, which was approved in the U.S.A. in 1995 and marketed in Japan in 2007, is a good example.[21, 22] [23]

The state of PEG on the liposome surface determines the stability and circulation lifetime of the liposome.[2, 24, 25] The effects of the PEG molecular weight, density, and flexibility were summarized by Vonarbourg et al.[26] Two conformations have been identified for PEG chains on a liposome surface depending on the PEG density.[16] The mushroom form is observed at low PEG densities on the liposome surface, whereas high PEG densities give brush forms. The interaction between PEG–liposome and blood components is different for the two different forms, affecting the liposome circulation time.[17, 27] The PEG molecular weight and the graft

density determine the PEG chain conformation on the liposome surface.[1, 17, 28, 29] PEG chain flexibility is an important factor in the liposome circulation time; a highly flexible PEG has been suggested to suppress opsonization and recognition by proteins and antibodies.[26, 30] However, the flexibility of PEG on the liposome surface has hardly been evaluated by experimental analysis, and the effect of PEG flexibility on liposome stability has mainly been discussed on the basis of computational simulations.[31]

Some analytical techniques, such as differential scanning calorimetry (DSC), ultrasound velocity, and zeta potential measurement, have been used to evaluate PEG on liposome surfaces.[23] Recently, PEG morphology and mechanical properties were studied using small-angle X-ray diffraction and atomic force microscopy.[25, 32] However, there have been no reports that provided insight into the PEG state on liposome surfaces at the molecular level. Understanding the PEG molecular state on the liposome surface is crucial for designing efficient liposomal formulations and controlling their quality. NMR spectroscopy is a powerful technique for studying polymers at the molecular level. The molecular states of polymers that have been modified to nanoparticles have been studied using NMR spectroscopy.[33-38] However, little NMR research has been conducted on the molecular states of PEG on liposome surfaces, although this potentially provides detailed information, such as the chemical environment and molecular mobility. So far, the evaluation of PEG-modified liposome surfaces using NMR methods has been limited to the quantification of PEG on the liposome surface.[36] The chemical shifts in the NMR spectra reflect the chemical environments around molecules.[33,

34] The NMR peak line-widths in one-dimensional spectra are related to the mobility: sharp peaks indicate high

mobility. [33-35] The NMR relaxation times provide quantitative information on molecular mobility.[39]

Spin–lattice (T_1) and spin–spin (T_2) relaxation times provide information on molecular motion. T_2, which is

widely used to evaluate polymer flexibility and conformation, is determined by the peak line-width.[40-42]

The purpose of this study was to investigate the molecular state of PEG on ca. 100-nm liposome surfaces,

focusing on flexibility, using variable-temperature ^{1}H NMR spectroscopy. The chemical shift, shape, and T_2 of

the PEG peak in the ^{1}H NMR spectrum were evaluated. Distearoylphosphatidylethanolamine (DSPE)–PEG was

modified on the surface of ca. 100-nm liposomes that were prepared using hydration and extrusion methods.

The effects of PEG molecular weight on PEG flexibility were evaluated using DSPE–PEG with PEG molecular

weights of 750, 2000, and 5000. The effect of cholesterol addition to the lipid bilayer on PEG chain flexibility

was also investigated.

2. Experimental

2.1. Materials.

1,2-Distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC), DSPE–PEG5000, and DSPE–PEG2000 were

purchased from the Nippon Oil & Fats Co., Ltd. (Japan). Cholesterol and DSPE–PEG750 were obtained from

Solvay (Belgium) and Avanti Polar Lipids, Inc. (USA), respectively. All of the materials were used without

further purification.

2.2. Preparation of PEG-liposomes.

The PEG-liposomes were prepared according to the post-modification method reported by Yoshino et al., [43] in order to modify the DSPE-PEG only on the outer aqueous side of liposome. Bare liposomes of sizes of ca. 100 nm were prepared by the hydration and extrusion methods (Scheme S1). DSPC and cholesterol at the required molar ratio (100/0, 90/10, 80/20, 70/30) were codissolved in chloroform. Chloroform was removed by rotary evaporation (60 °C, 150 rpm), and the resulting lipid film was placed under vacuum to completely remove the chloroform. The dried lipid films were hydrated with phosphate-buffered saline in D_2O (D-PBS, pH 7.4) at 70 °C at a lipid concentration (DSPC+cholesterol) of 33.3 mM and were extruded three times through a filter with 200-nm pores and 10 times through a filter with 100-nm pores at 70 ℃ using an extruder (Northern Lipids, Canada) to obtain a 100-nm liposome suspension. A DSPE–PEG micelle solution at a concentration that was adjusted to the definitive molar ratio of the PEG liposome (DSPC+cholesterol to DSPE-PEG = 100/3.33, 100/1.25, 100/0.50) was also prepared using similar methods to those above by a hydration method. The partitioning of DSPE-PEG to the outer liposome surface could be achieved by incubating the liposome suspension and DSPE-PEG micelle solution above the T_c and subsequent rapid cooling. [43] In this study, PEG–liposome suspensions were prepared by adding preformed DSPE–PEG micelle solutions to liposome suspensions with a ratio of 1:9 (v/v). The mixed suspension of liposome suspension and DPSE-PEG micelle

solutions was incubated at 70 ºC, which is above the T_c of DSPC, for 60 min and then rapidly cooled for the purpose of partitioning the DSPE-PEG onto the liposome membrane.

2.3. Particle size distribution.

The PEG–liposome particle size distributions were determined at 25 °C by a dynamic light scattering (DLS) method (MICROTRAC 9340-UPA (Ultrafine Particle Analyzer)–UT151, Nikkiso Co., Ltd., Japan). The PEG–liposome suspensions were diluted 100-fold with phosphate buffered saline in H_2O (H-PBS, pH 7.4) for measurements. Three independent measurements were performed for each sample suspension. The mean volume diameter was used as the mean particle size.

2.4. Zeta potential.

The zeta potentials of the PEG–liposomes were determined at 25 ºC using a laser Doppler method (ELSZ1 Otsuka Electronics Co., Ltd., Japan). The PEG–liposome suspensions were diluted 50-fold with H_2O for measurements. Smolowkovski's equation was used as a conversion equation. The measurements were repeated three times to obtain the mean zeta potential.

2.5. DSC.

The phase-transition temperatures of the liposomal bilayer membranes were determined using DSC (EXTAR6000 SEIKO6300, Seiko Instruments Inc., Japan). The PEG–liposome suspension (20 μL) was placed in a stainless-steel sampling pan, and the pan was sealed. D-PBS was used as a reference solution. The

measurement conditions were a scanning rate of 2 ℃ min^{-1} and scanning range of 30–70 ℃.

2.6. ^{1}H NMR spectroscopy.

^{1}H NMR spectroscopy was performed (JNM-ECA 500, JEOL Resonance Inc., Japan) at 500 MHz (11.74 T).

The following conditions were used: no spinning, 16,384 data points, 90º radio-frequency pulses at 12.44 µs, 10

s relaxation delay, and eight scans. The measurement temperatures were 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50

℃. The HDO signal, which was derived from residual H$_2$O, was used as a reference.[44] The PEG spin–spin

relaxation times (T_2) were determined from the half-line-width ($\nu_{1/2}$) of the PEG peak, using equation (1):[45]

$$\nu_{1/2} = \frac{1}{\pi T_2} \quad (1)$$

3. Results and discussion

3.1 Effect of PEG molecular weight on PEG chain flexibility.

Each PEG–liposome consisted of DSPC and DSPE–PEG with PEG molecular weights of 750, 2000, and

5000. The same ratio of DSPC to PEG monomer units (DSPC: monomer unit of ethylene glycol = 1:25

(mol/mol)) were used to compare each sample. The PEG–liposome compositions were DSPC/DSPE–PEG750 =

100/3.33 (mol/mol), DSPC/DSPE–PEG2000 = 100/1.25, and DSPC/DSPE–PEG5000 = 100/0.50. The PEG

chain conformation on the liposome surface depends on the DSPE–PEG modification ratio. The PEG2000 chain

conformation on the DSPC liposome surface changes from the mushroom to the brush form when the

DSPE–PEG2000 modification ratio to the liposome surface is above 5 mol%. This modification ratio assumed that

DSPE-PEG2000 is applied to both the outer and inner aqueous sides. In this study, we prepared liposomes that were modified with DSPE–PEG2000 only on the outer aqueous side so that the PEG2000 conformation could change from the mushroom to brush form at approximately 2.5 mol% DSPE–PEG2000. Because the PEG–liposome composition was DSPC/DSPE–PEG2000 = 100/1.25, the PEG chain was in the mushroom form.[46] According to a previous study [25], the PEG chains of the other PEG–liposomes, with PEG molecular weights of 750 and 5000, could also be in the mushroom form because the molar ratios of DSPC to the monomer unit of ethylene glycol were equal at 1:25.

Table 1 shows the mean particle sizes and zeta potentials of the PEG–liposomes. Every PEG–liposome suspension had a unimodal particle size distribution, with a mean particle size of approximately 100–110 nm as the hydrodynamic diameter. The zeta potential of the PEG–liposome suspension showed a negative charge derived from DSPE, and the negative value increased with decreasing PEG molecular weight. The PEG–liposomes with smaller PEG molecular weights had higher DSPE–PEG modification ratios, resulting in larger negative charges.

Table 1. Effect of PEG Molecular Weight on the Mean Particle Size and Zeta Potential of PEG–Liposome ($n = 3$, Mean ± Standard Deviation)

sample component (mol/mol)	mean particle size* (nm)	zeta potential (mV)
DSPC/DSPE–PEG750 (100/3.33)	104 ± 1.5	–32.3 ± 0.8
DSPC/DPSE–PEG2000 (100/1.25)	109 ± 7.0	–16.3 ± 1.7
DSPC/DSPE–PEG5000 (100/0.50)	112 ± 1.1	–6.3 ± 1.8

*Particle distribution pattern was unimodal.

The effect of PEG molecular weight on the phase transition of the lipid bilayer was evaluated using DSC (Figure 1).[47] Endothermic peaks of the phase transitions from a gel phase to a liquid crystalline phase were observed for all of the PEG–liposomes at approximately 53 °C, regardless of the PEG molecular weight.[48, 49] We confirmed that the PEG molecular weight barely affected the lipid bilayer fluidity, even if the modification ratio was different among the liposomes.
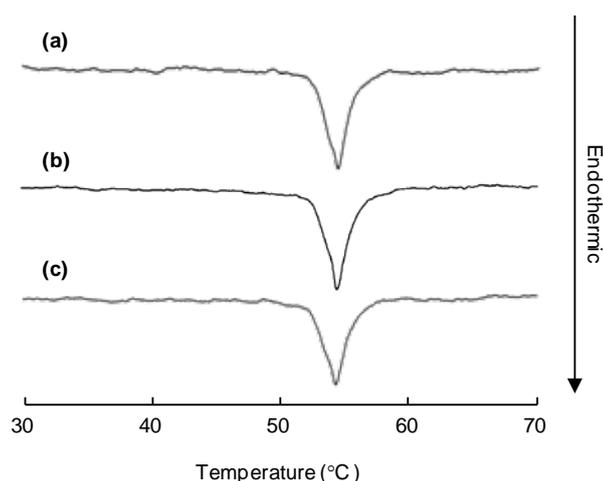
Figure 1. Effect of PEG molecular weight on the DSC curves of PEG–liposomes: (a) DSPC/DSPE–PEG750 (100/3.33), (b) DSPC/DSPE–PEG2000 (100/1.25), and (c) DSPC/DSPE–PEG5000 (100/0.5).

^{1}H NMR spectroscopy was used to investigate the effect of PEG molecular weight on PEG chain flexibility.

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Liquid-state NMR spectroscopy predominantly detects molecules with high molecular mobility, such as dissolved molecules.[50] Only peaks from the ethylene oxide group of DSPE–PEG were observed at 3.7 ppm, and no lipid bilayer peaks were observed (Figure S1). Figure 2 shows the expansion of the ^{1}H NMR spectra at 35 ℃ from 3.6 to 3.8 ppm to show the peaks of PEGs with different molecular weights.
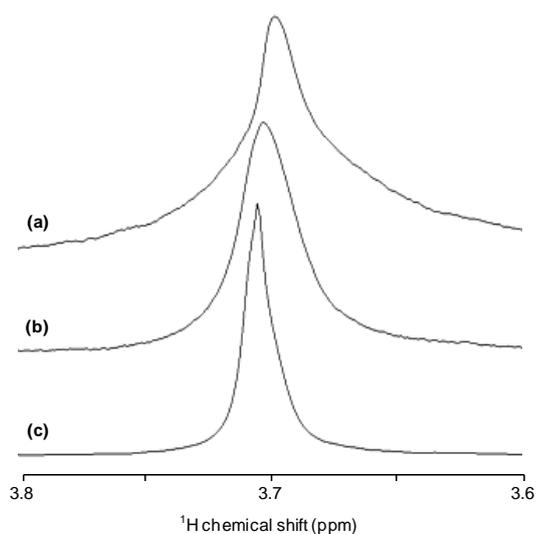
Figure 2. ^{1}H NMR spectra of a PEG-liposome at 3.6–3.8 ppm, showing a PEG peak (35 ℃): (a) DSPC/DSPE–PEG750 (100/3.33), (b) DSPC/DSPE–PEG2000 (100/1.25), and (c) DSPC/DSPE–PEG5000 (100/0.5).

The peak, especially for DSPE–PEG750 (Figure 2a), was a superimposition of overlapping broad and sharp peaks corresponding to the rigid and flexible components, respectively. Figure 3a shows a schematic representation of the PEG structure on the liposome surface.
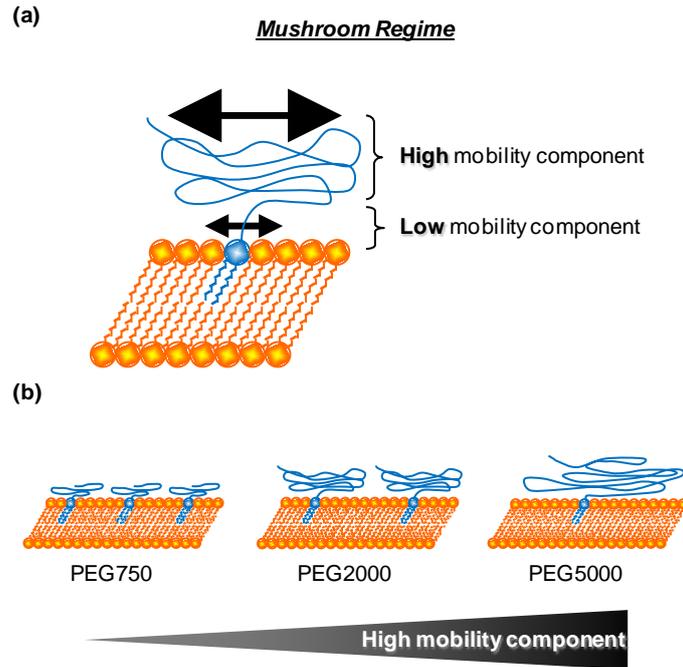
Figure 3. Schematic representation of (a) DSPE–PEG on a liposome surface and (b) DSPE–PEGs with different molecular weights.

Albert et al., reported that polymeric micelles of a block copolymer of PEG and poly(N-isopropylacrylamide) have rigid PEG segments close to the micelle core and more flexible distal segments by means of ^{1}H NMR spectroscopy. [51] Here, PEG on the liposome surface could consist of one component near the liposome surface and another component projecting into the water phase, although ca. 100 nm-sized liposomes were much different than those of the polymeric micelle, which were approximately 30-40 nm. The flexibility of the PEG component near the liposome surface was limited because DSPE was stuck in the lipid bilayer, whereas the PEG component projecting into the water phase was more mobile. In addition, increasing the PEG molecular weight sharpened the peak, and the half-line-width decreased. This change indicated that PEG with a longer chain was more flexible on the liposome surface (Figure 2a–c). In general, chemical compounds with large molecular weights show broader NMR peaks because of their low mobility. The PEG flexibility was therefore

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significantly changed by modifications on the liposome surface. Figure 3b shows possible structures of PEGs with different molecular weights on the liposome surface. The majority of PEG components in shorter PEG chains were rigid because they were close to the surface of the immobile liposome. The proportion of flexible PEG components projecting into the water phase increased for longer PEG chains. The PEG ^{1}H chemical shifts moved to a lower magnetic field with increasing PEG molecular weight (Figure 2a–c, Table S1). The chemical shift depends on the electron density distribution in the corresponding molecular orbitals; nuclei with lower electron densities have lower chemical shifts. The electron density of the PEG chain was therefore smaller when the PEG molecular weight was larger. Intramolecular interactions between ethylene glycols within a PEG chain on the liposome surface could be smaller in the flexible PEG component projecting into the water than in the rigid PEG component near the liposome surface.

Figure 4 shows the variation in T_2 of PEG on liposome surfaces as a function of temperature, from 5 to 50 ℃. The T_2 calculated from the half-line-width was used as the average flexibility of each PEG because the observed PEG peak was a superimposition of broad and sharp peaks. PEG with a longer chain had a larger T_2 at all measured temperatures. The T_2 of PEG750 on the liposome surface was constant, regardless of temperature change. The T_2 of PEG2000 was also almost constant below 45 ℃ and started to increase at approximately 50 ℃. The T_2 of PEG5000 gradually increased, even below 45 ℃, and started to dramatically increase at approximately 50 ℃. The increased bilayer fluidity near the transition temperature at approximately 53 ℃

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(Figure 1) could influence the flexibility of PEG on the liposome surface. We discussed why the temperature-dependent increase in PEG flexibility became more pronounced when the PEG molecular weight was larger. Intramolecular interactions between PEG components near the liposome surface could be stronger than those between PEG components projecting into the water phase. PEGs with shorter chains, which mainly consisted of rigid PEG components because of intramolecular interactions, were little affected by temperature increases. PEG on the liposome surface forms a hydration shell, where water molecules associate with hydrophilic PEG chains.[50] The water molecule mobility in the hydration shell could also influence PEG flexibility. PEGs with longer chains form larger hydration shells,[26] so the PEG flexibility is enhanced, accompanied by the increased mobility of water molecules in the hydration shell at higher temperatures.
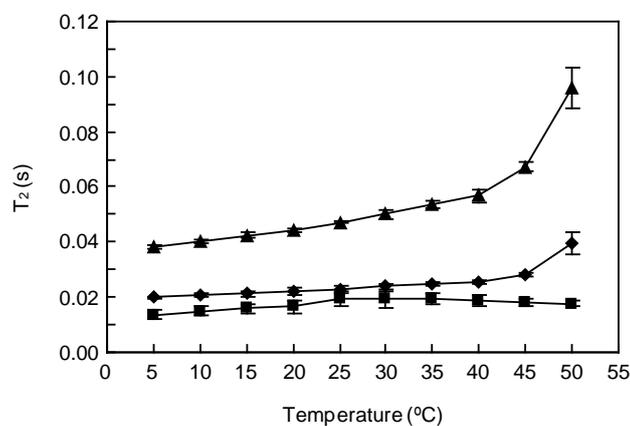
Figure 4. T_2 variations of PEG in PEG–liposomes with various PEG molecular weights as a function of temperature (n = 3, mean ± standard deviation): (■) DSPC/DSPE–PEG750 (100/3.33), (◆) DSPC/DSPE–PEG2000 (100/1.25), and (▲) DSPC/DSPE–PEG5000 (100/0.5).

The effect of the DSPE–PEG2000 modification ratio on PEG flexibility was also evaluated using ^{1}H NMR spectroscopy. Figure S2 shows the ^{1}H NMR spectra of DSPE–PEG2000 attached to liposome surfaces at

different modification ratios. The line-widths of the DSPE–PEG2000 peaks were almost the same, regardless of

the modification ratio. This showed that the DSPE–PEG2000 modification rate had little effect on PEG chain

flexibility. The temperature-dependent changes in the T_2 of the PEG peaks were not observed in all of the samples

that had a DSPE–PEG2000 different modification ratio (Figure S3). It was therefore confirmed that the PEG chain

length rather than the DSPE–PEG modification ratio contributes to the increased of PEG chain flexibility.

3.2 Effect of Cholesterol in the Lipid Bilayer on PEG Chain Flexibility.

Table 2 shows the mean particle sizes and zeta potentials of each PEG–liposome. All of the PEG–liposome

suspensions had a unimodal particle size distribution, with mean particle sizes with hydrodynamic diameters

100-120 nm. Cholesterol incorporation induced an increase of the PEG–liposome particle size. This is in

agreement with Tseng et al.'s report that the increase in lipid bilayer rigidity by cholesterol addition enhances

the resistibility of the particle size control in the extrusion method, resulting in increases in the liposome

size.[52] The zeta potentials of all of the PEG–liposome suspensions showed negative charges that were derived

from DSPE. The negative value became larger with increasing amounts of cholesterol in the lipid bilayer.

Makino et al. reported that the zeta potential of DSPC–liposomes changes with temperature because of

structural changes in the hydrophilic parts of the lipids.[53] Cholesterol in the lipid bilayer could affect the

electrostatic interactions between the hydrophilic parts of the lipids and change the structures of the hydrophilic

parts of DSPC and DSPE. The structural change in lipids caused by cholesterol could induce negative surface

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charges on PEG–liposomes.

Table 2. Effect of Cholesterol Incorporation into the Lipid Bilayer on the Mean Particle Size and Zeta Potential of PEG–Liposome ($n = 3$, Mean ± Standard Deviation)

sample component (mol/mol)	mean particle size* (nm)	zeta potential (mV)
DSPC/Chol/DPSE–PEG2000 (100/0/1.25)	109 ± 7.0	–16.3 ± 1.7
DSPC/Chol/DSPE–PEG2000 (90/10/1.25)	112 ± 3.0	–17.1 ± 1.2
DSPC/Chol/DSPE–PEG2000 (80/20/1.25)	116 ± 6.6	–18.1 ± 0.9
DSPC/Chol/DSPE–PEG2000 (70/30/1.25)	117 ± 4.8	–22.0 ± 1.9

*Particle distribution pattern was unimodal.

The effect of cholesterol on the phase transition of the lipid bilayer was evaluated using DSC (Figure 5). Endothermic phase-transition peaks were observed for liposomes with DSPC/cholesterol/DSPE–PEG2000 ratios of 100/0/1.25 and 90/10/1.25. The similarity of their phase-transition temperatures, at 53 and 52 °C, respectively, indicated that cholesterol had little effect on the phase-transition temperature. In contrast, no endothermic phase-transition peaks were observed for DSPC/cholesterol/DSPE–PEG2000 = 80/20/1.25 and 70/30/1.25 liposomes. The DSC results agree well with those reported by McMullen et al.[54-56] Cholesterol is localized in the DSPC bilayer at concentrations below ca. 20 mol% [57]. At this cholesterol concentration, cholesterol does not localize in the DSPC membrane but localizes in a formed miscible cholesterol-DSPC domain. Eventually, the lipid bilayer consists of two separate phases at lower cholesterol concentrations: the DSPC phase and the miscible cholesterol–DSPC phase. This led to endothermic peaks for the DSPC phase transition at lower

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cholesterol concentrations. In contrast, delocalization of cholesterol in the lipid bilayer occurs at concentrations

above ca. 20 mol%.[57] The lipid bilayer of the miscible-cholesterol phase at lower cholesterol concentrations lost

ordered DSPC packing and had no DSC endothermic peaks.
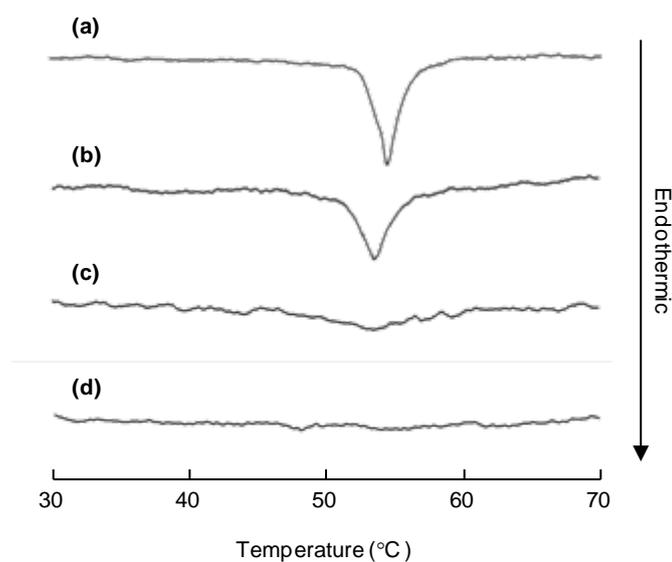
Figure. 5. Effect of the Chol content on the DSC curves of PEG-liposome; **(a)** DSPC/Chol/DSPE-PEG2000 (100/0/1.25), **(b)** DSPC/Chol/DSPE-PEG2000 (90/10/1.25), **(c)** DSPC/Chol/DSPE-PEG2000 (80/20/1.25), and **(d)** DSPC/Chol/DSPE-PEG2000 (70/30/1.25)

The effect of cholesterol in the lipid bilayer on PEG chain flexibility was investigated using ^{1}H NMR

spectroscopy. Figure 6 shows the ^{1}H NMR spectra of PEG in a sample with different amounts of cholesterol.

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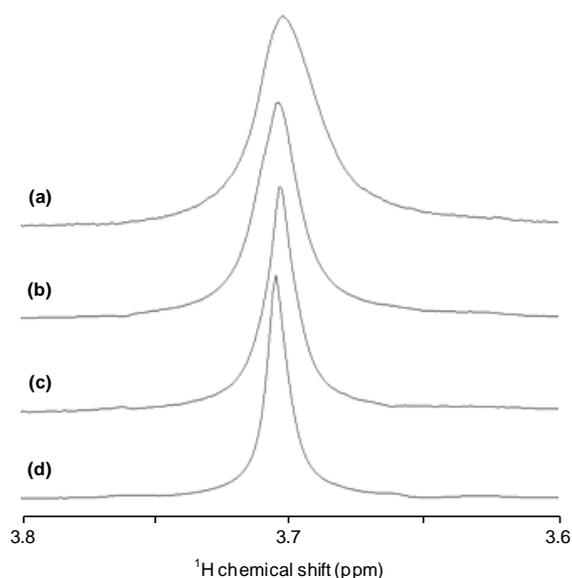
Figure 6. ^{1}H NMR spectra of PEG–liposomes at 3.6–3.8 ppm, showing the PEG peak (35 ºC): (a) DSPC/cholesterol/DSPE–PEG2000 (100/0/1.25), (b) DSPC/cholesterol/DSPE–PEG2000 (90/10/1.25), (c) DSPC/cholesterol/DSPE–PEG2000 (80/20/1.25), and (d) DSPC/cholesterol/DSPE–PEG2000 (70/30/1.25).

The PEG ^{1}H peak sharpened with the incorporation of cholesterol. Furthermore, the line-width significantly decreased with increasing cholesterol amount. This change clearly demonstrated that cholesterol incorporated into the lipid bilayer enhanced the PEG chain flexibility. The incorporation of cholesterol into the lipid bilayer increases the lipid lateral diffusion in the lipid bilayer in the gel phase as mentioned in the *Introduction*. Because DSPE–PEG was stuck in the lipid bilayer, enhanced lateral diffusion on cholesterol addition definitely affected the PEG chain flexibility (Figure 7).

without **cholesterol**　　　　　　with **cholesterol**
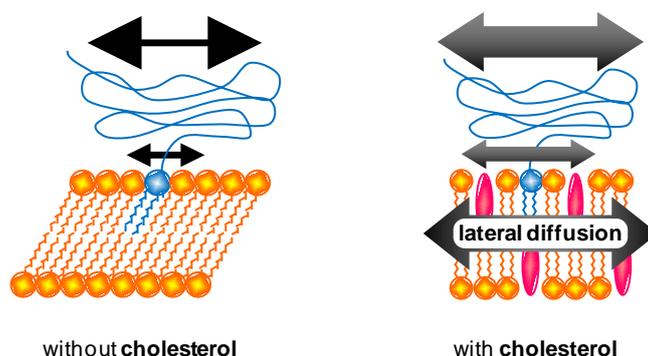
Figure 7. Schematic representation of the structures of the phosphatidylcholine bilayer membranes with DSPE–PEG and cholesterol.

Figure 8 shows the changes in T_2 for PEG on liposome surfaces as a function of temperature. The T_2 for the

PEG–liposomes without cholesterol was constant below 45 ℃ and started to increase at approximately 50 ℃. In

contrast, the T_2 for the PEG–liposomes with cholesterol increased with increasing temperature, especially for

the PEG–liposomes with DSPC/cholesterol/DSPE–PEG2000 compositions of 80/20/1.25 and 70/30/1.25. Wu

et al. evaluated the effect of cholesterol on the lateral diffusion of

1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) in lipid bilayers using fluorescence recovery after

photobleaching.[11] The DMPC diffusion coefficient in the lipid bilayer without cholesterol significantly

increases at the DMPC phase-transition temperature (23 ℃), whereas the diffusion coefficient above or below

the phase-transition temperature is almost constant. In contrast, the DMPC diffusion coefficient in the lipid

bilayer with cholesterol gradually increases with increasing temperature. The effect of cholesterol addition on T_2

changes as a function of temperature (Figure 8), which correlated well with those of the reported lateral

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diffusion coefficient changes in a previous article [11]. The suggestion, that the lateral diffusion of lipids determines the flexibility of PEG on the liposome surface could work in scientific theory. In addition, PEG chain flexibility significantly increased at a cholesterol incorporation of above 20 mol%. DSC (Figure 5) showed that cholesterol was localized in the lipid bilayer at concentrations below 20 mol%, but was delocalized at concentrations above 20 mol%. The small increase in T_2 for the PEG–liposome with 10 mol% cholesterol could be caused by increased local lateral diffusion. The T_2 for PEG–liposomes with more than 20 mol% cholesterol increased markedly because of an overall increase in lateral diffusion. The finding that cholesterol significantly enhances PEG chain flexibility by increasing lateral diffusion is important in understanding the effect of cholesterol incorporation on PEG–liposome stability in blood. It was suggested that the improvement in PEG–liposome stability by cholesterol incorporation could be explained not only by the enhancement of lipid bilayer fluidity but also by increased PEG chain flexibility. The composition of the lipid bilayer, other than cholesterol, possibly influenced the PEG–liposome stability by changing the PEG chain flexibility as well as lipid fluidity.[58, 59]
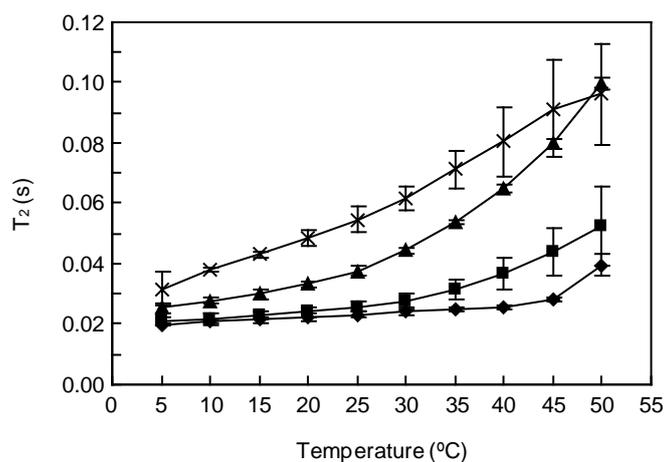
Figure 8. T_2 variations for PEG in PEG–liposomes with various cholesterol contents as a function of temperature ($n = 3$, mean ± standard deviation): (◆) DSPC/cholesterol/DSPE–PEG2000 (100/0/1.25), (■) DSPC/cholesterol/DSPE–PEG2000 (90/10/1.25), (▲) DSPC/cholesterol/DSPE–PEG2000 (80/20/1.25), and (×) DSPC/cholesterol/DSPE–PEG2000 (70/30/1.25).

4. Conclusions

^{1}H NMR studies revealed that the PEG chain flexibility on the surface of ca. 100-nm liposomes changed

depending on the PEG molecular weight and lipid composition. PEG chains with larger molecular weights were

more flexible because of the increased proportion of the flexible PEG component on the liposome surface.

Cholesterol incorporated into the lipid bilayer also enhanced the PEG chain flexibility. Enhanced lipid lateral

diffusion caused by the presence of cholesterol in the lipid bilayer increased the PEG chain flexibility.

Furthermore, the uniform distribution of cholesterol in the lipid bilayer significantly increased the PEG chain

flexibility.

In recent years, formulations with PEG–liposomes of sizes of approximately 100 nm have been developed as

drug delivery system carriers because PEG–liposome formulations improve the stability and circulation lifetime in blood. In this study, we succeeded in demonstrating the PEG chain flexibility on the surfaces of liposomes with several compositions, using variable temperature ^{1}H NMR spectroscopy, including relaxation time evaluation. This finding that cholesterol incorporation changed not only the lipid fluidity but also the PEG chain flexibility on the liposome surface is crucial. The next step is to clarify the relationship between the PEG chain flexibility and circulation lifetime of PEG–liposomes. We expect that the quantitative evaluation of the PEG chain flexibility on liposome surfaces will allow us to further understand the behavior of PEG–liposomes in blood.

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