Transformation from Multilamellar to Unilamellar Vesicles by Addition of a Cationic Lipid to PEGylated Liposomes Explored with Synchrotron Small Angle X-ray Scattering

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Abstract. PEGylated liposomes composed of a benzamidine derivative (TRX), hydrogenated soybean phosphatidylecholine (HSPC), and N-(monomethoxy-polyethylene glycol) carbamyl distearoyl phosphatidylethanolamine (PEG-PE) were examined in terms of how the addition of TRX affects their structures with small angle x-ray scattering (SAXS) as well as transmission electron microscopy (TEM). TEM images showed the presence of unilamella vesicles for both with and without TRX, though a small amount of multilamella vesicles were observed in absence of TRX. We analyzed SAXS profiles at contained TRX composition combined with contrast variation technique by adding PEG solution and unilamella vesicle model could be reproduced. Subsequently, we analyzed SAXS profiles at no TRX composition. The mixture model of unilamella and multilamella vesicle was reconstructed and we estimated about 10 % multilamella vesicles from a fitting parameter.

1. Introduction

Phospholipids have a distinctive propensity to form bilayer membranes in aqueous solutions [1]. This molecular character has been used to prepare lipid vesicles, or liposomes; defined as aqueous compartments enclosed by the bilayer membrane. Liposomes can be formed by sonicating lipid-dispersed solutions to give closed vesicles that are in most case quite uniform in size. The liposomes formed by this method are generally unilamellae and spherical in shape with a diameter of about 50 - 200 nm, denoted by ULV (unilamellar vesicle) [2]. In some cases, concentric multilamellar vesicles (MLV) with an onion-like layered membrane or a large unilamellar vesicle contains several smaller oligo-vesicles without concentric arrangement (OVV) can be formed [3]. On strict definition, only ULM should be called liposomes; however, we treat MLS and OVV as degenerated liposomes.

Liposomes have been used as a drug carrier because of capability to encapsulate hydrophobic compounds within their bilayers [4] as well as hydrophilic compounds within their internal aqueous areas [5]. Liposomes are only a protocol to use in vivo gene delivery system [6-8]. Recently, to prolong their blood circulation time, poly(ethylene glycol) modification (PEGylation) on the liposome surface is commonly applied [9]. PEGylation causes the liposome surface less susceptible to immunological attack and thus to reduce clearance by reticuloendothelial system [10]. The molecular mechanism of such effects is considered owing to steric hindrance or the excluded volume effect of
the hydrated PEG chains on the surface [11]. In addition, PEG has other advantages such as variability in chain length and abundance of protocols for its chemical derivatization [12].

It was reported that benzamidine and benzyle-amine derivatives (the former is denoted by TRX) can be used as a transfection regent with a better efficiency and less toxic than commercial products [13-16]. Among others, Kawahara et al [17] examined pharmacokinetics of PEGylated TRX/HSPC liposome and found that the addition of TRX to PEGylated liposomes induces drastic change in the half-life in the blood circulation. Here, HSPC is hydrogenated soybean phosphatidylcholine that is well-known to form liposomes [18]. Although this change may be due to the cationic nature of added TRX, it is important to examine how addition of TRX changes the liposome structures. This paper examines PEGylated TRX/HSPC liposome in terms of how the addition of TRX affects its structures by using small angle x-ray scattering (SAXS) as well as transmission electron microscopy (TEM).

2. Material and Methods

Material: TRX having two pentadecyloxy tails attaching at meta-position was synthesized with a reported method [19]. N-(monomethoxy-polyethyleneglycolcarbamyl) distearoyl phosphatidylethanolamine (PEG-PE) with a purity of more than 95% was obtained from Genzyme Company (Switzerland) and HSPC was purchased from Lipoid KG (the Netherlands). The chemical structures of these lipids were shown in Figure 1. The PEGylated liposome composed of TRX/HSPC (and of only HSPC for comparison) were prepared with the reported method described in elsewhere [17]. Here, non-PEGylated one was first prepared and secondary filtrated with an extrusion process (Extruder T-10; Lipex Biomembranes, Canada) using a 0.2 µm membrane filters (Whatman, U.S.A.) and PEG-PE was then added at 1.50 mol% and unencapsulated PEG-PE was removed by gel filtration. The compositions of liposomes and code names are shown in Table 1. Phosphate buffered saline (PBS) tablet was purchased from Sigma-Aldrich, Japan and 1/10 PBS at pH = 7.4 was used as a solvent. Homo-polyethylenegycol with the weight average molecular weight of 400 (PEG400) was purchased from Wako Pure Chemical Industries, Ltd.

Characterization of Liposomes: The particle size of PEG-liposome was determined dynamic light scattering (DLS) with a Zetasizer Nano Series (SYSMEX CORPORATION). In this instrument, the scattering angle is fixed at 173° and the obtained time correlation data was analyzed with the cumulant method by use of the attached program to obtain the size distribution profile. Transmission electron microscopy (TEM) was carried out for the specimens made with the following procedure; PEG-liposome solutions were diluted to 0.4 mM with 1/10 PBS then added with an equal volume of 2 % (NH4)2MoO4 solution and left for 1 h to negatively stain the samples.

Figure 1. Chemical structures of lipids used in this study.

Table 1. The compositions of liposomes and their code names.

| sample name | sample      | TRX / HSPC (wt%) |
|-------------|-------------|------------------|
| 0-TRX       | HSPC / PEG-PE | 0                |
| 12-TRX      | HSPC / TRX / PEG-PE | 11.6             |
| 19-TRX      | HSPC / TRX / PEG-PE | 18.7             |
The obtained solution was dropped onto a carbon-coated copper TEM grid and then placed onto the surface of a copper grid for 15 min and the excess solution was drawn off using a filter paper. After freeze drying, TEM images were observed with a JEOL JEM 3010 at an accelerating voltage of 200 kV.

**Small angle x-ray scattering:** SAXS measurements were carried out at 40B2 SPring-8 with 0.7 or 4 m camera using a Rigaku imaging plate (30×30 cm, 3000×3000 pixels) as a detector. The wavelength of the beam was 1.0 or 0.71 Å, the exposure time was 120 sec or 300 sec, and the relative X-ray intensity and the sample transmittance were determined with two ion-chambers located in front of and behind the sample. A bespoke chamber for SAXS measurements was constructed that enables a sample to be introduced in vacuum and scattering measured from it [20]. A quartz capillary cell (diameter of 2 mm, Hilgenberg GmbH) was used for all measurements.

Fitting Models and Electron Density Calculation: The form factor for multilayer spherical model ($P_{V}^{(Pc)}$) is given by Eq 1 [21] [22] as a summation of concentric multiple spheres with number of $k$.

\[
P_{V}(q) \propto \left[ \sum_{i=1}^{k} \frac{(\rho_{i} - \rho_{i+1})V_{i}j_{1}(qR_{i})}{qR_{i}} \right]^{2}
\]

Here, $j_{1}$ is the first order spherical Bessel function. $\rho_{i}$, $V_{i}$, and $R_{i}$ are the electron density, the volume, the radius of the $i$-th sphere, respectively. $\rho_{i+1}$ is the electron density of solvents (i.e., $\rho_{i+1} = \rho_{sol}$) in this equation, the width of the $i$-th layer ($t_{i}$) is given by $t_{i} = R_{i} - R_{i-1}$ except for $i = 1$, since the first layer is essentially the inner compartment of the liposome. When applied to the data, we assume the Gaussian distribution only for $R_{1}$. One serious problem to use the multilayer model is that there are too many adjustable parameters to obtain a unique solution. Therefore, in order to reduce mathematical degree of freedom in Eq 1, we need rules and relation to correlate the parameters to real molecules.

The solvent electron densities of PBS and after PEG 400 added were calculated from the following equation;

\[
\rho = d \sum_{i} \rho_{w,i}w_{i}
\]

Here, $d$ (g/nm$^3$) is the density of the mixed solvent, and $\rho_{w,i}$ (e/g) and $w_{i}$ are electron density per weight and the weight fraction for the $i$ component, respectively. We applied a contrast variation technique to SAXS by adding PEG 400 to the solvent [23, 24]. PEG has a larger electron density than water ($\rho_{water} = 334$ and $\rho_{PEG} = 370$ e/nm$^3$), since they contain oxygen atoms which have a relatively larger scattering length than carbon and hydrogen atoms [25].

3. Results

3-1: Particle size analyses and electron microscopy

The obtained autocorrelation functions are plotted against the delay time in Figure 2a and the size-distributions determined are plotted in Figure 2b for 0-, 12-, and 19-TRX. Although three samples showed the similar narrow distribution curves around 100 nm, 0-TRX was slightly larger than the others. The peak-top sizes are 101, 87 and 84 nm, respectively. The narrow distribution is attributed to sonicating and filtrating (with a 0.2 µm membrane) treatments after the lipids were dispersed. We added PEG-PE after the filtration. This treatment did not change the size [17]. The small but appreciable difference between the presence and absence of TRX suggests that TRX can differentiate the liposome structures.
Figure 3 compares the TEM images for the three liposomes. All of them showed a typical ring image for ULV liposome with a diameter around 100 nm. These sizes are consistent with the DLS results. When we carefully examined the images for 0-TRX, we found that a few of rings consisted of double line or their width broader than those of 12- and 19-TRX. The width of the ring for 12- and 19-TRX was about 7 nm, which is consistent with the molecular size of bilayers [26], while some of 0-TRX were about 13 nm as indicated in the Figure 3, almost twice of 12- and 19-TRX. This fact indicated that 0-TRX liposomes contain MLV. We sometimes clearly observed double ring images for 0-TRX although their population was seemed to be minor. However, based on TEM observation, it seemed difficult to obtain quantitative information.

3-2: Small angle X-ray scattering

Figure 4a compares the SAXS profiles for the three liposomes with the same concentration (10 mM) and Figure 4b and c show the concentration dependence of 0- and 12-TRX. The profiles of 12- and 19-TRX in Figure 4a show a typical scattering from ULV, i.e., the scattering from the outermost sphere at $q < 0.3 \text{ nm}^{-1}$ and the large undulations from bilayer membrane around $0.3 < q < 2 \text{ nm}^{-1}$ and smaller ones at $q > 2 \text{ nm}^{-1}$. The Ginner region must be observed below the measuring range judging from the size of the liposome of 70-80 nm (the Ginner region for this size is $q < 0.04 - 0.05 \text{ nm}^{-1}$). The large undulation is reflecting from the cross sectional structure of the liposome. The smaller ones at the larger $q$ region evidence that the structure is almost uniquely determined by the composition and the fluctuation and distribution are relatively small.

0-TRX has the almost same feature with 12- and 19-TRX, except for a different shape of the large undulation. It seems that there is a shoulder around $q = 1.0 \text{ nm}^{-1}$. This type of shoulder can be due to diffraction between liposomes due to infinite concentrations. Eguchi et al [27] extensively studied the concentration dependence of the SAXS from charged micelles and found that the similar shoulder
appeared on the large undulation with increasing the concentration due to diffraction (i.e., disappeared at low concentration and the scattering merged into the form factor around \( C = 7.6 \) wt % [27]). Figure 4b shows that the shoulder did not disappeared even at 0.22 mM (0.02 wt %), at which concentration should be low enough to eliminate the inter-particle diffraction. This fact leads that the shoulder can be ascribed to the MLV structure observed with TEM.

![Figure 4.](image-url) (a) Scattering profiles combined SAXS and USAXS. Comparison of the concentration dependence of the scattering profiles between 0-TRX (b) and 12-TRX (c). The arrows indicate the shoulder that was not observed in 12-TRX.

4. Discussions

4-1 : Contrast variation analysis of SAXS from TRX-12

A straightforward analysis might fit first for the 0-TRX scattering and then for 12-TRX by referencing the 0-TRX fitting parameters, because 0-TRX has less components than 12-TRX and thus might be simpler than 12-TRX. However, TEM showed presence of MLV in 0-TRX, meaning more fitting parameters, while 12-TRX presumably consists of only ULV, less fitting parameters. Therefore, we decided to analyze 12-TRX first.

As mentioned in the experimental section as well as depicted in Figure 5a, to describe ULV with a multilayer model, we need to set \( k = 5 \) in Eq 1 with having four geometric parameters; \( t_1 \): inner diameter of the liposome, \( t_2 \) and \( t_4 \) (assumed \( t_2 = t_4 \)): the headgroup domain width, \( t_3 \): the alkyl chain domain width, four electron densities (\( \rho_1, \rho_2, \rho_3 \) and \( \rho_4 \)), and \( \rho_{\text{sol}} \): the solvent density (assumed \( \rho_1 = \rho_{\text{sol}} \)), corresponding each layer. Additionally, since we can suppose the PEG block of PEG-PE forms a layer outside the outer head group, we introduced two parameters: the PEG layer width (\( t_5 \)) and its electron density, (\( \rho_5 \)) as presented in Figure 5a. Among these parameters, there are rules to follow in terms of molecular characters and Eq 2. The electron densities of bulk PEG and the PBS solvent are known as \( \rho_{\text{PEG}} = 370 \) e/nm\(^3\) and \( \rho_{\text{PBS}} = 335 \) e/nm\(^3\), respectively and thus \( \rho_{\text{sol}} < \rho_5 < \rho_{\text{PEG}} \) because of the PEG dissolving in PBS. Furthermore, \( \rho_{\text{sol}} < \rho_3 \), or \( \rho_1 \) and \( \rho_5 < \rho_{\text{sol}} \) should be hold owing to their constituent elements [23]. Although simple liposomes without PEG layers requires \( \rho_2 = \rho_4 \) due to symmetrical bilayer structures, this relation is not necessary maintained for PEGlyated ones since the phosphoric group or hydrophobic tails are contained only in the 4-th layer.

Even with those rules to reduce freedom of the fitting parameters, there were too may adjustable parameters to obtain a unique combination of them. To solve this problem, we used a contrast variation technique by adding PEG to solvent. If the addition of PEG did not alter the liposome...
structures, we should be able to fit the all data with the same parameters except for $\rho_{\text{sol}}$ that is determined by the solvent composition through Eq 2.

Figure 5b presents how the scattering profile was changed by addition of PEG 400. With adding PEG 400 to 12-TRX, a dent-like depression in the profile around $q = 0.4$ nm$^{-1}$ become less obvious and almost filled up at PEG = 9.5 M (39 wt%). The addition of PEG400 changed $\rho_{\text{sol}}$ from 335 to 385 e/nm$^3$. For each profile, we made an attempt to fit all the data with same parameters with titrating [28]. The resultant theoretical curves are compared with the data in Figure 5b and obtained parameters are listed in Table 2. Except for the smaller undulation region, the agreement is good, indicating the 5th-layer model is good enough to describe the 12-TRX. The smaller undulation region reflects more detailed atomic structures and thus the simple multilayer model may be too course to reproduce these region. We may need a more elaborate method such as molecular dynamic calculation.

In order to confirm the parameters in Table 2, Figure 5c compares the calculated values with the data when other parameters were used. In Table 2, we conclude $t_1 = 20 \pm 1$ nm. When we substitute $t_1 = 22$ nm at $C_{\text{PEG}} = 0$, it is clear that $t_1 = 22$ nm cannot reproduce the intensity around $0.03 \text{ nm}^{-1} < q < 0.4 \text{ nm}^{-1}$. In the case of $t_5 = 5$ at $C_{\text{PEG}} = 0$, the theory did not agree with the data around $0.03 \text{ nm}^{-1} < q < 0.4 \text{ nm}^{-1}$. The inner headgroup density was smaller than that of the outer one by about 24 e/nm$^3$ for all samples. If we fitted in the condition of $\rho_2 = \rho_3$, the resultant curve did not fit the data in the same level of $\rho_2 \neq \rho_4$. The disagreement of then may be explained that the outer headgroup incorporates the phosphor-ester moiety of PEG-PE or inner headgroup incorporates the hydrophobic tails. The width of the headgroup and its electron density, and those of the alkyl domain take reasonable values in terms of their molecular structures [26]. Since PEG400 was added after liposome formation, we expected that PEG400 cannot enter the inner solvent compartment. For this case, we have to fix $\rho_1 = \rho_{\text{PBS}}$ for all fitting, however, if do so, the agreement became poor. We assume that the added PEG 400 could enter the inside, probably through fusion between liposomes. Although PEG 400 can be at inside, we cannot observed any evidence that PEG-PE anchors on the inner layer. Based on these above discussions, we can conclude this section that the addition of PEG 400 did not alter the liposome structure and only change the solvent electron density. By taking advantage, all of the structural parameters were uniquely determined.

(a) (b) (c)

**Figure 5.** (a) A schematics for the multi spherical model ($k = 5$ in Eq 1) used to fit 12-TRX. (b) Changes of the scattering profiles of 12-TRX when PEG400 was added to solution at a fixed lipid concentration (gray lines) and their best fit curves (black line) calculated from Eq 1. (c) Theoretical curves when one of the fitting parameter was changed from the best fit combination, compared with the data.
According to the 7th-layer model, the origin of the shoulder is diffraction between the three layers. These results indicate that 0-TRX consist of the mixtures of UMV and double MLV and the volume than that of 12-TRX. This reason is not clear at this moment, but it probably due to our assumption the double lamella vesicle and the second and third headgroup domain are located close enough to give one layer for scattering. This assumption is reasonable since 0-TRX has no charged headgroup to induce repulsive interaction. We tried to fit the data with only the 5th-layer model (Figure 6c, bottom), but the shoulder at \( q = 1.0 \text{ nm}^{-1} \) was more enhanced than the experiments. According to the 7th-layer model, the origin of the shoulder is diffraction between the three layers. These results indicates that 0-TRX consist of the mixtures of UMV and double MLV and the volume fraction is defined by \( \psi \).

\[
I(q) = \Psi \left[ \sum_{i=1}^{5} \frac{V_i(\rho_{t_i} - \rho_{t+1})j_i(qR_i)}{qR_i} \right]^2 + (1 - \Psi) \left[ \sum_{i=1}^{7} \frac{V_i(\rho_{t_i} - \rho_{t+1})j_i(qR_i)}{qR_i} \right]^2
\]  

(3)

**Table 2. Characteristics of 12-TRX**

| Solvent            | \( t_1 \) | \( t_3 \) | \( t_2 \) or \( t_4 \) | \( t_5 \) | \( \rho_2 \) | \( \rho_3 \) | \( \rho_4 \) | \( \rho_5 \) | \( \rho_{\text{sol}} \) |
|--------------------|----------|----------|----------------|--------|----------|----------|----------|----------|----------|
| PBS                | 20       | 2.7      | 1.8            | 9      | 475      | 200      | 500      | 340      | 335      |
| 0.96M PEG, PBS     | 20       | 2.6      | 2              | 9      | 475      | 200      | 500      | 340      | 340      |
| 1.9M PEG, PBS      | 20       | 2.5      | 1.9            | 9      | 475      | 200      | 500      | 348      | 345      |
| 3.8M PEG, PBS      | 20       | 2.6      | 1.9            | 9      | 485      | 200      | 500      | 356      | 353      |
| 4.8M PEG, PBS      | 17       | 2.6      | 1.9            | 9      | 485      | 200      | 510      | 363      | 357      |
| 9.5M PEG, PBS      | 20       | 2.6      | 1.9            | 9      | 475      | 200      | 495      | 385      | 385      |

The units of \( t \) and \( \rho \) are nm and e / nm\(^3\), respectively.

### 4-2 : Analysis of MLV of 0-TRX

To describe MLV (in our case two lamellae), we supposed \( k = 7 \) as presented in Figure 6a, where we assume the double lamella vesicle and the second and third headgroup domain are located close enough to give one layer for scattering. This assumption is reasonable since 0-TRX has no charged headgroup to induce repulsive interaction. We tried to fit the data with only the 5th-layer model (Figure 6c, bottom), but the shoulder at \( q = 1.0 \text{ nm}^{-1} \) was more enhanced than the experiments. According to the 7th-layer model, the origin of the shoulder is diffraction between the three layers. These results indicates that 0-TRX consist of the mixtures of UMV and double MLV and the volume fraction is defined by \( \psi \).

\[
I(q) = \Psi \left[ \sum_{i=1}^{5} \frac{V_i(\rho_{t_i} - \rho_{t+1})j_i(qR_i)}{qR_i} \right]^2 + (1 - \Psi) \left[ \sum_{i=1}^{7} \frac{V_i(\rho_{t_i} - \rho_{t+1})j_i(qR_i)}{qR_i} \right]^2
\]  

(3)

**Figure 6.** A model used to fit 0-TRX (a). Comparison of the data and theoretical curve calculated from Eq 3 that is a combination of \( k = 5 \) and \( k = 7 \) in Eq 3 (b). The panel (c) shows that the data can be fitted with neither \( k = 5 \) nor \( k = 7 \).

When we use this combinational equation, the experimental data was well fitted as presented in Figure 6b and the used parameters are listed in Table 3. Compared these values with Tabe 3, it can be seen that the outer and middle headgroup’s electron densities for 0-TRX were larger than that of 12-TRX. This reason is not clear at this moment, but it probably due to our
assumption that the second and third head domains were treated on layer. Even though this, the scattering profile was well reproduced with this model and we can conclude that this combination model can describe the 0-TRX. According to our analysis, about 1-ψ = 0.071 of liposomes took MLV.

**Table 3. Characteristics of 0-TRX**

| Solvent | $t_1$ | $t_2$ or $t_3$ | $t_4$ | $t_5$ | $t_6$ | $\rho_2$ | $\rho_3$ or $\rho_5$ | $\rho_4$ | $\rho_6$ | $\rho_7$ | $\rho_{\text{sol}}$ |
|---------|-------|----------------|-------|-------|-------|----------|----------------------|----------|----------|----------|-------------|
| PBS     | 20    | 1.6            | 2.5   | 4     | 9     | 490      | 200                  | 550      | 530      | 340      | 335         |

The units of $t$ and $\rho$ are nm and e/ nm$^3$, respectively.

4-3 : Other general discussions

DLS showed that 0-TRX always showed a larger particle size than 12- and 19-TRX, even all of them were filtered with the same pore size. When liposomes go through the small pore, their spherical shape might deform to prolate to go through the pore easily. MLV may be harder to deform than UMV, and thus after filtration UMV may be larger than MLV. Nevertheless, the result is opposite. This difference may be due to decrease of membrane flexibility by adding TRX to HSPC.

A grafted PEG chain on interface can classify two types [29]. One is isolated chain on the surface called “mushrooms” and the other is closely-spaced chains called “brush”. The width of the PEG layer is 9 nm for our system. According to previous work [30], the radius of gyration of PEG 5000 is about 3.08 nm. By comparing these values, we can conclude the PEG on the liposome surface is in the brush state. From the $\rho_3$ and $\rho_{\text{PEG}}$, we can evaluate the PEG volume percent in the PEG layer and roughly estimated how many PEG-PE molecules anchored in one liposome. This average number is estimated to be about 850.

5. Conclusions

The present paper examined the structural changes of the PEG-liposomes when TRX was added. TEM showed the presence of ULV liposomes with a diameter around 100nm for all the compositions. These results were consisted with the DLS results. TEM also indicated that 0-TRX liposomes contained some MLVs, while 12- and 19-TRX did not. When we analyzed the SAXS profiles from 12-TRX combined with the contrast variation technique, the SAXS data was consistently explained with a ULV model. For 0-TRX, when we assumed a mixture of ULV and MLV, the data could be reconstructed.
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