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Competition of PEG Coverage Density and Con-A Recognition in Mannose/PEG Bearing Nanoparticles

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Abstract

Cell specific ligand molecules are attached to polyethylene glycol (PEG) modified nanoparticles to enhance the drug delivery efficiency. The tethered PEG would interfere in ligand recognition as well as providing biocompatibility to the nanoparticles. The denser PEG can give the greater biocompatibility, while should more hamper the ligand recognition. Therefore it is important to tune PEG density at an appropriate amount to compensate these two factors. In this study, we prepared a series of nanoparticles composed of α -mannose-bearing lipid, dioleoyltrimethyl ammoniumpropane (DOTAP) and 1,2-Distearoyl-*sn*-Glycerol-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000] (DSPE-PEG₂₀₀₀). The nanoparticles were characterized with dynamic light scattering (DLS), field flow fractionation (FFF), small angle X-ray scattering (SAXS), and quartz-crystal microbalance (QCM). Based on the structural parameter obtained from DLS, SAXS, and FFF, we determined the PEG crowding parameter (σ) quantitatively; when $\sigma = 1.0$, the PEG chain occupies the same value on the surface as when the chain is present in the unperturbed state, and at $\sigma > 1.0$, the PEG chains start to contact each other. We found that the recognition ability had the maximum around $\sigma \sim 0.75$ and there was the critical composition at $\sigma = 1.0$ for which the recognition was drastically reduced. The present results demonstrated that quantitative characterization and controlling the PEG density are key to designing effective targeting delivery system.

Keywords

PEG coverage density, field flow fractionation, dynamic light scattering, small angle X-ray scattering, quartz-crystal microbalance,

1. Introduction

Polyethylene glycol (PEG)-coated liposomal nanoparticles (PCNs) are used as efficient drug delivery systems because PEG provides stability in biological liquids and thus high circulation times in blood. [1, 2, 3] PEG on nanoparticles prevents opsonization, i.e., non-specific binding of immunoglobulins and other proteins such as C3b to the hydrophobic portions of the particles. The molecular mechanism of this prevention is presumably related to the combination of the steric hindrance of PEG and water molecules bound to PEG, which is called the stealth effect of PEG. Many studies have shown that the characteristic of PEG over-crowding on the PCN surface is important for the stealth effect. Despite these advantages, when compared to small molecule targeted drugs such as imatinib mesylate (Gleevec, also known as STI-571), the lack of selectivity for targeting PCNs still remains a major drawback. [4, 5] To provide selectivity to PCNs, conjugating ligands, such as antibodies, and targeting peptides has been proposed. However, such conjugations have not always shown successful outcomes. Recently, Stefanick et al [6] demonstrated that the appropriate peptide linker length together with the appropriate PEG coating and optimal ligand density is important to provide optimal drug efficacy. This is because the PEG chains interfere with the interaction between the ligands and the targeting receptor in addition to providing the stealth effect to PCNs. Their work clearly demonstrated that the recognition of ligands on the PCN surface is determined by the relationship between PEG density and ligand density. **Many pioneering studies have addressed the importance of PEG properties with respect to PCNs. [7, 8, 9] Recently, Rabanel et al. [10] reviewed the techniques used to qualitatively or quantitatively assess the effects of PEG chain coverage density, conformation, and layer thickness on polymeric nanoparticles. However, these previous studies have not quantitatively determined PEG coverage density using the scattering technique or compared it with ligand-recognition ability.**

PEG coverage density has been quantitatively studied in the field of surface chemistry. The parameter σ determined by the following equation is used to express the crowding nature of PEG. [11]

$$\sigma = \frac{\pi R_g^2}{S_c/N} \quad (1)$$

Here, R_g , S_c , and N are the radius of gyration of the free PEG chain with the same molecular weight of the PEG tethered on the surface, the surface (or interface) area covered by the PEG chains, and the number of PEG chains tethered on the surface. σ is the area ratio of the free PEG chains to the tethered PEG chains, meaning the number of tethered chains in the area πR_g^2 covered by one chain in an unperturbed conformation in the same solvent. [11] When $\sigma = 1$, a tethered chain occupies the same value on the surface as when the chain is present in the unperturbed state. At $\sigma > 1$, the adjacent chains start to contact each other and change their conformation and overlap. According to a previous study, [11] when σ exceeds 4, the PEG chains start to cross-over, and the change in the surface property caused by the cross-over can be observed experimentally. When the value exceeds 10, the PEG chains are highly stretched perpendicular to the interface, and the surface property shows different characteristic features from those at $\sigma \sim 1$. Tockary et al determined σ on a pDNA polyplex and found that σ is directly related to the lifetime in blood circulation. [12] More recently, Shiraishi et al [13] evaluated the structural features of PEG-bearing polymeric micelles, including core size and aggregation number (N_{agg}), using small-angle X-ray scattering (SAXS), field flow fractionation with multi-angle light scattering (FFF-MALS) analysis, and dynamic light scattering (DLS). They accurately determined σ and found that σ is directly related to the micelles' *in vivo* pharmacokinetic behavior.

The major aim of this paper is to determine σ and the ligand density and then quantitatively express the ligand recognition ability as a function of σ . In order to determine the PEG coverage density based on Eq 1, we need to determine the structural parameters of PCNs and N_{agg} . To do so, we combined SAXS, DLS, and FFF-MALS measurements. As a model system for PCNs, we used an

α -mannose-modified aromatic lipid (denoted α -Man), as shown in S1. In a previous paper,[14] we described the synthetic procedure for α -Man and demonstrated that a two-component micelle made from a cationic lipid, dioleoyltrimethylammoniumpropane (DOTAP), and α -Man formed an ion complex with pDNA. The resultant complex showed a high transfection efficiency to RAW264 cells through the mannose lectins of the cells. In the present paper, we add a PEG-bearing lipid to this DOTAP/ α -Man system and evaluate how the PEG density influences the α -mannose recognition.

2. Experimental Section

Materials. DOTAP and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG₂₀₀₀) were obtained from Sigma Aldrich (St. Louis, MO, USA) and Avanti (Alabaster, AL, USA), respectively, and used without further purification. α -Man was synthesized following methods described previously [14]; briefly, 2,3,4,6-tetra-*o*-benzyl-D-mannopyranose and diphenyl sulfoxide were reacted, and the resultant solution was added to trifluoromethanesulfonic anhydride, and then to trimethylsilyl azide to obtain 1-azido-2,3,4,6-tetra-*o*-benzyloxy-6-D-mannopyranose. The α -azido-mannose was attached to an aromatic ring with C12 in the meta-meta position using Crick chemistry to obtain α -Man. Concanavalin A (Con A) was purchased from WAKO (Tokyo, Japan).

Micelle Preparation. α -Man and DOTAP were mixed, dissolved in chloroform, and vacuum dried. Each mixture was dissolved in water and added to DSPE-PEG₂₀₀₀ at various ratios. The molar compositions of these ternary mixtures are described as DSPE-PEG₂₀₀₀/ α -Man/DOTAP = $\alpha/\beta/\chi$, e.g., 1.0/0.50/0.50, where $\beta = \chi$, for which the pDNA transfection efficiency was maximal [14]. After the addition of DSPE-PEG₂₀₀₀, the solution was maintained at 37°C for 8 h to stabilize the structure. These particles were denoted PMD(x) using the molar ratio, where $x = \alpha/\beta$, and the composition ratio X was determined as $X = M_w(\text{DSPE-PEG}_{2000}) \times \alpha / M_w(\alpha\text{-Man})$ using the molar weight of the particle.

Field Flow Fractionation (FFF) coupled with Light Scattering (LS), and Dynamic Light Scattering (DLS): An Eclipse 3+ separation system (Wyatt Technology Europe, Dernbach, Germany) was used for FFF, which was sequentially connected to a Dawn Heleos II Multi-Angle Static Light Scattering (SLS) Detector (Wyatt Technology) and an Optilab rEX DSP Differential Refractive Index (RI) Detector (Wyatt Technology) operating at a wavelength of 658 nm, in that order, from the upper stream. A Wyatt channel (Eclipse 3 channel LC) was used, which had a tip-to-tip length of 17.4 cm and a nominal thickness of 250 μm , and a membrane (Nadir Cellulose Membrane, 10 kDa LC) was attached to the bottom of the channel. Samples were injected into the system and the injected solution was kept at a single specified area on the membrane before fractionation. This process is called “sample focusing.” After focusing, two flows were applied: the laminar (or channel) flow, which carries the sample through the separation chamber, and the cross flow, which refers to the separation field applied perpendicular to the channel. The rate of cross flow can be kept constant or systematically changed; normally, it is gradually decreased. The cross flow pushes the particles toward the bottom of the channel where a membrane filter is attached. The extent to which particles can diffuse back into the channel is determined by the combination of the diffusion coefficient of the particles and the cross flow rate. For more details regarding FFF, see references [15, 16].

The specific refractive index increment ($\partial n / \partial c$) was determined for each sample with a DRM-1021 Differential Refractometer (Otuska Electronics, Osaka, Japan). The obtained value was used to determine the weight-averaged molar masses (M_w) and the radius of gyration (R_g). The hydrodynamic radius (R_H) was determined with a DLS (Malvern Instruments, Malvern, UK).

Quartz-Crystal Microbalance (QCM): ConA interactions were examined with QCM. ConA was immobilized on a gold substrate in a quartz-crystal microbalance sensor cell (AFFINIXQN μ ; INITIUM, Inc., Tokyo, Japan) at 10 $\mu\text{g/mL}$ for 1 h at room temperature. After blocking with 1% bovine serum albumin in phosphate-buffered saline for 1 h, the sensor cell was filled with 500 μL of 10 mM HEPES containing 2 mM CaCl_2 ; samples (36 $\mu\text{g/mL}$) were added and the frequency changes at 25°C was measured.

Synchrotron SAXS Measurements: SAXS measurements were performed at BL-40B2 of SPring-8, Japan. [17] A 30 cm $\times$ 30 cm imaging plate (Rigaku R-Axis VII; Tokyo, Japan) detector was placed at 1.65 m from the sample. The wavelengths of the incident beam (λ) were 0.10 nm. A modified SAXS vacuum sample chamber [18] was used and the X-ray transmittance of the samples was determined with an ion chamber located in front of the sample and a Si photodiode for X-rays (Hamamatsu Photonics S8193; Hamamatsu, Japan). The sample concentration was 1 mM in 150 mM NaCl and the exposure time was 300 s for all measurements. The scattering intensity $I(q)$ was measured as a function of the scattering angle 2θ and 2θ was converted to the magnitude of the scattering vector (q) according to $q = (2\pi/\lambda)\sin\theta$.

3. Results and Discussion

Synthesis of α -Man lipids:

An α -mannose-modified aromatic lipid (α -Man, Figure S1) was synthesized following methods described previously [14]. Based on the ^1H NMR spectrum and an elemental analysis, we confirmed that we indeed obtained the correct compound. Eleven samples with different X values were obtained, as presented in the first column of Table 1. All solutions (150 mM NaCl) were transparent and no precipitate was observed. Before DLS, FFF, and QCM, the solutions were filtered through a 1.0- μm membrane (GF013100, Glass Fiber).

Characterizing PMD particles:

DLS: Figures 1A and 1B show the first-order autocorrelation function $[G^2(\tau) - 1]$ for PMD(1.00) and the size distribution determined using a Cumulant analysis [19] implemented in Malvern software. The data points in Figure 1A can be fitted by a straight line, suggesting that the PMD(1.00) particles were almost monodisperse. From the slope of this fitted line, we evaluated the translational diffusion coefficient, and R_H was then obtained using the Stokes–Einstein equation.[19] This R_H value agreed with the maximal value in Figure 1B, which shows a rather broad distribution compared with the single exponential decay in Figure 1A. We suppose that the Cumulant analysis resulted in instrument-related overestimates of the size distribution by considering background noise as meaningful signal. The other samples showed almost the same features, and the results are summarized in the second column in Table 1. Figure 1C plots R_H against X . With an increase in X , R_H decreased from 30 nm to 14 nm. This can be ascribed to the increase in bulky DSPE-PEG₂₀₀₀ possibly reducing the aggregation power, which was confirmed with SAXS. This issue will be discussed later.

FFF coupled with SLS: Figure 2A shows a typical chromatogram of PMD particles measured using RI when the cross flow was 4.2 mL/min. At this flow rate, PMD particles were not eluted from the channel, and only free DSPE-PEG₂₀₀₀ was observed. The reason for this result is the free DSPE-PEG₂₀₀₀ molecule has a larger diffusion coefficient and thus can move back from the surface of the membrane and then be eluted by the channel flow, while due to the small diffusion coefficients of PMD particles, they never diffuse back from the bottom. By subtracting the baseline, we calculated the amount of free DSPE-PEG₂₀₀₀. S2 (A) plots the molar ratio of the free to bound DSPE-PEG₂₀₀₀ on PMD particles. Although with an increase in X the amount of free DSPE-PEG₂₀₀₀ slightly increased, most of the added DSPE-PEG₂₀₀₀ was incorporated into the PMD particles, because of the conjugation ratio was still around 80% at $X=8.0$. As mentioned in the experimental section, the solution was kept at 37°C for 8 hr after adding DSPE-PEG₂₀₀₀. This process was found to be important to reduce the free DSPE-PEG₂₀₀₀ and to obtain reproducible results.

Figure 2B shows chromatograms of PMD(1.00) measured using the reflective index and light scattering at a cross flow of 1.0 mL/min. When we lowered the cross flow to 1.0 mL/min, the PMD particles were eluted from the channel and became observable with RI and light scattering (LS). As shown in the supplementary information, the shape of the PMD peak depended on the flow rate. The reason for this relationship is that the PMD particles may be deformed during the focusing because of the interaction, mostly hydrophobic interaction, with the membrane attached to the bottom of the FFF device. During the focusing, an opposite current for the carrier solvent is used to focus particles to one specified area. This procedure is necessary to prevent peak broadening. However, the focusing push of all of the particles on the membrane and the local concentration are dramatically increased. Therefore, deformation of the micellar structures and/or lead secondary aggregation may be caused. This is a drawback of FFF called the “focusing problem”. As shown in Figure 2B, there was a shoulder at early time points in RI, and there were broad second peaks at later time points in both RI and LS. There was no LS signal for the first shoulder, meaning that these were

low-molecular-weight components. Therefore, the first shoulder can be ascribed to de-complexed lipid components due to focusing. The molar masses of the broad second peaks were much larger than that of the main peak, and their shapes were dependent on the focusing conditions and cross flow rate, indicating that these peaks can be ascribed to secondary aggregation formed during the focusing. We determined the molar masses for each fraction of the main peak and plotted them in **Figure 2B**. The molar masses ranged from 1.0×10^6 to 3.0×10^6 , and the average molar mass was calculated as M_W . The resultant values for all samples are summarized in Table 1. S2 (B) plots the obtained M_W against X , indicating that M_W increased first and reached the maximum at $X=2.0$ and then decreased with an increase in X . The initial increase may be ascribed to incorporation of the large molar mass of DSPE-PEG₂₀₀₀. The decreasing molar mass confirms that the addition of DSPE-PEG₂₀₀₀ reduces the aggregation power of the other lipids.

We found that the M_W values changed little with different cross flows or focusing conditions. We determined R_g from the slope of the angular dependence of LS intensities. It is well known that R_g values become z-averaged for polydisperse samples, and R_g is quite sensitive to the shape of the scattering objects. [19] In fact, its values changed with different cross flows or focusing conditions. Therefore, for the present system, the molar mass may be reliable, but the distribution and the R_g are not. For reasonably narrow disperse solid spheres (i.e., unique electron density), the relation of $R_h = \sqrt{5/3} R_g$ would hold. [20] This is not the case for the present system, probably because of the focusing problem and because the PMD particles do not form sufficient complexed structures to be regarded as solid sphere PMD particles.

Synchrotron SAXS: SAXS profiles for some of the PMD particles are compared in Figure 3. Before the addition of DSPE-PEG₂₀₀₀ (PMD(0.00)), shape diffraction peaks were observed, and the peak positions satisfied the relation of $1:\sqrt{3}:2:\sqrt{7}$, indicating formation of a hexagonally packed cylindrical structure.[20] When we added DSPE-PEG₂₀₀₀, the hexagonal diffraction peaks became small, and a broad maximum presumably ascribed to the form factor became large. At $x = 1.00$, there was still the first peak observed at $q = 1.16 \text{ nm}^{-1}$, and the peak position was almost the same as $x = 0.10$. From the peak position, we estimated the cylinder radius to be about 3 nm. At $x = 0.25$, the peak position seemed to shift to a large q by about 0.1 nm^{-1} , but we assume that this is due to experimental error since the others did not show any shift in the peak position. It should be mentioned that most SAXS profiles, especially for PMD(0.20)–PMD(1.00), showed an upward curvature at the low- q range. This upward curvature can be considered part of the form factor of isolated scattering objects, most likely the Guinier region (i.e., $I(q) = I(q \rightarrow 0)\exp(-\frac{q^2 R_g^2}{3})$) for spherical particles.[19,20] This feature suggests that the overall morphology of the PMD particles was spherical.

SAXS clearly showed that the particles were not vesicles, rods, plates, or nano-gels based on the low- q slope; each of these shapes should show a characteristic low- q behavior, for example, $I(q) \propto q^{-2}$ for plates [20]. Given their upward curvatures, it is safe to assume that they were spherical aggregates. The high- q SAXS data reflect the inner structure of the spherical PMD particles. The presence of the inter-cylinder diffraction indicates that cylindrical tubes were densely entangled within the particle, similar to a densely packed coiled thread, as shown schematically in Figure 3.

From the SAXS profiles and referring to the change in R_h and M_w , we can propose the following morphological changes induced by the addition of. Based on DLS and SLS, the PMD particles have an infinite size, and presumably, they have a spherical shape with a size ranging from 15 – 30 nm in radius. SAXS showed the presence of a cylinder with a size of about 3 nm in radius. Therefore, cylindrical tubes are closely packed inside the spherical PMD particle, just like a densely packed coiled thread. The addition of DSPE-PEG₂₀₀₀ reduced the overall size, but it did not change the cylinder distance. This result means that DSPE-PEG₂₀₀₀ was not highly incorporated inside the particles; otherwise, the inter-cylinder peak positions would have moved to a large q . The added DSPE-PEG₂₀₀₀ mainly attached to the outer side of the particles and enhanced the compatibility with water. This explains the decrease in particle size as X increased. We can also expect a small quantity of PEG to be incorporated into the inside of the particles. This causes a disordering of the hexagonal packing as well as a reduction in aggregation ability owing to the hydrophilicity of PEG. This is another reason for the inverse relationship between size and X .

Recognition assay with Con-A using QCM

Figure 4A plots the frequency time-changes (ΔF) of the Con A-coated QCM sensor after immersion in PMD particle solutions. For all samples, the frequencies immediately dropped after immersion and then reached an equilibrium after 800 s. Here, we confirmed that the changes due to non-specific interactions were negligibly small. Figure 4B plots the equilibrium frequency changes against X , showing that ΔF sharply dropped to around $X = 1.0$ because the addition of DSPE-PEG₂₀₀₀ interfered with the recognition of mannose by Con A.

Determination of the PEG coverage density and its relationship with recognition

In order to evaluate the PEG coverage density from Eq 1, we need to know both S_C and N . N can be calculated from the bound PEG ratio and M_W . If we knew the core radius (R_C) for when PMD particles are regarded as a core-shell sphere, S_C could be calculated from $S_C = 4\pi(R_C)^2$. The relation of $R_C < R_H < R_S$ is generally held, where R_S is the overall micelle size, including the PEG shell. Especially for less PEG-modified particles, we can suppose $R_C \sim R_H$. Therefore, we calculated our σ using the following equation:

$$\sigma = \frac{\pi R_g^2}{4\pi(R_H)^2/N} \quad (2)$$

Here, R_g is the radius of gyration of the unperturbed PEG chain with the same molecular weight as the PEG in mannose recognition, which we knew to be 1.65 nm. We know that there is a certain ambiguity in σ , but at present, there was no way to determine R_C . Figure 5A plots σ against X , indicating that there seems to be a linear relation between the two parameters. S4 and 5B plot ΔF_e and $\Delta F_e/N_{\text{man}}$ against σ , where N_{man} is the number of α -Man. Both plots show that the mannose recognition was dramatically suppressed at $\sigma = 1.0$. As mentioned in the introduction, the adjacent PEG chains start to contact each other at $\sigma = 1.0$, and at $\sigma < 1.0$, the tethered PEG chains are separated; thus, the mannose moiety also tethered to the core may stand alone without being shielded by the PEG and the core portion is exposed to solvents. This is the reason for the dramatic decrease in mannose recognition at $\sigma = 1.0$. Our results are quantitatively consistent with previous PEG surface chemistry results [11, 12, 21, 22] and confirm that controlling the PEG density is important to providing a targeting ability to DDS nanoparticles.

As mentioned above, there are several ambiguities when determining σ using Eq 1. The size of the core may be overestimated since we used R_H instead of R_C , and the error caused by this can be estimated to increase σ by approximately 10%, at most. This is because $R_S \cong R_C + R_g(\text{PEG})$ for fully covered states and $R_S \cong R_H$. [13, 23, 24, 25] However, the general trend for the dependence of σ on X in Figure 5A does not change, and thus, the above conclusion remains same. Figure 5B shows that there seems to be a maximum for $\Delta F_e/N_{\text{man}}$ around $\sigma \sim 0.75$. Since the frequency change is normalized by the number of mannose molecules in Figure 5B, the presence of the maximum suggests that the coexistence of mannose and PEG slightly enhances the recognition ability.

4. Concluding Remarks

In this paper, we evaluated the PEG coverage density for nanoparticles made from the ternary lipids DOTAP, α -Man, and DSPE-PEG₂₀₀₀ that have previously been studied as gene carriers. [14] The mannose recognition by Con A was examined using QCM, and we found that there was a critical composition for which recognition was drastically reduced. This critical composition was almost the same as $\sigma = 1.0$, at which the tethered PEG chains start to overlap. The results of the present study confirmed that careful control of the PEG density on DDS nanoparticles is essential for targeted delivery.

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Table 1. The obtained structural parameters for the **PMD** nanoparticles with FFF coupled with SLS and DLS.

sample	<i>X</i>	<i>R_H</i> (nm) ^a	<i>R_g</i> (nm) ^b	$\partial n/\partial c$ (mL/g) ^c	bound DSPE-PEG ₂₀₀₀ (μg)	<i>M_w</i> (g/mol) ^d	σ^e
PMD(2.00)	7.79	13.8	13.6	0.134	35.0	1.71×10^6	1.98
PMD(1.33)	5.20	14.9	9.3	0.138	39.7	1.80×10^6	1.33
PMD(1.00)	3.90	14.4	16.4	0.141	40.1	2.30×10^6	1.24
PMD(0.80)	3.12	16.0	13.0	0.143	43.6	2.37×10^6	1.12
PMD(0.66)	2.60	16.7	6.6	0.145	41.4	2.40×10^6	1.07
PMD(0.57)	2.23	20.6	7.0	0.146	41.3	2.40×10^6	0.783
PMD(0.50)	1.95	22.3	38.1	0.148	43.6	2.18×10^6	0.596
PMD(0.33)	1.30	26.0	7.1	0.152	43.3	2.03×10^6	0.384
PMD(0.25)	0.97	26.5	56.0	0.155	43.8	1.93×10^6	0.323
PMD(0.20)	0.78	26.9	15.0	0.157	43.8	2.07×10^6	0.333
PMD(0.10)	0.39	30.0	20.5	0.164	42.6	1.66×10^6	0.204

a: error range is $\pm 3 - 5 \%$ in S.D., *N* = 1 - 3

b: error range is $\pm 5 \%$ at the same FFF conditions, but it become larger (more than 20 %) for different focusing conditions.

c: error range is less than $\pm 2 \%$, *N* = 1.

d: error range is $\pm 5 - 8 \%$ in S.D. owing to how to take the base line, *N* = 1 or 2.

e: error range is about $\pm 5 \%$ in S.D. at most mainly owing to determination error in the number of PEG chains.

Figure(s)

Figure 1. A typical example of the first order autocorrelation function measured for PMD(1.00) (A), the size distribution (B), and the composition (X) dependence of R_H (C). Most of the measurements were repeated at three or four times and the slope of the $\ln[G^2(\tau) - 1]$ vs. τ was determined within $\pm 3 - 5\%$ at most for each sample.

Figure 2. FFF chromatograms for PMD(1.00). (A): Free DSPE-PEG₂₀₀₀ peaks observed at the cross-flow rate of 4.2 mL/min, (B): PMD particle became observable at the at the cross-flow rate of 1.0 mL/min although additional artificial peaks were appeared due to focusing.

Figure 3. SAXS profiles changes upon addition of DSPE-PEG₂₀₀₀ and a schematic illustration of morphological changes induced by DSPE-PEG₂₀₀₀.

Figure 4. The QCM frequency (ΔF) decays comparing three different PEG modifications (A) and equilibrium frequency changes (ΔF_e) against X (B). In QCM, ΔF_e was determined within about $\pm 3\%$ for each sample with the same QCM plate. However, different lots with the similar X showed a rather large error: about $\pm 8\%$ at lower X and about $\pm 15\%$ around $X = 2 - 4$, probably owing to the sample preparation conditions and difference in sensitivity of the QCM sensor.

Figure 5. Relation between σ and X (A) and σ dependence of ΔF_e (B).