Self-aggregates of Highly Hydrophobic Phospholipid Polymers in Aqueous Solution

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Abstract: In this study, the authors produced self-aggregates of highly hydrophobic phospholipid polymers consisting of 2-methacryloyloxyethyl phosphorylcholine (MPC) and stearyl methacrylate (SMA). MPC/SMA copolymers (PMS) self aggregated in a solvent consisting of 1-propanol and water (in 1:4 (w/w)). When alcohols other than 1-propanol were used, no homogeneous self-aggregate solution was obtained. The average diameter of PMS self-aggregates varied from 10 to 40 nm depending on the MPC content in PMS. The PMS self-aggregate was stable, and the diameter showed no changes even when the solution was stored at 60°C or 4°C for 3 months. The PMS showed high solubilization ability and solubilized hydrophobic compounds, such as tocopherol acetate and coenzyme Q10, in aqueous solutions. PMS that contained 50 unit mol% of SMA (PMS5050) showed the highest solubilization ability among PMS of different SMA contents. In spite of the high solubilization ability, PMS self-aggregates showed low surface activity and almost no cytotoxicity, showing that the PMS self-aggregates are promising biocompatible solubilizer.

Key words: self-aggregate, phosphorylcholine, MPC, nano, solubilization

1 Introduction

Molecular assemblies consisting of low-molecular-weight phospholipids and lipid microspheres can transport hydrophobic bioactive molecules into living tissues since they are highly compatible to the blood (1). Although many studies have been conducted to develop drug delivery systems using the assemblies, most efforts have failed since phospholipid assemblies are mechanically and chemically unstable under physiological conditions. Moreover, bioactive molecules entrapped in cells were prone to leaching shortly after injection. Thus, the stability of these phospholipid assemblies needs to be improved.

Ishihara et al. have synthesized various phospholipid polymers that have 2-methacryloyloxyethyl phosphorylcholine (MPC) units, aiming to develop blood-compatible biomaterials (2-5). MPC was originally synthesized by Nakabayashi in 1978 (6), and NOF Corporation established a mass production technology and started commercial production in 1997. MPC is methacrylate monomer that belongs to the same polar group (phosphorylcholine group) as that of the biomembrane, and can be copolymerized with all kinds of vinyl monomers using a conventional radical polymerization technique.

Ishihara synthesized water soluble copolymer, PMB3070, which consists of 0.3 unit mol fraction of...
MPC and 0.7 unit mol fraction of n-butyl methacrylate (BMA) (7). PMB3070 forms polymer aggregate in water through a hydrophobic interaction and can solubilize hydrophobic compounds. The polymer is called “polymeric lipids”, but in most low-molecular-weight phospholipids, alkyl chains are longer than n-butyl groups.

Konno et al. synthesized another MPC copolymer, PMS9010, which contained more hydrophobic alkyl groups than n-butyl groups and consisted of 0.9 unit mol fraction of MPC and 0.1 unit mol fraction of stearyl methacrylate (SMA) (8). However, the solubilization ability of PMS9010 was lower than that of PMB3070 since it contained less SMA units. The MPC copolymers consisting of higher SMA contents have been expected to show higher solubilization ability, but have not been examined due to their extremely low solubility to water.

With such a background, we developed a novel method for obtaining self-aggregates of highly hydrophobic MPC/SMA copolymer (PMS) using a quite simple process (9, 10). Preparation of PMS self-aggregates and evaluation of their physical and fundamental properties as biocompatible solubilizer are reported.

2 Experimental

2.1 Materials

2-Methacryloyloxyethyl phosphorylcholine (MPC), stearyl methacrylate (SMA), and t-butyl peroxynedecanoate (Perbutyl-ND) were supplied from NOF Corporation and were used as received. The other reagents and solvents were commercially available reagents of extra-pure grade and were used without purification.

2.2 Preparation of Polymers

MPC/SMA copolymers (PMSmn: “m” and “n” denote the unit mol% of MPC and SMA unit in PMS) were synthesized using a conventional radical polymerization technique. A total of 20 g combined of MPC and SMA was dissolved in 80 g of 1-propanol in a vessel and stirred. After removing oxygen from the solution by nitrogen bubbling, 0.4 g of Perbutyl-ND was added as an initiator. The reaction temperature was kept at 60°C for 8 h. The reaction mixture was precipitated in acetone, rinsed with acetone and dried in vacuo. The structure of PMS is shown in Fig. 1.

MPC/n-butyl methacrylate copolymer consisting of 30 unit mol% of MPC (PMB3070) was synthesized as reported (11).

2.3 Preparation of PMS Self-aggregates

Prior to the preparation of polymer self-aggregates, 0.1 g of PMS was dissolved in 0.4 g of 1-propanol at 40°C. The solution was colorless and transparent. PMS self-aggregates were prepared in three step procedures. At first, 1.6 g of ion-exchanged water was added to the PMS/1-propanol solution (“Step 1”). The mixture was stirred at 60°C for 20 minutes. Polymer precipitation disappeared gradually, and the solution became homogeneous (“Step 2”). The homogeneous solution was diluted with 7.9 g of ion-exchanged water at 60°C (“Step 3”). Then, the solution was dialyzed with ion-exchanged water.

2.4 Solubilization of Hydrophobic Compounds

Solubilization ability of the polymer was estimated by dissolving 10 mg or 50 mg of tocopherol acetate and 0.1 g of the polymer in 0.4 g of 1-propanol at 40°C and solubilizing as described for Steps 1 to 3.

The solubilization ability was compared between PMS5050 and PMB3070 by dissolving 5 mg of coenzyme Q10 (CoQ10) and 0.1 g of the polymer in 0.4 g of 1-propanol and solubilizing as described for Steps 1 to 3.

2.5 Cytotoxicity Study

Neutral Red uptake assay (NRU) was performed using rabbit-derived corneal (SIRC) cells as described by Tani et al. (12). The SIRC cells were cultured in media containing various concentrations of the test compounds for 24 h. The cultured cells were rinsed and
incubated for three hours in a medium containing Neutral Red, which was taken up by viable cells. After rinsing, the dye present in the cell population was liberated, and the amount was quantified using a spectrophotometer to count viable cells. The number of cells in the presence of the test compounds was compared to that of control cultures, and the percentage inhibition of growth was calculated.

2.6 Measurements

Conversion of monomers were determined by HPLC analysis using a mixture of methanol/n-hexane (5/5 v/v) as eluent (flow rate: 1.0 mL/min.) and ODS-3 (GL Science) column. Detection was performed using UV-970 (Jasco; \( \lambda = 260 \text{ nm} \)).

The molecular weight of PMS was determined by gel permeation chromatography-low angle laser light scattering (GPC-LALLS) analysis, by eluting with a mixture of tetrahydrofuran/1-butanol (8/2 v/v), 5 mM of lithium chloride and 0.1 % (w/v) of phosphoric acid at a flow rate of 0.7 mL/min., and using two TSKgel-GMHXL (Tosoh) columns and one TSKgel-G2500HXL (Tosoh) column in series. LALLS detection was performed with KMX-6 (Chromatix). The number average molecular weight (Mn) and poly dispersity index (Mw/Mn) are shown in Table 1.

The particle size of PMS self-aggregate was measured by dynamic light scattering (DLS). DLS was performed with Nicomp 380ZLS particle sizer (Particle Sizing System) using an argon ion laser source. The concentration of the sample was kept constant at 1 mg/mL.

Cryo-transmission electron microscopy (cryo-TEM) was carried out by depositing 0.1 mg/mL of dialyzed PMS3070 self-aggregate solution onto a holey carbon film supported by a copper grid, removing the excess using a piece of filter paper, and plunging the grid into a liquid ethane bath cooled with liquid nitrogen (FEI Vitrobot). The specimens were observed under FEI Tecnai Polara electron microscope operating at 300 kV at approximately -195°C.

The surface tension of dialyzed PMS self-aggregate solution was measured using the Wilhelmy plate method and CBVP-A3 tensiometer (Kyowa Interface Science) at 25°C.

3 Results and Discussion

3.1 Preparation of PMS Self-aggregates

PMS self-aggregate was prepared in three step procedures. The mixture was kept at 60°C and mildly stirred throughout the process. The obtained solution was clear and colorless, or slightly opaque and pale blue. Figure 2 shows the procedure for PMS5050.

Most low-molecular-weight phospholipids require high pressure homogenization or ultra sonic radiation for forming lipid microspheres or liposomes. In 1973, Batzri and Korn proposed a simple method for preparing small liposomes, which involved injecting a stock

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Synthetic Result of PMS.</th>
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<tbody>
<tr>
<td>Code</td>
<td>mol % of monomers (in feed)</td>
</tr>
<tr>
<td></td>
<td>MPC</td>
</tr>
<tr>
<td>PMS7030</td>
<td>70</td>
</tr>
<tr>
<td>PMS5050</td>
<td>50</td>
</tr>
<tr>
<td>PMS3565</td>
<td>35</td>
</tr>
<tr>
<td>PMS3070</td>
<td>30</td>
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<sup>a</sup>M<sub>n</sub> represents the number average molecular weight.  
<sup>b</sup>M<sub>w</sub> represents the weight average molecular weight.
solution of phospholipids in ethanol into an aqueous solution rapidly (13). Using this technique, small liposomes with narrow distributions have been prepared (14-18). Ishii \textit{et al.} examined lipid/alcohol/water ternary systems in detail and obtained homogeneous and unilamellar liposomes using 1- and 2-propanol (19). Essentially, these techniques are based on the self-aggregation of hydrophobic phospholipids in aqueous solutions, and the kind and composition of mixed solvents are quite important.

In case of PMS, no homogeneous self-aggregate solution could be obtained when methanol, ethanol or 2-propanol was used instead of 1-propanol. This suggests that the self-aggregation of PMS was affected by the chemical structure of solvents, and alcohols with higher and more linear alkyl groups are more interactive to PMS than those with lower and branched alkyl groups.

The optimum ratio of water to 1-propanol for obtaining the smallest particles of PMS was 4 to 1 in “Step 1” of the self-aggregation process (Fig. 3). When the amount of water was too large or too small, no homogeneous self-aggregate solution was obtained. 1-Butanol was expected to behave similarly to 1-propanol, but a mixture of water and 1-butanol (4:1) became heterogeneous because 1-butanol is little soluble to water.

When the temperature during “Step 2” dropped below 40°C, no homogeneous self-aggregate solution was obtained.

The PMS3070 self-aggregate solution left at the “Step 2” stage for 1 week at room temperature became gel, but the diluted sample (“Step 3”) did not. This was probably because the hydrophobic association of stearyl group was loosened by 1-propanol at high concentrations, and the loosening of hydrophobic association led to the formation of physical crosslinks among the PMS self-aggregates.

3.2 Characterization of PMS Self-aggregates

The volume-average diameter of PMS self-aggregate was determined by DLS. The average diameter

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**Fig. 3** Volume-average Diameter of PMS3070 Self-aggregates as a Function of Water:1-propanol ratio in “Step 1” of the Self-aggregation process. The solution was; (a) homogeneous, (b) heterogeneous.

**Fig. 4** Volume-average Diameter of PMS Self-aggregates as a Function of MPC content in PMS.

**Fig. 5** Cryo-transmission Electron Micrograph of PMS3070 Self-aggregate.
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decreased as the MPC content in PMS increased as shown in Fig. 4. Since there were small differences in molecular weight among the PMS samples, the diameter probably depended on the aggregation number of polymers.

An angular-shaped particle image was obtained from cryo-TEM observation of the PMS3070 self-aggregate (Fig. 5). The dark region around each particle suggested that phosphorylcholine groups associated with each other near the surface of the PMS self-aggregate.

The dialyzed self-aggregate solutions of PMS3565 and PMS5050 were quite stable under both chilled and heated conditions (Fig. 6), suggesting that the hydrophobic domains formed by the association of stearyl groups bound the polymer chains strongly.

3.3 Solubilization Ability of PMS

To estimate the solubilization ability of PMS, 10 mg and 50 mg of tocopherol acetate (TA) were solubilized with 0.1 g of PMS. In each concentration, TA was solubilized completely. The TA solution solubilized with PMS5050 was the most transparent of all as shown in Fig. 7a and 7b, suggesting that the PMS5050 has the highest solubilization ability among the series of PMS.

For further estimation, coenzyme Q10 (CoQ10) was solubilized, and the stability of the resultant solution was estimated. CoQ10, also known as ubiquinone or ubidecarenone, is a fat-soluble, vitamin-like substance.

![Fig. 6](image1.png)

**Fig. 6** Volume-average Diameter Changes of PMS Self-aggregates. (●) PMS3565 at 60°C, (▲) PMS3565 at 4°C, (○) PMS5050 at 60°C, (△) PMS5050 at 4°C.

![Fig. 7](image2.png)

**Fig. 7** Solubilization of Tocopherol Acetate (TA) with 1% of PMS: (a) 0.1% of TA, (b) 0.5% of TA; (1) PMS3565, (2) PMS5050, (3) PMS7030.

![Fig. 8](image3.png)

**Fig. 8** Stability of 0.05% of co-enzyme Q10 (CoQ10) Solubilized Solution: (a) and (b) are before and after storage at 60°C for 24 h; (1) 1% of PMB3070, (2) 1% of PMS5050 (”→” denotes phase-separated CoQ10).
found in the cells of many organisms. CoQ10 is essential for producing cellular energy in the form of adenosine triphosphate and acts as an antioxidant (20). However, CoQ10 is so hydrophobic that it is very difficult to prepare aqueous solutions of CoQ10.

The CoQ10 solution solubilized with PMB3070 separated within 24 h at 60°C (Fig. 8b). On the other hand, the CoQ10 solution solubilized with PMS5050 was more transparent and more stable than that with PMB3070 (Fig. 8a). The affinity of stearyl groups to CoQ10 was likely to be stronger than n-butyl groups, thus the CoQ10 solution solubilized with PMS5050 was more stable.

3.4 Cytotoxicity and Surface-activity

The Neutral Red uptake assay (NRU) using rabbit-derived corneal (SIRC) cells is known as a useful and high-sensitive method for evaluating cytotoxicity in vitro. In this study, cytotoxicity of the PMS self-aggregates and lysolecithin was examined.

Lysolecithin is an acyl chain hydrolyzed form of lecithin and has higher solubilization ability than the original lecithin. However, lysolecithin is cytotoxic due to its surface-active property (21). In this study, monostearoyl phosphatidylycholine (MSPC), which is a kind of lysolecithin, showed cytotoxicity at approximately 0.2 mg/mL as shown in Fig. 9.

From the viewpoint of the chemical structure, MSPC has phosphatidylycholine groups and stearyl groups in a ratio of 1:1, thus PMS5050 could be compared with MSPC. Although they are structurally similar, PMS5050 showed lower surface-activity than MSPC (Fig. 10) and caused substantially no damage to the SIRC cells (Fig. 9). The results suggest that the polymeric structure reduced the surface-activity and the cytotoxicity of PMS was extremely low.

4 Conclusion

In this study, self-aggregates of PMS in aqueous solution were obtained using a solvent of an optimum composition of 1-propanol and water (1/4 w/w). Solvent composition was the key of this technique. The average diameter of PMS self-aggregates determined by DLS and cryo-TEM were smaller than 50 nm. The diameter was likely to depend on the aggregation number of polymers. The PMS self-aggregate solutions were stable, and the diameter showed no changes even when the solutions were stored at 60°C or 4°C for 3 months. We also investigated the solubilization ability of PMS using tocopherol acetate. PMS5050 showed the highest solubilization ability. CoQ10 solution solubilized with PMS5050 was more stable than that solubilized with PMB3070. PMS self-aggregates showed low surface-activity and extremely low cytotoxicity compared to lysolecithin.

From these results, we concluded that PMS self-aggregates are a promising biocompatible solubilizer,
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which can be applied to medicines and cosmetics.

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