Critical Molecular Weight Effects in the Aggregation of Phospholipid Vesicles Triggered by Water-Soluble Polymers and an Integrated Glycolipid

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ABSTRACT: The intervesicular aggregation of phospholipid vesicles is induced by the addition of water-soluble polymers such as poly(ethylene glycol), dextran, etc. due to the interaction between the vesicular surface and the water-soluble polymers. The interaction can be expressed by the critical molecular weight (M_c) of the water-soluble polymers for the aggregation of vesicles. The surface modification of vesicles with glycolipids (O^1,O^5-bis(octadecyl) *N*-maltooligonoyl-L-glutamate) accelerates the aggregation of vesicles induced by dextran; therefore, M_c significantly decreased due to the surface modification. No dependence of phospholipid concentration and dextran concentration in an aqueous phase on the M_c indicates that dextran does not act as a cross-linking agent among the vesicles. A clear dependence of the density of the saccharide chains on the vesicular surface on the M_c suggests that dextran should adsorb on the surface of the vesicles by the interaction with the oligosaccharide chains on the surface and cause vesicular aggregation. A lower critical solution temperature was observed for this kind of interaction, and the critical temperature was controlled by changing the molecular weight of dextran.

1. Introduction

Interaction or recognition at the molecular level has been vigorously studied not only to understand the mechanisms of general reactions, especially catalytic or enzymatic reactions[1] and host−guest complexation[2] but also to construct the supramolecular assemblies.[3,4] We have therefore studied the complex formation between synthetic water-soluble polymers with simple repeating units. Polyelectrolyte complexes between polycations and polyanions with electrostatic interaction or polymer complexes with hydrogen-bonding have been studied in detail.[5,6] Interactions of water-soluble polymers with micelles or vesicles were also studied.[7−10] In these cases, the effects of hydrophobic interaction were significant besides electrostatic or hydrogen-bonding interactions. The molecular interaction of such polymers becomes higher with increasing molecular weight because of the cooperative effect of neighbor repeating units in the polymer. That is to say, the interaction between repeating units can be amplified by the increment of the molecular weight. Such complexation can be simply analyzed by the turbidimetry of the formed aggregates.[11,12] When polymers with various molecular weights are added to the counter polymers, a drastic increase in the turbidity is observed for a certain molecular weight of the polymers. Polymers are useful counterparts to study the molecular interaction because of the amplifying effect by the subsequence of the reactive monomer units. This critical molecular weight for complexation (M_c) is a useful parameter to express the strength of the interaction between polymers. Lower M_c means a stronger molecular interaction.

Amphiphilic molecules such as phospholipids spontaneously form a vesicular structure with a bilayer membrane when dispersed into aqueous solutions.[13] The vesicles with a bimolecular membrane (bilayer) are considered to be applicable molecular assemblies; they can encapsulate water-soluble molecules in the aqueous interior[14] and incorporate water-insoluble or amphiphilic molecules in the bilayer membrane.[15] They are also sensitive to stimuli such as temperature, pH, ionic strength, ion species, and various kinds of molecules which can interact with the surface of the vesicles, or can change the packing state of the bilayer membranes,[16] and form aggregates which can be monitored by turbidimetry. In turbidimetry, the particles of which the surface is modified with host molecules are also used to study antigen−antibody reactions in order to enhance the sensitivity of the molecular interaction.[17] We have reported the incorporation effect of synthetic glycolipids with an oligosaccharide chain on the restriction of vesicular aggregation.[18,19] The intervesicular aggregation is induced by the addition of water-soluble polymers, and the aggregation depends on the surface properties such as charges or steric effects of the incorporated molecules. Recently, we found that the surface modification with oligosaccharide chains promoted the aggregation for the addition of polysaccharides, indicating an interaction between saccharide chains, which has not been recognized yet. This would be due to the amplifying effect of particles on weak interactions other than the polymer effects, as already mentioned.

In this paper, we analyze the interaction of oligomers extending from the surface of the vesicle and adding polymers of various molecular weights. The critical molecular weight (M_c) of the polymers for the aggregation of vesicles was clearly obtained from turbidimetry. The M_c was used to characterize the surface properties of the surface-modified vesicles.

2. Experimental Section

Materials. All of the water-soluble polymers were commercially available: poly(ethylene glycol) [PEG (M_w 600, 1000, 1540, 2000, 6000, 20 000), Kanto], poly(vinyl alcohol) [PVA (M_w 40 000), Wako], poly(styrenesulfonate) sodium salt [PSSNa (M_w 70 000), Aldrich], dextran [Dex (M_w 980, 2500, 11 000,

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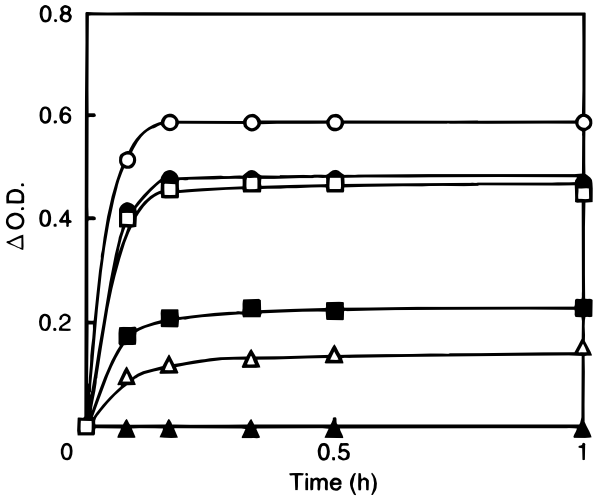

Figure 1. Aggregation of vesicles by the addition of various kinds of polymers at 25 °C: PSS−Na (○); PVA (●); PEG (□); Dex (■); HES (△); dextrin (▲).

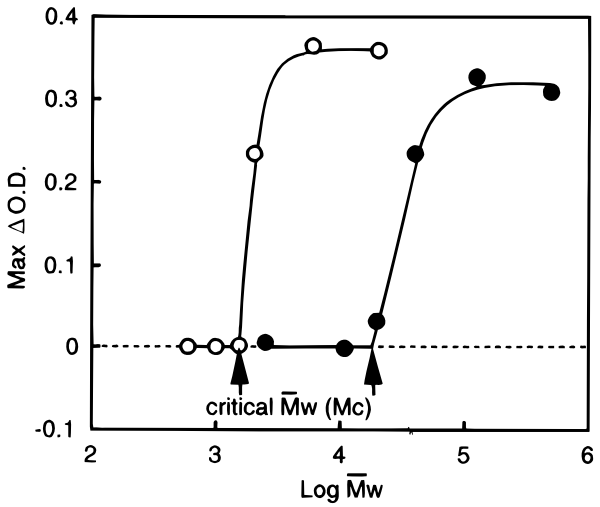

Figure 2. Maximum ΔO.D. change (maxΔO.D.) of the vesicle dispersions after the addition of PEG (○) and Dex (●) at 25 °C.

19 600, 40 000, 124 000, 487 000), Sigma], hydroxyethylstarch [HES (M_w 70 000), Sigma].

Methods. Synthesis of Glycolipids. Maltooligotose (50 mM) was dissolved in pure water in the presence of calcium carbonate (50 mM). To this mixture was added Br_2 (100 mM) dropwise for 10 h. The obtained carboxylate was converted to acid-form by a cation exchange resin (Amberlite IR-120B) and dehydrated *in vacuo* at 80 °C to form maltooligotose lactone.[20] The lactone was reacted with bis(tetra-*tert*-butylammonium) glutamate, and subsequently, two alkyl chains are connected to the dicarboxylate using 1-bromooctadecane. Crude glycolipids (O^1,O^5-bis(octadecyl) *N*-maltooligonoyl-L-glutamate (DOMOGlu (n = 2−7)) were purified by recrystallizations (twice) from $CHCl_3/CH_3OH/H_2O$ (3/4/1) to yield the white solid (overall yield: 30−50%). The characterizations were carried out by ^{1}H-NMR (JEOL, EX-270) and IR (JASCO, FT-IR5500).

Vesicle Preparation. The mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, and palmitic acid of 7, 7, and 2 as a molar ratio was dissolved in $CHCl_3/CH_3OH/H_2O$ (=65/25/4 by vol). The DOMOGlu was mixed with the lipid mixture and dried *in vacuo* to form a film. After the film was hydrated into a Tris−HCl buffer solution ([Tris] = 20 mM, [NaCl] = 140 mM, pH = 7.4), it was extruded through the polycarbonate membrane filters (the final pore size of the filter: 0.2 μm). The diameter of the resulting vesicles was determined to be 190 nm (standard deviation 50 nm) by a Coulter submicron analyzer (Model N4SD). The nonincorporating glycolipid was removed by ultracentrifugation (300000*g*, 1 h, Beckman). The incorporation ratio was determined to 92−95% by a phenol−sulfunic acid method. The precipitate was redispersed into a buffer in the same ratio as described above, adjusting the concentration of lipid to 0.075 g/dL.

Turbidimetry. To the vesicle dispersion (0.075 g/dL, 1.5 mL) in a cuvette (l = 1 cm), the water-soluble polymer solution (6 g/dL, 1.5 mL) in the same buffer was added at 25 °C, and the turbidity change at 600 nm was monitored after the addition of polymers by a UV−vis spectrophotometer (Shimadzu, MPS-2000).

3. Results and Discussion

Figure 1 shows the turbidity changes of the vesicle dispersions after the addition of water-soluble polymers: sodium poly(styrenesulfonate) (PSS−Na), poly(vinyl alcohol) (PVA), poly(ethylene glycol) (PEG), dextran (Dex), dextrin, and hydroxyethylstarch (HES). The turbidity increases with time and reaches a constant value, depending on the kinds of polymers. Such a turbidity increase indicates the aggregation of the vesicles induced by the addition of polymers.[21] This was influenced by the properties of the polymers such as charge, affinity, molecular weight, flexibility, and so on. Anionic polymers such as PSS−Na cause significant turbidity increases in spite of the electrostatic repulsion between the negative charges of the polymers and the surface of the vesicles. On the other hand, PVA and PEG show similar turbidity increases; intermediate between polyanions and polysaccharides. When polysaccharides were used as additives, the turbidity increases are considerably smaller in comparison with these of other polymers. Especially, no turbidity increases were observed for dextrin.

The aggregation of vesicles caused by the addition of PEG and polysaccharides were studied. In some cases, these polymers were studied as the triggers of the fusion of the vesicles.[22,23] The difference in the aggregation of the vesicles induced by various polymers can be explained from the entropy change by the release of water molecules accompanied with the adsorption of polymers to the surface of the vesicles and by the decrease in the hydrophilicity of the polymer-adsorbed surfaces. The number of water molecules associated with anion sites of the anionic polymers and the dissociated countercation is significantly high in comparison with nonionic polymers. Dehydration during the adsorption of the polymers to the surface of the vesicle through the countercation would result in a significant entropy increase of water molecules, and vesicles tend to aggregate in order to decrease the contact area of the resulting hydrophobic surface with the surrounding water molecules. Nonionic polymers such as PEG or PVA would adsorb by hydrogen-bonding, and the resulting surface is also hydrophobic.[22] In the case of polysaccharides, the adsorbed polymers still have the remaining hydrophilic sites and are hydrated. Therefore, the induction of the vesicle aggregation should be weak for polysaccharides.

The difference in the turbidity of the vesicle dispersion before the addition of polymers and the turbidity saturated after the addition of polymers was indicated as maxΔO.D. PEG and Dex are representative polymers of which various molecular weights are easily available. Figure 2 shows the molecular weight dependence in the turbidity change (maxΔO.D.) for PEG and Dex. An increase in maxΔO.D. for a molecular weight of a polymer was observed in both polymers. The starting point of the maxΔO.D. increase is defined as a critical molecular weight (M_c) of the polymers for the aggregation of vesicles. It means that the polymers with

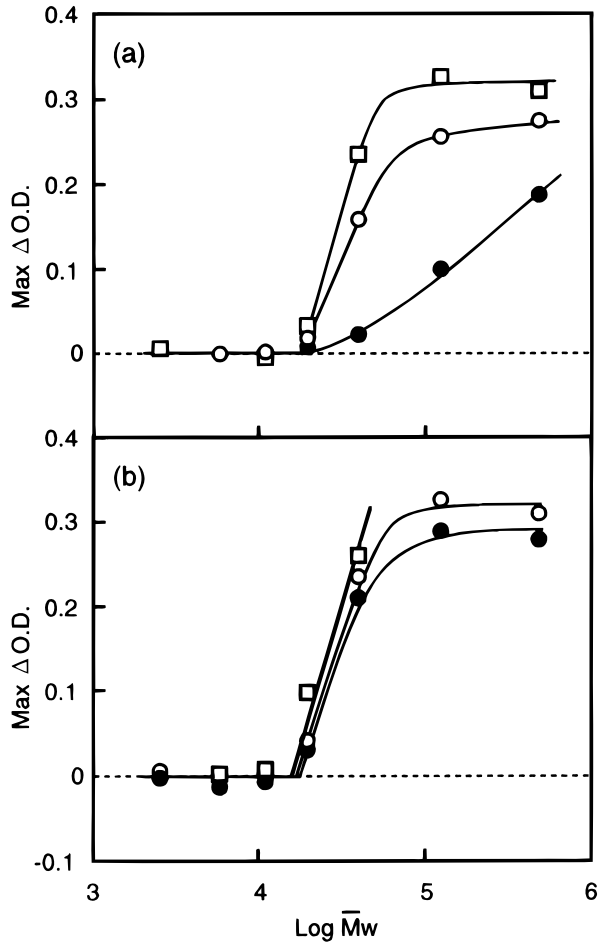

Figure 3. Maximum ΔO.D. change (maxΔO.D.) of vesicle dispersions after the addition of Dex at 25 °C. (a) The concentrations of Dex were 3 g/dL (□), 2 g/dL (○), and 1 g/dL (●). [mixed lipids] = 0.075 g/dL (constant). (b) The concentrations of mixed lipids were 0.3 g/dL (□), 0.15 g/dL (●), and 0.075 g/dL (○). [Dex] = 3g/dL (constant). maxΔO.D. of the samples of which the lipid concentrations were 0.3 and 0.15 g/dL were depicted as one-quarter and one-half of the measured values, respectively.

a molecular weight smaller than the M_c have no ability to promote the aggregation of vesicles. With increasing the molecular weight of the polymers above the M_c, the maxΔO.D increases steeply with the molecular weight of the polymers and reach a constant value for the polymers with a significantly larger molecular weight.

M_c for the aggregation of vesicles by the addition of the polymers is represented by the change in the interaction of the polymers on the surface of the vesicles. The critical points of PEG and Dex were 1500 and 15 000, respectively. This means that PEG has a stronger interaction with the surface of the vesicles than Dex which has been confirmed from the maxΔO.D. shown in Figure 1. M_c provides more information than Figure 1 because the molecular weights of PEG and Dex used in Figure 1 were 20 000 and 40 000, respectively. The molecular weight of PEG is sufficient to allow aggregation of the vesicles, and that of Dex is just above the M_c. Therefore, a large difference in turbidity was observed, as shown in Figure 1. However, if the molecular weight of Dex were higher, such a significant difference would not be observed. The strength of the interaction of the polymer with the vesicular surface is actually measured from the M_c.

Figure 3 shows the relationship between the molecular weight of dextran and the turbidity change (ΔO.D.) for various concentrations of polymers (a) and vesicles (b). When the concentration of dextran added in the vesicle dispersion becomes high, the turbidity increases faster and saturates at the higher values. The figure shows that maxΔO.D. increases at the same M_c, even though the concentration of the polymers increases. The increasing ratio of maxΔO.D. versus the increase in the molecular weight above M_c increases with the polymer concentration. The same phenomenon was observed for vesicles when the concentration of the vesicles was increased. Namely, maxΔO.D. becomes double or triple at a concentration of the vesicles 2 or 3 times higher. Therefore, in Figure 3b max ΔO.D. was depicted as one-half for 0.15 wt % and one-fourth for 0.3 wt % vesicles. It is noted that there is no dependence of the vesicle concentration on the M_c. These results would deny the cross-linking of dextran among vesicles to make a floc because the strong concentration dependence of vesicles on the M_c is predicted in this case. Such cross-linking should not be the first requisite for the aggregation of vesicles, and aggregation would occur as a result of destabilization in the dispersibility of vesicles by polymer adsorption.

The adsorption of a polymer on the surface of the vesicle would consist of two main processes: adsorption and desorption. The predominant process should be the desorption of the adsorbed polymer from the surface of the vesicle because there is no concentration dependence of dextran and vesicles on the M_c. When one site of a polymer binds to the surface of the vesicle, the next site can bind to the surface easier than the first process because of entropic reasons. The next several sites should continuously bind to the surface following the conformational change in the polymer, resulting in the formation of the *train* structure. The other intramolecular binding sites should bind to the surface to form the other *train* structure, forming a *loop* structure between the *train* structures. The entire structure of the adsorbed polymer is generally considered to be a *loop*−*train*−*tail* structure.[24] These processes, except for the first binding process, are included in the desorption process. The binding strength of the entire polymer becomes stronger with the increasing number of binding sites (*trains*), i.e., the molecular weight. In other words, the desorption process of the bound polymer should become more difficult with the molecular weight because the desorption of the polymer from the surface would become possible only when all of the binding sites dissociate completely and simultaneously. This leads to the low desorption constant for the entire polymer from the surface of the vesicles. The molecular weight corresponds to the length of a zipper. Two cloths connected by the zipper are difficult to separate if the length of the zipper becomes longer and can be separated only when the zipper is sliced at its edge. This suggests how difficult the separation process is for the long polymer bound on the surface with several sites and why there is no concentration dependence on the polymer and the vesicles. The polymer with a low molecular weight has the smaller number of binding sites and has high molecular movement; collision, adsorption, and desorption would occur frequently. Vesicles which have a surface covered with adsorbed polymers tend to aggregate because the surface potential becomes small. The above description explains the existence of the critical molecular weight of the polymer for the aggregation of vesicles. The critical molecular weight should be the lowest molecular weight of the polymer which can adsorb on the surface of the vesicles.[25] The critical molecular weight should depend on the properties of the polymer and the vesicular surface,

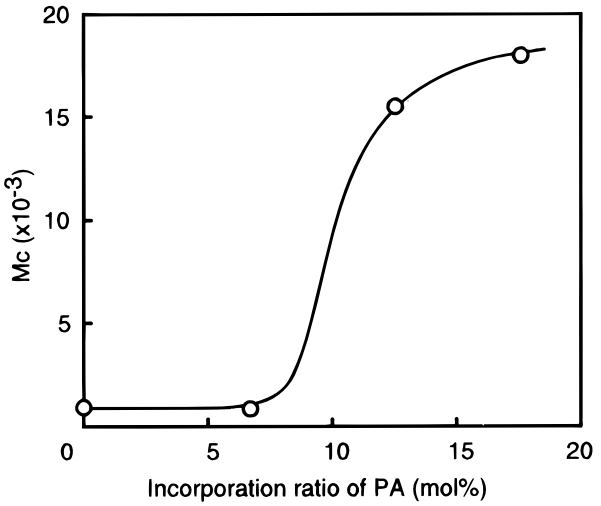

Figure 4. Relationship between the ratios of palmitic acid in the mixed lipids of vesicles and the critical molecular weights of Dex for the aggregation of the vesicles at 25 °C.

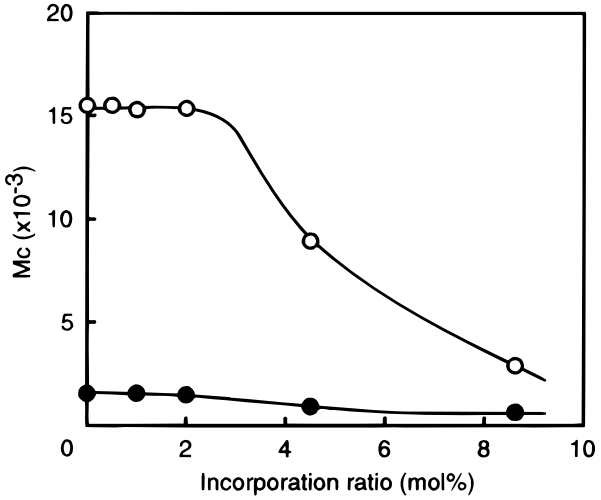

Figure 5. Influence of the incorporation ratios of DOMPGlu to the vesicles on the critical molecular weights of Dex (○) and PEG (●) for the aggregation of vesicles at 25 °C.

namely, the interaction energy between the polymer and the surface of the vesicle.

Figure 4 shows the relationship between the incorporation ratios of palmitic acid on the bilayer membrane and the M_c for the aggregation of the vesicles. The M_c for the aggregation of vesicles significantly depends on the surface states. The increase in the M_c means that the aggregation of vesicles is suppressed by the electrostatic repulsion between vesicles due to the introduction of a negative charge on the surface of the vesicles. The vesicles with no negative charge are not generally stable and tend to aggregate by the addition of polysaccharide with a lower molecular weight. The distance from the surface which electrostatic repulsion reaches increases with the incorporation ratio of palmitic acid. This can be generally explained by the Derjaguin−Landau−Verwey−Overbeek (DLVO) theory.[26] The thickness of the adsorbed polymer layer increases with the molecular weight of the polymer because the polymer would bind to the surface making a longer *loop* and *tail* structure. The thickness of the adsorbed polymer layer increases in proportion to the square root of the molecular weight of the polymer based on the calculation[27] and experiment.[28] When the thickness of the adsorbed polymer layer exceeds the thickness of the electrostatic layer, the aggregation of the vesicles would occur.[29] This would be one reason why the M_c increases with the incorporation ratio of palmitic acid.

The M_c also depends on the surface modification of the vesicles. Figure 5 shows the dependence of the incorporation ratio of the O^1,O^5-bis(octadecyl) *N*-maltopentaonoyl-L-glutamate (DOMPGlu) to the bilayer membrane on the M_c of PEG or Dex. At first, the M_c indicates a constant value until the incorporation ratio is 2 mol % and then it decreases with the increasing incorporation ratio. At a low density of oligosaccharide chains, the effect of the glycolipid modification is negligible. This effect becomes significant if the incorporation ratio is above 2 mol %. A similar phenomenon was observed for a PEG adding system though the M_c values and the incorporation effect were significantly smaller in comparison with a Dex system. The lowering effect of M_c by the surface modification would be due to the interaction of adding polymers with the oligosaccharide chains extending from the surface of the vesicles.

The modification with oligosaccharide chains enhances the aggregation of the vesicles if PEG or Dex is added. However, it is considered that, in general, polymers cannot access the surface of the vesicles in the case for no specific interaction of polymers with the modified surface, and the exclusive volume effect of the oligosaccharide should prevent the aggregation.[18] Therefore, the promotion of the aggregation of the oligosaccharide-modified vesicles by the addition of polysaccharides indicates the interaction between oligosaccharide chains on the surface and polysaccharide chains such as hydrogen-bonding. The aggregation of vesicles may be due to the dehydration of water molecules surrounding the vesicular surface by replacement with PEG or polysaccharide.

Table 1. Incorporation Ratios of Glycolipids with Different Chain Lengths to the Vesicles and Critical Molecular Weights of Dex for Aggregation of Vesicles at 25 °C [Saccharide Unit = 43 mol % (Constant)]

no. of saccharide unit (−)	incorporation ratio (mol %)	M_c (−)
2	21.5	850
3	14.3	1000
5	8.6	2900
7	6.2	5800

Second, the M_c was measured for the glycolipids with different saccharide chain lengths under a constant unit concentration on the vesicular surface. It was expected that the longer oligosaccharide chain length would have the stronger interaction with polysaccharide, and M_c would become small. However, reverse results were obtained, as shown in Table 1. In this experiment, the concentration of the glucose unit is constant, and the number of glycolipid molecules incorporated into the surface is higher for the glycolipids of shorter saccharide chains. The results of Figure 5 and Table 1 suggest the hypothesis that the density of the oligosaccharide chain rather than the length of saccharide chain would be an important factor to determine the M_c. The concentrations of the saccharide chains on the surface of the vesicles are also depicted in Table 1. The clear dependence of the chain density on the M_c supports this hypothesis. It would be considered that added polymer would interact with more than two oligosaccharide chains on the vesicular surface in order to adsorb onto the surface of the vesicles.

Figure 6 shows the temperature dependence of M_c. The M_c decreased with increasing temperature. It means that the vesicle aggregation with adding Dex or PEG has a low critical solution temperature (LCST). The same phenomenon was observed in the polymer complexes.[30,31] For instance, PMMA−PEO is a polymer

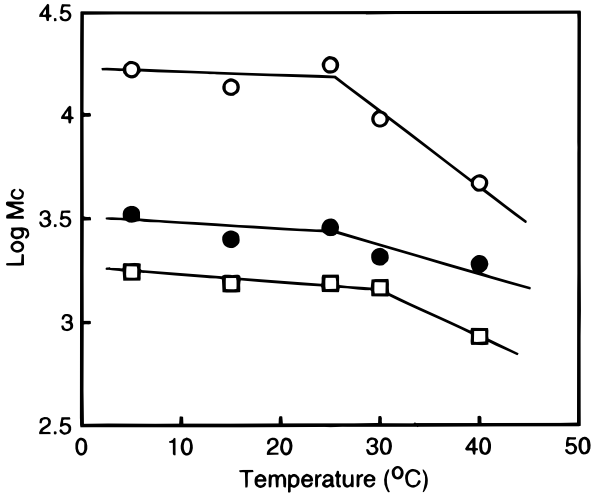

Figure 6. Temperature dependence of the critical molecular weights of Dex for the aggregation of the vesicles (○), oligosaccharide-modified vesicles (●), and PEG for the aggregation of the vesicles (□).

complex made by hydrogen-bonding above the critical molecular weight (M_c) of constituting polymers and the M_c decreased with increasing temperature.[30] It indicates that not only the hydrogen-bonding but also hydrophobic interaction plays an important role in the polymer complex. In general, a hydrophobic interaction increases as the temperature increases. Furusawa et al. reported that the amount of polymers with LCST adsorbed on the surface of latex particles increased above the LCST, because these polymers were dehydrated above the LCST.[32] Dex or PEG were considered to be dehydrated as the temperature increased and were adsorbed more strongly on the surface of the vesicles.

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