Poly(ethylene glycol)-mediated Steric Stabilization of Complexes Formed between Negatively Charged Liposomes and Folate-conjugated Poly(amidoamine) Dendrimers in Water

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In order to develop a new supramolecular complex for use as an active drug carrier, we prepared complexes of an anionic liposome comprising poly(ethylene glycol) ($PEG_{n=50}$) monooleyl ether with folate-conjugated poly(amidoamine) dendrimers (FA-PAMAM). Regardless of the ratio of FA-PAMAM to liposome, the complexes were stably dispersed in water. These results suggest that the PEG chains play an important role in the dispersion stability of the supra-complexes, preventing the complexes from aggregating.

Liposomes are nano-sized artificial lipid vesicles used to encapsulate bioactive molecules such as proteins, DNA, and drugs, and have been extensively employed as drug carriers. However, both in vivo and vitro, liposomes tend to fuse and/or aggregate with one another and leak their encapsulated contents, which restricts their potential advantages as drug carriers. A number of recent papers[1–5] aimed at improving the steric stabilization of liposomes have investigated the complexation of negatively charged liposomes with oppositely charged linear polyions. Linear polyions are physically adsorbed in loop-train type onto the vesicle surface by electrostatic interactions. However, the adsorption of polyions often causes liposome aggregation, induced by neutralization of the charges.[5] As a strategy for steric stabilization, Bronich et al.[1] utilized graft copolymers composed of comb-like polyions and water-soluble polymer chains. Using this approach, the polyions are anchored onto the vesicle surface, leaving the hydrophilic chains dangling in the aqueous medium, resulting in repulsion between liposomes. As a result, there is neither fusion nor aggregation of the liposomes.

In view of the topological effects of polyions, we have paid attention to dendrimers with a three-dimensional architecture and terminal functional groups.[6] Poly(amidoamine) dendrimer (PAMAM), which is a polyion with large charge density, has attracted attention as a promising functional nanomaterial for use as a drug carrier and as a cell transfection agent.[7,8] Using PAMAM, the drug is retained inside the dendrimer, and the terminal amino groups of the dendrimer are involved in the introduction of target ligand molecules such as folic acid.[9,10] Folate receptors are overexpressed in cancer cells. Thus, it is expected that folate-conjugated PAMAM (FA-PAMAM) would recognize only specific cancer cells and be incorporated into these cells through endocytosis. In order to develop a new active drug carrier on the basis of the foregoing viewpoints, we have proposed to design supramolecular complexes composed of anionic liposomes with cationic FA-PAMAM. Therein, to prevent the aggregation of the complexes through intermolecular cross-linked FA-PAMAM and elevate the dispersion stability of the complexes, we have introduced poly(ethylene glycol) monooleyl ether composed of hydrophilic PEG and a hydrophobic alkyl chain as components of the anionic liposome. It is expected that the PEG chain would weaken the strong interaction between cationic PAMAM and the anionic liposome owing to the PEG chains dangling from the outer surface of the liposomes. In this paper, the dependence of the dispersion stability of the complexes in water on the ratio of FA-PAMAM to liposome was investigated by light transmittance, dynamic light scattering (DLS), ζ-potential, and TEM measurements.

We prepared anionic liposomes with conventional thin film hydration methods by mixing egg yolk L-α-phosphatidylcholine (PC), phosphoric acid di-*n*-decyl ester (PD), cholesterol (CHOL), and PEG monooleyl ether (C_{18}-PEG: the degree of polymerization of the ethylene oxide units was 50, and the number of carbons in the alkyl chain was 18) in a 1:2:1:1 molar ratio, followed by extrusion through a polycarbonate filter with 80-nm-diameter pores.[11] On the other hand, amino-terminated G6.0 PAMAM branched from a cystamine core was synthesized according to Tomalia's previous paper.[12] Folate was bound to the amino groups at the free end of the G6.0 PAMAM by *N*,*N*′-dicyclohexylcarbodiimide (DCC)-mediated coupling.[9] The resulting dendrimer was characterized by ^{1}H NMR, ^{13}C NMR, IR, size-exclusion chromatography (SEC), UV spectroscopy, and potentiometric titration (data not shown). From these results, the numbers of terminal amino groups of G6.0 PAMAM and of the binding folate molecules were estimated to be 244 and 26, respectively. Finally, the complexes composed of the anionic liposomes with FA-PAMAMs (named PAMAM-LIPs) were prepared at room temperature in pH 7.4 phosphate buffer at the various weight concentration ratios of FA-PAMAM to PC (R = [FA-PAMAM]/[PC] (w/w)). The sizes of the prepared FA-PAMAMs and PAMAM-LIPs were determined by DLS measurements using Ar^+ laser (488 nm) and He–Ne laser (633 nm), respectively. The final lipid (PC) concentration in the prepared liposome solution was determined using a Phospholipid C-Test Wako kit (Wako Pure Chemicals Industries Ltd., Osaka).

The mean diameter of the FA-PAMAMs was determined to be 7.4 ± 1.8 nm (±standard deviation; S.D.) as cumulant diameter by DLS measurement. Likewise, the mean diameter was also estimated from TEM images to be 6.4 ± 1.3 nm (±S.D.).[11] Figure 1 shows the change in the optical transmittance of the liposomes comprising C_{18}-PEG in water for the complexation with FA-PAMAMs at a given value of $R = 9.7$ with 0.1 mg/mL PC concentration, compared to that of liposomes without C_{18}-PEG, as a function of time after addition of FA-PAMAM. In the case of the liposome without C_{18}-PEG, a steep increase in the turbidity of the liposomal dispersion occurred with time after addition of FA-PAMAM (see the inset lower photograph show-

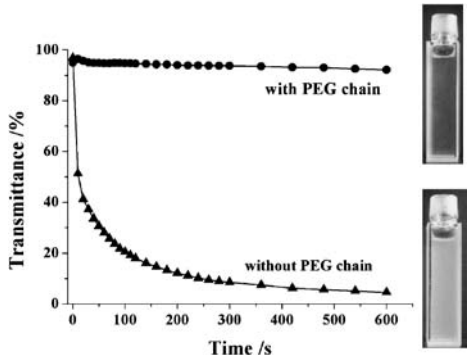

Figure 1. Changes in the optical transmittance of the anionic liposomes without PEG chain (triangle mark) and with PEG chain (circle mark) in water as a function of incubation time after FA-PAMAM addition at a given value of $R = 9.7$ (=[FA-PAMAM]/[PC] (w/w)) with 0.1 mg/mL PC concentration. The inset photographs (upper photo: the liposome with PEG chain; lower photo: the liposome without PEG chain) show the liposome suspensions after incubation of FA-PAMAM for 3 h.

ing the liposome suspension after incubation for 3 h) and precipitation was observed after 24 h incubation (data not shown). These results imply that the neutralization of anionic liposomes induced by complexation with FA-PAMAM causes abrupt aggregation. On the other hand, in the case of the liposome comprising C_{18}-PEG, the turbidity of the suspension remained unchanged against addition of FA-PAMAM (see the inset upper photograph showing the liposome suspension after incubation for 3 h). The liposomal sizes scarcely changed[11] after incubation for 3 h and no precipitation was observed after 24 h incubation (data not shown). These findings suggest that steric repulsion between the PEG chains plays an important role in maintaining the stability of aqueous liposome dispersions, preventing the complexes from aggregating.

Figure 2 shows the effect of the concentration of FA-PAMAM on the cumulant average diameter and ζ-potential of liposomes with C_{18}-PEG ($n = 3$, ±S.D.). The diameter of the liposomes slightly increased with FA-PAMAM concentration up to $R \approx 1.2$, and then levelled off. Size increased in increments of about 7–8 nm, and the polydispersity index was less than 0.1 (data not shown) despite the addition of FA-PAMAM. With increasing cationic FA-PAMAM concentration up to $R \approx 4.0$, the ζ-potential of the anionic liposomes was shifted from a negative (−9 mV) to a positive value (+10 mV), across a value of zero occurring at $R \approx 1.2$.[11] No further shifts were observed at higher FA-PAMAM concentrations. The TEM image in the inset shows that the outer surface of the liposome is coated with a monolayer of dendrimers.[11] Consequently, the dispersion behavior of the liposomes against the change in FA-PAMAM concentration, in particular, the neutralized particles at $R \approx 1.2$, reveals that the steric repulsion between PEG chains prevents liposome aggregation and plays an important role in the stability of the liposome/dendrimer dispersion in water. It should be noted that from the study with regard to the leakage of sulforhodamine B entrapped in the liposomes, the vesicle structure of the liposomes was confirmed to be stable during the adsorption processes for FA-PAMAM.[11]

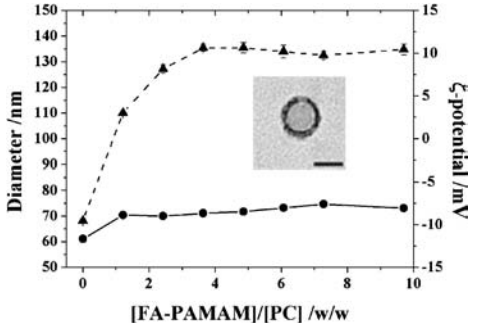

Figure 2. Cumulant diameter (circle) and ζ-potential (triangle) of liposomes with PEG as a function of the weight concentration ratio of FA-PAMAM and PC ($n = 3$, ±S.D.). The inset shows a TEM image of a liposome at a ratio [FA-PAMAM]/[PC] of 9.7. Scale bar in the TEM image is 50 nm.

In conclusion, we have prepared stably dispersed complexes of an anionic liposome with a cationic dendrimer in water by using PEG monooleyl ether as one component of the liposomes. Introduction of ligand molecules on the liposome outer surface via the dendrimers enables control of the amount and distribution of the ligand. It is therefore expected that the complexes would be effective for active drug targeting. The effect of the complexes on accelerating drug uptake into tumor tissues is currently being investigated.

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