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Design of Biocompatible Dendrimers with Environment Sensitivity

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Dendrimers have attracted much interest because of their unique structures and properties.[1] Their size, structure, and surface properties are highly controllable. Also, their interiors are capable of encapsulating small molecules.[2] Therefore, dendrimers are highly attractive materials for drug delivery applications.[3]

Recently, efforts have been made to prepare dendrimers with poly(ethylene glycol) (PEG) chains at the chain ends.[4] Because the dendrimer moieties of PEG-attached dendrimers are covered with highly hydrophilic PEG chains, they may act as nanocapsules with a biocompatible surface. In a previous study, we successfully attached PEG chains to all of the chain ends of third- and fourth-generation polyamidoamine (PAMAM) dendrimers and showed that these dendrimers have the ability to retain anticancer drugs.[4a]

In this study, we have attempted to provide the dendrimers with stimuli-sensitive properties because such properties are useful for site-specific or cytoplasmic drug delivery. Here, we report an effective strategy for the sensitization of dendrimers against oxidative or reductive environments using cysteine (Cys).

PEG-attached PAMAM dendrimers with Cys residues were synthesized according to Scheme 1. The PAMAM G4 dendrimer, which has *N*-*tert*-butoxycarbonyl-*S*-acetamidomethylcysteine [Boc-Cys(Acm)] residues, was prepared by reacting Boc-Cys(Acm) with the PAMAM G4 dendrimer. After the removal of the Boc groups using trifluoroacetic acid (TFA), a PEG monomethyl ether with an average molecular weight of 2000 was attached to the Cys(Acm) residues of the dendrimer using methoxy PEG-*p*-nitrophenyl carbonate to obtain the PEG-Cys(Acm)-G4 dendrimer. The Acm groups of the dendrimer were removed using I_2, and the PEG-Cys-G4 dendrimer was obtained.

The ^{1}H NMR spectrum of the PEG-Cys(Acm)-G4 dendrimer showed peaks corresponding to the PAMAM dendrimer, the Cys(Acm) residue, and PEG. From the integral ratios of these peaks, the average number of PEG chains and Cys(Acm) residues in the dendrimer were estimated to be 61 and 64, respectively, both of which agree with the number of chain ends (64) in the parent dendrimer. Therefore, this result indicates that essentially every chain end of the dendrimer was combined with a Cys(Acm) residue and a PEG chain. Also, the ^{1}H NMR spectrum for the PEG-Cys-G4 dendrimer exhibited no peak corresponding to the Acm group. The removal of the Acm group by the I_2 treatment results in the formation of disulfide bonds for the Cys residues.[5] In fact, no thiol group remained in the PEG-Cys-G4 dendrimer as detected by Ellman's method,[6] indicating that all of the Cys residues formed disulfide bonds. Because Cys residues are concentrated at the periphery of the dendrimer, they might readily react with each other and dimerize in the presence of I_2. In addition, it is known that exchange reactions occur between thiols and disulfides, such as between an SH group and a disulfide linkage of Cys residues in a protein.[7] Thus, the disulfide−thiol exchange reaction between Cys residues should enable rearrangement of their disulfide bridges, resulting in the efficient disulfide bond formation of the Cys residues.

The molecular weights of the PEG-Cys(Acm)-G4 and PEG-Cys-G4 dendrimers estimated by GPC using 200 mM Na_2SO_4 and 10 mM phosphate buffer (pH 7.4) as an eluent are listed in Table 1. The values estimated by GPC were much lower than the calculated molecular

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Scheme 1. Synthetic Route for PEG-Cys-G4 Dendrimer

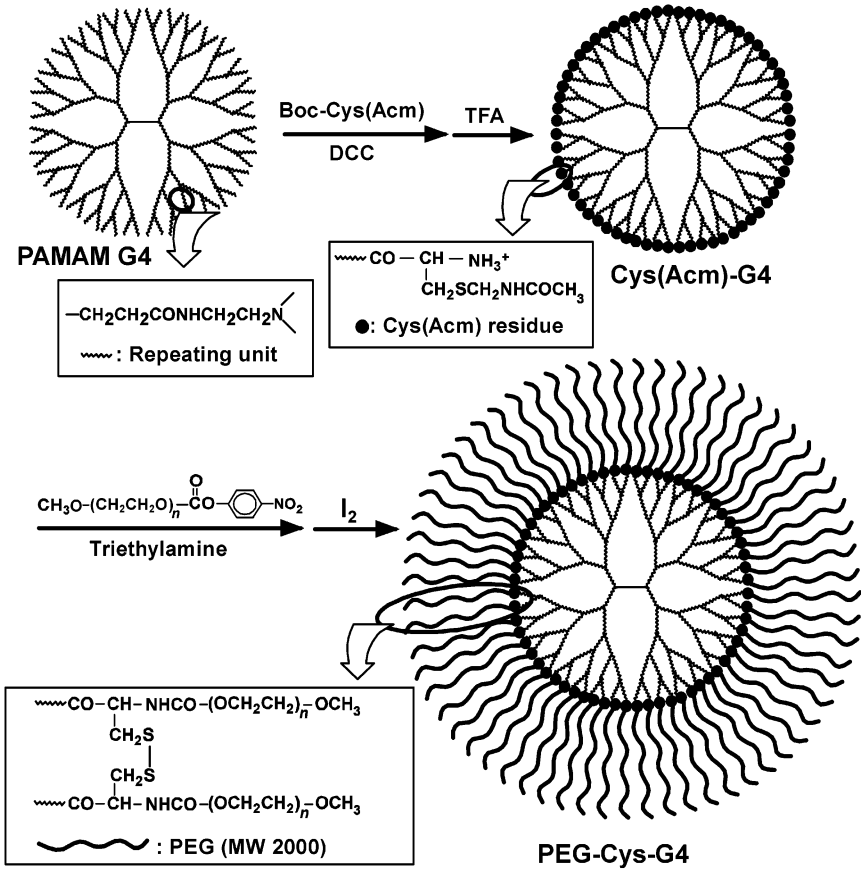

Table 1. Molecular Weights of PEG-Attached Dendrimers Having Cys Residues

dendrimer	molecular weight			
	calcd[a]	M_n	M_w	M_w/M_n
PEG-Cys(Acm)-G4	155 079	24 230	33 510	1.38
PEG-Cys-G4	150 471	18 450	25 720	1.39
PEG-Cys(SH)-G4[b]	150 535	20 350	24 860	1.22

[a] Calculated using 2000 for the molecular weight of PEG. [b] PEG-Cys(SH)-G4 represents the dendrimer after treatment with DTT (13 mM).

weight of the corresponding dendrimer because of the difference in molecular shape between the dendrimers and the PEG used as the standard.

The molecular weight of the PEG-Cys-G4 dendrimer was estimated to be lower than the PEG-Cys(Acm)-G4 dendrimer. Therefore, it is likely that disulfide bonds were formed within the single PEG-Cys-G4 dendrimer molecule. In addition, the molecular weight of the PEG-Cys-G4 dendrimer was measured after incubation in the same buffer containing 13 mM dithiothreitol (DTT) for 90 min. The resultant dendrimer showed almost the same molecular weight, suggesting that the cleavage of disulfide bonds hardly affected its molecular size. The polydispersity indexes of these dendrimers are not very close to unity, probably because of intermolecular cross-linking of dendrimers due to the contamination of PEG diol of the commercially available methoxy PEG.[4a]

The association of the dendrimers with rose bengal was investigated (Figure 1). Rose bengal has been shown to be a useful guest molecule to investigate dendrimer-small molecule interactions because this compound yields a bathochromic shift in the maximum absorption wavelength when bound to dendrimers.[2c,d] The absorption spectra of rose bengal in the absence or presence of various dendrimers and DTT are shown in Figure 1A. In the absence of dendrimers, a strong peak is observed at 549 nm, regardless of the presence or absence of DTT. In the presence of the PEG-Cys-G4 dendrimer, rose bengal showed a slightly smaller peak at the same wavelength in the absence of DTT, suggesting a weak interaction with the dendrimer under the oxidative condition. However, in the presence of DTT, the peak of rose bengal exhibited an apparent red shift, indicating that rose bengal molecules were bound to the dendrimer under the reductive conditions. In the presence of the PEG-Cys(Acm)-G4 dendrimer, the spectra showed similar peaks at 551 nm, irrespective of the presence or absence of DTT, indicating that the alteration of the environment does not affect the binding of rose bengal to the dendrimer, which cannot form disulfide bonds.

The differential absorption spectra of rose bengal in the presence of various dendrimers were obtained by subtracting the spectra of free rose bengal (Figure 1B). These spectra showed negative and positive peaks at 545 and 568 nm, respectively. The former corresponds to the decrease in free rose bengal molecules, whereas the latter is attributable to the increase in the bound molecules. The number of rose bengal molecules bound to the PEG-Cys-G4 dendrimer was estimated from Δabsorbance at 545 nm and the molar absorption coefficient of free rose bengal. It was found that 2.1 rose bengal molecules were bound to the PEG-Cys-G4 dendrimer in the absence of DTT at pH 7.4, but the number of bound dye molecules increased to 6.0 when DTT was present in the medium, which is a reductive environment. For the PEG-Cys(Acm)-G4 dendrimer, however,

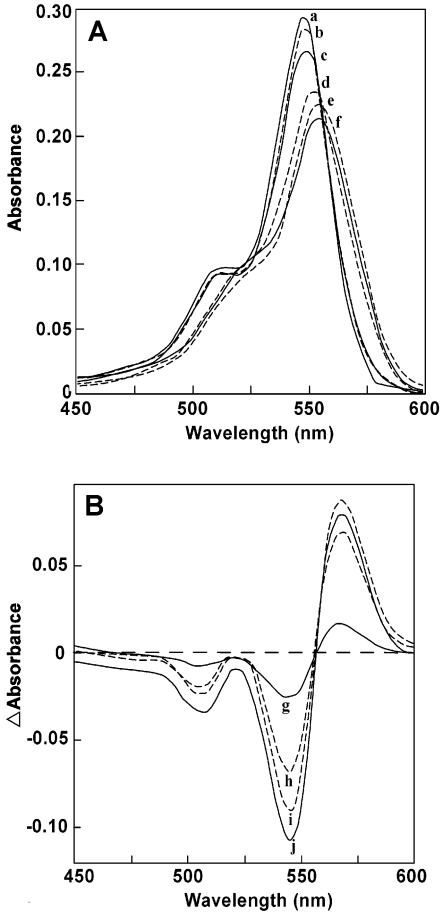

Figure 1. (A) Absorption spectra of rose bengal in the absence (a, b) or presence of PEG-Cys-G4 (c, d) and PEG-Cys(Acm)-G4 (e, f) dendrimers in 0.1 M phosphate buffer (pH 7.4) containing (dotted lines) or not containing (solid lines) 13 mM DTT. The concentrations of rose bengal and the dendrimers were 3 and 0.3 μM, respectively. (B) Differential absorption spectra of rose bengal in the presence of PEG-Cys-G4 (g, h) and PEG-Cys(Acm)-G4 (i, j) dendrimers in 0.1 M phosphate buffer (pH 7.4) containing (dotted lines) or not containing 13 mM DTT (solid lines).

the number of bound dye molecules slightly decreased from 8.7 to 8.0, responding to the same environmental change, probably due to reduced hydrophobic interaction in the presence of DTT. Comparing the numbers of bound dye molecules between the PEG-Cys-G4 dendrimer with the reduced form [PEG-Cys(SH)-G4 dendrimer] and the PEG-Cys(Acm)-G4 dendrimers, the latter has a higher ability to bind to the dye molecules. This may be because the Acm groups of Cys residues enhance interaction between the dendrimer and the dye molecule by hydrophobic interaction. A similar enhancement of rose bengal binding was observed for the PEG-attached PAMAM G4 dendrimer having phenylalanine residues (unpublished result). These results indicate that only the dendrimer that has Cys residues with disulfide bonds exhibits sensitivity to the reductive or oxidative condition of the environment.

Figure 2 depicts an influence of pH on the environment-sensitive binding of rose bengal to the PEG-Cys-G4 dendrimer. In the presence of DTT, where the dendrimer takes on a reduced form (PEG-Cys(SH)-G4), a remarkable enhancement of rose bengal binding to the dendrimer was observed, as the pH of the medium decreased from 7.4 to 6.0. Essentially all of the rose

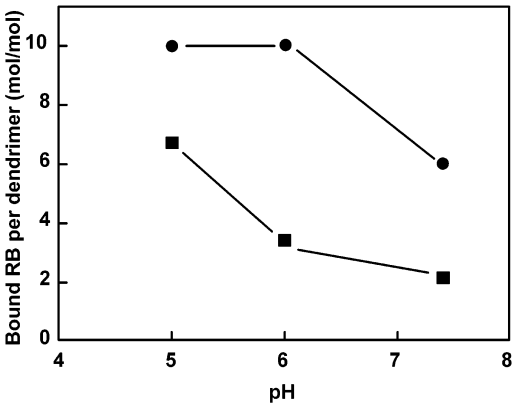

Figure 2. Numbers of rose bengal (RB) molecules bound to the PEG-Cys-G4 dendrimer in the presence (circles) and in the absence (squares) of DTT at varying pHs. The dendrimer (0.3 μM) and rose bengal (3 μM) were mixed in 0.1 M phosphate buffer of a given pH either containing or not containing DTT (13 mM) at 25 °C.

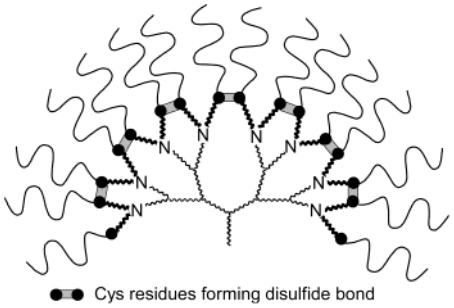

Figure 3. Schematic illustration of network structure formed by disulfide bonds of Cys residues at the periphery of PEG-Cys-G4 dendrimer.

bengal molecules were bound to the dendrimer under this condition. Because a larger fraction of the tertiary amino groups of the dendrimer interior was protonated at pH 6.0 than at neutral pH, it is likely that electrostatic interaction plays an important role in the enhancement of the interaction between the dendrimers and rose bengal.[4a] However, in the absence of DTT, where the dendrimer takes on an oxidized form (PEG-Cys(S-S)-G4), the dendrimer still exhibited much lower ability to interact with rose bengal at pH 6.0. Their interaction was enhanced to some extent, when the pH was decreased to 5.0, where significant protonation of the interior amino groups should cause expansion of the dendrimer chain.[1a,b]

As shown in Figure 3, cross-linking of the dendrimer chain ends by the Cys residues might generate a network structure at the periphery of the dendrimer. This network could reduce freedom of conformation of the dendrimer chain and make it compact, resulting in formation of a dense shell at the periphery of the dendrimer, which effectively suppresses the ingress of rose bengal into its interior. However, in the presence of DTT, the cleavage of disulfide bonds results in the disappearance of the shell and enables access into the interior for the dye molecules, resulting in the strong interaction between them.

To obtain information about the mechanism for the generation of the environment-sensitive property of the dendrimer, we performed titration experiments by adding increasing amounts of dendrimer to rose bengal in the presence or in the absence of DTT. The Klotz plot, which is widely used to analyze host−guest interac-

tions,[8] for the obtained data revealed that the numbers of the binding sites were 22 and 21 for PEG-Cys(SH)-G4 and PEG-Cys(S-S)-G4 dendrimers, respectively. Because the number of the binding site is not affected by the formation or cleavage of disulfide bonds, it is likely that locations of the binding sites are the same for both dendrimers and are presumably in the interior of the dendrimer. However, the intrinsic binding constants were estimated to be 7.6×10^5 and 4.5×10^4 for PEG-Cys(SH)-G4 and PEG-Cys(S-S)-G4 dendrimers, respectively. The affinity of the dendrimer with disulfide bonds for the dye molecule is remarkably lower than that of the dendrimer with SH groups, presumably because the shell formed by the cross-linked Cys residues on the periphery of the dendrimer hides the binding sites in the interior of the dendrimer and suppresses access of the dye molecule to the binding sites.

We demonstrated here that the PEG-attached PAMAM G4 dendrimer having Cys residues exhibits environment-sensitive associating property against rose bengal. The network structure formed by disulfide linkages of the Cys residues at the periphery of the dendrimer could act as an effective barrier which controls access of the small molecules into the dendrimer interior. The dendrimer with the network structure bears a resemblance to the shell-cross-linked nanoparticles developed by Wooley and co-workers.[9] Since their size can be controlled in the nanometer dimension, these particles are expected to have potential usefulness as nanotechnological devices. Further studies on the functionality of the PEG-attached dendrimer with Cys residues as an environment-sensitive nanocapsule and on the mechanism of the environment-sensitive switching of the dendrimer are currently in progress.

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Supporting Information Available: Synthesis and characterization of the dendrimers, ^{1}H NMR spectra of PEG-Cys-(Acm)-G4 and PEG-Cys-G4 dendrimers, analytic techniques, and Klotz plot for binding of rose bengal to PEG-Cys-G4 dendrimer. This material is available free of charge via the Internet at http://pubs.acs.org.

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