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Preparation of poly(ethylene glycol)-modified poly(amidoamine) dendrimers with a shell of hydrophobic amino acid residues and their function as a nanocontainer

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ABSTRACT

We studied the use of poly(ethylene glycol) (PEG)-modified dendrimers as a nanocapsule with a biocompatible surface. We designed PEG-modified dendrimers having a shell of hydrophobic amino acid residues in the peripheral moiety of the dendrimer to increase their encapsulation ability. Subsequently, L-phenylalanine or γ-benzyl-L-glutamate residues were introduced to all chain ends of the poly(amidoamine) G4 dendrimers. Furthermore, PEG (MW 2000) chains were attached to the amino acid residues. These hydrophobic amino acid residues rendered the PEG-modified dendrimers as more compact. After binding of Rose Bengal (RB) guest molecules to dendrimers, an assay using the Klotz plot showed that the hydrophobic amino acid layer slightly affected the guest site number, but significantly increased intrinsic binding of the dendrimers to guest molecules. The PEG-modified dendrimers with the hydrophobic amino acid layer were better able to retain guest molecules than the dendrimer without the layer: they are therefore useful for drug delivery.

1. Introduction

Dendrimers are attractive polymers for holding guest molecules: they have monodispersed molecular weights, well-defined structures, and inner spaces. Many researchers have used these attractive properties for applying dendrimers to drug delivery systems (DDSs) [1–4]. Exploitation of dendrimers for drug delivery presents the advantages of improved solubility and controllable release of drug molecules. Drug-conjugated dendrimers can act as potent prodrugs, for example poly(amidoamine) (PAMAM) and polyallyl ether dendrimers carrying anticancer drugs such as cisplatin, 5-fluorouracil, methotrexate, and doxorubicin [5]. Effective drug behavior in these systems, however, requires cleavage of the drug molecules from the dendrimers. In contrast, drug-encapsulating dendrimers are thought to be useful because the drug molecules remain intact inside the dendrimers. Some groups, including ours, have reported several types of drug-encapsulating dendrimers [5,6].

We have prepared poly(ethylene glycol) (PEG)-modified dendrimers as nanocapsules with a biocompatible surface and studied their feasibility as drug carriers [6]. These dendrimers can be targeted passively to tumor tissues, without diffusion throughout the body, because of their enhanced permeability and retention (EPR) effects [1–4]. We have reported PEG-attached dendrimer encapsulating anticancer drugs such as adriamycin and methotrexate [6], but these dendrimers have defective complex stabilities. In fact, anticancer drugs were rapidly released from these dendrimers under isotonic conditions [6]. To modify and improve the dendrimers' encapsulation capabilities, we have incorporated network structures to the dendrimer periphery using polymerization of methacryloyl groups [7] and disulfide bond formation of cysteine residues [8] at dendrimer chain ends, which imparted stable retention of guest molecules and environment-sensitive encapsulation properties to the dendrimers.

Introduction of a shell to the periphery of dendrimers might be another efficient approach to increase their functions as nanocapsules. Meijer and co-workers built a shell of bulky *N-tert*(I)-butoxycarbonyl-protected amino acid residues at the periphery of poly(propyleneimine) dendrimer and demonstrated that the shell behaved as a barrier that suppresses the diffusion of entrapped guest molecules outside of the dendrimer [9,10]. Recently, we combined hydrophobic amino acids, such as L-phenylalanine and L-leucine, to all chain ends of PAMAM dendrimers and demonstrated that close contact among these amino acid residues generated a shell in the dendrimer periphery [11].

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Considering their use as drug carriers, it is necessary that PEG-modified dendrimers possess high ability to retain drug molecules to carry them to the target site of the body. Based on the results of previous studies, we expected that incorporation of a shell consisting of hydrophobic amino acid residues might provide high retention ability to PEG-modified dendrimers, enabling their use as drug carriers. In this study, we attached amino acids with bulky and hydrophobic benzyl side groups, such as L-phenylalanines (Phe) and γ-benzyl L-glutamate [Glu(OBzl)], to all chain end moieties of dendrimer moiety of the PEG-modified PAMAM G4 dendrimer. Significant improvement of their ability as nanocapsules by incorporation of the hydrophobic amino acid shells is described herein.

2. Experimental

2.1. Materials

Poly(amidoamine) (PAMAM) G4 dendrimer and poly(ethylene glycol) monomethyl ether with a number-average molecular weight of 2000 were purchased from Aldrich Chemical (St. Louis, MO). *N*-(*tert*(I)-butoxycarbonyl) L-phenylalanine (Boc-Phe) and γ-benzyl *N*-(*tert*(I)-butoxycarbonyl) L-glutamate (Boc-Glu(OBzl)) were purchased from Peptide Institute (Osaka, Japan). 1,3-Dicyclohexycarbodiimide (DCC) and *N*-hydroxysuccinimide (HNS), trifluoroacetic acid (TFA) and triethylamine (TEA) were obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and Kishida Chemical (Osaka, Japan), respectively. Rose Bengal (RB) was purchased from Wako (Osaka, Japan).

2.2. Synthesis of PEG-attached dendrimers with hydrophobic moieties

We have already shown that various kinds of amino acid residues were able to combine to essentially every chain end of amine-terminated PAMAM dendrimers using a condensing agent DCC [7,8,11–13]. The PEG-modified dendrimer without amino acid residues (PEG-G4) or with Glu(OBzl) residues (PEG-Glu(OBzl)-G4) were synthesized as previously reported [6,13]. As previously reported, we synthesized PEG-modified PAMAM dendrimers having Phe residues (PEG-Phe-G4) (Scheme 1). Boc-Phe-dendrimer was synthesized as reported previously [12]. The removal of Boc groups by TFA and the reaction with PEG chains were carried out as reported previously [7,8,13]. Yield of PEG-Phe-dendrimer was 28%. ^{1}H NMR spectrum is shown in Fig. 1. Ninhydrin test was performed using 0.2% ninhydrin-containing solution on TLC plates [14].

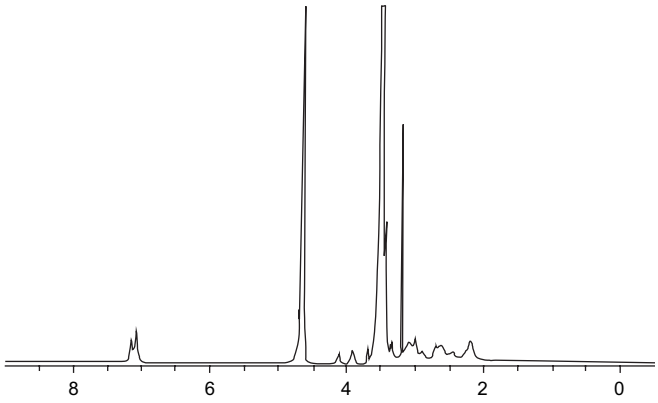


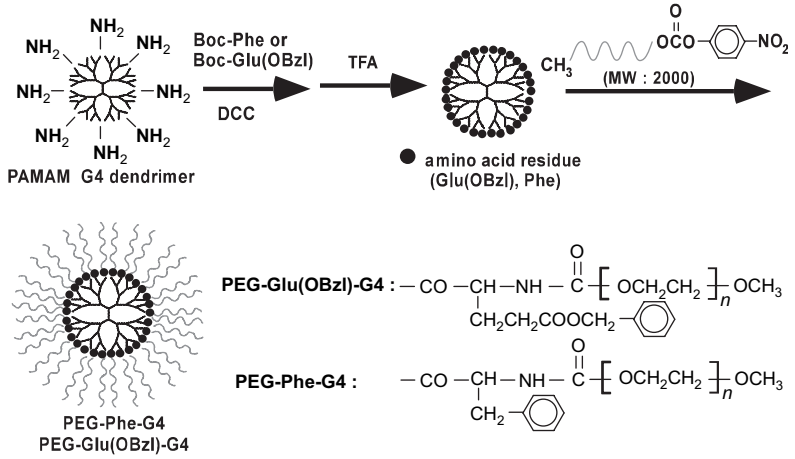
Fig. 1. ^{1}H NMR spectrum of PEG-Phe-G4 dendrimer in D_2O.

2.3. Analytical techniques

The average molecular weights of dendrimers were evaluated from gel permeation chromatography (GPC) using poly(ethylene glycol) as a standard. GPC was performed using a Shodex SB-2004 column (Showa Denko) with differential refractive index detection (Jasco, RI-930), using an eluent of 10 mM phosphate buffer at pH 7.4 containing 0.2 M sodium sulfate. Hydrodynamic diameters of dendrimers were evaluated by dynamic light scattering (DLS) at 25 °C using a Nicomp ZLS380 instrument (Particle Sizing Systems).

Transmission electron microscopic (TEM) analysis was performed as follows [15]. Synthesized dendrimers incubated with 2% (w/v) phosphotungstic acid for an hour were placed on a collodion-coated grid and drawn off with filter paper. The stained sample was allowed to dry. The grid was viewed under an electron microscope (JEOL Ltd., JEM-2000FEX II).

The measurements were performed at 25 °C using a SAXS beamline (BL40B2) at Spring 8, Harima, Japan. The concentration of the aqueous solutions of dendrimers was 20 wt%. The air scattering was subtracted. UV–vis absorption spectra were recorded on a spectrometer (Jasco V-520) at 25 °C. The binding of RB to the dendrimer was measured as reported previously [7,8]. In brief, varying amounts of the dendrimers were mixed to RB (final concentration: 1.91×10^{-6} M) in 10 mM phosphate and 0.1 M NaCl solution of a given pH. Then, concentrations of free and bound RB were determined from absorption spectra of the solutions. The binding of RB to the dendrimers was analyzed using the Klotz plot, which is widely used for analysis of host–guest



Scheme 1. Synthetic route for PEG-modified PAMAM G4 dendrimers with hydrophobic amino acid residues. PAMAM G4 dendrimer has 64 terminal primary amino groups.

interactions under equilibrium conditions [7,16,17]. The Klotz equation is as follows:

$$1/r = (1/nk)(1/C) + 1/n \quad (1)$$

where r, k, C and n indicate the number of moles of bound RB per 1 mol of dendrimer, the intrinsic binding constant, the concentration of free RB and the number of binding sites of the dendrimer [16,17].

3. Results and discussion

3.1. Synthesis of dendrimers with hydrophobic amino acid residues

Through previous studies, we found that various amino acid residues, such as Glu(OBzl), Phe, Lys, and Leu, were combined to all chain ends of PAMAM dendrimers using condensing agent DCC [7,11–13]. Additionally, we showed that PEG chains were attachable to every chain end of PAMAM dendrimers [6] or Glu(OBzl)-terminated PAMAM dendrimers [13] by reaction with PEG–4-nitrophenyl carbonate. In this study, we synthesized PEG-Glu(OBzl)-G4 and PEG-Phe-G4 dendrimers in addition to PEG-G4 dendrimer, which is a control of these shell-incorporated dendrimer (Scheme 1). As described above, PEG-G4 and PEG-Glu(OBzl)-G4 were already synthesized [7,13]. The PEG-Phe-G4 dendrimer was synthesized according to the same procedure [7,13]. The PEG-Phe-G4 dendrimer was characterized using ^{1}H NMR (Fig. 1). The quantities of Phe residues and PEG chains combined to the dendrimer were evaluated from the integrated ratio of typical signals of the PAMAM dendrimer (2.2 ppm), Phe (7.1 ppm), and the PEG chain (3.2 ppm) [7,8,13]. The compositions of these dendrimers, which were used for the following experiments, are described in Table 1. The quantities of PEG chains and amino acid residues per dendrimer agree with the quantities of chain terminals of PAMAM G4 dendrimer for all dendrimers, indicating that a PEG chain and an amino acid residue were combined to every chain end of the dendrimer. Ninhydrin tests of these dendrimers, by which primary and secondary amines are observed, were negative, indicating that these dendrimers contained negligible residual terminal amino groups, which is consistent with the results of prior characterization by ^{1}H NMR.

3.2. Size of dendrimers with hydrophobic amino acid residues

We examined the sizes of PEG-G4, PEG-Phe-G4, and PEG-Glu(OBzl)-G4 dendrimers to determine the effects of the hydrophobic amino acid residues on their respective sizes. The molecular weights of these dendrimers were evaluated using GPC. The number-average molecular weights, the weight-average molecular weights and polydispersity indexes for these dendrimers are presented in Table 2. These molecular weights, as estimated by GPC, are much less than the theoretical values. The globular shape of the dendrimers is well known to cause underestimation of molecular weight by GPC using linear polymers, such as PEG, as standards [6–8].

In general, the molecular weight distribution of dendrimers is known to be narrow. However, the polydispersity indexes for these PEG-modified dendrimers seem to be somewhat large. It is known that commercially available PEG monomethyl ether is frequently contaminated with telechelic PEG diol. The PEG diol contained in the starting material might have become derivatized with two 4-nitrophenyl carbonate groups on both terminals of its chain, which generates crosslinked dendrimers [6,18]. It is also possible that the absorption of these dendrimers to the column gel caused

Table 1
Characterization of various PEG-modified dendrimers

Dendrimer[a]	The number of amino acid residues per dendrimer	The number of PEG chains per dendrimer
PEG-G4	–	63
PEG-Phe-G4	65	65
PEG-Glu(OBzl)-G4	63	66

Estimated by ^{1}H NMR.
[a] PAMAM G4 dendrimer has 64 terminal primary amino groups.

Table 2
Molecular weights and hydrodynamic diameters of PEG-modified dendrimers

Dendrimer	Molecular weight				Diameter ± SD (nm) estimated by DLS	
	Calculated	Estimated by GPC				
		M_n	M_w	M_w/M_n	Number average	Weight average
PEG-G4	143,879	35,300	46,400	1.3	15.3 ± 1.1	16.2 ± 2.2
PEG-Phe-G4	153,297	29,300	36,200	1.2	14.2 ± 2.5	15.5 ± 2.0
PEG-Glu(OBzl)-G4	157,910	26,900	40,200	1.5	12.0 ± 1.8	13.0 ± 2.1

Molecular weights of dendrimers were calculated assuming that molecular weight of PEG is 2000. Phosphate (10 mM) and 0.2 M Na_2SO_4 solution (pH 7.4) were used as eluent. Poly(ethylene glycol)s were used as standards.

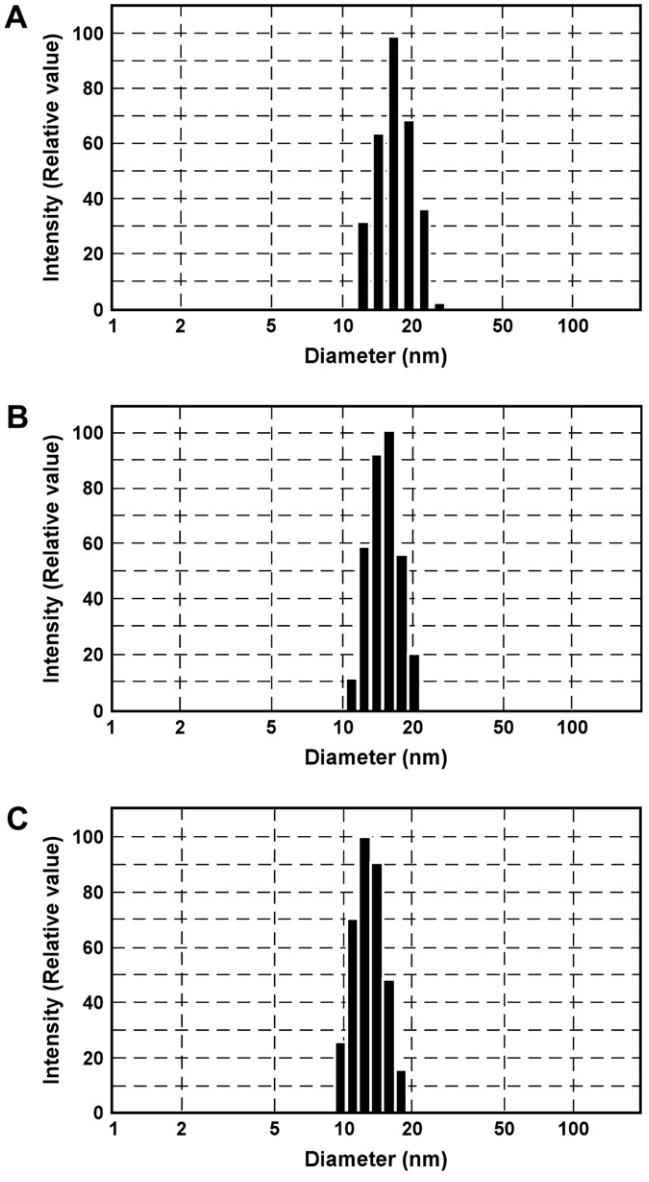


Fig. 2. Size distribution profiles of PEG-G4 (A), PEG-Phe-G4 (B), and PEG-Glu(OBzl)-G4 (C) dendrimers estimated by DLS. Weight-average size distributions are shown.

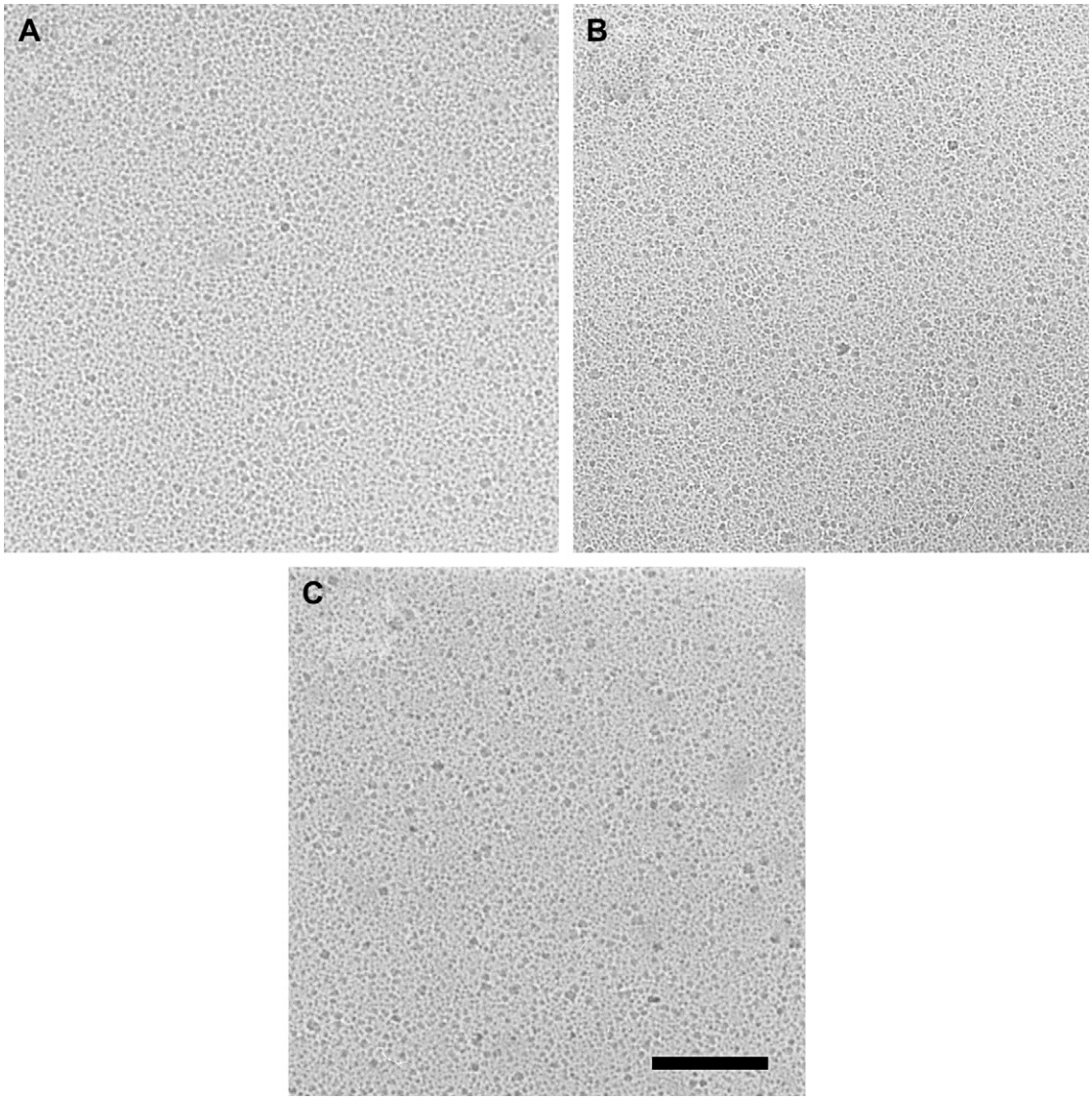


Fig. 3. TEM images of PEG-G4 (A), PEG-Glu(OBzl)-G4 (B), and PEG-Phe-G4 (C) dendrimers, which were positively stained with phosphotungstic acid. Bar, 200 nm.

the polydispersity. Nevertheless, the molecular weights of the dendrimers having amino acid residues were evaluated as less than those of dendrimers without amino acid residues, although the dendrimers having amino acid residues theoretically possess higher molecular weight than the dendrimer without amino acid residues. This result suggests that the amino acid residues decreased the dendrimer size.

The sizes of these dendrimers were further estimated using DLS. Typical examples of size distributions for these PEG-modified dendrimers are presented in Fig. 2. These dendrimers exhibited a narrow size distribution. The number-average diameters and the weight-average diameters evaluated by DLS are also presented in Table 2. The smaller sizes of the amino-acid-containing dendrimers are observed for the result of DLS, consistent with the result of GPC. It is likely that the amino acid residues attached in the periphery of the dendrimers are mutually associated through hydrophobic interaction. They make the conformation of the dendrimers more compact than the dendrimer without amino acid residues. Comparing these two types of dendrimers with amino acid residues, PEG-Glu(OBzl)-G4 dendrimer showed a smaller size than PEG-Phe-G4 dendrimer, probably because of the higher hydrophobicity of Glu(OBzl) residues than that of Phe residues.

The smaller size of the amino acid-shell-bearing dendrimers was further confirmed using small-angle X-ray scattering. Aqueous solutions of these PEG-modified dendrimers with a high concentration of 20 wt% exhibited scattering profiles, each with a peak around a scattering vector at 0.5 nm^{-1}. It is likely that, in the concentrated solution, closely contacting dendrimers might form an ordered structure with a repeat distance, which corresponds to the dendrimer diameter. The distances of PEG-G4, PEG-Phe-G4, and PEG-Glu(OBzl)-G4 dendrimers were estimated, respectively, as 15.1, 12.8, and 13.3 nm. These values are mostly consistent with their diameters evaluated using DLS (Table 2).

We also performed TEM analyses of these dendrimers by staining with phosphotungstic acid, as shown in Fig. 3. No aggregates were observed. Therefore, these dendrimers can act as a nanocontainer.

3.3. Interaction of dendrimers with hydrophobic amino acid residues with RB

To elucidate the effect of the amino acid residues of the PEG-modified dendrimer on its properties as a nanocontainer, we examined their binding ability to RB, which has been widely used to investigate dendrimer–small molecule interactions [11,19,20]. We

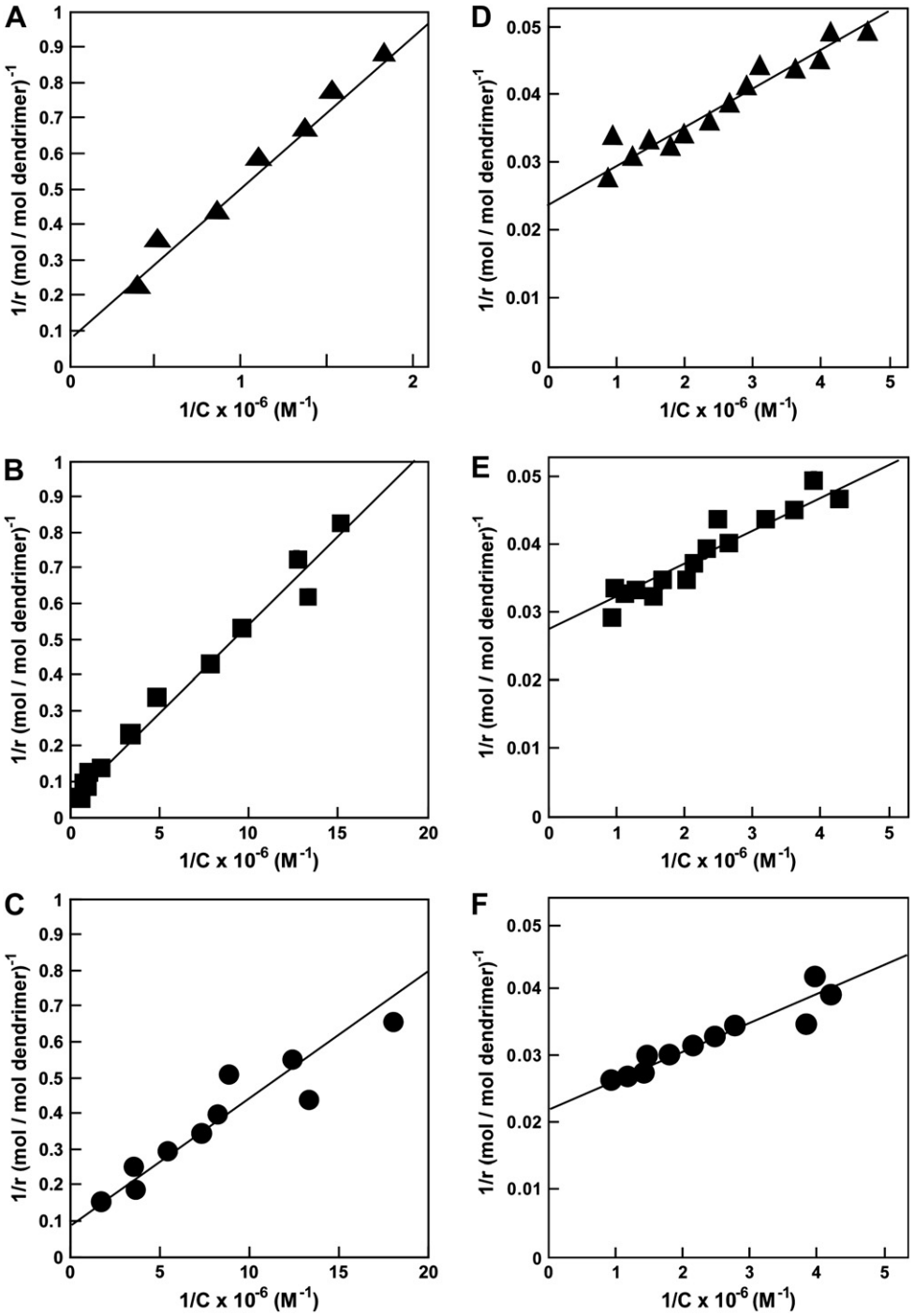


Fig. 4. Relationship between $1/r$ and $1/C$ for binding of RB to PEG-G4 (A and D), PEG-Phe-G4 (B and E) and PEG-Glu(OBzl)-G4 (C and F) dendrimers at pH 7.4 (A–C) and 5.0 (D–F).

have already shown that the concentrations of free and dendrimer-bound RB can be determined from differential absorption spectra obtained by subtracting the spectra of free RB from those in the presence of dendrimers [7,8]. For that purpose, we performed titration experiments by adding increasing amounts of dendrimer to RB at pH 7.4 and 5.0. The results were analyzed using a Klotz plot, which is widely used to study host–guest interactions [7,16,17]. As presented in Fig. 4, plots of $1/r$ against $1/C$ (Klotz plot) for the binding of RB to the dendrimers were fundamentally linear, with correlation coefficients R^2 of not less than 0.94 (Table 3) [21]. The apparent binding constant nk, the number of binding sites per dendrimer n, and the intrinsic binding constant k were estimated from Fig. 4 using the Klotz equation; they are also presented in Table 3.

It is apparent that the dendrimers with hydrophobic amino acid residues exhibited more than 10 times higher binding ability than the dendrimer without amino acid residues at pH 7.4. It is noteworthy that the dendrimers with the amino acid shell exhibited much higher k values than the dendrimer without the shell, although these dendrimers possess almost the same n values: 9–12. A similar situation was also apparent in their binding at pH 5.0. These dendrimers showed one or two orders of magnitude higher k values at pH 5.0 than at pH 7.4, indicating that their binding ability greatly increased in the mildly acidic pH. Results of previous studies demonstrated that the affinity of RB to the PAMAM dendrimers increased considerably at mildly acidic pH [7,8,22]. Negatively charged RB molecules interacted strongly with the dendrimer

Table 3
Binding constants and number of binding sites of dendrimers for RB

Dendrimer	pH 7.4				pH 5.0			
	nk (M^{-1})	n	k (M^{-1})	R^2	nk (M^{-1})	n	k (M^{-1})	R^2
PEG-G4	2.0×10^6	9	2.2×10^5	0.98	1.8×10^8	43	4.2×10^6	0.95
PEG-Phe-G4	2.1×10^7	11	1.9×10^6	0.99	2.8×10^8	37	7.6×10^6	0.95
PEG-Glu(OBzl)-G4	3.1×10^7	12	2.6×10^6	0.99	3.3×10^8	44	7.5×10^6	0.94

interior, of which tertiary amine groups become protonated below pH 7.0 [23–25].

As presented in Table 3, these dendrimers exhibited similar n values between 37 and 44, irrespective of possession of amino acid shells at pH 5.0. However, the dendrimers with the shell showed much higher k values than the dendrimer without the shell, as is true for pH 7.4. Apparently, the hydrophobic amino acid residues attached at the periphery of PAMAM dendrimer only slightly affected the number of binding sites but greatly affected the binding constant. This result strongly suggests that the guest molecules are bound to the interior of the dendrimer and that the shell of amino acid residues strengthened their binding. Electrostatic interaction plays an important role in the dendrimer–RB interaction. Therefore, it is likely that positive charges control the number of binding sites. In contrast, the amino acid residues in the dendrimer periphery might enhance binding of guest molecules through hydrophobic interaction. Compact conformation of the amino acid-containing dendrimers (Table 2) might also enhance interaction between the dendrimer backbone and guest molecules. Consequently, the dendrimers with the amino acid residues represented a stronger affinity to the guest molecules.

3.4. Stability of dendrimer–RB complexes

Absorption spectra of RB are known to change when its chemical structure changes from a quinoid form to a lactonic form in liquids with acidic pH [26]. In fact, RB on a quinoid form shows an absorption peak at 549 nm in an aqueous solution at pH greater than 4.0, but this absorption disappears at pH less than 3.0 upon its structural change to a lactonic form, which loses negative charge (Fig. 5). To elucidate the influence of the hydrophobic shell on stability of the dendrimer–RB complexes, we therefore examined the stability of the dendrimer–RB complexes by following the absorbance of RB encapsulated in the PEG-modified dendrimers.

Fig. 6A presents the time course of the decrease in absorbance at 549 nm for RB in the presence or absence of dendrimers upon changing pH of the medium from 4.0 to 3.0. The absorption of free RB was lost immediately after changing pH, indicating that the quinoid form quickly transformed to the lactonic form. However, the profiles of change in absorbance differed greatly among these dendrimers. The PEG-modified dendrimer without amino acid residues showed a rapid decrease in absorbance to 50% immediately after the pH change; the absorbance continued to decrease gradually with time. In contrast, for the PEG-modified dendrimers with the amino acid shells, a small decrease in absorbance was observed initially, but the absorbance remained constant thereafter.

Considering that RB molecules not complexed with dendrimers change from quinoid form to lactonic form, the observed decrease in absorbance might be attributable to dissociation of RB from the dendrimer. Therefore, this result suggests that dendrimers with the amino acid residues have higher capabilities of retaining guest molecules in their interior. PAMAM dendrimers possess almost all-protonated inner tertiary amines at pH lower than 4.0 [25]. Probably, the shell of the hydrophobic amino acid residues in the periphery of the dendrimer causes a strong interaction between the

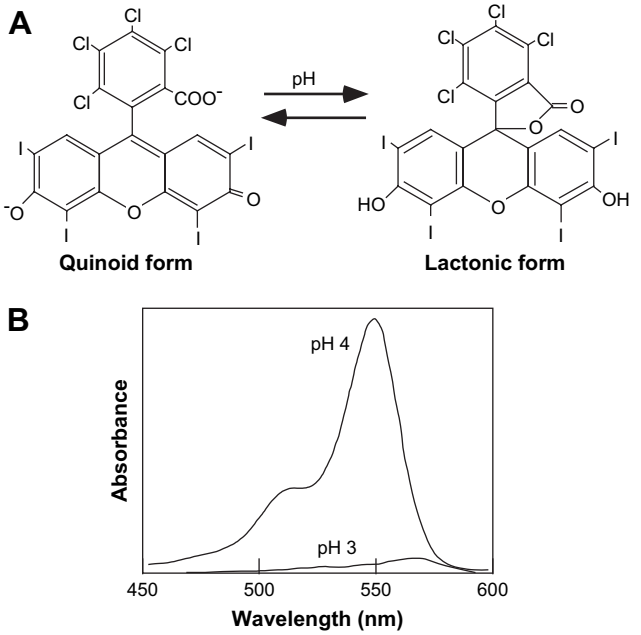


Fig. 5. Conformational change of RB from quinoid form to lactone form (A) and the UV–vis spectra at pH 4.0 and 3.0 (B).

dendrimer backbone and RB. Therefore, PEG-Glu(OBzl)-dendrimer, i.e., dendrimers having amino acid residues with highly hydrophobic side chains, displayed the strongest ability to retain guest molecules in the interior among these dendrimers. However, when the pH of the medium was decreased to 2.0, absorbance of RB disappeared immediately (Fig. 6B). This result indicates that RB molecules can be released, even from dendrimers with the hydrophobic shell, by decreasing pH.

Electrostatic interaction plays a crucial role in binding between the dendrimer and RB. For that reason, we next examined the pH-induced dissociation of the dendrimer–RB complexes at varied salt concentrations (Fig. 7). Dissociation of RB from the dendrimers was enhanced with increasing ionic strength of the medium for all the dendrimers. However, dissociation of RB was apparently suppressed by the hydrophobic amino acid shells, even at high NaCl concentrations, although their effect is rather weak at 1.0 M of NaCl, where electrostatic interaction might be weakened significantly. These results indicate that the hydrophobic amino acid residues in the dendrimer periphery were able to stabilize RB molecules bound to the dendrimer interior and strongly suppress dissociation of RB molecules from the dendrimer interior.

4. Conclusions

We prepared PEG-modified PAMAM G4 dendrimers with hydrophobic amino acid residues, such as Glu(OBzl) and Phe. Results showed that incorporation of the shell of hydrophobic amino acid residues into the periphery of PEG-modified dendrimers increases their performance as nanocapsules. These dendrimers were capable of serving as biocompatible containers with tightly controlled size in a nanometer dimension and high ability to retain guest molecules. Therefore, they are inferred to be highly attractive as carriers for drugs and bioactive molecules, such as RB, which generate singlet oxygen under light irradiation and which are used for photodynamic therapy [14,27]. Information obtained through this study might be of great importance for the development of dendrimer-based drug delivery systems and will contribute to expanding the range of dendrimer applications in fields of biomedicine.

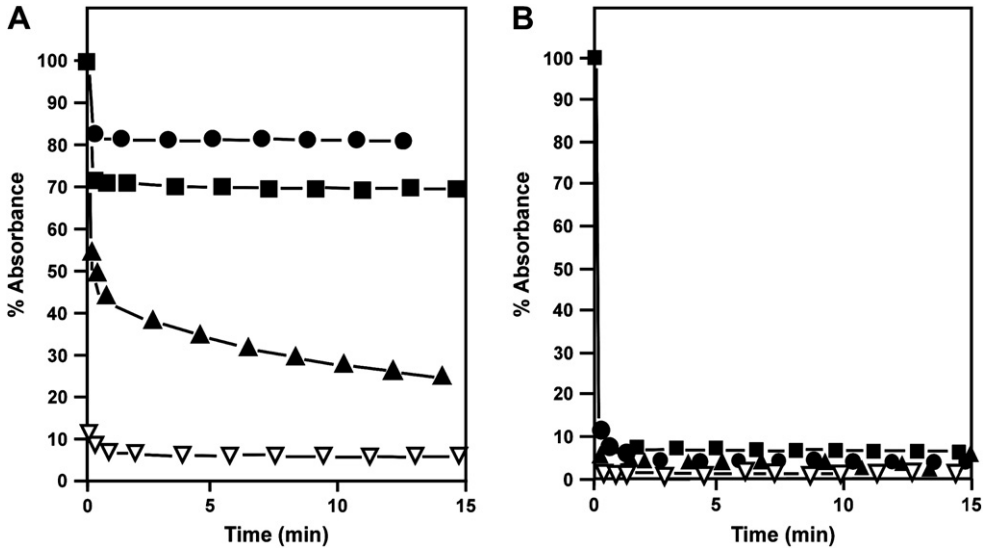


Fig. 6. Time course of change in absorbance of RB bound to PEG-Glu(OBzl)-G4 (●), PEG-Phe-G4 (■), and PEG-G4 (▲) dendrimers and free RB (▽) in 10 mM phosphate and 0.1 M NaCl solution after pH changes from 4.0 to 3.0 (A) and 2.0 (B). [RB] = 1.91×10^{-6} M. Absorbance (%) was evaluated as the ratio to absorbance at pH 4.0.

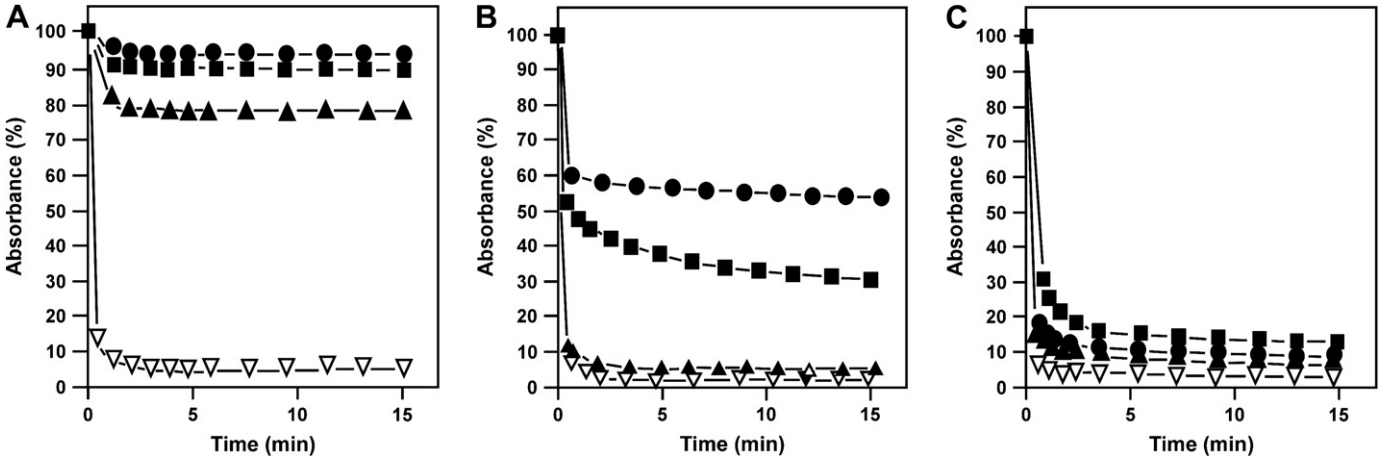


Fig. 7. Time course of change in absorbance of RB bound to PEG-G4 (▲), PEG-Phe-G4 (■) and PEG-Glu(OBzl)-G4 (●) dendrimers in 10 mM phosphate solution containing 0 (A), 0.1 (B) or 1 M (C) NaCl. Free RB (▽) was also shown as a control. Absorbance (%) was evaluated as the ratio to absorbance at pH 4.0.

Acknowledgements

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