HPLC analysis of functionalized poly(amidoamine) dendrimers and the interaction between a folate-dendrimer conjugate and folate binding protein

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Poly(amidoamine) (PAMAM) dendrimers of different generations with carboxyl, acetyl, and hydroxyl terminal groups and a folic acid (FA)-dendrimer conjugate were separated and analyzed using reverse-phase high performance liquid chromatography (HPLC). Analysis of both the individual PAMAM derivatives and the separation of mixed generations can be achieved using a linear gradient 0–50% acetonitrile (ACN) (balance water) within 40 min. We also show that PAMAMs with defined acetylation and carboxylation degrees can be analyzed using HPLC. Furthermore, a generation 5 dendrimer-FA conjugate (G5.75Ac-FA_4; Ac denotes acetyl) was analyzed and its specific binding with a bovine folic acid binding protein (FBP) was monitored. The HPLC and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) results indicate the formation of three complexes after the binding of G5.75Ac-FA_4 with FBP. Dendrimers with FA moieties show much higher specific binding capability with FBP than those without FA moieties. Findings from this study indicate that HPLC is an effective technique not only for characterization and separation of functionalized PAMAM dendrimers and conjugates but also for investigation of the interaction between dendrimers and biomolecules.

Introduction

Poly(amidoamine) (PAMAM) dendrimers are highly branched, narrowly dispersed synthetic macromolecules with well-defined structure and composition.[1] The terminal amine groups of PAMAM dendrimers can be modified with different functionalities and can be linked with various biomolecules.[2,3] These unique structural features of PAMAM dendrimers make them ideal nanoplatforms to conjugate biologically important substances (*e.g.*, imaging agents, targeting molecules, and drugs) for subsequent imaging, targeting, and treatment of biological systems.[4–8] The multiple surface modifications of PAMAM dendrimers always result in the structural complexity of the final products, which gives rise to great challenges in the analysis of their structures, molecular distribution, and surface functionalities. Various techniques have been utilized to analyze PAMAM dendrimers and derivatives, including but not limited to NMR spectroscopy,[9,10] matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry,[11,12] size exclusion chromatography (SEC),[10] polyacrylamide gel electrophoresis (PAGE),[9,13–17] and capillary electrophoresis (CE).[9,13,16–22] In our group, CE has been used extensively as one of the important tools to analyze various dendrimer derivatives and dendrimer-based nanodevices.[20] We have shown that the combination of CE with other techniques is important for us to understand the structural characteristics of dendrimer derivatives and nanodevices. However, the development of different analytical techniques for analyzing various dendrimeric nanostructures still remains a great challenge.

As an alternative analytical tool, high performance liquid chromatography (HPLC) has recently been used for the analysis of PAMAM dendrimers and derivatives[23,24] and of other dendritic polymers such as poly(propylene imine) and polyether dendrimers.[25–27] In our previous report, we have shown that reverse phase HPLC (RP-HPLC) is a vital tool to analyze dendrimers with various generations and terminal groups.[23] We have achieved generational separation of amine-terminated PAMAM dendrimers and separation of PAMAM dendrimers of the same generation but with different terminal groups.[23] However, generational separation of PAMAM dendrimers with functionalities other than amine groups and separation of dendrimers with defined partial modifications have not been reported.

Targeting moieties are important components in multifunctional PAMAM dendrimer-based nanodevices. It has been demonstrated that folic acid (FA)-modified generation 5 (G5) PAMAM dendrimer nanodevices can be specifically internalized by tumor cells expressing FA receptors.[8,28,29] As the soluble form of FA receptor, that is overexpressed on the surface of many tumor cells, folic acid binding protein (FBP) has been used to detect the specific binding with FA-modified nanoparticles using the surface plasmon resonance (SPR) technique.[30–32] The molecular association can be detected on a gold surface immobilized with FBP. To the best of our knowledge, there is no reported literature regarding HPLC analysis of the binding between FA-modified nanoparticles and FBP.

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In this study, we report generational separation of PAMAM dendrimers with carboxyl, acetyl, and hydroxyl groups. G5 PAMAM dendrimers with defined acetylation and carboxylation substitutions were also analyzed using RP-HPLC. These studies not only expand the demonstrated analytical capability of HPLC for dendrimers but also provide new insights into the understanding of the separation mechanism as well as the structural characteristics of PAMAM derivatives. In addition, the binding of a G5.75Ac-FA_4 (Ac denotes acetyl) conjugate with FBP was analyzed using RP-HPLC. Extra peaks are expected to appear in the HPLC chromatogram due to the formation of complexes between G5.75Ac-FA_4 and FBP. The HPLC results were also compared with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) measurements. The monitoring of the specific and nonspecific binding events of FA-modified dendrimer nanoparticles with FBP using HPLC will be useful for one to design and synthesize FA-modified dendrimer-based nanodevices for *in vitro* and *in vivo* tumor targeting studies.

Experimental

Materials

Ethylenediamine (EDA)-cored PAMAM dendrimers of generations 1–6 (G1–G6) were purchased from Dendritech (Midland, MI) in methanol solution. The surfaces of amine-terminated PAMAM dendrimers of selected generations were converted to carboxyl, acetyl, and hydroxyl groups by reacting with succinic anhydride, acetic anhydride and glycidol, respectively. The carboxyl, acetyl, and hydroxyl-terminated PAMAM dendrimers formed are denoted as Gn.SAH, Gn.Ac, and Gn.NGlyOH, respectively ("*n*" represents the number of the dendrimer generation). Details of the conversion procedure can be found elsewhere.[10,18] G5 PAMAM dendrimers with defined acetylation (25%, 50%, and 75%, denoted as G5.25Ac, G5.50Ac, and G5.75Ac, respectively) and carboxylation (25%, 50%, and 75%, denoted as G5.25SAH. G5.50SAH, and G5.75SAH, respectively) degrees were synthesized and characterized elsewhere.[16] FBP (extracted from bovine milk, Mw = 30 000) and FA were obtained from Sigma-Aldrich. G5.75Ac-FA_4 conjugate was synthesized and characterized according to the reported procedure.[5,33] Briefly, amine-terminated G5 dendrimers were partially (75%) acetylated, followed by FA conjugation using EDC chemistry. The final product contained on average four FA moieties per dendrimer molecule as confirmed by ^{1}H NMR.

Size exclusion chromatography (SEC)

SEC was used to determine the absolute molecular weights of the as-prepared PAMAM dendrimer derivatives. SEC experiments were performed using an Alliance Waters 2690 separation module (Waters Corp., Milford, MA) equipped with a Waters 2487 UV absorbance detector (Waters Corp.), a Wyatt Dawn DSP laser photometer (Wyatt Technology Corp., Santa Barbara, CA), an Optilab DSP interferometric refractometer (Wyatt Technology Corp.), and TosoHaas TSK-Gel Guard PHW 06762 (75 × 7.5 mm, 12 μm), G 2000 PW 05761 (300 × 7.5 mm, 10 μm), G 3000 PW 05762 (300 × 7.5 mm, 10 μm), and G 4000 PW (300 × 7.5 mm, 17 μm) columns. Column temperature was maintained at 25 ± 0.2 °C with a Waters temperature control module. Citric acid buffer (0.1 M) with 0.025% sodium azide in water was used as a mobile phase. The pH of the mobile phase was adjusted to 2.74 using NaOH, and the flow rate was maintained at 1 mL min^{-1}. A sample concentration was kept at 2 mg mL^{-1}, and 100 μL was injected for all measurements. Molar mass moments of the PAMAM dendrimers were determined using Astra software (version 4.7) (Wyatt Technology Corp.).

High performance liquid chromatography (HPLC)

The RP-HPLC system (Beckman Coulter, Fullerton, CA) consisting of a System GOLD 126 solvent module, a model 507 autosampler equipped with a 100 μL loop, and a model 166 UV detector was used in this work. A Jupiter C5 silica-based RP-HPLC column (250 × 4.6 mm, 300 Å) was purchased from Phenomenex (Torrance, CA). Two Phenomenex Widepore C5 safety guards (4 × 3 mm) were installed ahead of the Jupiter column. The mobile phase for elution of PAMAM dendrimer derivatives of different generations was a linear gradient beginning with 100 : 0 (v/v) water/acetonitrile (ACN) at a flow rate of 1 mL min^{-1}. Trifluoroacetic acid (TFA) at 0.14% (v/v) concentration in water as well as in ACN was used as a counterion to neutralize the dendrimer surface charges. All the samples were dissolved into the aqueous mobile phase (water containing 0.14% TFA) at the concentration of 1 mg mL^{-1}. The detection of eluted samples was performed at 210 nm. The analysis was performed using Beckman's System GOLD Nouveau software.

For the binding of dendrimers with FBP, the mobile phase used was a linear gradient beginning with 90 : 10 (v/v) water/acetonitrile (ACN) at a flow rate of 1 mL min^{-1}. All the samples were dissolved into the 90 : 10 (v/v) water/ACN mobile phase (both water and ACN contain 0.14% TFA) at the concentration of 1 mg mL^{-1}. The detection of eluted samples was performed at 210 nm and/or 280 nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Analysis of FBP and its binding with PAMAM dendrimers by SDS-PAGE was performed on a Micrograd vertical electrophoresis system Model FB-VE10-1 (FisherBiotech, Pittsburgh, PA). Precast 4–20% gradient express gels for SDS-PAGE were obtained from ISC BioExpress (Kaysville, UT). A commercial power supply Model EC135-90 (Thermo Electron Corp., Milford, MA) was used. Tris-Glycine SDS buffer (pH = 8.3) was purchased from Invitrogen (Carlsbad, CA). It was diluted by 10 times for use as the running buffer. SDS-PAGE separations typically required 30–40 min at 200 V. Into each sample well, 10 μL of a sample solution containing 5 μL FBP or FBP/dendrimer mixtures and 5 μL bromophenol blue sucrose dye (50% sucrose, 1% bromophenol blue) was injected. Developed gels were stained overnight with 0.025% Coomassie Blue R-250 in a 40% methanol and 7% acetic acid aqueous solution. The gels were destained with an aqueous solution containing 7% (v/v) acetic acid and 5% (v/v) methanol.

Table 1 Theoretical and experimental molar masses of PAMAM dendrimers with carboxyl, acetyl, and hydroxyl termini

PAMAM	Theoretical molar mass, Mn/g mol^{-1}	Experimental molar mass[a], Mn/g mol^{-1}	Polydispersity (Mw/Mn)[a]	Peak elution time/min[b]	$W_{H/2}$/min
G1.SAH	2230	2063	1.070	16.27	0.306
G2.SAH	4856	4062	1.011	18.96	0.365
G3.SAH	10 109	9790	1.022	20.81	0.349
G4.SAH	20 615	19 000	1.071	22.50	0.561
G5.SAH	41 626	40 330	1.045	23.67	0.659
G6.SAH	83 648	70 321	1.201	24.51	1.219
G1.Ac	1766	1744	1.019	15.09	0.313
G2.Ac	3928	3791	1.016	17.95	0.317
G3.Ac	8253	8428	1.064	19.80	0.461
G4.Ac	16 903	15 373	1.161	21.32	0.486
G5.Ac	34 202	30 990	1.060	22.66	0.623
G1.NGlyOH	2614	2385	1.011	12.81	0.794
G2.NGlyOH	5624	5045	1.036	16.02	0.590
G3.NGlyOH	11 645	9198	1.070	18.96	0.733
G4.NGlyOH	23 687	18 916	1.113	20.30	0.852
G5.NGlyOH	47 770	38 382	1.131	22.17	1.025

[a] Measured using SEC. [b] Peak elution time is at the HPLC conditions of Fig. 2.

Table 2 Theoretical and experimental molar masses of G5 PAMAM dendrimers with defined acetylation and carboxylation degree and a G5.75Ac-FA_4 conjugate

PAMAM	Theoretical molar mass, Mn/g mol^{-1}	Experimental molar mass[a], Mn, g mol^{-1}	Polydispersity (Mw/Mn)[a]	Peak elution time/min[b]	$W_{H/2}$/min
G5.NH2	28 826	26 010	1.104	22.03	0.577
G5.25Ac	30 170	27 510	1.086	21.73	0.579
G5.50Ac	31 514	28 680	1.086	21.73	0.528
G5.75Ac	32 858	29 240	1.100	21.73	0.560
G5. Ac	34 202	30 990	1.060	22.18	0.498
G5.25SAH	36 058	32 910	1.045	22.48	0.594
G5.50SAH	37 914	36 050	1.072	22.62	0.746
G5.75SAH	39 770	37 650	1.096	22.92	0.691
G5.SAH	41 626	40 330	1.054	23.22	0.520
G5.75Ac-FA_4	34 973	31 400	1.027	—	—

[a] Measured using SEC. [b] Peak elution time is at the HPLC conditions of Fig. 3.

Results and discussion

Size exclusion chromatography (SEC)

The SEC apparatus was equipped with three detectors: a multiangle laser light scattering (MALLS), a differential refractive index, and a UV-visible. The use of a MALLS detector allows determination of absolute molar mass without any calibration standards, which is of great importance in the case of dendrimers since no calibration standards are currently available. The absolute molecular weights of various functionalized PAMAM dendrimer derivatives of different generations measured using SEC display deviation from the theoretical ones (see Table 1 and Table 2), suggesting the existence of structural imperfections composed of generational (trailing generations and dimers), skeletal (missing arms and intramolecular loops), and substitutional dispersities (variance between substituted dendrimer molecules).[9,23] The polydispersities of all dendrimer derivatives are close to 1, regardless of their generation numbers and surface functionalities. Fig. 1 shows the typical differential mass fraction profiles of carboxyl-terminated PAMAM dendrimers of different generations. All dendrimer samples show a single symmetric peak although larger generation PAMAMs exhibit a much broader peak. Neither the FA-modified dendrimer conjugate nor G5 PAMAM dendrimers functionalized with different degrees of acetylation and carboxylation show a significant change in their polydispersities (Table 2). It is generally known that the performance of the SEC technique is based on the difference of the molecular volumes of macromolecules. After successful surface modifications, the volume of the same generation dendrimer derivatives does not change significantly as

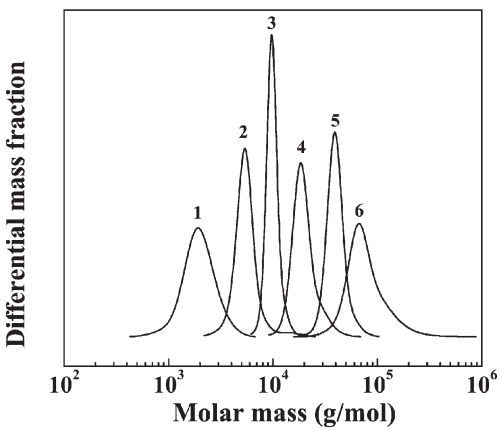

Fig. 1 Differential mass fraction curves of G1.SAH (peak 1), G2.SAH (peak 2), G3.SAH (peak 3), G4.SAH (peak 4), G5.SAH (peak 5), and G6.SAH (peak 6) measured by SEC.

compared to the starting amine-terminated dendrimers; therefore, the polydispersity of PAMAM derivatives of the same generation does not change significantly. Other chromatographic techniques such as HPLC and CE are based on the interaction between the column and the analytes. This leads to a significant difference in the homogeneity evaluation for PAMAM derivatives of different generations modified with various surface functionalities as observed in previous CE studies.[16] It is also noted that the trailing generations and dimers do not appear in the SEC mass diffraction profiles (Fig. 1) but rather appear in the HPLC chromatograms (see below). This suggest that the SEC technique is not sensitive enough to detect impurity species (<10% in the case of single generation dendrimers) based on the difference of the molecular volumes.

High performance liquid chromatography

Analysis and generational separation of PAMAM derivatives. Both the purity and distribution of dendrimers can be analyzed using HPLC. The primary factor governing the elution of dendrimers through a hydrophobic column is the density of dendrimer-TFA ion pairs, which increases as a function of the number of dendrimer generations. For carboxyl-, acetyl-, and hydroxyl-terminated dendrimers, the number of tertiary amine groups mainly determines their elution times. The elution time of dendrimer derivatives (*i.e.*, carboxyl-, acetyl-, and hydroxyl-terminated dendrimers) increases with the number of dendrimer generations (Table 1). Both the individual dendrimer analysis and the generational separation were performed using HPLC. Fig. 2 shows HPLC chromatograms of both the individual generation (Fig. 2a) and the separation of mixed generations (Fig. 2b) of acetyl-terminated PAMAM dendrimers. HPLC chromatograms of carboxyl- and hydroxyl-terminated PAMAM dendrimers are not shown. All dendrimer derivatives contain both trailing generations and dimers (totaling <10%). At each generation, the dimer peak very closely overlaps with the next higher generation, while the trailing generation overlaps with the next lower generation (*e.g.*, in Fig. 2a). The results are consistent with the fact that PAMAM dendrimers always contain a small portion of both trailing generations and dimers, resulting from divergent synthetic technology.[1] This is in agreement with previous PAGE analytical results.[13,21,34] The elution time of each generation dendrimer derivative does not change in both individual HPLC analysis and generational separation runs, indicating the high reproducibility of the HPLC technique.

The polydispersity of PAMAM dendrimers can be estimated by assessing the peak width at half height ($W_{H/2}$).[23] Note that the polydispersity reflected from $W_{H/2}$ in HPLC chromatograms is different from the one calculated from SEC experiments, which is the ratio of Mw and Mn. Accordingly, the polydispersity derived from $W_{H/2}$ is not necessarily correlated to that derived from SEC experiments. It is clear that the polydispersity of carboxyl-, acetyl-, and hydroxyl-terminated PAMAMs increases as a function of the number of generations (Table 1). For instance, $W_{H/2}$ of carboxyl-terminated dendrimers increases from 0.3 (G1.SAH) to 1.2 min (G6.SAH) with the increase of the number of dendrimer generations (Table 1). The reported data in Table 1 are only relative to the operative conditions used to generate the HPLC chromatograms of the dendrimers used in this study. A similar situation applies to the separation of G5 dendrimers with different degrees of acetylation and carboxylation (*vide infra*). It is not surprising to observe the increase of polydispersity of PAMAM dendrimers with the increase of the number of dendrimer generations, which is believed to be ascribed to successive iteration sequences involved during synthesis.[11]

Analysis of G5 PAMAM derivatives with defined degrees of acetylation and carboxylation. G5 PAMAMs with defined degrees of acetylation and carboxylation (0, 25%, 50%, 75%, and 100%) were also analyzed individually using HPLC (Fig. 3). All the materials are pure and well characterized.[16] The elution time and $W_{H/2}$ do not change significantly after the defined substitutions (Table 2). It indicates that within the same generation PAMAMs, defined modifications of PAMAMs cannot be significantly differentiated under the current HPLC condition using a C5 column, unlike our previous work with CE analysis, which is based on the change of charge/mass ratios.[16] However, the advantage of HPLC is

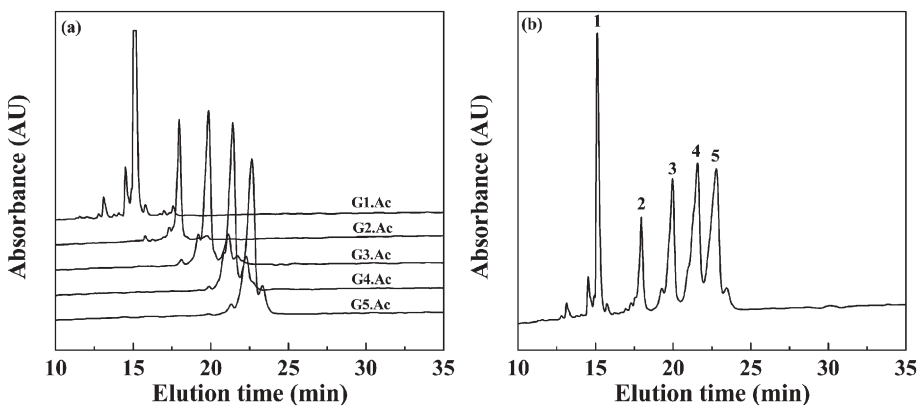

Fig. 2 (a) HPLC chromatograms of acetyl-terminated dendrimers of different generations injected separately. The gradient used was 0–50% ACN (balance water) within 40 min. Injection: 30 μL. (b) HPLC chromatogram of the mixture of G1.Ac-G5.Ac dendrimers obtained after injecting 50 μg of the mixture generation sample (the concentrations of each generation of acetyl-terminated dendrimer in the mixture are ~0.2 mg mL^{-1}). Peaks 1, 2, 3, 4, and 5 are identified as G1.Ac, G2.Ac, G3.Ac, G4.Ac, and G5.Ac, respectively.

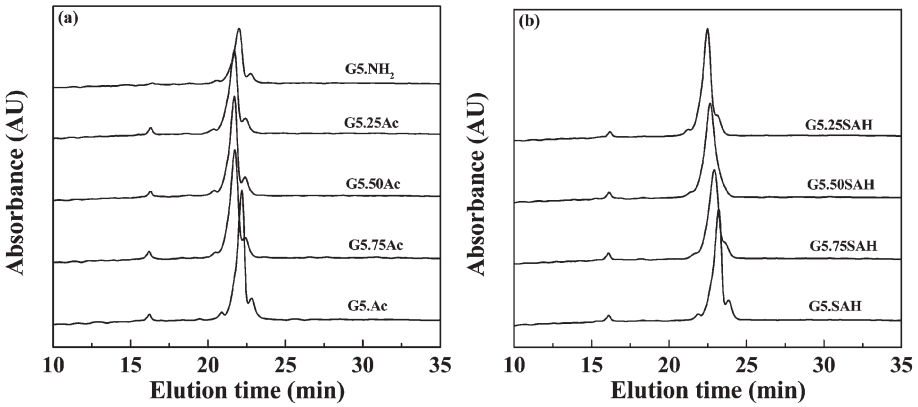

Fig. 3 HPLC chromatograms of generation 5 dendrimers with defined (a) acetylation and (b) carboxylation degrees. The gradient used was 0–50% ACN (balance water) within 40 min. Injection: 20 μL.

that both small molecules and macromolecules can be separated under the same conditions, which allows us to evaluate the purity as well as the interaction between dendrimer nanodevices and biomacromolecules (*e.g.*, proteins, DNA, and enzymes).

Analysis of the binding of G5.75Ac-FA_4 conjugate with FBP. Compared with other surface analytical techniques such as SPR, elliposometry, *etc.*, HPLC is a simple, fast, and routine method, and pre-immobilization of proteins is not necessary. In addition, HPLC allows the detection to be performed in solution which is close to the real biological conditions. The sensitivity of HPLC is not as high as the SPR technique, but the binding kinetics can be evaluated by comparing the HPLC peak area of target proteins before and after binding. We investigated the binding of a G5.75Ac-FA_4 dendrimer conjugate with FBP in solution using HPLC and compared it with PAMAM derivatives without FA modification.

Fig. 4a–c show the HPLC chromatograms of free FA, FBP, and G5.75Ac-FA_4 PAMAM conjugate. The starting material used to synthesize G5.75Ac-FA_4 is G5 PAMAM dendrimer with 75% acetylation, G5.75Ac (for molecular weight data, see Table 2). The detection wavelength was selected at 280 nm, which is the characteristic maximum absorption wavelength of FA. After conjugation with FA, G5.75Ac-FA_4 displays monomodal distribution (elution time is 14.06 min, Fig. 4c) and keeps a similar dispersity to the starting material G5.75Ac (HPLC chromatogram of G5.75Ac separated at the same gradient is not shown), suggesting a homogeneous conjugation reaction. In addition, we observe that FA and FBP have similar elution times (10.23 min, Fig. 4a and 4b) although their molecular weights are enormously different. There are two minor impurity peaks shown in the chromatogram of FBP (one at 15.14 min and one at 29.01 min, Fig. 4b).

We simply mixed FA, G5.75Ac, and G5.75Ac-FA_4 with FBP in a volume ratio of 10 : 90 in parallel for 30 min before HPLC analysis. Fig. 4d shows the HPLC chromatograms of G5.75Ac-FA_4 (1), G5.75Ac (2), and FA (3) after binding with FBP. It is interesting to see that after the FBP binding process, three individual additional peaks appeared in all the chromatograms in the same peak position (elution times at 18.07, 21.47, and 23.74 min), suggesting the formation of three complexes. The three complexes formed in all three cases (FA, G5.75Ac, and G5.75Ac-FA_4) may display similar analogue structures (related to the HPLC separation condition) that can be eluted out in similar time sequences. The peak related to free FBP (10.23 min) in the chromatogram of G5.75Ac-FA_4, after binding with FBP, disappeared. In contrast, in the chromatogram of G5.75Ac after binding with FBP, there is still a peak related to free FBP (10.23 min, as indicated by an arrow in Fig. 4d). This indicates that G5.75Ac-FA_4 exhibits much higher specific binding affinity than G5.75Ac without FA modification. Therefore, under similar conditions, G5.75Ac-FA_4 conjugates are able to bind all the free FBP to form complexes, which is believed to result from both ligand-receptor and physical interactions. The ligand-receptor interaction is much stronger than the physical interaction.

In order to determine the stability of the formed complexes between G5.75Ac-FA_4 and FBP, free FA was added into the mixture solution for the competitive binding. Shown in Fig. 4e is the chromatogram of G5.75Ac-FA_4 bound with FBP, followed by addition of free FA. The shape and position of the three peaks (labeled as 1, 2, and 3 in Fig. 4e) related to binding complexes did not change after the addition of free FA, suggesting that the formed complexes between G5.75Ac-FA_4 and FBP are stable and not influenced by the post-addition of free FA. We note that under the current HPLC condition, all studied species (dendrimers, dendrimer-FA conjugates, and proteins) are acidic (pH = 2.4) because of the presence of TFA in both mobile phases. In order to test the binding behavior between dendrimer-FA conjugates with FBP at different pH conditions, SDS-PAGE was utilized (see below).

SDS-PAGE analysis

The formation of complexes between dendrimer-FA conjugate and FBP was also verified using SDS-PAGE. All the samples were dissolved in PBS buffer (pH 7.4) and their concentrations were all kept at 1 mg mL^{-1}. FBP was mixed with all dendrimer samples and free FA in the same manner as the HPLC experiment. It is clear that after mixing G5.75Ac-FA_4 and G5.75Ac dendrimers with FBP in parallel, three PAGE bands appeared (see horizontal arrows in Fig. 5) in both cases,

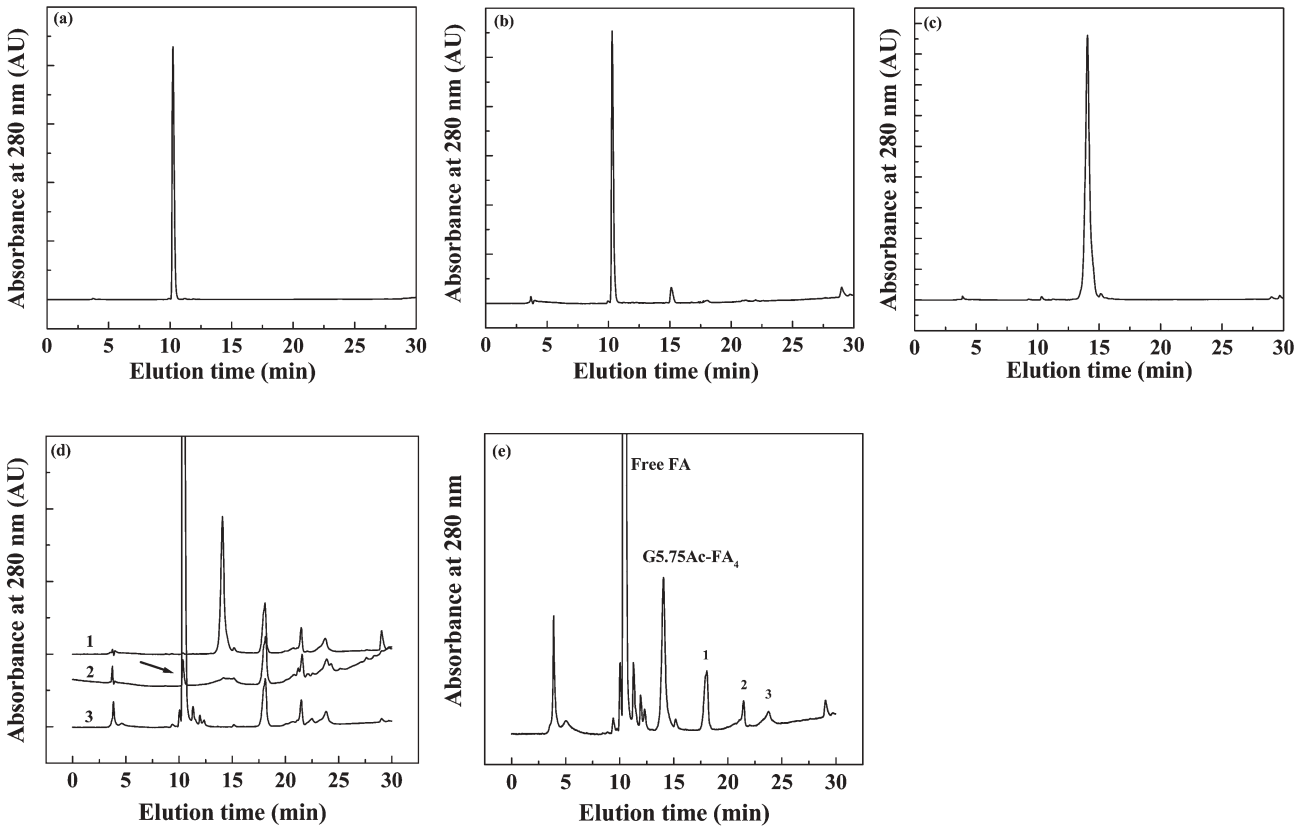

Fig. 4 HPLC chromatograms of (a) FA, (b) FBP, and (c) G5.75Ac-FA_4 dendrimer conjugate. FA, FBP, and G5.75Ac-FA_4 were dissolved in 1 : 9 (v/v) ACN/H_2O mobile phase at the concentration of 1 mg mL^{-1}. Injection volume: FA, 5 μL; FBP, 20 μL; and G5.75Ac-FA_4, 30 μL. (d) HPLC chromatograms of FA, G5.75Ac, and G5.75Ac-FA_4 after binding with FBP. Injection: 30 μL. 1. G5.75Ac-FA_4 + FBP; 2. G5.75Ac + FBP; 3. Free FA + FBP. (e) HPLC chromatogram of the mixture solution of G5.75Ac-FA_4 and FBP (1 : 9, v/v) after addition of free FA. The final volume ratio of G5.75Ac-FA_4/FBP/FA is 1 : 9 : 1. Injection: 30 μL. In all cases, the gradient used was 10–80% ACN (balance water) within 30 min.

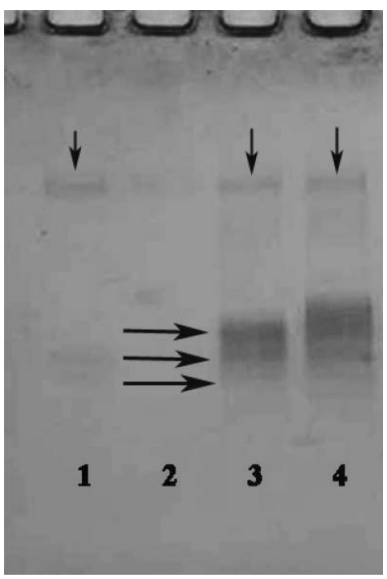

Fig. 5 PAGE electropherograms of the binding of FBP with dendrimer-FA conjugates. Horizontal and vertical arrows indicate the 3 formed binding complexes and free FBP, respectively. Lane 1: free FBP; Lane 2: FA + FBP; Lane 3: G5.75Ac-FA_4 + FBP; Lane 4: G5.75Ac + FBP. Injection volume: 10 μL.

indicating the formation of three complexes between dendrimers and FBP. This is consistent with the HPLC results; however, the specific and non-specific binding of FBP between FA-modified dendrimer and dendrimer without FA moieties cannot be quantitatively differentiated using SDS-PAGE. We also see that the three complexes formed between dendrimers and FBP migrate faster than free FBP (see vertical arrows in Fig. 5). This is probably due to the formation of more compact structures after binding. It is interesting to note that the binding between free FA and FBP using SDS-PAGE (Fig. 5, Lane 2) under similar conditions was undetectable. We think that the structures of the three formed complexes between FA and FBP are significantly different than those formed between dendrimers and FBP, although the three formed complexes in the binding between free FA, dendrimers and FBP show three HPLC peaks at the similar elution times (*vide supra*).

Conclusions

In summary, PAMAM dendrimers of different generations with various surface modifications (carboxylation, acetylation, hydroxylation, defined acetylation and carboxylation) were analyzed and separated using HPLC. The binding between G5.75Ac-FA_4 and FBP was monitored in comparison with dendrimers without FA conjugation. The HPLC results show

that three complexes formed after the binding of G5.75Ac-FA_4 with FBP, which corroborates the SDS-PAGE data. Dendrimers with FA moieties show much higher specific binding capability with FBP than those without FA moieties. The structures of the three formed complexes are as yet still not clear. In our ongoing studies, preparative HPLC will be used to collect the samples related to the three peaks for further molecular weight and structural characterization. We also anticipate investigating other dendrimer-FA conjugates with different dendrimer generations or different numbers of FA moieties in the same generation dendrimer for the binding with FBP. The results in the present study indicate that HPLC is an effective technique for characterization and separation of PAMAM dendrimer derivatives and conjugates and for investigation of the interaction between dendrimers and biomolecules. This will be helpful for our future development of dendrimer-based nanodevices for targeted cancer therapy.

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