

Understanding the Binding Interactions between Dendrimer and 18 Common Amino Acids by NMR Techniques

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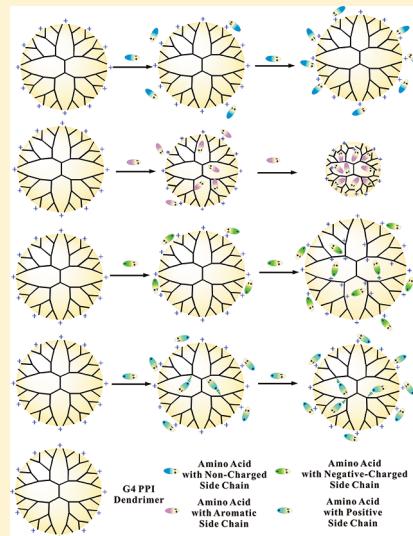
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Supporting Information

ABSTRACT: In the present study, we focus on the interactions between poly(propylene imine) (PPI) dendrimer and 18 of the 20 common amino acids by several NMR techniques, including NMR titrations and NOESY analysis. Surface ionic interactions and interior encapsulations were observed, and the binding behavior of amino acids with PPI dendrimer depends much on the side-chain properties of the amino acid, such as charge and hydrophobic/hydrophilic properties. The ¹H NMR titration results show that the formation of PPI dendrimer–amino acid complexes are driven mainly by ionic interactions for all the amino acids except tryptophan, which is involved in strong hydrophobic interactions with the interior pockets of PPI. The hydrophobic encapsulation of tryptophan in PPI pockets is confirmed by NOESY analysis. Amino acids with negatively charged residues much more easily saturate the surface charges on PPI than amino acids with uncharged residues, whereas amino acids with positively charged residues are the most difficult to bind with the surface amine groups on the PPI dendrimer. A simultaneous occurrence of interior encapsulation (hydrophobic, hydrogen bond, or ionic interactions) and surface binding (ionic interactions) was observed for tryptophan, phenylalanine, arginine, lysine, histidine, cysteine, and asparagine, and a preferential surface ionic binding on the PPI surface rather than encapsulations in the interior was obtained for the other amino acids.



1. INTRODUCTION

Dendrimers are synthetic macromolecules with globular or ellipsoidal shapes, nanoscale sizes, tree-like topological structures, excellent monodispersity, empty cavities, well-defined molecular weights, and large numbers of surface functionalities.^{1–3} The first commercialized dendrimer became available in 1993 when Meijer and co-workers used a divergent method for an industrial-scale preparation of kilogram quantities of poly(propylene imine) (PPI) dendrimer.^{4,5} PPI dendrimer was synthesized by Michael addition of amine to acrylonitrile, followed by the reduction of nitrile groups to primary amine groups, which provides new branching points.⁴ Up to now, PPI dendrimers have been widely used as carriers for catalysts,⁶ drugs,⁷ genes,⁸ and imaging agents,⁹ as templates for the synthesis of nanomaterials,¹⁰ as scaffolds for constructing multifunctional nanodevices,¹¹ and especially, as hosts in host–guest systems.¹² Since 1994, the empty cavities within the PPI dendrimer have been proposed to encapsulate guest molecules such as nitroxide radicals and Bengal rose.^{13,14} Afterward, the PPI dendrimer was chosen as a preferable host candidate in the design of novel host–guest systems.^{6,12,15,16}

Dendrimer-based host–guest systems have attracted increasing attentions in the past decade.^{17–20} It is well-known that the

physicochemistry of dendrimer-based host–guest systems is of central importance in the miscellaneous applications of dendrimers.^{21–24} Previous studies have reported large numbers of guests involved in host–guest interactions with different types of dendrimers through ionic, hydrophobic, and hydrogen bond interactions.^{19,20,25–27} However, these results lack comparability because of different analysis methods, experimental procedures, and types of dendrimers and guests used in these studies. Thus we are prevented from getting a clear understanding of the host–guest interactions of dendrimers.

In this study, we chose the 20 common amino acids as model guests to investigate the influences of different physicochemical parameters on the binding of guests with the PPI dendrimer and to rule out the effect of different analysis and experimental methods used in the references. The 20 common amino acids contain an amine group, a carboxylic acid group, and a side chain that varies among the amino acids. These amino acids can be sorted into different families by their charge properties (negatively charged, positively charged, or noncharged), and their hydrophilic/hydrophobic

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properties (polar and nonpolar) of the side chains. In addition, these amino acids have different pK_a values for the amine and the carboxylic acid groups and also have different isoelectric points (pI_s) for the amino acids due to the different side chains. Furthermore, the 20 common amino acids have different side-chain lengths, thus, possess different molecular weights. These differences allow us to evaluate the effects of the different physicochemical parameters on the binding of dendrimers with different amino acids.

In addition, the interactions of dendrimer with protein can reveal the fate of dendrimers in biological systems that contain lots of proteins, thus providing helpful information for the design of delivery systems for macromolecular drugs such as proteins and peptides.^{28–32} First, the interactions of dendrimers with proteins in human plasma may influence the biodistributions of dendrimers in the tissues and decrease the circulation half-life of the dendrimers in the blood.^{31,32} In addition, the interactions may alter the conformations of proteins, causing them to lose their inherent biological functions, and lead to unexpected toxicity of the administered dendrimers.³⁰ Second, proteins, peptides, and DNA cannot be effectively delivered by conventional tablets and injections.^{33,34} Therefore, there is an urgent demand for the design of vehicles that can effectively deliver these macromolecular drugs.³⁴ Dendrimers have been reported as promising carriers for proteins, peptides, and DNA by several groups.^{35,36} The investigation of protein/peptide interactions with dendrimers is essential for the design and optimization of such delivery systems. Current studies on protein/peptide–dendrimer interactions have focused on the variations of protein secondary structures and have provided macroscopic views by circular dichroism and fluorescence studies.^{28,37} The information on protein–dendrimer interactions at the amino acid level are limited. Therefore, the interactions between dendrimer and different amino acids are useful for us to understand the physicochemistry of dendrimer-based host–guest systems and to predict the protein/peptide interactions by a bottom-up strategy.

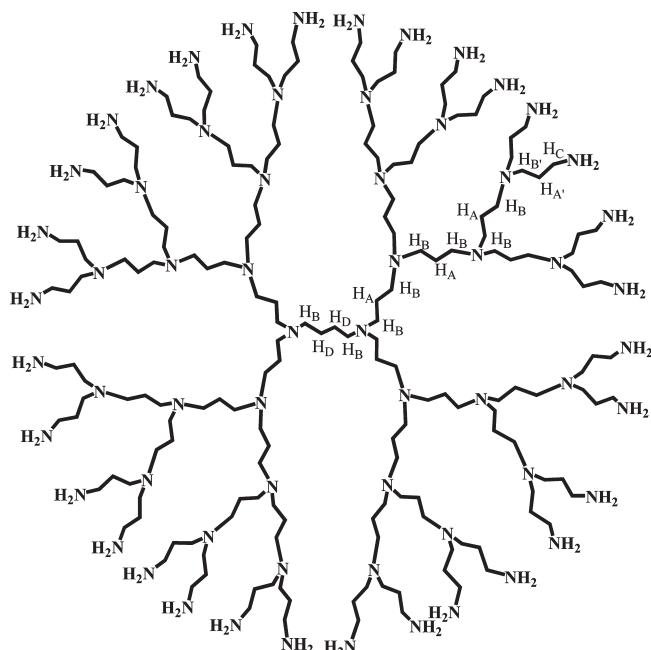
In this study, we use NMR techniques, including proton NMR titrations and nuclear Overhauser effect spectroscopy (NOESY) to investigate the interactions between dendrimers and amino acids. NMR techniques are powerful, sensitive, and noninvasive tools in the analysis of host–guest interactions, as demonstrated in our previous studies.^{21–23,38} G4 PPI with 32 surface amine groups was used as a representative dendrimer. Common amino acids were used, except for aspartic acid and tyrosine, which have poor aqueous solubility.

2. EXPERIMENTAL SECTION

2.1. Materials. G4 Diaminobutane (DAB)-cored and amine-terminated PPI dendrimer with a molecular weight of 3513 Da was purchased from Sigma-Aldrich (St. Louis, MO). All the amino acids were obtained from Biotech & Bio Basic Inc. (Shanghai, China). Deuterium oxide (D_2O) was purchased from Beijing Chongxi High-Tech Incubator Co., Ltd. (Beijing, China). PPI dendrimer stock solution was prepared at 20 mg/mL in D_2O and stored at 4 °C before use.

2.2. Solution Preparations. Titrations of PPI dendrimer with different amounts of amino acids were conducted. Generally, 0.5 mg of G4 PPI dendrimer (28.4 μM) was used; the molar ratios of amino acid and PPI dendrimer were 0, 8, 16, 24, 32, 40, 48, 64, 80, 100, and 120. The solutions were kept at a constant volume of 500 μL , and the amino acid molar concentration ranged from

Scheme 1. Molecular Structure and Proton Labeling of G4 PPI Dendrimer



0 to 34.2 mM. The samples were transferred into the NMR tubes before 1H NMR and 1H – 1H NOESY studies.

2.3. 1H NMR and NOESY Studies. 1H NMR experiments for PPI dendrimer/amino acid solutions in D_2O were conducted on a Varian 699.804 MHz NMR spectrometer at 298.2 ± 0.1 K. The heater and cooling unit was switched on to reach and stabilize the set temperature, which avoids the influence of temperature variation on chemical shifts in the 1H NMR spectra. The G4 PPI dendrimer concentration in the 1H NMR titration experiments was fixed at 1 mg/mL.

The 1H – 1H NOESY spectra of the G4 PPI and amino acid (glycine, arginine, glutamic acid, glutamine, tryptophan) solutions were obtained on a Varian 699.804 MHz instrument using standard pulse sequences at 298.2 ± 0.1 K in D_2O .³⁹ The G4 PPI dendrimer concentration was kept at a concentration of 1 mg/mL. The molar ratio of amino acids and G4 PPI dendrimer was set at 16. Generally, the 1H – 1H NOESY experiments were performed with a 1 s relaxation delay, 150 ms acquisition time, and a 7.6 μs 90° pulse width, and 300 ms was chosen as the mixing time for the optimization of cross-peak intensities with minimum distortions during the period for NOE establishment. Thirty-two transients were averaged for 300 complex t_1 points. All the data were processed with NMRpipe software on a Linux workstation with standard Lorentz–Gauss window function and zero-filling in both dimensions.

3. RESULTS AND DISCUSSION

3.1. Chemical Shift Assignments and NOESY Spectrum of the G4 PPI Dendrimer. The G4 PPI dendrimer has six groups of protons that are assigned as protons H_A , $H_{A'}$, H_B , $H_{B'}$, H_C , and $H_{D'}$. H_A and $H_{A'}$ correspond to protons on the methylene groups in the center of each repeated unit in the interior pockets and the outermost layer of G4 PPI, respectively ($-NCH_2-CH_2CH_2N-$, 56H_A, 1.50 ppm; and $-NCH_2CH_2CH_2NH_2$,

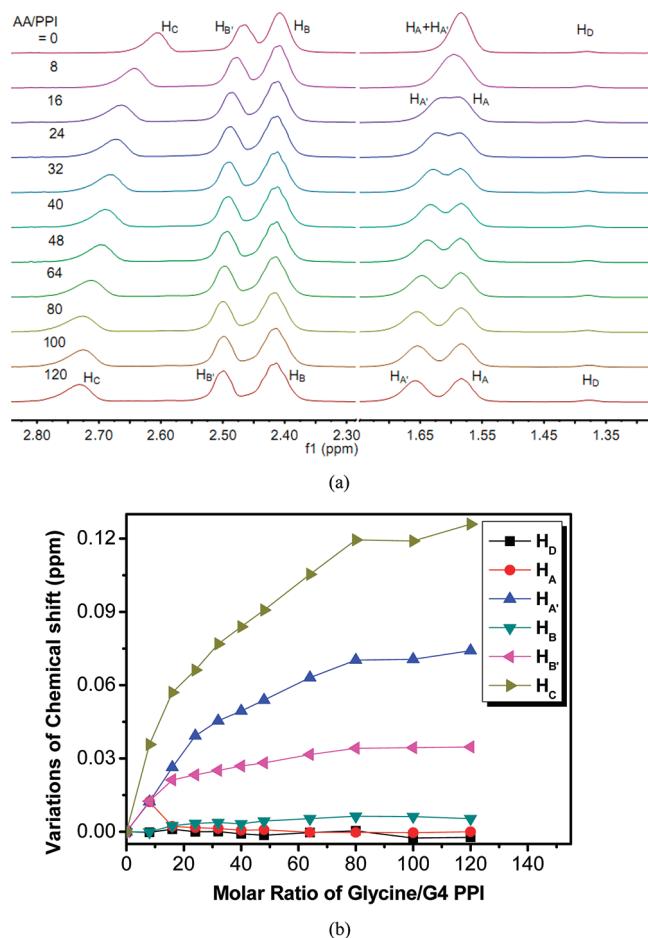


Figure 1. ^1H NMR spectra of G4 PPI at different molar ratios of glycine/PPI (a) and chemical shift variations of PPI protons during the titrations (b).

64 $H_{A'}$, 1.50 ppm). H_B is the 116 methylene protons adjacent to the interior tertiary amine groups ($-\text{NCH}_2\text{CH}_2\text{CH}_2\text{N}-$, 2.33 ppm), and $H_{B'}$ represents the 64 protons on the methylene groups that are adjacent to tertiary amines and located on the outermost layer of the G4 PPI dendrimer ($-\text{NCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$, 2.37 ppm). H_c are the 64 methylene protons adjacent to the 32 surface amine groups ($-\text{NCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$, 2.49 ppm). H_D are the 4 methylene protons located in the center of the PPI core ($-\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N}-$, 1.30 ppm). The chemical shift assignments of the G4 PPI dendrimer (Scheme 1) are confirmed by the ^1H - ^1H NOESY spectrum of G4 PPI shown in Figure S2, in which the absence of cross-peaks for H_c - H_D and $H_{B'}$ - H_D and the presence of other cross-peaks were observed.

3.2. Titrations of PPI Dendrimer with Glycine, Alanine, Isoleucine, Leucine, Methionine, Proline, and Valine. The ^1H NMR spectrum of glycine in D_2O has one peak corresponding to the methylene protons (Figure S3a, $\text{NH}_2\text{CH}_2\text{COOH}$, 3.48 ppm). The addition of glycine molecules into the G4 PPI solution causes a significant downfield shift of the methylene protons ($H_{A'}$, $H_{B'}$, and H_c) located on the outermost layer of G4 PPI dendrimer (Figure 1a). The most obvious change in the chemical shifts was observed for the resonance of protons (H_c) adjacent to the surface amine groups, suggesting the presence of ionic interactions between the surface amine groups of G4 PPI and the carboxylic group of glycine (Figure 1b). The order of $\Delta\delta$

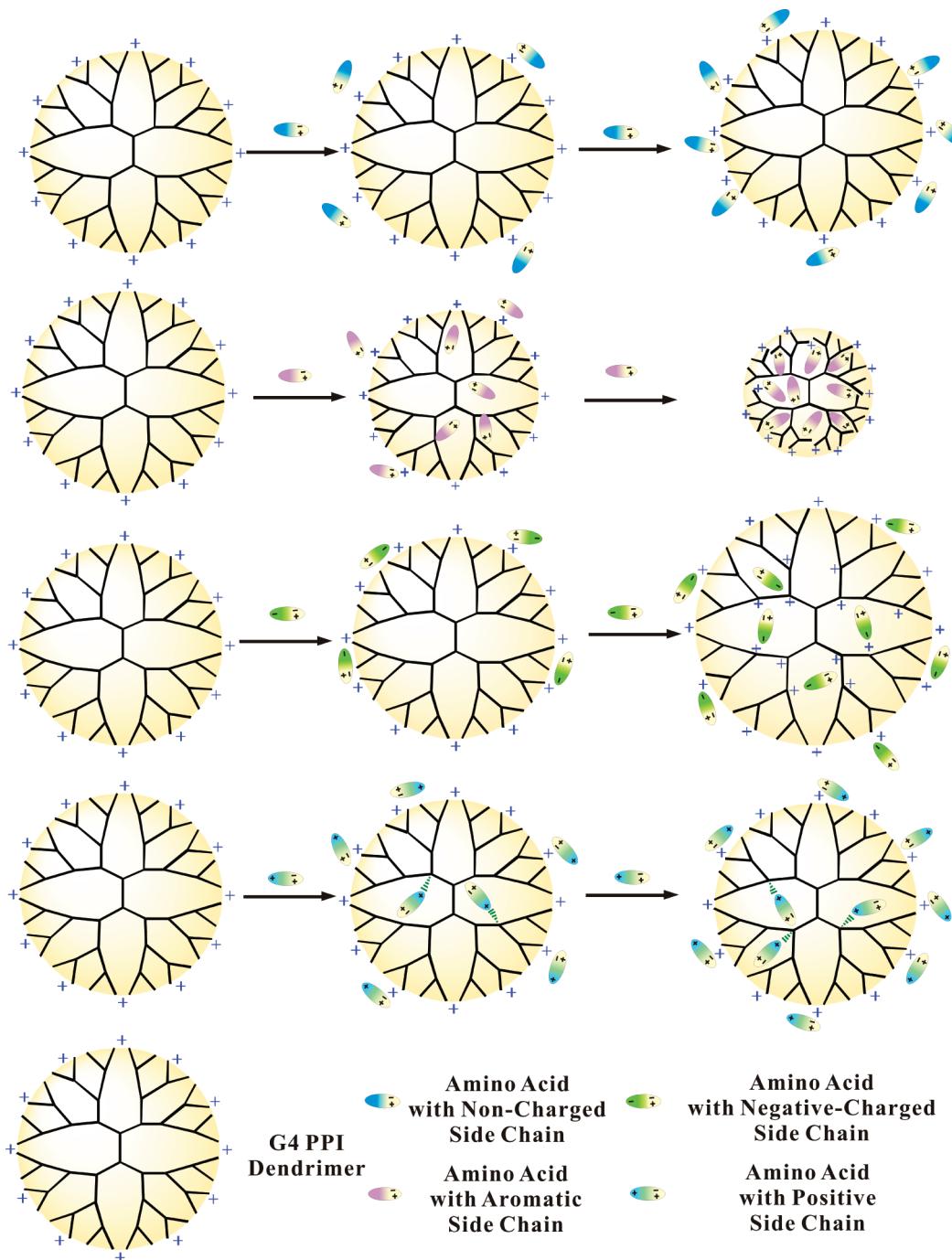
for $H_{A'}$, $H_{B'}$, and H_c ($H_c > H_{A'} > H_{B'}$) revealed that the downfield shift of peaks for protons $H_{A'}$ and $H_{B'}$ is attributed to surface ionic interactions. No shift was observed for the resonance of protons located in the interior of G4 PPI dendrimer, suggesting the selectivity of ionic bindings of glycine with surface amine and tertiary amine groups, and the lack of glycine encapsulations in the inner cavities during the titrations (Scheme 2a). The lack of glycine encapsulation is confirmed by the absence of cross-peaks between glycine protons and the PPI interior protons in the NOESY spectrum of the glycine/G4 PPI complex (Figure S4). The pH values of these samples ranged from 10.94 to 9.05 during the titration experiment, and the pK_a values of tertiary amine groups of PPI dendrimer were around 6.10,⁴⁰ further proving the absence of tertiary amine quaternization during the titrations.

The glycine proton exhibits a significant upfield shift at a glycine/G4 PPI ratio of 8, and the peak gradually shifts back to the chemical shift of free glycine in the absence of PPI upon addition of more glycine molecules into the dendrimer solution (Figure S3b), suggesting a fast exchange occurs between the free and bound states for the glycine molecules.^{21,35} The chemical shift of H_c is scarcely changed above a glycine/PPI ratio of 80, which can be recognized as the saturation point for surface binding (Figure 1b).¹⁵ For the G4 PPI dendrimer with 32 primary amine groups, at least 80 glycine molecules are needed to saturate the surface amine groups on each PPI dendrimer, indicating competitive bindings between amine groups of PPI and glycine with the carboxylic acid of glycine. Similar results were obtained for amino acids with uncharged aliphatic side chains, including alanine (Figures 2a and S5), leucine (Figures 2b and S6), isoleucine (Figure S7), valine (Figure S8), methionine (Figure S9), and proline (Figure S10). In the case of these amino acids, no saturation point was observed for the surface ionic binding of amino acid on G4 PPI dendrimer, probably due to the steric hindrance of the side chains as compared with glycine.¹⁵ A common phenomenon for these amino acids is that no encapsulation within the PPI dendrimer was observed, even at a amino acid/PPI molar ratio of 120 (Scheme 2a).

3.3. Titrations of PPI Dendrimer with Tryptophan and Phenylalanine. In Figure 3, the titration of G4 PPI dendrimer with tryptophan shows a much different shift behavior of the PPI protons. The peaks for protons (H_c and $H_{A'}$) of PPI dendrimer exhibit mild downfield shifts upon the addition of tryptophan until the amino acid/PPI ratio reaches 80, after which the peaks exhibit an upfield shift. On the other hand, peaks for the protons located in the inner cavities of G4 PPI show a significant upfield shift throughout the titration experiment. As demonstrated in our previous studies, an upfield shift of protons in the interior of dendrimer is attributed to hydrophobic interactions of the guest with the dendritic scaffold,^{21,41} and the insertion of tryptophan aromatic rings into the PPI pockets results in shielding effects.

The results in Figure 3b suggest that hydrophobic encapsulation of tryptophan in G4 PPI occurred as soon as this amino acid was titrated into the dendrimer solution. Some of the tryptophan molecules were bound on the surface of PPI via ionic interactions, and no selectivity of tryptophan binding with the PPI surface and interior is observed at the early stages of titration (Figures 3b and S11). The protons (H_c and $H_{A'}$) are also involved in hydrophobic interactions with tryptophan above the amino acid/PPI ratio of 80, indicating that hydrophobic interactions are the major force that drives the formation of tryptophan/PPI complexes

Scheme 2. Proposed Interactions (Surface Ionic Binding versus Interior Encapsulation) between G4 PPI Dendrimer and Common Amino Acids



(Scheme 2b). The encapsulation of tryptophan molecules within the cavities of PPI dendrimer is proved by the presence of cross-peaks between the aromatic protons of tryptophan and the scaffold protons of the G4 PPI (Figure 4).^{23,24,38,42,43}

The distinct binding behavior of tryptophan and glycine with PPI dendrimer is caused by the aromatic rings on the side chain of tryptophan that are involved in strong hydrophobic interactions with the PPI dendrimer. Part of the driving force might be the need to get the tryptophan aromatic rings out of the polar water solvent environment, and the zwitterionic end of the amino

acid might then remain at the surface to interact with the surface amine groups of PPI and the solvent. This result can be used to design novel PPI dendrimer-based delivery systems, especially for the delivery of tryptophan-containing peptides. Similar results were observed for phenylalanine, which also contains an aromatic side chain (Figure 5 and Figure S12). A slight upfield shift of the pocket protons (H_A , H_B , and H_D) of PPI dendrimer was observed when phenylalanine molecules were titrated into the PPI, suggesting hydrophobic encapsulation of phenylalanine in dendrimer cavities. The difference between phenylalanine and

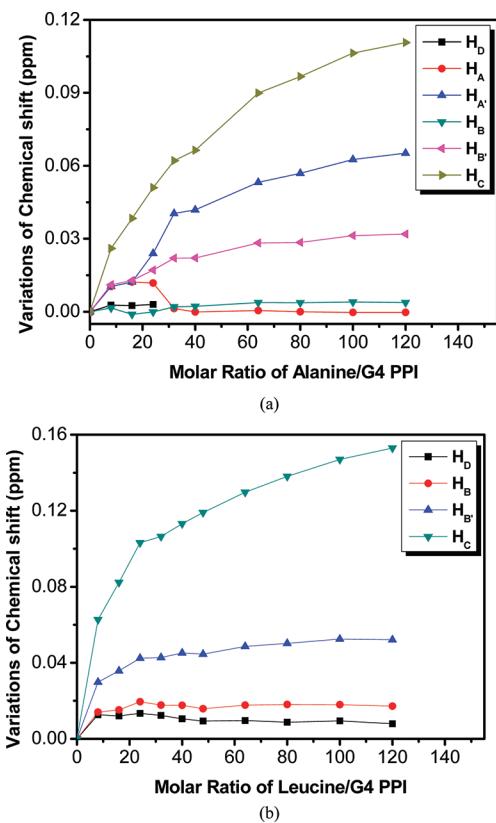


Figure 2. Chemical shift variations of PPI protons at different molar ratios of amino acid/PPI, (a) alanine, and (b) leucine.

tryptophan binding is that the surface ionic interaction is the predominant force driving the formation of PPI/phenylalanine complexes, rather than hydrophobic interactions focused in the PPI/tryptophan system (Figure 5).

3.4. Titration of PPI Dendrimer with Glutamic Acid. When G4 PPI dendrimer is titrated with glutamic acid, a significant downfield shift of the H_C protons is observed (Figures 6 and S13). A saturation point for the surface binding of G4 PPI with glutamic acid is achieved at an amino acid/PPI ratio of 32, which appears much earlier than that for glycine and approximates the surface amine number of a G4 PPI dendrimer. Glutamic acid has two carboxyl groups, with pK_a values of 2.16 and 4.15. Both of the carboxyl groups are deprotonated during the titrations (pH value ranges from 9.78 to 7.49 in the ratio amino acid/PPI range of 0–32). Therefore, less glutamic acid is needed to saturate the surface amines of G4 PPI than that of glycine. In addition, a significant downfield shift of the protons (H_D) located at the core of G4 PPI dendrimer is observed in the amino acid/PPI ratio range of 8–48, which is the direct evidence that glutamic acid is encapsulated and located near the core of the PPI dendrimer. The peak for protons (H_B) also exhibits a significant downfield shift in the ratio range of 24–64, suggesting there is a selective protonation of the tertiary amine groups in the different layers of PPI dendrimer. The pH value of the PPI/glutamic acid solution ranges from 4.29 to 3.67 in the ratio range of 64–120, proving that high percent of the interior tertiary amines were protonated (Scheme 2c). However, a second downfield shift of protons (H_C and H_{B'}) was observed above a glutamic acid/PPI ratio of 80, probably due to the formation of supramolecular structures between PPI dendrimer and glutamic acid during this period.

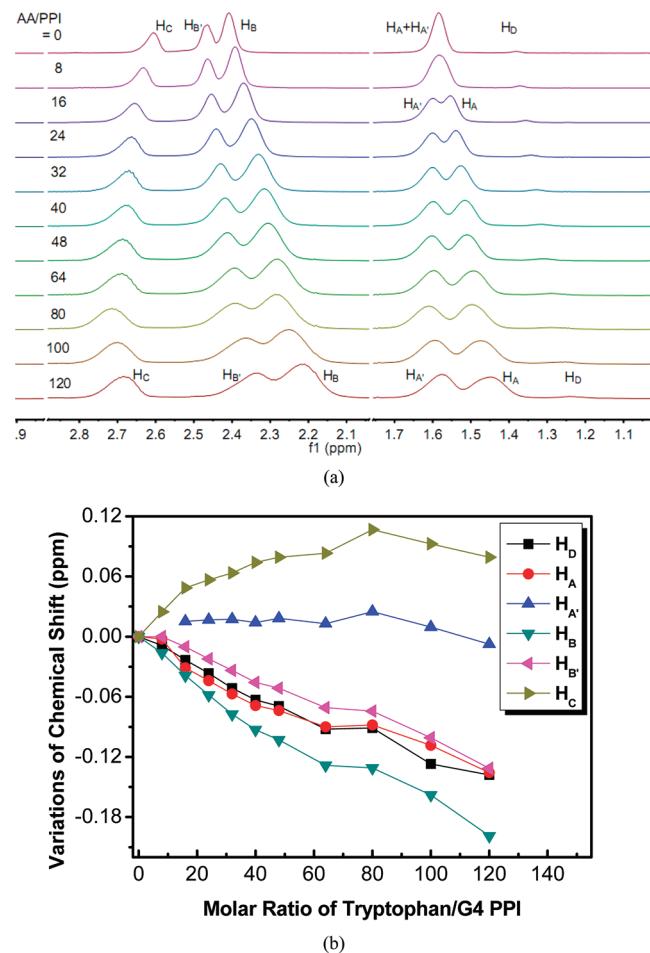


Figure 3. ¹H NMR spectra of G4 PPI at different molar ratios of tryptophan/PPI (a) and chemical shift variations of PPI protons during the titrations (b).

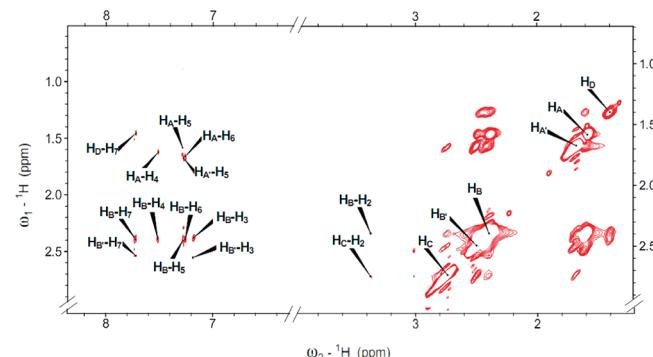


Figure 4. ¹H-¹H NOESY spectrum of tryptophan/G4 PPI complex in D₂O at a mixing time of 300 ms.

Although the encapsulation of glutamic acid within PPI is deduced from ¹H NMR titration results, no cross-peaks between the glutamic acid protons and PPI protons were observed in the NOESY spectrum in Figure S14, suggesting ionic interaction-mediated encapsulations of glutamic acid within the PPI dendrimer (Scheme 2c).

3.5. Titrations of PPI Dendrimer with Arginine, Lysine, Asparagine, Histidine, and Cysteine. Arginine has two amine

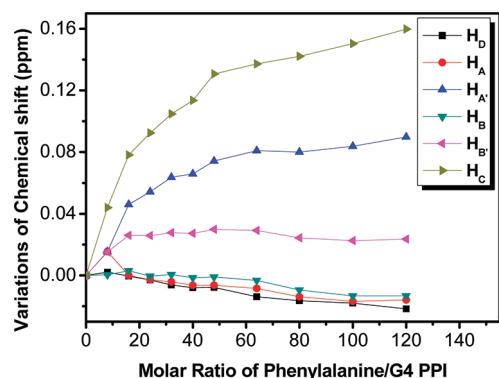
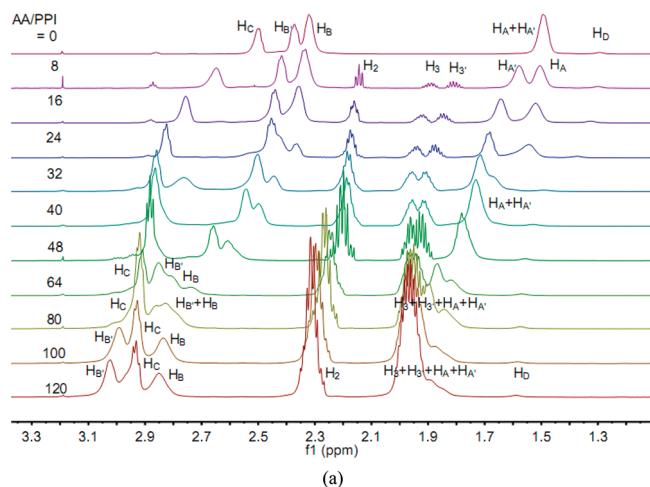


Figure 5. Chemical shift variations of PPI protons at different molar ratios of phenylalanine/PPI dendrimer.



(a)

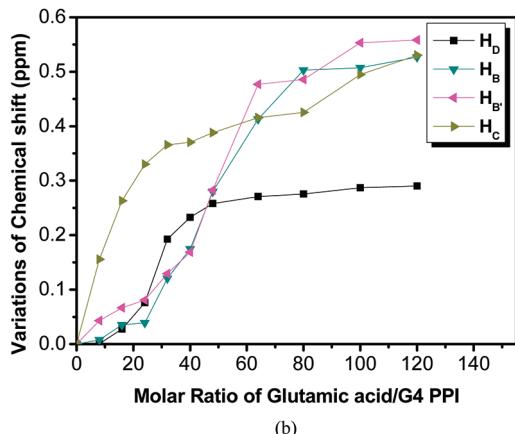
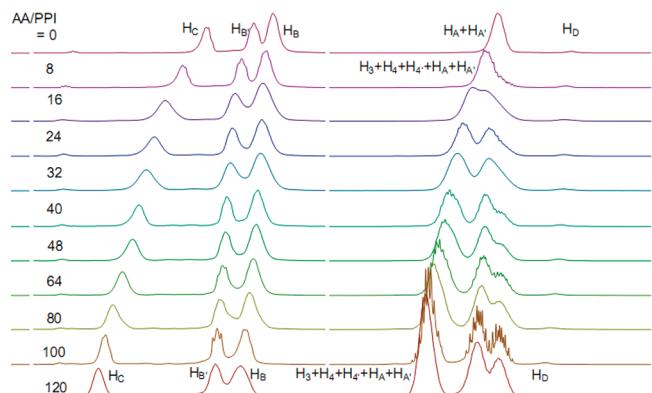


Figure 6. ^1H NMR spectra of G4 PPI dendrimer at different molar ratios of glutamic acid/PPI (a) and chemical shift variations of PPI protons during the titrations (b).

groups, with $\text{p}K_a$ values of 9.0 and 12.1. These two amine groups are partially or fully protonated in the presence of the G4 PPI dendrimer and may bind with the carboxylate group of arginine in a competitive manner. As a result, no saturation points were observed for the surface amine groups, even at an arginine/PPI ratio of 120 (Figure 7, Figures S15, S16). Interestingly, downfield shifts of protons (H_B and H_D) were observed at the beginning of arginine titration, which means that surface binding and interior



(a)

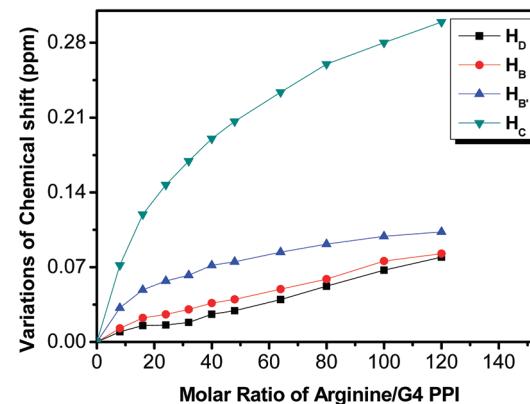


Figure 7. ^1H NMR spectra of G4 PPI dendrimer at different molar ratios of arginine/PPI (a) and chemical shift variations of PPI protons during the titrations (b).

encapsulation occur simultaneously (Scheme 2d). The surface binding is much more significant than the interior encapsulation throughout the titration. According to the pH values of samples during the titration (10.69–9.46), the tertiary amine groups within PPI ($\text{p}K_a \sim 6.10$) are not protonated, suggesting that the inclusion of arginine is driven by hydrogen bond interactions.

Similar binding behavior was obtained with lysine (Figure S17, $\text{pH} \sim 10.52$ –9.57, the interior tertiary amines are noncharged, hydrogen bonding drives the encapsulation) and asparagine (Figure S18, $\text{pH} \sim 10.27$ –7.27, <10% of the tertiary amines are protonated at the end of titration, hydrogen bonding and ionic interaction together contribute to encapsulation). Amino acid encapsulation driven by ionic interaction and hydrogen bond interaction is also observed with histidine having an imidazole side chain (Figure S19, $\text{pH} \sim 10.3$ –6.55), and the interactions of this amino acid within PPI cavities need further investigations. Surprisingly, the encapsulation of cysteine with a thiol side chain by hydrogen bond interactions is observed (Figure S20, $\text{pH} \sim 9.79$ –9.15), probably due to the formation of a disulfide bond between two cysteine molecules, yielding a cystine molecule with two carboxylic and amine groups.

3.6. Titrations of PPI Dendrimer with Threonine, Serine, and Glutamine. For amino acids with a polar uncharged side chain, such as threonine, serine, and glutamine, a selective ionic binding of amino acid on the PPI surface and a lack of interior

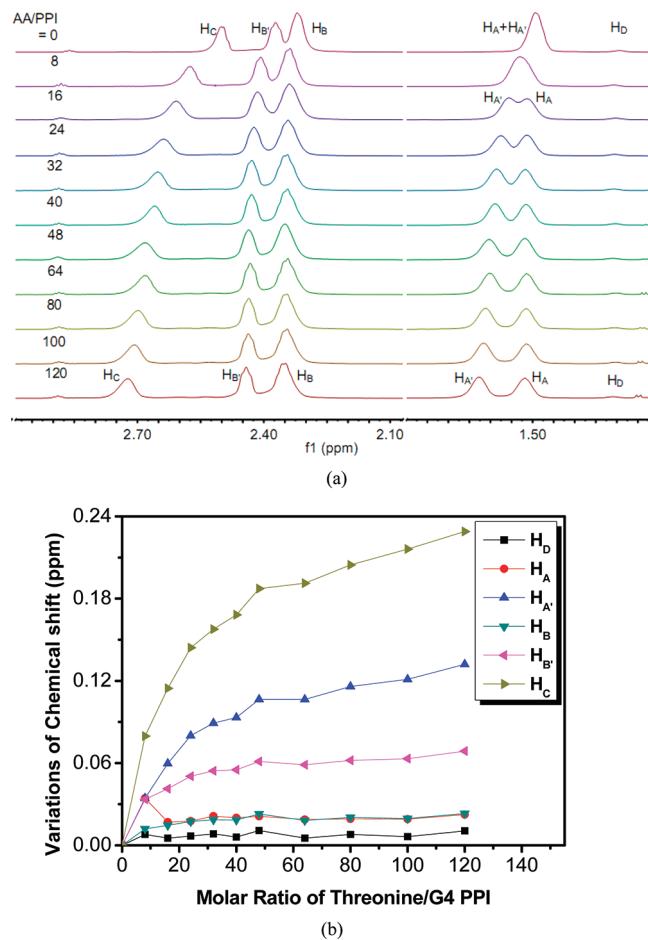


Figure 8. ¹H NMR spectra of G4 PPI dendrimer at different molar ratios of threonine/PPI (a) and chemical shift variations of the PPI protons during the titrations (b).

encapsulations are observed (Figures 8, S21, S22, S23), which is similar to that observed for amino acids with a nonpolar uncharged side chain, such as alanine and leucine (Scheme 2a). The saturation point for the surface binding of threonine (Figure 8) and serine (Figure S21) appears at 48 and is well before that of alanine and leucine. This is due to the presence of hydroxyl groups on the side chain of these two amino acids, which forms strong hydrogen bond interactions with the surface amines on the PPI dendrimer, thus accelerating the saturation of the surface binding.

4. CONCLUSIONS

The interactions of common amino acids with G4 PPI dendrimer in D₂O were investigated by ¹H NMR titration and NOESY analysis. The amino acids interact with the surface of PPI dendrimer via ionic interaction, which is found to be the predominant force driving the formation of PPI–amino acid complexes. The only exception is tryptophan. Hydrophobic interaction contributes more to the formation of the PPI–tryptophan complex than ionic interaction. Amino acids with negatively charged residues, such as glutamic acid, much more easily saturate the surface charges on PPI than amino acids with uncharged residues, such as glycine and alanine, whereas amino acids with positively charged residues, such as arginine and lysine,

have the most difficulty in saturating the surface amine groups on the PPI dendrimer. For instance, the titration of G4 PPI dendrimer, having 32 primary amine groups, with 120 mol equiv of arginine or lysine shows no obvious saturation point. The amount of amino acids needed to saturate the surface of each PPI dendrimer is much higher than the theoretical amount (32 mol equiv), which is due to the competitive binding between amino groups in PPI and amino acid and the steric effect of amino acid residues (Table S1 in the Supporting Information). Apart from tryptophan, phenylalanine, glutamic acid, arginine, lysine, histidine, cysteine, and asparagine, the encapsulations of amino acids within the pockets of PPI dendrimer are not initiated until the saturation point on PPI surface is achieved. The encapsulation and surface binding of arginine or lysine molecules by PPI dendrimer occur at the same time, and the encapsulation of glutamic acid starts after the occurrence of surface binding but before the saturation point. Hydrogen bond interaction drives the encapsulation of arginine, lysine, and cysteine, and hydrophobic interaction plays an important role in the encapsulation of tryptophan and phenylalanine, whereas ionic interaction and the hydrogen bond together contribute to the inclusion of histidine and asparagine within the cavities of PPI dendrimer. This study provides new insights into the dendrimer-based host–guest systems. ¹H NMR titrations and NOESY are powerful tools in the comprehensive analysis of dendrimer–amino acid interactions.

■ ASSOCIATED CONTENT

S Supporting Information. Further information on the ¹H NMR and NOESY spectra of the PPI dendrimer and its complexes with the 18 common amino acids. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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