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Fast Screening of Dendrimer-Binding Compounds by Diffusion NMR Techniques

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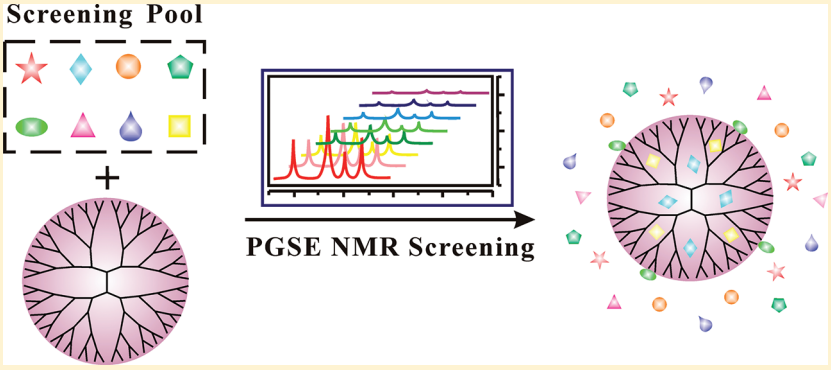
Supporting Information



ABSTRACT: High-throughput screening of dendrimer-binding drugs is essential for the design and optimization of dendrimer-based drug delivery systems. In this study, pulsed gradient spin echo (PGSE) NMR was used for fast screening dendrimer-binding compounds using common amino acids as a screening pool. Diffusion coefficients of the amino acids before and after the addition of poly(propylene imine) (PPI) dendrimer were used to rank the binding affinities of the amino acids to the dendrimer. Among the common amino acids, cysteine, glutamic acid, aspartic acid, and tryptophan show strong binding affinity to PPI dendrimer. Tryptophan forms inclusion complexes with PPI dendrimer through hydrophobic interactions, while other amino acids mainly bind with PPI dendrimer via ionic and hydrogen-bond interactions. The PGSE NMR-based screening method provides new insights into high-throughput screening of dendrimer-binding compounds and should facilitate the study of dendrimer-based host−guest systems.

INTRODUCTION

Rapid advances in organic and pharmaceutical chemistry speed up the synthesis of lead compounds and their derivatives for medicine.[1] The advent of combinatorial chemistry has further revolutionized the process of drug discovery and generated an exponentially increasing number of biological active compounds annually.[2] However, most of the drug candidates face various challenges before entering the market, such as poor aqueous solubility and stability, unpredictable toxicity, low bioavailability, and unsatisfactory pharmacokinetic and pharmacodynamic properties.[3] One of the most effective solutions is using suitable carriers in the delivery of therapeutic compounds.[4,5] In addition to an ever-growing library of drug candidates, advances in drug delivery systems have produced a growing list of potential drug carriers, which make it difficult for a pharmaceutist to design effective drug formulations in a relatively short time.[6,7] Therefore, high-throughput screening (HTS) technologies should be developed in order to keep pace with the vast library of drug and carrier candidates.

Dendrimers are a new architectural class of macromolecules with hyperbranched structures, globular shapes, nanoscale size, and well-defined molecular weights and numbers of surface functionalities.[8−10] These versatile polymers have proved to be promising carrier candidates in drug delivery.[6,11−13] Lots of drugs were reported to benefit from dendrimer-based drug delivery systems.[14−17] These drugs were randomly chosen as model drugs of dendrimers, thus such dendrimer−drug formulations were reported in a "one drug at a time" fashion.[18−22] Since this one-by-one strategy is time-consuming and labor-intensive, there is an urgent need to develop a method for HTS of dendrimer-binding drugs from the vast library of drug candidates. In a recent study, our research group reported a method for HTS of dendrimer-binding drugs using NMR techniques including Hadamard-encoded transferred nuclear Overhauser enhancement (Hadamard-trNOE) and

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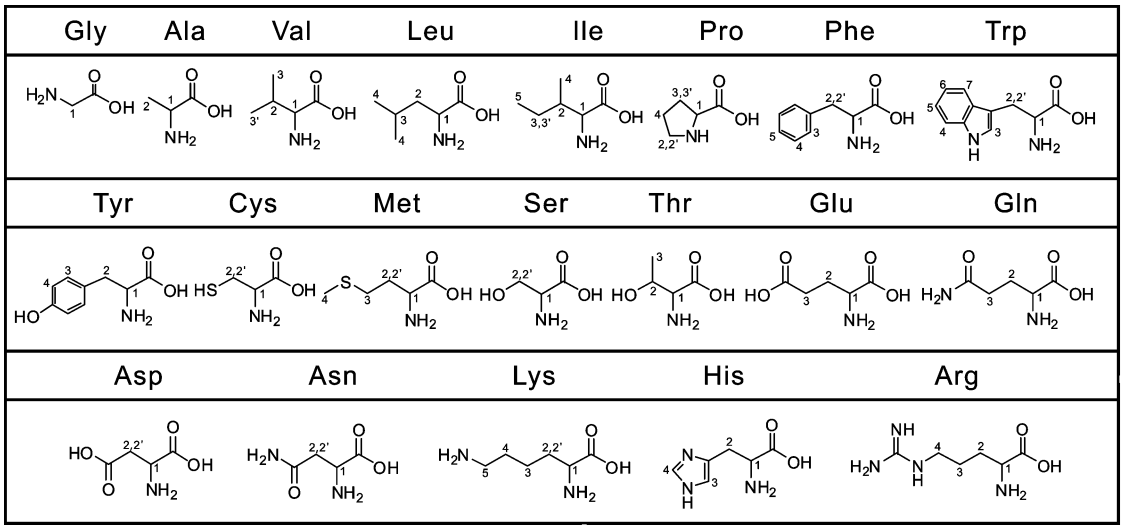
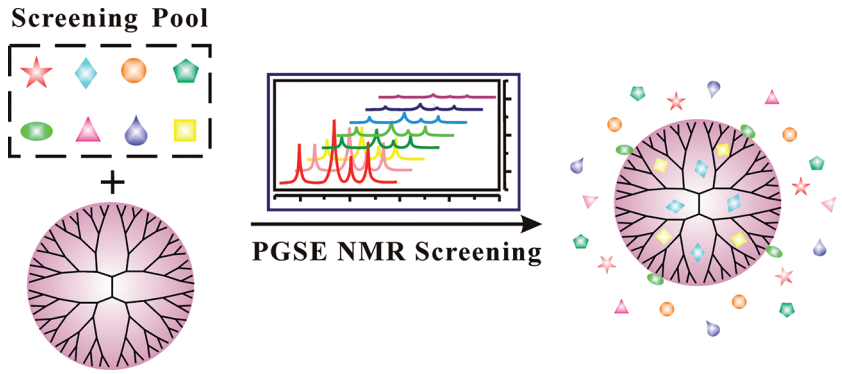
Scheme 1. Chemical Structures of the Common Amino Acids Used in the Screening Experiments



Scheme 2. The Use of PGSE NMR Techniques in the Fast Screening of Dendrimer-Binding Compounds



saturation transfer difference (STD).[23] This method provided informative hits to identify and optimize dendrimer-based drug delivery systems, and was effective to narrow the field of candidate therapeutic agents in primary screening. However, it cannot give the order of binding affinity within a group of drug candidates toward a specific dendrimer, which is important in HTS. Also, the trNOE NMR is limited to screen insoluble drugs that were encapsulated within dendrimer interior cavities.[23]

On this basis, we developed a method for fast screening of dendrimer-binding compounds using a pulsed gradient spin echo (PGSE) NMR in this study. The binding affinities of the drug candidates toward dendrimer were ordered by measuring the diffusion coefficient variations of the compounds in a screening pool before and after the addition of poly(propylene imine) (PPI) dendrimers. Diffusion NMR measurement interests us in HTS because the diffusion coefficient of a small molecule can be changed by more than 1 order of magnitude if it is bound to a macromolecular target.[24,25] The diffusion coefficient can be obtained with little deviation, and the data before and after dendrimer binding can be used to reveal the binding affinity between dendrimer and drug candidates. Here, 20 common amino acids were chosen as the screening pool. Each amino acid has the same fundamental structure, differing only in the side-chain (Scheme 1). These amino acids can be sorted into different families by the side-chains, such as charge properties (negatively charged, positively charged, or noncharged), and hydrophilic/hydrophobic properties (polar and nonpolar).[26] In addition, each amino acid has different isoelectric points and side-chain lengths. The diversity of amino acids in their properties perfectly meets our need as a screening pool in the fast discovery of dendrimer-binding compounds (Scheme 2). PPI dendrimer with a hydrophobic interior and a cationic surface was used as a model dendrimer.[27]

EXPERIMENTAL SECTION

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). The common amino acids were obtained from Bio Basic, Inc. (Shanghai, China). Deuterium oxide was purchased from Beijing Chongxi High-Tech Incubator Co., Ltd. (Beijing, China). All the chemicals were used as received without further purification. PPI dendrimer was prepared at 20 mg/mL in D_2O as stock solution and stored at 4 °C before further use.

^{1}H NMR Studies. All the NMR spectra were acquired at 298.2 ± 0.1 K on a Varian 699.804 MHz NMR spectrometer, equipped with a 5 mm standard probe. The ^{1}H NMR spectra were obtained with 32 scans and a 2 s relaxation delay. The samples were maintained for at least 10 min to avoid the fluctuation of temperature before each acquisition. In each screening pool, eight amino acids were chosen, and a total of seven screening groups were used in this study. For each screening pool, three repeats of the amino acids in the absence

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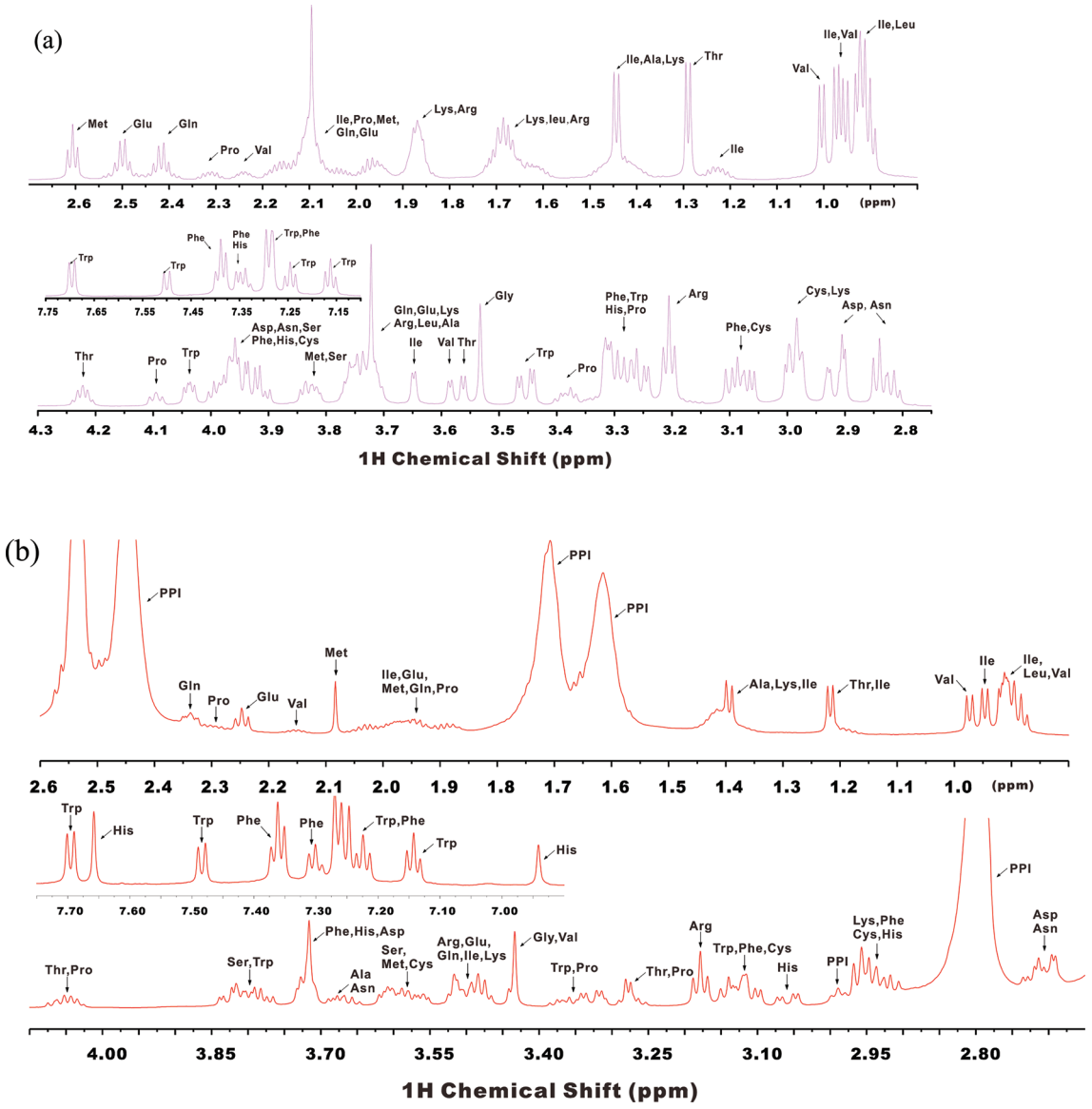

Figure 1. ^{1}H NMR spectra of the 19 amino acids in the absence (a) and presence (b) of G4 PPI dendrimer.

and presence of G4 PPI dendrimer were conducted. The G4 PPI dendrimer concentration in each NMR tube was 0.89 mM, and the molar ratio of each kind of amino acid and PPI dendrimer was 4.

Two-Dimensional Correlation Spectroscopy (2D-COSY) Studies. Since a lot of the NMR peaks of amino acids overlap in the ^{1}H NMR spectra, 2D-COSY was used to assist the chemical shift assignments of amino acids before and after the addition of PPI dendrimers. The COSY spectra were obtained by the standard pulse program within a Varian 699.804 MHz NMR spectrometer, with 256 × 1024 data points. The relaxation delay was 1 s. Eight scans were averaged. A sine-bell squared window function and zero filling were applied to both dimensions.

PGSE NMR Studies. The self-diffusion coefficients of the amino acids in the screening pool were measured by a standard PGSE sequence on the same NMR instrument at 298.2 ± 0.1 K. The heater and cooling unit was switched on to reach and stabilize a desired temperature, which avoids the influence of temperature variation on diffusion measurement. The time interval (Δ) between gradient pulses was chosen as 400 ms, while the duration time of gradient pulses (δ) was 3 ms. The recycle time was 10 s. The pulse gradients (g) linearly increased in 16 steps to attenuate the spin−echo signal, and the maximum gradient strength was 70 G/cm. The gradient pulse was calibrated on a mixture of D_2O and H_2O (10% D_2O and 90% H_2O) under the same experimental conditions. The diffusion coefficients (D) of the amino acids were obtained by fitting the spin−echo signal and gradient strength by the following equation:

$$I_n = I_0 \exp[-\gamma^2 D\delta^2(\Delta - \delta/3)g^2]$$

where I_n and I_0 are the intensities of spin−echo signal when the sine-shaped field gradient is present and absent, respectively, and γ represents the proton magnetogyric ratio (2.68×10^8 s^{-1} T^{-1}).

Two-Dimensional Nuclear Overhauser Effect Spectroscopy (2D-NOESY) Studies. 2D-NOESY experiment was conducted to check whether the amino acids were encapsulated within the interior pockets of PPI dendrimer or just bound on the dendrimer surface via ionic/hydrogen-bond interactions.

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The ^{1}H−^{1}H NOESY experiments for the PPI/amino acid complexes were conducted on the same NMR instrument using standard pulse sequences. A 1 s relaxation delay, 146.63 ms acquisition time, a 6.5 μs 90°pulse width, and a 300 ms mixing time were chosen. Thirty-two transients were averaged for 512 × 1024 complex points. All the data were processed with NMRpipe software on a Linux workstation.

RESULTS AND DISCUSSION

Chemical Shift Assignments of Amino Acids in the Screening Pool. Figure 1a,b shows the ^{1}H NMR spectra of the 19 amino acids in the absence and presence of PPI dendrimer, respectively. Tyrosine (Tyr) was not included in the screening pool due to its poor aqueous solubility. ^{1}H NMR peaks for the amino acids overlap with each other in the spectra. To assist the chemical shift assignments of the amino acids, 2D-COSY experiments were conducted. On the basis of the COSY spectra in Figure 2 and Figure S1 (Supporting Information), the accurate chemical shift for each amino acid was determined. It was observed that most of the H_α peaks of amino acids locate in the chemical shift range of 3.20−4.10 ppm. These H_α signals do not overlap with the broad resonance signals of PPI dendrimer and thus were used to differentiate the amino acids in the screening pool. All the H_α resonances of amino acids shift to a lower frequency after the addition of PPI dendrimer. The peaks for aromatic protons of tryptophan (Trp) and phenylalanine (Phe) were located in the range of 6.80−7.80 ppm.

In our PGSE NMR experiments, gradient pulses increased in 16 steps were applied to attenuate the spin−echo signal (Figure 3). Generally, signal intensities of the peaks were used to calculate the diffusion coefficient in a PGSE NMR experimen, and the signal intensity was usually determined by integrating the area under selected lines of the NMR spectrum.[28,29] Overlapping peaks in the spectrum may interfere with the determination of diffusion coefficient. To avoid the influence of overlapping peaks, we properly chose eight amino acids in each group, and seven groups in total were designed based on the knowledge of ^{1}H NMR assignments of individual amino acid (Table 1). The COSY spectra with chemical shift assignments for the seven groups in the absence or presence of PPI dendrimer are shown in Figures S2−S8, respectively.

Screening Dendrimer-Binding Compounds by Diffusion NMR Studies. The diffusion coefficient (D) of an amino acid is dependent on its molecular size, shape, and solution viscosity and temperature.[28] The solution viscosity and temperature are the same for all the amino acids in a screening pool, while the shape differences of common amino acids can be neglected due to their similar chemical structures. Therefore, the diffusion coefficient of amino acid in this system is size-dependent. Dendrimers, amino acids, and dendrimer/amino acid complexes in the system can be regarded as spheres, and we can describe their diffusion coefficients using an Einstein-Stokes equation.[28]

$$D = kT/6\pi\eta R_s$$

where k is the Boltzmann constant, T is the systematic temperature, η is the viscosity of the solution, and R_s is the hydrodynamic radius of the particle.

If an amino acid in the screening pool binds to PPI dendrimer, it partially obtains the motional rate of the dendrimer, thus developing a much larger hydrodynamic radius and a lower diffusion coefficient.[24] As a result, the ratio of

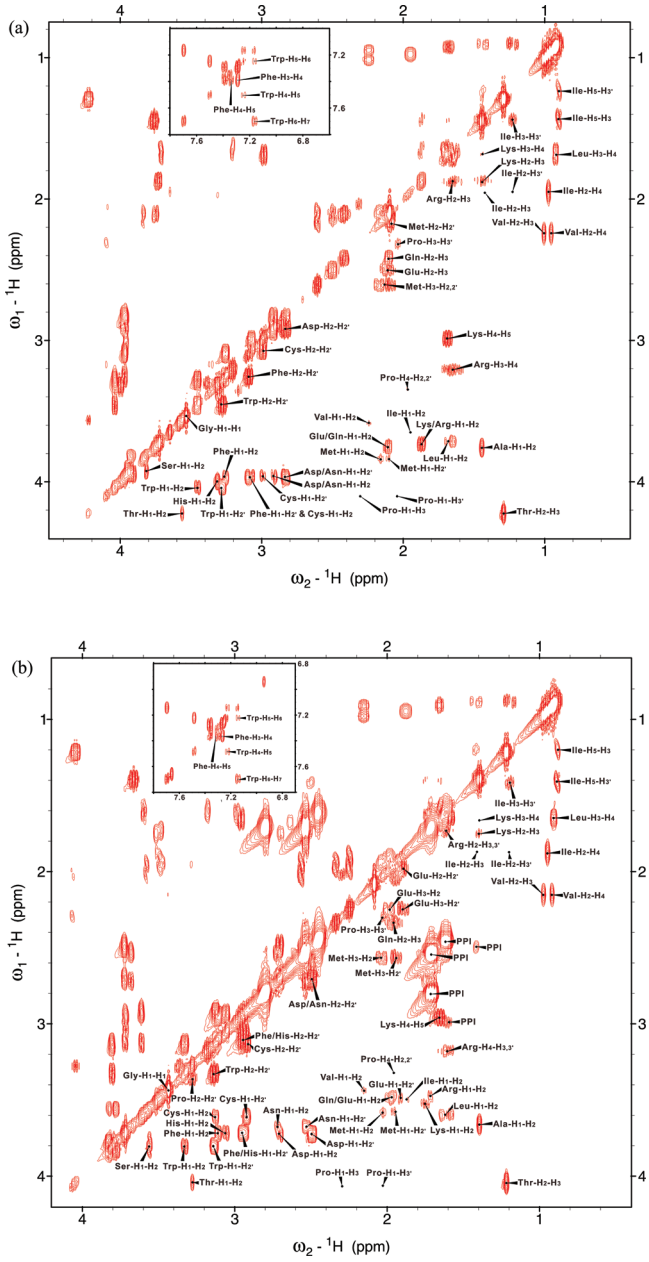

Figure 2. Expanded 2D-COSY spectra of the 19 amino acids in the absence (a) and presence (b) of G4 PPI dendrimer.

diffusion coefficient of an amino acid before and after the addition of PPI dendrimer can be used to reveal its binding affinity to PPI dendrimer. We define D_1 and D_2 as diffusion coefficients of amino acids in the absence and presence of PPI dendrimer, respectively. The corresponding viscosities of the amino acid solutions are η_1 and η_2, respectively. At a constant temperature, the diffusion coefficients of the amino acids before and after the addition of PPI dendrimer are in inverse proportion to the solution viscosity and hydrodynamic radius.[28] Assuming that the effect of viscosity on the diffusion coefficient of each amino acid is equal in the screening system, we can calibrate the diffusion coefficient of amino acid after the addition of PPI dendrimer as

$$D_2' = \alpha D_2 \sim D_2\eta_1/\eta_2$$

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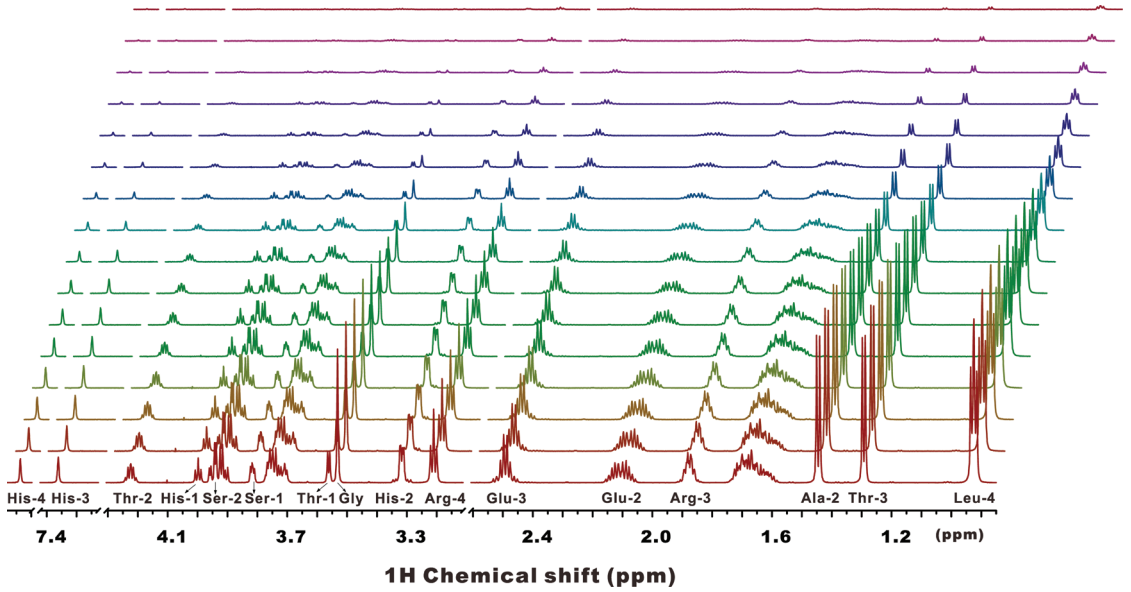

Figure 3. A representative ^{1}H PGSE NMR decay profile for the amino acids in screening experiments.

Table 1. The Seven Groups with Eight Amino Acids in Each Group Used in the Screening Experiments

Group	Amino Acids
1	Thr, Ala, Glu, Arg·HCl, His, Gly, Ser, Leu
2	Pro, Ala, Glu, Arg·HCl, His, Gly, Met, Ile
3	Pro, Val, Trp, Lys·HCl, Asp, Gly, Met, Thr
4	Thr, Ala, Gln, Trp, His, Val, Ser, Leu
5	Pro, Phe, Glu, Ala, Ile, Gly, Met, Thr
6	Pro, Asn, Glu, Lys·HCl, Ile, Gly, Val, Thr
7	Pro, Cys, Glu, Lys·HCl, Ile, Gly, Val, Thr

In a screening system, the calibration factor α (η_1/η_2) is a constant for the amino acids. Therefore, we can directly use D_2/D_1 to rank the binding affinities of amino acids in a screening pool to PPI dendrimer.

Figure 4a–g shows the D_2/D_1 values of the amino acids in the seven groups. In the first group (Figure 4a), the D_2/D_1 value of 0.649 for glutamic acid (Glu) is much lower than those of the other seven amino acids (>0.900), indicating that Glu has a much stronger binding ability than other amino acids in the screening pool. This is probably due to the fact that Glu has two carboxylic groups while the other amino acids such as histidine (His), threonine (Thr), serine (Ser), arginine (Arg), glycine (Gly), alanine (Ala), and leucine (Leu) in this group have only one carboxylic group in their molecular structures. The carboxylic group in the side chain of Glu can also interact with the cationic surface of PPI dendrimer through ionic interactions, thus strengthening its binding affinity to the dendrimer.[26] Also, the ^{1}H NMR titration results in Figure S9 show that Glu simultaneously interacts with the interior and surface of PPI dendrimer. Among the eight amino acids in the first group, Ala shows the lowest binding affinity to PPI dendrimer with a D_2/D_1 value of 1.000.

The second group also confirmed the high binding affinity of Glu to PPI dendrimer (Figure 4b). In this group, the D_2/D_1 values of the amino acids are lowest at 0.653 for Glu and highest at 1.030 for Arg. Arg shows low binding affinity to PPI dendrimer due to the guanidine group on the side chain of Arg. This guanidine group has a high pK_a value of 12.10 and thus is positively charged in the screening system. As a result, the ionic repulsion between the cationic surface of PPI and the two positively charged groups of Arg leads to low binding affinity of Arg to PPI dendrimer. Just like Glu with two carboxylic groups, aspartic acid (Asp) shows strong binding affinity to PPI dendrimer in the third group with a D_2/D_1 value of 0.720 (Figure 4c). Lysine with two cationic groups shows the lowest binding affinity to the dendrimer ($D_2/D_1 \sim 0.996$).

In the fourth group (Figure 4d), tryptophan (Trp) shows the lowest D_2/D_1 value of 0.813. The high binding affinity of Trp to PPI dendrimer is caused by a combination of several interaction mechanisms of this amino acid with the dendrimer. First, the carboxylate group of Trp can interact with the cationic surface of PPI via ionic interaction, which is proved by the shift of PPI surface protons to a higher frequency in the ^{1}H NMR titration results in Figure S10. Second, the aromatic region on the side chain of Trp is involved in strong hydrophobic interactions with the lipophilic pockets of PPI dendrimer.[26] This mechanism is also proved in Figure S10 in which the peaks for interior protons as well as the surface protons of PPI dendrimers are shifted to a lower frequency at higher Trp/PPI molar ratios. This encapsulation is further confirmed by NOE measurement (Figure S11). His and Glutamine (Gln) in the fourth group show medium binding affinities to PPI dendrimer with D_2/D_1 values of 0.857 and 0.882, respectively. His forms an inclusion structure with PPI dendrimer by ionic and/or hydrogen-bond interactions, while the hydrogen-bond donors on the side-chain of Gln are involved in hydrogen-bond interactions with the interior and surface of PPI dendrimer, thus strengthening the interactions of these amino acids with the dendrimer. It is worth noting that the D_2/D_1 value of His in the first group is 0.929, which is much higher than that in the fourth group (0.857), indicating that Glu has a stronger binding affinity to PPI dendrimer than Trp.

Similarly, Glu in the fifth and sixth groups maintained its highest binding affinity with PPI dendrimer ($D_2/D_1 \sim 0.633$ (Figure 4e,f). Phenylalanine (Phe) showed a medium binding affinity to the dendrimer due to the encapsulation of this amino acid within the interior pockets of PPI dendrimer through hydrophobic interactions. However, cysteine (Cys) exhibited

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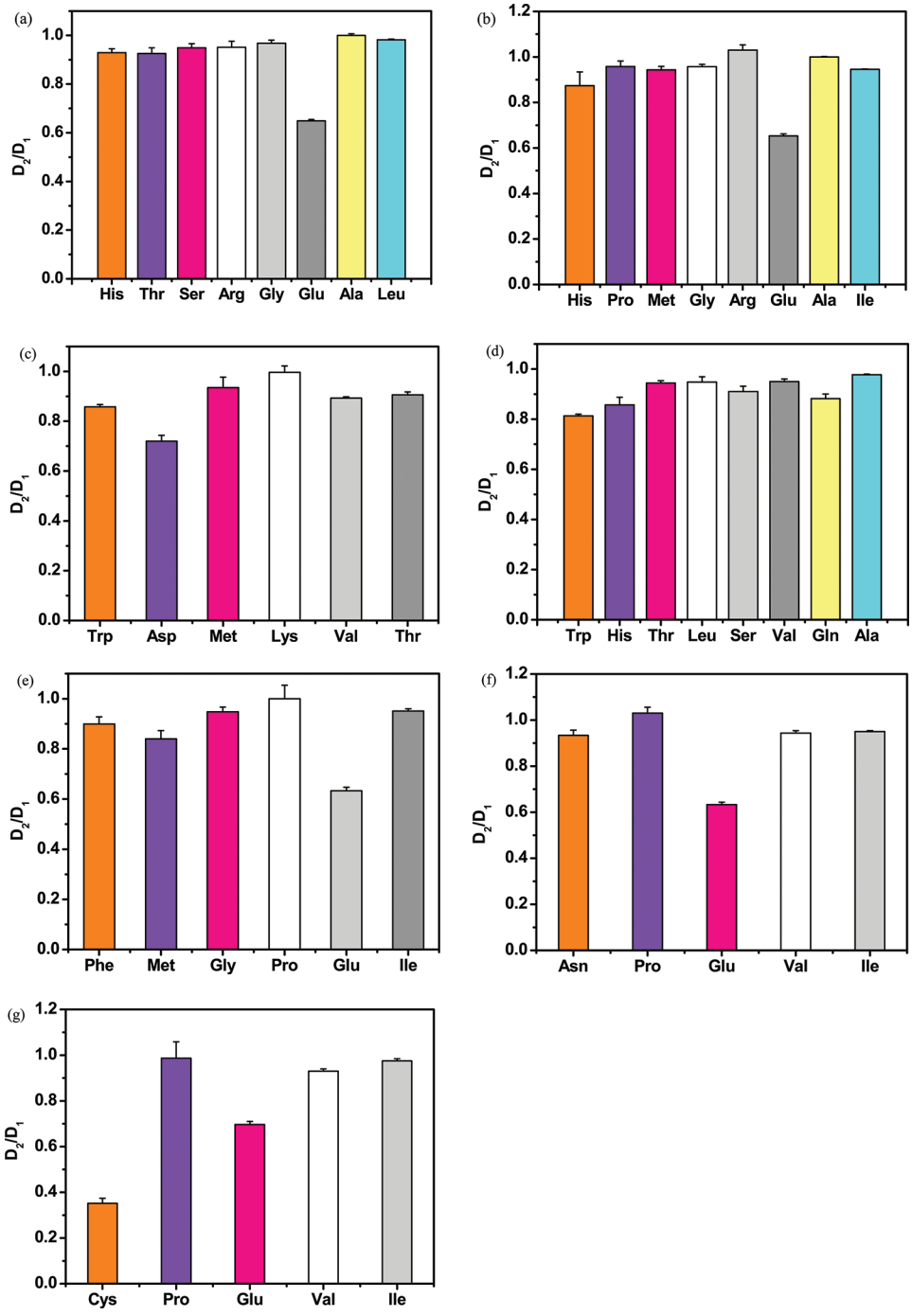

Figure 4. D_2/D_1 values of the amino acids in the screening pool obtained from PGSE NMR screening. Panels a–g represent the D_2/D_1 values of the amino acids in the first to the seventh group, respectively.

an extremely low D_2/D_1 value of 0.352 in the seventh group (Figure 4g), while that of Glu is 0.697. This is probably due to the fact that Cys is easily oxidized into cystine, and the yielded cystine has two carboxylic groups and a flexible linker between the two carboxylic groups, thus exhibiting a strong binding affinity to PPI dendrimer.[30]

Throughout the screening experiment, it is found that the 19 common amino acids show distinct binding behavior with PPI dendrimer. The binding affinity orders of these amino acids with the dendrimer were exhibited in a quantitative way. Here, we divide the binding behaviors of the amino acids with PPI dendrimer in a screening pool into three categories according to their D_2/D_1 values: strong binding affinity ($D_2/D_1 < 0.85$), medium binding affinity ($0.85 < D_2/D_1 < 0.95$), and weak binding affinity ($D_2/D_1 > 0.95$). The screening results for the seven groups are shown in Table 2. This is a competitive binding system, and the binding affinity orders of the candidates may vary with the variation of components in the screening pool. However, this method is sufficient for fast screening of dendrimer-binding compounds. It is worth noting that the common amino acids have seriously overlapping peaks in the ^{1}H NMR spectra, reducing the efficiency of PGSE NMR-based screening experiments. This takes extra time to assign the overlapping resonances and to arrange the candidates into

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Table 2. Screening Results of the PPI Dendrimer-Binding Amino Acids in the Seven Groups

PPI dendrimer-binding amino acids			
group	strong	medium	weak
1	Glu	Thr, His, Ser	Arg, Gly, Leu, Ala
2	Glu	His, Met, Ile	Gly, Pro, Ala, Arg
3	Asp	Trp, Val, Thr, Met	Lys
4	Trp	His, Gln, Ser, Thr, Leu, Val	Ala
5	Glu	Met, Phe, Gly	Ile, Pro
6	Glu	Asn, Val	Ile, Pro
7	Cys, Glu	Val	Ile, Pro

several groups. However, when this PGSE NMR-based screening method was used to screen dendrimer-binding drugs in the libraries, the screening efficiency can be highly improved. Furthermore, the use of cryogenic probe system and high-sensitive NMR instrument can further accelerate the screening experiment,[31] which can increase the signal-to-noise ratio by several times, and reduce the NMR experimental period and the amount of target and drug candidates in the HTS experiments.

In the PGSE NMR-based screening experiments, we found that Glu, Asp, Trp, and Cys have high binding affinities with PPI dendrimer. It is also important for us to identify the binding sites of amino acids on the dendrimer. To reveal the binding mechanisms of the amino acids, a 2D-NOESY experiment of the sample containing PPI dendrimer and the 19 common amino acids was conducted.[28] As shown in Figure 5, only negative cross-peaks between Trp and PPI dendrimer

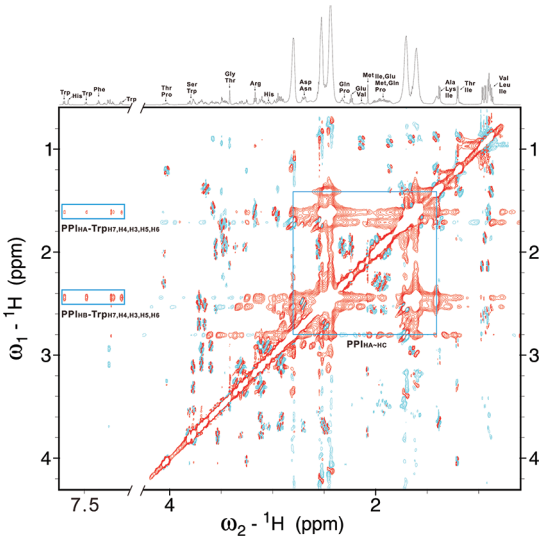

Figure 5. 2D-NOESY spectrum of the 19 amino acids in the presence of PPI dendrimer.

can be observed, suggesting that Trp molecules are most likely to be encapsulated within the PPI pocket among the 19 amino acids. Other amino acids in the screening pool should be bound with PPI dendrimer through ionic interactions or hydrogen-bond interactions.[26]

Besides the significance in the HTS of dendrimer-binding drugs, the screening results in this study can be used to design and optimize dendrimer-based drug delivery systems, especially for the delivery of peptides.[32] Glu, Asp, Trp, and Cys with strong binding affinity to PPI dendrimer can be used to design dendrimer-binding peptides. A peptide with a hydrophobic binding region (Trp) followed by an ionic binding region (Glu or Asp) should have strong binding affinity with PPI dendrimer. This peptide is likely to insert its hydrophobic region into the interior pockets of PPI dendrimer, and then its ionic binding region interacts with the cationic surface of the dendrimer, which strengthens the binding of peptide to the dendrimer. Such a peptide can be modified with a degradable peptide linker and followed by the linkage of therapeutic peptides. We are now in the process of evaluating such a proof-of-concept system for the design of polymeric peptide delivery systems.

CONCLUSIONS

In the present study, PGSE NMR was used to screen dendrimer-binding compounds using common amino acids as a screening pool. Compared to previously developed trNOE and STD experiments for the HTS of dendrimer-binding drugs, the PGSE NMR strategy is a facile, quantitative, and accurate method for the fast discovery of dendrimer-binding compounds. Cys, Glu, Asp, and Trp showed strong binding affinity to PPI dendrimer, and the results are useful for the design of dendrimer-based peptide delivery systems. This study is expected to provide a fast screening method for dendrimer-binding drugs and will play an important role in the design and optimization of dendrimer-based drug delivery systems. Moreover, this PGSE NMR method is applicable to different families of dendrimers and surface-engineered dendrimers as well as a vast library of drug candidates.

ASSOCIATED CONTENT

Supporting Information

Further information on the ^{1}H NMR, COSY, and ^{1}H–^{1}H NOESY spectra of the complexes of G4 PPI dendrimer with the common amino acids. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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