External Electrostatic Interaction versus Internal Encapsulation between Cationic Dendrimers and Negatively Charged Drugs: Which Contributes More to Solubility Enhancement of the Drugs?

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Relationships of electrostatic interaction and encapsulation between poly(amidoamine) (PAMAM) dendrimers and negatively charged drug molecules have been investigated by aqueous solubility and NMR (^{1}H NMR and two-dimensional nuclear Overhauser effect spectroscopy (2D-NOESY)) studies. PAMAM dendrimers significantly increased the solubilities of phenobarbital and sulfamethoxazole, but scarcely influenced those of primidone and trimethoprim. Moreover, ^{1}H NMR and 2D-NOESY measurements indicated that few phenobarbital or sulfamethoxazole molecules were entrapped in the cavities of low-generation dendrimers (generation 3, G3). These results suggest that external electrostatic interaction contributes more to the solubility enhancement of drugs than internal encapsulation.

1. Introduction

Dendrimers are hyperbranched three-dimensional macromolecules with globular or ellipsoidal shapes, nanoscale sizes, hydrophobic or hydrophilic cavities in the interior, a large number of controllable functionalities at the periphery, and extremely low polydispersity.[1–4] Since their successful synthesis by Tomalia et al. in the mid-1980s,[5] dendrimers have attracted considerable attention because of the wide and almost limitless variations on their chemistry, availability of numerous synthetic methodologies, and their unique structures, featured properties, versatility, and so forth, which render them a reliable alternative to traditional polymers in a wide range of applications, especially in the design of new nanocontainers and nanodevices for biomedical purpose.[1,4,6–12] Noncovalent or covalent attachment of drugs to dendrimers was reported to significantly influence the dissolution rate, the aqueous solubility, the stability, and other physicochemical properties of the drugs in physiological conditions.[13–15]

The investigation of interactions between dendrimers and drugs is one of the most active areas of biomedical and pharmaceutical sciences today.[6,13,15–19] The interactions between dendrimers and drugs can be subdivided into the following three types: internal encapsulation (involving physical encapsulation, hydrophobic interaction, and hydrogen-bond interaction), external electrostatic interaction, and covalent conjugation.[13,14,20,21] Although numerous references reported these interaction mechanisms between dendrimers and drugs (involving anti-inflammatory drugs, antimicrobial drugs, and anticancer drugs)[14,15,18,22] and the applications of such systems to enhance drug solubility,[22,23] stability, and bioavailability,[24,25] little work has been done to clarify the relative importance of these interaction types. In the present work, we first compared the contributions of total encapsulation (physical encapsulation, hydrophobic interaction, and hydrogen-bond formation) and electrostatic interaction to the solubility enhancement of four model drugs with extremely low aqueous solubility (phenobarbital, primidone, sulfamethoxazole, trimethoprim). Two-dimensional nuclear Overhauser effect spectroscopy (2D-NOESY) was used to investigate the host properties of poly(amidoamine) (PAMAM) dendrimers and to provide credible evidence of hydrophobic encapsulation of these guests within the dendritic architectures. Finally, which type of interaction between dendrimers and drug molecules contributes more to the solubility enhancement of these drugs was fully discussed.

2. Experimental Section

2.1. Materials. Generation 2−6 (G2−G6) PAMAM dendrimers were purchased from Dendritech, Inc. (Midland, MI). Phenobarbital was obtained from Bio Basic, Inc. (Shanghai, China). Primidone was a gift from School of Life Sciences, University of Science and Technology of China. Sulfamethoxazole and trimethoprim were obtained from Shouguang Fukang Pharmacy Factory (Shandong, China). Deuterium oxide (D_2O) was purchased from Beijing Chongxi High-Tech Incubator Co., Ltd. (Beijing, China). All the chemicals were used as received without further purification. Double-distilled water was used in the aqueous solubility studies.

2.2. Aqueous Solubility Studies. The aqueous solubilities of the four model drugs were determined by the equilibrium solubility method described as follows. Excess drugs were added to 500 μL of each test solution (0−10 mg/mL PAMAM dendrimers, double-distilled water was used as a blank) to ensure the drug solution reached saturation. The solution was mechanically shaken for 24 h at 37 °C, and then the solutions were centrifuged at 10 000 rpm for 5 min. The saturated solutions obtained were then diluted to a proper concentration with boric acid buffer (0.1 M boric acid, 0.1 M KCl, and adjusting the pH condition to 10.0 using 0.1 M NaOH) for phenobarbital and primidone, and with distilled water for sulfamethoxazole and trimethoprim. The diluted samples were analyzed at the

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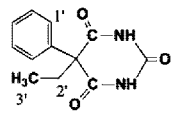
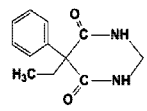
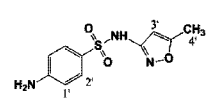
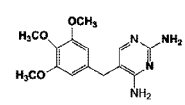
TABLE 1: The Characteristics of the Four Investigated Model Drugs

Drug name	Phenobarbital	Primidone	Sulfamethoxazole	Trimethoprim
Molecular Structure				
Class	Anticonvulsant		Anti-bacterial drugs	
Molecular Formula	$C_{12}H_{12}O_3N_2$	$C_{12}H_{14}O_2N_2$	$C_{10}H_{11}O_3N_3S$	$C_{14}H_{18}O_3N_4$
Molecular Wight (g/mol)	232	218	253	290
pKa value	7.2-7.4	>13	5.7	7.3-7.4

characteristic wavelengths of the four drugs (240 nm for phenobarbital, 257 nm for primidone, 265 nm for sulfamethoxazole, and 271 nm for trimethoprim) by a Perkin-Elmer UV−vis spectrometer to estimate the amount of drugs entrapped by PAMAM dendrimers. Three repeats of each sample were conducted.

2.3. NMR Studies. 2D-NOESY spectra were obtained for the solutions of dendrimers (2 mg dissolved in 1 mL of D_2O) and drugs (2 mg each), and acquired at 500.132 MHz, using 0.3 s mixing time and 8.2 μs ^{1}H 90° pulse width. The experiments were done with a 2 s relaxation delay and 205 ms acquisition time. Eight transients were averaged for each 400 × 1024 complex t_1 increments. The data were processed with Lorentz-to-Gauss window function and zero filling in both dimensions to display data on a 2048 × 2048 2D-matrix. All data were processed with NMRPipe software on a Linux workstation (see: Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. *J. Biomol. NMR* **1995**, *6*, 277). ^{1}H NMR spectra of dendrimers, drugs, and the mixtures of dendrimers and drugs (dissolved in D_2O) were also obtained on a 500.132 MH_Z NMR Spectrometer (Bruker, Germany).

3. Results and Discussion

3.1. Aqueous Solubility Studies. As shown in Figure 1, the aqueous solubility of phenobarbital was significantly increased in the presence of G3−G5 amine-terminated PAMAM dendrimers. However, the solubility of primidone was scarcely changed in dendrimer solutions under the same experimental conditions. Previous studies suggested that the enhanced solubilities of insoluble drugs in cationic dendrimer solutions were due to several interaction mechanisms between dendrimers and drugs. First, the existence of a large number of relative nonpolar cavities in the interior of dendrimers provide dendrimers the ability to encapsulate guests in the cores by hydrophobic interactions.[5,13,17,18,26] Second, the high density of cationic functional groups on the surface of dendrimers endues these dendritic architectures with the capability for electrostatic attachment of negatively charged guests.[22,27–30] Third, tertiary amines and amide groups in internal cavities of dendrimers can interact with specific atoms (nitrogen or oxygen atoms) or functional groups (hydroxyl or carboxyl groups) of the guest molecules by hydrogen-bond formation.[23,27] Last but not least, the aggregation of dendrimers into assembled clusters due to dendrimer−dendrimer interactions further provides amphiphilic "micelles" to load drug molecules.[24] Phenobarbital and primidone, which are the most often used drugs in the treatment of epilepsy, have extremely similar properties (involving the molecular structure, weight, and size). Both drugs have an ethyl group and a phenyl group on C_5 of the barbituric ring. The only difference between the two drugs is the lack of an oxygen atom on C_2 in the primidone molecule (Table 1). On the basis of the overall similarities of the structures of phenobarbital and primidone, the influence of molecular size on the host−guest interactions between dendrimers and drugs can be ignored. In addition, the primidone molecule is more hydrophobic than the phenobarbital molecule, suggesting that primidone is even easier to be encapsulated in the relative nonpolar cavities of PAMAM dendrimers. However, the total amount of phenobarbital molecules entrapped by dendrimers (both internal and external) is much more than that of primidone, as shown in Figure 1. It was reported that phenobarbital and primidone molecules have different forms in equilibrium in basic solutions: noncharged forms and negatively charged forms (Scheme 1). However, the pK_a value of primidone (pK_a > 13) is much higher than that of phenobarbital (pK_a ∼ 7), indicating that primidone molecules in dendrimer solutions (pH ∼ 10) exist in noncharged form, and that the negatively charged forms of phenobarbital can be attached to the surface of positively charged PAMAM dendrimers by electrostatic interaction. Therefore, we suppose that the difference of the solubilization behavior of dendrimers to phenobarbital and primidone is predominantly due to the distinct contributions of electrostatic interaction, or that the electrostatic interaction contributes more to the solubility enhancement than

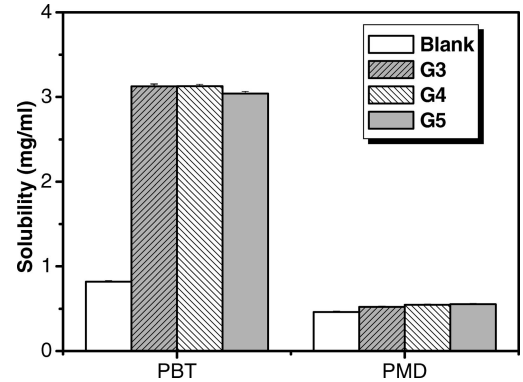
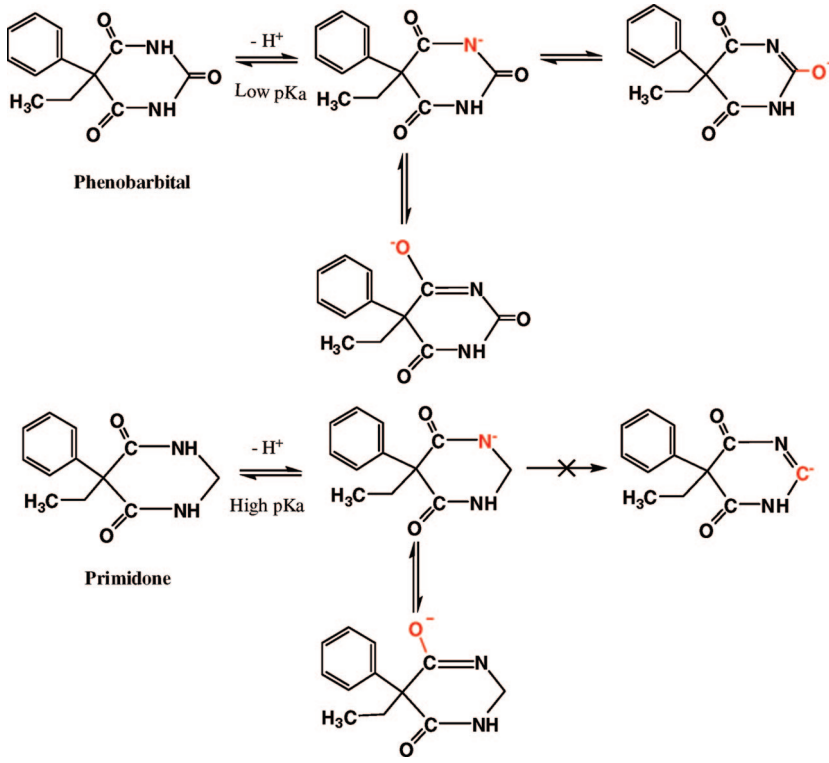


Figure 1. Solubilities of phenobarbital (PBT) and primidone (PMD) in the presence of different generations of amine-terminated PAMAM dendrimers (4 mg/mL).

SCHEME 1: Equilibrium of Noncharged and Negatively Charged Forms of Phenobarbital and Primidone Molecules in Solutions



total encapsulation, including hydrophobic interaction, hydrogen-bond interaction, and physical encapsulation.

Further validation of the proposed relationship between electrostatic interaction and total encapsulation was conducted by using sulfamethoxazole and trimethoprim as model drugs. Both sulfamethoxazole and trimethoprim are synthetic folate antagonists with extremely low aqueous solubilities. The combination of them is frequently used in clinical trails to treat different types of bacterial infections. Although the molecular sizes and original solubilities of the two drugs are similar, they exhibit distinct solubility profiles in dendrimer solutions. As shown in Figure 2, the aqueous solubility of sulfamethoxazole significantly increased with the help of PAMAM dendrimers, while that of trimethoprim remained approximately unchanged, regardless of the presence or absence of dendrimers. In dendrimer solutions, the acidic sulfamoyl group ($-SO_2NH-$) in sulfamethoxazole molecule generates a negatively charged form which can be attached to the surface of dendrimers via electrostatic interaction (Scheme 2). However, the lack of negatively charged groups in trimethoprim limits the solubility enhancement of trimethoprim by PAMAM dendrimers. It is worth noting here that the solubilities of these hydrophobic drugs are slightly changed in buffers with the same pH condition (take sulfamethoxazole for example: its solubility in 2 mg/mL, pH 9.9 G4 PAMAM dendrimer solutions is 2.96 ± 0.04 mg/mL; however, its solubility in a pH = 10 sodium hydroxide solution is around 0.52 ± 0.03 mg/mL, just a bit higher than its aqueous solubility of 0.35 ± 0.02 mg/mL in neutral conditions), suggesting that the pH effect of the dendrimer is not an important factor in the solubilization of these drugs with solubility problems. These results together with the solubilization data obtained from phenobarbital and primidone suggest that the electrostatic interaction contributes more to the solubility enhancement of drugs than total encapsulation.

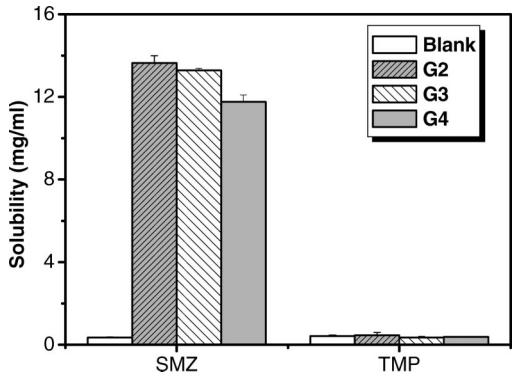
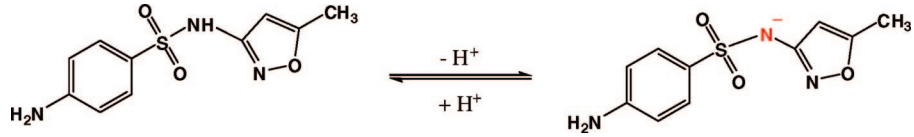


Figure 2. Solubilities of sulfamethoxazole (SMZ) and trimethoprim (TMP) in the presence of different generations of amine-terminated PAMAM dendrimers (10 mg/mL).

The effects of dendrimer concentration and generation on the enhanced solubilities of phenobarbital and sulfamethoxazole were also investigated. Taking phenobarbital for example, it was observed that the solubility of phenobarbital increased linearly as a function of dendrimer concentration (Figure 3). Higher dendrimer concentration means higher cationic group concentration and more opportunities for electrostatic attachment of negatively charged guests.[29] In addition, the numbers of both hydrophobic cavities and primary amine groups increase with dendrimer generation at a fixed dendrimer molar concentration.[31] In this way, we could explain why higher-generation dendrimers could enhance the solubility of phenobarbital more effectively than lower ones at the same molar concentration.

3.2. NMR Studies. In order to further demonstrate the reasons for the different behaviors of solubility enhancement of guests in dendrimer solutions, the interactions between dendrimer and drug molecules (phenobarbital and sulfamethoxazole) was investigated by ^1H NMR spectroscopy. The experiments performed in D_2O were carried out on free dendrimer,

SCHEME 2: Ionization of the Sulfamethoxazole Molecule in Solutions



phenobarbital and sulfamethoxazole molecules, and their mixtures, respectively. Generally, the encapsulation of guest molecules (phenobarbital and sulfamethoxazole) into the hydrophobic interior of hosts (dendrimers) causes the displacement of the chemical shift of guest protons, which is an evidence of inclusion.[32,33] However, as shown in Figure 4, the lack of significant shifts for phenobarbital in the presence of G3 or G6 PAMAM dendrimer was observed, suggesting that the hydrophobic encapsulation is not a predominant interaction type between dendrimers and the guests.

2D-NOESY measurements can provide information on the distance between protons in close spatial proximity within a given molecule and can also be used to detect the host−guest interactions in solutions.[33–35] Figure 5 shows the 2D-NOESY spectra of G3 or G6 PAMAM dendrimer and phenobarbital mixtures at a weight ratio of 1 and a concentration of 2 mg/mL in D_2O. No cross peaks between protons of phenobarbital and that of G3 PAMAM dendrimer at different experimental conditions were observed (Figure 5a), indicating that few phenobarbital molecules were entrapped in the cavities of a G3 dendrimer. However, the solubility of phenobarbital was significantly increased in the presence of G3 dendrimer, as shown in Figure 1, suggesting that the increased solubility of phenobarbital was predominantly due to the electrostatic attachment of the drugs to the surface of dendrimers. The existence of NOE cross-peaks between protons of phenobarbital (1′-H and 3′-H) and all the protons of G6 dendrimer (1-H, 2-H, 3-H, and 4-H) in Figure 5b indicates that phenobarbital and dendrimer scaffolds in the complex were close enough (less than 5 Å) to make the dipole−dipole interactions between these protons observed. These results demonstrated that G6 dendrimer is more capable of encapsulating phenobarbital molecules into its interior than G3 dendrimer. This can also explain the reason why G6 PAMAM dendrimer can enhance the solubility of phenobarbital more effectively than G3 dendrimer (Figure 3).

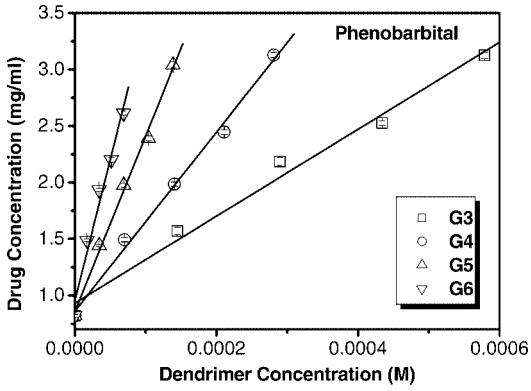


Figure 3. Solubilities of phenobarbital at different concentrations and generations of PAMAM dendrimers.

The 2D-NOESY spectra of sulfamethoxazole in the presence of G3 and G6 PAMAM dendrimer are shown in Figure 6a,b, respectively. It clearly shows the interactions between phenyl residue protons (1′-H and 2′-H) of sulfamethoxazole and methylene protons (1-H and 3-H) of G6 dendrimer. Also, the cross-peaks for the interactions between heteroaromatic proton (3′-H) of sulfamethoxazole and the methylene protons (1-H, 3-H, and 4-H) of G6 PAMAM dendrimer can be observed in Figure 6b. However, the 2D-NOESY spectrum in Figure 6a does not show any signs of these interactions between sulfamethoxazole and G3 dendrimer, which agrees well with the 2D-NOESY spectrum of phenobartital and G3 dendrimer.

In Figures 5b and 6b, there are three kinds of cross-peaks: A, B, and C. A represents the cross-peaks between the methylene protons (1-H, 2-H, 3-H, and 4-H) of dendrimer, B peaks are the cross-peaks between the protons of guests, while C peaks are the cross-peaks between protons of dendrimer and that of the guests. Surprisingly, although the spatial distance between the methyl protons (4′-H) and phenyl protons (1′-H and 2′-H) of sulfamethoxazole is larger than 5 Å, cross-peaks for the NOE interactions between these protons are clearly observed in Figure 6b, which is due to the binding of sulfamethoxazole molecules to G6 PAMAM dendrimer. Overall, the NOESY spectra of the dendrimer and the drugs clearly demonstrated that the electrostatic interaction contributes more to the solubility enhancement

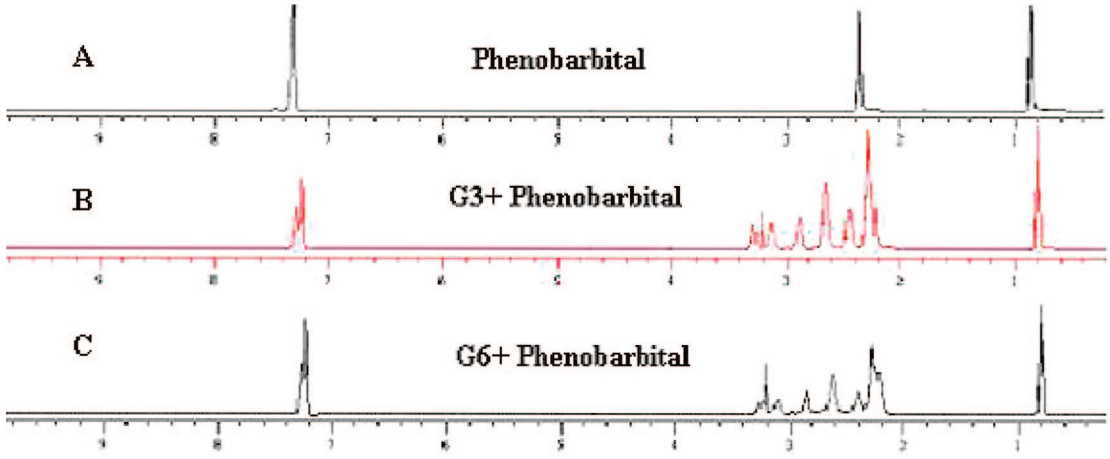


Figure 4. ^1H NMR spectra of phenobarbital and its complexes with PAMAM dendrimers in D_2O: (A) phenobarbital alone, (B) phenobarbital and G3 PAMAM dendrimer, and (C) phenobarbital and G6 PAMAM dendrimer.

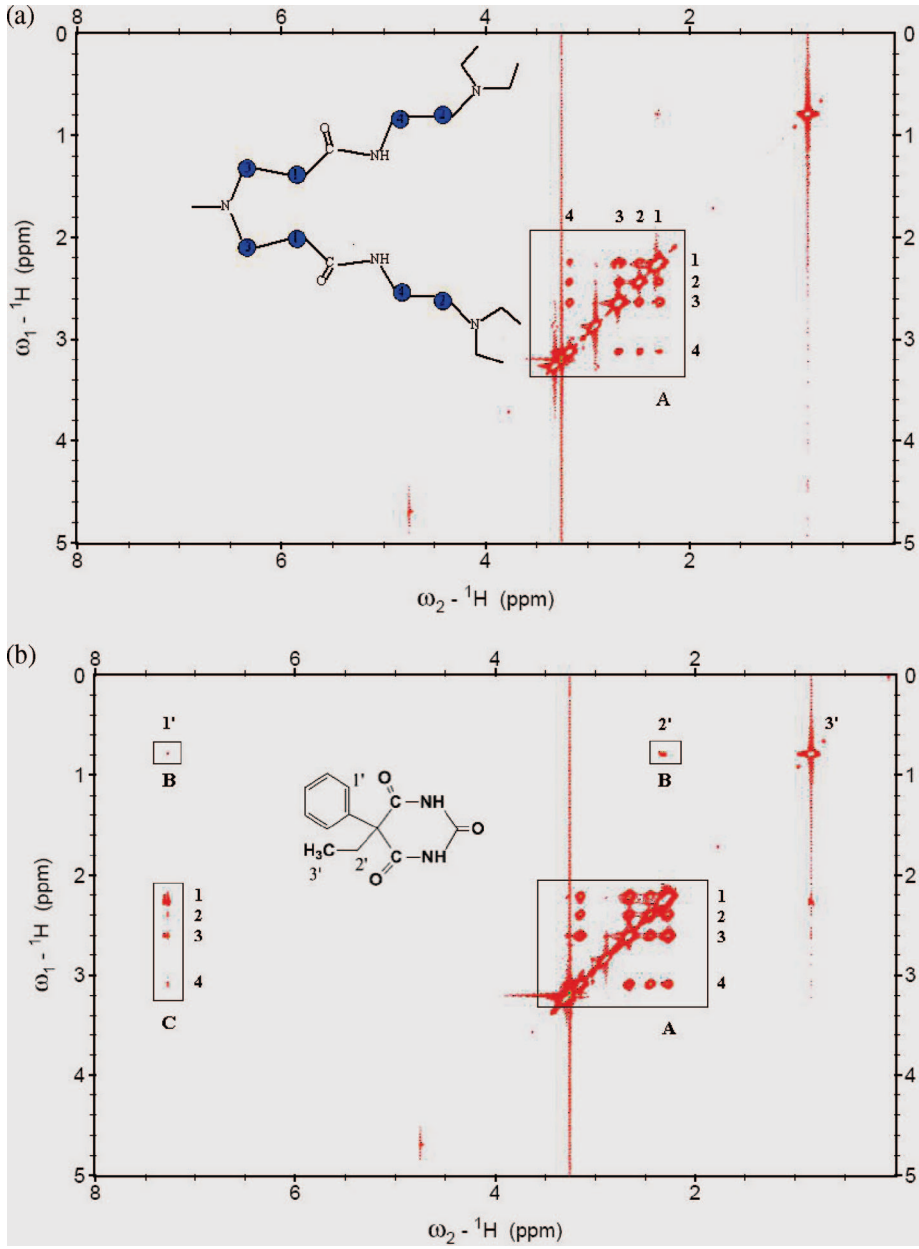


Figure 5. 2D-NOESY spectra of phenobarbital and (a) G3 or (b) G6 PAMAM dendrimers in D_2O.

of drugs than encapsulation, especially for low-generation dendrimers (such as G3 PAMAM dendrimer).

3.3. Discussions of Previously Investigated Drugs. We have demonstrated the relationships between electrostatic attachment and encapsulation of negatively charged drugs to PAMAM dendrimers by aqueous solubility and NMR studies. However, a theory proposed here is that not all the guest molecules have negatively charged groups. In order to validate the conclusions we have made, a list of drugs that have been previously employed as guests of the most investigated dendrimers (PAMAM and poly(propyleneimine) (PPI)) was surveyed (dendrimer−drug conjugates are not included). Interestingly, these drugs involving anti-inflammatory drugs,[22,24,28,32] antimicrobial drugs,[29,36] and anticancer drugs[37−40] are mostly insoluble drugs possessing negatively charged groups and can be classified into four types according to the source of negative charge: (a) drugs with carboxyl groups[13,24,25,27,28,32,36,37,41−48] (Scheme S1 in the Supporting Information); (b) drugs with other negatively charged groups,[29,39,49] such as phenolic hydroxyl or mercapto groups, acidic sulfamoyl groups, phosphate groups, and sulfonic groups (Scheme S2 in the Supporting Information; doxorubicin,[50] niclosamide,[30] and rifampicin[51] are not included); (c) drugs that have no negatively charged groups but a tautomerism (keto−enol) in their molecular structures,[40,52] which can generate negatively charged forms of these drugs (Scheme S3 in the Supporting Information; the tautomerization of the drug molecule is critical for its solubility enhancement in dendrimer solutions, but the stabilization of the deprotonized form of the drug via tautomerization is every important); and (d) drugs that can be transformed into negatively charged forms by chemical reactions,[38,53] such as the lactone ring-opening (Scheme S4 in the Supporting Information).

Over 50% of the surveyed drugs belong to type (a), and most of them are nonsteroidal anti-inflammatory drugs. The exceptions not included in Schemes S1−S4 are nifedipine,[23] fluorouracil,[54] and chloroquine phosphate.[55] Nifedipine, an insoluble drug without negatively charged groups in the molecule, was employed as a guest molecule of low-generation (up to G3) PAMAM dendrimers by Devarakonda et al.[23] Although much high dendrimer concentrations (up to 40 mg/mL) were used,

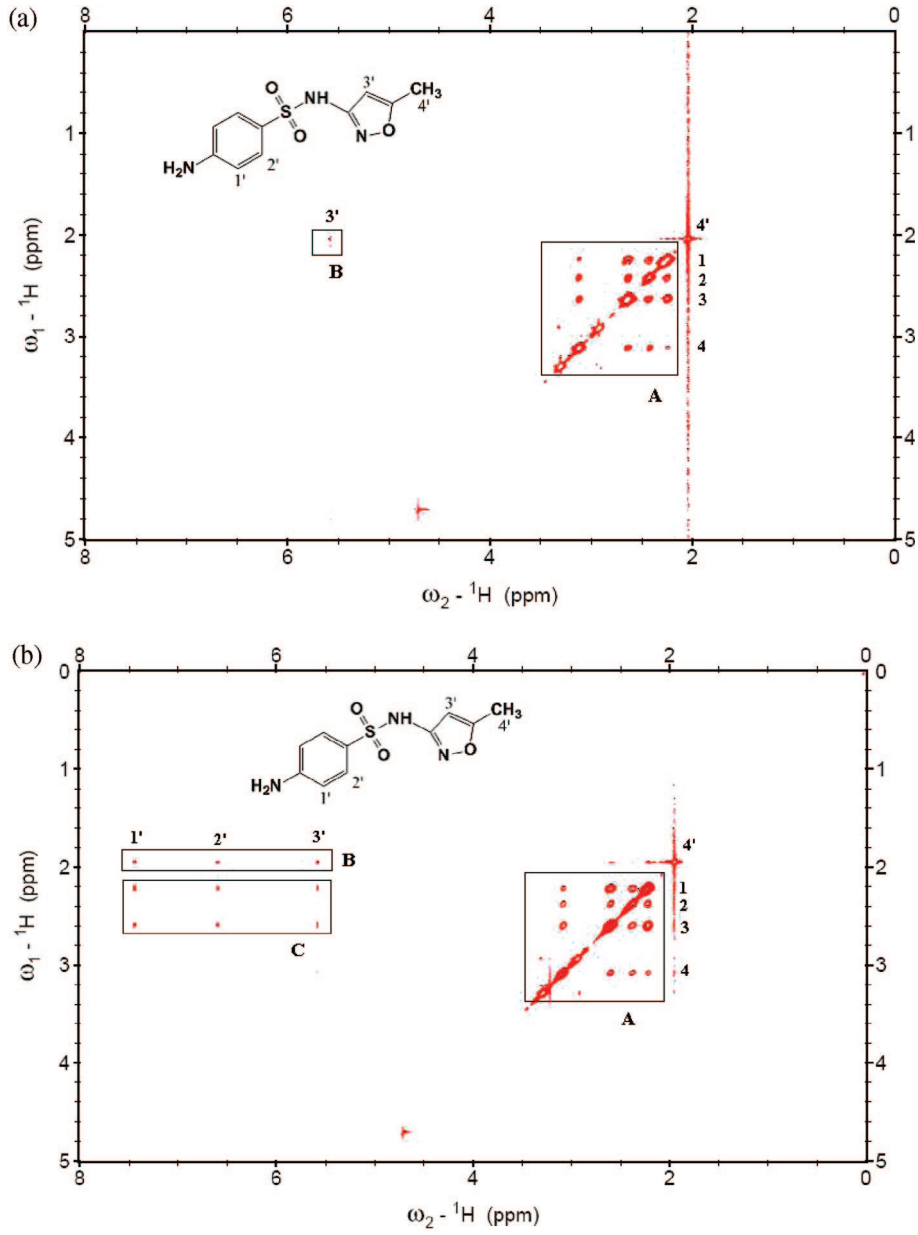


Figure 6. 2D-NOESY spectra of sulfamethoxazole and (a) G3 or (b) G6 PAMAM dendrimers in D_2O.

the solubility enhancement of nifedipine was not as sufficient as that of the drugs with negatively charged groups, indirectly suggesting the importance of electrostatic interactions to the solubility enhancement of drugs. Fluorouracil[54] and chloroquine phosphate[55] are water-soluble drugs, therefore discussing the solubility enhancement of them by dendrimers makes no sense. Altogether, the structures of previously investigated drugs confirmed that the conclusion made from aqueous solubility and NMR studies (electrostatic interaction contributes more to the solubility enhancement than total encapsulation) is not limited to phenobarbital and sulfamethoxazole in this study, but a wide spectrum of drugs with negatively charged groups.

Although cationic dendrimers such as PAMAM and PPI exhibit promising host−guest properties toward a wide range of charged drugs, several observations are worth noting here. The first observation is that the potential to transfer a neutral and hydrophobic drug to a charged drug that has stronger interaction with the carrier is more significant in some cases. It is known that the loading/encapsulating ability of a dendrimer is dependent on not only the properties of a drug molecule but also the architecture of the dendrimer. Dendrimers with more hydrophobic repeated units or cavities will encapsulate more neutral and hydrophobic drugs in the interior rather than adsorb them on the surface. Currently, new synthesized dendrimers are reported to sufficiently encapsulate neutral and hydrophobic drugs. We think it will not be a problem for dendrimers to deliver all kinds of drugs in the future. The second question is, although external electrostatic interaction predominantly enhances the solubility of the drugs, is it beneficial for the effective delivery of the drugs using this inclusion complex formed through electrostatic interaction? Generally, we can benefit a lot from this electrostatic interaction-mediated drug formulation in different routes of drug administration, such as in the transdermal, intravenous, and ocular routes. There are currently many literature reports related to the delivery of dendrimer/drug complexes, and most of these trials exhibited excellent in vitro behaviors and in vivo pharmacodynamic and pharmacokinetic profiles.[13–15,17,18] Our previous experiments using dendrimer−drug complexes mainly formed by electrostatic interactions also showed that they are good choices in clinical

practice.[29,36,44] However, we also got unexpected data when administrating the dendrimer−drug complexes to rats in an oral administration route.[46] Not so good pharmacodynamic and pharmacokinetic profiles were obtained because of the degradation of the complexes in the stomach where the pH condition is extremely low. Although the electrostatic interaction is much stronger than hydrophobic encapsulation in dendrimer−drug formulations, this will not prevent the release of the drug from the dendritic matrix. This is because the electrostatic interactions between dendrimers and drugs are easily influenced by the pH condition, salt concentration, and many other factors in the administration systems. Either way, the general rule in this study will have a significant contribution to the design of new dendrimer-based drug delivery systems and to guide the administration routes of these dendrimer-based drug formulations.

4. Conclusions

The interactions between PAMAM dendrimers and four model drugs were investigated by aqueous solubility and NMR studies. The cationic dendrimers significantly increased the solubilities of phenobarbital and sulfamethoxazole, but scarcely influenced that of primidone and trimethoprim. These results together with 2D-NOESY data suggest that electrostatic interaction contributes more to the solubility enhancement than encapsulation. The validity of this conclusion is not limited to phenobarbital and sulfamethoxazole, but a wide spectrum of drugs. Of course, the ability of dendrimers to interact with drugs also depends on the nature of dendrimers (surface functional groups, hydrophobic properties of the cavities, and polarity of dendrimer surface). Anyway, on the basis of this study, it is possible to design new drug delivery systems by appropriate selection of dendrimers and drug molecules with different functionalities.

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Supporting Information Available: Schemes depicting the four types of drugs that have been employed as guests of cationic dendrimers. This material is available free of charge via the Internet at http://pubs.acs.org.

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