Macromolecules
ARTICLE

Macromolecules **2010**, *43*, 3011–3017 **3011**
DOI: 10.1021/ma902670q

Dendrimers as Drug Carriers: Dynamics of PEGylated and Methotrexate-Loaded Dendrimers in Aqueous Solution

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Received December 2, 2009; Revised Manuscript Received February 12, 2010

ABSTRACT: An investigation of the dynamics of aqueous solutions of PEG, PEGylated dendrimers, methotrexate, and PEGylated and drug (methotrexate)-loaded dendrimers was carried out with broadband dielectric relaxation spectroscopy (DRS). Two dielectric dispersions were observed in all systems, and the explanation of their molecular origin was offered. The lower frequency process, characterized by an activation energy of 52.2 kJ/mol, was attributed to motions in ice. The higher frequency process is of the Cole−Cole type and is assigned to bound water around PEG and methotrexate molecules. The effect of the average number of attached PEG chains and the average number of encapsulated methotrexate molecules was probed. Our results indicate that the dynamics are independent of these numbers but are a strong function of the composition of the complex. Spectral characteristics and temperature dependence of all relaxation processes are described.

Introduction

Dendrimers are nanoscopic globular macromolecules with unique architecture in that they have well-defined, compartmentalized structure and narrow polydispersity. They have been extensively investigated as drug delivery carriers[1−7] due to the presence of nanocompartments that can encapsulate guest molecules.[8−10]

Methotrexate or amethopterin is an antimetabolite drug that works by competitively inhibiting an enzyme in the folic acid metabolism. It is a weak acid, with two carboxylic groups. Encapsulation of methotrexate into PAMAM dendrimers is believed to result from the acid−base interaction between methotrexate and dendrimers interior.[11]

Methotrexate has been encapsulated in PAMAM dendrimers, but the drug was found to be released readily in the cell culture.[12] To slow down the release rate, Kojima et al. modified PAMAM dendrimers by attaching PEG chains[13] and reported that the encapsulation efficiency increased with increasing generation of dendrimers and molecular weight of PEG. Pan et al. investigated the influence of surface coverage of dendrimers with PEG. They found that the surface coverage had little influence on the encapsulation efficiency of methotrexate, but it affected the release rate.[11] The principal objective of this work is to gain a deeper insight into the nature of methotrexate-loaded PEGylated dendrimers by conducting an investigation on their molecular dynamics in aqueous solutions.

Studies of molecular dynamics of neat dendrimers have been previously reported. Few dendrimer families were investigated: phosphorus-containing dendrimers[14−16] and carboxilane dendrimers,[17,18] including our study on the first six generations of PAMAM dendrimers.[19] Reports on dendrimer dynamics in complex environment are scarce. Recently, we conducted a comprehensive investigation on dynamics of dendrimers in complex hydrophobic/hydrophilic environment[20,21] and dynamics of PEGylated dendrimers.[22] To the best of our knowledge, however, this study marks the first report on the dynamics of aqueous solutions of PEGylated and methotrexate-loaded dendrimers as studied by DRS.

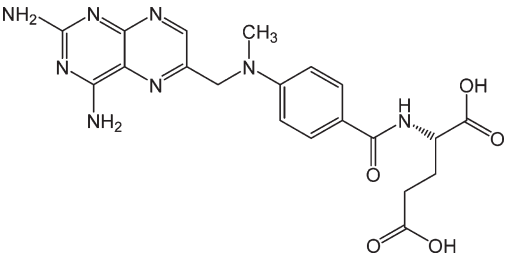

Figure 1. Molecular structure of methotrexate.

Experimental Section

Materials. *Dendrimers.* Generation 3 poly(amidoamine) PAMAM dendrimer with amino surface groups in methanol solution (20 wt %) was obtained from Aldrich.

PEG. Poly(ethylene glycol), PEG, with molecular weight of 2000 Da and different end groups, one nonreactive (methyl group) and the other reactive (epoxy group), was obtained from SunBio, Inc.

PEGylated Dendrimers. PEGylated dendrimers were synthesized by adding various amounts of PEG to a solution of dendrimers in methanol. Three molar ratios of PEG to dendrimer were used: 32:1, 16:1, and 8:1. The mixtures were stirred for 24 h at room temperature, using a high-speed stirrer. The solutions were then placed in a vacuum oven for 7 days at 80 °C. The completion of reactions was confirmed by near-infrared (NIR) spectroscopy. The reaction was considered complete when no peak was detected at the epoxy absorption frequency (4520 cm^{-1}).

Encapsulation of Methotrexate. The molecular structure of methotrexate is shown in Figure 1. Methotrexate was encapsulated by adding PEGylated dendrimers in solution of methotrexate in water, as described by Pan et al.[11] The molar ratios of methotrexate to the PEGylated dendrimers were 13:1, 8:1, and 4:1.

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Published on Web 02/23/2010

Table 1. Investigated Samples

description	no. of PEG per dendrimer	no. of drug per dendrimer	code
D + PEG + M	8	13	8P-13M
D + PEG + M	16	4	16P-4M
D + PEG + M	16	8	16P-8M
D + PEG + M	16	13	16P-13M
D + PEG + M	32	13	32P-13M

All solutions were made in deionized water. In the sample codes for PEGylated and methotrexate-loaded dendrimers (#P-#M) the first number represents the average number of the attached PEG chains and the second one corresponds to the average number of the encapsulated drug molecules. All investigated systems and their codes are summarized in Table 1.

Techniques. *Dielectric Relaxation Spectroscopy* (*DRS*). Dielectric measurements were performed in the frequency range from 10^{-1} to 10^{6} Hz using Novocontrol α Analyzer, which was interfaced to computers and connected to a heating/cooling unit (modified Novocontrol Novocool System) that can control temperature from 173 to 523 K with a precision of ±0.5 K. Samples were placed in a cell, specially designed for liquid media, composed of two stainless steel electrodes separated by a Teflon ring, whose cavity was filled by the sample. The diameter of the electrodes is 10 mm, and the thickness of the sample is 635 μm. Further details about our experimental facility for dielectric measurements are given elsewhere.[23]

Fourier Transform Infrared Spectroscopy (*FTIR*). FTIR spectroscopy was performed using a Nicolet Magna-IR system 750 spectrometer with spectral range coverage from 15 800 to 50 cm^{-1} and the Vectra interferometer with resolution better than 0.1 cm^{-1}. Near-infrared (NIR) data were obtained using a calcium fluoride beam splitter, a white light source, and a mercury−cadmium−tellurium (MCT) detector. The details of this technique can be found elsewhere.[24]

Results and Discussion

Theoretical and experimental aspects of DRS are well established and will not be reviewed here. Instead, the interested reader is referred to several excellent books.[25−27] The presentation of our results is divided into four parts: (1) dynamics of aqueous solutions of PEG, (2) dynamics of aqueous solutions of PEGylated dendrimers, (3) dynamics of aqueous solutions of methotrexate, and (4) dynamics of aqueous solutions of PEGylated and methotrexate-loaded dendrimers.

Dynamics of Aqueous Solutions of PEG. We begin by examining the dielectric response of aqueous solutions of PEG. Dielectric permittivity and loss in the frequency domain of PEG at 3 wt % in the aqueous solution below the freezing point with temperature as a parameter are shown in Figure 2. Two relaxation processes are observed between 193 and 233 K, and they are indicated with arrows in the figure. Both processes shift to higher frequency with increasing temperature, but they differ in that the lower frequency process (LFP) increases in intensity while the higher frequency process (HFP) slightly decrease in intensity with increasing temperature. In agreement with published results,[28−30] LFP is attributed to pure ice. The HFP originates from the bound water around PEG. Previous DRS results of aqueous solution of DNA[29] and protein solutions,[30] where observed process was associated with movement of water around studied system, corroborate this conclusion. Another possible origin of HFP lies in the phenomenon of interfacial polarization, though the absence of evident interfaces in these systems would not support such hypothesis. The solid lines in the figure are the combined fits of the sum of ionic conductivity and the Havriliak−Negami

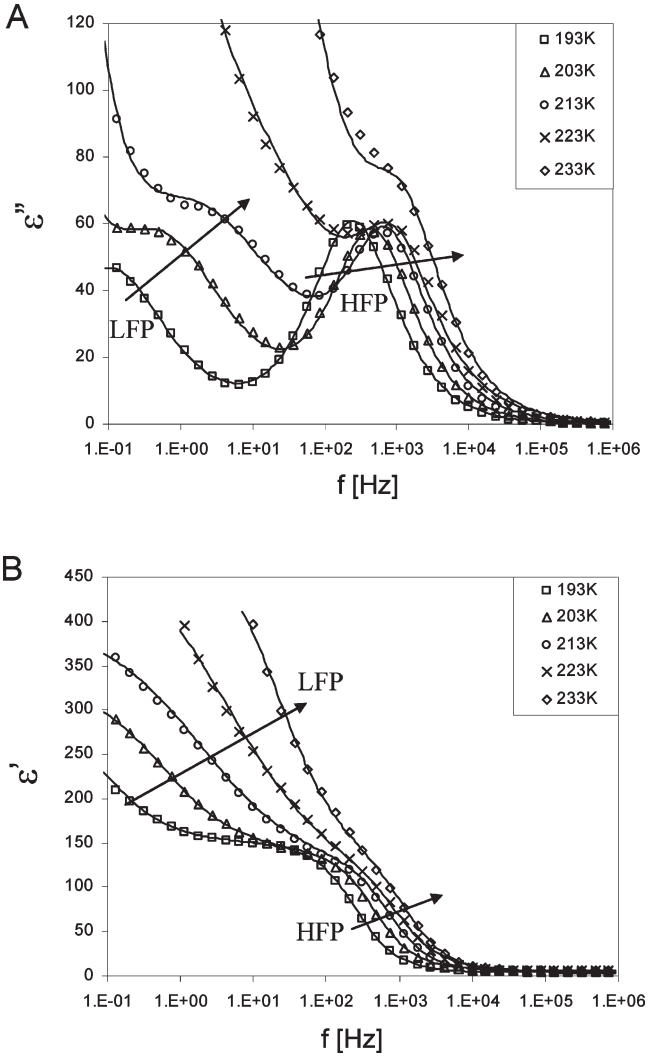


Figure 2. Dielectric loss (A) and permittivity (B) in the frequency domain with temperature as a parameter for the water/PEG solution (3%).

(HN) functional form:[31]

$$\varepsilon^*(\omega) = \varepsilon' - i\varepsilon'' = -i\left(\frac{\sigma_c}{\varepsilon_0\omega}\right)^N + \sum_{k=1}^{n}\left[\frac{\Delta\varepsilon_k}{\left(1+(i\omega\tau_k)^{a_k}\right)^{b_k}} + \varepsilon_{\infty k}\right] \quad (1)$$

where ω is the angular frequency ($2\pi f$), ε_0 is the vacuum permittivity (8.85×10^{-12} F/m), σ_c is the conductivity, a and b are the shape parameters that define the breadth and the symmetry of the spectrum, respectively, and τ is the average relaxation time. The best results are obtained by setting the HN parameter $b = 1$ for both processes, implying a symmetry of the observed relaxations. That reduces the HN function to the Cole−Cole function.[32]

We next turn attention to the dielectric loss spectra with different PEG concentration (3%, 5%, and 10%). Figure 3 shows dielectric loss in the frequency domain for the aqueous solution of PEG at 213 K, with PEG concentration as a parameter. The increase in PEG concentration results in a significant decrease of LFP. Spectral deconvolution reveals that the dielectric relaxation strength of LFP decreases from 284 at PEG 3 wt % to 79 at PEG 10 wt %, while that of HFP increases from 115 at PEG 3 wt % to 120 at PEG 10 wt % at 213 K. These results are in agreement

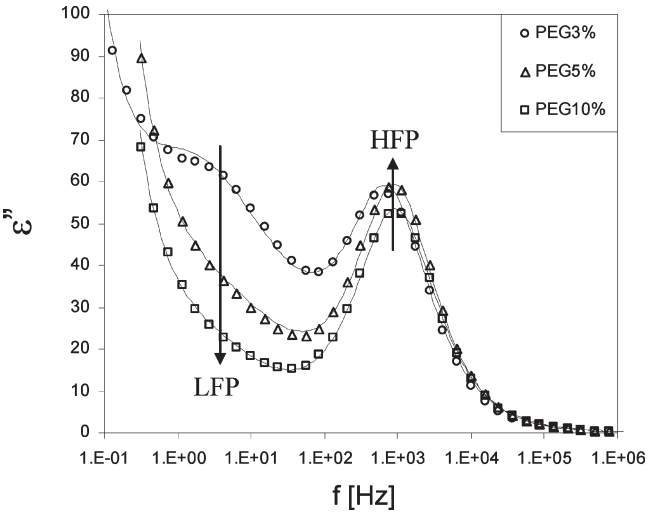


Figure 3. Dielectric loss in the frequency domain for the aqueous solution of PEG at 213 K with PEG concentration as a parameter.

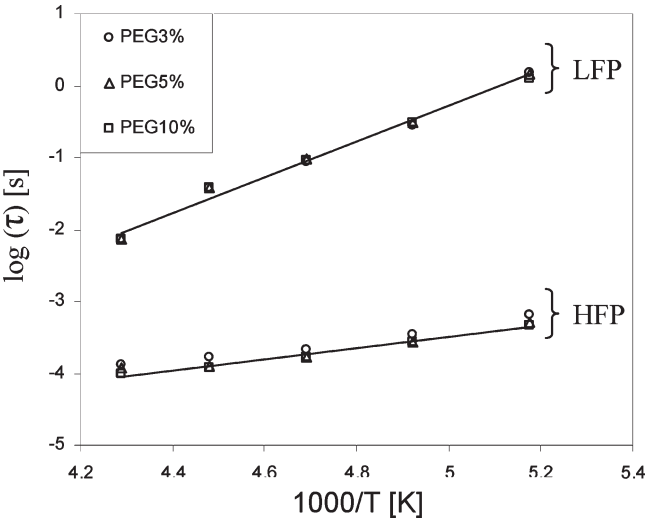


Figure 4. Temperature dependence of the average relaxation time for low- and high-frequency process for aqueous solution of PEG.

with the proposed origins of both processes since the content of ice decreases and that of bound water increases with increasing concentration of PEG, causing the dielectric relaxation strength of LFP to decrease and that of HFP to increase.

We examine next the temperature dependence of the average relaxation time for LFP and HFP. The average relaxation time for each process, obtained from HN fits, is shown in Figure 4. Both processes are Arrhenius-like with activation energy independent of PEG concentration. The LFP has activation energy of 52.2 kJ/mol. This value is similar to that previously reported by Auty and Cole[28] for pure ice. The HFP has an activation energy of 14.7 kJ/mol.

Dynamics of Aqueous Solutions of PEGylated Dendrimers. We proceed with the analysis of dynamics of aqueous solutions of PEGylated dendrimers. The real and imaginary part of dielectric permittivity in the frequency domain with temperature as a parameter for PEGylated dendrimers with eight PEG chains attached are shown in Figure 5. Aqueous solutions of PEGylated dendrimers are highly conductive, and relaxation due to pure ice is masked by high-conductivity or low-frequency dispersion such as hopping of charge carriers.[33] As a consequence, only bound water relaxation

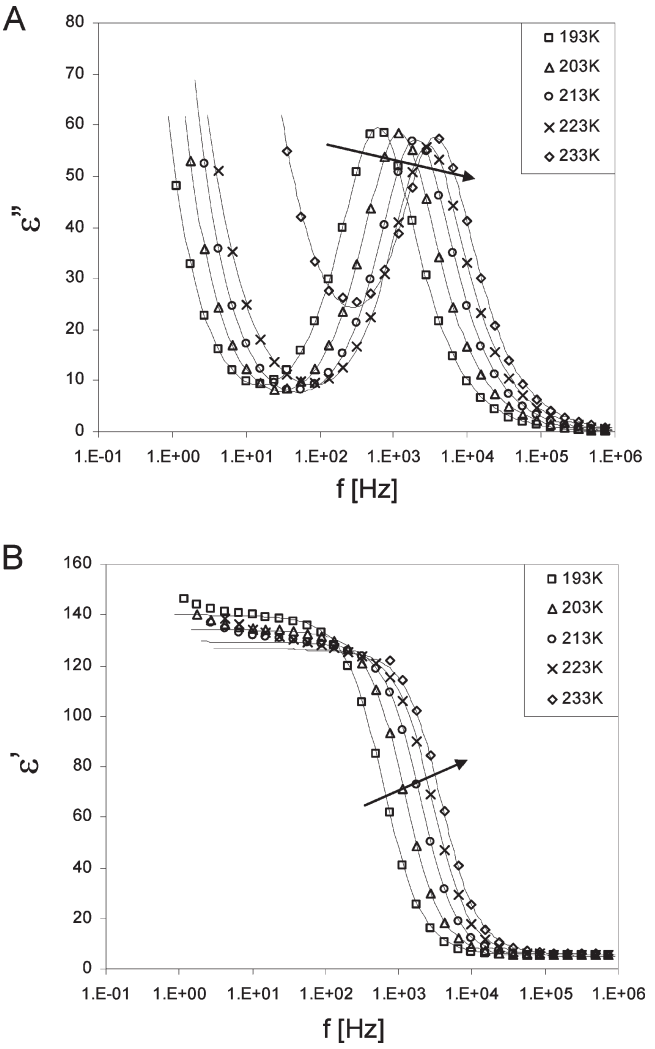


Figure 5. Dielectric permittivity (A, loss part; B, real part) in the frequency domain with temperature as a parameter for aqueous solution of P8.

(HFP) is observed. HFP shifts to higher frequency and decreases in intensity with increasing temperature, as in the aqueous solution of pure PEG. This process is further characterized by symmetric, Cole−Cole type relaxation spectra. The HN breadth parameter decreases slightly from 0.94 at 193 K to 0.90 at 233 K. The temperature dependence of the average relaxation time is Arrhenius-like with activation energy of 17.1 kJ/mol.

To probe the effect on dynamics of the average number of attached PEG chains in aqueous solution, we compare the results for PEGylated dendrimers with 8, 16, and 32 PEG chains. In Figure 6 we show dielectric loss in the frequency domain of aqueous solutions of PEGylated dendrimers with the number of attached PEG chains as a parameter at 213 K. All samples are at a concentration of 3 wt % of complex in the water. Interestingly, the average number of attached PEG chains does not have an effect on the dynamics of bound water. Intuitively, one would expect that an increase in the number of attached PEG chains would result in an increase in the amount of bound water and consequently the peak intensity. But our results do not show that. We attribute our results to the mushroom conformation of PEG chains. In this conformation, the amount of PEG exposed to water is independent of the number of attached chains, and hence the amount of bound water remains unchanged. However, the

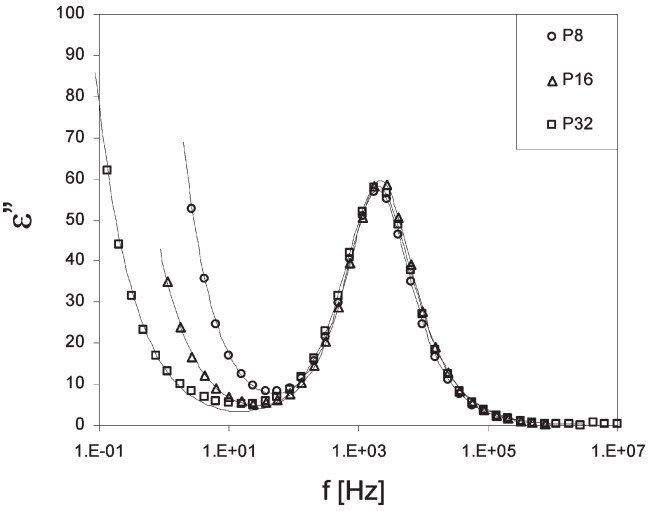


Figure 6. Dielectric loss in the frequency domain of aqueous solutions of PEGylated dendrimers with number of attached PEG as a parameter at 213 K.

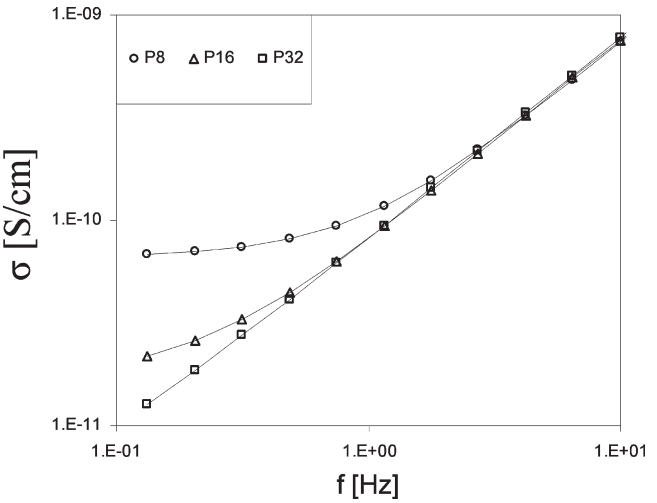


Figure 7. Conductivity in the frequency domain with number of attached PEG chains as a parameter.

average number of attached PEG chains affects conductivity, and that is also seen in Figure 6.

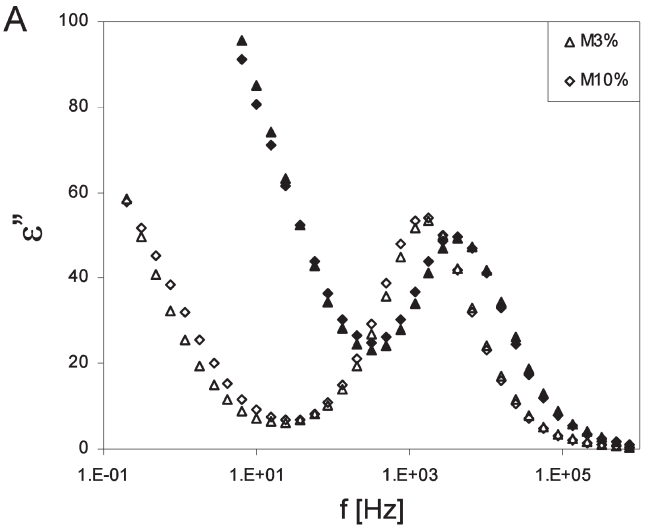
Ionic conductivity is defined by the following relationship:[34]

$$\sigma = \varepsilon'' \omega \varepsilon_0 \tag{2}$$

Figure 7 represents conductivity in the frequency domain with the average number of attached PEG chains as a parameter at 213 K. Conductivity is characterized by a frequency-independent region at low frequencies and, above some critical frequency (f_c), a frequency-dependent region. This is in agreement with previous studies which show that conductivity, $\sigma(\omega)$, is constant at low frequencies (at the dc value) but increases at higher frequencies according to a power law: $\sigma(\omega) \sim \omega^p$.[35−37] Hence, the frequency dependence of conductivity can be expressed as the sum of the dc component and the frequency power law terms according to[38,39]

$$\sigma(\omega) = \sigma_{dc} + \sum_i A_i \omega^{p_i} \tag{3}$$

where A_i is a temperature-dependent parameter in ith frequency domain and p_i is the frequency exponent in ith



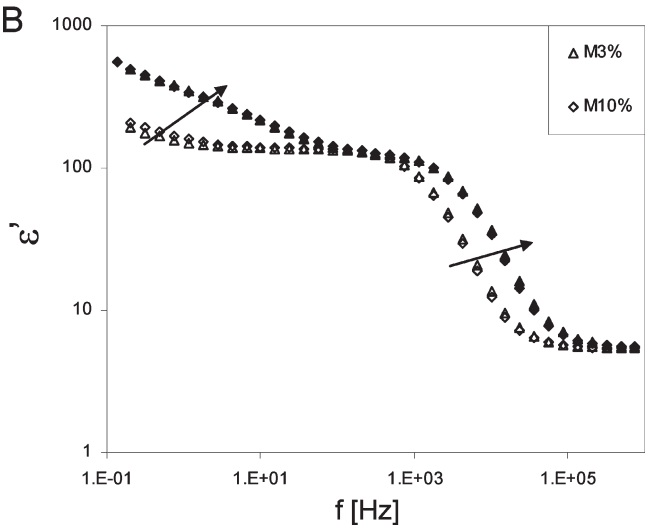


Figure 8. Dielectric loss (A) and permittivity (B) of aqueous solutions of methotrexate with concentration of methotrexate as a parameter at 203 K (open symbols) and 223 K (filled symbols).

frequency domain. The exponent p is obtained by fitting eq 3 for each frequency domain. For our samples, p lies between 0.95 and 1 and is an increasing function of the average number of attached PEG chains. Further, dc conductivity decreases with increasing average number of attached PEG chains, as shown in Figure 7.

Dynamics of Aqueous Solutions of Methotrexate. We continue by describing molecular dynamics of methotrexate in water. The effect of concentration (wt %) of methotrexate in aqueous solutions on the dielectric loss in the frequency domain at 203 and 223 K is presented in Figure 8A. As shown in this figure, dielectric spectra of methotrexate in aqueous solutions are also dominated by conductivity which masks the relaxation of ice. The lone process observed in dielectric loss spectra originates from the bound water around methotrexate, shifts to higher frequency, and decreases in intensity with increasing temperature. The process is of the Cole−Cole type. The temperature dependence of the average relaxation time follows the Arrhenius form with the corresponding activation energy of 20.0 kJ/mol.

It is well-known that conductivity contributes only to the dielectric loss. Because of that we show in Figure 8B, real permittivity in the frequency domain for two concentrations

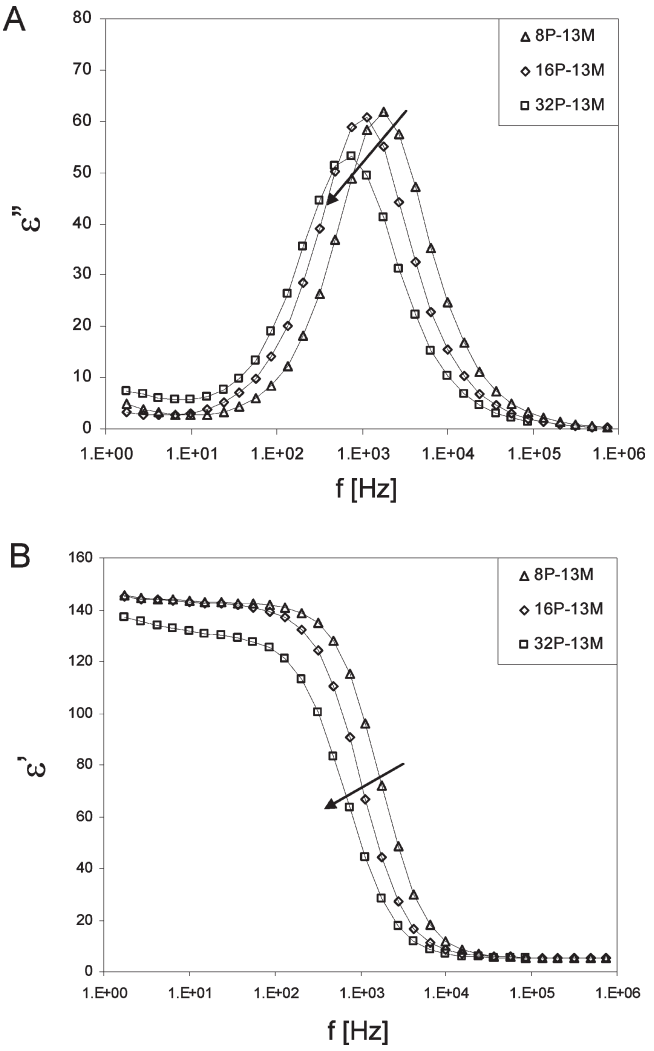


Figure 9. Dielectric loss (A) and permittivity (B) of aqueous solution of PEGylated and methotrexate-loaded dendrimers with number of attached PEG chains as a parameter at 203 K.

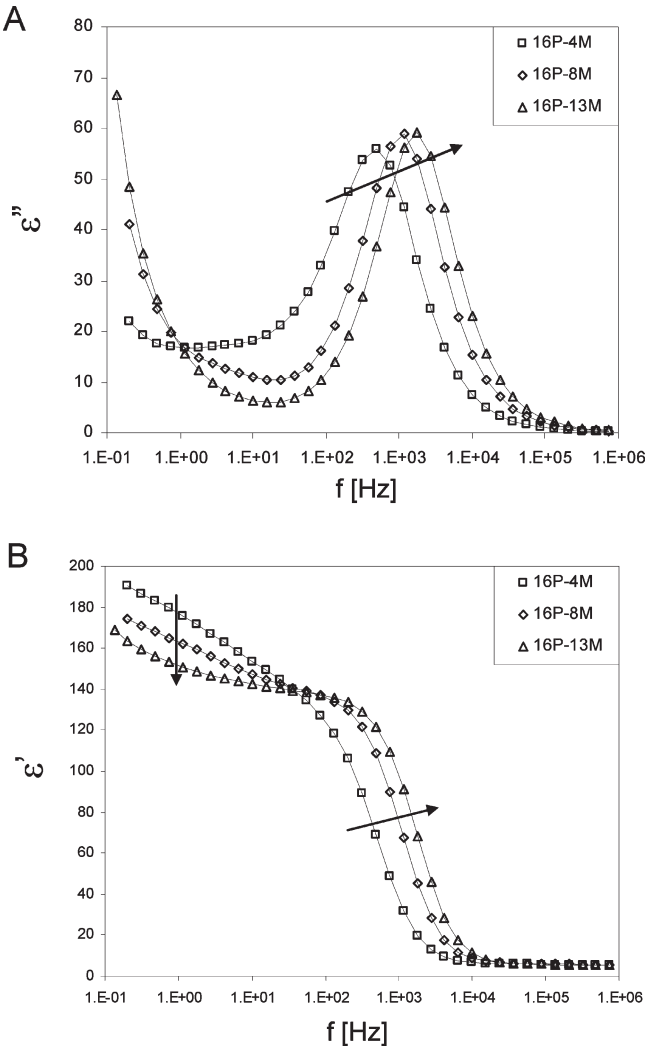


Figure 10. Dielectric loss (A) and permittivity (B) of aqueous solution of PEGylated and methotrexate-loaded dendrimers with number of encapsulated methotrexate as a parameter at 213 K.

of methotrexate in water at 203 and 223 K. Two processes are observed, and they are marked with arrows in the figure. Apart from the relaxation that stems from the movement of bound water around methotrexate, the relaxation of ice is also visible at low frequency.

The effect of methotrexate concentration can also be deduced from Figure 8. We observe no change in the time scale of relaxation, the peak intensity, or the conductivity as a function of methotrexate concentration.

Dynamics of Aqueous Solutions of PEGylated and Methotrexate-Loaded Dendrimers. We proceed with the discussion of the results for PEGylated and methotrexate-loaded dendrimers. In the text below we focus on the key parameters that define dynamics: real and imaginary part of dielectric permittivity, shape of the relaxation spectra, dielectric relaxation strength, average relaxation time, and their dependence on temperature, average number of attached PEG chain, and average number of encapsulated methotrexate. All investigated complexes described below are at concentration of 3 wt % in deionized water. We reiterate that in the sample code the number in front of the letter M represents the average number of encapsulated drug molecules.

Here we pose key questions about the nature of the complex systems composed of a solution of methotrexate-loaded PEGylated dendrimers and seek to provide answers. How are the system dynamics affected by the number of PEG chains? How does the number of loaded methotrexate molecules influence dynamics? What is the molecular origin of the dielectric response of methotrexate when loaded into PEGylated dendrimers? What parameters govern the relaxation response of these systems?

We begin by examining the effect on dynamics of the number of attached PEG chains. Dielectric permittivity and loss in the frequency domain at 203 K, with average number of attached PEG molecules as a parameter (8P, 16P, 32P), are shown in Figure 9. We observe only one process (HFP) that shifts to lower frequency and decreases in intensity with increasing average number of attached PEG molecules. Spectra generated at different temperatures (not presented here) show that HFP shifts to higher frequency and decreases in intensity with increasing temperature. It is important to note that the frequency dependence of HFP with respect to the average number of PEG attached to dendrimers changes significantly with the presence of methotrexate, clearly indicating an effect on the system's dynamics due to encapsulation.

The effect on dynamics of the average number of encapsulated methotrexate molecules (4M, 8M, 13M) is considered next. Figure 10 shows the real and imaginary dielectric permittivity with the number of encapsulated drug molecules

Table 2. Spectral Breadth Parameter as a Function of Temperature for HFP in PEGylated and Drug-Loaded Dendrimers

	T [K]				
code	193	203	213	223	233
8P-13M	0.94	0.93	0.93	0.92	0.91
16P-4M	0.98	0.93	0.89	0.89	0.86
16P-8M	0.97	0.95	0.95	0.92	0.90
16P-13M	0.96	0.95	0.94	0.93	0.91
32P-13M	0.90	0.90	0.89	0.89	0.89

as a parameter at 213 K. It is evident that the HFP process shifts to higher frequency and increases in intensity with increasing amount of drug. Interestingly, LFP, which is attributed to ice, decreases in intensity with increasing average number of encapsulated methotrexate molecules. A possible reason for this observation is that some water molecules inside the PEGylated dendrimers are substituted with methotrexate. As the content of methotrexate increases, the content of ice decreases, causing the dielectric relaxation strength, which is proportional to the height of loss peak at maximum, to decrease.

The shape of the relaxation spectra is determined by evaluating the HN parameters *a* and *b*, which describe the breadth and the symmetry of the spectrum, respectively. The NH symmetry parameter is independent of the average number of attached PEG chains and encapsulated methotrexate molecules and is constant at 1 for all temperature. Thus, the relaxation of bound water in all systems studied is characterized by the Cole−Cole functional form. The breadth parameter decreases with increasing temperature as shown in Table 2. This is not surprising because the same behavior was found for the individual components in aqueous solution. Furthermore, no apparent trend as a function of the average number of attached PEG chains or encapsulated methotrexate molecules is observed.

We next turn our attention to dielectric relaxation strength ($\Delta\varepsilon$). This parameter is defined by the following relationship: $\Delta\varepsilon = \varepsilon'(0) - \varepsilon'(\infty)$, where $\varepsilon'(0)$ and $\varepsilon'(\infty)$ represent the limiting low- and high-frequency dielectric permittivity, respectively. $\Delta\varepsilon$ is proportional to concentration of dipoles and mean-squared dipole moment per molecule. The effect of temperature, average number of attached PEG chains, and average number of encapsulated methotrexate molecules was studied, and the results are plotted in Figure 11. This figure depicts $\Delta\varepsilon$ as a function of temperature with the average number of attached PEG chains and the number of encapsulated methotrexate molecules as variables. An examination of trends in $\Delta\varepsilon$ leads to the following observations. First, $\Delta\varepsilon$ decreases with increasing temperature for all systems. This is expected because the same behavior for HFP was observed for methotrexate and PEGylated dendrimers in aqueous solution. Second, with increasing average number of attached PEG chains, dielectric relaxation strength decreases. And third, with increasing average number of encapsulated methotrexate molecules, $\Delta\varepsilon$ increases. To explain the $\Delta\varepsilon$ dependence on PEG and methotrexate, we calculated weight percent of PEG and methotrexate in the complex, and these results are shown in Table 3.

From Figure 11 and Table 3, we conclude that dielectric relaxation strength increases with increasing wt % of methotrexate in the complex, i.e., decreases with increasing wt % of PEG. This signifies that dipoles from bound water around methotrexate and around PEG contribute to this process.

Finally, the average relaxation time obtained from the HN fit is examined, and the results are plotted versus reciprocal temperature in Figure 12. Note that the relaxation time of HFP increases with increasing number of attached PEG chains

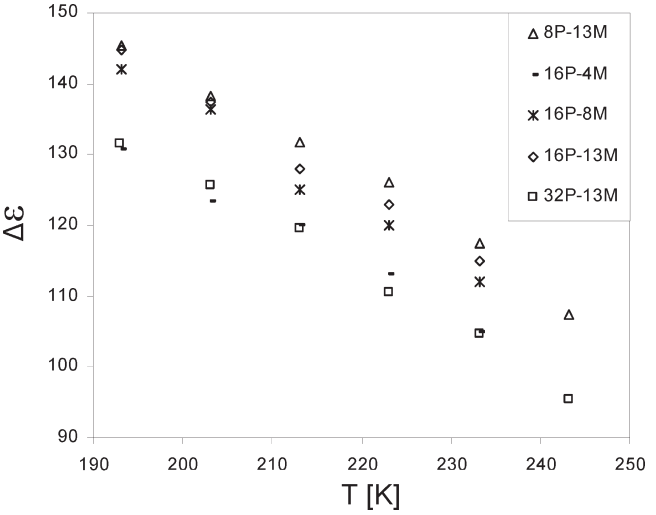


Figure 11. Dielectric relaxation strength as a function of temperature with the average number of attached PEG chains and number of encapsulated methotrexate as a parameter.

Table 3. Weight Percent of Methorexate and PEG in Complex

code	wt % (methotrexate)	wt % (PEG)
8P-13M	20	57
16P-4M	4	79
16P-8M	8	76
16P-13M	13	72
32P-13M	7	84

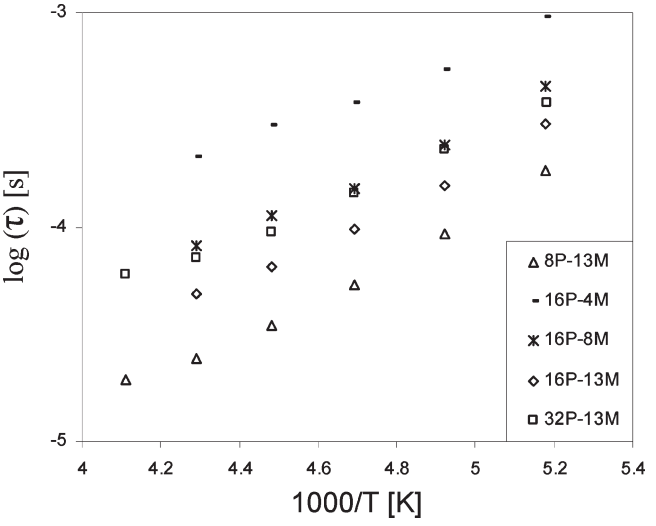


Figure 12. Average relaxation time as a function of reciprocal temperature for aqueous solutions of PEGylated and methotrexate-loaded dendrimers.

and decreasing number of encapsulated methotrexate molecules. An explanation for this behavior lies in the weight percent of methotrexate present in the complex. As the weight percent of the drug increases, the time scale of the process decreases. From the relaxation time and dielectric relaxation strength dependence on the weight percent of methotrexate and PEG in the complex, we can say that the observed relaxation in PEGylated and methotrexate-loaded dendrimers presents a combination of processes that encompass relaxations of bound water around methotrexate and around PEG.

The data in Figure 12 are fitted to the Arrhenius equation, and the calculated activation energies are summarized in Table 4. One important observation is that the activation

Table 4. Activation Energies for HFP

code	E_a [kJ/mol]	wt % (methotrexate)	wt % (PEG)
8P-13M	19.3	20	57
16P-4M	13.7	4	79
16P-8M	15.8	8	76
16P-13M	17.0	13	72
32P-13M	14.7	7	84
P	14.7	0	100
M	20.0	100	0

energy of HFP increases with increasing weight percent of methotrexate. It is also interesting to note that the values of activation energy for HFP in PEGylated and drug-loaded dendrimers lie between those for HFP in aqueous solutions of PEG and methotrexate. This further confirms that HFP represents a combination of different bound water processes.

Based on the fact that the observed HFP in PEGylated and methotrexate-loaded dendrimers originates from the bound water around methotrexate and PEG, our results suggest that methotrexate molecules encapsulated in PEGylated dendrimers carry bound water molecules.

Conclusions

We have completed an investigation of the dielectric properties of aqueous solutions of PEG, PEGylated dendrimers, methotrexate, and PEGylated and methotrexate-loaded dendrimers. Dynamics were studied by dielectric relaxation spectroscopy (DRS) over the frequency range from 10^{-1} to 10^{6} Hz and at temperatures from 193 to 243 K. The major conclusions are as follows.

Aqueous solutions of PEG are characterized with two dielectric dispersions. The lower frequency process (LFP) is attributed to the motions of ice, while the higher frequency process (HFP) originates from the bound water around PEG. Both processes are Arrhenius-like with activation energy independent of PEG concentration.

Solutions of PEGylated dendrimers in water are highly conductive, and only bound water relaxation is observed in these systems. The average number of attached PEG chains does not have a significant effect on the dynamics of bound water. This observation is credited to the mushroom conformation of PEG chains. Nonetheless, this number was found to affect conductivity, which decreases with increasing average number of attached PEG chains.

The effects of the average number of attached PEG chains and the average number of encapsulated methotrexate molecules on the dynamics of PEGylated and drug-loaded dendrimers yielded interesting observations regarding the dielectric relaxation strength and the time scale of the HFP. The dielectric relaxation strength increases, while relaxation time decreases with decreasing average number of attached PEG chains and increasing average number of encapsulated methotrexate molecules. By considering the weight fraction of the components in the complex, we were able to conclude that the observed relaxation in the PEGylated and methotrexate-loaded dendrimers presents a combination of relaxation processes due to water bound to methotrexate and PEG. The value of activation energy for HFP in PEGylated and drug-loaded dendrimers lies between those for HFP in aqueous solution of PEG and methotrexate. This further confirms that HFP represents a combination of relaxations in bound water. The implication is that methotrexate molecules encapsulated in PEGylated dendrimers carry bound water molecules.

Acknowledgment. This material is based on work supported by National Science Foundation under Grant DMR-0346435.

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