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Investigation of the Hydration of Nonfouling Material Poly(ethylene glycol) by Low-Field Nuclear Magnetic Resonance

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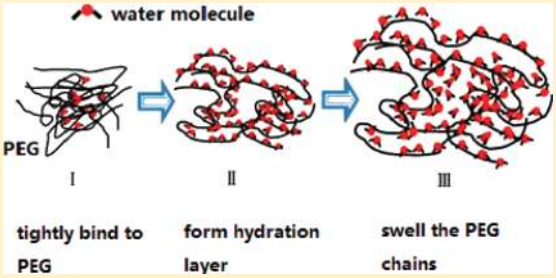
ABSTRACT: The strong surface hydration layer of nonfouling materials plays a key role in their resistance to nonspecific protein adsorption. Poly(ethylene glycol) (PEG) is an effective example of materials that can resist nonspecific protein adsorption and cell adhesion. Thus, the strong interaction between water molecules and PEG was investigated through each T_2 component in water/PEG mixtures using multiexponential inversion of T_2 relaxation time measured by the Carr−Purcell−Meiboom−Gill (CPMG) sequence of low-field nuclear magnetic resonance (LF-NMR). Results show that about one water molecule is tightly bound with one ethylene glycol (EG) unit, and additional water molecules over 1:1 ratio mainly swell the PEG matrix and are not tightly bound with PEG. This result was also supported by the endothermic behavior of water/PEG mixtures measured by differential scanning calorimetry (DSC). It is believed that the method developed could be also applied to investigate various interactions between macromolecules and other small molecules without using deuterium samples, which might open a novel route to quantitatively measure guest−host interactions in the future.



INTRODUCTION

It is becoming more and more important to prevent protein adsorption on surfaces, as nonfouling materials show great potential in applications such as drug delivery, surface coatings, boat hulls,[1−3] and so forth. Thus, it is essential to understand the mechanism of the protein resistance for developing new materials with superlow fouling as well as biocompatibility.[4−6] It is commonly believed that a strong surface hydration layer formed on the nonfouling materials generates the net repulsive force on proteins;[7−12] for example, water molecules could form a hydration layer on poly(ethylene glycol) (PEG) chains through hydrogen bonds while the rest of the water molecules are in a bulk-like phase.[7,13,14] In recent years, many efforts have been made to investigate those tightly bound water molecules around materials by various methods: ^{2}H NMR,[15] X-ray adsorption spectra,[16,17] attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR),[13] molecular simulation,[7,18] and differential scanning calorimetry (DSC).[14] Since the amount of tightly bound water molecules might be an important criterion for not only evaluating the resistance of nonfouling materials against protein adsorption but also understanding the mechanism of nonfouling properties, it is worth investigating further. However, the amount of tightly bound water molecules on nonfouling materials is still unclear because the information obtained by these methods is always mixed with other properties. For example, when the amount of interfacial water molecules on phosphorylcholine (PC) groups was estimated by DSC,[14] the results relied on two assumptions: (1) the cold crystallizable water is composed of freezable water (bulk water or bulk-like water in hydrogel that is able to freeze) and nonfreezable water (tightly bound water to the polymers that is not able to freeze) and (2) the heat of fusion of the freezable water is equal to that of pure water (334 J g^{-1}) at 0 °C. However, these assumptions might cause systematic error due to the high concentration of strong hydration groups (PC or PEGs) in hydrogels and the difference of freezing/melting temperature regimes. Thus, it is observed that there are ∼23 nonfreezable water molecules on one PC group by DSC,[14] which is far more than the average eight bound water molecules on one sulfobetaine (SB) group by molecular simulation.[18] Such a big difference might indicate the entropic effect observed by DSC due to water molecules adsorbed on two kinds of zwitterionic groups with different hydrophobicity. This exemplifies why it is necessary to investigate the hydration of nonfouling materials accurately in order to understand the mechanism behind nonfouling properties.

NMR spectroscopy is a powerful technique for investigating the structure, mobility and hydration properties of various polymeric systems, which is the most direct approach to evaluate the mobility changes of water molecules on polymer hydration layer.[19−23] In solid/liquid coexisting systems, Zimmerman and Brittin[24] investigated the fast-exchange relationship to explain the water molecules in different statuses characterized by different T_2 relaxation time components, of which each T_2 component reflects the mobility and anisotropy[25−30] of a subpopulation of water and its exchange

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with the other water populations. With the quick development of LF-NMR and the T_2 inversion method for analyzing multicomponent T_2 relaxation times through the Carr–Purcell–Meiboom–Gill (CPMG) sequence, a simple way to quantify the amount of small molecules in different environments/or statuses is developed. However, few works have been done in polymeric solution because the signal from free polymer may interfere with the signal of water molecules. Thus, it is necessary to develop a systematic method to investigate the interaction between water molecules and PEG without the influence from protons of the PEG.

Various molecular weight (MW) PEG polymers[31,32] have been widely used to resist protein adsorption due to their excellent nonfouling capability.[33] Currently, the nonfouling property of PEG is often explained by the "steric repulsion model",[34] which only fits long PEG chains, or by the "water barrier mechanism",[2,35−38] which suggests that a layer of tightly bound water molecules around PEG chains via hydrogen bonds might play a key role for the nonfouling property. Therefore, it is vital to characterize both the details of water molecules around PEG chains and the statuses of PEG chains for understanding the mechanism of nonfouling materials. In this work, multicomponent T_2 relaxation times of water molecules in different environments at a sequence of hydration levels of PEG were investigated by applying the T_2 inversion method. Additionally, the statuses of water and PEG molecules represented by each T_2 component are discussed.

MATERIALS AND METHODS

PEG2000 (Polyethylene glycol, 2000 Dalton) were purchased from Aladdin Reagent (China). PEG20000 and PEG200000 were purchased from J&K Chemical. D_2O (99.9% atom% D) used in experiments was purchased from J&K Chemical. Water used in experiments was purified using a Millipore water purification system with a minimum resistivity of 18.0 MΩ·cm.

Sample Preparation. *Deionized (DI) H_2O.* Different amounts of DI water from 200 to 1400 μL were added into each glass tube (φ15 mm).

Polymer in Different Amounts of D_2O or H_2O. The polymer (PEG2000, PEG20000, and PEG200000) water mixtures were prepared by the same procedure. In brief, 0.44 g of polymer with varied amounts of D_2O (99.9%) or H_2O was added into each glass tube (φ15 mm). The amounts of D_2O or H_2O added were 79.2, 118.8, 158.4, 178.2, 198, 237.6, 277.2, 316.8, 356.4, 396, 435.6, and 475.2 μL, respectively. The samples were sonicated for 1 h to allow complete mixing and then were incubated overnight in an oven at 32 °C to reach equilibrium.

NMR Spin–Spin Relaxation (T_2) Measurements. NMR relaxation measurements were performed on a Niumag Bench-Top Pulsed NMR Analyzer PQ001 (Niumag Electric Corporation, Shang, China) operating at a resonance frequency for protons of 21 MHz (0.5T). Samples were inserted into the NMR probe. Spin–spin relaxation time (T_2) was measured using the CPMG sequence.[39,40] The maximum point of every second echo was accumulated using a 90° pulse of 17 μs and a 180° pulse of 34 μs. The delay between the 180° pulses, τ, was 500 μs. The amplitude of every second echo was measured, a total of 18 000 points were collected, and scans were coded using a repetition time (TR) of 3 s. The relaxation measurements were performed at 32 °C.

With regard to almost all samples, the relaxation signal can often be expressed by multiexponential form. In this paper, the spin–spin T_2 relaxation time inversion spectrum was performed using the equation below:[24]

$$M(t) = \sum_i P_i \exp\left(-\frac{t}{T_{2i}}\right) \quad (1)$$

Here P_i is the signal intensity of the i component of which spin–spin relaxation time is T_{2i}.

DSC Measurement. The polymer–water mixtures in 1:2, 1:1, and 2:1 ratios of EG units to H_2O molecules were characterized in order to compare with the results obtained by the NMR method. Polymer without water was used as a control sample.

Samples from 6 to 9 mg were placed in an aluminum pan, and then the pan was hermetically sealed. An empty aluminum pan was used as the control. Measurements were performed using TA Q200. During the cooling and heating experiments, the sample cell was purged with nitrogen gas at a flow rate of 50 mL/min. The samples were initially cooled from room temperature to −40 °C at a rate of 5 °C/min and then heated to 70 °C at the same rate.

RESULTS AND DISCUSSION

T_2 Relaxation Time Inversion Spectra of DI H_2O. To discuss the mobility of water molecules upon addition to materials, it is necessary for us to investigate the properties of DI water by low-field NMR (LF-NMR). The protons of DI water were observed in only one status since it is a homogeneous single content. As seen in Figure 1, the inversion spectra of the T_2 relaxation time of DI water show only one peak, which occur at the same T_2 relaxation time of 2877.5 ms. Figure 2 shows the integrated signal intensity of the inversion spectra versus varied amounts of water. The integrated signal intensity is linear with the amount of water and can directly represent all the proton numbers in each sample. The degree of fitting is 0.999, indicating that the inversion algorithm for T_2 relaxation time is very reliable and accurate.

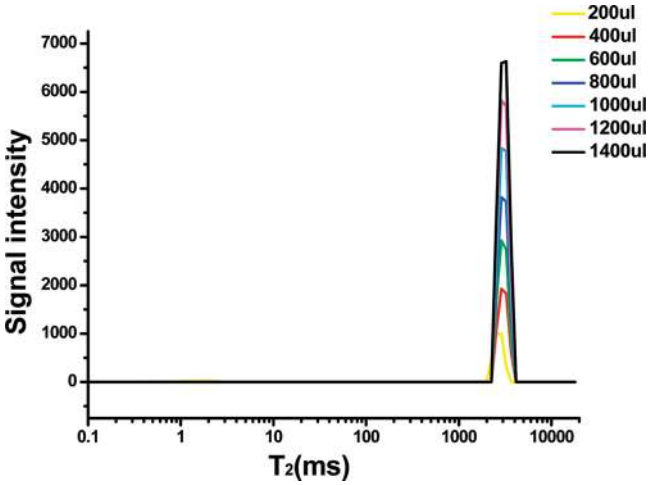

Figure 1. T_2 inversion spectra (signal intensity versus corresponding relaxation time) for varied amounts of DI H_2O from 200 μL to 1400 μL. The signal intensity represents the number of protons in the corresponding relaxation time.

T_2 Relaxation Time Inversion Spectra of PEG with Different Amounts of D_2O and H_2O. In contrast to only one peak appearing for the DI H_2O (Figure 1), two key peaks appear when different amounts of water were added to each PEG sample (Figure 3). The total signal of the second peak gradually increases with the increasing number of H_2O

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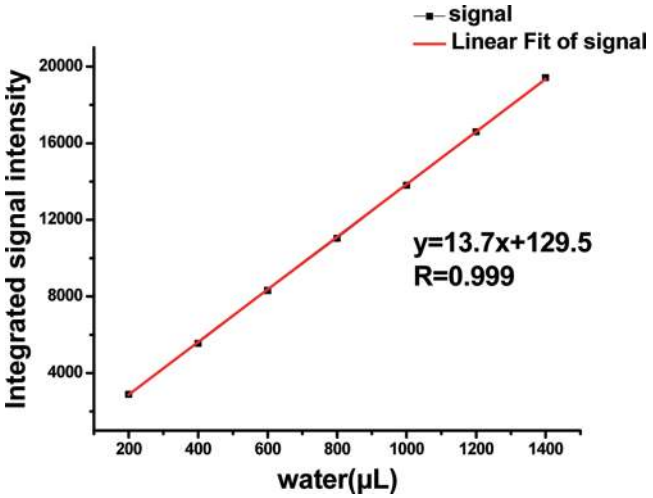

Figure 2. The integrated signal intensity of water peak in the inversion spectrum versus the amount of DI H_2O.

molecules, while the total signal of the first peak stops increasing after the water/polymer molar ratio reaches 1.56:1. It is clear that the second peak belongs to the water molecules since the first big peak is given by PEG in solution when the H_2O is replaced by D_2O (Figure 4). Beside the PEG peak, there is a small peak occurring at the same location of the second peak in Figure 3, which mainly comes from the small amount of water left in the PEG samples and also the signals from the free segments of PEGs when the amount of D_2O increases. Thus, these two key peaks belong to PEG and water molecules, respectively.

In addition, the total signal of the water peak in Figure 3 linearly increases with water amount (Figure 5) and slightly deviates from the area of DI water when the molar ratios of H_2O/EG are higher than 1.56. It is believed that such deviations are a result of the deconvolution method used for multiple components in our system. For example, the deviation of the water signal of the PEG2000 solution is much larger than that of the PEG200000 solution since the interference of proton signals between PEG2000 and the water molecules occurs more easily than that between the larger PEG200000 and water molecules in deconvolution. In conclusion, the deconvolution method is rather reliable for further data analysis.

To detect the different statuses of the hydrated water on PEGs, the T_2 relaxation time of both PEGs and H_2O are fully investigated since the T_2 relaxation times strongly depend on the mobility of the molecules involved, especially on their rotational motion. First, there is a clear difference between T_2 components of PEG and water molecules (Figure 6) from Figure 3, especially for high MW PEG. This clear difference ensures the accuracy of T_2 components of water molecules. Second, the T_2 components of three MW PEG polymers are in the order $T_{2\ \mathrm{PEG2000}} > T_{2\ \mathrm{PEG20000}} > T_{2\ \mathrm{PEG200000}}$ at the same H_2O/EG ratio. This order clearly shows the high mobility of smaller PEG molecules. The T_2 components of water molecules associated with three different MW PEG polymers are in the order $T_{2\ \mathrm{H2O/PEG20000}} > T_{2\ \mathrm{H2O/PEG2000}} > T_{2\ \mathrm{H2O/PEG200000}}$. The value for $T_{2\ \mathrm{H2O/PEG2000}}$ being smaller than $T_{2\ \mathrm{H2O/PEG20000}}$ might be caused by more tightly bound water molecules to the small MW PEG molecules. Such a reverse in capability of hydration was also observed by other experiments.[41] Third, all T_2 components of PEG polymers and water molecules rise with the increase of the H_2O/EG molar ratio. After all, those water molecules and polymer molecules in lower PEG concentrations show longer T_2 components, and the bound water molecules are slowed down by PEG molecules. These results indicate that these T_2 components of PEG polymers and water molecules obtained from the multiexponential inversion algorithm for T_2 relaxation time through the CPMG sequence well reflect the mobility of the protons in different environments.

Quantify Hydrated Water Molecules on EG Units. When water is added to PEG, partial water molecules will be tightly bound to PEG polymers through hydrogen bonds, and

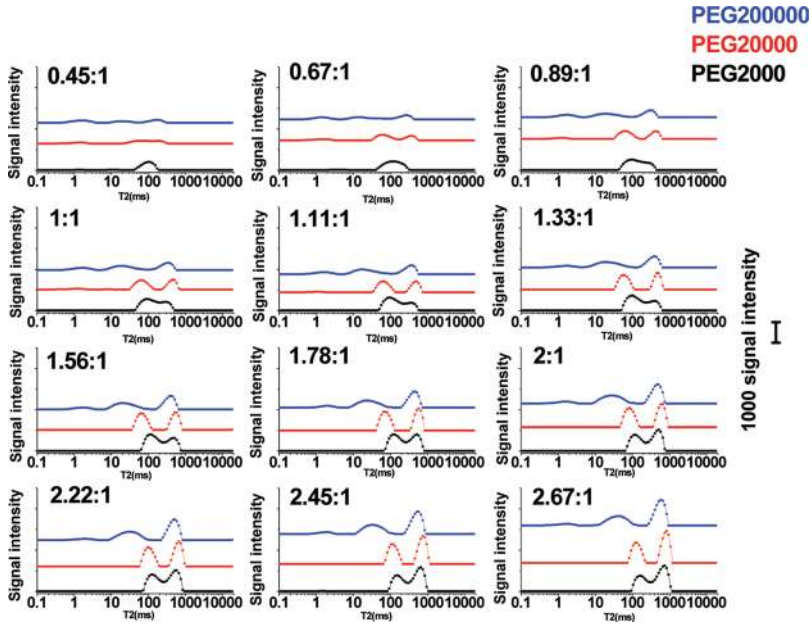

Figure 3. T_2 inversion spectra (signal intensity versus corresponding relaxation time) for H_2O/PEG mixtures. The N_w, which is the number of added water molecules per EG unit, increases from 0.45:1 to 2.67:1.

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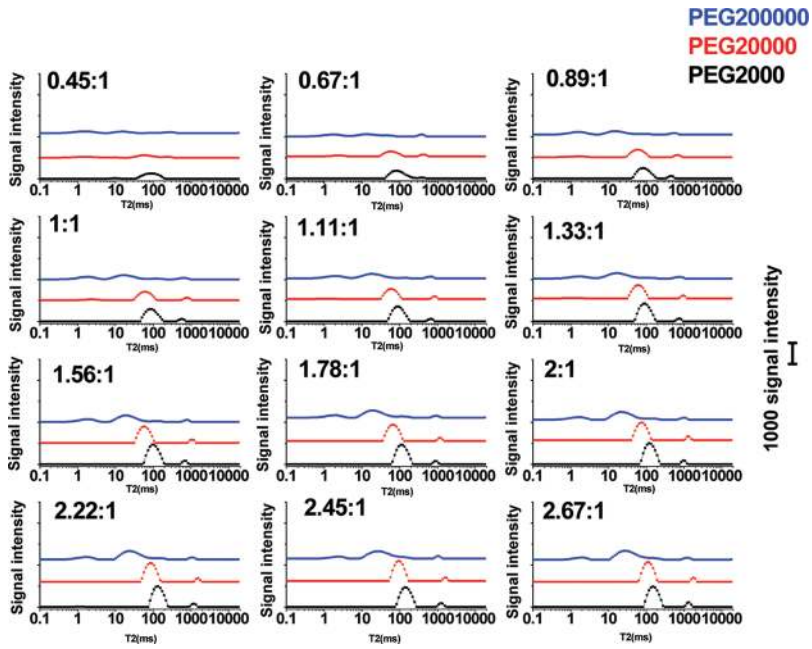

Figure 4. T_2 inversion spectra (signal intensity versus corresponding relaxation time) for D_2O/PEG mixtures. The N_w, which is the number of added D_2O water molecules per EG unit, increases from 0.45:1 to 2.67:1.

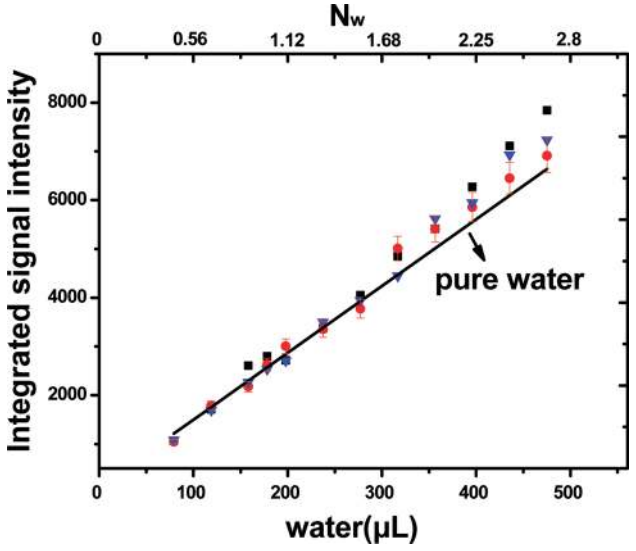

Figure 5. The integrated signal intensity of the water component in the inversion spectra of H_2O/PEG (PEG2000 ■, PEG20000 ●, and PEG200000 ▼) mixtures versus different amounts of water and N_w. Error bars on PEG20000 are typical ones.

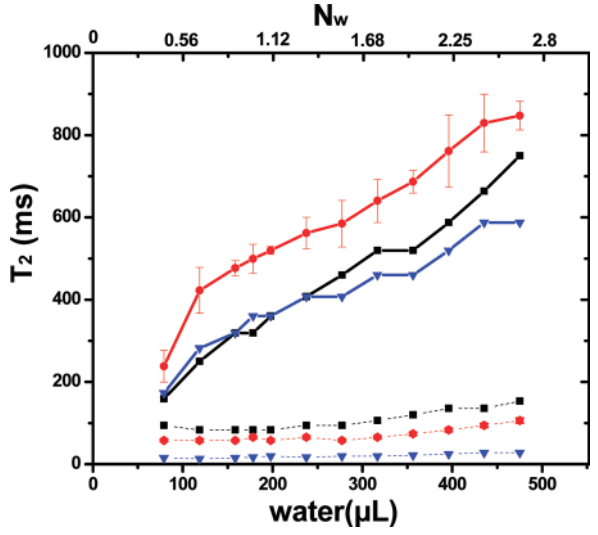

Figure 6. The peak values of T_2 inversion spectra of the polymer (dashed line) component and water (solid line) component in H_2O/PEG (PEG2000 ■, PEG20000 ●, PEG200000 ▼) mixtures with varied amounts of water. Error bars on PEG20000 are typical ones.

the fluctuations of water molecules consequently slow down, which cause the bound water molecules to have a shorter T_2 than bulk water. Thus, there are two types of water molecules described as "bound" and "free" (or unbound) water when water molecules come into contact with solid surfaces or polymer hydrogel matrices.[23,42−45] These two kinds of water proton relaxation times can be described in terms of two T_2 components, which represent two kinds of mobile water molecules in different environments when the fast-exchange model is used.[21,42] Thus, the T_2 components of water molecules can be described by the following equation:

$$\frac{1}{T_2} = \frac{m_{bw}}{m_w T_{2b}} + \frac{m_{fw}}{m_w T_{2f}} = \frac{1}{T_{2f}} + \frac{m_{bw}}{m_w}\left(\frac{1}{T_{2b}} - \frac{1}{T_{2f}}\right) \quad (2)$$

Here $m_{fw} = m_w - m_{bw}$.

T_{2b} is the short proton relaxation time of bound water, T_{2f} is the long proton relaxation time of "free" water, m_{bw} is the mass of bound water to PEG, m_{fw} is the mass of free water within the PEG−water mixture, and m_w is the total mass of added water. In fact, T_{2b} and T_{2f} are the intrinsic relaxation times of the bound and free water components, respectively. T_{2f} is equal to 2877.5 ms measured from bulk water by LF-NMR.

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From eq 2, the $1/T_2$ observed in experiment is mainly determined by m_{bw}/m_w. According to Baumgartner and Blinc's work,[21,23] it is found that the bound water fraction is proportional to the polymer concentration $m_p/(m_w + m_p)$ after the binding sties of polymer chains are close to saturated. Thus, $1/T_2$ should be proportional to the polymer concentration $m_p/(m_w + m_p)$ within a certain range of PEG concentrations, mainly in the range of PEG almost saturated by water molecules. In fact, after the $m_p/(m_w + m_p)$ value is below 0.7 or the H_2O/EG ratio is above 1.06, the $1/T_2$ versus $m_p/(m_w + m_p)$ shows a linear relationship in Figure 7, and the

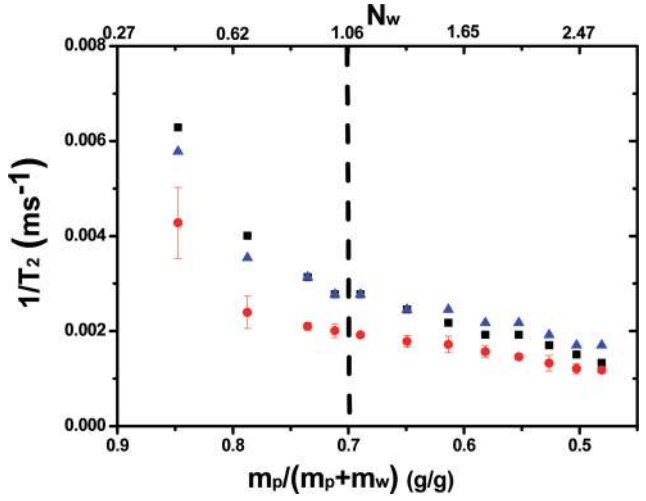

Figure 7. The reciprocal of T_2 of the water component in the inversion spectrum, $1/T_2$ (ms^{-1}), of water/PEG (PEG2000 ■, PEG20000 ●, PEG200000 ▼) mixtures versus polymer concentration ($m_p/(m_w + m_p)$ (g/g)). Error bars on PEG20000 are typical ones.

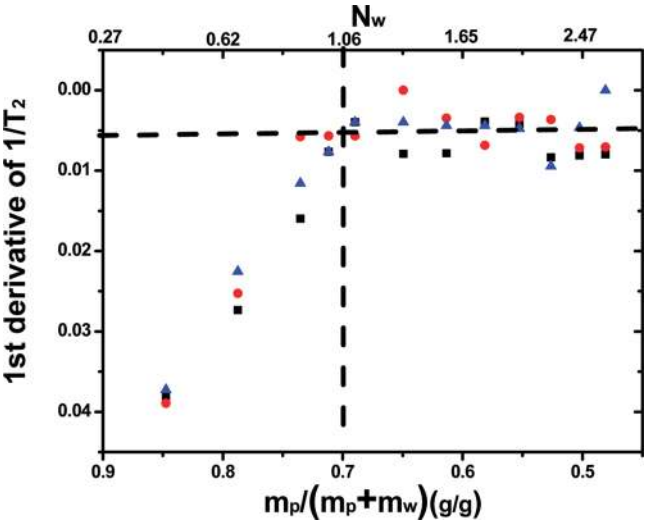

Figure 8. First derivative of $1/T_2$ (ms^{-1}) of water/PEG (PEG2000 ■, PEG20000 ●, PEG200000 ▼) mixtures to polymer concentration ($m_p/(m_w + m_p)$ (g/g)).

first derivative of $1/T_2$ versus $m_p/(m_w + m_p)$ in Figure 8 shows a constant value. This indicates that the surfaces of PEG chains are close to being saturated by water molecules when the H_2O/EG ratio reaches around 1.06. Moreover, $1/T_2$ deviates from the linear relationship when the H_2O/EG ratio is below 1.06. This is mainly caused by very low concentrations of free water molecules at high PEG concentrations, and the value of $1/T_2$ is controlled by the first term, m_b/m_wT_{2b} in eq 2, which also suggests that the association constant between water molecules and PEG chains is rather high. After all, the cross area of the two dashed lines in Figure 8 shows a rough turning range, which indicates that the maximum capacity of bound water molecules to PEG chains is about 1.06 H_2O per EG unit. This value agrees well with the result from computer simulations. The two oxygen atoms on both sides of the EG unit could form two H-bonds with one water molecule.

To determine the accuracy of the above estimation, the trend of m_{bw} change versus the water/EG ratio is examined by evaluating the number of bound water molecules per EG unit (N_{bw}). The change of N_{bw} is determined by $N_w\cdot(1/T_2 - 1/T_{2f})$ from eq 3, as both T_{2b} and T_{2f} are constants and are defined as the intrinsic relaxation times of the bound and unbound water. As shown in Figure 9, the $N_w\cdot(1/T_2 - 1/T_{2f})$ values rapidly rise

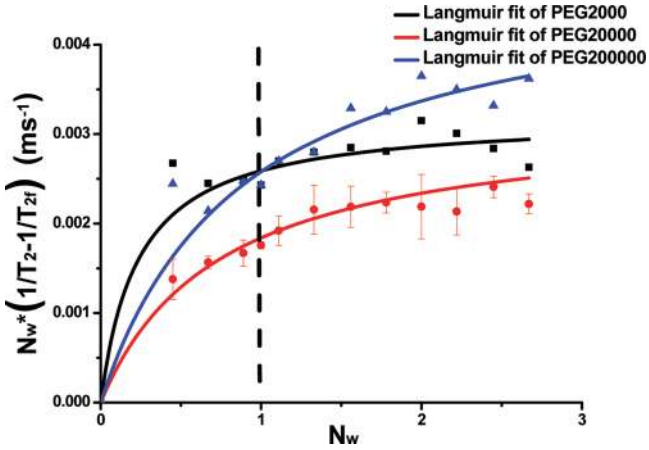

Figure 9. The value of $N_w\cdot(1/T_2 - 1/T_{2f})$ as a function of N_w (PEG2000 ■, PEG20000 ●, PEG200000 ▼). The first point of N_w at 0.45:1 deviates from the Langmuir–Blodgett fit, which is mainly caused by the error measured by LF-NMR due to rather small amounts of free water molecules in very large amounts of PEG. Error bars on PEG20000 are typical ones.

with the increase of amount of water added and level off after the number of total water molecules per EG unit (N_w) reaches around 1. Furthermore, the change in N_{bw} fits well with the Langmuir–Blodgett adsorption equation. In fact, the change in N_{bw} reflects the change in θ since m_{bw} is equal to $m_s{*}\theta$, where m_s is a constant for a saturate water mass of the given amount of PEG, and θ is the ratio of surface coverage. Thus, the flat range over $N_w = 1$ shows that the amount of water on PEGs is close to the saturated value. It is reasonable to believe that all the bonding sites of polymer chains were saturated after N_w exceeds 1. This result agrees well with the result obtained by the first method.

$$N_w\cdot\frac{\left(\frac{1}{T_2} - \frac{1}{T_{2f}}\right)}{\left(\frac{1}{T_{2b}} - \frac{1}{T_{2f}}\right)} = N_{bw} \quad (3)$$

Here $N_w = m_wM_p/m_pM_w$.

M_p is the molecular weight of the EG unit and M_w is the molecular weight of water.

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In addition, the maximum hydration capacity of the EG unit detected by NMR could be supported by typical DSC heating thermograms of PEG20000 with different water contents (Figure 10), which are above (H_2O:EG = 2:1), around (1:1),

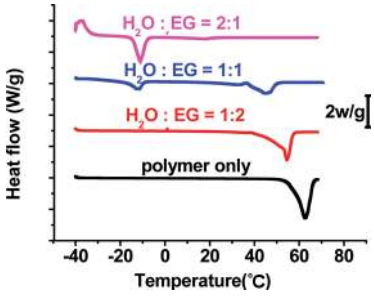

Figure 10. DSC thermo-grams of water/PEG20000 mixtures at a heating rate of 5 °C/min for different water contents. The values indicate the molar ratios of H_2O:EG.

and below (0.5:1) the saturated value. Also, PEG20000 without water was chosen for comparison. It is observed that two major peaks occur during the heating experiments, which are close to the ice-to-water transition and the solid-to-liquid transition of PEG20000, respectively. Furthermore, there is no endothermic peak observed near the phase transition of PEG20000 when the H_2O:EG ratio is 2:1. This suggests that each molecule of PEG20000 is surrounded by freezable water molecules, and the endothermic behavior is controlled mainly by water. However, when the H_2O:EG ratio was lowered to around saturated value (1:1), both endothermic peaks of the phase transition of water and PEG20000 were observed simultaneously. This indicates that low hydrated PEG20000 keeps partial endothermic behavior of polymer and freezable water molecules in the water/PEG20000 mixture. The endothermic peak near the phase transition of PEG20000 should be caused by the melting of water/PEG20000 complexes. In contrast, when the H_2O:EG ratio was lowered to 0.5:1, the phase transition of freezable water molecules disappeared and only the endothermic behavior of water/PEG20000 complexes occurred. This indicates no freezable water molecules in the water/PEG20000 mixture at a H_2O:EG ratio of 0.5:1, suggesting that most water in this water/PEG20000 mixture is so-called nonfreezable water, [14,42] and a H_2O:EG ratio of 0.5:1 is the lowest estimated value of tightly bound water molecules to PEGs. It is also believed that this kind of nonfreezable water is caused by tightly bound water molecules to PEGs via hydrogen bonds. Thus, the turning point of different phase transition behaviors of water/PEG20000 mixtures is between H_2O:EG ratios of 0.5:1 and 1:1. In conclusion, these heating thermo-grams of water/PEG20000 mixtures give a similar range of H_2O:EG ratios to form saturated hydration layers on PEG20000, which is consistent with the results obtained by the NMR method.

The Mobility of PEG Chains. The increasing pattern of total signals of D_2O/PEG mixtures also provides information about the statuses of the PEG chains. When the area of PEG peaks versus total D_2O amount were plotted in Figure 11, it clearly shows that the total signal of PEG increases dramatically first and then reaches a maximum. This reflects that increasing PEG chains are in liquid status and detectable by LF-NMR when more D_2O is added. In addition, there is a clear turning point of the signals of PEG2000 and PEG20000 seen at a D_2O/

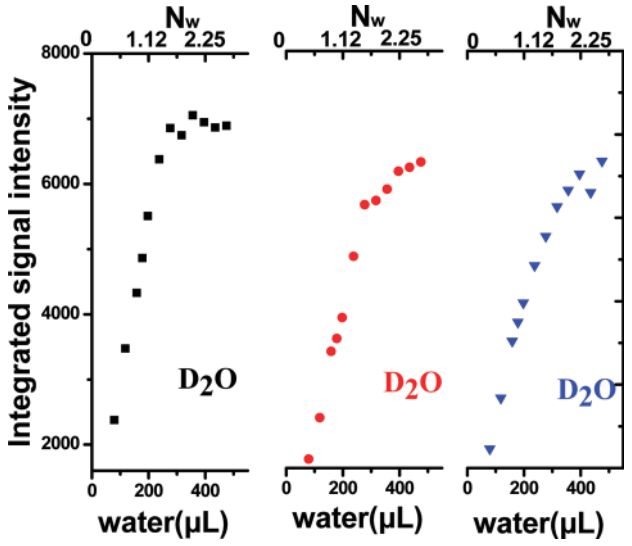

Figure 11. Integrated signal intensity of polymer component in water/PEG (PEG2000 ■, PEG20000 ●, PEG200000 ▼) mixtures versus varied amounts of D_2O.

EG ratio of 1.56:1, which indicates the complete formation of PEG/water solutions. This result suggests that water molecules are very tightly bound to PEG chains before the binding sites on PEG are saturated by water molecules, and extra water molecules are required for free rotation of PEG chains. Thus, this distinct turning point could be a second way to estimate the maximum capacity of tightly bound water on PEG chains, which is also close to the value estimated by both our NMR and DSC methods.

Model of the Hydration Process of PEGs. Based on the results of NMR and DSC, a model of the hydration of PEG chains is proposed (Figure 12). The hydration of PEGs might

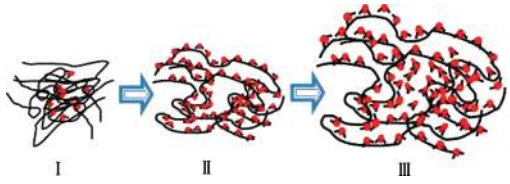

Figure 12. A proposed model of the hydration process of PEG. The red dots represent water molecules, while the black lines represent linear polymer chains. The hydration of PEG might go through three stages with increase of water content: water molecules tightly bind to PEG, saturate binding sites of PEG, and finally swell and dissolve PEG.

go through three stages. First, large amounts of water binding sites on PEG chains are available for water molecules (H_2O:EG < 1.06, Stage 1). These water molecules in the water/PEG mixtures are tightly bound to PEGs, and few water molecules are loosely bound, leading to a dramatic decrease of $1/T_2$ of the water component and an increase of the bound water (m_{bw}). This corresponds to N_{bw} values rising with the increase of water content and only polymer-like endothermic peaks observed in DSC. Then, the amount of water is close to the maximum available sites on the PEG chains (H_2O:EG ratio in the range of 1.06−1.56, Stage 2). A full hydration layer on PEG chains is formed, and certain segments of PEG chains with tightly bound water molecules become mobile caused by a small amount of unbound water molecules. As a result, the first derivative of $1/T_2$ of the water component gradually reaches the turning point,

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and the amount of bound water begins to level off, as observed by both polymer-like and ice-to-water-like phase transition peaks in DSC. Finally, water molecules fully saturate the binding sites, and extra water molecules begin to swell the PEG matrix (H_2O:EG > 1.56, Stage 3), which leads to a slow linear decrease of $1/T_2$. This is indicated by the amount of bound water reaching a steady range with the increase of water content and only ice-to-water-like transitions as observed in DSC. At this stage, the water/PEG mixtures have become PEG water solutions.

CONCLUSIONS

The method for multiple components deconvolution of the T_2 signals measured by the CPMG sequence of LF-NMR can be successfully applied to investigate the interaction between different MW PEGs with water molecules. The results show that this method not only characterizes the statuses of water molecules around the PEG chains but also reveals the mobility of the PEG chains in D_2O or H_2O. Water molecules first very tightly bind to PEG chains before most binding sites are saturated by water molecules, and then additional water molecules are required to swell the PEG/water complexes. Furthermore, this method provides a rather accurate estimation of the binding ratio of water molecules to EG units, which is about one water molecule per EG unit. Also, the association between water molecules and PEG chains is very stable, especially when the water-to-EG unit ratio is below 0.5:1, which is also supported by the results obtained from thermal analysis. We believe that the method developed can be a powerful tool to explore novel nonfouling materials through analyzing water–polymer interactions in the future.

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