Characterization of Biomedical Hydrated Polymers by NMR and ESR Spectroscopic Techniques

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ABSTRACT: The spin lattice relaxation time ($T_1$) and the rotational correlation time ($\tau_1$) have been measured in poly(2-hydroxyethyl methacrylate), pHEMA. Higher degrees of crosslinking lead to reduced water content, and this is correlated with shorter spin lattice relaxation values and lower mobility of the probe molecules. The shorter $T_1$ of water protons in the hydrogels, which acts as a function of water content in both uncrosslinked pHEMA and crosslinked pHEMA systems, suggests that the water in the hydrogel is less mobile than in pure water. Rotational correlation times are 10-100 times longer in the hydrogels than in aqueous solution. Changes in solvent environment were monitored using the nitrogen hyperfine coupling constants, $A_0$, which show a steady decrease as the water content of the hydrogels decreases. Variable temperature ESR studies showed a gradual reduction in probe mobility on cooling, but complete immobilization did not occur until temperatures were as low as 195 K.

Introduction

Water swelling polymer networks, generally referred to as hydrogels, have important uses as biomaterials and have also been investigated for clinical sensor applications due to their resemblance to living soft tissue. The most widely studied polymer in this class is poly(2-hydroxyethyl methacrylate), pHEMA. PHEMA has hydrophilic binding properties due to the hydroxy groups as well as hydrophobic properties associated with the methyl substituents and the backbone methylene groups. However, the existence of separate hydrophilic and hydrophobic phases within the gel has been refuted.

Two approaches have been adopted for dynamic studies of polymers using resonance spectroscopy: the first involves the introduction of a spin probe molecule into the polymer matrix, in which case the mobility behavior reflects the interaction between the probe and the surrounding environment. The second involves the chemical combination of the probe with the polymer, in which case the mobility being monitored reflects the local motion of the labeled polymer sites. The most popular types of spin probe, both for biological and synthetic membranes are the nitroxide radicals in ESR spectroscopy. The ESR spectra of these radicals give informations on the mobility of the probe and the polarity of its environment as well as being sensitive to anisotropic effects. ESR spectroscopy has high sensitivitiy and the technique of ESR imaging has recently been applied to polymeric materials using nitroxides as the probes.

Nitroxides have been extensively used to investigate the properties of hydrated polymer systems, including poly(vinyl alcohol), dextran-based hydrogels and ion-exchange membranes. There is one recent ESR study on pHEMA and pHEMA blends, where amino substituted nitroxide radicals were used to look for evidence of phase separation in the gels. The pH dependence of the ESR spectra showed that in pHEMA, PVA and poly(acrylamide) hydrogels, there was no evidence for separate hydrophilic and hydrophobic phases.

This work is aimed to characterize the mobility...
of the aqueous phase within the pHEMA hydrogels and how this is influenced by the composition of the gels using ESR and NMR spectroscopy. The pHEMA hydrogels are modified by the change of the crosslinking degree.

**Experimental**

**Polymers.** In these experiments, polymers were prepared by free-radical polymerization of monomers.9

**Uncrosslinked pHEMA:** Monomer HEMA (Aldrich) was degassed with N2(g) and mixed with azo-bis-isobutyronitrile (AIBN: 0.5% by weight of monomer) as the initiator. The mixture was added dropwise to degassed ethanol. The solution was stirred continuously for 9 hours in N2(g) at 60°C. The resultant polymeric solution was filtered under reduced pressure. The pHEMA was obtained by precipitation from a mixture of diethyl ether and solid CO2. Unchanged monomer was removed by soaking the resulting polymer in water for several days.

**Crosslinked pHEMA:** Crosslinked polymer was prepared using the method described above for uncrosslinked pHEMA. However, initially the mixture of monomer HEMA and 0.5% of AIBN was dissolved with ethylene glycol dimethacrylate (EGDMA) added as a crosslinker. A series of crosslinked polymers were prepared by adding different concentrations of EGDMA in the range 1%–5% by weight.

**NMR Instrumentation.** The NMR spin lattice relaxation times were measured by utilizing Varian XLFT-100 pulsed NMR spectrometer operating at a magnetic field of 23490 gauss and an RF frequency of 100 MHz for proton resonance. The XL-199 NMR was designated to operate in a frequency-sweep mode. Long-term stability was attained by locking a resonance line in the spectrum, or to the deuterium resonance of a deuterated solvent added to the sample. In this system, the magnetic field strength was kept constant and the transmitter frequency was swept. Using a π-π/2 pulse sequence, the spin lattice (longitudinal) relaxation time (T1) was measured by following the return of magnetization to its equilibrium value after it had been perturbed by a radio frequency field at the resonance frequency field at resonance frequency. The relaxation delay time was a measure of the time of fluctuation of the local environment. The data of T1 was determined from the slopes of the semilog plots. The temperature of the samples was maintained at 34±1°C during the measurements.

**ESR Instrumentation.** ESR spectra were obtained using a Bruker ESP300 spectrometer at X-band (9.3–9.7 GHz). For measuring variable temperature spectra in the range 160 K–50 K, the Bruker variable temperature unit ER 4111 VT was used. The accurate microwave frequency was measured using a Hewlett-Packard microwave frequency counter. ESR spectra were recorded using the following instrumental parameters: scan range, 100 G; time constant, 0.25 s; scan time, 4 min; modulation amplitude, 0.1 G; microwave power, 10 mW; modulation frequency, 100 Hz. The modulation amplitude and the microwave power were adjusted to avoid line broadening and saturation.

**Probe Radicals:** Two nitroxide molecules, 2,2,6,6-tetramethyl-1-oxyl TEMPO and 4-hydroxy-2,2,6,6-tetramethyl-1-oxyl TEMPO were used as probe molecules in ESR experiments. TEMPO and TEMPOL were purchased from Aldrich and used without further purification.

**Sample Preparation.** The water swollen polymers were kept in 1×104 mol L–1 of nitroxide aqueous solution for 3 days. The surface was washed with water five times in order to remove any residue of nitroxide. Then, the samples were dried gently using filter papers. The final polymers were introduced into a quartz flat cell or a cylindrical quartz tube of 2 mm diameter for ESR measurements.

**Rotational Correlation Times:** The rotational correlation time (τc), for isotropic rotation of the spin probe can be calculated using the ESR narrowing formalism:10

\[
\tau_c = \frac{\sqrt{\pi} \Delta \nu}{2C} \times \left( \frac{H_{e0}}{H_{e1}} + \frac{H_{m0}}{H_{m1}} - 2 \right)
\]

where the subscripts -1, 0, and +1 refer to the nitrogen nuclear magnetic quantum number, H_{e1}, H_{e0} are the individual peak heights, Δ ν0 is the peak-top-peak line width of the mid-field line and C is the
constant based on the hyperfine interaction tensors of the spin label in solution. Thus, the approximate \( \tau \) value for the TEMPO probe can be calculated directly from Equation 2.

\[
\tau = 5.95 \times 10^{-10} \times \Delta W_0 \times \left( \sqrt{\frac{H_{\Delta, 0}}{H_{\Delta, 0}}} + \sqrt{\frac{H_{\Delta, 0}}{H_{\Delta, 0}}} - 2 \right)
\]

\( \Delta W_0 \) is the line width of the central (0) line in gauss on the first derivative absorption spectrum. Also, the approximate \( \tau \) value for the TEMPOL probe can be calculated from Equation 3.

\[
\tau = 6.51 \times 10^{-10} \times \Delta W_0 \times \left( \sqrt{\frac{H_{\Delta, 0}}{H_{\Delta, 0}}} + \sqrt{\frac{H_{\Delta, 0}}{H_{\Delta, 0}}} - 2 \right)
\]

As the above quadratic term is essentially unaffected by microwave power saturation effects, \( \tau \) is used throughout this work.

**Immobilization Measurements:** A Varian Cary 5 UV-Vis-Near IR spectrophotometer was used to confirm the presence of immobilized nitroxide in the polymers by measuring the absorbance at 445 nm.

**Equivalent Water Content.** The polymers were soaked in distilled water for three days until the polymers reached a constant weight. They were then weighed after the surface water was removed by filter paper. Dehydrated polymers were obtained by keeping the samples under vacuum at 60 °C to achieve constant weight. The equivalent water content (EWC) was calculated as the ratio of the weight of water in the hydrogel to the weight of the hydrogel at equilibrium hydration, expressed as a percentage.

**Results and Discussion**

The uncrosslinked PHHEMA prepared here shows typical hydrogel swelling with an EWC of 42%. A range of PHHEMA derivatives were then prepared by cross-linking HEMA with ethyleneglycol dimethacrylate (EGDMA). These modifications led to reduced water content and loss of flexibility. Table I gives the equivalent water contents of the cross-linked PHHEMA and these results are consistent with those.

**NMR Measurements.** The proton NMR spectra obtained from pure water and from the PHHEMA with 40% H2O water content are shown in Figure 1. The proton NMR spectrum of pure water shows a narrow and high peak, while the proton NMR spectrum of the water in the hydrogel system shows a broadened peak of decreased height in Figure 1. These results indicate that the water proton environment in the hydrogel is very different from that in pure water. Similar observations were obtained for hydrated proteins and for the water in sintered glass. It was observed that the spin lattice relaxation time \( T_1 \) of ice was 0.829 sec at 16.40 °C. Table II represents the \( T_1 \) data as a function of water content in PHHEMA hydrogel systems at 34°C and 100 MHz. Comparing the data with the measured spin lattice relaxation time of pure water protons, 4.5 sec at 34°C, the average \( T_1 \) values of water protons in the hydrogels were greatly reduced. These results indicate that there are considerable interactions between the water...
molecules and the polymer networks in the hydrogel systems. The short $T_1$ of water protons in the hydrogel suggests that the water in the hydrogel is less mobile than in pure water. This can be interpreted in terms of the structure ordering of water molecules in the hydrogel networks. The most probable binding positions of water molecules are the polar sites, such as the hydroxyl and carbonyl groups in the methacrylate polymers. However, the measured values of the proton spin-lattice relaxation times, $T_1$, cannot be considered an average of three states of water in the hydrogels.19

**ESR Measurements.** The TEMPO and TEMPOL probe radicals were introduced from dilute aqueous solution into the fully hydrated polymers and the ESR spectra were measured. Figure 2 shows the spectra of TEMPO in pHEMA as a function of the degree of crosslinking with EGDMA. In aqueous solution, the three lines of a nitroxide ESR spectrum have equal intensities and linewidths, characteristic of rapid isotropic rotation. The spectra of TEMPO in the pHEMA polymers show unequal linewidths and amplitudes. At high EWC, the spectral line shape approaches that of the aqueous solution. However, as the water content is reduced, the intensity of the high field line $H_{n+}$ is seen to decrease remarkably and broaden (Figure 2). As the rate of rotation slows down and becomes comparable to the hyperfine splitting and the g-tensor anisotropy, there is a differential broadening of the spectrum with the high-field line broadening the most.

A convenient way to analyze the resultant spectra is to use the relative peak heights of the central and high field lines $H_{n+}/H_{n-}$. The spectrum of TEMPO in uncrosslinked pHEMA shows some asymmetry with $H_{n+}/H_{n-}$=1.24 (Table III). At 5% cross-linking, this ratio increased to 3.87, consistent with a substantial reduction in probe mobility.

The correlation times, $\tau_\alpha$, for the rotation of the probe nitroxides can also be derived from the spectra using Equations (1)~(3). These are relative rather than absolute correlation times and assume an isotropic rotation. The correlation times for TEMPO and TEMPOL are given in Table I, along with the values in solution. TEMPO in uncrosslinked pHEMA has a correlation time $\tau_\alpha$ of 0.15 ns, about eight times the value of 0.02 ns in aqueous solution. At 5% cross-linking, the correlation time for TEMPO rotation increased by another order of magnitude to 1.92 ns. The behaviour of TEMPOL very closely parallels that of TEMPO, with the correlation times being slightly longer, ranging from 0.28 to 2.45 ns. At the highest degrees of crosslinking, a new broadband spectral component appears as shoulders on the low field sides of the peaks, indicative of anisotropic rotation of the TEMPO molecule. The correlation times quoted for these samples are therefore only an approximation since they do not take this anisotropy into account.

The nitrogen hyperfine coupling constant, $A_\alpha$, is

**Table II. Proton NMR Spin Lattice Relaxation Times for Hydrgels Based on pHMA of Different Equilibrium Water Content at 100 MHz and 34°C (unit: sec)**

<table>
<thead>
<tr>
<th>wt% of total water content in the hydrogel</th>
<th>$T_1$ uncrosslinked pHMA</th>
<th>$T_1$ crosslinked pHMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.189</td>
<td>0.186</td>
</tr>
<tr>
<td>30</td>
<td>0.214</td>
<td>0.198</td>
</tr>
<tr>
<td>35</td>
<td>0.280</td>
<td>0.230</td>
</tr>
<tr>
<td>40</td>
<td>0.300</td>
<td>0.259</td>
</tr>
<tr>
<td>45</td>
<td>0.340</td>
<td>0.296</td>
</tr>
</tbody>
</table>

**Figure 2. ESR spectra of TEMPO in hydrated pHMA as a function of % crosslinking with EGDMA.**
Characteristic of Biomedical Hydrated Polymers

Table III. ESR Parameters for TEMPO and TEMPOL in Crosslinked pHEMA and in Solutions

<table>
<thead>
<tr>
<th>Amount of Crosslinker</th>
<th>TEMPO A(0) (G)</th>
<th>H&lt;sub&gt;1&lt;/sub&gt;/H&lt;sub&gt;4&lt;/sub&gt;</th>
<th>TEMPO A(0) (G)</th>
<th>H&lt;sub&gt;1&lt;/sub&gt;/H&lt;sub&gt;4&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>17.27</td>
<td>0.15</td>
<td>17.03</td>
<td>0.28</td>
</tr>
<tr>
<td>1%</td>
<td>17.25</td>
<td>0.21</td>
<td>17.03</td>
<td>0.47</td>
</tr>
<tr>
<td>2%</td>
<td>17.23</td>
<td>0.30</td>
<td>17.01</td>
<td>1.72</td>
</tr>
<tr>
<td>3%</td>
<td>17.20</td>
<td>0.31</td>
<td>17.00</td>
<td>1.94</td>
</tr>
<tr>
<td>4%</td>
<td>17.19</td>
<td>0.16</td>
<td>16.98</td>
<td>2.16</td>
</tr>
<tr>
<td>5%</td>
<td>17.17</td>
<td>0.32</td>
<td>16.95</td>
<td>2.45</td>
</tr>
<tr>
<td>Water</td>
<td>17.28</td>
<td>0.02</td>
<td>16.94</td>
<td>0.46</td>
</tr>
<tr>
<td>Methanol</td>
<td>16.32</td>
<td>0.03</td>
<td>16.18</td>
<td>0.04</td>
</tr>
</tbody>
</table>

dependent on the structure of the radical, the temperature and the polarity of the solvent. In particular, A(0) increases with increasing solvent polarity and hydrogen bonding due to stabilization of the resonance structure having greater spin density at the nitrogen nucleus. 18-19 A(0) values measured from the pHEMA spectra are listed in Table III. Increased hydrophobicity at lower EWC results in a corresponding decrease in A(0) for both TEMPO and TEMPOL.

Temperature Dependence. The ESR spectra were measured as a function of temperature in the range 310 to 195 K. A sample set of data for TEMPO in 2% crosslinked pHEMA is given in Figure 3. The spectrum at 195 K is characteristic of a completely immobilized system, having a typical powder-pattern lineshape. As the temperature is raised, a two component spectrum appears, due to a strongly anisotropic rotation of the probe. There is further gradual change up to about 275 K, the motion is close to isotropic. It is also clear that there is no sudden transition between a freely mobile and an immobilized probe. Note that it is difficult to keep the EWC fixed during the variable temperature experiments and this is why the spectrum at the highest temperature in Figure 3 is still somewhat broadened.

Comparing the results of hydrogels by NMR and ESR experiments, it is evident that there is no simple overall correlation between NMR spin lattice relaxation time and rotational correlation time for the nitroxide probes. Recently, similar behaviour was found for dextran-based hydrogels 16 of varying porosity where the effects of compartmentalization due to cross-linking had a strong effect on correlation times, above the threshold value.

The nitrogen hyperfine splitting constant A(0) has been shown to depend not only on polarity but also on hydrogen bonding effects. A(0) can be expressed as the sum of three factors: 18,19

\[
A(0) = A_{\text{iso}} + A_{\text{dipol}} + A_{\text{dip-pair}}
\]

where A(0) is the hyperfine splitting of the probe in a non-proton donating (aprotic) medium with a dielectric constant ε = 1. A(0)polar is the component determined in polar aprotic solvents and A(0)dip-pair is related to hydrogen bonding effects at the nitroxide N-O group. The relative magnitudes of these effects can be seen by comparing A(0) for TEMPO in water (17.28) and methanol (16.32). The hydrogen bonding contribution in methanol solution is about half that in water and this accounts for about 0.6 of the decrease in A(0), the re-

Figure 3. Variable temperature ESR spectra of TEMPO in 2% crosslinked pHEMA. Temperature range 195-325 K.
drom en bonding of the nitrooxide to the gel-bound water. In the crosslinked pHEMA series, the decrease in TEMPO $A_0$ from 17.27 to 17.17 (Figure 5) represents the level of change observed for TEMPO in solution on changing the solvent from water to ~15% ethanol-water or ~25% methanol-water. Comparing the changes in $A_0$ for TEMPO and TEMPOL, the trends are practically identical. The effect of hydrogen bonding at the 4-hydroxy group in TEMPOL accounts for its lower $A_0$ values but there is no evidence obtained from the ESR data for stronger interactions between the more hydrophilic TEMPOL probe and the pHEMA polymers.

Conclusions

The hydrogels has been characterized by NMR and ESR spectroscopy. The chemical and physical properties of hydrogels are strongly influenced by the composition of hydrophobicities and hydrophilicities. This led to a decrease in water content.

With a decrease in the water content within hydrogels, the spin lattice relaxation times ($T_1$) have shorter values. The shorter $T_1$ of water protons in the hydrogels indicates that the water behaviour within hydrogels is less mobile and is influenced by polymeric environments.

ESR studies have been carried out on the mobility of the small hydrophilic probe radicals TEMPO and TEMPOL as a function of hydrogel composition for cross-linked pHEMA. Rotational correlation times are 10-100 times longer in the hydrogels than in aqueous solution. The hyperfine splitting constants are consistent with a relatively high local hydrophilic character in all samples, although there is a steady decrease as the EWC decreases. The temperature dependence of the ESR spectra shows a gradual change from a freely rotating to an immobilized probe over the temperature range 310–195 K. The TEMPO and TEMPOL radicals used in this study act as probes of hydrophilic interactions in the hydrogels. Since the EWC of hydrogels can be manipulated by many different factors, ESR probe studies provide a valuable method of comparing solute mobilities for different materials.
There is no simple correlation between EWC and mobility, however, there is reverse relation between NMR-relaxation time and ESR rotational correlation time of probe molecules. As the water contents in hydrogels decrease, the T1 values become shorter, while ESR rotational correlation time of probe molecules increases. This result demonstrates that the mobility is restricted by the polymeric environment and is strongly dependent on the water contents.

References

(1) O. Wichterle, and D. Lim, Nature, 185, 117 (1960).


Functionality of Polypeptide by Induction of Specific Tertiary Structure

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ABSTRACT: Two different approaches for arranging functional groups regularly in space are presented. One way is based on the covalently bonding chemistry as exemplified by proteins. Here, a helix-bundle structure was elaborately prepared by connecting two or three helices to a template molecule. The peptide carrying three helices in the molecule formed ion channel at lower concentration than the peptides of the helical part alone or carrying two helices in the molecule. The other way is based on the self-assembling system. The amphiphiles carrying a chromophore-containing amino acid formed a bilayer membrane in water. The membrane showed efficient photoenergy migration between chromophores and effectively reduced cytochrome c by photo-irradiation.

Introduction

Functionality of proteins is exhibited on the basis of the specific tertiary structure because the efficacy of the protein function is generally impaired by denaturation. Therefore, construction of a well-defined structure should be important in terms of developing novel functional polymers where the functional groups are fixed in space to realize efficient cooperation between them. However, the problem is that we do not know the mechanism by which a polypeptide chain folds into a corresponding tertiary structure. In the present report, two ways to dissolve the difficulty are presented for the regulation of the topological arrangement of the functional amino acids.

One way is to synthesize polypeptides with graft chains connected to a template molecule, so-called template-assisted synthetic proteins (TASPs). In the TASP, the spatial arrangement of the graft chains is regulated entropically and energetically by connecting it to the template molecule. Here this method is applied to the synthesis of ion-channel-forming peptides which take a bundle structure of α-helices.

The other way is to fix functional amino acid residues in a molecular assembly. Recently, the number of reports on molecular assembly is increasing. It has been shown that amphiphilic molecules construct a bilayer membrane in a shape of a regular spherical vesicle in water. Functional amino acid residues will be fixed regularly in the bilayer membrane by incorporation of the amino acid residue into the amphiphile.

Experimental

Materials. Peptides were synthesized by a conventional liquid-phase method. The synthesis of one of the amphiphiles is previously reported, and the other was prepared similarly.

Methods. CD, fluorescence, and transition absorption measurements were carried out on a JASCO J600 spectropolarimeter, a Hitachi MPF-4 fluorophotometer, and an Osaka Electronics IMU-7000 using a XeF excimer laser (351 nm, 54 mJ).

Ion-channel formation of the peptides was examined by BLM measurement A thin Teflon film (0.25 mm thick) with an aperture of 0.2~0.3 mm diameter was clamped between two halves of a Teflon trough. The hole was precoated with hexadecane/hexane (6/4 v/v). The soybean-lectin mem-