



The state of water in thermoresponsive poly(acryloyl-L-proline methyl ester) hydrogels observed by DSC and $^1\text{H-NMR}$ relaxometry

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Abstract

Hydrogels that reversibly swell or shrink in water with decreasing or increasing temperature, respectively, were obtained by γ ray-induced polymerization of acryloyl-L-proline methyl ester in the presence of different amounts of a crosslinking agent. The role of water in the hydrated polymers was investigated by DSC and $^1\text{H-NMR}$ relaxometry. From the curves of fusion of water determined by the former it was possible to ascertain that the amount of both freezing and non freezing water decreased with increasing the crosslinker percentage and/or swelling temperature. Moreover, at the temperatures higher than 37°C , the water absorbed by the different hydrogels is mostly present as non freezing water. The $^1\text{H-NMR}$ relaxometry study enabled the spin-spin and spin-lattice relaxation curves to be analyzed. It was possible to distinguish three "populations" of protons and identify two of them with protons of freezing and non freezing water determined by DCS. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Thermoresponsive hydrogels are hydrophilic polymeric networks, both natural and synthetic, which

swell below a lower critical solution temperature (LCST) and shrink above it. These physical changes are reversible and this property has been advantageously considered for a number of biomedical applications, including drug delivery systems (Gehrke, 1993; Kaetsu, 1996).

α -amino acid-containing thermosensitive hydrogels, i.e., acryloyl and methacryloyl derivatives, have been synthesized by radiation-induced polymerization and

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their swelling–deswelling behaviour in a wide range of temperature, as well as their use as matrices for the controlled release of drugs, have been described (Yoshida et al., 1989; Safranji et al., 1993).

Copolymer hydrogels based on acryloyl-L-proline methyl ester (A-ProOMe), suitable for a sustained drug release, have been recently obtained (Yoshida et al., 1991; Martellini et al., 1998). In particular, a proper balance of hydrophilic and hydrophobic comonomers in the polymer chains allowed a shift of the LCSTs to be attained (Martellini et al., in press). The polymerization kinetics of aqueous solutions of A-ProOMe has been studied by pulse radiolysis (Takaacs et al., 1996).

In biomaterials science, the requirement of biocompatibility is of greatest importance since it means the acceptance of an artificial implant by the surrounding tissues or, in short, the absence of any harmful interaction with organism. When hydrogels are concerned, since they are designed to be in contact with the body fluids, the biocompatibility is closely related to the adsorption of a considerable amount of water inside their network structure. It is therefore not surprising that a great deal of work has been devoted to the nature of the water in hydrogels since a pioneer work in which a three-state model was developed (Jhon and Andrade, 1973). According to this hypothesis, three kinds of water exist: non freezing or bound water, freezing interfacial or intermediate water and free (“normal”) water, whose relative importance increases as the water content in the hydrogel progressively increases.

Later on, other work accumulated on this subject and a number of hydrogels have been studied such as poly(2-hydroxyethyl methacrylate) (Sung et al., 1981; Smyth et al., 1988; Allen et al., 1993), poly(vinyl alcohol) (Hatakeyema et al., 1984) and poly(N-vinyl-2-pyrrolidone-co-methyl methacrylate), (Quinn et al., 1988). In those studies use was made of differential scanning calorimetry (DSC) and ^1H pulse nuclear magnetic resonance (NMR), which are powerful and complementary tools for investigating the physical state and structure of water in hydrogels.

We report here on DSC and low resolution NMR results concerning hydrogels based on poly(A-ProOMe) obtained by radiation polymerization.

2. Experimental

2.1. Materials

A-ProOMe was synthesized at JAERI, Takasaki, according to the procedure elsewhere described (Yoshida et al., 1991). The crosslinking agent trimethy-

lolpropane trimethacrylate (TMPTMA), an Aldrich Chemical Co. product, was used as received.

2.2. Synthesis of thermosensitive hydrogels

Hydrogels were obtained by radiation-induced polymerization of the mixtures A-ProOMe with TMPTMA (percentages 1, 5, 10 and 15%) using γ -rays from a Co-60 source at the dose rate of 0.49 Gy/s and at the temperature of 25°C after flushing nitrogen.

The samples were swollen in water at equilibrium at different temperatures, weighed after gently wiping the surface water excess, dried in a vacuum heater for 48 h and weighed again.

2.3. DSC characterization

The hydrogel specimens with different degrees of crosslinking were allowed to swell in water at the temperatures of 4°, 22° and 37°C until the equilibrium swelling for each temperature was reached. The samples were wiped with a filter paper to remove the surface water excess and sealed in previously weighed aluminum pans similar to those used for volatile samples. The weight of swollen samples was 2–6 mg. To homogenize the samples, the pans with each series of samples were maintained at the respective controlled temperature. Before the experiments, all samples were kept in the DSC at each equilibrium temperature for 10 min. After the measurements, the pans were weighed again to be sure that any loss of water did not occur during the DSC run. The water content of the different samples was determined by the weight difference of the pans before and after drying which was performed by heating the punched pans in the apparatus at 150°C until total water elimination. The drying temperature is far below the onset temperature of degradation of the four polymers (~300°C).

The thermograms were obtained using a Perkin–Elmer differential scanning calorimeter DSC-7, filled with a subambient intracooler accessory working down to –60°C. The temperature calibration was carried out with *n*-decane and benzene, while indium was used for ΔH calibration. The cooling and heating rates were 5°C/min in the temperature range from –55° to 37°C.

The amount of non freezing water, W_{nf} , was calculated as:

$$W_{\text{nf}} = W_{\text{c}} - W_{\text{f}} \quad (1)$$

where W_{c} is defined as:

$$W_{\text{c}} = \text{weight of water absorbed/weight of dry polymer} \quad (2)$$

and W_f is the amount of freezing water determined from the total peak area of endotherms.

2.4. $^1\text{H-NMR}$ characterization

The hydrogel samples with different degrees of cross-linking, R , were swollen at equilibrium in distilled water at the temperatures, T , of 21.5°, 25.0°, 28.5°, 34.5°, and 41.0°C. Subsequently, they were gently surface dried and set in NMR sample tubes of 10 mm diameter which were immediately sealed with parafilm.

The NMR measurements for each specimen were carried out at the corresponding swelling temperature. Therefore we performed the $^1\text{H-NMR}$ relaxation study on 20 different (R , T) situations.

The proton magnetic relaxation measurements were carried out, at the selected temperatures, at 20 MHz by a pulsed low-resolution Minispec P20 spectrometer (Bruker, Karlsruhe, Germany) equipped with a system for pulse-programming control and data acquisition (Stelar, Mede, Italy) and with a thermostat.

The 90° pulse length was 4.6 μs and the dead time was 8 μs .

The spin–spin relaxation curves were detected by the CPMG sequence (Carr and Purcell, 1954; Meiboom and Gill, 1958). About 2000 echo values were collected and the measurements were carried out at different time intervals τ between echoes, i.e., $\tau = 0.4, 1.0, 2.0$ and 4.0 ms.

The spin–lattice relaxation curves were obtained by the inversion recovery (IR) sequence. We selected 55 increasing τ values, in geometrical progression. The signal, after every 90° pulse, was acquired at different times along the free induction decay (FID) curve (Abragam, 1961).

The full relaxation curve was obtained both in spin–lattice and spin–spin relaxation measurements, i.e., we analyzed data covering a range of values up to a time that was long enough to reduce the signal until it was comparable with the background noise (Senatra et al., 1990).

The CPMG and IR measurements were repeated eight and four times, respectively. Each CPMG sequence was repeated eight times for signal averaging. IR sequences were repeated four times and a delay of 5 s always allowed the nuclear magnetization to return to its equilibrium value.

The FID curves were acquired with ≈ 8000 points at 2 μs intervals. Every FID was obtained from 128 repeated signals.

The spin–spin relaxation curves, detected by CPMG sequence, were analyzed, using the iterative procedure supplied by Curfit program (Bevington, 1969), via a single-exponential model:

$$M_t(t) = M_0 e^{-t/T_2} \quad (3)$$

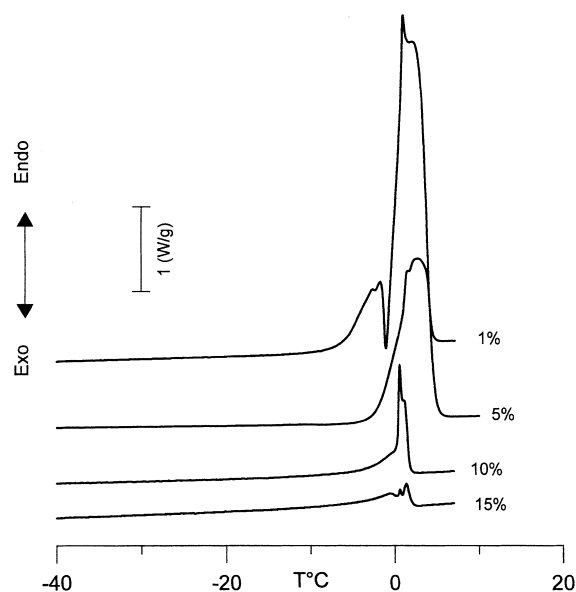


Fig. 1. Curves of fusion of water for poly(A-ProOMe) hydrogels crosslinked with TMPTMA at the concentrations of 1, 5, 10 and 15% swollen at equilibrium at 4°C.

where $M_t(t)$ is the transverse magnetization at t time, M_0 is the equilibrium magnetization ($t = 0$) and T_2 is the spin–spin relaxation time constant.

The analysis of the spin–spin decay curves at the different τ values did not show any significant variation. Thus we will report the average T_2 values obtained from the analysis of the whole measurements carried out on the same (R , T) situation.

The spin–lattice relaxation curves were fitted, using the same procedure, to the function:

$$M_1(t) = M_0(1 - 2e^{-t/T_1}) \quad (4)$$

where $M_1(t)$ is the longitudinal magnetization at t time and T_1 is the spin–lattice relaxation time constant.

The validity of the models was determined by the χ^2 test ($P < 0.05$) and the T_2 , T_1 values were confirmed by the continuous distribution of relaxation times analysis (Provencher, 1982).

3. Results and discussion

It was previously found that the equilibrium water content of crosslinked poly(A-ProOMe) hydrogels decreased as both the concentration of the crosslinking agent TMPTMA and swelling temperature increased (Martellini et al., in press). In the present work the

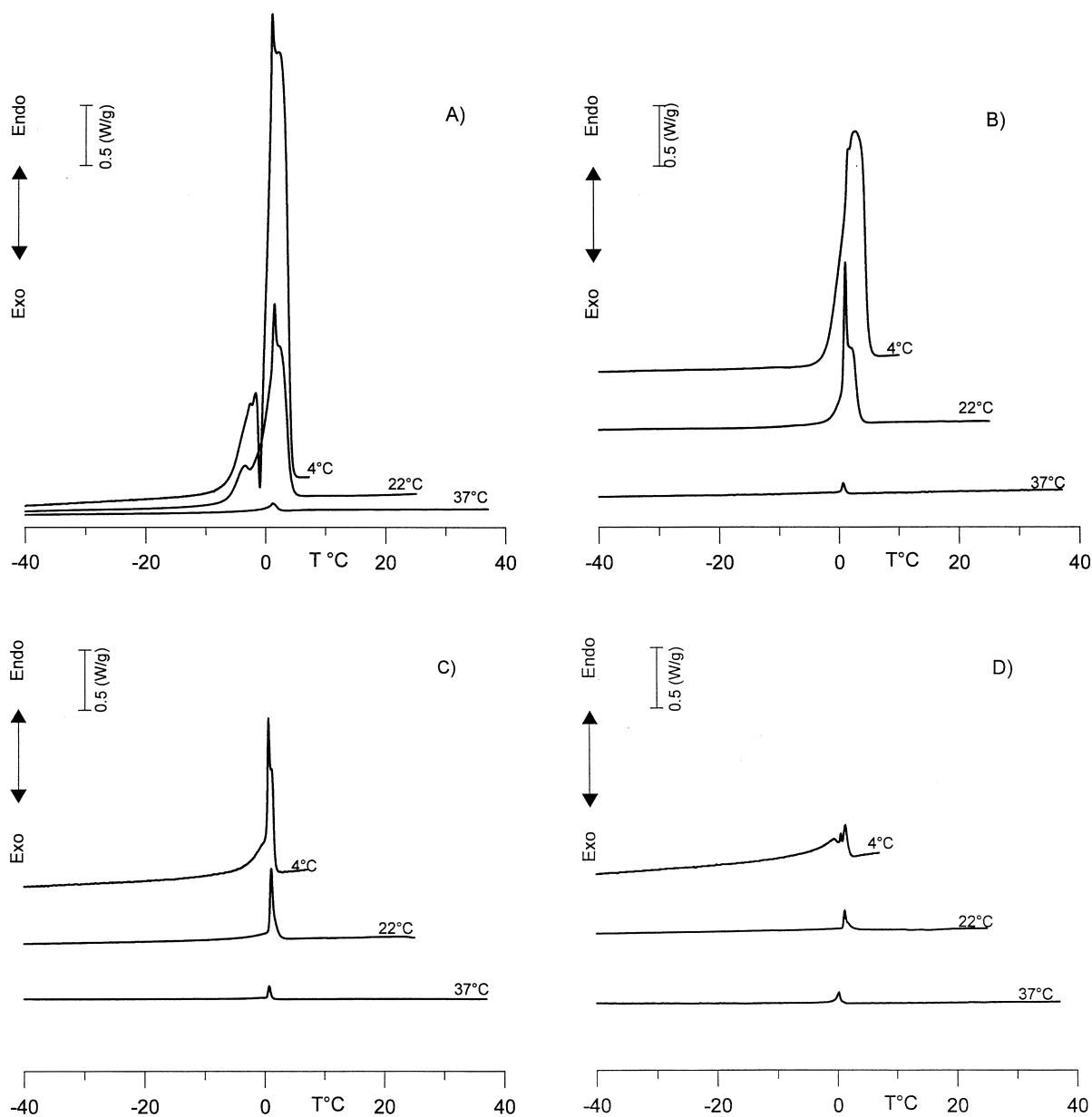


Fig. 2. Curves of fusion of water for poly(A-ProOme) hydrogels swollen at equilibrium at the temperatures of 4°, 22° and 37°C. The percentage of TMPTMA was 1% (A), 5% (B), 10% (C) and 15% (D).

properties of water-hydrogels systems as a function of both parameters have been investigated.

The curves of fusion of water for poly(A-ProOme) specimens crosslinked at different concentrations of TMPTMA and swollen at equilibrium at 4°C are reported in Fig. 1. It can be seen that the different peak areas, normalized with respect to the weight of the sample under examination, progressively decrease as the percentage of the crosslinking agent increases,

thus indicating a decrease of the amount of freezing water.

Fig. 2 shows the curves of fusion of water for hydrogels with different extents of crosslinking and swollen at the temperatures of 4°, 22°, and 37°C. It can be noted that the peak area of freezing water decreases not only as the degree of crosslinking increases, as reported in Fig. 1 for the temperature of 4°C, but also as the swelling temperature increases. The latter result

Table 1

Values of W_c and W_f (g H₂O/g dry polymer) for hydrogels obtained at different degrees of crosslinking and swollen at the temperatures of 4°, 22° and 37°C

$T(^{\circ}\text{C})$	$R(\%)$	1	5	10	15
4	W_c	3.26	1.07	0.46	0.32
	W_f	2.55	0.58	0.06	0.01
22	W_c	0.88	0.51	0.37	0.27
	W_f	0.38	0.10	0.02	0.01
37	W_c	0.37	0.28	0.27	0.18
	W_f^*	0.004	0.005	0.03	0.002

* Rough evaluation from the thermograms of Fig. 2.

is not unexpected if one bears in mind that poly(A-ProMe) is an “LCST” hydrogel, i.e., it collapses with increasing temperature. To this regard, a decrease of the pore size of the polymer matrices with increasing temperature has been recently observed by scanning electron microscopy (Martellini et al., in press).

The values of W_c and W_f for the samples at different degree of crosslinking and swollen at 4°, 22° and 37°C are reported in Table 1.

The amount of non freezing water, W_{nf} , as a function of swelling temperature (see Fig. 3) shows a trend similar to that observed for freezing water. It can be seen that W_{nf} decreases in a different way with increasing the swelling temperature, depending on the crosslinker amount. That is, the less crosslinked polymer (1%) shows the highest amount of non freezing water

which markedly decreases as the swelling temperature increases. On the contrary, for samples with higher crosslinking agent percentages, W_{nf} decreases to a lesser extent.

Fig. 4 shows the change of the ratio W_f/W_c as a function of the crosslinker amount. One can observe that, while at $T = 4^{\circ}\text{C}$ and $T = 22^{\circ}\text{C}$ by increasing the percentage of crosslinking agent the amount of freezing water decreases more and more until it reaches a value very close to zero, at $T = 37^{\circ}\text{C}$ it approaches zero in all the range of crosslinking agent investigated. Such a behaviour indicates that at $T \geq 37^{\circ}\text{C}$ most of the water absorbed from the polymers differently crosslinked would be in the state of non freezing water W_{nf} .

Moreover, from the data of Table 1 and by using Eq. (1), one can estimate that at $R = 1\%$ $W_{nf} < W_f$

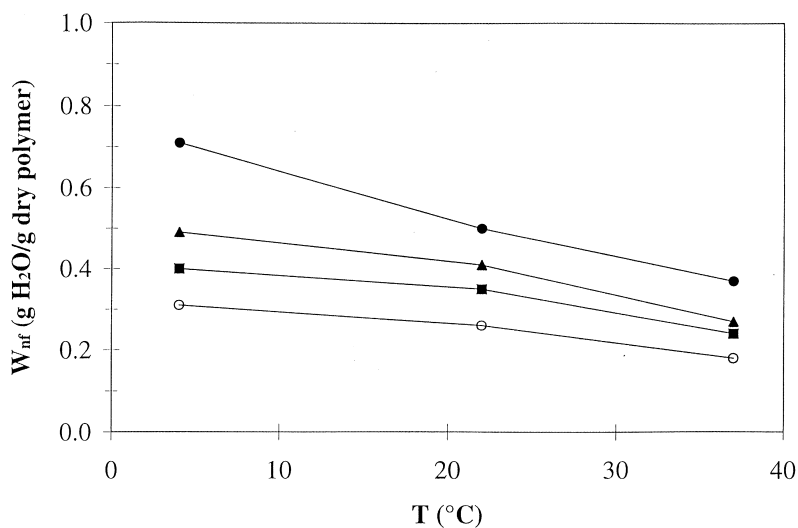


Fig. 3. Amount of non freezing water, W_{nf} , as a function of swelling temperature for poly(A-ProMe) hydrogels crosslinked with TMPTMA at the concentrations of 1% (●), 5% (▲), 10% (■) and 15% (○).

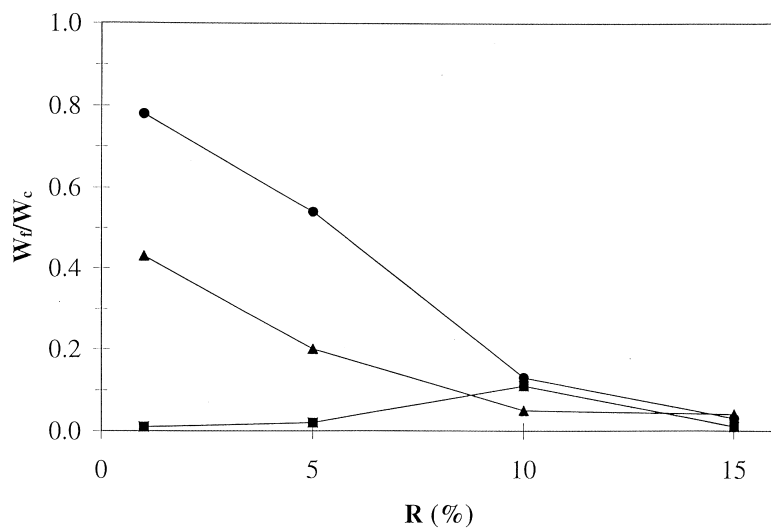


Fig. 4. W_f/W_c ratio as a function of the crosslinking agent percentage for the poly(A-ProOMe) hydrogels swollen at equilibrium at the temperatures of 4° (●), 22° (▲) and 37°C (■).

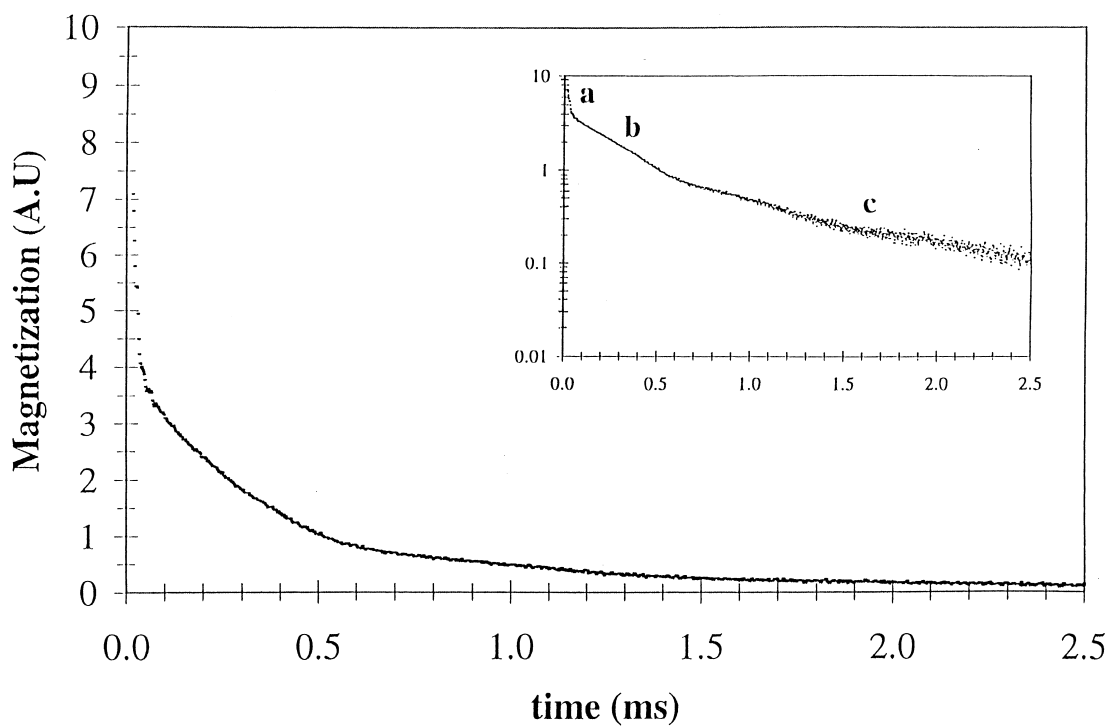


Fig. 5. Experimental FID curve determined at 41°C from the poly(A-ProOMe) hydrogel crosslinked with TMPTMA at the percentage of 1% and swollen at equilibrium at 41°C. In the inset the experimental data are shown as $\text{Log } M_t$ vs t .

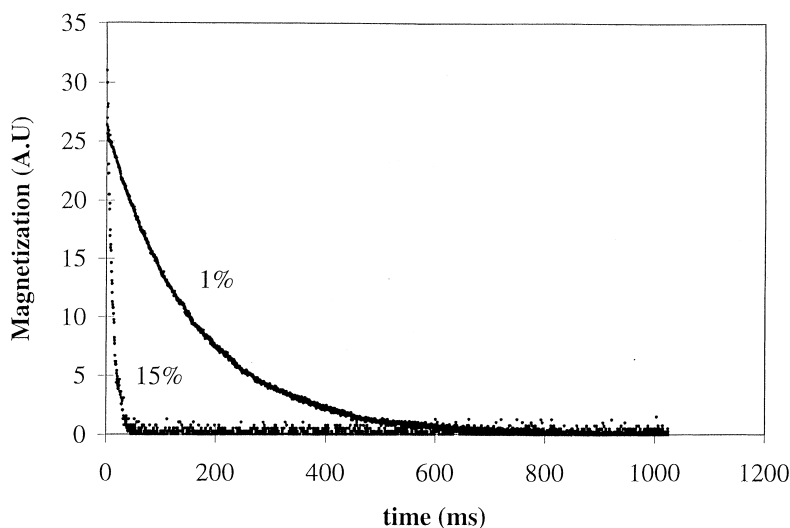


Fig. 6. Experimental spin–spin relaxation curves (by CPMG sequences) of the $^1\text{H}_c$ water protons from the hydrogels crosslinked at R of 1% and 15% and swollen at equilibrium at the NMR measurement temperature of 21.5°C .

at the swelling temperature of 4°C , $W_{\text{nf}} \approx W_f$ at 22°C while $W_{\text{nf}} \approx W_c$ at 37°C . For the polymer with $R = 15\%$, $W_{\text{nf}} \approx W_c$ at all swelling temperatures analyzed. It can be therefore inferred that at the highest crosslinking agent percentage of 15%, the effect of temperature on the polymer is very small while it is very pronounced at the lowest crosslinking degree of 1%.

From these results one can conclude that for crosslinking percentages $< 15\%$ the effect of temperature on swelling is the same as that of the crosslinking agent but with the former only the phenomenon is reversible.

The ^1H -NMR relaxation study was performed in three steps:

1. Analysis of the FID (free induction decay) curves in order to evidenciate the presence of ^1H characterized by T_2 time constants lower than a millisecond (“solid-like” protons) (Bloembergen et al., 1948), not detectable by CPMG sequences.
2. Analysis of the CPMG curves, in order to see the behaviour of ^1H , characterized by a T_2 time constant higher than a millisecond (“liquid-like” protons), as the percent of crosslinking, R , and the temperature, T , change.
3. Analysis of the IR curves, in order to evaluate the sensitivity of the spin–lattice relaxation (T_1 time constant) to the R and T variations.

The FID signals show, at a first sight, a progressively decreasing slope (corresponding to an increase of the T_2 time constant) as the time, t , increases. As an example, in Fig. 5 the experimental FID signal of the

sample with $R = 1\%$ and swollen at $T = 41^\circ\text{C}$ is reported. A more thorough analysis of all FID curves (Log M_t vs t , as reported in the inset of Fig. 5) suggests the presence of three roughly straight lines (**a**, **b** and **c**) with different slopes: the first one with the maximum slope, the second one with the intermediate slope and the last one with the minimum slope. This behaviour could be explained by assuming that:

- three “populations” of protons ($^1\text{H}_a$, $^1\text{H}_b$ and $^1\text{H}_c$) are present;
- the M_t magnetizations due to each population (M_a , M_b and M_c) exponentially decay;
- the corresponding relaxation times are in the following order: $T_{2a} < T_{2b} < T_{2c}$.

In this hypothesis the above straight lines would be explained as follows:

- In the region **a** there is the decay of the magnetization due to the protons as a whole: $M_t(t) = M_a(t) + M_b(t) + M_c(t)$. The prevailing pattern is due to $M_a(t)$ which decays very quickly (maximum slope);
- In the region **b**, $M_a(t) \approx 0$, so that $M_t(t) \approx M_b(t) + M_c(t)$ and the prevailing pattern is due to $M_b(t)$ (intermediate slope);
- In the region **c** we can suppose that also $M_b(t) \approx 0$, so that $M_t(t) \approx M_c(t)$ (minimum slope).

Therefore, the intercepts at $t=0$ of the straight lines (**a**, **b** and **c**) provide an assessment of the values: $M_a(0) + M_b(0) + M_c(0)$, $M_b(0) + M_c(0)$ and $M_c(0)$, re-

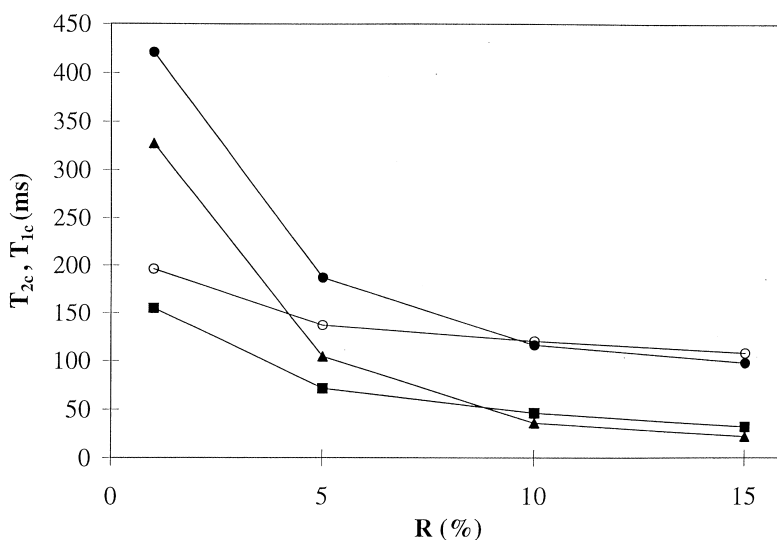


Fig. 7. T_2 and T_1 NMR relaxation times of the $^1\text{H}_c$ water protons vs crosslinking agent percentage R for the hydrogels swollen at equilibrium at the temperatures of 21.5°C (▲ T_2 ; ● T_1) and 41°C (■ T_2 ; ○ T_1). Relaxation measurements were always performed at the equilibrium temperature.

spectively. These values are proportional to $N_a + N_b + N_c$, $N_b + N_c$ and N_c , respectively, where N_a , N_b and N_c are the number of $^1\text{H}_a$, $^1\text{H}_b$ and $^1\text{H}_c$ protons, respectively.

From the analysis of FID curves it was also possible to evaluate the transverse relaxation times associated to the three populations of protons:

- $T_{2a} \approx 10^{-2}$ ms;
- $T_{2b} \approx 10^{-1}$ ms;
- $T_{2c} >$ ms.

The $^1\text{H}_a$ are ascribable to the protons of the polymer matrices and it is difficult to obtain further informations on them by our NMR spectrometer. The small T_2 values of $^1\text{H}_b$ protons prevent from a CPMG study. Only $^1\text{H}_c$ protons are investigable by CPMG methods.

Looking at the curves obtained by CPMG sequences, one can immediately recognize that the signals are strongly dependent on the percentage R of the crosslinking agent in the hydrogels (Fig. 6). Furthermore, the signals corresponding to the same percentage of crosslinking have been found to be dependent on the temperature.

The spin–lattice relaxation curves were analyzed at ~ 70 μs on the FID (when both $^1\text{H}_b$ and $^1\text{H}_c$ are present while $^1\text{H}_a$ are not present any more) and at $t \approx 600$ μs when practically only $^1\text{H}_c$ are present. A further graphical analysis enabled an evaluation of T_{1b} (≈ 100 ms).

The T_2 and T_1 values of the $^1\text{H}_c$ water protons (T_{2c} ,

T_{1c}) decrease with increasing the crosslinking percentage R , as shown in Fig. 7, and the rate of decrease of T_{2c} and T_{1c} is higher the lower the temperature is. One can also see that a certain parallelism exists between T_{2c} and T_{1c} curves. We observe that, as the crosslinking percentage exceeds 10%, variations of the relaxation times are not relevant any more.

Let us now take into consideration the dependence of T_{2c} and T_{1c} relaxation times on temperature. It can be seen from Fig. 8 (a, b) that for the samples with a crosslinker percentage of 5, 10 and 15% a significant dependence on temperature is not discernible. On the contrary, for the sample with $R = 1\%$ a noticeable decrease of both T_2 and T_1 with increasing temperature can be observed at $T \leq 25^\circ\text{C}$. Such a behaviour may seem anomalous at first, because usually the relaxation times increase with temperature. However, this phenomenon is not surprising because the amount of water absorbed in these samples is an inverse function of temperature (Martellini et al., in press) and, moreover, $T_{2,1}$ values of water protons, obtained at $T = \text{constant}$, in heterogenous systems decrease with decreasing hydration (Senatra et al., 1990). Therefore, the proton relaxation times in the hydrogels analyzed are subjected to two opposite effects as the temperature varies.

From the results obtained one can conclude that:

1. At $R \geq 5\%$ the two opposite effects are comparable in the temperature range investigated;
2. at $R = 1\%$ one observes the same behaviour as before for $T \geq 25^\circ\text{C}$ while, for $T \leq 25^\circ\text{C}$, the “hy-

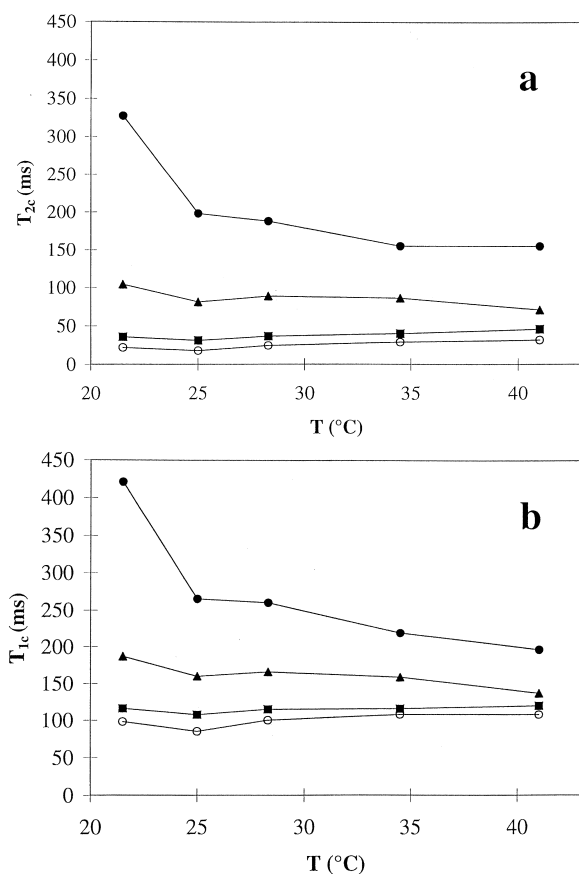


Fig. 8. T_2 (a) and T_1 (b) NMR relaxation times of the $^1\text{H}_c$ water protons vs “relaxation measurement temperature” for the hydrogels crosslinked at $R = 1\%$ (●), 5% (▲), 10% (■) and 15% (○) and swollen at equilibrium at the five selected temperatures.

dration effects” are prevailing.

From the above analysis of the data, we obtained both the (T_{2c}, T_{1c}) and (T_{2b}, T_{1b}) values for every (R, T) sample. The comparison between the spin–lattice and spin–spin relaxation data indicates that the (T_{2c}, T_{1c}) and (T_{2b}, T_{1b}) pairs are always physically consistent, i.e., $T_1 \geq T_2$ (Abragam, 1961) and therefore ascribable to two protons “populations”, i.e., two water proton types.

If protons can exchange between two different environments (**b**, **c**, in which they have τ_b and τ_c lifetimes), with increasing exchange rate the observable parameters (T_2, T_1) deviate from those “inherent” to **b** and **c**. In the “fast exchange” regime ($T_{2,1} \gg \tau_{b,c}$) the measured relaxation rate $(1/T_{2,1})$ is an average relaxation rate:

$$\frac{1}{T_{2,1}} = \frac{b(\%)}{T_{2,1b}} + \frac{c(\%)}{T_{2,1c}} \quad (5)$$

while, in the limit where the proton lifetimes τ_b, τ_c are infinite (“slow exchange” regime), the measured relaxation curve is the sum of the curves “inherent” to the environments **b** and **c** (Zimmerman and Brittin, 1957).

Our NMR results indicate that the possible exchange between $^1\text{H}_b$ and $^1\text{H}_c$ water protons is not fast compared to their T_{2b} ($10^{-1} \div 1$ ms), T_{2c} (> 10 ms) relaxation times.

Looking at the values of the ratio T_1/T_2 we estimated:

1. $T_{1b}/T_{2b} \approx 10^2\text{--}10^3$ for all situations analyzed, thus indicating a reduced microscopic mobility;
2. T_{1c}/T_{2c} varies from ~ 1.3 up to 3–4 as the equilibrium water content decreases, i.e., as the surface/volume ratio of the water microenvironments increases.

These values suggest a liquid-like dynamic for the water **c**. The reduced T_{2c}, T_{1c} values with respect to those of free water can be coherently explained by considering the “geometrical” confinement (restricted diffusion) experienced by the water molecules (Villa et al., 1983).

As previously described, from the FID analysis it is possible to get a graphical evaluation of the ratios $r = N_b/(N_b + N_c)$. As an example, we obtained $r = (90 \pm 10)\%$ for the sample with $R = 1\%$ and $T = 41^\circ\text{C}$ (see Fig. 5) and $r = (60 \pm 10)\%$ for $R = 1\%$ and $T \approx 20^\circ\text{C}$. It is noteworthy that the r values are in agreement with the values of the ratio W_{nf}/W_c obtainable from the data of Table 1 or Fig. 4 by using Eq. (1).

All these results suggest to identify the **b** and **c** fractions with non freezing and freezing water states determined by the DSC study.

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