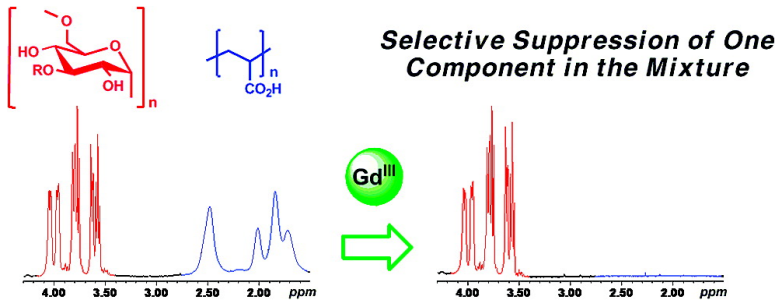
Article

Paramagnetic NMR Relaxation in Polymeric Matrixes: Sensitivity Enhancement and Selective Suppression of Embedded Species (H and C PSR Filter)

Eduardo Fernandez-Megia, Juan Correa, Ramon Novoa-Carballal, and Ricardo Riguera

J. Am. Chem. Soc., **2007**, 129 (49), 15164-15173 • DOI: 10.1021/ja0737117



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES
Published on Web 11/16/2007

Paramagnetic NMR Relaxation in Polymeric Matrixes: Sensitivity Enhancement and Selective Suppression of Embedded Species (^{1}H and ^{13}C PSR Filter)

Eduardo Fernandez-Megia,† Juan Correa, Ramon Novoa-Carballal, and Ricardo Riguera*

Contribution from the Departamento de Química Orgánica, Facultad de Química, and Unidad de RMN de Biomoléculas Asociada al CSIC, Universidad de Santiago de Compostela, Avenida de las Ciencias, S.N. 15782 Santiago de Compostela, Spain

Received May 23, 2007; E-mail: ricardo@usc.es

Abstract: A study of the practical applications of the addition of paramagnetic spin relaxation (PSR) ions to a variety of polymers (PLL, PAA, PGA, PVP, and polysaccharides such as hyaluronic acid, chitosan, mannan, and dextran) in solution (D_2O and DMSO-d_6) is described. Use of Gd^{III}, Cu^{II}, and Mn^{II} allows a reduction of up to 500% in the ^{1}H longitudinal relaxation times (T_1), and so in the time necessary for recording quantitative NMR spectra (sensitivity enhancement) neither an increase of the spectral line width nor chemical shift changes resulted from addition of any of the PSR agents tested. Selective suppression of the ^{1}H and ^{13}C NMR signals of certain components (low MW molecules and polymers) in the spectrum of a mixture was attained thanks to their different sensitivity [transverse relaxation times (T_2)] to Gd^{III} (PSR filter). Illustration of this strategy with block copolymers (PGA-*g*-PEG) and mixtures of polymers and low MW molecules (i.e., lactose−hyaluronic acid, dextran−PAA, PVP−glutamic acid) in 1D and 2D NMR experiments (COSY and HMQC) is presented. In those mixtures where PSR and CPMG filters alone failed in the suppression of certain components (i.e., PVP−mannan−hyaluronic acid) due to their similarity of ^{1}H T_2 values and sensitivities to Gd^{III}, use of the PSR filter in combination with CPMG sequences (PSR−CPMG filter) successfully resulted in the sequential suppression of the components (hyaluronic acid first and then mannan).

Introduction

A well-recognized handicap of the NMR spectroscopy of polymers in solution resides on the long time necessary to record quantitative spectra with an acceptable signal-to-noise ratio (S/N). This difficulty relies on the necessity of acquiring multiple pulses and the substantial differences in longitudinal relaxation times (T_1) between backbone and side chains,[1] which force the use of long repetition times (at least five times the longest T_1).[2] Although efforts to develop less time-consuming multidimensional experiments have recently appeared in the protein arena,[3] alternatives for routine NMR spectra of polymers are still awaited.

With the aim of developing faster NMR experiments for polymers in solution, we focused on use of paramagnetic spin relaxation (PSR) agents as a source of additional relaxation. Although it is known that nuclei relax faster [shorter T_1 and transverse (T_2) relaxation times] in the presence of PSR agents,[4,5] application of this concept for shortening repetition times and hence the time required to obtain good quality spectra has been limited so far to a few reports dealing with proteins exclusively. Thus, Wüthrich and co-workers reported that addition of a Gd^{III} chelate enhances the S/N of labile amide protons in large

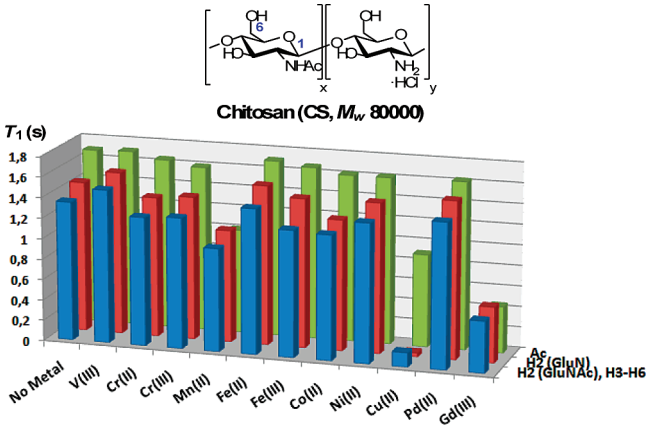


Figure 1. ^{1}H T_1 of CS (8 mg/mL in D_2O, 500 MHz) in the absence/presence of various PSR agents (1 mM).

† Dr. Eduardo Fernandez-Megia is a Ramón y Cajal fellow.

(1) Bovey, F. A.; Mirau, P. A. *NMR of Polymers*; Academic Press: San Diego, 1996; pp 17−18 and 358−360.
(2) Rabenstein, D. L.; Keire, D. A. Quantitative Chemical Analysis by NMR. In *Modern NMR Techniques and their Application in Chemistry*; Popov, A. I., Hallenga, K., Eds.; Marcel Dekker: New York, 1991; pp 323−369.
(3) (a) Frydman, L. *C. R. Chimie* **2006**, *9*, 336. (b) Pervushin, K.; Vögeli, B.; Eletsky, A. *J. Am. Chem. Soc.* **2002**, *124*, 12898.
(4) Bertini, I.; Luchinat, C.; Aime, S. *Coord. Chem. Rev.* **1996**, *150*, 77.
(5) (a) Helm, L. *Prog. Nucl. Magn. Reson. Spectrosc.* **2006**, *49*, 45. (b) Bertini, I.; Luchinat, C.; Parigi, G. *Prog. Nucl. Magn. Reson. Spectrosc.* **2002**, *40*, 249. (c) Sharp, R.; Lohr, L.; Miller, J. *Prog. Nucl. Magn. Reson. Spectrosc.* **2001**, *38*, 115.

10.1021/ja0737117 CCC: $37.00

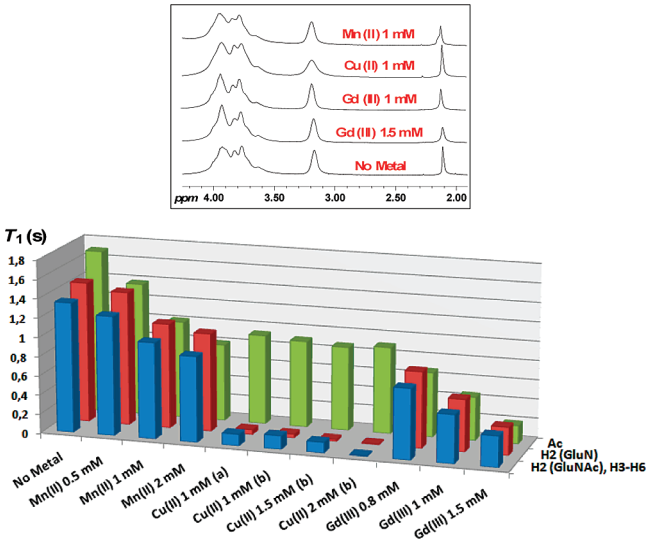


Figure 2. Influence of the PSR agent concentration on the ^{1}H T_1 of CS (8 mg/mL in D_2O, 500 MHz): (a) $CuCl_2$ and (b) $CuSO_4$.

proteins by selectively reducing the ^{1}H T_1 of water.[6] In other example, Pervushin and co-workers used a Gd^{III} chelate for reducing the ^{13}C T_1 in 2D ^{13}C NMR of proteins.[7] More recently, Chen and co-workers used a Ni^{II} chelate to enhance the sensitivity of multidimensional experiments by reducing ^{1}H T_1 of proteins.[8]

In the case of polymers with a molecular weight above 10 000 Da (rotational correlation times $\tau_r = 10^{-5}-10^{-7}$ s)[9] placed in a paramagnetic environment, τ_r is expected to be larger than the electronic relaxation time (τ_s). Under these circumstances, the Solomon−Bloembergen−Morgan equations indicate that τ_s should have a sizable contribution to the overall correlation time (τ_c).[10] As a consequence, comparable enhancements in longitudinal and transverse relaxation rates (R_1 and R_2) should develop for polymers interacting through a fast chemical exchange with a PSR agent in molar defect.[4,8] This positive paramagnetic relaxation effect should result even with PSR metal ions having long τ_s, such as Gd^{III} ($\tau_s \approx 10^{-8}$ s), leading to significant reductions on T_1 and smaller effects on T_2 and on the line width of the otherwise typically broad signals of polymers.

The presence of functional groups with metal complexing ability along the polymer chain (hydroxyl, ether, amino, amide, or carboxylic acid groups for example) favors polymer−PSR metal-ion complexation through a fast chemical exchange, whose magnitude will determine the extent of the paramagnetic effect.[4,11] Consequently, from a practical point of view, the PSR agent concentration should certainly have to be tuned for each polymer when pursuing a specific PSR enhancement.

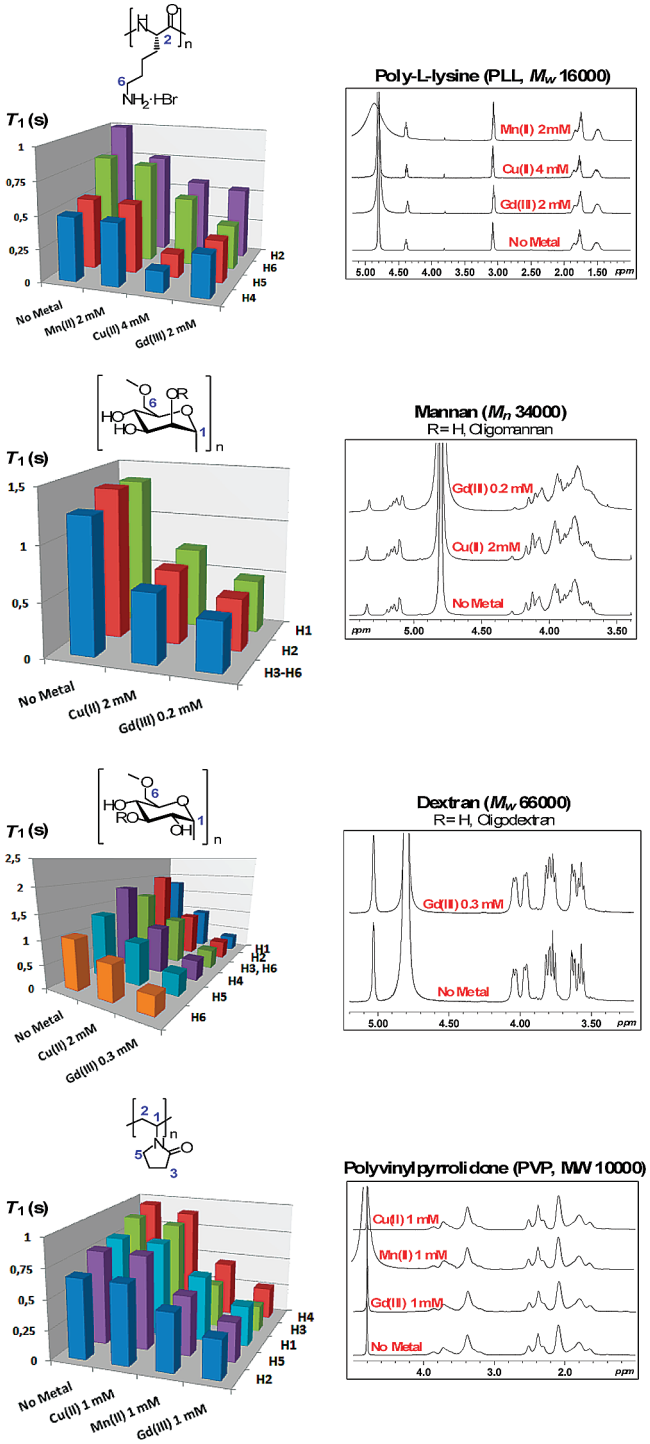


Figure 3. ^{1}H T_1 and ^{1}H NMR spectra of representative polymers (8 mg/mL in D_2O, 500 MHz) in the absence/presence of various PSR agents.

Herein we report the reduction of relaxation times (T_1 and T_2) of polymers by PSR metal ions and its practical application to effectively shorten the instrumental time for recording good quality quantitative ^{1}H NMR spectra (sensitivity enhancement). In addition, we will show that the different sensitivity of the species embedded in a polymeric matrix/mixture to the action of the PSR ions can be used to selectively suppress the signals (^{1}H and ^{13}C in 1D and 2D NMR experiments) of the more sensitive component in what constitutes a ^{1}H and ^{13}C PSR filter.

(6) Hiller, S.; Wider, G.; Etezady-Esfarjani, T.; Horst, R.; Wüthrich, K. *J. Biomol. NMR* **2005**, *32*, 61.
(7) Eletsky, A.; Moreira, O.; Kovacs, H.; Pervushin, K. *J. Biomol. NMR* **2003**, *26*, 167.
(8) Cai, S.; Seu, C.; Kovacs, Z.; Sherry, A. D.; Chen, Y. *J. Am. Chem. Soc.* **2006**, *128*, 13474.
(9) Dais, P.; Tylianakis, E.; Kanetakis, J.; Taravel, F. R. *Biomacromolecules* **2005**, *6*, 1397.
(10) (a) Kowalewski, J.; Nordenskiöld, L.; Benetis, N.; Westlund, P.-O. *Prog. Nucl. Magn. Reson. Spectrosc.* **1985**, *17*, 141. (b) Bloembergen, N.; Morgan, L. O. *J. Chem. Phys.* **1961**, *34*, 842. (c) Bloembergen, N. *J. Chem. Phys.* **1957**, *27*, 572. (d) Solomon, I. *Phys. Rev.* **1955**, *99*, 559.
(11) Bakhmutov, V. I. *Practical NMR Relaxation for Chemists*; John Wiley & Sons Ltd.: Chichester, 2004; Chapter 12.

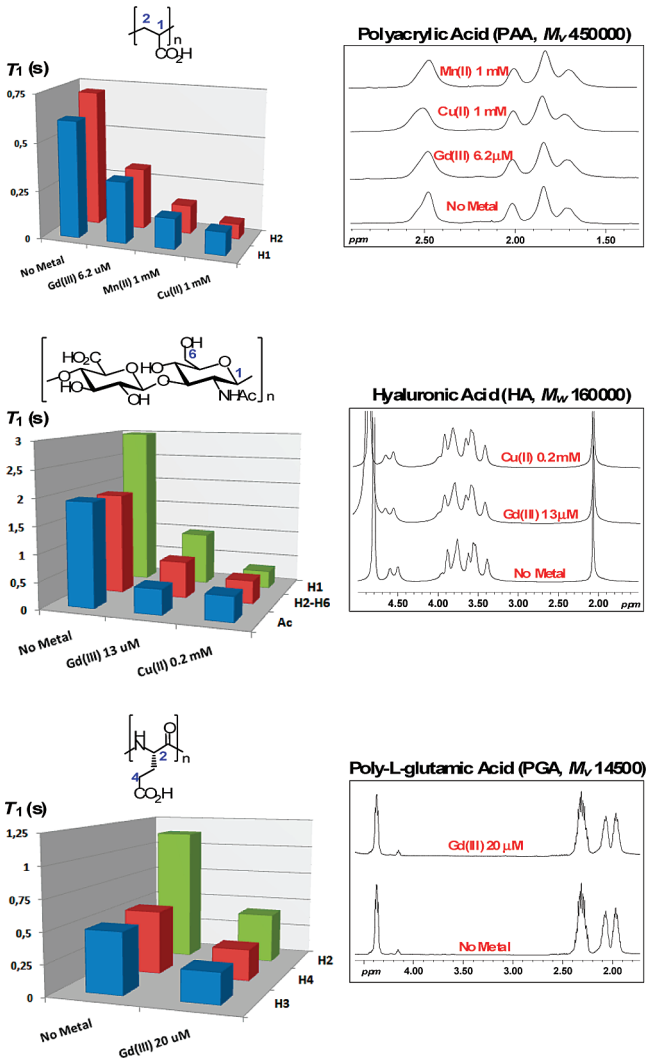


Figure 4. ^{1}H T_1 and ^{1}H NMR spectra of representative acidic and polyanionic polymers (8 mg/mL in D_2O, 500 MHz) in the absence/presence of various PSR agents.

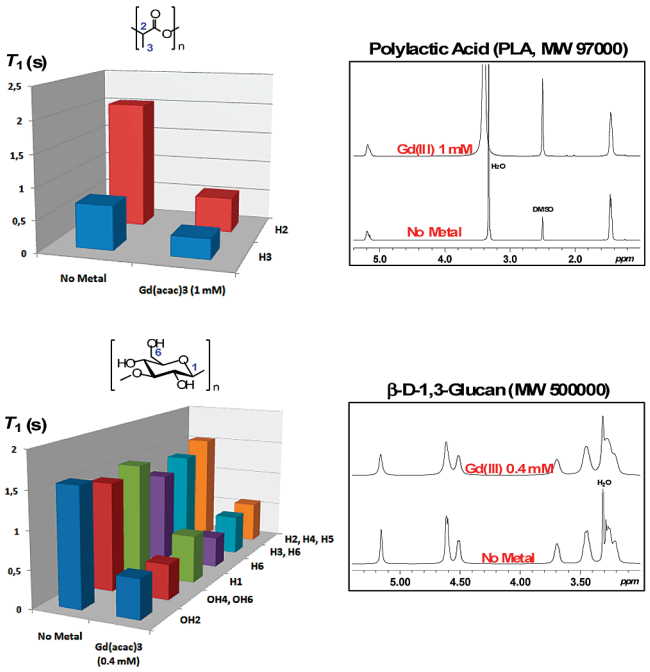


Figure 5. ^{1}H T_1 and ^{1}H NMR spectra of PLA and β-D-1,3-glucan (8 mg/mL in DMSO-d_6, 500 MHz) in the absence/presence of Gd(acac)$_3$.

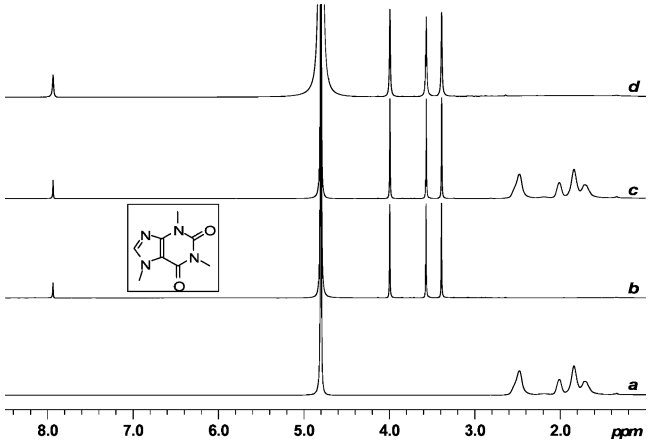


Figure 6. ^{1}H PSR filter. ^{1}H NMR spectra (D_2O, 500 MHz) of PAA (a), caffeine (b), and a mixture of PAA (1.5 mg/mL) and caffeine (1 mg/mL) in the absence (c) and presence of Gd^{III} (25 μM) (d).

Results and Discussion

Sensitivity Enhancement of Polymers by Paramagnetic Relaxation. In order to check the feasibility of reducing the T_1 of polymeric systems in general by addition of a PSR agent, we screened first the effect of a fixed concentration of various paramagnetic metal ions (V^{III}, Cr^{II}, Cr^{III}, Mn^{II}, Fe^{II}, Fe^{III}, Co^{II}, Ni^{II}, Cu^{II}, Pd^{II}, Gd^{III}; 1 mM in D_2O) on the ^{1}H T_1 and line width of chitosan (CS, M_w 80 000), a biocompatible and biodegradable biopolymer taken as a representative example (Figure 1).[12] For these experiments we selected the ^{1}H nucleus because of its high gyromagnetic ratio.[11] As for the PSR agent, nonchelated metal ions were tested based on their greater capacity for enhancing the relaxation of macromolecular protons.[6]

From these assays Gd^{III}, Cu^{II}, and Mn^{II} were the only effective ions (at the 1 mM concentration of the experiments) in reducing the ^{1}H T_1 of CS, in agreement with their highest τ_s.[4] In contrast, the other ions tested led to T_1 values similar to those obtained in the absence of the PSR agent. The marked paramagnetic enhancement of the CS backbone protons in the case of Cu^{II} results from the well-known strong and selective CS complexation of Cu^{II}.[13]

Subsequently, the effect of Gd^{III}, Cu^{II}, and Mn^{II} at different concentrations on the ^{1}H T_1 of CS was studied (Figure 2). By increasing the concentration of the three PSR agents a significant reduction of all ^{1}H T_1 was always achieved with Gd^{III} (1.5 mM) leading to T_1 values around 0.2−0.3 s, which stand for a reduction of more than 500% in the NMR time necessary to get a quantitative ^{1}H NMR spectrum of CS with comparable S/N. Significantly, neither an appreciable increase of the spectral line width nor chemical shift changes resulted from addition of any of the PSR agents tested. In addition, no influence of the counterion on the relaxation enhancement was observed.

Application of this strategy to a variety of polymers/biopolymers representative of different classes is depicted in

(12) Ravi Kumar, M. N. V.; Muzzarelli, R. A. A.; Muzzarelli, C.; Sashiwa, H.; Domb, A. J. *Chem. Rev.* **2004**, *104*, 6017.

(13) (a) Skorik, Y. A.; Gomes, C. A. R.; Podberezskaya, N. V.; Romanenko, G. V.; Pinto, L. F.; Yatluk, Y. G. *Biomacromolecules* **2005**, *6*, 189. (b) Varma, A. J.; Deshpande, S. V.; Kennedy, J. F. *Carbohydr. Polym.* **2004**, *55*, 77.

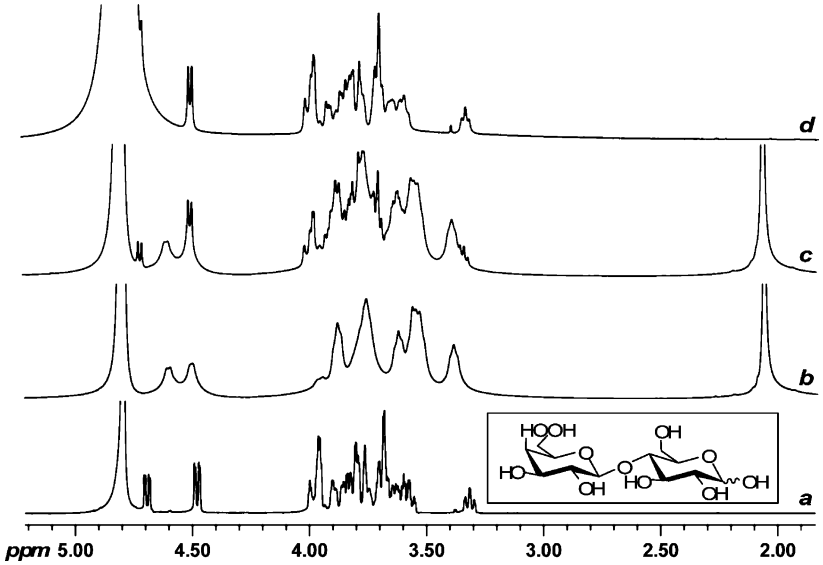


Figure 7. ^{1}H PSR filter. ^{1}H NMR spectra (D_2O, 500 MHz) of lactose (a), HA (b), and a mixture of lactose (1 mg/mL) and HA (8 mg/mL) in the absence (c) and presence of Gd^{III} (0.4 mM) (d). Similar results were obtained in the COSY of the same lactose−HA mixture, indicating the suitability of the present approach for 2D NMR experiments (Figure 8). Thus, while the anomeric and H2 cross-peaks of HA are clearly seen (red cycles) in the COSY spectrum of the mixture (Figure 8b), these peaks are completely removed after addition of Gd^{III} (0.4 mM, Figure 8c), leading to a COSY spectrum identical to that of lactose in Figure 8a.

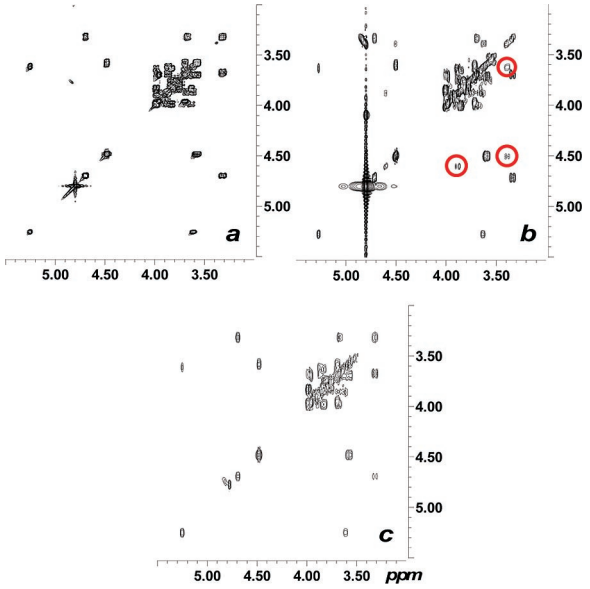


Figure 8. COSY PSR filter. COSY spectra (D_2O, 500 MHz) of lactose (a) and a mixture of lactose (1 mg/mL) and HA (8 mg/mL) in the absence (b) and presence of Gd^{III} (0.4 mM) (c). Red cycles indicate the HA cross-peaks in the mixture.

Figures 3 and 4: basic polymers [poly-L-lysine (PLL)], acidic and polyanionic polymers [poly(acrylic acid) (PAA), hyaluronic acid (HA), and poly-L-glutamic acid (PGA)], neutral polysaccharides (mannan and dextran), and neutral synthetic polymers [polyvinylpyrrolidone (PVP)]. In each case, the PSR agent concentration (Gd^{III}, Cu^{II}, and Mn^{II}) was pursued for a maximum reduction of T_1 ($T_1 \leq 0.5$ s to allow recording of quantitative ^{1}H NMR spectra with repetition times of 2.5 s, a standard value in routine experiments) while leaving unaffected the line width and chemical shifts.

Indeed, addition of minute concentrations of Gd^{III}, Cu^{II}, and Mn^{II} proved to be quite practical for reduction of all the ^{1}H T_1 of these polymers in D_2O. Gd^{III} led again to the highest relaxation enhancements with concentrations in the range 0.3−2.0 mM typically affording ^{1}H $T_1 \leq 0.5$ s with line widths and chemical shifts unaffected. This PSR efficiency of Gd^{III} results from its high τ_s value and the large number of unpaired electrons.[4]

These results indicate that in order to get the same PSR enhancement as Gd^{III}, higher concentrations of Cu^{II} and Mn^{II} are required. Their relative PSR efficiency seems to be polymer dependent with Mn^{II} being very effective with PVP and Cu^{II} with PAA and CS.

Interestingly, acidic and polyanionic polymers (PAA, HA, and PGA) were much more sensitive to the effect of the PSR agent due to electrostatic interactions (Figure 4).[14] Thus, for these polymers the concentration of Gd^{III} required for an effective relaxation could be reduced down to 5−20 μM, 100 times lower than for the other polymers studied.

We will show (vide infra) that this huge difference of sensitivity to the paramagnetic ion can be profitably utilized in the NMR of mixtures of species embedded in polymeric matrixes.

The paramagnetic sensitivity enhancement attained by addition of PSR agents has also been demonstrated for polymers dissolved in organic solvents (DMSO-d_6) (Figure 5). For this purpose, Gd^{III} was selected as the PSR metal ion of choice based on the above results in aqueous media. An organosoluble Gd^{III} complex, such as gadolinium(III) acetylacetonate [$Gd(acac)_3$], was selected based on its favorable solubility properties, commercial availability, and low cost. Indeed, when solutions of two representative organosoluble polymers, polylactic acid (PLA) and β-D-1,3-glucan, dissolved in DMSO-d_6 were treated with $Gd(acac)_3$, again a reduction of up to 360% in all the ^{1}H T_1 of these polymers was achieved, leading to ^{1}H T_1 values in the range of 0.5 s without significantly affecting line widths and chemical shifts.

(14) Yoshioka, N.; Nishide, H.; Tsuchida, E. *Inorg. Chim. Acta* **1987**, *128*, 135.

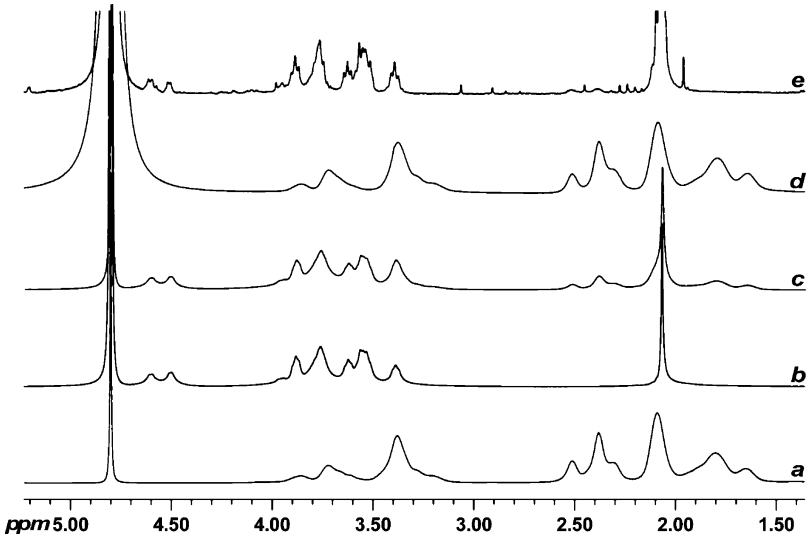


Figure 9. ^{1}H PSR filter. ^{1}H NMR spectra (D_2O, 500 MHz) of PVP_{360000} (a), HA (b), and a mixture of PVP_{360000} (1 mg/mL) and HA (2.2 mg/mL) in the absence (c) and presence of Gd^{III} (0.2 mM) (d). A mixture of PVP_{360000} (4 mg/mL) and HA (20 mg/mL) after a CPMG filter (225 ms) (e).

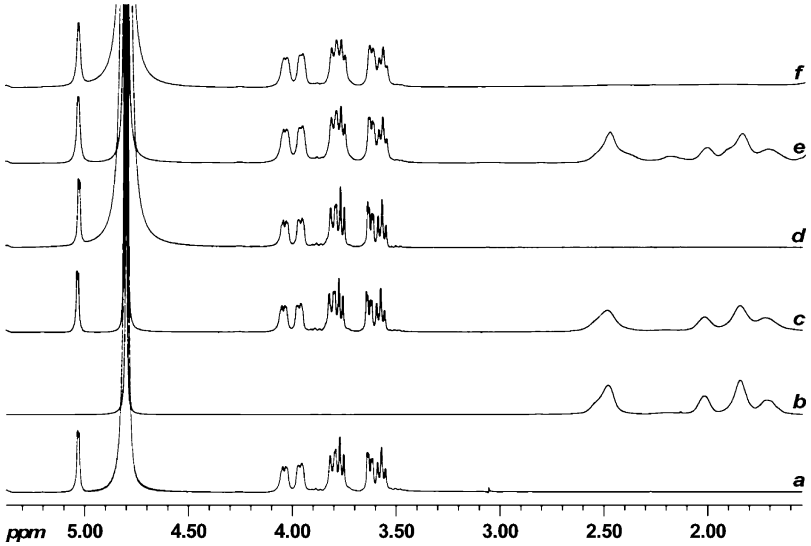


Figure 10. ^{1}H PSR filter. ^{1}H NMR spectra (D_2O, 500 MHz) of dextran (a), PAA_{450000} (b), and a mixture of dextran (1.5 mg/mL) and PAA_{450000} (1.5 mg/mL) in the absence (c) and presence of Gd^{III} (0.1 mM) (d). A mixture of dextran (1.5 mg/mL) and PAA_{1800} (1.5 mg/mL) in the absence (e) and presence of Gd^{III} (0.1 mM) (f).

Selective Suppression of the ^{1}H NMR Signals of Species Embedded in Polymeric Matrixes (^{1}H PSR Filter). The different response of the polymers to the PSR ions causes certain polymers to require a concentration of PSR ion up to 100−300 times higher than others for pursuing comparable sensitivity enhancements. This fact has prompted us to explore the use of PSR agents for selective suppression of signals of species within polymeric matrixes and mixtures (PSR filter). The basis for this idea resides on the inverse proportionality between the line width and T_2. Thus, by addition of PSR metal ions at concentrations higher than those required for the above sensitivity enhancement experiments, a drastic reduction not only of T_1 but also of T_2 (with a concomitant line width broadening) should develop for the constituents of a mixture with the highest sensitivity to the PSR agent. Eventually, at higher PSR agent concentrations these signals could embed in the baseline[15] while leaving unaffected those of the other less sensitive components.

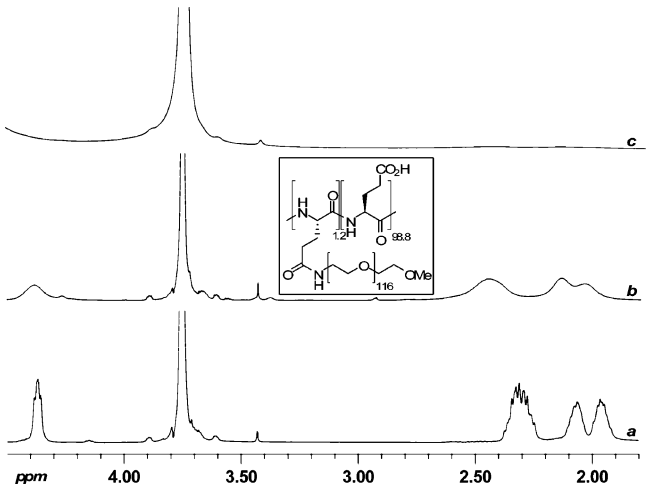


Figure 11. ^{1}H PSR filter. ^{1}H NMR spectra (D_2O, 500 MHz) of a mixture MeO-PEG-OH_{5000} (2.1 mg/mL) and PGA (3.75 mg/mL) (a) and of the block copolymer PGA-*g*-PEG (6 mg/mL) in the absence (b) and presence of Gd^{III} (1.0 mM) (c).

(15) La Mar, G. N.; de Ropp, J. S. In *Biological Magnetic Resonance*; Berliner, L. J., Reuben, J., Eds.; Plenum Press: New York, 1993; Vol. 12, pp 1−78.

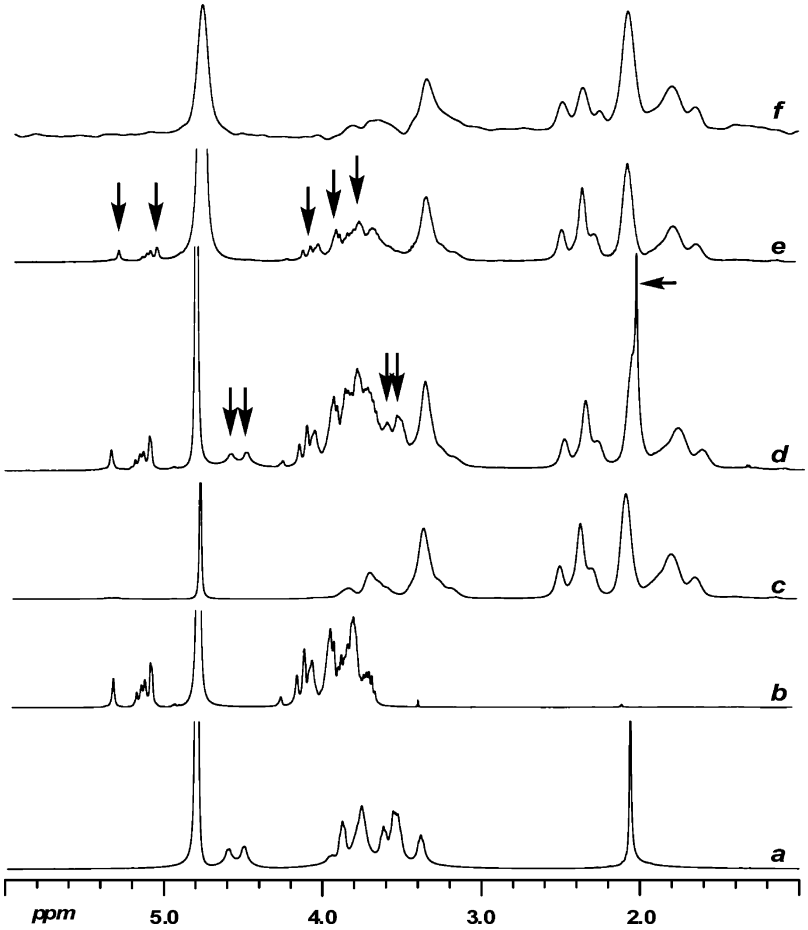


Figure 12. ^{1}H PSR−CPMG filter. ^{1}H NMR spectra (D_2O, 500 MHz) of HA (a), mannan (b), PVP_{10000} (c), a mixture of HA (1 mg/mL)−mannan (3 mg/mL)−PVP_{10000} (1 mg/mL) (d), the same mixture with Gd^{III} (0.05 mM)−CPMG filter (3 ms) (e), and the same mixture with Gd^{III} (0.35 mM)−CPMG filter (125 ms) (f). Arrows point to the signals to be removed by the PSR−CPMG filters.

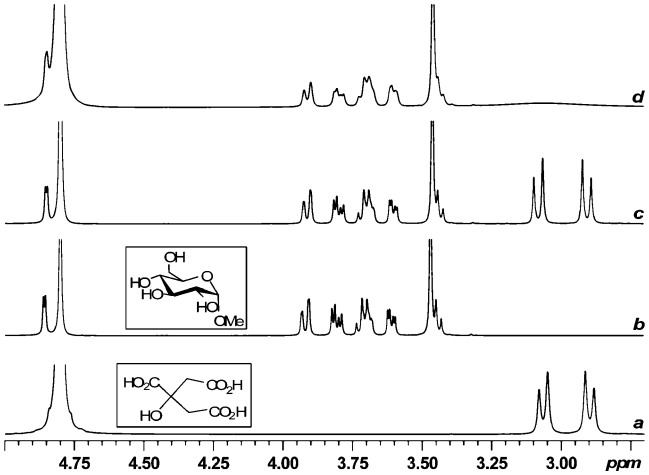


Figure 13. ^{1}H PSR filter. ^{1}H NMR spectra (D_2O, 500 MHz) of citric acid (a), methyl-α-D-glucopyranoside (b), and a mixture of citric acid (4 mg/mL) and methyl-α-D-glucopyranoside (4 mg/mL) in the absence (c) and presence of Gd^{III} (0.4 mM) (d).

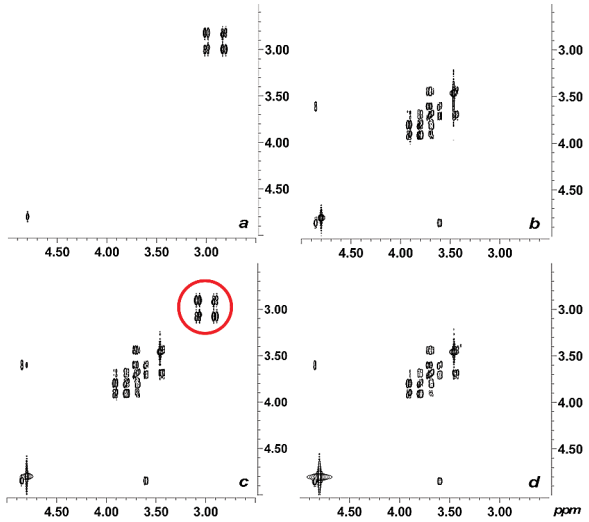


Figure 14. COSY PSR filter. COSY spectra (D_2O, 500 MHz) of citric acid (a), methyl-α-D-glucopyranoside (b), and a mixture of citric acid (4 mg/mL) and methyl-α-D-glucopyranoside (4 mg/mL) in the absence (c) and presence of Gd^{III} (0.4 mM) (d). Red cycle indicates the citric acid cross-peaks in the mixture.

This hypothesis was confirmed when Gd^{III} was added to the NMR tube containing a mixture of caffeine and PAA in D_2O. Figure 6c shows the spectrum of a caffeine−PAA mixture where the three methyl and CH signals of caffeine and those of PAA are clearly identified. After addition of Gd^{III} (25 μM) the resonances corresponding to PAA (the most sensitive species due to the carboxylic acids) were completely removed and only those of caffeine (the less sensitive species) were clearly visualized (Figure 6d).

In another example a mixture of lactose and HA was treated with Gd^{III}. In the spectrum of the mixture (Figure 7c) the lactose signals cannot be easily identified due to its low proportion and

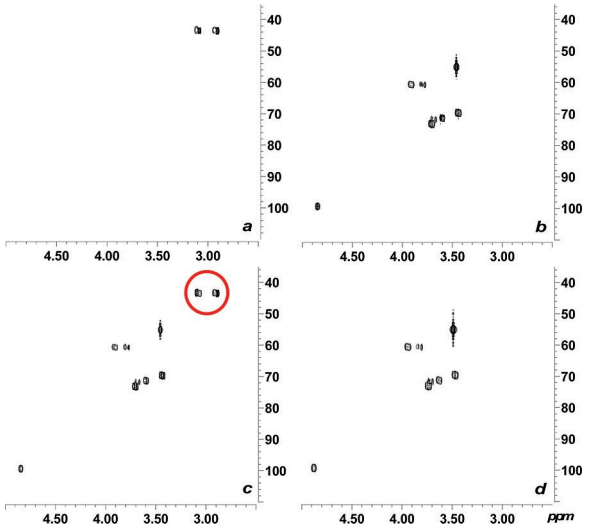


Figure 15. HMQC PSR filter. HMQC spectra (D_2O, 500 MHz) of citric acid (a), methyl-α-D-glucopyranoside (b), and a mixture of citric acid (4 mg/mL) and methyl-α-D-glucopyranoside (4 mg/mL) in the absence (c) and presence of Gd^{III} (0.4 mM) (d). Red cycle indicates the citric acid cross-peaks in the mixture.

similar chemical shifts to HA. However, after addition of Gd^{III} (0.4 mM) the resonances corresponding to the most sensitive species to Gd^{III} (HA) were completely removed and only those of lactose clearly visualized (Figure 7d), leading to a spectrum of comparable quality and S/N to the one of lactose shown in Figure 7a.

Proof of the generality of the PSR filter was obtained when applied to mixtures of polymers having more alike ^1H T_2 values but different metal complexing abilities, such as polyacids and neutral polymers. Again, in this case effective removal of the resonances due to the polyacid was produced after addition of Gd^{III}, resulting in a clean spectrum of the neutral polymer independent of the polyacid molecular weight.

Thus, Figure 9c shows the spectrum of a mixture $HA_{160000}-PVP_{360000}$ where the PVP signals are not clearly visualized due to its lower proportion in the mixture. After addition of Gd^{III} (0.2 mM), the HA signals were completely removed, leading to a spectrum of PVP_{360000} (Figure 9d) with identical line width, chemical shifts, and S/N to the one of PVP_{360000} alone in Figure 9a. When a lower molecular weight PVP_{10000} was employed in the mixture ($HA_{160000}-PVP_{10000}$), effective removal of the HA signals was again produced by addition of Gd^{III}, in accordance with its higher sensitivity to Gd^{III} independent of molecular weight.

Interestingly, a standard CPMG filter (225 ms) applied to the $HA_{160000}-PVP_{360000}$ mixture was revealed complementary to the PSR filter, allowing selective and complete removal of PVP_{360000}, the species with shorter ^1H T_2 values (Figure 9e). It should be emphasized, however, that the CPMG filter produces a HA spectrum with a different relative intensity of the signals and lower S/N than that of HA in Figure 9b.

When the molecular weight of the PVP in the mixture was reduced ($HA_{160000}-PVP_{10000}$), application of the CPMG filter was shown to be nonselective in this case (due to its molecular weight dependence), contrary to the success shown again by the PSR filter.

Application of the same concept to mixtures of PAA−dextran was also possible. Thus, when Gd^{III} (0.1 mM) was added to mixtures of PAA of various molecular weights (PAA_{1800} or PAA_{450000}) and $dextran_{66000}$ (Figure 10c and 1e), removal of the resonances due to PAA was always obtained independent of its molecular weight, resulting in a clean spectrum of dextran with unaffected line width and chemical shifts (Figure 10d and f). These results contrast again with the outcome of standard CPMG filters over the same mixtures, which selectively removed the PAA_{450000} signals, but was shown to be nonselective with the PAA_{1800}/$dextran_{66000}$ mixture. In brief, while CPMG filter depends on differences of T_2 which are greatly dependent on molecular weight, PSR filter depends on differences of sensitivity to the PSR agent, which are nearly insensitive to molecular weight.

Using the same approach, we reasoned that block copolymers could benefit from application of PSR strategies by simplifying spectra, allowing visualization of only the less sensitive of the blocks. This could be of interest for end group analysis or when dealing with blocks showing overlapping resonances.

Figure 11b shows the spectrum of a graft copolymer PGA-*g*-PEG (degree of PEGylation 1.2%), where the PEG block is easily identified by the presence of an intense signal at around 3.7 ppm. Formation of a strong IPN[16] between the blocks is revealed by the broader signals of the PGA block in the ^1H NMR of the copolymer (Figure 11b) when compared with the NMR results of a mixture of the two polymers (Figure 11a). Application of the PSR technology to PGA-*g*-PEG was possible by addition of Gd^{III} (1.0 mM). The signals corresponding to PGA (the block with the highest complexing ability) were completely removed from the spectrum of the copolymer, allowing visualization of the PEG block only (Figure 11c).

Combination of PSR and CPMG Filters. When dealing with polymers having not only similar ^1H T_2 values but also similar sensitivities to Gd^{III}, application of PSR and CPMG filters alone was impracticable. However, selective removal of the components with the highest sensitivities to Gd^{III} could be accomplished with the aid of a CPMG filter in conjunction with addition of Gd^{III} (PSR−CPMG filter). Application of this strategy to a three-component mixture (HA/mannan/PVP_{10000}, Figure 12) was possible and allowed sequential removal of the signals of the polymers according to their sensitivity to Gd^{III}. Thus, the signals of HA were selectively removed by simultaneous addition of Gd^{III} (0.05 mM) and application of a short CPMG filter of 3 ms (Figure 12e). By increasing the concentration of Gd^{III} (0.35 mM) and length of the CPMG filter (125 ms), the signals of mannan were then removed, ultimately allowing visualization of a clean spectrum of the less sensitive PVP (Figure 12f).

Interestingly, use of CPMG filters alone was unfeasible owing to its lack of selectivity. Thus, application of the first CPMG filter (3 ms) without Gd^{III} over the original mixture has affects similar to the three components. While application of the second CPMG filter (125 ms) in the presence of 0.05 mM Gd^{III} affected was more pronounced for the PVP signals than the mannan ones (although without complete selectivity).

PSR Filter in the Suppression of Low Molecular Weight Components. Application of the PSR filter methodology for

(16) (a) Park, Y.-J.; Liang, J.; Yang, Z.; Yang, V. C. *J. Controlled Release* **2001**, *75*, 37. (b) Tanaka, T.; Mori, T.; Tsutsui, T.; Ohno, S.; Tanaka, R. *J. Macromol. Sci. Phys.* **1980**, *B17*, 723. (c) Mori, T.; Tanaka, R.; Tanaka, T. *J. Appl. Polym. Sci.* **1978**, *22*, 2817. (d) Tsutsui, T.; Tanaka, T. *Chem. Lett.* **1976**, 1315.

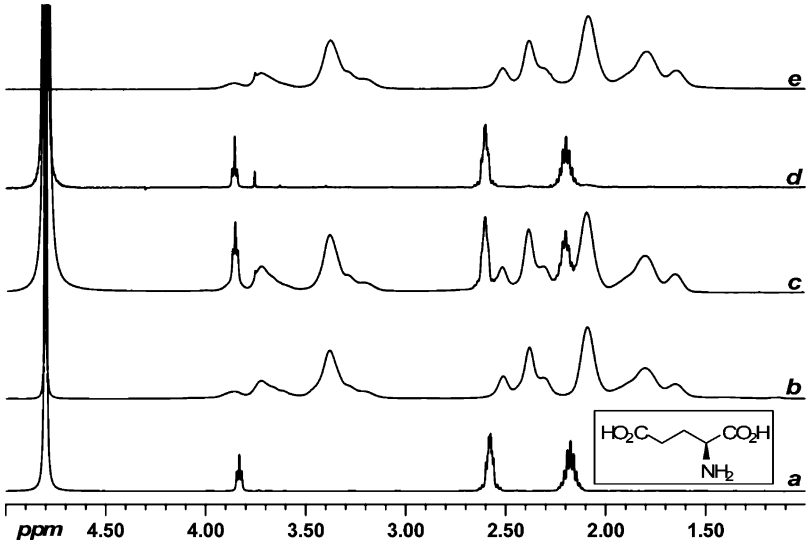


Figure 16. ^{1}H PSR filter. ^{1}H NMR spectra (D_2O, 500 MHz) of glutamic acid (a), PVP_{360000} (b), a mixture of glutamic acid (1.5 mg/mL) and PVP_{360000} (1.5 mg/mL) (c), the same mixture after a CPMG filter (225 ms) (d), and the same mixture after addition of Gd^{III} (0.6 mM) (e).

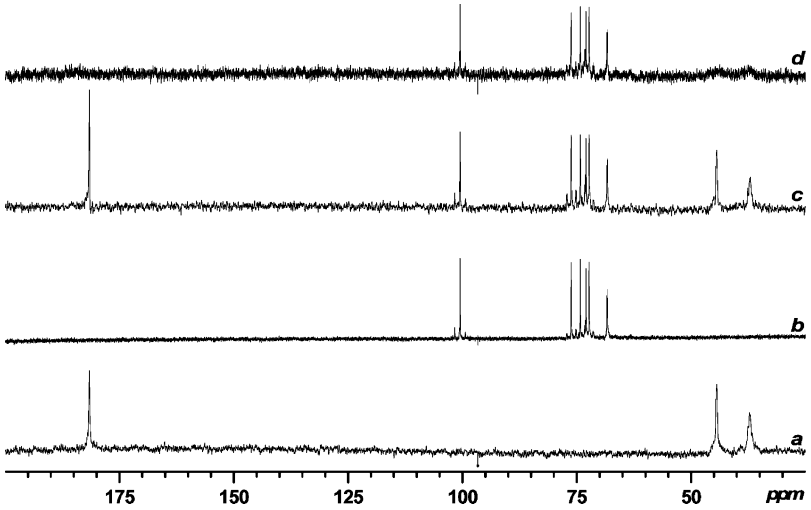


Figure 17. ^{13}C PSR filter. ^{13}C NMR spectra (D_2O, 300 MHz) of PAA (a), dextran (b), and a mixture of PAA (20 mg/mL) and dextran (20 mg/mL) in the absence (c) and presence of Gd^{III} (2.0 mM) (d). Likewise, when a mixture of citric acid and methyl-α-D-glucopyranoside was treated with Gd^{III} (3.4 mM), the resonances due to citric acid could be completely removed from the ^{13}C NMR of the mixture (Figure 18d).

the selective suppression of the ^{1}H signals of low molecular weight molecules was envisioned as a more challenging task than when applied to polymers. Thus, in molar defect of the PSR ion, simultaneous complexation of various ions to a single polymer chain facilitates the spread of the paramagnetic relaxation along the chain in contrast to low molecular weight species, where the paramagnetic effect is restricted only to the atoms within the molecules complexed at any time. Also, the inherent multidentate nature of polymers increases the stability of the polymer−metal complex (slows down the chemical exchange) and so enhances the extent of the paramagnetic effect. In addition, low molecular weight molecules are characterized by higher ^{1}H T_2 values than polymers.

In accordance with all these shortcomings, the PSR filter was indeed more demanding for suppression of the signals of low molecular weight molecules than for polymers, although it was completely successful when dealing with highly complexing multidentate molecules.

Thus, for example, when a mixture of citric acid and methyl-α-D-glucopyranoside (Figure 13c) was treated with Gd^{III} (0.4 mM), the two characteristic doublets of citric acid at around 3.0 ppm completely disappeared from the NMR spectrum of the mixture, as a consequence of its preferential complexation to Gd^{III} (Figure 13d). This resulted in a clean spectrum of methyl-α-D-glucopyranoside with comparable resolution and S/N to the one shown in Figure 13b.

The same mixture of citric acid and methyl-α-D-glucopyranoside serves to illustrate again the suitability of the PSR filter for 2D NMR experiments. Thus, the citric acid cross-peaks (red cycles) in the COSY and HMQC spectra of the mixture (Figures 14c and 15c) are completely removed after addition of Gd^{III} (0.4 mM), resulting in clean spectra showing the signals of the sugar exclusively (Figures 14d and 15d).

In a particularly difficult case because of the huge difference of T_2 of the components, the ^{1}H signals of glutamic acid embedded in a PVP_{360000} matrix could be selectively suppressed from the ^{1}H NMR spectrum of the mixture (Figure 16c) after addition of Gd^{III} (0.6 mM). A clean spectrum of PVP_{360000} (the species with the originally shortest ^{1}H T_2 values) was obtained

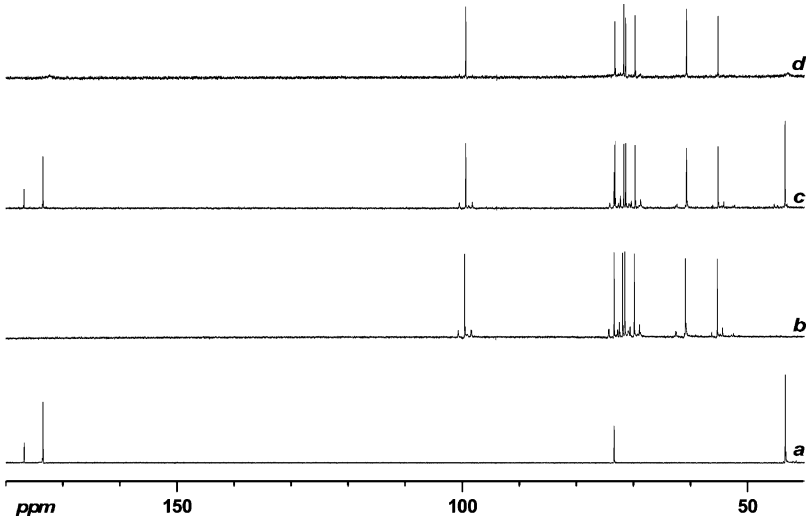


Figure 18. ^{13}C PSR filter. ^{13}C NMR spectra (D_2O, 300 MHz) of citric acid (a), methyl-α-D-glucopyranoside (b), and a mixture of citric acid (32 mg/mL) and methyl-α-D-glucopyranoside (52 mg/mL) in the absence (c) and presence of Gd^{III} (3.4 mM) (d).

(Figure 16e) with identical resolution and S/N to the one of PVP_{360000} alone shown in Figure 16b.

The complementarity between PSR and CPMG filters mentioned before is shown again in this case thanks to the high difference of ^1H T_2 values between PVP_{360000} and glutamic acid. Thus, when a CPMG filter (225 ms) was applied to the mixture, removal of the signals of PVP_{360000} was observed, allowing clean visualization of glutamic acid (Figure 16d).

Selective Suppression of the ^{13}C NMR Signals of Species Embedded in Polymeric Matrixes and Mixtures (^{13}C PSR Filter). Finally, application of the PSR filter for removal of ^{13}C resonances was considered. In this case and due to the 4-fold smaller gyromagnetic ratio of ^{13}C than ^1H, higher concentrations of the PSR agent were expected to be necessary for efficient ^{13}C signal suppression.

Indeed, ^{13}C PSR filter was successful when applied to mixtures of both polymers and low molecular weight molecules. Herein we present the results of the addition of Gd^{III} to the PAA−dextran and citric acid−methyl-α-D-glucopyranoside mixtures.

Figure 17c shows the ^{13}C NMR of a mixture PAA−dextran where the signals of both polymers are easily identified due to their characteristic chemical shifts. Application of a ^{13}C PSR filter to this mixture was possible by addition of Gd^{III} (2.0 mM), which led to removal of the three resonances due to PAA (the most sensitive species) and allowed visualization of only the dextran signals (Figure 17d).

Conclusions

Addition of minute concentrations of Gd^{III} and other transition-metal ions to the NMR tube containing a polymer in solution (D_2O and DMSO-d_6) leads to a substantial reduction (up to 500%) of all the ^1H T_1 in the sample and hence of the time necessary for recording quantitative ^1H NMR experiments (sensitivity enhancement). ^1H T_1 values in the range of 0.5 s were typically obtained, allowing use of repetition times of 2.5 s, a standard value in routine experiments. Significantly, neither an appreciable increase of the spectral line width nor chemical shift changes resulted from addition of the PSR agent. Acidic and polyanionic polymers were especially sensitive to the effect of the PSR agent due to their favorable metal complexing abilities through electrostatic interactions.

The differences of sensitivity to the PSR ions shown by the polymers studied were exploited to allow selective suppression of the NMR signals of the most sensitive components (PSR filter). Application of this concept for removal of ^1H and ^{13}C resonances of block copolymers and mixtures of polymers and low molecular weight molecules is presented. This approach has also been extended to 2D NMR experiments (COSY and HMQC), allowing selective visualization of the cross-peaks due to the less complexing components. In those cases dealing with polymers having similar ^1H T_2 values and sensitivities to Gd^{III} application of the PSR filter alone was impracticable while its combination with a standard CPMG filter (PSR−CPMG filter) led to clean and selective removal of the components with highest sensitivities to Gd^{III}.

Employment of the PSR filter to other polymer−molecule systems such as those present in many pharmaceutical presentations, natural extracts, biological fluids, and others is envisioned as a complement to standard CPMG and diffusion filters.

Experimental Section

All NMR experiments were performed on a 300 or 500 MHz spectrometer at 25 °C in D_2O (99.999% D). Some of the samples were lyophilized (D_2O) before the experiment was performed. T_1 values were measured using a standard inversion recovery pulse sequence (16 different relaxation delay times) at a polymer concentration of 8 mg/mL. All polymer samples were used as received unless otherwise noted. The following salts were used as the source of PSR agent: VCl_3, CrF_2, $Cr(NO_3)_3 \cdot 9H_2O$, $MnCl_2 \cdot xH_2O$, $FeSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $NiSO_4 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $CuCl_2$, $PdCl_2$, and $Gd_2(SO_4)_3 \cdot 8H_2O$.

Materials. Chitosan·HCl (CS) was obtained from Pronova Biomedical A.S. (M_w 80 000 by SEC-MALLS, degree of acetylation 14% by ^1H NMR). Poly-L-lysine·HBr (PLL) was purchased from Fluka (M_n 12 400, M_w 16 100, DP = 77, by LALLS; M_n 19 800, M_w 25 700, DP = 123, by viscosity). Mannan from *Saccharomyces cerevisiae* was obtained from Fluka (M_n 34 000, M_w 36 000, M_z 37 000, by SEC-MALLS). Dextran from *Leuconostoc mesenteroides* was purchased from Fluka (M_n 33 698, M_w 65 794, M_p 46 998, M_z 126 780 by GPC). Polyvinylpyrrolidone (PVP) samples were obtained from Fluka (average molecular weight 360 000) and Sigma (average molecular weight 10 000). PVP 10 000 was purified by precipitation from MeOH−Et_2O

before the NMR experiments. Poly(acrylic acid) (PAA) samples were purchased from Aldrich: PAA_{450000} (M_v ~450 000) and PAA_{1800} (M_n 1022, M_w 1773, M_z 3229). Hyaluronic acid (HA) was obtained from Bioiberica (M_w 160 000). Poly-L-glutamic acid (PGA) was obtained from Fluka (M_v 14 500). Polylactic acid (PLA) was purchased from Aldrich (molecular weight 75 000−120 000). β-D-1,3-Glucan from *Euglena gracilis* was obtained from Fluka (average molecular weight 500 000).

Synthesis of PGA-*g*-PEG. PGA (25 mg, 0.194 mmol of repetition unit, M_v 14 500) and MeO-PEG-NH_2 (14 mg, 2.69 μmol, M_n 5200 by MALDI-TOF) were dissolved in H_2O (1 mL). HOBt (3 mg, 22.2 μmol) and EDC (3 mg 19.7 μmol) were added. The reaction was allowed to stir overnight. Then, it was purified by ultrafiltration (Amicon YM30, H_2O, 15 × 30 mL) to afford 30.6 mg of PGA-*g*-PEG (degree of PEGylation 1.2% by ^{1}H NMR, 87% yield).

^{1}H NMR Data. *CS.* ^{1}H NMR (500 MHz, D_2O, 25 °C): δ 2.11 (s, Ac), 3.17 [br s, H2 (GluN)], 3.42−4.35 [m, H2 (GluNAc), H3−H6].

PLL. ^{1}H NMR (500 MHz, D_2O, 25 °C): δ 1.49−1.59 (m, H4), 1.75−1.93 (m, H3, H5), 3.07 (t, H6), 4.37 (t, H2).

Mannan. ^{1}H NMR (500 MHz, D_2O, 25 °C): δ 3.64−4.02 (m, H3−H6), 4.03−4.29 (m, H2), 5.06−5.37 (m, H1).

Dextran. ^{1}H NMR (500 MHz, D_2O, 25 °C): δ 3.57 (t, H4), 3.63 (dd, H2), 3.74−3.85 (m, H3, H6), 3.96 (d, H5), 4.04 (dd, H6), 5.03 (d, H1).

PVP_{10000}. ^{1}H NMR (500 MHz, D_2O, 25 °C): δ 1.55−1.89 (m, H2), 1.99−2.15 (m, H4), 2.34−2.59 (m, H3), 3.12−3.44 (m, H5), 3.55−3.77 (m, H1).

PAA_{450000}. ^{1}H NMR (500 MHz, D_2O, 25 °C): δ 1.52−2.12 (m, H2), 2.32−2.63 (m, H1).

HA. ^{1}H NMR (500 MHz, D_2O, 25 °C): δ 2.08 (s, Ac), 3.22−4.15 (m, H2−H6), 4.50 (br s, H1), 4.61 (br s, H1).

PGA. ^{1}H NMR (500 MHz, D_2O, 25 °C): δ 1.90−2.02 (m, H3), 2.03−2.14 (m, H3), 2.23−2.39 (m, H4), 4.37 (dd, H2).

PLA. ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C): δ 1.44−1.47 (m, H3), 5.09−5.20 (m, H2).

β-D-1,3-Glucan. ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C): δ 3.14−3.30 (m, H2, H4, H5), 3.37−3.52 (m, H3, H6), 3.64−3.76 (m, H6), 4.47−4.55 (br s, H1), 4.57−4.65 (m, OH4, OH6), 5.13−5.18 (br s, OH2).

Acknowledgment. This work was financially supported by the Spanish and Galicia Governments (NAN2004-09230-C04-03, CTQ2006-12222/BQU, and PGIDIT06PXIB20-9058PR). E.F.-M. and R.N.-C. thank the Spanish Government (MEC) for "Ramón y Cajal" and FPU fellowships, respectively.

JA0737117