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Seeing through Macromolecules: T_2-Filtered NMR for the Purity Assay of Functionalized Nanosystems and the Screening of Biofluids

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Once established as the standard technique for the determination of organic structures, NMR has rapidly evolved into one of the most powerful tools for investigating different properties of molecules and materials. High resolutions achieved nowadays ultimately have made it possible to obtain quality spectra even on complex matrices or crude mixtures, which often provide an overwhelming amount of information. For this reason, much effort in modern NMR spectroscopy has been devoted to the simplification of crowded spectra that invariably result from complex systems. Ranging from multiplicity editing to signal suppression and selective filtration, this area of research has generated such sophisticated techniques that a proper "spectral editing" is often preferable to more time-consuming preprocessing of the sample.

A common problem in the analysis of complex mixtures is the presence of large chemical entities such as macromolecules, polymers, or even nanoparticles. In this case, the broad signals of these species dominate the NMR spectra and impair the quantification, or even the detection, of small molecules. Samples with such features are commonplace in the analysis of biofluids because of the presence of proteins, lipids, and other macromolecules.[1] Another relevant but scarcely addressed field where similar problems are encountered is the purity assay of nanoparticles, whose use in medicine is now attracting enormous interest.[2] However, possible applications of nanoparticles as drugs must comply with strict requirements of chemical purity or at least with the determination of any contaminants, neither of which is an easy task. Many of the analytical methods (GC, HPLC, etc.) used to check the purity of organic compounds are not suitable for nanoparticles, while the most common techniques used for the characterization of nanosystems (TEM, SEM, etc.) give scarce information on chemical purity, particularly in regard to the presence of small-molecule contaminants. NMR spectroscopy could provide the solution to this problem, since it allows for the direct identification and quantification of the organic species present in the sample. To date, the problem of assessing the purity of functionalized nanoparticles by NMR has been tackled qualitatively by the use of simple pulse−acquire experiments[3] or more sophisticated translational diffusion filters.[4] In this latter case, taking advantage of the nanoparticle's reduced mobility, a diffusion filter of proper strength is calibrated to remove the signals of fast-diffusing species, so a visual inspection of the filtered and unfiltered spectra can tell whether any impurities or detached molecules are present.[5] Nonetheless, the opposite requirement applies in order to attempt any purity assay, namely, to obtain the spectrum of the impurity rather than that of the "clean" nanoparticle.

In this communication, we propose a new approach based on transverse relaxation (T_2) spectral editing that allows a reliable analysis of small molecular species in solutions of nanoparticles and macromolecules. Since large molecules in solution possess small rotational diffusivity and short T_2 spin relaxation times, it turns out that their broad spectral lines can be "filtered out" by keeping the magnetization in the transverse plane for relatively short periods of time. In our case, the efficacy of such a filter rests on the evidence that nuclear spins anchored to nanoparticles/macromolecules with little or no conformational degrees of freedom lose their phase coherence more rapidly than the slowly relaxing spins of small-sized, fast-tumbling molecules.

In order to refocus the chemical shift evolution during the wait period, T_2 filters exploit spin-echo trains of the kind $(\tau - \pi_\varphi - \tau)_n$, also known as Carr−Purcell or Carr−Purcell−Meiboom−Gill (CPMG) sequences, depending on the shift of the phase φ with respect to the first pulse.[6]

Unfortunately, J couplings generally lead to extra signal modulations when such echo trains are applied on a homonuclear spin system. These modulations can severely affect the spectra, since they convert in-phase to anti-phase magnetization that distorts the line shape and alters the integrated signal intensity. This phenomenon is clearly evident when a standard CPMG sequence is applied to the simple two-spin system of cytosine. As the echo trains increase in number (Figure 1, trace A), the shape and integrated intensity of the observed signals are modulated in such way that not only the quantification but even the identification of the resonant spins can be seriously compromised.

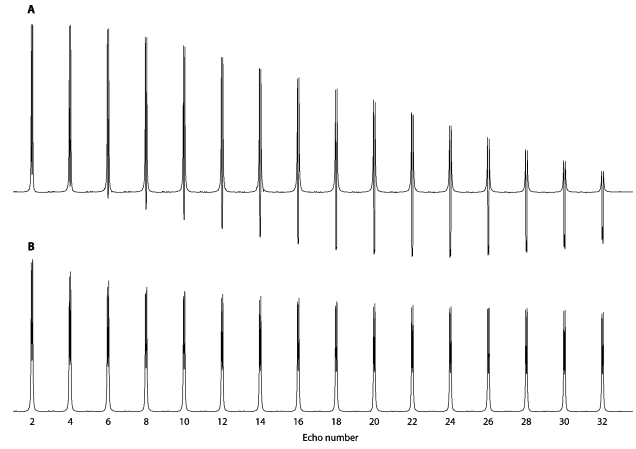

Figure 1. Even-numbered echoes obtained on a sample of 50 mM cytosine in D_2O with (A) the standard CPMG pulse sequence and (B) the CPMG-z pulse sequence described in the text. In both (A) and (B), τ is set to 1.5 ms and the low-field signal is observed ($J_{IS} = 7.2$ Hz). The echoes obtained in (A) display a rapidly growing contribution of anti-phase magnetization that distorts the line shape and alters the integrated signal intensity.

The spin dynamics of echo trains has recently been investigated in detail.[7] In the simple case of a two-spin system *IS*, such as that of cytosine, we may distinguish between slow and fast repetition rate limits, depending on the values of $1/(2\tau)$ and Ω_S (the offset frequency of S when I is set on-resonance with the radio-frequency carrier). In the former case, the average Hamiltonian reduces to

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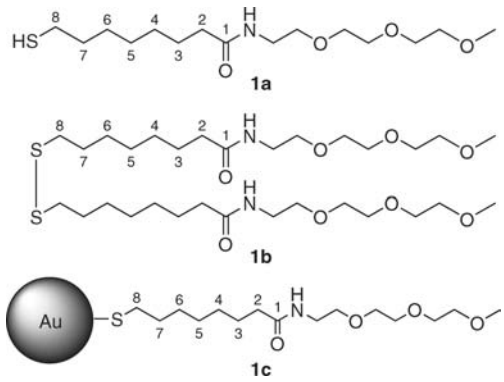
weak coupling and the echoes are *J*-modulated, while in the latter case, the average Hamiltonian resembles the mixing Hamiltonian of a TOCSY experiment,[8] where the echo modulations are removed because all of the spins behave as magnetically equivalent. The repetition rate employed in T_2 filters typically falls between the two aforementioned limits, so that the unwanted modulations may not be completely suppressed, as shown before. On the basis of this evidence, we devised a modified spin-echo train, which we have called CPMG-*z*, where the anti-phase magnetization generated during each $(\tau - \pi_\varphi - \tau)$ cycle is purged by means of a *z* filter[9a] in such way that the magnetization is reset to pure in-phase before the new echo starts.[9b] More specifically, if $k(J,\Omega,\tau)$ is the amplitude of the in-phase magnetization generated in a single echo $(\tau - \pi_\varphi - \tau)$, then the same magnetization emerging from the $(n + 1)$th echo will be $\sigma_{n+1} = k(J,\Omega,\tau)\sigma_n$. When this is recognized as the recursive relation of a geometric sequence, imposing the initial condition $\sigma_0 = I_x + S_x$ directly gives $\sigma_n = k^n(J,\Omega,\tau)[I_x + S_x]$. Since $-1 < k < 1$, the CPMG-*z* sequence should finally lead to an exponential decay of the echo signal, which adds to the transverse relaxation (Figure 1, trace B).[10]

Chart 1. Tri(ethyleneglycol) Thiol Derivatives **1a** and **1b** and Functionalized Gold Nanoparticle **1c**



In the context of purity assays, the use of the new CPMG-*z* sequence may have dramatic effects, as we report here. We selected 2 nm gold nanoparticles **1c** (Chart 1) protected with a monolayer of the tri(ethyleneglycol) thiol derivative **1a** as a typical representative of nanosystems for biomedical applications, where a surface passivating layer is used to promote nanoparticle stability toward aggregation along with water solubility and, in the case of poly(ethyleneglycol) derivatives, to ensure prolonged circulation times in vivo.[11] The excess of molecules used to assemble the organic monolayer may not be completely removed in the purification procedures, or the thiols may detach from the grafting surface with time, thereby becoming a potential contaminant of the preparation. Moreover, thiols easily form disulfides under oxidative conditions (including air exposure), so solutions of nanoparticles **1c** may also contain **1b** as a byproduct.

In a sample of nanoparticles **1c**, the diagnostic ^{1}H NMR signals of detached or unreacted **1a** and **1b** are provided by the methylene protons of C8: in $CDCl_3$ solutions, these protons appear as a quartet ($\delta = 2.5$ ppm) in the monomer **1a** and a triplet ($\delta = 2.7$ ppm) in the dimer **1b**. In contrast, the same protons disappear completely from the spectrum in **1c** because of their low mobility and ensuing fast relaxation.[12] With the aim of investigating the effect of T_2 filtration on the line shape of these target signals, we first applied both the CPMG and CPMG-*z* sequences to a sample containing **1a** and **1b** alone (Figure 2). As anticipated, not only did the standard CPMG sequence (trace C) drastically reduce the signal-to-noise ratio for both species, but also the quartet signal of **1a** almost disappeared from the spectrum. With this sequence, not only would the amount of the two species have been underestimated, but the very presence of **1a** might not have been noticed at all. On the contrary, the same T_2 filtration obtained with the CPMG-*z* sequence (Figure 2, trace B) produced a spectrum that was almost indistinguishable from the reference (trace A), thus ensuring a correct identification and quantification of both species.

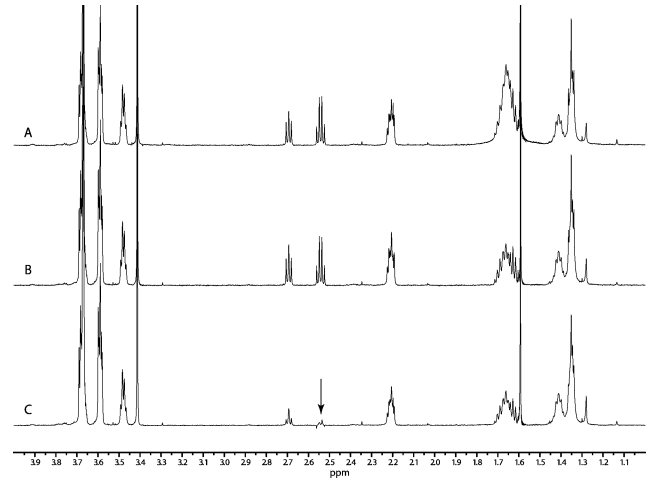


Figure 2. Mixture of **1a** and **1b** (1 mM in $CDCl_3$): unfiltered reference spectrum (A) and T_2-filtered spectra obtained with CPMG-*z* (B) and standard CPMG (C) sequences ($n = 100$, $\tau = 1$ ms). The vertical scales of the spectra have been adjusted to match the amplitudes of the $-OCH_3$ singlet ($\delta = 3.4$ ppm). The disappearing signal in spectrum C (indicated by the arrow) should be noted.

Following this result, the identification and quantification of contaminants in a sample of aged **1c** nanoparticles was attempted. With the aim of assessing their chemical stability, the nanoparticles were prepared and purified following a previously reported procedure[5a] (see the Supporting Information). As expected, no impurities were detected immediately after this process. After 3 months of storage as a solid, the same sample was analyzed again to detect possible traces of detached **1a**. Apart from the sharp peaks of residual solvents, the unfiltered NMR spectrum (Figure 3, trace A) again suggested a relatively clean sample, with only the broad signals typical of the nanoparticle-grafted species. However, the triplet at 2.7 ppm was suspect, possibly indicating the presence of **1b**. This hypothesis was fully confirmed by application of a 200 ms CPMG-*z* filter, whereby the resonances of the octyl chain protons of **1b**, which are completely masked by the broad signal of the same protons in **1c**, clearly emerged in the spectral region going from 1.0 to 2.2 ppm. Moreover, the baseline flattening resulting from T_2 filtration allowed for a reliable quantification of **1b**, whose concentration was estimated to be 0.46 mM (2.5% w/w) by integration of the triplet signal against the singlet of a reference substance.[13]

The triplet of **1b** used to quantify the impurity amount in the previous example lies in a relatively flat region of the spectrum, and one may argue that its integration would have been just as effective in the unfiltered spectrum. In order to test a more tricky situation and showcase the versatility of our method, we turned our attention to an example from metabolic profiling, namely, the glucose content in fetal bovine serum (FBS, GIBCO, cat. no. 10270-106). In dealing with biofluids, ^{1}H NMR-based metabolomics makes frequent use of CPMG sequences to remove the background signals of macromolecules,[1] which is sometimes followed by automatic

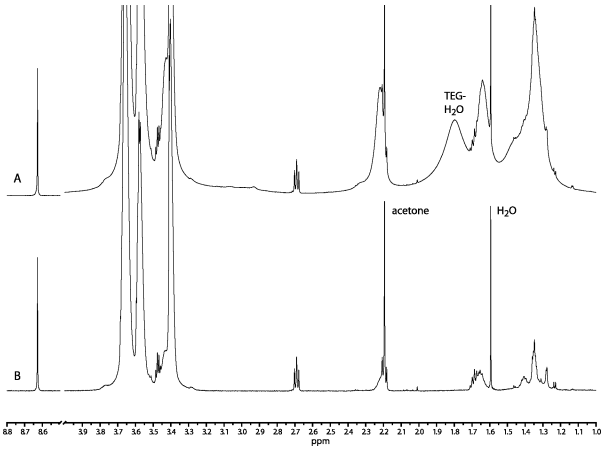


Figure 3. Aged sample of **1c** (7 mg in 600 μL of $CDCl_3$): (A) unfiltered reference spectrum; (B) T_2-filtered spectrum obtained with a CPMG-z sequence ($n = 100$, $\tau = 1$ ms). The vertical scales of the spectra have been adjusted to match the amplitudes of the reference signal ($\delta = 8.6$ ppm). The peak marked as TEG-H_2O originates from residual water bound to the tri(ethyleneglycol) chains.[14]

integration of the resulting spectra for the quantification of small metabolites. The need for spectral editing was evident when the signals of the anomeric protons of α- ($\delta = 5.2$ ppm) and β-glucose ($\delta = 4.6$ ppm) were tentatively integrated in the unfiltered spectrum (Figure 4, trace A): even by applying an automated slope correction, the integrated intensities still differed by 35% from the expected equilibrium populations of α- and β-glucose in water (α:$\beta = 0.37$: 0.63).

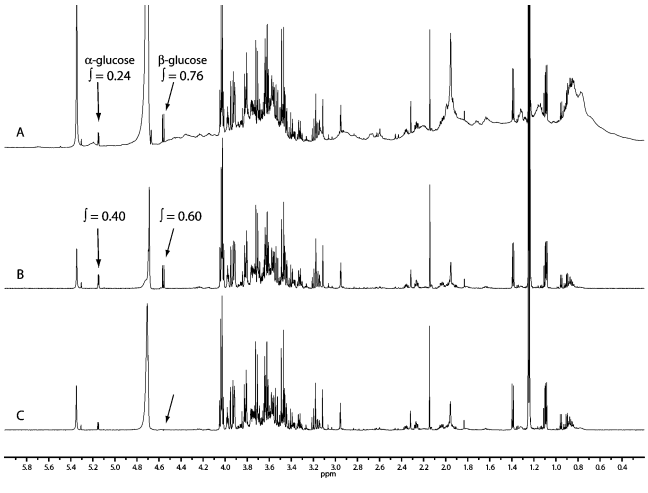


Figure 4. FBS (500 μL + 100 μL of D_2O): unfiltered reference spectrum (A) and T_2-filtered spectra obtained with CPMG-z (B) and standard CPMG (C) sequences ($n = 200$, $\tau = 0.5$ ms). All of the spectra were acquired with water presaturation at $\delta = 4.70$ ppm, and the vertical scales have been adjusted to match the amplitudes of the signal at $\delta = 2.14$ ppm (acetone). The disappearing signal in spectrum C (indicated by the arrow) should be noted.

What is worse, application of a 200 ms CPMG filter virtually canceled the signal of the anomeric proton of β-glucose (Figure 4, trace C) in much the same way as already seen for compound **1a**. In contrast, not only did the equivalent CPMG-z sequence (Figure 4, trace B) recover the missing signal, but in addition, the integrated signal intensities of the two anomeric protons yielded values close the expected populations of the two anomers.

In conclusion, we have developed a convenient method for obtaining T_2-filtered spectra with maximum sensitivity, regardless of the repetition rate adopted for the spin-echo train. The advantages of the new method with respect to a conventional CPMG sequence have been highlighted in a purity assay of monolayer-protected gold nanoparticles and in the determination of glucose in fetal bovine serum. Implications for the characterization of nanosystems and for metabolomic studies are evident. In the near future, we plan to investigate the efficacy of this method in the study of the structure and conformational organization of nanoparticle monolayers, along with possible applications in multidimensional NMR spectroscopy.

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Supporting Information Available: Details on the implementation of the CPMG-z pulse scheme (experimental and theoretical) and on the synthesis of gold nanoparticles. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) It is worth pointing out that while the "z-purging" process takes place inside the spin-echo train, the classic z filters used in TOCSY or other similar sequences operate external to the mixing event. In the case of CPMG, an external z filter would not affect the spin dynamics of the echo train, and as a consequence, it would not recover any signal loss due to J modulation. For the use of z filters in TOCSY, see: (a) Rance, M. *J. Magn. Reson.* **1987**, *74*, 557–564. (b) Thrippleton, M. J.; Keeler, J. *Angew. Chem., Int. Ed.* **2003**, *42*, 3938–3941.
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