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Residual Dipolar Couplings (RDCs) Analysis of Small Molecules Made Easy: Fast and Tuneable Alignment by Reversible Compression/Relaxation of Reusable PMMA Gels**

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With the relatively recent access to aligning media compatible with organic solvents, residual dipolar couplings (RDCs)[1–4] are becoming a key NMR parameter for the structural analysis of small organic molecules.[5,6] Since RDCs provide information about non-local character, the relative configuration of remotely located stereocenters can be determined with relative ease, particularly for those cases where conventional NMR experiments such as nuclear Overhauser enhancement (NOE)[7,8] or ^3J coupling constants analysis[9,10] fail to provide a solution. However, the application of RDC analysis to small molecules is not yet as widespread as it is for biological macromolecules. One of the reasons is the lack of access to a user-friendly and affordable experimental setup.

RDCs of small molecules are usually collected by partially aligning them either in liquid crystal solutions of chiral homopolypeptides[11–15] or strained aligning gels (SAGs). The SAG method, the main focus of the work discussed herein, was originally and independently proposed in 2000 by Tycko et al.[16] and Grzesiek and co-workers[17] for aligning biopolymers in strained water-swollen polyacrylamide gels, either by mechanically stretching or compressing the gels. This approach was conceptually related to earlier experiments by Deloche and Samulski.[18] In 2006, Kuchel et al.[19,20] developed an ingenious device for the rapid and reversible gelatin gel stretching applied to the discrimination between enantiomers using RDCs and other anisotropic NMR parameters. However, all of these approaches were only applicable to water-soluble compounds. In 2004, the SAG concept was extended by Luy and co-workers to organic solvents,[21,22] and novel gels compatible with organic solvents were mainly developed by the same group.[23–29] Recently, Luy and co-workers extended the application of Kuchel's apparatus to poly(acrylamide) (PAAm) (aqueous) and polyacrylonitrile (PAN) ([D_6]DMSO) gels.[30] It involves the use of silicone tubing (outer diameter 4 mm and inner diameter 2.4 mm), placed inside an open-cut NMR tube and fixed with a PTFE plug at the bottom that permits the precise measurement of RDCs with rapid and reversible variation of the alignment strength.

We have introduced the use of poly(methyl methacrylate) (PMMA) gels to measure RDCs, mainly for applications to small molecules soluble in $CDCl_3$ and CD_2Cl_2.[31,32] PMMA gels have shown very good aligning properties when swollen in these solvents and they present little polymer background signal in the HSQC experiments used for the RDCs measurement. We have been employing the traditional SAG method,[21] but it has several experimental shortcomings. The swelling and equilibration time is too long, particularly in PMMA (20–30 days). Samples can be dissolved in the swelling solvent and then allowed to diffuse into the gel as the latter swells but with the risk of sample decomposition over the long swelling period. An alternative option is to let the sample diffuse into the gel after the equilibration period, but this process takes about 48–72 h. Another major problem of chemically crosslinked gels is the presence of residual monomer. In the case of PMMA, attempts to wash it with $CDCl_3$ failed, since the dry material is very brittle and cracks as the solvent evaporates, leaving unusable gel sticks.

Here we present a SAG approach that not only overcomes all these experimental problems but also presents several additional advantages. Instead of using a traditional polymer rod of about 4 mm diameter and 10 mm length, we synthesized cross linked PMMA in 3 mm NMR tubes resulting in dry polymer rods of about 2 mm (see the Experimen-

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[**] PMMA = poly(methyl methacrylate).

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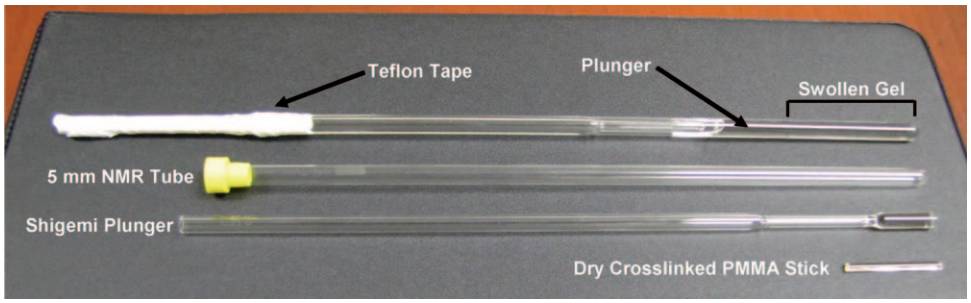


Figure 1. The materials needed for this experiment and how the plunger is locked at the desired position using Teflon tape.

tal Section). The polymer sticks were cut into 25-mm-long pieces and placed into a 5-mm NMR tube (Figure 1). The sticks were allowed to swell in $CDCl_3$ only in the radial direction by blocking the vertical growth by using a Shigemi tube plunger (Figure 2A). After 24–48 h, the gels reached the NMR tube walls (Figure 2B), as revealed by a quadrupolar splitting ($\Delta\nu_Q$) of 53 Hz in the ^{2}H NMR signal of $CDCl_3$. If the Shigemi tube plunger is removed, the gel releases the tension in the vertical direction, leading to a fully decompressed gel of about 42 mm in length (Figure 2D). (The final swollen gel length depends upon the degree of crosslinking and the friction between the expanding gel and the NMR tube walls.) Consequently, the $\Delta\nu_Q$ of the solvent peak becomes zero. The process is reversible and the original $\Delta\nu_Q$ of 53 Hz can be recovered if the gel is compressed back to the original length of 25 mm (Figure 2A). The observed $\Delta\nu_Q$ is linearly scalable as a function of the degree of compression ε, defined as $\varepsilon=(l_r-l)/(l_r-l_i)$, where l_r, l, and l_i are the lengths of the relaxed gel, the gel at any position of the restricting plunger, and the initial (dry and fully compressed) gel, respectively (Figure 2). Measuring the displacement of the plunger with a regular ruler in millimetres involves more errors than just using the $\Delta\nu_Q$, since 1 mm in plunger displacement is equivalent to about 4 Hz in $\Delta\nu_Q$. Hence, the use of $\Delta\nu_Q$ is more accurate and it can be considered as an excellent "spectroscopic ruler". Depending on the length of the dry polymer stick, the maximum value of the $\Delta\nu_Q$ will change; that is, the longer the gel, the lower the value of $\Delta\nu_Q$, at the same crosslink density. The latter parameter is critical to convey the right flexibility to the gel. We are using a crosslink density of 0.27 mol% of ethylene glycol dimethacrylate (EGDMA) (see the Supporting Information). A higher crosslink density produces a less flexible gel, which is characterized by a higher maximum $\Delta\nu_Q$ value but is rather brittle.

If solvent (100–200 μL) is added to the tube and the gel is repeatedly compressed and decompressed with a gentle pumping action of the plunger, the gel behaves like an elastic sponge and the monomer can be easily washed out of the gel in 5–7 washes (see the Supporting Information). In the same way, the compound to be analyzed can be sucked in and squeezed out of the gel in a few minutes. The methodology is clean and fast because it allows the determination of RDCs by tuning the alignment in easy to reuse gels. Although we can not claim that the gels can be reused unlimitedly, one of them was successfully used to collect RDCs from four different compounds over four months (Figure 3).

To remove the monomers or to recover the samples from the gel, usually 5–7 washings with about 200 μL of fresh solvent are sufficient. The PMMA background signal was not filtered with a CPMG experiment to prove that the sample can be put in and removed from the gel easily. The background signal goes away in 30 ms in a CPMG experiment and it is hardly observed in the HSQC experiments without using any filter as previously shown.[32]

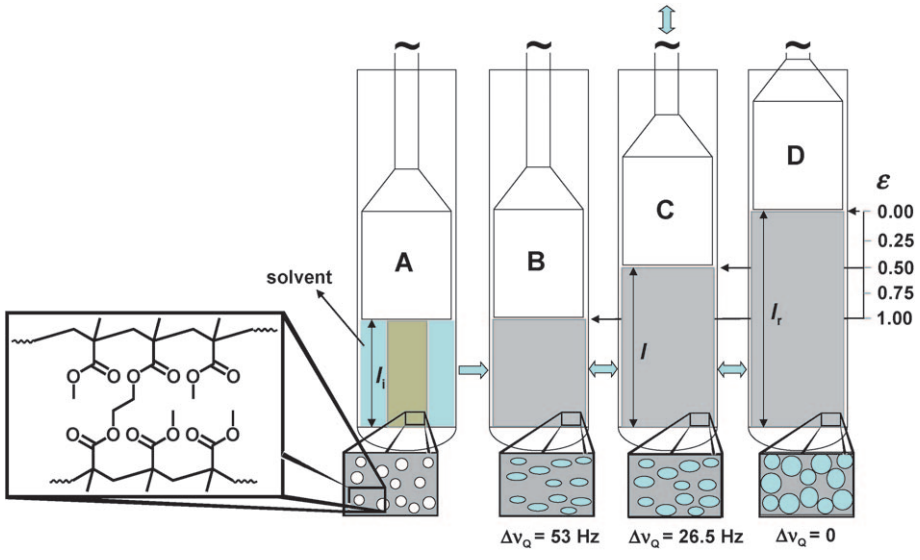


Figure 2. Representation (not to scale) of the reversible gel compression/relaxation experimental setup: A) dry crosslinked PMMA stick radially swells in $CDCl_3$ under the restriction of a Shigemi plunger and the gel "cavities" adopt an oblate character (B) that conveys anisotropy to the gel, showing a ^{2}H quadrupolar splitting $\Delta\nu_Q$ of 53 Hz . C) As the plunger is released, this oblate character decreases gradually to give an isotropic gel (D) for which the $\Delta\nu_Q$ collapsed to zero. The process is linearly reversible from D to B as a function of the compression factor ε.

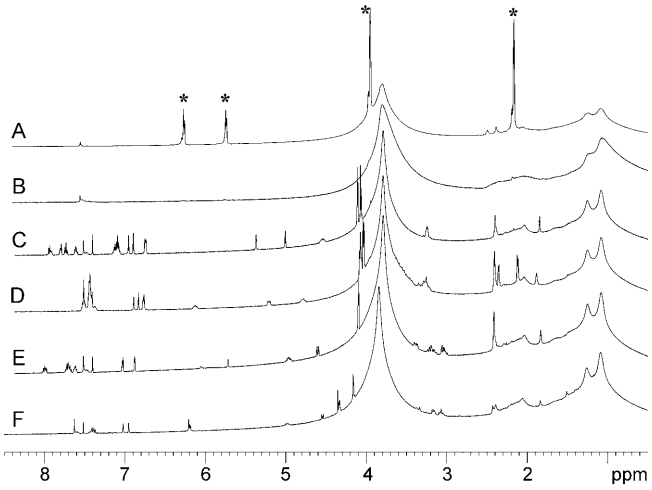
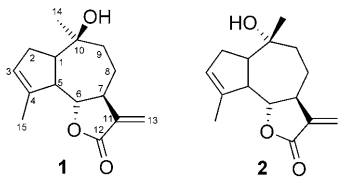


Figure 3. ^{1}H NMR spectra of the same PMMA gel in the same NMR tube right after the first swelling (A) with monomer marked with asterisks, and after five washes each with 200 μL of $CDCl_3$ (B; no monomer), and suctioning in and squeezing out four consecutive benzazepine derivative samples (C–F; see Supporting Information).

To show the aligning properties of our gels, the natural sesquiterpene lactone 10-epi-8-deoxycumambrin B (**1**)[33] was selected as a sample compound. Its configuration is well established and it is the epimer at C-10 of 8-deoxycumambrin B (**2**; Scheme 1).[34–36] The only major structural difference between **1** and **2** is the orientation of the CH_3 and OH groups at C-10. The geometry of their carbon skeletons is slightly different with an RMS fit error of 0.33 Å. This serves as a very good example problem to show the power of using average methyl groups RDCs[37] to discriminate between the configurations of a chiral center lacking a CH bond (quaternary). Compound **1** (2.8 mg) was dissolved in $CDCl_3$ (200 μL), poured into the NMR tube containing the swollen gel, and the gel was gently compressed and decompressed several times (pumping action) with the Shigemi plunger. A quick 1D ^{1}H CPMG NMR spectrum showed that the sample was inside the gel in a matter of minutes. A series of proton-coupled HSQC experiments were collected at different degrees of compression ε (see the Supporting Information), as well as a ^{2}H NMR spectrum to measure the $\Delta\nu_Q$ of the solvent at each compression stage. The plunger was held at each compression position using Teflon tape (see Figure 1). The ^{1}H–^{13}C one-bond heteronuclear total couplings $^1T_{CH} = {}^1J_{CH} + D_{CH}$ were extracted from the HSQC F2 slices as described earlier.[32] A clear linear relationship was observed for the $^1T_{CH}$ values versus the $\Delta\nu_Q$ at each compression stage, as previously observed by Luy and co-workers for the stretching apparatus.[30] The results are summarized in Table S1 in the Supporting Information. Instead of using a single set of RDCs to perform the singular value decomposition (SVD) fitting of RDC data to structure, we used the slopes obtained from the variable degree of compression experiments. Using the slopes presents some advantages: i) fitting the data to the structure is more precise since the calculation already includes the statistical average from the linear regression analysis of the data; and ii) it is not necessary to run a control sample in isotropic solution. This is particularly important when only small amounts of samples are available, which can be lost over extensive manipulations. The slopes themselves are proportional to the RDCs of each individual bond and the plot intercepts at zero $\Delta\nu_Q$ represent the isotropic $^1J_{CH}$ values.[30] It is important to highlight that by using slopes in the SVD fittings, the values of the principal components of the calculated alignment tensor (A_{xx}, A_{yy}, A_{zz}) do not represent the degree of alignment and consequently, the GDO (generalized degree order) values become meaningless.[2] The slopes for C–H bonds at carbons 1, 3, 5, 6, 7, 13, 14, and 15 were SVD fitted to the structures of **1** and **2** in MSpin (http://www.mestrelabs.com) using the option of averaging CH_3 groups as free rotors. Quality factors (Q)[38] of 0.105 and 0.199 were obtained for structures **1** and **2**, respectively. If the slopes of the CH_3 groups are excluded from the fitting, the Q factors obtained for **1** and **2** are 0.084 and 0.121, respectively. Although structure **1** fits better to the data in both cases, the addition of the CH_3 data produced a better structural discrimination in which the Q factor for **2** is almost twice as high as the Q factor for **1**.



Scheme 1. Structures of 10-epi-8-deoxycumambrin B (**1**)[33] and 8-deoxycumambrin B (**2**).[34–36]

The fundamental difference between stretching and compressing the gel is that the directions of the main axis of the ellipsoidal gel "cavities" are perpendicular.[17] This is almost equivalent to physically rotating the gel by 90° in the magnetic field from the z axis to the x,y plane, leading to a rotation of the molecule probability tensor **P**.[2] This indicates that the combined use of both methodologies (stretched and compressed gels) clearly provides valuable complementary structural information. Although this was shown on biomolecules in PAAm gels,[17] the same behaviour should be observed in PMMA with organic compounds. To experimentally show this, RDCs data were also collected for compound **1** aligned in a stretched PMMA gel using Luy's original approach.[21] For this case it was also necessary to collect a proton-coupled HSQC spectrum under isotropic conditions ($CDCl_3$) to calculate the RDCs in the traditional way.[32] On the other hand, the RDCs values for **1** in the compressed gel were calculated by the difference from the data at full compression ($\varepsilon = 1.00$) and the data under isotropic conditions. The corresponding alignment tensors **A** for compound **1** in the compressed and in the stretched gel were calculated from the data analysis in MSpin,[39] and in turn were converted to their respective probability tensors **P**. Figure 4 shows a representation (not to scale) of the preferred partial

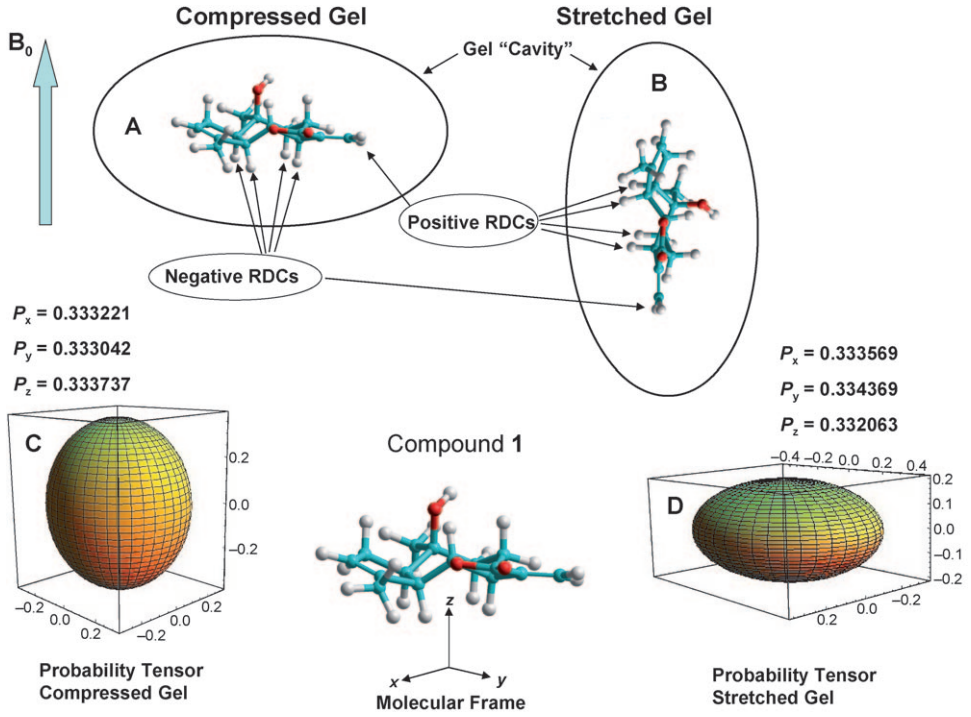


Figure 4. Comparison of the preferred orientation of compound **1** in the compressed gel (A) and in the stretched gel (B) with the corresponding probability tensors **P** (C and D) calculated from the RDCs data. The 3D probability ellipsoids plots of the tensors **P** (C and D) are represented such as the principal components values of the tensor (P_x, P_y, and P_z) are rescaled as (P–0.333) × 1000, otherwise the differences in the axis are barely noticed.

alignment of the molecules of compound **1** inside the "cavities" of the gel when it is stretched (Figure 4A) and when it is compressed (Figure 4B). A quick comparison of the RDCs in each data set (see the Supporting Information) shows that the pseudoaxial protons of the seven-membered ring (H-1, H-5, H-6 and H-7) (Figure 4) have a negative sign in the compressed gel and a positive sign in the stretched gel. The opposite trend is observed for the protons of the double bond (H-13a,b), whose C–H bonds are aligned with the plane of the molecule. This indicates at first glance that the C–H bonds of the above pseudoaxial protons orient parallel to $\mathbf{B_0}$ in the compressed gel and perpendicular to $\mathbf{B_0}$ in the stretched gel. This is clearly depicted in the plots of the corresponding **P** tensors (Figure 4C and 4D). The probability distribution of the direction of the external magnetic field $\mathbf{B_0}$ in the molecular frame of **1** is higher ($P_z = 0.333737$) along the z axis in the compressed gel, while it is higher along the y axis in the stretched gel ($P_y = 0.334369$).

In conclusion, we have presented a novel scalable methodology to measure anisotropic NMR parameters of small organic molecules that does not require a complex device but just a PMMA polymer gel stick of 2 mm diameter and 25 mm length, a regular NMR tube, a Shigemi tube plunger (or any glass plunger), and Teflon tape. The methodology is clean, without the interference of residual monomer. It is fast, since the gel can be swollen and equilibrated within 24–48 h, the sample can be diffused into the gel in a matter of a few minutes, and after the collection of the data it can be easily recovered also in a matter of a few minutes. Although the preparation of the gels is easy and recycling is not necessarily needed, the same gel can be reused several times as long as it is thoroughly washed after each experiment. In addition, it is not necessary to carry out a control experiment under isotropic conditions since slopes can be used to fit the data to the structure. PMMA does not swell in methanol, but the current experimental setup can be extended to other nonpolar solvents (i.e., to all good solvents for PMMA). The experiment has significantly facilitated the collection of RDCs in our research group and we are currently using it for the conformational and configurational analysis of natural and synthetic small organic molecules. RDCs are propelling the structural analysis of small molecules to a higher level of structural information and in the present case, since the experiment is performed in a regular 5-mm NMR tube, the sensitivity of the experiment is only limited by a combination of the sensitivity of current of NMR probes and magnetic field strengths.

Experimental Section

General and more specific experimental procedures for gel preparation, preparation of alignment, NMR measurements, experimental and computed RDCs values for **1**, and probability tensors **P** calculations are provided in the Supporting Information.

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Keywords: gels · NMR spectroscopy · residual dipolar couplings · small molecules · stereochemistry

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