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ELISE NMR: Experimental liquid sealing of NMR samples

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Abstract

We present a simple, generally applicable approach to prevent sample evaporation when working at elevated temperatures in high resolution NMR. It consists of experimentally sealing the NMR sample by a second liquid (Experimental Liquid Sealing, ELISE). For aqueous samples, we identified the mineral oil commonly used in PCR application as the best candidate, because it contains only a very limited amount of water-soluble contaminants, is stable over time and heat resistant. The procedure does not interfere with shim settings, and is compatible with a wide variety of samples, including oligosaccharides and proteins. For chloroform samples, a simple drop of water allows to efficiently seal the sample, avoiding solvent evaporation even over lengthy time periods.
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1. Introduction

Solvent evaporation in NMR samples can be a real problem, especially when the measurement requires high temperatures, volatile solvents, and long accumulation times because of sensitivity requirements. If the initial size of the sample is large, and the NMR tube is carefully sealed, an equilibrium will set in between the liquid and the saturated vapour, and shim settings will not severely suffer as long as the measurement is not overly long. For size limited samples, such as encountered when one uses a Shigemi tube, solvent evaporation can easily lead to the creation of air bubbles below the plunger, and hence severely deteriorate the magnetic field homogeneity.

Because we encountered this problem in several cases that will be described below, we have explored different solutions, and come up with an Experimental LIquid SEaling (ELISE) approach that obeys all criteria that we had set out from the beginning. Requirements were (i) technical simplicity, (ii) general use for most if not all commonly used samples (iii) independence of NMR conditions (field strength, regular or cryogenic probe, regular or Shigemi tube, ...), and (iv) time stability of the solution. The general principle of our method consists of introducing a small volume of a second solvent on top of the first one. Oils are a natural choice for aqueous samples, because they stay above the water level due to their lesser density, and do in principle not mix with water. We have investigated several commonly found oils, and identified the Nujol mineral oil of common use during the PCR amplification of DNA as a close to ideal candidate. We will describe different applications, including NMR analysis of a concentration limited vaccinal dose at high temperature in a Shigemi tube, several protein samples where both concentration and temperature were a problem, and the case of ligand screening, where one would like to avoid the use of susceptibility matched plungers because of the time requirement to condition correctly the NMR tube. Finally, we extend our ELISE approach to the case of samples in organic solvent, and demonstrate that aqueous sealing of chloroform samples can lead to a previously unseen sample stability.

2. Identification of the sealing oil

Commonly used oils were tested in the following manner. We prepared a reference solution of 1 mM TMSP in

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D_2O, and filled several new NMR tubes with 600 μl of this solution. To a first tube, we added 100 μl of the same solution, and sealed it by parafilm as a reference, whereas for the other tubes, we placed 100 μl of a given oil on top of the initial reference solution and sealed it in the same manner. In this manner, we replace the water/air interface of the original sample, already sufficiently far from the detection coil, by a water/oil interface, without any other change. An initial 1D spectrum was recorded with 64 scans on a 600 MHz spectrometer equipped with a cryogenic probe head, and the samples were then stored at room temperature in the laboratory, and re-examined over a period of 6 months. In the reference spectrum, when we scale the spectrum by a factor of 1024, we can see some impurities, including the peak at 1.92 ppm of acetate, and several other resonances between 0.5 and 1.5 ppm. Whereas we do not know whether these contaminants come from our D_2O, the TMSP stock solution or incomplete cleaning of the NMR tubes, the reassuring fact is that these impurities hardly evolve over time (Fig. 1).

We then tested first several oils from vegetable origin, commonly used in the kitchen. Aliphatic signals coming from organic molecules that leak from virgin olive oil in the aqueous phase, for example, were visible when scaling the sample by a factor of 128, indicating that they are far more numerous and concentrated than in the reference solution. Moreover, these signals evolve over time, making the olive oil a less suitable candidate to seal a real NMR sample. When we tried a sodium oleate, the situation was even worse. Already with a eightfold spectral scaling, the impurities were clearly visible, and they increased significantly over time. We equally remarked that the TMSP signal decreased over time in this sample, possibly because of its selective partitioning inside the lipid phase.

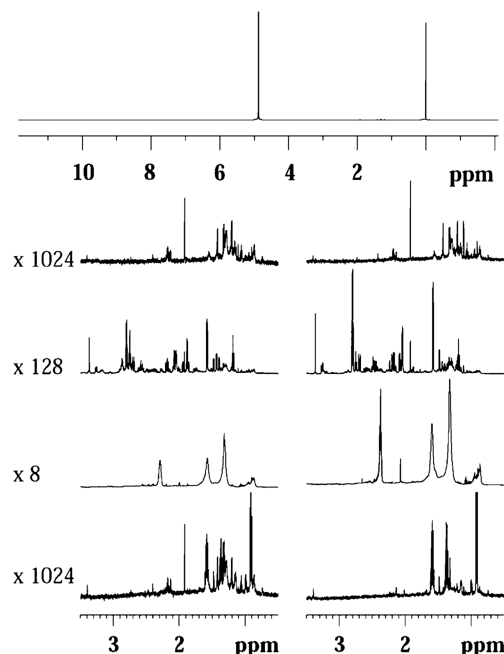

Fig. 1. (top) Reference spectrum of 1 mM TMSP in D_2O. From top to bottom, blow-ups of the reference solution capped by (i) the same solution, (ii) the olive oil, (iii) sodium oleate, and (iv) the Nujol mineral oil. The spectra recorded immediately after the preparation of the samples (left column) are compared with the spectra of the same samples after 6 months (right column). The scaling factor is given at the left.

Without going through all other oils that we tested, the Nujol mineral oil, commonly used to seal DNA samples during the thermocycles of the PCR procedure, proved to be an excellent candidate. Spectral impurities were only twofold larger than the signals in the reference spectrum, were limited in number and did not evolve significantly over time of a period of 6 months. We therefore decided to pursue with this product for testing with real samples.

2.1. Spectral recording of a vaccinal dose

As an application, we wanted to record 2D homo- and heteronuclear spectra on a single vaccine dose, composed of a mixture of polysaccharides. The estimated content of every polysaccharide is 2 μg/dose, so sensitivity is a real problem. Dissolving the sample in 300 μl through the use of a Shigemi tube allows an increase of the concentration by roughly a factor of two, compared to the 500 or 600 μl volumes used in a regular tube. However, we wanted to work at 50 °C to separate the residual water resonance from the anomeric protons [1]. Both requirements proved incompatible, because air bubbles invariably formed at the liquid/plunger interface already during the first increments of the lengthy TOCSY and HSQC spectra, and this despite extensive degassing of the sample and/or pre-equilibration of the Shigemi tube at 50 °C. We therefore filled the Shigemi tube with the 300 μl aqueous solution, and added on top of this aqueous phase 70 μl of the mineral oil. Only at this moment, the plunger was introduced to below the water/oil interface, thereby ensuring that the lower part of the plunger was completely immersed in the aqueous phase. Because the water/oil interface is easily visible, the tube assembly in the presence of the oil seal is very similar as for a regular sample. To investigate whether this procedure would not deteriorate spectral quality over time, we first prepared a sucrose sample in this manner, and record 1D spectra over time. As shown in Fig. 2 on the sucrose anomeric proton, resolution did not degrade even after 12 h.

Because this experiment indicates that spectral quality does not deteriorate over time, we applied it to seal efficiently the vaccin sample and thereby avoid any

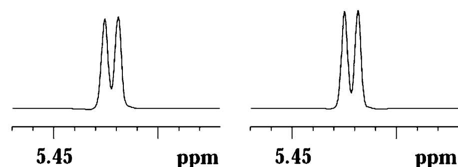

Fig. 2. 1D spectrum of 10 mM sucrose in 300 μl of D_2O, with 70 μl of Nujol oil above the plunger. Spectra were recorded at 50 °C on a 600 MHz spectrometer, and are separated by a 12 h interval.

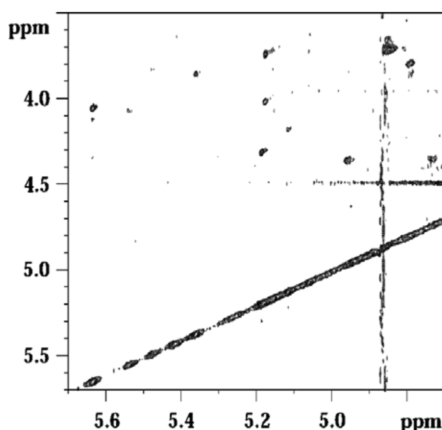

Fig. 3. Anomeric region of the TOCSY spectrum of a vaccinal dose in a Shigemi tube, recorded at 50 °C on a 600 MHz spectrometer.

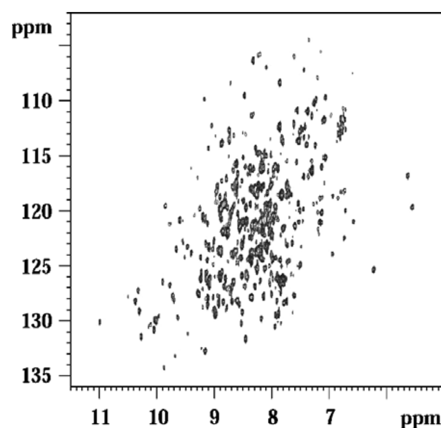

Fig. 4. TROSY spectrum of the OpgG protein. The sample was 400 μl of aqueous solution in a Shigemi tube, sealed by 100 μl of Nujol oil. Spectra were recorded at 800 MHz and at 37 °C.

evaporation of the aqueous solution. High resolution TOCSY spectra with 144 scans per increment could be acquired on this sample at both 600 and 800 MHz. Although both experiments required over 62 h of measurement time at 50 °C, no spectra deterioration due to air bubbles occurred, illustrating the efficacy of the sealing even at high temperature (Fig. 3).

2.2. Working with protein samples

A source of problems might be a non-specific interaction of protein samples with the oil, with protein denaturation or other problems as potential pitfalls. Without claiming generality for all proteins, we show here our results for two cases that distinguish themselves in the size range. The first protein sample is the 56 kDa *Escherichia coli* OpgG protein involved in the biosynthesis of osmoregulated periplasmic glucans by the bacteria. Although we have solved ourselves the crystal structure of this enzyme [2], we have failed to cocrystallize it with any oligosaccharide ligand, and have attempted therefore an NMR approach. Because of the important size of the protein, we prepared a triply labeled ($^{2}D/^{15}N/^{13}C$) sample, and dissolved it in 400 μl of Tris buffer. Spectral quality being significantly better at 37 °C than at 20 °C, all triple resonance spectra were recorded at 800 MHz and 37 °C. Because we wanted to add in a second phase several oligosaccharides to the sample, we did not use the Shigemi plunger for this case, and only exploited the susceptibility matched tube bottom. The sample was introduced in a Shigemi tube without plunger, and was sealed by 100 μl of mineral oil. Importantly, the TROSY spectrum was identical as that of a sample in a regular 600 μl sample, and did not vary in the control TROSY experiments that we ran between the triple resonance spectra (Fig. 4). Finally, it should be added that sample recovery can be very simply done by pippetting the aqueous phase from the tube, and halting just before the oil phase.

In the second application, we set up a systematic screen of small molecular ligands in order to detect their interaction with the Pin1 WW domain [3]. Whereas a screening setup with an LC-NMR installation has been described [4], the unavailability of such a device often forces the preparation of individual samples in regular NMR tubes before spectral recoding of a standard HSQC spectrum. Shigemi tubes could limit the amount of labeled sample, but are rarely used in this approach. Their elevated cost, albeit a one-time investment, is one reason, but the time requirement associated with the tube assembly equally is an obstacle to their use in a screening effort.

Liquid sealing of a 300 μl sample without the plunger might combine sample gain with the rapidity or even possible automation of a regular sample tube. When we first tested this for a sample containing only TMSP and D_2O, we found not unexpectedly that the mineral oil contributes importantly to signal intensity between 1.5 and 0.5 ppm. When we adjusted the tube position such that the aqueous solution was covered precisely by the detection coil (this corresponds to raising the tube by 2 mm from its regular position), these signals did decrease in intensity, but remained nevertheless of the same intensity as the TMSP signal (Fig. 5). Whereas this seemingly might limit the usefulness of the approach, the ^{1}H–^{15}N HSQC spectrum on the ^{15}N labeled WW domain did not suffer at all from these spurious signals, as the magnetization transfer through the INEPT stages combined with the pulsed magnetic field gradients efficiently purge all non-^{15}N coupled proton

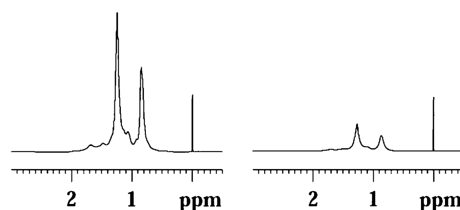

Fig. 5. 1D spectra of a 300 μl TMSP sample in a Shigemi tube, sealed by 100 μl of Nujol oil. The sample insertion height was varied such that the detection coil optimally covered the aqueous phase in the right spectrum.

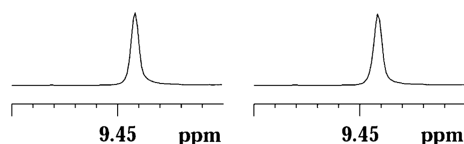

Fig. 6. Aldehyde proton of myrtenal in chloroform, in a Shigemi tube sealed by 100 μl of water. Spectra were recorded at one week interval at 20 °C on a 300 MHz spectrometer.

magnetization. Therefore, whereas proton derived NMR based screening methods such as Saturation Transfer Difference spectra [5] would suffer from the presence of the oil near the detection coil, HSQC based experiments could indeed benefit from the ELISE approach.

2.3. Working with organic solvents

Sample evaporation is enormously enhanced when working with volatile organic solvent such as chloroform. Even though the magnetic susceptibilities of chloroform and D_2O are equally close to that of H_2O, very few NMR laboratories would dare to use Shigemi tubes with chloroform samples, because air bubbles invariably form below the plunger and deteriorate the shim settings. We reasoned that a similar ELISE approach should work with these samples as well, at the condition that another sealing substance is used. Water is actually an excellent candidate, because its lesser density than chloroform, and its closely matched magnetic susceptibility. We thus prepared a chloroform sample containing 1 mM of myrtenal in a Shigemi tube, prepared it as usual, and added 100 μl of water above the plunger. The spectral quality was excellent, but more impressively, even after one week, the spectrum of this sample has not changed at all (Fig. 6). When we prepared the same sample with 100 μl of D_2O rather than H_2O on top of it, we did notice that the residual water signal initially present in the chloroform decreases over time, indicating that there is some $^1H/^2D$ exchange over the interface.

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