NMR Spectroscopy

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Chromatographic NMR Spectroscopy with Hollow Silica Spheres

*Tania González-García[+], Tommaso Margola[+], Adriano Silvagni, Fabrizio Mancin, and Federico Rastrelli**

In memory of Alessandro Bagno

Abstract: *The use of micrometric hollow silica spheres is described as a strategy to reduce magnetic field inhomogeneities in the context of NMR chromatography. When employed as a stationary phase, hollow silica microspheres allow the use of common solution-state NMR instruments to measure the diffusion coefficient perturbation induced by the interaction of the analytes with the silica surface.*

The importance of NMR spectroscopy in the analysis of complex mixtures has been steadily increasing over the last years mostly driven by emerging disciplines such as metabolomics, or under the impulse of pharmaceutical and food industry, both urged by strict product quality regulations.[1] With respect to other techniques, the advantages of NMR spectroscopy include the easy preparation of the sample, the detailed (structural) information provided on the analytes and the possibility to detect several species simultaneously.

Notwithstanding the increase of magnetic field strengths in standard instrumentation, NMR spectra of complex mixtures still result in very crowded patterns of resonances. This may not be a problem when some of the mixture components are already known (e.g. in metabolomics or in most pharmaceutical analyses) since one may try to extract specific target signals from the spectrum and quantify them. In the unfortunate cases where the relevant NMR signals are concentrated in a small frequency range, methods based on multiple quantum editing have proven to be useful for unravelling the spectral complexity.[2]

The need for "broad scope" mixture analysis has led to the advent of hyphenated techniques such as LC-NMR spectroscopy, which sequentially couples liquid chromatography with solution-state NMR spectroscopy in much the same way as HPLC is coupled with mass spectrometry in HPLC-MS instruments. Hyphenated methods for on-line separation are however time consuming and quite difficult to implement (special probes are needed), up to the point that off-line chromatography followed by classic NMR analysis of the single fractions often appears as a better option.

Indeed, a chromatographic separation process results from a combination of a net convective flow and a partition equilibrium between the mobile and the stationary phase. If the dominant transport process is diffusion rather than convection, a net separation among the analytes is never attained, but an alteration in their apparent diffusion rates can, in principle, be observed by virtue of the same partition equilibrium. Based on this idea, Caldarelli and co-workers proposed the use of NMR diffusometry in the presence of a stationary phase to improve the signal separation in diffusion-ordered NMR spectra.[3]

Owing to the intrinsic sample inhomogeneity, the practical implementation of this method, dubbed chromatographic NMR spectroscopy, relies on magic-angle spinning (MAS) to remove the anisotropy of magnetic susceptibility across the sample. It so turns out that such a technique requires spectrometers equipped with high-resolution MAS probes, which join the line-narrowing advantages of MAS with high-resolution capabilities of solution-state probes, including pulsed magnetic field gradients (PFG). The setup of these experiments requires much care: as pointed out by the same authors, diffusion measurements of liquids under MAS conditions are prone to artefacts stemming from mechanical sample vibrations.[4] In addition, as a result of the centrifugal force, spinning of liquid suspensions at the magic angle can cause aggregation either near the rotor perimeter or in a central vortex, most likely in low viscosity samples.[5]

An alternate approach to reduce the magnetic field inhomogeneity across the sample, proposed by Hoffman and co-workers, consists in matching the magnetic susceptibility of the stationary phase with that of properly mixed deuterated solvents.[6] A notable advantage of this approach is that standard solution-state probes can be used in place of high-resolution MAS probes. Nonetheless, the strict requirements on the susceptibility matching entail the use of uncommon (and often commercially unavailable) deuterated solvents to make effective mobile phases. Even more, mixtures which satisfy the susceptibility matching condition do not necessarily provide the correct polarity to observe a sizeable chromatographic effect. In order to circumvent the problem of heterogeneous samples, the use of shift reagents,[7] microemulsions,[8] or soluble stationary phases such as micelles,[9] polymers,[10] and nanoparticles[11] has also been suggested. Indeed, while soluble stationary phases do address the heterogeneity problem, they also bring some disadvantages. First, broad residual signals from the stationary phase

[*] Dr. T. Margola,[+] Dr. A. Silvagni, Prof. F. Mancin, Dr. F. Rastrelli
Department of Chemical Sciences, Università degli Studi di Padova
via Marzolo 1, 35131 Padova (Italy)
E-mail: federico.rastrelli@unipd.it
Dr. T. González-García[+]
Organic and Inorganic Chemistry Departament, University of Oviedo
c/ Julián Clavería, no. 8, Oviedo 33006 (Spain)
[+] These authors contributed equally to this work.

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may overlap with those of the analytes, making the data processing in the diffusion dimension much more challenging. Second, the use of such phases is limited by their solubility, and screening different solvent mixtures to optimize the "chromatographic" effect may not always be possible.

In this challenging framework we propose an alternate solution to the stumbling block of sample heterogeneity in chromatographic NMR spectroscopy. Making use of hollow silica microspheres, our approach retains the advantages of a solid stationary phase while reducing the field inhomogeneities, which allows the use of any solvent mixture and of standard solution-state probes.

Our idea is based on the observation that chromatographic silica suspended in a liquid can be approximated by a dense random packing of non-penetrating spheres.[12] This ensemble can be viewed as a superposition of single spheres of permeability μ_{int} and radius r, each embedded in an isotropic medium of permeability μ_{ext}. In the presence of a uniform magnetic field $\mathbf{H}_0$, the effect of a single isolated sphere on the external region is equivalent to that of a dipole with moment **m,** located at the center of the sphere and proportional to the field itself [Eq. (1)].

$$\mathbf{m} = \frac{\mu_{int} - \mu_{ext}}{\mu_{int} + 2\mu_{ext}} 4\pi\, r^3 \mathbf{H_0} \qquad (1)$$

In the above and similar equations, the magnetic susceptibility χ is sometimes used in place of the permeability: the two quantities are related as $\mu/\mu_0 = \chi - 1$, where μ_0 is the permeability of free space. When an NMR tube filled with silica and a solvent is put into the magnet, the resulting field will be the sum of the external field and a myriad of dipolar fields originating from the silica spheres. As a consequence, the magnetic field becomes inhomogeneous within small domains across the sample, a situation which is beyond the correction capabilities of the spectrometer shim system. Just as clearly, when the internal and external magnetic susceptibilities are close to each other (i.e., when $\mu_{ext} \approx \mu_{int}$), the magnitude of **m** decreases and the field inhomogeneities become smaller, which is the pathway pursued by Hoffman and co-workers.[6]

If we now consider a hollow sphere of internal radius a and external radius b, it can be shown that Equation (1) is modified into[13] Equation (2),

$$\mathbf{m} = \left[\frac{4\pi(2\mu' + 1)(\mu' - 1)}{(2\mu' + 1)(\mu' + 2) - 2a^3/b^3(\mu' - 1)^2}\right](b^3 - a^3)\mathbf{H_0} \qquad (2)$$

where the quantity $\mu' = \mu_{int}/\mu_{ext}$ has been introduced to simplify the equation (for a sphere of silica 60H immersed in $CDCl_3$ the value $\mu' = 1.12$ is obtained).[6a] Equation (2) reduces to Equation (1) when $a = 0$ and $b = r$, namely when the hollow sphere becomes a full sphere. In this respect, Equation (1) can be conveniently used as a normalization factor to obtain the relative magnetic moment as a function of the ratio a/b (Figure 1).

Inspection of Figure 1 reveals that a significant reduction of the magnetic dipole is expected for hollow spheres with rather thin shells. More precisely, in order to reduce the magnetic dipole of a full sphere by more than 90%, a/b ratios larger than 0.95 are required. The field inside the cavity filled by the solvent is uniform and parallel to the external magnetic field; moreover, for materials of low magnetic permeability such as silica, its magnitude is virtually identical to that of the external field.

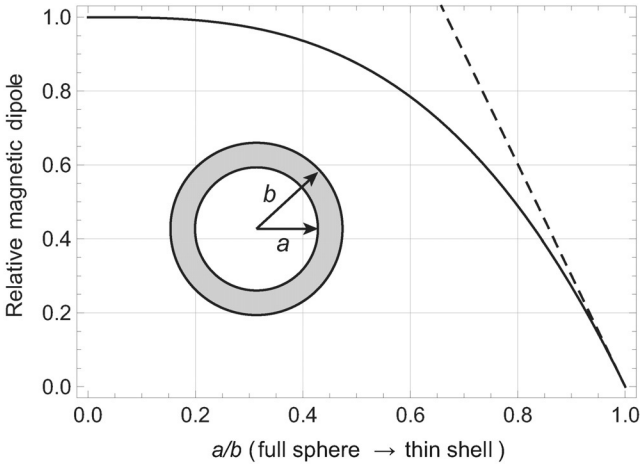

Figure 1. Magnetic dipole of a hollow sphere [Eq. (2)] relative to the magnetic dipole of a full sphere with the same radius, as a function of the ratio a/b (solid line). a = internal radius; b = external radius; $\mu' = 1.12$. The case $a/b = 0$ corresponds to the full sphere, whereas the limit $a/b \to 1$ corresponds to a thin shell. The rate of collapse of the relative magnetic dipole approaches a limit value of about −3 when $a/b \to 1$ (dashed line).

Intrigued by such a theoretical outcome, we decided to put it at test on a physical sample: to this aim, silica hollow microspheres (HMS) were prepared by template polymerization of tetraethyl orthosilicate (TEOS) on polystyrene microspheres, as described in the Experimental Section. Depending on the amount of employed TEOS (see the Supporting Information), micrometric silica HMS with a shell thickness ranging from approximately 70 to 220 nm were obtained and characterized by TEM and thermogravimetric analysis (TGA).

Several batches were prepared and tested by filling 5 mm NMR tubes with silica HMS and deuterated solvents. The samples were sonicated and centrifuged repeatedly to remove air bubbles. Solution-state NMR spectra of such samples were collected at 25 °C on a Bruker AVANCE III spectrometer operating at 500.13 MHz ^{1}H Larmor frequency and equipped with a 5 mm z-gradient broadband inverse (BBI) probe. The deuterium resonance was sharp enough to allow for sample lock: for HMS with the thinnest shells (Figure 2) suspended in $CDCl_3$, a typical line width of 7 Hz could be obtained after shimming on the residual $CHCl_3$ signal. A tube prepared using commercial silica for chromatography did not allow for sample lock, and the resulting spectrum was uninformative (see the Supporting Information).

As representative test analytes for chromatographic NMR spectroscopy we first selected benzoic acid, benzaldehyde and benzyl alcohol, a mixture that can result from an incomplete disproportionation of benzaldehyde in the well-known Cannizzaro reaction.[14] Owing to their similar molecular weights,

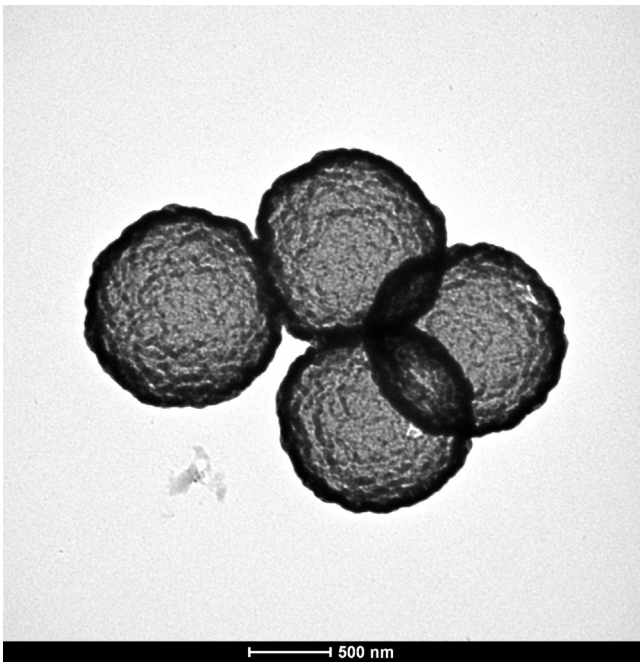

Figure 2. Representative TEM image of silica hollow microspheres prepared as described in the text. The estimated shell thickness is about 75 nm and the average diameter is about 1200 nm ($a/b = 0.87$).

these three molecules are difficult to resolve in a standard DOSY spectrum. Figure 3 illustrates the effect of silica HMS with variable shell thickness. For HMS with a/b ratios of 0.87 the line width of residual $CHCl_3$ is close to 6 Hz, a value low enough to allow a good resolution for each signal of the three molecules. In agreement with the proposed model, HMS with smaller a/b ratios give rise to broader signals because of their associated larger magnetic dipoles [Eq. (2)]: eventually, some peaks are even broadened beyond detection. We also observe no NMR signal splitting due to solvent molecules residing inside and outside the cavity, likely because the inner and outer magnetic fields are very similar to each other as discussed before.

Encouraged by the above results, we selected silica HMS with the largest a/b ratio (ca. 0.87 depending on the batch) as the best candidate for chromatographic NMR experiments. In order to interpret the diffusion measurements it is worth to remind that, in the NMR fast exchange limit, the apparent diffusion coefficient D of a molecule is an average weighted over the populations of the bound and free states, the latter having larger D values. When such interactions become increasingly weak, the diffusion coefficients appear as virtually unperturbed. In this respect the outcome of a DOSY experiment is very different from a true chromatographic process, where tiny affinities for the stationary phase are amplified (up to physical separation) by forced convection driven by capillarity, gravity or high-pressure pumping.

Figure 4 (top) highlights that a standard DOSY spectrum cannot fully resolve the Cannizzaro mixture whereas, in the presence of silica HMS (bottom), the three analytes are completely separated in the diffusion dimension. As expected on the basis of the chemical functionalities, benzoic acid

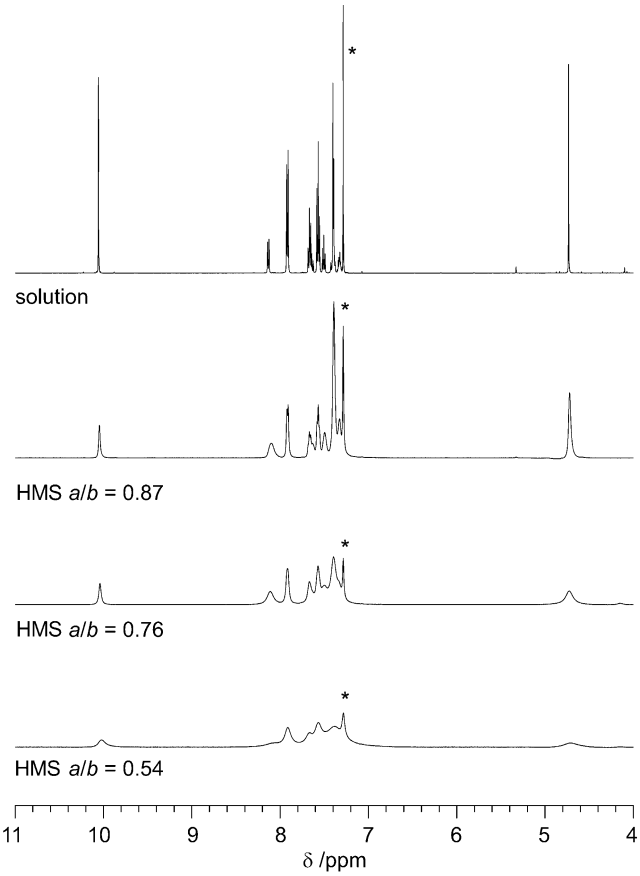

Figure 3. Top: Solution-state ^1H NMR spectrum of 5 mM benzoic acid, benzaldehyde and benzyl alcohol in $CDCl_3$. Other traces: same mixture in the presence of silica HMS with variable shell thickness and a/b ratios. All the traces are normalized by the area of the peak at 10 ppm. The line width of residual $CHCl_3$ (asterisk) in the sample with $a/b = 0.87$ is about 6 Hz. Different signals within the same spectrum exhibit different linewidths depending on the affinity of the parent molecules for the silica.

interacts the most with silica, followed by benzyl alcohol. Relative to the self-diffusion coefficient of $CHCl_3$, the diffusion coefficient of benzaldehyde appears unperturbed, which accounts for a weak interaction with silica. Furthermore, addition of a small quantity of [D_{12}]cyclohexane was found to improve the separation in the diffusion dimension. This result proves that the outcome of the analysis can be effectively altered by adjusting the solvent composition, in much the same way as the retention times are optimized by gradient elution in liquid chromatography.

As a second test of the effectiveness of silica HMS we chose a mixture of hexane, citric acid and lactic acid. To add a further complication, lactic acid was a mixture contaminated with approximately 20% of other oligomers, mostly the dimer lactoyl lactate (2-(2-hydroxypropanoyloxy)propanoic acid; see the Supporting Information). The higher polarity of the analytes requires the use of [D_3]acetonitrile as a solvent. Similarly to the case of the Cannizzaro mixture, a standard DOSY spectrum is barely able to resolve the components in the diffusion dimension, whereas the separation improves significantly in the presence of silica HMS (Figure 5).

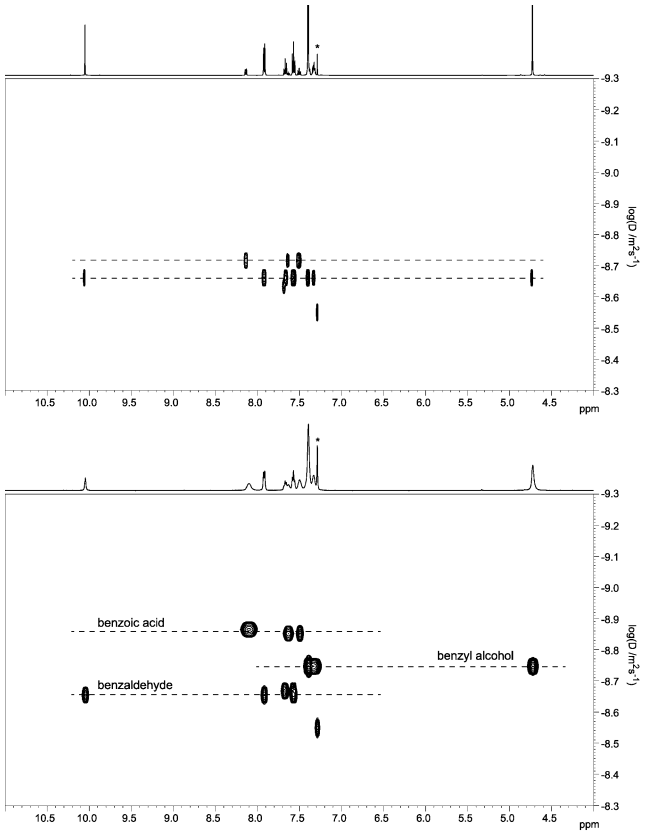

Figure 4. Top: Solution state DOSY spectrum of 5 mM benzoic acid, benzaldehyde and benzyl alcohol in $CDCl_3$. Bottom: same mixture in the presence of silica HMS (see text for details on sample preparation) and $CDCl_3$:[D_{12}]cyclohexane = 6:1.

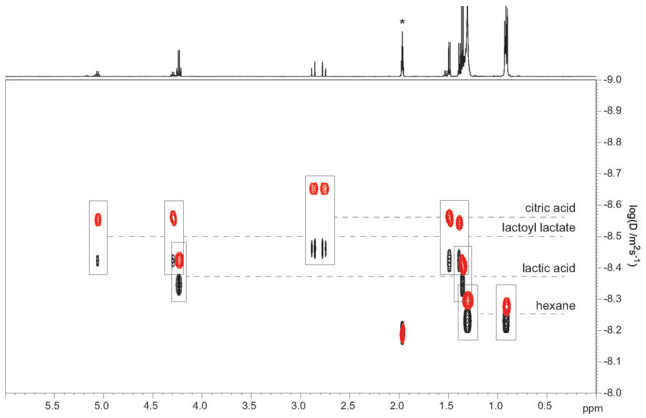

Figure 5. Black and top trace: Solution state DOSY spectrum of 5 mM hexane, citric acid and lactic acid in [D_3]acetonitrile. Red: same mixture in the presence of silica HMS. The two spectra have been aligned in the diffusion dimension taking the residual solvent signal (in the absence of microspheres, asterisk) as a reference.

In the context of chromatographic NMR spectroscopy, the above results indicate that silica HMS clearly provide some major advantages over conventional stationary phases. First, a good field homogeneity is obtained with standard 5 mm tubes fit for solution-state NMR probes. Such probes benefit from an established cryogenic cooling technology, which increases the sensitivity and opens access to detection of analytes in the micromolar range. Second, virtually any mixture of (deuterated) solvents can be used to fine-tune the separation of the analytes, in close parallel to the paradigm of classic chromatography. Such encouraging results indeed call for further investigation. In particular, we plan to quantify the effect of different solvent mixtures and investigate how the efficiency of silica HMS can be improved by varying their geometry or their surface properties (e.g. by functionalization). We also plan to seek a possible correlation between the apparent diffusion coefficients in the presence of silica HMS and the retention times observed in chromatographic runs, with silica HMS as a stationary phase. Eventually, this comparison would be useful to provide a deeper insight into the partition phenomena that underlie any successful chromatographic separation.

Experimental Section

Representative procedure for the synthesis of silica hollow microspheres (see Chen et al.[15] and the Supporting Information for details). As a first step, template monodispersed polystyrene (PS) microspheres were prepared by dispersion polymerization in an ethanol/water medium. To this purpose, we employed polyvinylpyrrolidone as a stabilizer (PVP K30, 1.5 g), azobisisobutyronitrile as a radical initiator (AIBN, 200 mg, 1.22 mmol), monomeric styrene (11 mL, 96 mmol), and the comonomer 2-(methacryloyl)ethyltrimethylammonium chloride (MTC, 80 % *w*/*w* H_2O, 424 μL). Mixing these reagents at 70 °C for 24 h led to the polystyrene latex template (positively charged). The second step involved the addition of NH_4OH and tetraethyl orthosilicate (TEOS, 6.4 mL, 30.87 mmol). Homogenous stirring of this mixture for approx. 1 h at 50 °C provided PS-core silica-shell spheres with an approximate 1000 nm diameter (estimated by TEM). The so obtained spheres were first washed with ethanol and water, and then dried at 60 °C in vacuum overnight. The PS template was finally removed by calcination at 550 °C.

General procedure for NMR samples preparation and spectra acquisition: 40 mg of microspheres were weighted and transferred into a 5 mm NMR tube. Then, 700 μL of a mixture of the analytes dissolved in proper deuterated solvents were added. The resulting suspensions were mildly sonicated for 30 minutes and centrifuged repeatedly to remove possible bubbles. DOSY spectra were obtained with a standard stimulated echo pulse sequence, featuring bipolar gradient pulses and a longitudinal eddy current delay (BPP-LED). The diffusion delay and the encoding gradient duration were set to 100 and 2 ms, respectively. The encoding gradients were ramped in 32 increments, providing a total experimental time of about 36 minutes.

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