Matrix-assisted DOSY

Article (Accepted Version)

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*Iain J. Day**

School of Life Sciences, University of Sussex, Falmer, Brighton, BN1 9QJ, UK

* *To whom correspondence should be addressed:*

Telephone: +44 01273 876622

Fax: +44 01273 876687

Email: i.j.day@sussex.ac.uk

Keywords: Diffusion NMR, Chromatography, Micelle, Surfactant, Polymer

Abstract

The analysis of mixtures by NMR spectroscopy is challenging. Diffusion-ordered NMR spectroscopy enables a pseudo-separation of species based on differences in their translational diffusion coefficients. Under the right circumstances, this is a powerful technique; however, when molecules diffuse at similar rates separation in the diffusion dimension can be poor. In addition, spectral overlap also limits resolution and can make interpretation challenging. Matrix-assisted diffusion NMR seeks to improve resolution in the diffusion dimension by utilising the differential interaction of components in the mixture with an additive to the solvent. Tuning these matrix-analyte interactions allows the diffusion resolution to be optimised. This review presents the background to matrix-assisted diffusion experiments, surveys the wide range of matrices employed, including chromatographic stationary phases, surfactants and polymers, and demonstrates the current state of the art.

1. Introduction

Nuclear magnetic resonance spectroscopy is unsurpassed in its utility for structure determination in the solution state. For example, NMR spectroscopists regularly utilise scalar couplings to determine through-bond connectivities between sites with different chemical shifts. However, NMR spectroscopy has additional tricks up its sleeve. By incorporating magnetic field gradients into the experiment, NMR spectra can become sensitive to a number of bulk parameters such as diffusion and flow, via the spatial encoding of the spins in the sample. In fact, some early NMR experiments utilised the inhomogeneous background magnetic field, arising from the magnets used, to measure self-diffusion coefficients [1]. These experiments were subsequently refined into the pulsed gradient spin echo experiments (PGSE) which form the basis of diffusion and flow measurements by NMR spectroscopy and magnetic resonance imaging [2,3]. It should also be noted that experiments which use pulsed field gradients for coherence pathway selection can be sensitive to the effects of diffusion and flow, resulting in some signal loss under unfavourable conditions [4].

The analysis of mixtures by NMR spectroscopy is typically very challenging. The overlap of resonances from different components of a mixture can render interpretation or quantitation difficult or even impossible, depending on the degree of spectral overlap. In fact, significant effort is usually employed in the sample preparation process in order to separate and purify samples prior to analysis [4,5]. This separation is typically performed using some form of liquid chromatography, or by solid-phase extraction. Depending on the sample, considerable time may be required for the development of a suitable chromatographic method prior to analysis by NMR spectroscopy [5].

Modern diffusion NMR experiments are typically performed using some variation of the pulsed gradient stimulated echo (PGSTE) experiment [6,7]. This experiment comprises three 90° pulses with diffusion-encoding gradients incorporated after the first and third RF pulses, allowing diffusion to be monitored over a time Δ. A pulse sequence timing diagram is shown in Fig. 1(a). The initial 90° pulse generates transverse magnetisation, which is spatially encoded by the initial pulsed field gradient. This encoded magnetisation is then restored to the *z*-axis by the second RF pulse. After an appropriate delay, the final 90° pulse returns this stored magnetisation to the transverse plane and the second gradient pulse refocuses the magnetisation, with the signal acquisition starting at the maximum of the stimulated echo [3]. This pulse sequence serves as the basic building block for most modern diffusion NMR experiments, with various elaborations and modifications included to address various experimental imperfections by partially cancelling eddy currents formed in the probe body [8], ensuring constant power dissipation in the probe and sample during the application of different gradient strengths [9], and cancelling the effects of convection [10,11]. An alternative pulse sequence is the spin echo, shown in Fig. 1(b), however, the stimulated echo is usually preferred as the magnetisation is stored along the *z*-axis during the diffusion labelling period, rather than in the *xy*-plane as in the case of the spin-echo experiment. This is at the cost of a loss in sensitivity of a factor of 2 [2,3]. For small molecules, T_1 is almost always equal to or longer than T_2, so relaxation losses during the stimulated echo pulse sequence are reduced compared to the spin echo [3], but the main reason for preferring the former is that it minimises the time for which magnetization is transverse, reducing both *J* modulation and susceptibility to the effects of magnetic field disturbance by the gradient pulses.

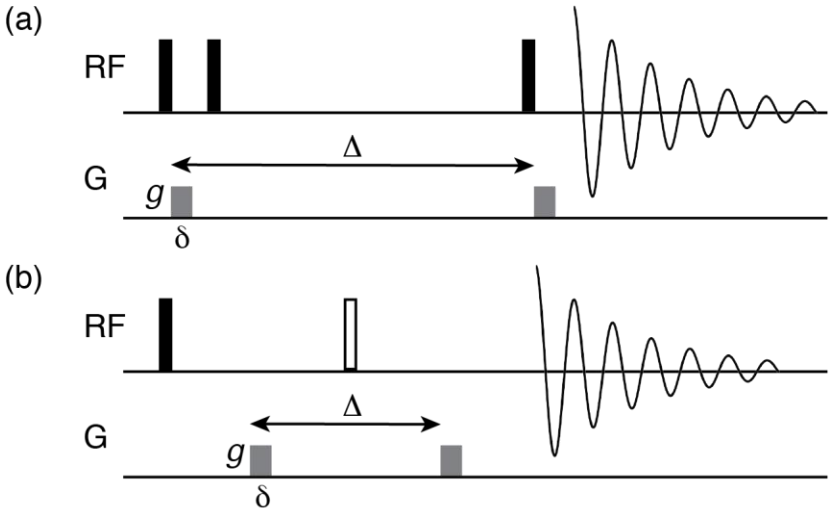

Figure 1: (a) Pulse sequence timing diagram for the prototypical pulsed gradient stimulated echo diffusion NMR experiment. Elaborations to correct for various experimental imperfections, such as eddy currents and lock refocusing can be incorporated into this pulse sequence and are used routinely. These modifications are discussed in more detail in Refs. [8–10]. (b) shows the timing diagram for the pulsed gradient spin echo experiment. The open rectangle represents a 180° pulse. The other symbols have the same meaning as in (a).

In order to determine the diffusion coefficient, a series of spectra are obtained varying one of three parameters: the diffusion-encoding gradient duration (δ), the diffusion-encoding gradient strength (g) or the diffusion labelling period (Δ). In principle any of these parameters can be varied, however, in reality it is almost always the diffusion-encoding gradient strength which is varied as so to ensure that the effects of relaxation on the echo attenuation are the same for each increment of the gradient

strength. The observed echo attenuation is related to these pulse sequence parameters via the following equation, known as the Stejskal-Tanner equation [12]:

$$s(g) = s(0)e^{-\gamma^2 g^2 \delta^2 \Delta'} \quad (1)$$

where γ is the magnetogyric ratio of the nucleus which is used to encode the diffusion, and $s(0)$ is the signal in the absence of any applied pulse field gradient. Δ' is the diffusion labelling time corrected to account for diffusion during the application of the gradient pulses [3,12,13]. This correction is pulse sequence dependent, and is determined by the exact timing and shapes of the RF and gradient pulses used [13]. Further modification to include the effect of spatial gradient non-uniformity, via the expansion of Eq. 1 as a power series is also possible [14]. The Stejskal-Tanner equation is used to fit the measured echo attenuation and the diffusion coefficient is obtained as a parameter. In DOSY presentation, a pseudo-2 dimensional spectrum is synthesised as a function of chemical shift and diffusion coefficient; the peak positions in the diffusion dimension are determined from the fitted diffusion coefficients, with the width determined by the error estimated in the fit. A Gaussian line shape is typically used in the diffusion dimension. This simple processing of diffusion NMR experiments is included in most modern spectrometer vendor and third party software packages, such as MNova [15], and is available via other tools such as the DOSYToolbox [16] and its successor [17].

Species which overlap in the chemical shift dimension present a challenge to the analysis of diffusion NMR data in that the measured echo attenuation is now multiexponential, as each species has its own diffusion coefficient. Fitting multiexponential data is known to be a very challenging problem [18]. A number of other methods are available to analyse diffusion NMR data, including (S)CORE [19–

21], DECRA [22–24] and constrained regularisation [25,26]. A discussion of these methods and their applications is beyond the scope of this work, however, more details can be found in a review by Toumi et al. [27].

An additional spectral processing tool which is often applied to diffusion NMR data is reference deconvolution [28,29], which aims to remove line shape distortions which affect all signals equally, such as those arising from B_0 field inhomogeneity/poor shimming, or phase or lineshape distortions [29]. The aim here is impose an idealised line shape onto those obtained experimentally, the process is sometimes known by the acronym FIDDLE (Free Induction Decay Deconvolution for Line shape Enhancement) [28]. Typically, an isolated singlet, e.g. from the solvent or an added reference compound, is used to determine the transformation needed to obtain a given target Lorentzian or Gaussian line shape, and this transformation is then applied to the whole spectrum [28]. When applied to diffusion NMR data, greater precision in the measured diffusion coefficients is typically obtained [30]. Reference deconvolution is also used in metabolomics and mixture analysis [31].

With a high level of chemical detail available, NMR spectroscopy has found widespread application in the analysis of mixtures [32], especially for so-called "omics" samples [33]. Diffusion NMR methods have also been applied to the study of other types of mixtures, however, somewhat less extensively. The main difficulty with these samples is the extensive overlap in the spectral dimension which complicates the analysis of the spin echo data. For example, Nilsson et al. have demonstrated the application of high resolution DOSY processing of diffusion NMR data to the analysis of various port wine samples [34]. These samples are complex

mixtures of mono- and disaccharides, phenolic flavour compounds, various short chain alcohols and their oxidation products, amongst other species. In total, over 35 individual species were identified, although not every signal in the spectrum was assigned to a component of the mixture. Interpretation of the HR-DOSY spectra of the port samples is complicated by the fact that resolution in the diffusion dimension is limited due to the extensive spectral overlap in the frequency dimension. Fitting to a single exponential decay is used to extract the diffusion coefficient (see equation 1), hence when there is spectral overlap the diffusion coefficient observed is intermediate between those for the overlapping species. Similar spectral complexity has been observed in other beverage samples, such as beer [35]. In this latter case, the use of higher dimensionality NMR experiments, such as DQF-COSY, improves the ability to resolve individual signals, as does increasing the static magnetic field. For the beer samples, various trisaccharides were identified using DOSY spectra recorded at a proton frequency of 800 MHz [35].

1.1 Alternative diffusion NMR methods

The robustness of the analysis of complex mixtures by diffusion NMR can be improved by incorporating an additional dimension. For example, a combination of DOSY and selective TOCSY transfer has allowed the analysis of mixtures from biomass-derived materials [36]. Diffusion NMR data is bilinear, that is it depends on two independent variables, namely frequency (i.e. chemical shift) and diffusion coefficient (via variation of the pulsed field gradient amplitude). Analysis of this bilinear data is complicated by the problem of rotational ambiguity, in that many valid solutions can be found to the matrix form of Eq. 1 [37]. Typical approaches to avoiding this problem include incorporating additional constraints such as non-

negativity, or imposing a known functional form on the echo attenuation. An alternative which avoids the rotational ambiguity problem is to extend the data to be dependent on a third independent parameter, producing a trilinear dataset. In the context of a diffusion NMR experiment, this additional dimension is often a T_1 encoding [37], or a time series for monitoring chemical kinetics [38]. The analysis of trilinear data is typically performed using parallel factor analysis (PARAFAC) [39] and has been implemented in the GNAT package from the Morris and Nilsson group [17,40]. Björnerås et al. have demonstrated the clean separation of a mixture of quinine, camphene and geraniol using PARAFAC analysis of T_1-DOSY data [37]. The strongly overlapped spectra of the three components rendered the analysis by more routine DOSY methods challenging, with particularly poor resolution in the diffusion dimension in the aliphatic region below 2 ppm [37]. PARAFAC analysis was used to monitor the acid-catalysed hydrolysis of maltose to glucose [38]. In this case the ^{1}H spectra of both carbohydrates are heavily overlapped and impossible to separate by diffusion NMR. The trilinear analysis allowed not only complete spectral separation, but also information on the hydrolysis kinetics, without the incorporation of any prior information into the analysis [38]. These ideas have been extended further by combining selective TOCSY transfer, diffusion and relaxation encoding, followed by a modified PARAFAC analysis, to separate strongly overlapped components in stout beer [41].

A number of standard two-dimensional NMR experiments have been modified to incorporate diffusion-encoding elements with the aim of improving the precision of the diffusion analysis by increasing the resolution in the frequency domain [42,43]. For example, the ^{13}C DEPTSE experiment allows carbon-13 chemical shifts to be

used as an alternative method to circumvent the spectral overlap problem [44]. This is similar to the ^{13}C INEPT DOSY experiment [45], however, there is a sensitivity penalty of a factor of 0.5 as the diffusion-encoding is performed using a ^{1}H stimulated echo. In contrast, the DEPTSE employs a spin echo following the DEPT transfer, allowing longer diffusion-encoding gradient pulses to be used to compensate for the lower magnetogyric ratio of ^{13}C [44]. Higher dimensionality diffusion NMR experiments are also useful for mixture analysis, for example Trefi et al. have used DOSY-COSY experiments to determine fingerprints for various genuine and fraudulent formulations of sildenafil [46]. Typically, the main drawbacks of these experiments are extended acquisition times and non-standard processing requirements, however, this is becoming less of an issue with packages such as GNAT [17] and MAGNATE [40] becoming available.

An alternative approach to the production of a pseudo-separation of a mixture of species, which can give a similar presentation to that of a DOSY spectrum is maximum-quantum (MaxQ) NMR [47]. Here species are separated based on the highest order quantum coherence which can be established within a spin system in a given molecule, with the MaxQ signal being a singlet in the multiple quantum dimension [47]. MaxQ and DOSY have been combined to give improved separation in a three dimensional experiment with a given projection able to reduce the spectrum to a single peak per species [48]. Filtering spectra based on other spin-dependent parameters is also possible, for example, complex mixtures may be analysed based on their sensitivity to paramagnetic relaxation [49].

1.2 Additives for diffusion NMR

The use of additives to modify the diffusion properties of mixtures is not a new technique. In the mid-1990s Morris et al. demonstrated that micelles could be used to differentially affect the observed diffusion coefficients of a mixture of various short chain alcohols [50]. In these systems 150 mM dodecyltrimethylammonium bromide (DTAB) was added, causing a retardation of the measured diffusion coefficients. These measured diffusion coefficients were interpreted in terms of a partitioning between free species and those associated with the micelles in solution. The Lindman law was used to describe the observed diffusion coefficient [3]:

$$D_{obs} = p_j D_j^{mic} + (1 - p_j) D_j^{free} \quad (2)$$

where p_j is the mole fraction for the species associated with the micelle and D_j^{mic}, D_j^{free} are the diffusion coefficients of the micelle and free species respectively [50]. The Lindman law, a weighted average, is valid provided the exchange between free and micelle-association species is rapid on the time scale of the diffusion labelling time Δ [3]. The observation of a single set of resonances in the NMR spectrum indicates that exchange is fast on the NMR chemical shift time scale. This latter time scale is typically much faster than the diffusion time scale, hence the observation of a single set of resonances generally implies that analysis in terms of Lindman's law is valid [2,3]. Morris et al. also demonstrated the use of sodium dodecylsulfate micelles for the modulation of the diffusion properties of a mixture of toluene, benzyl alcohol and tetraethylene glycol [50]. These results are shown in Fig 2. They noted that the interaction with the micelles depends on the hydrophobicity of the analyte species, however, detailed analysis of the nature of these interactions and the influence of micelle composition on the observed change in diffusion coefficient would come much later (see Surfactants, below).

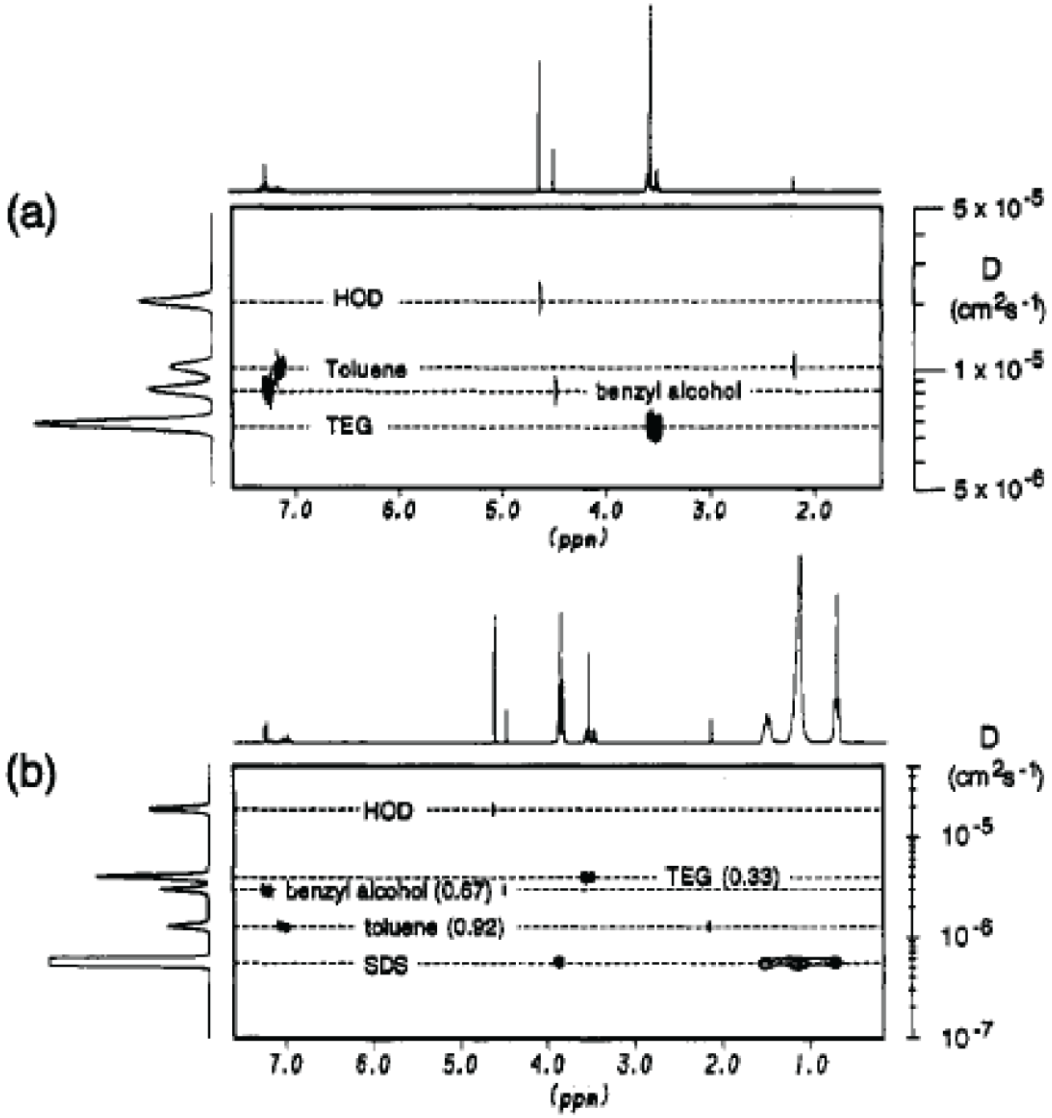

Figure 2: DOSY presentation of an equimolar mixture of toluene, benzyl alcohol and tetraethylene glycol (TEG) in D_2O. (b) Shows the same mixture in the presence of 150 mM SDS [50]. Reproduced with permission from Morris et al., *Anal. Chem.*, **66** (1994) 211–215. Copyright 1994 American Chemical Society.

The idea of using the interaction of a small molecule with a second species has also been demonstrated by Shapiro and colleagues in the development of affinity NMR. The aim here is to measure the binding of small molecule ligands to receptor proteins

as part of the drug discovery process [51,52]. Similar arguments, based on Lindman's law, are used to determine which species is binding to the target of interest. A diffusion-weighted experiment is performed and signals which arise from non-binding species are strongly attenuated as these molecules retain their rapid diffusion behaviour since they are small. Binding ligands, conversely, diffuse more similarly to the protein and hence suffer less attenuation. The combination of the degree of attenuation and the concentration of the ligand can be used to determine the binding affinity of the small molecule [51,52]. This then provides an alternative ligand screening experiment [53] to more common approaches such as saturation transfer difference [54]. When processed and plotted using the conventional HR-DOSY representation, it is straightforward to identify the binding species in a mixture of compounds [53].

Matrix-assisted diffusion (MAD) NMR is the general term given to the use of an additional species to modulate the diffusion properties of an analyte or analytes of interest. The choice of additive to use as the matrix has a number of requirements. Clearly, there must be some interaction between the analyte(s) and the matrix in order to affect a modulation of the observed diffusion coefficient. This interaction needs to be finely balanced, ensuring that exchange between free and interacting state is rapid both on the NMR chemical shift and diffusion labelling timescales [3]. If this is not the case, then there will be an increase in spectral complexity arising from intermediate and/or slow exchange regimes. The effects of exchange on diffusion NMR spectra, while potentially complex, have been well studied [3,55,56]. The Kärger equations can be used to describe the signal intensity as a function of exchange rates, diffusion coefficients and various experimental parameters [57,58]. In

the majority of cases, MAD experiments are most easily performed and interpreted within the fast exchange limit [59].

Addition of the matrix should only cause limited disruption to the NMR spectrum, at the same time as being chemically compatible with the analytes and solvent system in use. As will be seen below, a number of matrices can cause line broadening or introduce a significant number of additional signals to the NMR spectrum. Ideally, these effects should be minimised or ameliorated in some way. Often signals from the matrix itself are localised to part of the spectrum, and usable analyte signals can be found in other regions. Lindman's law (Eq. 2) suggests that in MAD the matrix should have significantly slower diffusion than the analytes, in order for the interactions with the matrix to effect the greatest change in the measured diffusion coefficient. The common matrices, such as surfactant or polymer micelles and chromatographic stationary phases, satisfy this criterion. The practical challenge is to find experimental conditions under which approximately half of the analyte is bound to the matrix, since this is when the measured diffusion coefficient will be most sensitive to differences in binding strength.

At this stage, it is also worth making the comparison between matrix-assisted DOSY (or chromatographic NMR) and NMR chromatography, an on-line technique in which the output from a (liquid) chromatography separation is fed into an NMR spectrometer via a flow probe as the detector (also known as LC-NMR, or with further hyphenation as LC-NMR-MS) [60]. This technology has been available since the early 1990s, but has had somewhat limited uptake due to various technical challenges related to spectrometer stability, the difficulty of shimming changing

multicomponent solvent systems (e.g. as the solvent composition changes along a D_2O / acetonitrile gradient elution method) [60,61] and the volumes of expensive solvent required due to the LC system having to be outside of the magnet 5 G line. Lindon et al. have presented a review of the field, including a comparison with off-line chromatography and automated sample preparation [62]. More recently, with the resurgence of low-field benchtop spectrometers some of these challenges have become less severe due to weaker and more compact stray fields, and the direct coupling of a chromatographic separation to an NMR spectrometer is more viable, especially when high spectral resolution is either not required or not possible, e.g. in the case of polymer analysis [63,64].

In general, any suitably large species which is going to modulate the observed analyte diffusion coefficients depending on some differential interaction between the matrix and analyte(s) can be used to effect matrix-assisted DOSY. Modern implementations of matrix-assisted DOSY can be broadly categorised according to the nature of the matrix employed: chromatographic stationary phases, surfactants, or polymers. This review presents the current state of the art, broadly organised around the choice of matrix.

2. Chromatographic Stationary Phases

Diffusion NMR, and in particularly the high resolution DOSY presentation of the data as a pseudo 2-dimensional spectrum is often described in terms of an analogy with chromatography [6]. It is fitting then, that the resurgence in interest in matrices or additives to modify the observed diffusion coefficients by interaction with analyte species was with the addition of chromatographic stationary phases, reported in 2003

[65]. The early work in this area was performed independently by groups in France and Israel, using silica matrices.

2.1 Silica Supports

In high performance liquid chromatography (HPLC) separations, a mobile phase (solvent) is flowed over a stationary phase contained within a metal column. The differential interaction of analyte species with the stationary phase results in their retardation, and hence elution at different times after injection onto the column. In a variant of matrix-assisted DOSY, termed chromatographic NMR, an HPLC stationary phase is added to the NMR sample with the aim that the interaction of the analytes with the stationary phase will modulate the observed diffusion coefficients in a similar manner to the observed retardation in a traditional on-flow chromatography experiment. Caldarelli and Viel first demonstrated this approach using bare silica to cause a pseudo-separation of dichlorophenol, ethanol and heptane, as shown in Fig. 3. In the absence of the stationary phase these three species show similar diffusion coefficients, severely overlapping in the diffusion dimension (Fig. 3(a)). On addition of fused silica, a separation is observed with the ordering of species being in line with that expected for a normal phase HPLC separation of the species (Fig. 3(b)) [65]. The use of silica in traditional NMR solvents was demonstrated by the separation of a mixture of four hydroxyl containing species in D_2O, including methanol, propan-2-ol and phenol [66]. Similarly, a C_{18}-functionalised silica was used to separate three less polar species, naphthalene, dec-1-ene and ethanol [65]. One drawback of functionalised silica stationary phases is that additional signals related to the functionalisation are introduced into the NMR spectrum. These additional signals have the potential to obscure the signals of interest. Subsequently Caldarelli

demonstrated that reverse phase HPLC conditions can also be used to similar effect. A mixture of anthracene, naphthalene and benzene was separated using C_{18}-functionalised silica (Lichromspher100 C18) with an acetone/water solvent system, again with the ordering of species being comparable to that obtained using traditional on-flow HPLC under the same stationary and mobile phase conditions [67]. Interesting, the same mixture of species shows a similar pattern in its separation under chromatographic NMR conditions when the stationary phase is replaced with a bare silica (Lichromspher100 Si), despite the three species co-eluting in HPLC run under comparable conditions [67]. Clearly therefore explanation of the separation observed in the diffusion dimension of a chromatographic NMR experiment does not have a simple one to one mapping with the observations in the corresponding HPLC experiment under similar conditions.

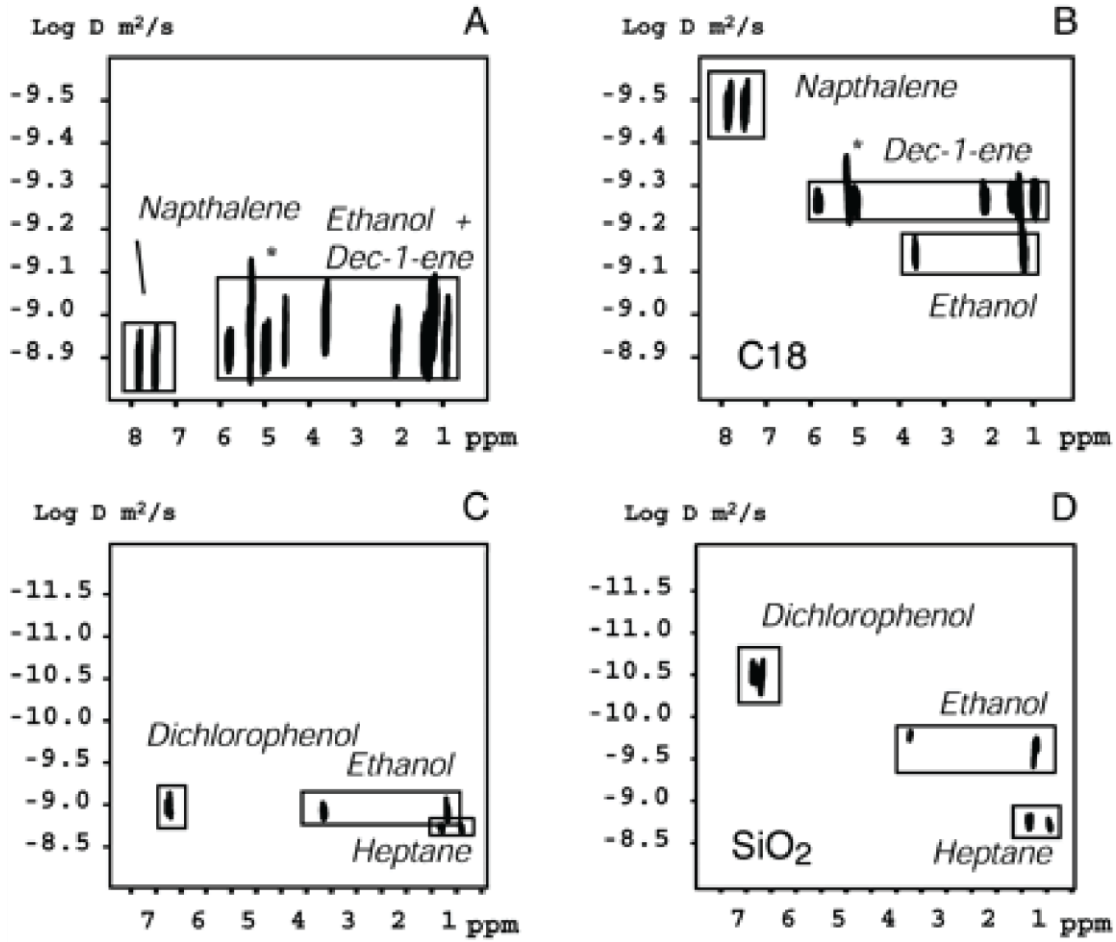

Figure 3: DOSY spectra for two mixtures demonstrating the influence of a chromatographic stationary phase on the measured diffusion coefficients. Naphthalene, ethanol and dec-1-ene (A) are clearly separated using C_{18}-functionalised silica (B), while dichlorophenol, ethanol and heptane (C) are separated using the more hydrophilic fused silica (D) [65]. Reproduced from Viel et al., *Proc. Natl. Acad. Sci. U.S.A.*, **100** (2003) 9696–9698. Copyright (2003) National Academy of Sciences, USA.

One of the key factors in both traditional chromatography and chromatographic NMR is how the analyte interacts with the stationary phase. The nature and strength of this interaction determines whether a separation (or pseudo-separation) will be effective.

In an on-flow chromatography column it is difficult to study the transport of the analyte between the stationary and mobile phases directly. This is, however, possible with pulsed field gradient NMR spectroscopy. Caldarelli and colleagues have investigated the transport of benzene in deuterated chloroform over a porous silica stationary phase under MAS conditions [68]. Analysis of the diffusion behaviour of the benzene required the inclusion of the benzene vapour phase within the pores in addition to the diffusion in the bulk liquid. Diffusion at the surface of the silica was neglected due to rapid relaxation occurring at the surface. The diffusion of the benzene was found to be strongly dependent on the filling factor of the intraparticle voids, with changes in the effective diffusion coefficient spanning a factor of 2-3 over a range of filling factors [68]. These results demonstrate that there are a number of factors which influence the observed diffusion coefficient beyond just the interaction between the analyte and the stationary phase, but that the influence of the solvent, and of solvent-solute and solvent-stationary phase interactions, should also be properly considered. Subsequent work by Carrara and Caldarelli showed that mass transport effects are extremely important and are strongly affected by the loading of the system, as expressed by the solution/solid phase ratio [69]. Chromatographic NMR samples can be prepared in a continuum of loading ratios, from where the stationary phase is a suspension (or settled suspension) in the solvent [70] through to effectively dry powders to which a small volume of solvent is added [69]. Carrara and Caldarelli demonstrated that changes in the loading ratio can induce separation under chromatographic NMR conditions in situations where no such separation was apparent using HPLC with similar stationary and mobile phases. For example, a mixture of three aromatic species, benzene, naphthalene and anthracene, can be separated using LiChromspher Si100 at low phase ratio (typical solution to solid

volume ratio below 3) with 4 kHz MAS, whereas the components of this mixture co-elute under similar conditions on-flow [67,68]. Conversely, a mixture containing some more polar species, e.g. naphthalene, aniline and phenol, requires significantly higher phase loadings (solution to solid volume ratio above 4) to achieve separation in the chromatographic NMR experiment, despite clear baseline resolution in the HPLC trace with the same stationary phase [69]. The implication of these results is not only that the nature of the interaction with the stationary phase is important, but also that there is an interplay between this and transport between the intra- and interparticle voids. This is especially important for volatile species, which can undergo condensation-evaporation at the surface of the pores in the stationary phase, such as benzene. Caldarelli has presented a detailed review of chromatographic NMR using silica stationary phases, including a comparison with on-line chromatography [71]. The shape and connectivity of the pores within a stationary phase will also influence the diffusion behaviour of analyte molecules within the stationary phase.

The description of matrix-assisted DOSY as chromatographic NMR, when silica stationary phases are used in conjunction with the analogy between chromatography and DOSY spectra, immediately brings to mind whether chromatographic NMR can be used to predict liquid chromatography performance or at least assist in the time-consuming and somewhat empirical process of chromatographic method development. The strength of the interaction between the analyte species and the silica surface has a huge influence on the separation achievable for a given stationary phase. Hoffman and co-workers analysed the impact of H60 silica on the diffusion properties of 55 small molecules with a wide range of functionalities, and broadly categorised them into three groups of compounds depending on the degree of change

in the observed diffusion coefficient [72]. Here, the ability to form hydrogen-bonding interactions with free hydroxyl groups on the surface of the silica was postulated as the driving force behind the different levels of interaction. Intra- and intermolecular interactions were also found to be important, for example methylamine was found to have only limited interaction with the silica due to the formation of large scale intermolecular hydrogen-bonded chains in solution [72]. Carrara et al. have developed an implementation of the chromatographic NMR methodology in which the pore volume is largely occupied, i.e. the solution to solid volume phase ratio is high, in which case the ratio of the observed diffusion coefficient in the presence of the stationary phase to that in its absence, D/D_0, is proportional to the porosity of the stationary phase. As long as the fast exchange regime is in effect for the analyte, i.e. exchange between the stationary phase and the solution is rapid, then the process is under thermodynamic control and the chromatographic NMR results should mirror those of the on-flow HPLC analysis [73]. Carrara et al. demonstrated that a linear correlation was observed between HPLC retention time and D/D_0 for a series of polycyclic aromatic compounds using two stationary phases: Acclaim 120 C18 and Acclaim Polar Advantage. This indicates that the fast exchange approximation is valid, as expected from the NMR diffusion data, and that under these conditions, chromatographic NMR can be used to predict chromatographic shape selectivity.

The influence of stationary phase functionalisation, and the ability of chromatographic NMR to provide useful information about the potential LC behaviour of a given phase, was investigated by Lopez et al. across eight stationary phases [74]. These stationary phases were all based on C_{18}-functionalised silica but employed different bonding strategies and end-capping. The pore sizes for these

matrices were in the range 100-120 Å, with total surfaces areas in the range 320 to 425 m^2 g^{-1} [74]. Five different analytes with basic functionality, such as benzylamine, quinine and carvedilol, were studied under HR-MAS conditions, with varying solution to solid ratios. The factors affecting the kinetics of interaction between the analyte and stationary phase were found to include the stationary phase silanol density, the net charge on the analyte, and its overall hydrogen bond acceptor capacity. The role of the solvent was found to be particularly important, especially at lower solute concentrations where slower kinetics were observed [74]. The authors also pointed out that the absence of flow in the chromatographic NMR experiment is a major difference compared with liquid chromatography, and that this has a large potential impact on the interaction kinetics, and hence separation capability [74]. The behaviour of the solvent, particularly water/acetonitrile mixtures in reversed-phase chromatographic media, has been investigated by ^{14}N quadrupolar relaxation, allowing a thermodynamic characterisation of the free and bound acetonitrile in terms of a two-state fast exchange model, and allows the thickness of the solvent layer around the stationary phase particles to be determined [75].

2.2 Bulk Magnetic Susceptibility issues

NMR spectroscopists typically take great care to ensure that particulate matter is not present in their samples. Particulate matter within the sample tube generally results in line broadening and line shape distortions due to differences in the bulk magnetic susceptibility between the particulate matter and the solvent [76,77]. It is generally not possible to "shim out" these distortions as they occur on a microscopic scale. The addition of a chromatographic stationary phase, typically added such that the material completely fills the coil region, therefore results in significant line broadening being

observed. In certain cases, this renders spectral interpretation extremely difficult. Two approaches have been presented to alleviate the issues of susceptibility broadening in chromatographic NMR experiments. The first approach was developed by Caldarelli and takes advantage of the fact that the susceptibility broadening contribution to the Hamiltonian has a dependence on the second Legendre polynomial [77], mathematically similar to the chemical shift anisotropy or dipolar coupling, and can therefore be removed by magic angle spinning. Relative to solid state NMR only low spin rates (1-4 kHz) are required to remove this susceptibility broadening from the spectrum. Clearly, this has additional hardware requirements in that in order to undertake these chromatographic NMR experiments a magic angle spinning probe equipped with a pulsed field gradient is required. This requirement is usually satisfied in so-called HR-MAS probes, which are designed for moderate spinning speeds and typically include a magic angle gradient coil, and are typically used for the analysis of tissue samples [78], slurries and semi-solids [79]. Caldarelli and Viel have pioneered the use of HR-MAS in chromatographic NMR, typically with silica stationary phases [65,67,80] while Day has utilised dextran-based phases [81,82].

Rapid rotation of the NMR sample has the potential to lead to vortexing effects which can influence the observed diffusion coefficient as these effects superimpose coherent motion onto the incoherent motion detected by the spin-echo experiment [80,83]. Bradley et al. demonstrated that increased coherent motion, as detected via an asymmetric double spin-echo experiment [10], was present with an HR-MAS probe compared to a standard solution state probe, especially for low viscosity solvents such as chloroform, and at higher spin rates (>2 kHz) [83]. An increase in scan-to-scan intensity “jitter” was also noted; however, the effects of this could be reduced by

increased signal averaging, at the cost of extended acquisition times [83]. Interestingly, other groups reported little variation in the observed diffusion coefficient under MAS, especially for more viscous solvents [80]. Issues relating to gradient uniformity, temperature stability and rotor volume were found to have a great influence on the measured diffusion coefficient. Selecting regions of uniform gradient and small volume rotors generally showed better correspondence between the diffusion coefficients measured under MAS with those determined in standard 5 mm NMR tubes. In all cases, spinning rates below 5 kHz were found not to be problematic [80]. The experimental differences between the observations of Bradley et al. and Viel et al. may, at least in part, be related to differences in probe construction, particularly around sample rotation (spinning) control and drive tips etc., as the two groups used probes from different instrument vendors. Rotor synchronisation of the diffusion labelling period, the gradient pulses and other delays can reduce the errors associated with measuring diffusion coefficients under MAS conditions [82–84]. Additionally, there is the possibility that the centrifugal forces induced by the rapid rotation of the sample can cause sedimentation of the stationary phase to the walls of the rotor, particularly if high solvent/stationary phase ratios are used [85–87]. Bertini and colleagues have used this idea, albeit with significantly faster spinning rates, to acquire NMR spectra of partially soluble proteins, where the NMR spectrum is only available under MAS conditions, using material sedimented against the wall of the rotor [85,87]. In the context of matrix-assisted DOSY it is therefore possible that under MAS there is a higher proportion of stationary phase at the walls of the rotor compared to closer to the rotation axis, and hence the modulation of the observed diffusion coefficient is not uniform radially across the sample [82]. The influence of

this on the final analysis should be small since this sedimentation effect is generally orthogonal to the diffusion-encoding gradient direction.

An alternative approach suggested by Hoffman does not require any additional hardware and can therefore be performed on a standard liquid state NMR spectrometer. The Hoffman approach is to systematically vary the magnetic susceptibility of the solvent, using a combination of various solvents, searching for the minimum linewidth as the susceptibility of the solvent approaches that of the stationary phase [88]. This is demonstrated in Fig. 4(a), where the minimum line width found is close to 12 Hz using a mixture of halogenated solvents and DMSO. Hoffman's method requires careful tuning of the solvent system used, and can be slow as a range of samples needs to be prepared. In addition, the stationary phase must be compatible with the solvent system, and not undergo any chemical changes or degradation. For example, agarose or dextran stationary phases cannot tolerate more than small volume fractions of DMSO, whereas bare silica stationary phases are more robust (functionalisation, however, will limit the solvent compatibility range). Typically, for silica-based stationary phases, a high proportion by volume of a halogenated solvent is required [88] as these have the large magnetic volume susceptibilities which are needed to match that of the silica. Using a susceptibility matched mixed solvent system of chloroform-*d* and diiodomethane, Hoffman and co-workers were able to effect the separation of a mixture of DSS, propylene glycol, hexanol and hexamethyl disiloxane, as shown in Fig. 4(b). Good separation was obtained for most of the mixture in the diffusion dimension, however, the latter two compounds showed little separation in the diffusion dimension, despite being clearly resolved in the chemical shift dimension. In fact, the observed diffusion coefficients

for hexanol and hexamethyl disiloxane showed little influence of the matrix, indicated a lack of interaction between the matrix and these analytes [88].

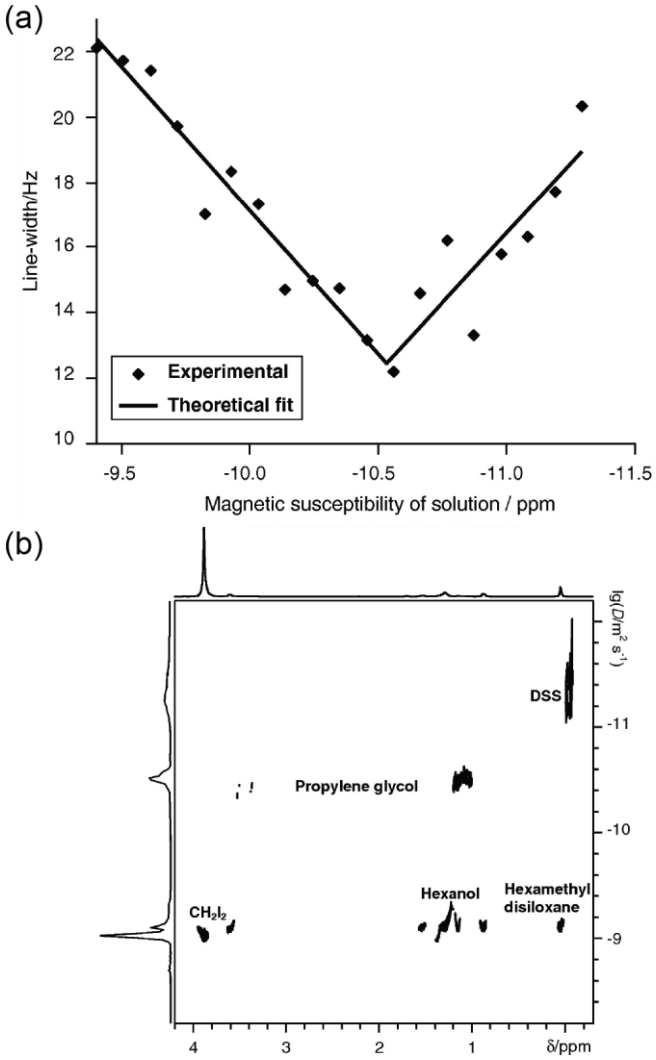

Figure 4: (a) shows the variation in line width for the CH_2I_2 solvent signal as a function of magnetic susceptibility, controlled by varying the ratio of $CDCl_3$ to CH_2I_2. (b) DOSY plot of a representative mixture in the presence of a silica 60H stationary phase, using a susceptibility matched solvent [88]. Reproduced from J. Magn. Reson., 194, R. Hoffman, H. Arzuan, C. Pemberton, A. Aserin and N. Garti, High-resolution NMR "chromatography" using a liquids spectrometer, 295-299, Copyright (2008) with permission from Elsevier.

Despite this, both approaches are inconvenient, requiring the use either of relatively uncommon HR-MAS hardware, or of halogenated solvents with their associated sample compatibility issues. Yang et al. have addressed these problems by replacing the use of microscale silica stationary phase with a nanoscale silica sol [89]. This material has a solid content of 40.9% and a particle size of only 19 nm. At this small diameter, susceptibility broadening issues will be significantly reduced, avoiding the line width problems associated with silica stationary phases reported by Caldarelli [65] and Hoffman [88]. Yang et al. have reported the effective separation of fumaric and maleic acids, molecules which differ only in the isomerisation state of a central double bond. In fact the separation was shown to be improved with a change in solvent system from silica sol in water (1:2 v/v) to silica sol in acetone (1:2 v/v) [89]. The line width observed in the spectral dimension was comparable to that obtained in the absence of the stationary phase. Geometric isomers of more complex structures have also been separated under similar conditions, with the isomers of 1,2-cyclehexane dicarboxylic acid separated in a chromatographic NMR experiment using silica sol in deuterated dimethyl sulfoxide (1:2 v/v) [89].

2.3 Restricted diffusion effects

In the context of MAD, the nature of the matrix may result in restricted diffusion effects coming into play under certain conditions, for the bound or interacting component. This is most likely to occur when using chromatographic stationary phases which are porous such as fumed silicas, or dextrans.

Accurate determination of the true diffusion coefficient depends on the ability of a spin to undergo free translational diffusion during the diffusion labelling period Δ [2,3,6]. If there is a restriction, for example if the diffusing species is inside a pore and undergoes collisions with the walls during the diffusion labelling period, then the diffusive behaviour is no longer governed pure by random Brownian motion. The measured echo attenuation no longer reflects the free diffusive behaviour, but is more or less strongly affected by the restrictions [3]. Under these circumstances, the measured diffusion coefficient becomes a function of the diffusion labelling period. Ultimately, if the diffusion labelling time is long enough, or the restriction small enough, compared with the free diffusion coefficient, then the measured diffusion coefficient depends only on the size of the pore or void being explored [3]. The exact dependence of $D(\Delta)$ is complicated, being a function of the pore geometry [90,91] and, when measured by NMR spectroscopy, pulse sequence [3,92]. These effects can be used to probe the nature of a porous material [93], and have found great utility in NMR diffusometry [94]. Rottreau et al. have investigated two silica phases typically used in catalysis [95], that have different, but well defined port architectures. Diffusion NMR measurements were used to determine the tortuosity (effectively the degree of hinderance due to the stationary phase) and its relationship both to the pore geometry within the stationary phase, and to the nature of the analyte, in this case the length of fatty acid carbon chain. Greater tortuosity was observed for the more highly interconnected KIT-6 stationary phase compared to the cylindrically isolated pores of SBA-15 [95]. These results may help to inform the choice of silica phases for chromatographic NMR.

2.4 Size-exclusion phases

Cross-linked dextran stationary phases are typically used in size exclusion chromatography (SEC) and gel permeation chromatography (GPC), for the size determination of large biomolecules and polymers respectively. In reality, the techniques are broadly similar, with the difference in nomenclature arising due to the origin of the techniques in different fields. In both, separation occurs due to partitioning of the analyte into pores within the stationary phase. As the analyte explores these pores, smaller molecules get stuck and spend more time trapped inside these pores than larger molecules, and hence large species are eluted before smaller species, which are retarded by the stationary phase [96]. Clearly, there is also a high molecular weight cut off, where extremely large species cannot access the pores at all and are eluted with the solvent front. Initially, this would suggest that size exclusion phases would not be well suited as stationary phases for chromatographic NMR since the size-dependent influence of the stationary phase is exactly opposite to that arising naturally for diffusion. Smaller species will be retarded more by the stationary phase than larger species, while smaller species have faster diffusion coefficients than larger species.

Despite the potential limitations, Day and co-workers have demonstrated the use of size exclusion stationary phases for chromatographic NMR with polymer analytes [70,81]. This implementation is essentially the same as chromatographic NMR with silica stationary phases, except that due to the typically larger line widths observed when dealing with polymers, HR-MAS or susceptibility matching is not normally required, although it can be readily incorporated if needed [82]. The stationary phase is allowed to settle under gravity such that it fills the coil region, and the solution to solid volume ratio is typically very high [70]. The presence of the stationary phase has

been shown to cause a retardation of the observed diffusion coefficient for a series of polymer molecular weight reference standards such as poly(styrene sulfate), with the degree of retardation dependent on the molecular size [70]. The effect of the stationary phase has been characterised and interpreted in terms of size exclusion behaviour, with similar phenomenological expressions to those used by Anderson and Stoddart [97,98] and Determann and Michel [99] to analyse the influence of the stationary phase:

$$\log M = a_0 - a_1 D \quad (3)$$

where a_0 and a_1 are dependent on the stationary phase used. Trends in a_1 with varying stationary phase are consistent with size exclusion behaviour [70].

Size-exclusion chromatographic NMR operates with a similar mechanism to other forms of chromatographic NMR and MAD in general, with an equilibrium established between the in-pore and free components [70]. The effect on the observed diffusion coefficient upon addition of the stationary phase can be used to investigate the partition coefficient (the ratio between free and in-pore components), with typical values in the range 0.1-0.75 [100]. Restricted diffusion effects are observed when the product of the diffusion coefficient D and diffusion labelling period Δ is greater than the square of the effective pore size (a), i.e. when the parameter ξ is much greater than 1 [3,101]:

$$\xi = \frac{D\Delta}{a^2} \quad (4)$$

In the case of size-exclusion chromatographic NMR, with typical experimental parameters $\xi > 10^5$ therefore the diffusion coefficient for species within the pores should be solely dictated by the size and geometry of the pore and porous network [3,101]. This leads to the conclusion that the observed diffusion coefficient in the

presence of an SEC stationary phase is governed by the free diffusion coefficient and the partitioning between the pores and free solution [70]. This is illustrated graphically in Fig. 5. More recently, Elwinger et al. have presented a similar methodology using fully equilibrated polymer solutions in the presence of porous media to determine the equilibrium distribution coefficients, by the joint analysis of the echo attenuation curves in the absence and presence of the stationary phase [102]. This is then presented in terms of a selectivity curve and provides details of the pore sizes without the need to numerically invert the echo attenuation, a procedure which is known to be numerically unstable [18,26]. Good agreement between the diffusion NMR and regular inverse size-exclusion chromatography was reported [102].

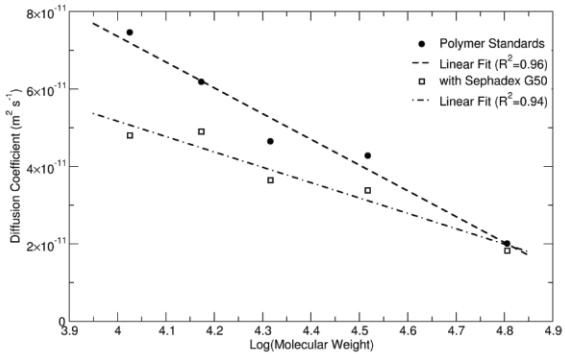

Figure 5: Observed diffusion coefficients for a range of poly(styrene sulfonate) molecular weight reference standards in the absence and presence of Sephadex G-50 stationary phase. The straight lines are fits to Eq. 3 [70]. Reproduced from J. Magn. Reson., 220, R. E. Joyce and I. J. Day, Chromatographic NMR with size exclusion chromatography stationary phases, 1-7, Copyright (2012) with permission from Elsevier.

Size exclusion chromatographic NMR has also been employed in the context of small molecule aggregation driven by π-π stacking interactions. Joyce and Day have demonstrated a partitioning of aggregates of sunset yellow (a food dye) into in-pore and free components based on the size of aggregates [81]. Identification of the two components was made based both on chemical shift arguments [103] and on the influence of the MAS on the observed line widths. The in-pore component showed little improvement in resolution under HR-MAS whereas the free component signals, broadened by susceptibility differences as described above, became narrow and a liquid-like line shape was restored upon spinning [81]. This allowed the relative proportions of the in-pore and free components to be quantified and correlated with other assembly behaviour [103–105].

2.5 Other chromatographic phases

Chromatography has a rich variety of implementations, including thin-layer chromatography, liquid-liquid separation and the use of supercritical fluids. In the context of chromatographic NMR, it is the stationary phase which yields the greatest potential variety in separation ability. While the majority of HPLC columns use silica stationary phases, other materials can be used, with zirconia being particularly common [106]. The use of an alternative base support can impart differences in selectivity and hence separation due to differences in pH, surface interactions and polarizability [106,107]. Solid-state ^{13}C NMR has been used, in conjunction with other characterisation methods and molecular dynamics, to investigate the chain ordering of C_{30} chains assembled on silica, titania and zirconia surfaces, with greater ordering observed on the group 4 oxides, albeit with large amplitude motions present at the termini of the alkyl chains [108]. Alternatives phases to silica have not yet

found application as matrices in diffusion NMR experiments, however, zirconia would be an ideal candidate for investigation as it has a similar magnetic susceptibility to D_2O [109].

3. Surfactants

Micelles formed by the aggregation of amphiphilic molecules are ideal candidates as matrices for MAD experiments as they will typically be much larger, and hence diffuse more slowly, than the small molecule analytes. Interaction with these micelles will then lead to a large change in the observed diffusion coefficient upon addition of the matrix, via the Lindman law (Eq. 2). The use of sodium dodecylsulfate (SDS) micelles to modify the diffusion properties of small molecules has a long history in NMR spectroscopy [110], with a focus on the effects of short chain alcohols on the micellar structure [111]. Cationic micelles based on long chain trimethylammonium halides such as CTAB and DTAB have also been used as mentioned earlier [50]. More recently, Morris, Nilsson and colleagues have elegantly demonstrated that SDS micelles can be used to effect separation of structural isomers in the diffusion dimension; for example, the three isomers of dihydroxybenzene have the same diffusion coefficient in D_2O solution, however, can be separated using 150 mM SDS with the diffusion coefficients varying by nearly 40% [112]. This is demonstrated in Fig. 6. The order of separation was explained by the strength of interaction between the dihydroxybenzene isomer and the SDS micelle [112]. Addition of a matrix clearly has the potential to alter other bulk properties of the sample such as viscosity, ionic strength etc, however, the fact that the diffusion coefficient of the solvent signal was not significantly affected by the presence of the micelles lends support to the argument that it is a specific interaction between the analytes and SDS micelle matrix

which is responsible for the diffusion modulation effect, rather than bulk sample changes [112].

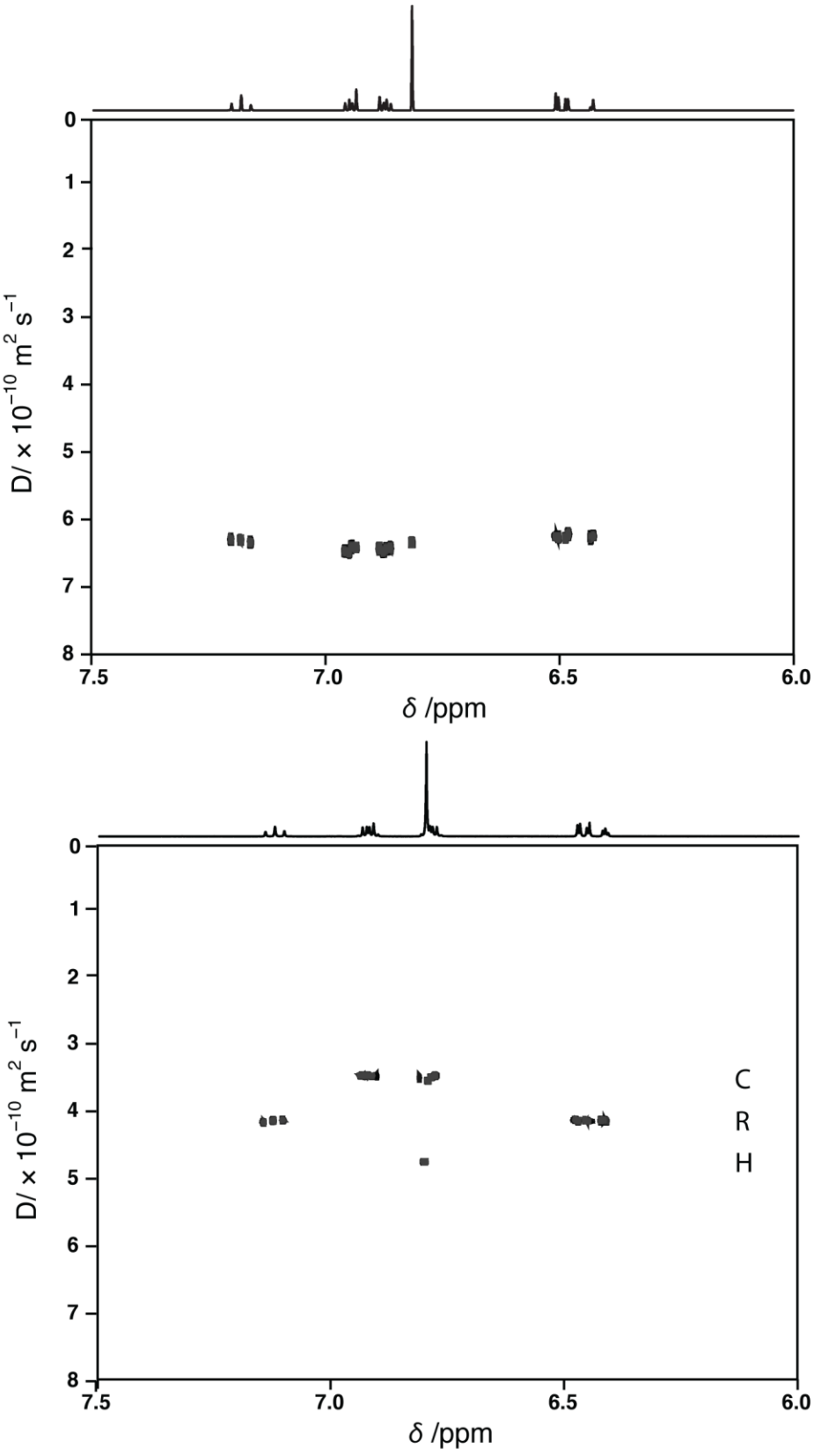

Figure 6: Oneshot DOSY spectra of an equimolar mixture of the dihydroxybenzenes catechol (C), resorcinol (R) and hydroquinone (H). Top is in D_2O, below is the same spectrum recorded in the presence of 150 mM SDS micelles [112]. Reproduced with permission from Evans et al., *Anal. Chem.*, **81** (2009) 4548–4550. Copyright 2009 American Chemical Society.

Further, more detailed investigation by the Manchester group demonstrated that other species could be separated using SDS micelles in a similar manner, for example, the isomers of methoxyphenols showed similar separation using 36 mM SDS [113]. Tormena et al. also investigated the influence of SDS concentration on the effectiveness of isomer separation, for a range of SDS concentrations from 30 to 230 mM, all well above the critical micelle concentration. The results of these experiments demonstrated that effective separation was possible using relatively low concentrations of SDS, and high sample loadings [113], i.e. large amounts of analyte compared to matrix; this can be thought of in a similar manner to the solvent to solid phase ratio discussed in the context of chromatographic stationary phases [68]. The use of SDS micelles as an effective matrix (in a mixed D_2O – DMSO-d_6 solvent) was demonstrated using a more complex mixture of the flavonoids: flavone, fisetin, (+)-catechin and quercetin [114]. Álvarez et al. utilised SDS micelles in MAD experiments as part of a larger investigation into two flavonoids from *Persea caerulea* which differ only in the additional of an extra hydroxyl group and the nature of the carbohydrate moiety [115]. In the absence of SDS micelles the two flavonoids diffuse at the same rate, whereas on addition of 180 mM SDS, quercetin-3-O-α-L-arabinofuranoside shows greater retardation [115]. The additional hydroxyl group

compared to kaempferol-3-O-α-L-rhamnopyranoside is suggested to be responsible for the greater interaction with the SDS micelles, and hence for the greater retardation [115]. Cassani and coworkers also demonstrate that SDS micelles have a wide range of utility in matrix assisted DOSY in that they can be used in the presence of relatively large amounts of a co-solvent, such as DMSO [114]. Under these conditions, SDS micelles have been shown to be stable [116], enabling the investigation of compounds with limited solubility in aqueous solution [114].

Tormena et al. have presented a simple model to account for the changes in the observed diffusion coefficients upon addition of SDS micelles to a solution [117]. In this model the micelles are assumed to be of uniform size, comprising *n* surfactant molecules, and with an association constant which is independent of the number of surfactant molecules comprising the micelle or the number of solute molecules bound to the micelle:

$$nS \rightleftharpoons S_n$$

$$A + nS \rightleftharpoons AS_n$$

$$A + AnS \rightleftharpoons A_2S_n$$

…

This approach has some similarity with the isodesmic indefinite association models used to model self-assembly [118]. The Tormena model allows the fraction of bound and free solute to be formulated in terms of the critical micelle concentration *cmc* as:

$$\frac{[A]_b}{[A]_0} = \frac{K([S]_0 - cmc)}{1 + K([S]_0 - cmc)} \quad (5)$$

$$\frac{[A]_f}{[A]_0} = \frac{1}{1 + K([S]_0 - cmc)} \quad (6)$$

where $[S]_0$ is the total surfactant concentration. The observed diffusion coefficient is given by the Lindman's law in the fast exchange limit [3], hence:

$$D_{obs} = f_{free}D_{free} + f_{bound}D_{bound} \quad (7)$$

$$= \frac{D_{free,0} + D_{bound}K([S]_0 - cmc)}{1 + K([S]_0 - cmc)} \quad (8)$$

This expression can be used to determine the association constant and the free and bound fractions, provided that the micelles are well defined and do not change significantly in size or structure upon association with the solute molecules. SDS micelles typically fall into this category, and the equations above have been used successfully to model the observed diffusion behaviour of various small chain linear and branched alcohols, and isomers of methoxyphenol. The association constants obtained from these analyses correlate strongly with the octanol:water partition coefficients (log *P*), which provides a useful quantitative index of relative hydrophobicity [117] and hence by implication of tendency to incorporate into the hydrophobic core of a surfactant micelle.

SDS micelles are reasonably well-understood surfactant systems, however, their use is typically limited to aqueous or strongly polar solutions such as DMSO or methanol [116]. A common alternative when encapsulation of an aqueous environment in a hydrophobic medium is required is the concept of a reverse micelle, in which an aqueous medium is encapsulated inside a hydrophilic core, with the surfactant hydrophobic tails protruding out into the hydrophobic solvent. Reverse micelles have previously been used in biomolecular NMR as a means of reducing the rotational correlation time of large proteins encapsulated in a water layer inside a reverse micelle in a low viscosity solvent such as liquid ethane or propane [119,120]. In the context of matrix assisted DOSY, reverse micelles allow the range of solvents used to

be expanded, for example to typical NMR solvents such as chloroform [112,113]. Evans et al. have demonstrated that sodium bis(2-ethylhexyl) sulfosuccinate (known as AOT) reverse micelles in chloroform solution can be used to separate the same mixture of dihydroxybenzenes previously separated using SDS micelles in D_2O [112]. Reverse micelles have a central aqueous core and are dispersed in a hydrophobic solvent, in this case chloroform. The main difference observed in the presence of AOT is that the order of analyte separation is partially reversed, with catechol and resorcinol swapping order in the diffusion dimension relative to that in aqueous SDS. This is attributed to the differences in interaction with solvent-exposed hydrophobic tails in the reverse micelles compared to the hydrophilic headgroups in SDS micelles. Interestingly, the effects of the micelles on the diffusion of hydroquinone was similar in the case of both micelle systems [112].

The model developed by Tormena et al. to characterise the diffusion of SDS micelles and association of solutes, given in Eq. 8, can also be applied to AOT reverse micelles [117]. However, AOT reverse micelles are typically less well-structured than SDS micelles, being much more heterogeneous, and are dependent on the relative proportions of surfactant to water used to prepare the micelle solutions. This led to the suggestion that the hydrophilic core of the reverse micelles is not the main driver of the association, and that therefore the analytes may be interacting with the outer layer of the AOT rather than the core areas [117]. Law and Britton have employed a combination of NMR diffusion studies and dynamic light scattering to characterise the nature of AOT reverse micelles under a range of conditions [121]. The structural heterogeneity observed, with a weakly defined critical micelle concentration, often results in often poorer performance as a matrix for DOSY compared with more

homogeneous, well-defined micelles such as those of SDS. The correlation between association constant and log *P* is essentially non-existent for AOT reverse micelles, in contrast to the strong linear correlation observed for SDS micelles [117]. The origin of this lack of correlation is postulated to be that the association of the solutes or analytes is relatively insensitive to the polar component of the reverse micelle [117].

The surfactants presented thus far have all been ionic in nature. Electrostatic interactions are typically important both in micelle stability and in the interactions responsible for diffusion separation in MAD experiments. An alternative in situations where electric charges may be problematic is to utilise a non-ionic surfactant, such as the Brij or Triton families. Brij surfactants are comprised of a polyoxyethylene hydrophilic headgroup and polymethylene long alkyl chain hydrophobic tail. Brij surfactant micelles typically have an extremely low critical micelle concentration in water solution (generally < 0.05 mM [122]). In mixed solvent systems, such as D_2O/DMSO, the critical micelle concentration is raised [123], however, it is still in the same range as that observed for SDS [124]. This allows Brij surfactants to be used as matrices for diffusion NMR experiments in mixed solvents, when analyte solubility is low in pure aqueous solvent. Vieira et al. demonstrated that Brij 78 was a suitable matrix for the separation of a mixture of three monoterpenes (carvacrol, thymol and L-(-)-carvone) in 80:20 D_2O/DMSO (v/v) solution [123], shown in Fig. 7. The alkyl region of the spectrum is significantly more crowded, with additional contributions from the alkyl and alkoxy groups arising from the surfactant. Interestingly, separation of these terpenes is evident, despite the Brij 78 concentration (5 mM) being below the reported critical micelle concentration (8 mM) [123]. This presumably because the presence of the solutes encourages micelle or premicellar aggregate formation. The

flavanoids quercetin, fesein and (+)-catechin were also separated using the longer chain Brij 98, above its critical micelle concentration, in 50:50 D_2O/DMSO (v/v). The separation using Brij 98 is generally better under these conditions than when using SDS micelles, as discussed above [114], presumably due to the increased strength of interaction between the analytes and micelles. In both cases, a correlation between the binding strength of the analyte and micelle and log *P* was observed [123].

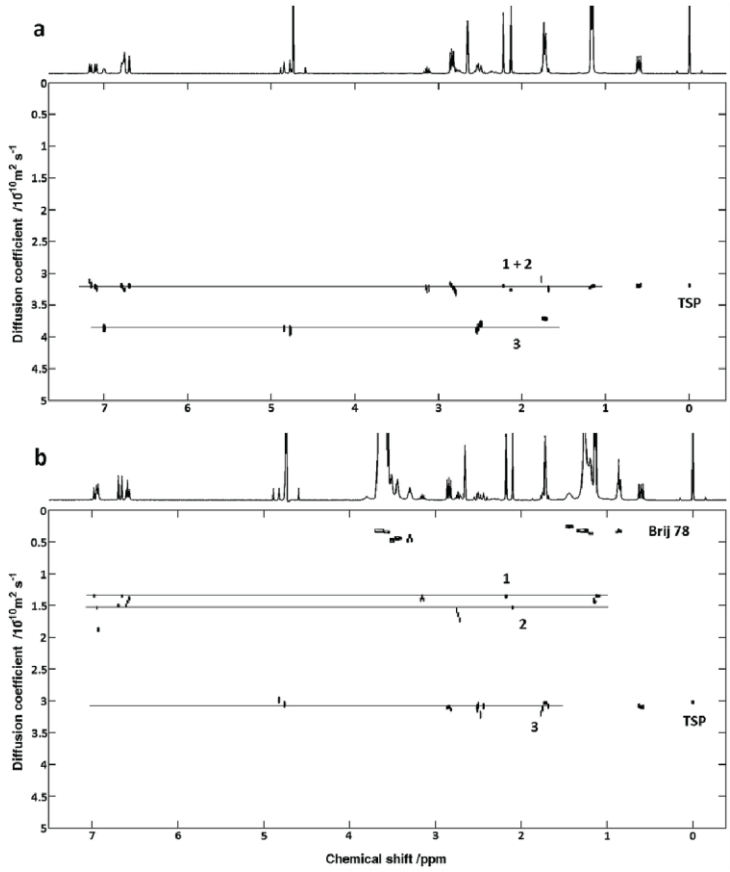

Figure 7: (a) Oneshot DOSY spectra of a mixture of three monoterpenes: carvacrol (1), thymol (2) and L-(-)-carvone (3) in 20% DMSO-d_6 / 80% D_2O. (b) shows the same sample recorded in the presence of 5 mM Brij 78 non-ionic surfactant demonstrating separation of the mixture in the diffusion dimension [123].

M. G. S. Vieira, N. V. Gramosa, N. M. P. S. Ricardo, G. A. Morris, R. W. Adams and M. Nilsson, *RSC Adv.*, 2014, **4**, 42029 – Published by The Royal Society of Chemistry.

Non-ionic inverse micelles formed by the commercial Igepal CA-520 series of compounds have been shown to demonstrate MAD like effects when dispersed in cyclohexane, with 0.1 M HCl present as the polar phase [125]. Under these conditions, a stable microemulsion forms, which when investigated by diffusion NMR results in a separation in the diffusion dimension according to the number of monomer units within the polyethylene oxide polar head groups. The fractional intensity of the peaks in the DOSY plot was found to follow a Poisson distribution, with the derived log *K*c values depending linearly on the ethylene oxide number [125].

The use of fluorine substituents in organic and medical chemistry has seen a large increase in interest recently, with around 25% of commercial drugs containing one or more fluorine atoms or fluorinated groups (e.g. CF_3) [126,127]. As such, mixture analysis by fluorine-19 NMR spectroscopy is also gaining attention, for example, in the investigation and identification of impurities in formulations of pharmaceutically active species [128]. ^{19}F NMR has a number of challenges relative to ^{1}H NMR spectroscopy, firstly, the need for uniform excitation over the large spectral widths typical for ^{19}F species [129], and secondly, larger homonuclear and heteronuclear couplings resulting in more complex spectra [126,130]. One solution to some of these challenges is presented by the CHORUS (CHirped, ORdered pulses for Ultra-broadband Spectroscopy) technique, which employs a carefully selected sequence of chirped pulses to afford uniform excitation across bandwidths in excess of 250 kHz using minimal RF power [129]. The combination of CHORUS and the Oneshot

DOSY experiment has resulted in CHORUS-Oneshot allowing ^{19}F diffusion NMR to be performed on mixtures of fluorinated species containing a range of fluorine functional groups [131]. Effective separation of rosuvastatin, a precursor, fluticasone propionate and fluconazole was possible using CHORUS-Oneshot with clear, well-behaved signals for both the aromatic and aliphatic fluorine signals despite their being over 200 ppm apart [131], shown in Fig. 8.

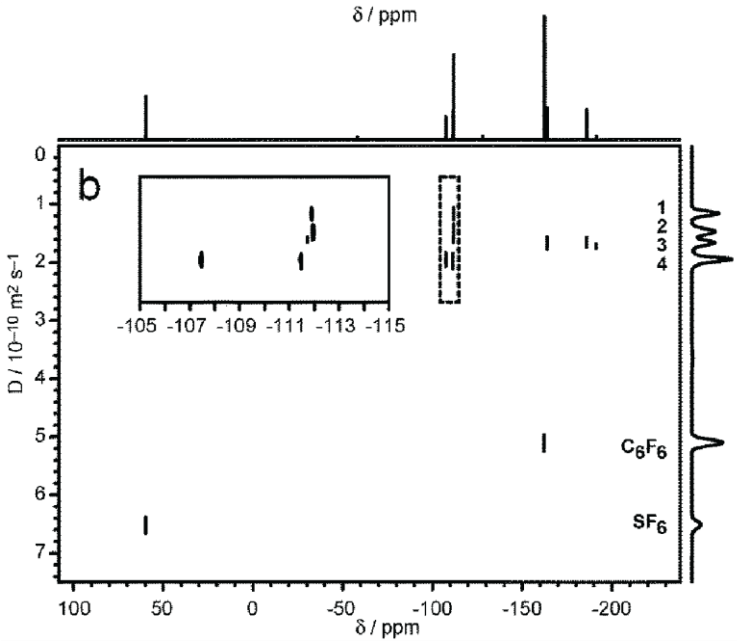

Figure 8: ^{19}F CHORUS Oneshot DOSY spectrum of a mixture of rosuvastatin (1), its precursor (2), fluticasone propionate (3) and fluconazole (4). SF_6 and C_6F_6 are also included as reference standards. J. E. Power, M. Foroozandeh, P. Moutzouri, R. W. Adams, M. Nilsson, S. R. Coombes, A. R. Phillips and G. A. Morris, *Chem. Commun.*, 2016, **52**, 6892 – Published by The Royal Society of Chemistry.

To demonstrate the applicability of MAD methods to fluorinated species, Dal Poggetto et al. took mixtures of fluorophenol and fluoroaniline isomers using both aqueous SDS or CTAB micelles and AOT in $CDCl_3$ reverse micelles as matrices

[132]. Some of these species had previously been used when developing ^{19}F DOSY in the presence of large ^{19}F-^{19}F homonuclear couplings [130]. Separation of the isomers of fluorophenol or fluoroaniline was achieved using all of the micelle systems, with small differences in the effects attributed to differences in binding strength between the analytes and the micellar surfaces [132]. The use of ^{19}F MAD allows much clearer separation of a mixture of all six fluorophenol and fluoroaniline species using 50 mM CTAB micelles than the corresponding ^{1}H DOSY spectrum as there is significantly less spectral crowding in the frequency dimension, due to the greater chemical shift dispersion observed in the ^{19}F frequency dimension, and simpler spectra due to the lack of numerous homonuclear couplings [132]. The ^{19}F DOSY plots are easily interpretable, in contrast to the ^{1}H DOSY plots, as demonstrated in Fig. 9, where it is extremely difficult to determine the number of species present, let alone attempt any more detailed interpretation of the spectrum. A curious feature, however, was the observation of a particularly broad ^{19}F signal for the *p*-fluoroaniline isomer in the presence of AOT reverse micelles in $CDCl_3$. This broadening was attributed to the protonation state of *p*-fluoroaniline due to the fact that the pK_a for the para-isomer is around 1 log unit higher than for the other isomers. The addition of a small amount of base, in this case triethylamine, resulted in a ^{19}F spectrum with comparable line widths across the three isomers [132].

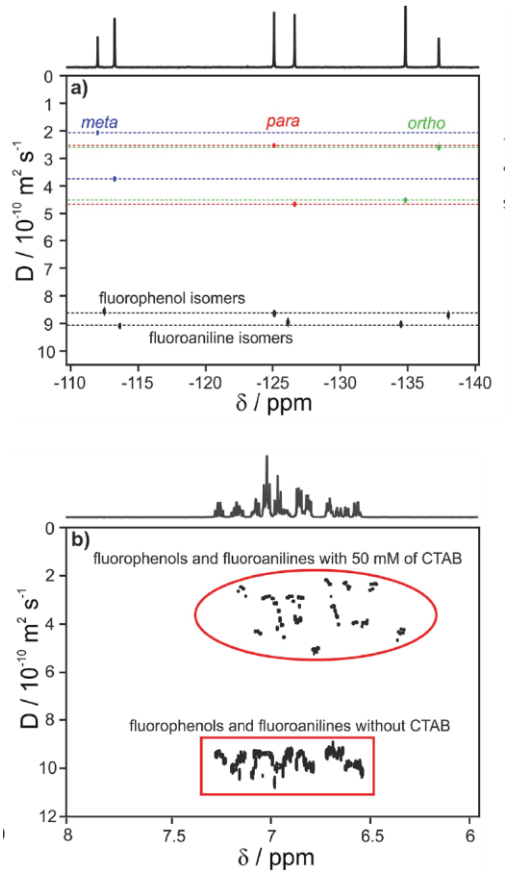

Figure 9: (a) shows overlaid ^{19}F Oneshot DOSY spectra recorded on a mixture of fluorophenol and fluoroaniline isomers (black traces), while the coloured traces are recorded in the presence of 50 mM CTAB. (b) shows the ^{1}H Oneshot DOSY spectrum for the sample samples as in part (a) [132]. Reproduced with permission from Dal Poggetto et al., *Magn. Reson. Chem.*, **55** (2017) 323–328. Copyright (2017) John Wiley and Sons Ltd.

3.1 Microemulsions

Hoffman has taken the concept of using surfactants as matrices in diffusion NMR experiments a step further, extending the idea to using microemulsions [133]. These are multicomponent systems employing both hydrophilic and hydrophobic (co)solvents and surfactants. Depending on the balance of components they can be

described as water-in-oil (W/O), oil-in-water (O/W) or continuous microemulsions [134,135]. These systems are stable for long periods of time, typically years [134], unlike silica suspensions, and do not have the sample inhomogeneity and magnetic susceptibility issues associated with chromatographic stationary phases. Hoffman and coworkers have presented a number of different systems based on perdeuterated or perfluoronated species as surfactants and cosolvents; for example, a matrix comprising lithium perfluorododecanate, propan-2-ol-d_8, D_2O and perfluorohexane was used to separate the components of a commercial available pharmaceutical sample Dexamol [133]. Clear separation of all seven components: the active ingredient paracetamol and various excipients, including magnesium stearate, carbohydrates, and PEG binder, was obtained in the DOSY spectrum, allowing subspectra of the components to be extracted [133].

A further demonstration of the use of microemulsions is the separation of 11 species (including some components of the emulsion). In this case, a mixture of broadly hydrophobic compounds, including *trans*-*trans*-farnesol, geraniol, β-citronellol, linolool and jasmine were effectively separated using a W/O microemulsion [136]. In this study, Hoffman et al. demonstrated a correlation between lipophilicity and hydrophilicity and the ability of a microemulsion to effect a separation. W/O microemulsions tend to separation based on lipophilicity, while O/W systems order species based on hydrophilicity [136]. The exact details and modelling are still to be elucidated, but are expected to be more complex than purely micellar systems such as SDS.

3.2 Invisible matrices

Surfactants are typically good matrices for modulating diffusion properties, enabling effective resolution in the diffusion dimension, as the sample conditions are readily tuneable and compatible with a wide range of systems. However, a major drawback is that they contribute significantly to the NMR spectrum, for example the long tails of SDS and Brij surfactants obscure the low shift alkyl region of the spectrum [113,117]. One obvious solution to this problem is to deuterate the surfactant, hence removing its signals from the chemical shift dimension. Zielinski and Morris have presented the use of deuterated SDS micelles (SDS-d_{25}) as a matrix to separate a mixture of two dipeptides, tryptophan-glycine and leucine-methionine [137]. Effective separation was obtained using 25 mM SDS-d_{25} [137]. Performance of the deuterated micelles for separation should be very similar to that of normal SDS, however, the major drawback with this approach is that deuterated SDS is approximately 500-fold more expensive per gram than regular SDS-h_{25}!

Surfactants and micelles typically tumble reasonably quickly and are sufficiently chemically diverse that their additional signals are both well dispersed and well resolved. Since the signals are reasonably narrow, with line widths only 2-3× those of the analytes, they cannot always easily be removed by T_2 filters [138,139]. T_2 filters employ a CPMG echo train allowing the selective attenuation of broad, i.e. more rapidly relaxing, signals [139]. The downside of this pulse sequence element is that systems with homonuclear couplings can give rise to line shape distortions if the interpulse interval is not small compared to the inverse of chemical shift differences between coupled spins. These distortions arise due to evolution of *J*-couplings during the echo train, and results in dispersive contributions to the line shape being present during acquisition. In the context of a diffusion experiment these distortions can result

in large errors in the determination of the diffusion coefficient. The Oneshot-45 sequence was developed to reduce such dispersive components using a 45° purging pulse prior to acquisition [140], but is not a general solution where *J* modulation is severe. Aguilar et al. have presented an alternative solution to this problem using the Periodic Refocusing of *J* Evolution by Coherence Transfer (PROJECT) experiment, in which the *J*-modulation is refocused by the inclusion of a 90° pulse in the centre of each evolution period within the CPMG train [141], in an analogous manner to the perfect echo experiment [142]. This approach has been used to improve diffusion NMR experiments, which can be affected by line shape distortions if homonuclear couplings are allowed to evolve during the various delays, and to remove chemical exchange effects [143,144]. Evans et al. have combined the PROJECT [141] and Oneshot [9] sequences enabling the acquisition of T_2-filtered diffusion spectra, allowing the broad, rapidly relaxing signals of a surfactant matrix to be edited out of the final spectrum [145]. In order to accomplish this, CTAB micelles were induced to form much larger structures in a saturated solution of sodium chloride, with concomitantly reduced T_2 times, and hence broader micelle signals. Evans et al. demonstrated clear resolution of isobutanol and *sec*-butanol using CTAB in NaCl solution, where overlap with the matrix signals in the absence of the sodium chloride caused poor resolution in the diffusion domain [145]. Additionally, they were able to demonstrate the improved separation of nicotinic acid and 4-aminobenzoic acid using a similar matrix of 150 mM CTAB in saturated sodium chloride (98:2 D_2O/DMSO) [145]. The improvement in separation in the diffusion dimension in this case was a factor of nearly 5 compared with the absence of the invisible matrix. The tunability of the separation capability and the invisibility of the matrix make this approach

particularly attractive, providing that the analytes are soluble in the high ionic strength solvent system in question.

An alternative to deuteration, which is only practical (if costly) for a few common surfactants, or the salt-induced aggregation of micelles, is to use fluorinated surfactants, originally suggested by Zielinski and Morris [137], and elegantly demonstrated by Hernandez-Cid et al. [146]. In this case sodium perfluorooctanoate (PFO) is used as the matrix, being inexpensive, readily available, and invisible in the ^{1}H NMR spectrum, just as protonated micelles are invisible when used in ^{19}F matrix-assisted diffusion NMR experiments [130,132]. Perfluorinated surfactants are not "drop-in" replacements for common micelle forming systems, such as SDS, CTAB or AOT. The differences in polarity, electronegativity etc between a proton and fluorine atom means that the nature of the interactions [147], and the selectivity as a DOSY matrix, will be very different [146]. This is illustrated by the fact that PFO does not cause effective separation of common test mixtures such as dihydroxybenzenes, which are effectively separated by SDS micelles. Charge-charge interactions dominate the interaction between highly fluorinated species and PFO has a lower affinity for long alkyl groups [148], hence it should be a good matrix for the separation of charged species, and should have a reasonable range of tunability by virtue of pH adjustment of the bulk solution [146]. Separation of a quaternary mixture of amino acids at neutral *p*H has been demonstrated using 100 mM PFO micelles [146]. The models used to described the diffusion coefficients of the various species in a micelle matrix diffusion experiment, as describe above [117], can be modified to account for the charge state of the analyte species as a function of *p*H. This model has been used to construct ionisation state curves for a range of amino acids and hence

provides an alternative approach to the determination of the ionisable group pK_a [146].

4. Polymers

The use of simple polymers as matrices has the ability to avoid the susceptibility effects associated with chromatographic stationary phases and the potential thermodynamic stability issues common with surfactants and colloidal suspensions. The ability to modify the polymer functionality also has the benefit of being able to tune the matrix to influence the separation achievable in the diffusion dimension. The use of diffusion NMR to investigate polymer behaviour in solution is also well established [149].

Heikkinen and colleagues have presented MAD experiments utilising two different polymers: poly (vinyl pyrrolidone) (PVP) [150] and poly (ethylene glycol) (PEG) [151]. Typically, 50 mg of commercial PVP was added to a standard 0.6 mL chloroform sample and allowed the separation of signals from a mixture of *p*-xylene, benzyl alcohol and *p*-methoxyphenol [150]. The influence of the polymer on the overall viscosity of the sample was accounted for by the use of standard conditions for all samples, and a range of polymer molecular weight profiles was screened. In total, a series of nine compounds was investigated using PVP [150] and 17 using PEG [151]. In the latter case, maximum entropy processing in the diffusion dimension was used to resolve overlapping signals [152] allowing closely related structures, such as those of the sex hormones β-estradiol and testosterone to be separated [151]. In both cases, residual signals from the polymer matrix provide a complicating factor when analysing the DOSY plots, however, is it generally possible to identify at least one

analyte signal sufficiently removed from the polymer to allow characterisation. The influence of physical parameters, such as viscosity, polymer concentration and molecular weight, along with temperature, was investigated, and it was found that variation of these parameters allows the separation of signals from some substituted aromatic species to be tuned [153]. The mechanism for this tunability was suggested to be the influence of polarity on the strength of the interaction between the analytes and the polymer matrix [153].

In order to ameliorate the complicating presence of the polymer signals in the NMR spectrum, Huang and co-workers have suggested the use of silicon-containing polymers, particularly polydimethylsiloxanes, as matrices for diffusion NMR studies [154]. In this case, separation of a mixture of hydroxyl-containing species and epoxides was achieved. The benefit of the siloxane polymer was that signals from the polymer are all clustered around very low chemical shifts, typically in the region 0.0 - 0.5 ppm and hence well removed from typical analyte signals [154]. PDMS has a wide range of analyte compatibilities, and has been demonstrated as general matrix, effective in separating not just oxygen-containing species, but also boronic acids, aromatic and halogenated species, typically those formed from standard organic transformations, being the likely reagents and products [154].

A hybrid method, utilising micelles prepared from polymeric systems, has been used to investigate the interaction of chiral species with the micelle surface [155]. Poly (sodium *N*-undecanoyl-L-leucylvalinate) (polySULV) micelles are used in electrokinetic chromatography (EKC) to enable enantiomeric resolution since enantiomers will interact differently with the chiral centres along the polymer.

Diffusion NMR was used to determine the strength of the interaction between a range of species including propranolol, binaphthols, warfarin and Troger's base with polySULV micelles. The binding equilibria were determined from Lindman's law and difference in diffusion coefficient in the presence of the micelles [155]. This allows details of the interactions to be elucidated and provide further information related to the mechanism of separation occurring in EKC separations.

A logical extension of the use of polymers as matrices is the application of polymer engineering, to enable formation of heterogeneous hydrogels, in this case comprising a propylene-imine core, surrounded by extensive pegylation. In order avoid signals from the nanoparticles, ^{19}F NMR was utilised. Inclusion of selective ^{19}F-labels into the hydrogels allowed their diffusive behaviour to be characterised [156]. These nanoparticles were studied with a combination of diffusion NMR and diffusion-relaxation correlation NMR [157] in order to determine the size and composition of the nanoparticles. Matrices of this type have excellent tunability and could provide a rich source of new diffusion-modulating effects, induced for example by variation of cross-linking strategies causing differences in porosity or mesh sizes [158].

5. Other matrices

A more unusual demonstration of MAD has been made by Ning et al. who were able to separate the two anomers of D-glucopyranose in the diffusion dimension by the simple addition of 250 mM $CaCl_2$. The α-anomer was reported to have faster diffusion than the β form, presumably due to differences in hydrodynamic radius upon complexation with Ca^{2+} [159]. A similar separation was also observed using calcium ions in a saline solution, suggesting that there was little interference from the

sodium ions present. Interestingly, despite the decrease in diffusion coefficient on addition of the metal salt indicating the likely formation of a complex, separation of the anomers was not achieved using magnesium chloride. The separation of other carbohydrates and amino sugars, such as fructose, xylose and glucosamine, was also not achieved with calcium, again despite evidence of complex formation [159].

Lanthanide shift reagents, and their chiral analogues, have a long history in the NMR analysis of systems with poor chemical shift resolution [160]. Resolution is obtained by close association of the analyte and shift reagent resulting in a paramagnetic contribution to the observed chemical shift, which is dependent on the distance between the functional group interacting with the lanthanide ion and the group of interest [161]. Shift reagents are a commonly used alternative to chiral HPLC for the determination of enantiopurity [162] and as agents to probe protein interactions [163]. Diffusion NMR studies have been shown to complement traditional chemical shift titration experiments in the latter case [164]. Rogerson et al. have demonstrated how the common lanthanide shift reagent europium (III) tris(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedianato) ($Eu(fod)_3$) can be used to improve both the chemical shift and diffusion resolution of a mixture of *n*-hexane, hexan-1-ol and *n*-heptanal [165]. Resolution in the chemical shift dimension arises from the normal paramagnetic-derived lanthanide shift effects [160], while the differential interaction of an alkane, alcohol and aldehyde with the europium (III) centre results in resolution in the diffusion dimension [165]. The larger size of $Eu(fod)_3$ compared with the analytes results in effective separation along the diffusion axis as the difference between the free and bound diffusion coefficients is large. Salome and Tormena have shown that simple axially chiral BINOL and BINOL-derived additives can be used as

matrices for enantiodiscrimination [166]. A change in diffusion coefficient for both enantiomers of 1-phenylethanol was observed upon addition of the *S*-BINOL. While the differences in diffusion coefficient between the two isomers was small, around 1×10^{-10} m^2 s^{-1}, clear resolution was observed in the diffusion dimension, in addition to a splitting in the chemical shift dimension indicating the formation of a diastereomeric complex [166].

Salvia et al. have demonstrated the use of surface functionalised gold nanoparticles as matrices [167]. This is based on the idea of NMR chemosensing in which a diffusion filter and NOE transfer are used to detect binding of an analyte to a functionalised gold nanoparticle [168,169].This is similar to ligand binding experiments used to find small molecules which bind to a protein receptor [54,170]. The surface functionality of the nanoparticle can be tuned to detect binding of different species via coupling of different "receptor" moieties to thiols on the surface of the nanoparticle via alkyl linkers [167,168]. These nanoparticles can be used to simplify the spectra of complex mixtures of small organic molecules. Salvia et al. demonstrated the effective separation of sodium salicylate, sodium benzoate, potassium tosylate and tyramine in aqueous buffer using only 90 μM gold nanoparticles with a trimethylalkylammonium receptor group [167]. A degree of overlap was observed at around 6.8 ppm for some of the signals from sodium salicylate and tyramine, however, clear resolution in the spectral dimension was established for other resonances of these species, allowing their separation and identification in the diffusion domain. The tunability of the surface functionalisation and low matrix loading suggest that these nanoparticles are particularly well suited as matrices for diffusion NMR experiments.

Other chemosensing materials can be used for matrix-assisted DOSY experiments. Adams et al. have shown that cyclodextrins can be used as a matrix to separate epimers of the flavanone glycoside naringin. These epimers differ solely in the absolute configuration at the 2 position, and hence the two isomers are almost identical in size, yielding the same diffusion coefficient. Cyclodextrins are well known to form inclusion-type complexes with small molecules [171] and have been used as pore-formers and sensors in a range of applications [172,173]. Addition of 4.7 mM β-cyclodextrin to a solution of 1.4 mM naringin in D_2O results in modest separation in the diffusion dimension, with the 2*S* epimer diffusing slightly more slowly than the 2*R* isomer [174]. Overlap of some naringin resonances with the cyclodextrin signals in the region 3.2-4.0 ppm precluded analysis of this region. The corresponding aglycone did not show any diffusion resolution, suggesting that it is the interaction of the glycoside with the β-cyclodextrin which is responsible for the resolution [174]. The similarity in size between the analyte and matrix means that the overall modification of the diffusion behaviour will be small, however, Adams et al. note that decorating a polymer chain with cyclodextrins would produce greater diffusion resolution at the expense of increased line width due to the presence of the polymer [174].

6. Choosing a matrix

The most effective matrix for a given problem will clearly depend on the analytes under investigation, what information is required from the investigation and potentially what spectrometer hardware is available. Having said that, some general guidance is possible. If comparison with on-line chromatographic separation is required, or details of analyte-support interactions are under investigation, then the

use of a chromatographic support, such as a functionalised silica, would be appropriate. Clearly there are potential issues such as line broadening and hardware requirements, however, there are approaches to circumvent these problems, as described above. These systems are reasonably general, and tolerant of a wide range of solvents, similar to that of on-line chromatography. Tunability is provided via changing the solvent to solid ratio, i.e. the loading of the chromatographic phase.

A more general approach to MAD experiments is the use of a soluble matrix, such as surfactant micelles or a suitable polymer. Clearly the choice of matrix here will be based on the nature of the interactions between the matrix and analyte and chosen so as to effect the desired separation. Tunability is possible as there is a huge range of surfactants and solvent systems available. The most suitable for a given separation is not easy to predict *a priori*. Gramosa et al. have undertaken an wide-ranging study of potential matrices for separating a mixture of dihydroxybenzene isomers, studied extensively by the Manchester group [175]. In this study, matrices including Brij family surfactants, SDS and AOT micelles were combined with polymers, mixed micelles and cyclodextrins were investigated, alongside mixed solvent systems [175]. The effective separation of the dihydroxybenzene isomers was highly tunable depending on the exact composition of the matrix, be it the concentration, combination of surfactants, including the relative proportions, or use of a mixed solvent, again with a range of exact compositions. The degree of separation obtained is in line with the arguments proposed above, in that it is the degree of interaction between the analyte and matrix, and the differences in this interaction between species which affords the improved separation in the matrix-assisted DOSY experiment.

Gromosa et al. conclude with the statement that "*we lack a clear understanding of all the underlying mechanisms, and further study would be desirable*".

7. Conclusions

Diffusion NMR is an extremely powerful analytical technique, which has only recently become a more mainstream part of the spectroscopist's toolbox for the analysis of complex mixtures. The principal benefit of diffusion NMR is that the analysis can be undertaken without the need for physical separation of the mixture. Matrix-assisted diffusion NMR affords the ability to improve the separation of signals in the diffusion dimension by adding a suitable sample modifier. A wide range of sample modifiers, including chromatographic stationary phases, surfactant micelles, polymers and nanoparticles, have been demonstrated as suitable matrices and have enabled significantly improved separation of challenging mixtures. However, the exact choice of matrix and sample conditions, e.g. solvent(s), pH etc., remains empirical. There is, therefore, significant scope for further developments in matrix-assisted diffusion NMR, particularly, around the mechanisms of action, and therefore choice of matrices and sample conditions.

Acknowledgements

The author thanks Robert Evans and previous members of the Day group: Rebecca Joyce, Guillermo Lucena Alcalde, Jordann Wells, Natalie Anderson and Maisie Pearcey for helpful discussions. The EPSRC (EP/H025367/1 and EP/P015735/1) and the University of Sussex are acknowledged for financial support.

This work is dedicated to the memory of Professor Stefano Caldarelli.

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Abbreviations

AOT	sodium bis(2-ethylhexyl) sulfosuccinate
CHORUS	CHirped, ORdered pulses for Ultra-broadband Spectroscopy
CTAB	Dodecyltrimethylammonium chloride
DOSY	Diffusion-Ordered SpectroscopY
DQF	Double Quantum Filter
DTAB	Dodecyltrimethylammonium bromide
EKC	ElectroKinetic Chromatography
$Eu(fod)_3$	europium (III) tris(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedianato)
FIDDLE	Free Induction Decay Deconvolution for Line shape Enhancement
HPLC	High-Performance Liquid Chromotography

GNAT	General NMR Analysis Toolbox
GPC	Gel-Permeation Chromatography
LC-NMR	Liquid Chromatography NMR
LC-NMR-MS	Liquid Chromatography NMR Mass Spectrometry
MAD	Matrix-Assisted DOSY
MAGNATE	Multidimensional Analysis for the GNAT Environment
PARAFAC	PARAllel FACtor analysis
PEG	Poly (ethylene glycol)
PFO	Perfluorooctanoate
PGSE	Pulsed Gradient Spin Echo
PGSTE	Pulsed Gradient STimulated Echo
polySULV	Poly (sodium *N*-undecanoyl-L-leucylvalinate)
PROJECT	Periodic Refocusing of *J* Evolution by Coherence Transfer
PVP	Poly (vinyl pyrrolidone)
SDS	Sodium DodecylSulfate
SEC	Size-Exclusion Chromatography
TOCSY	TOtal Correlation SpectroscopY