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Chromatographic NMR with size exclusion chromatography stationary phases

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ABSTRACT

Chromatographic NMR describes the use of stationary phases or solvent additives, such as polymers, to modify the diffusion properties of analyte molecules and thereby improve the observed resolution in the diffusion domain. This paper demonstrates similar ideas using size exclusion chromatographic media and characterises the changes in the observed diffusion coefficient using a series of polymer molecular weight reference standards of known polydispersity. The results are interpreted in terms of a simple description of the size exclusion phenomena.

1. Introduction

The influence of translational motion on nuclear magnetic resonance (NMR) experiments is well understood [1,2]. In general, analyte motion is monitored using gradient spin echoes, with diffusive incoherent motion resulting in a signal attenuation, while bulk flow and coherent motion results in a phase shift proportional to the flow velocity [2]. The spectral separation of molecular species depending on their diffusion behaviour is a powerful technique, exemplified by High-Resolution Diffusion Ordered Spectroscopy (HR–DOSY) [3]. In this experiment a pseudo two dimensional spectrum is obtained correlating chemical shift with observed diffusion coefficient [3] and thereby allowing the spectral separation of complex mixtures [3–5]. More recently, techniques have been developed which seek to enhance the observed separation in the diffusion dimension via modification of the solvent system using polymer additives [6,7] and surfactant micelles [8,9]. The principle behind these approaches is that the various species comprising the mixture will have differing interactions with these additives, and hence the observed separation in the diffusion dimension will be influenced by the strength of these interactions. Similar in concept to this is the so-called "chromatographic NMR" approach of Caldarelli and co-workers [10,11] and of Hoffman et al. [12] where the diffusion properties of the molecules under investigation are modified by the use of a chromatographic stationary phase within the NMR tube, such as fused silica [10,11]. This approach has utility in the spectral separation of complex mixtures, as well as for gaining access to information regarding the nature of the interaction between the analyte(s) and the stationary phase [6,8,11,13].

An unfortunate side effect of using an insoluble stationary phase, such as a silica, to modify the analyte diffusion properties is that the sample inhomogeneity results in significant broadening of the spectral resonances, potentially leading to peak overlap and a loss of information even for small particle sizes [10–12]. In order to utilise this chromatographic approach in high-resolution diffusion NMR methods, two techniques have been presented to lessen this observed line broadening. The first is high resolution magic angle spinning (HR-MAS) to reduce the contributions from sample heterogeneity and susceptibility broadening, as has been successfully demonstrated by Calderelli and co-workers when using HPLC stationary phases [10,14]. The second, alternative, approach relies on reducing broadening caused by differences in magnetic susceptibility by aiming to match the susceptibility of the solvent to that of the stationary phase employed [12]. This works well for phases such as silica, which can tolerate wide variations in solvent composition [12], however, is of limited use if the stationary phase is soluble or degraded in organic solvents.

The behaviour of polymers in solution depends critically, amongst other things, on the overall molecular weight and its distribution [15–18]. Size-exclusion or gel-permeation chromatography (SEC/GPC) is commonly used to determine the molecular weight (and distribution) for polymer samples in solution [15,19,20]. This is a robust technique with wide ranging applicability [15,20]. Other techniques available for determining a molecular weight profile include osmometry, dynamic light scattering (DLS) and mass spectrometry [15,20]. Depending on the method chosen and the detector technology employed, different aspects of the molecular weight profile may be highlighted. For example, osmometry measures the number average molecular weight M_n,

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whereas the use of light scattering techniques results in measurement of the weight average molecular weight M_w [15,20]. Access to the molecular weight distribution is also possible with the use of MALDI-TOF mass spectrometry [15].

NMR spectroscopy has also been used to determine molecular weights via diffusion coefficients obtained from the analysis of gradient spin-echo attenuation experiments [21–23]. In the case of distributions of molecular weights, typical of synthetic polymers in solution, i.e. distributions of diffusion coefficients, this process is non-trivial due to the ill-posed nature of the required inverse Laplace transform [2,23]. A number of approaches to improve the numerical stability based on regularization [23–25] and maximum entropy methods [26] have been demonstrated, however, to date these have not found wide ranging application. More recently, a simple diffusion NMR method has been presented to determine the polydispersity of simple polymers utilising the differences in mobility between end groups and the main chain of the polymer [27].

In this paper we extend the idea of in situ chromatographic NMR to the use of mesoporous size-exclusion chromatography stationary phases in aqueous solvent, and demonstrate the modification of the diffusion parameters by two cross-linked dextran-based phases. Comparison with traditional on-flow gel permeation chromatographic data for one of the phases is also presented. The results are interpreted in terms of an empirical relationship similar to Determann's equations relating the molecular weight of the analyte to properties of the stationary phase [28–30].

2. Methods

2.1. Materials

Poly(styrene 4-sulphonate) molecular weight reference standards of low polydispersity (typically < 1.20) were purchased from Kromatek (Essex, UK) and used as obtained. The weight average molecular weights M_w of the five polymer standards were: 10.6, 14.9, 20.7, 32.9 and 63.9 kDa. Sephadex G-50, Superdex 75 and 200 stationary phases were obtained from Sigma Aldrich (Dorset, UK). Sephadex G-50 was supplied as a dry powder and swelled for 3 h using 11 mL of buffer solution per gram of stationary phase, then diluted to a suspension of 60 mg mL^{-1} prior to use. Superdex 75 and 200 differ only in their fractionation range, 3–70 and 10–600 kDa (quoted for globular proteins) respectively, [31] with important parameters being listed in Table 1. The Superdex phases were supplied as pre-swollen suspensions in 20% (v/v) ethanol in water and were washed with buffer three times before use. The buffer solution used was either 50 mM sodium phosphate and 150 mM sodium chloride at pH 9, or 150 mM sodium chloride (pH 7). Deuterium oxide was obtained from Goss Scientific (Cheshire, UK). All other chemicals were purchased from Sigma Aldrich and used as obtained.

2.2. NMR Spectroscopy

All NMR data were obtained on a Varian VNMRS 600 spectrometer (Agilent Technologies, Yarnton, UK) operating at a ^1H frequency of 599.7 MHz, using an $X\{1H\}$ broadband probe equipped with an actively-shielded z-gradient coil capable of producing 0.7 T m^{-1}. The sample temperature was maintained at 298 K throughout.

Poly(*N*-isopropyl acrylamide) (poly(nipam)) and poly(allyl amine) hydrochloride (poly(AA)) samples were prepared from 2 mM stock solutions with 150 mM sodium chloride and were diluted with 150 mM sodium chloride in D_2O or a Sephadex G-50 stationary phase in 150 mM sodium chloride, as appropriate, to give a final polymer concentration of 0.2 mM. The nominal molecular weights of these polymers are given in Table 2. Stock samples of the Poly(styrene 4-sulphonate) molecular weight reference standards were prepared as 1.0 mM solutions of the polymer in D_2O with 50 mM sodium phosphate (pH 9) and 150 mM sodium chloride. Samples containing the stationary phases were prepared by allowing 1 mL of stationary phase suspension to settle under gravity. 200 μL of supernatant was then removed and replaced by 200 μL of a 1 mM solution of the polymer standard to give a final polymer concentration of 0.2 mM. The samples were thoroughly mixed, then placed in standard 5 mm NMR tubes and allowed to settle under gravity for at least 30 min prior to use such that the settled stationary phase filled, and extended beyond, the RF coil region.

Diffusion NMR spectra were acquired using the Oneshot sequence [32] with a typical diffusion delay Δ being 200 ms. The diffusion encoding gradients were 5 ms in duration, with 16 amplitudes equally spaced in g^2, from 0.0450 to 0.5625 T m^{-1}. Typically 32 transients were recorded per gradient increment, over a spectral width of 8 kHz. Solvent suppression was performed using the excitation sculpting approach [33,34]. The spectra were processed using the DOSY Toolbox [35] with mono-exponential fitting of the Stejskal–Tanner equation [2] to the observed echo attenuation, appropriately modified for the Oneshot sequence [32]. Given the narrow molecular weight distributions of the polymer standards (PDI < 1.20) this is a reasonable approximation.

2.3. Gel permeation chromatography

GPC data were acquired using an Agilent 1200 LC instrument at the University of Greenwich, with a Superdex 200 10/300 GL column. Peak detection was performed using a photodiode array, with the absorption wavelength set to 254 nm. The mobile phase used was 50 mM sodium phosphate (pH 9) and 150 mM sodium chloride. The polymer standards were prepared as 10 mg mL^{-1} samples in mobile phase and a 20 μL injection was used for each chromatographic run. A flow rate of 0.5 mL min^{-1} was used throughout.

Table 1
Selected properties of the size exclusion chromatography stationary phases used in this study [31].

Stationary phase	Particle size (μm)	Fractionation range (kDa)	
		Globular proteins	Dextrans
Sephadex G-50	20–80	1.5–30	0.5–10
Superdex 75	22–44	3.0–70	0.5–30
Superdex 200	22–44	10–600	1.0–100

3. Results and discussion

3.1. Proof of concept

Two polymers were used for the initial proof of principle experiments, poly(*N*-isopropyl acrylamide) with a nominal molecular weight of ~25 kDa and two samples of poly(allyl amine) hydrochloride with molecular weights of ~15 and ~56 kDa. All three samples were prepared both in the absence and presence of the Sephadex G-50 size exclusion stationary phase. This phase has a reported fractionation range spanning 1.5–30 kDa for globular proteins [31]. The properties of this and the other stationary phases investigated are detailed in Table 1. The DOSY spectra obtained using the Oneshot pulse sequence are shown in Fig. 1, with the observed diffusion coefficients reported in Table 2. It is apparent that there is a deterioration of the NMR line shape upon addition of the insoluble stationary phase. This decrease in resolution seen on the