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Size-Exclusion Chromatographic NMR of Polymer Mixtures

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Abstract

The use of chromatographic stationary phases or solvent modifiers to modulate diffusion properties in NMR experiments is now well established. Their use can be to improve resolution in the diffusion domain or to provide an insight into analyte-modifier interactions and hence the chromatography process. Here we extend previous work using size-exclusion chromatographic (SEC) stationary phases to the investigation of polymer mixtures. We demonstrate that similar diffusion modulation behaviour is observed with an SEC stationary phase which can be understood in terms of size-exclusion behaviour.

Introduction

Diffusion ordered spectroscopy is often described as "NMR chromatography" in that it enables the pseudo-separation of mixtures based on the relative diffusion coefficients of the various constituent components.[1, 2] Over the past ten years or so, this concept has been extended and enhanced by the addition of chromatographic stationary phases[3-6] or other sample modifiers.[7-10] These additives modulate the observed diffusion coefficients as a result of some favourable interaction between the additive and the analyte of interest. In some cases, this can significantly improve the attainable resolution in the diffusion dimension.[4, 7, 8] The exact nature of the interactions responsible for the diffusion modification is not fully understood. However, recent studies have shown that in the case of chromatographic NMR, the loading of the stationary phase, that is the relative amount of stationary phase to "mobile phase", plays an important role.[11, 12] Indeed high mobile phase to stationary phase ratios are required to reproduce results which are consistent with on-flow liquid chromatography.[12, 13] One minor drawback of adding a stationary phase to the NMR sample is the increased line width observed as a result of susceptibility broadening due to the mismatch in magnetic susceptibilities of the solvent and the stationary phase.[3-6, 14] Previous studies with small molecules have either utilised magic angle spinning[5] or matching of the solvent magnetic susceptibility to that of the stationary phase[6] to reduce the line broadening to acceptable levels.

Recently, we have adapted this chromatographic NMR approach to the use of size-exclusion chromatographic stationary phases.[15] We have demonstrated that the observed changes in diffusion properties of a series of polymer molecular weight reference standards is consistent with size-exclusion behaviour, in which smaller

polymers show greater retardation in their diffusion than larger polymers.[15] These systems have also been used to investigate aggregating systems, with assemblies of the azo-dye sunset yellow partitioning between the pores in the stationary phase and the free solution surrounding the particles, depending on the overall aggregate size.[16] In both cases, the large size of the analyte molecules or assemblies means that the addition of the stationary phase causes only minor additional line broadening.[15, 16]

In this paper we demonstrate extending of the range of applicability of in-situ size-exclusion chromatographic NMR to polymer mixtures. We show that under dilute or near-dilute conditions, the size-exclusion behaviour is similar for polymer mixtures as for the individual components using pairs of polymers which are closely matched in molecular weight and a "mismatched" pairing. We interpret the results using the same framework as previously.[15]

Materials and Methods

Materials

Poly(styrene sulfonate) (PSS) and polymethacrylate (PMA) molecular weight reference standards with low polydispersity (typically < 1.20) were purchased as their sodium salts from Kromatek (Essex, UK) and used as obtained. Sephadex G-50 size-exclusion chromatographic stationary phase (dry bead size 20-50 μm, fractionation range: 1.5-30 kDa for globular proteins, 05-10 kDa for dextrans, pore size ~3 nm)[17, 18] was obtained from Sigma-Aldrich (Dorset, UK). Deuterium oxide was purchased from Goss Scientific (Cheshire, UK).

The samples were prepared following the previously published procedure.[15] Briefly, 1 mL of a stationary phase suspension (at 60 mg mL^{-1}) in 50 mM sodium phosphate (pH 9) and 150 mM sodium chloride was allowed to settle under gravity. 200 μL of supernatant was then removed and replaced with 200 μL of a 1 mM solution of the required polymer or polymer mixture, resulting in a final total polymer concentration of 0.2 mM. The suspension was then thoroughly mixed and transferred to a 5 mm NMR tube where it was allowed to settle under gravity for at least 30 mins prior to use. The sample position was adjusted such that the stationary phase filled the RF coil region.[15] The sample therefore has a high solution to solid volume ratio.[12]

NMR Spectroscopy

All NMR data were acquired using a Varian VNMRS 600 spectrometer (Agilent Technologies, Yarnton, UK) equipped with an X{^{1}H} broadband probe and *z*-gradient coil capable of up to 0.7 T m^{-1}. All ^{1}H data were obtained at 599.7 MHz and a temperature of 298 K.

Diffusion NMR data was obtained using the Oneshot sequence of Pelta et al.,[19] with 16 gradient points spanning 0.0020-0.6066 T m^{-1}, equally spaced in g^2. The diffusion encoding time was 100 ms, with 2 ms gradient pulses. The spectral window was 9.6 kHz over 16k complex data points. The resulting data were processed with either 4 or 8 Hz exponential line broadening prior to Fourier transformation and the resulting peak intensities were fitted to the appropriately modified Stejskal-Tanner equation[19] using the Levenburg-Marquardt algorithm within DOSY Toolbox.[20]

Results and Discussion

Previous proof of concept studies using two different size-exclusion stationary phases to modulate the diffusion properties of a single polymer, poly(styrene sulfonate), showed a sized-dependent effect, with smaller polymers showing larger changes in the observed diffusion coefficient.[15] This behaviour was interpreted in terms of the polymers accessing the pores of the stationary phase resulting in size-exclusion behaviour.[15] Since the overlap of spectral signals presents a challenge for diffusion experiments,[21, 22] for this work, two polymers with different functional groups were chosen, polymethacrylate and poly(styrene sulfonate) as used previously.[15] These have signals from the side-chains in very different regions of the spectrum. There should also be little overlap between the methyl group of the polymethacrylate and the CH_2 fragments of the dextran supports of the stationary phase.

Fig. 1 shows the results of diffusion measurements of a series of polymethacrylate molecular weight reference standards in the presence and absence of Sephadex G-50 size-exclusion stationary phase. The data clearly show similar trends to those reported previously with poly(styrene sulfonate).[15] In the absence of the stationary phase, the diffusion coefficient decreases with increasing molecular weight as is expected.[2] Upon the addition of the stationary phase, changes in the diffusion coefficient are clearly observed, with those changes being more dramatic for the smaller polymers. This behaviour is consistent with size-exclusion behaviour occurring in the presence of the Sephadex G-50 stationary phase, in that the smaller polymers spend longer inside the pores of the stationary phase and hence show greater retardation in their diffusion coefficients.[15, 23] As previously, the data are interpreted in terms of an empirical equation,[15] similar to that used by Anderson and Stoddart[24, 25] and Determann and Michel.[26] The equation is of the form:

$$\log M_w = a_0 - a_1 D \quad (1)$$

where M_w is the weight-average molecular weight and D the measured diffusion coefficient. This is a phenomenological expression, and more precise models exist for describing the diffusion properties of homologous polymer series.[27] The results of fitting this expression to the data in Fig. 1 are given in Table 1. For comparison, the previous results obtained using poly(styrene sulfonate)[15] are also included. The polymethacrylate samples chosen span a similar range of molecular weights to the PSS samples used previously, and as such, reveal similar a_0 and a_1 parameters. As seen before, the addition of the chromatographic stationary phase causes changes principally in the a_1 parameter, which is consistent with size-exclusion effects.[23] The small difference in the changes between the two polymers is likely related to differences in the interaction of polymer with the stationary phase, i.e. differences between aromatic PSS and alkyl PMA side-chains.[28-31]

In order to investigate whether the presence of multiple species influences the ability of the size-exclusion stationary phase to modulate diffusion properties, an equimolar mixture of two polymers was used. The binary mixture was prepared using pairs of polymers with similar molecular weights, e.g. 32.9 kDa poly(styrene sulfonate) was paired with 36.3 kDa polymethacrylate, etc. Diffusion NMR experiments were then performed on mixtures in the presence and absence of Sephadex G-50. The results are presented in Fig. 2. In the absence of the stationary phase, a similar trend to that shown in Fig. 1 for PMA, and reported previously for PSS,[15] is observed. The measured diffusion coefficients are in broad agreement with those obtained for the individual polymers at the same concentrations. The parameters obtained from fitting Eqn. (1) are given in Table 2. The values are altered slightly in the case of the

polymer mixture as opposed to solutions of the individual polymers, which suggests that there may be some interaction between the polymers.[32, 33] On addition of the Sephadex G-50, there is a noticeable change in the observed diffusion coefficients, which as expected, is more pronounced for the smaller polymers. This is consistent with size-exclusion behaviour, and indicates that the presence of the polymer mixture does not alter the gross diffusion modulating effect. Fitting the observed data to Eqn. (1) results in the straight lines shown in Fig. 2 and the parameters reported in Table 2. While there is a clear change in the parameters on addition of the stationary phase, the magnitude of the change is not as large as in the case of the individual polymers only. Differences in the interactions between polymers and stationary phase,[28-31] in addition to small changes in the viscosity of the solution may be a contributing factor here.[23] In traditional on-flow size-exclusion chromatography, these effects can be removed via universal calibration methods.[23]

In order to further generalise the results presented, a "mismatched" sample comprising polymethacrylate with a molecular weight of 20.3 kDa, and poly(styrene sulfonate) with a molecular weight of 63.9 kDa was prepared in a similar manner to those used above. Fig. 3(a) shows the ^{1}H spectra of this mixture in the absence and presence of the stationary phase, demonstrating that the addition of the stationary causes only minor increases in the observed line width. Fig. 3(b) shows the DOSY spectrum of the polymer mixture in the absence of Sephadex G-50. There is clearly a separation between the two polymers in the diffusion dimension. The CH_2 region around 1-2 ppm is clearly very crowded, and shows extensive overlap of signals from both polymers and the stationary phase dextran support. The methyl groups of the polymethacrylate at 0.8 ppm, however, are resolvable and show as a distinct signal in

the diffusion dimension, with a larger diffusion coefficient to that of the poly(styrene sulfonate). The measured diffusion coefficients in the presence and absence of Sephadex G-50 are shown in Fig. 3(c). In the case of the polymers in the absence of the stationary phase, the values are similar to those expected for the individual polymers, although the diffusion coefficient for PSS is slightly larger than observed previously. When the Sephadex G-50 stationary phase is added to the mixture, the observed diffusion coefficients are reduced as expected, broadly in line with the effects seen in Fig. 2. The effect of the stationary phase is greatest for the smaller polymer as its diffusion is hindered more by the pores of the stationary phase than the larger polymer. Overall, similar effects are observed as with the matched-weight polymers, however, the PSS sample used here has a molecular weight above the cut off of the stationary phase and hence its diffusion properties should be unaffected by the addition of the stationary phase. This was observed previously in the case of the single polymers,[15] and in the case of the weight-matched pairs discussed above. In this case, there is a reduction in the observed diffusion coefficient. This may be the result of some interaction between the PSS and PMA, or due to reduced space for self-diffusion due to the presence of the stationary phase. It is currently unclear as to why this would be observed here, but not previously.

Conclusions

The use of chromatographic stationary phases[3-6, 14] or solvent modifiers[7-10, 34] to modulate diffusion properties is becoming well established. We have previously demonstrated that size-exclusion media can be used in a similar manner, that is, they can induce a change in the observed diffusion coefficient which is consistent with size-exclusion behaviour.[15] Here, we have demonstrated that these techniques can be

applied to mixtures of polymers with similar results. Modification of the observed diffusion coefficients upon addition of Sephadex G-50 is found whether the polymers are close in molecular weight or not. The size of the modification is again consistent with size-exclusion behaviour occurring in the NMR sample, with the smaller polymers showing a much greater change in diffusion coefficient than the larger polymers. The application of in-situ size-exclusion chromatographic NMR to other systems, such as biopolymers, is currently under investigation.

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Tables and Table Captions

Table 1: Parameters returned from fitting Eqn. (1) to the data in Fig. 1. The data for poly(styrene sulfonate) is from Joyce and Day.[15]

Sample	a_0	a_1 / 10^{10} s m^{-2}	R^2
PMA only	5.05	1.68	0.99
PMA + Sephadex G-50	5.36	3.19	0.98
PSS only[15]	5.11	1.50	0.96
PSS + Sephadex G-50[15]	5.30	2.52	0.94

Table 2: Parameters returned from fitting Eqn. (1) to the data in Fig. 2.

Sample	a_0	a_1 / 10^{10} s m^{-2}	R^2
PMA free	5.02	1.72	0.99

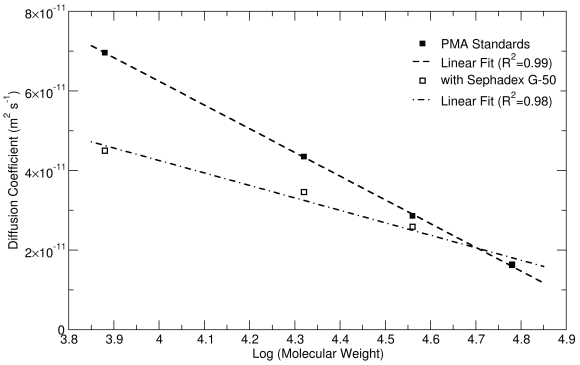
PSS free	5.01	1.57	0.94
PMA + Sephadex G-50	5.18	2.72	0.97
PSS + Sephadex G-50	5.00	1.90	0.97

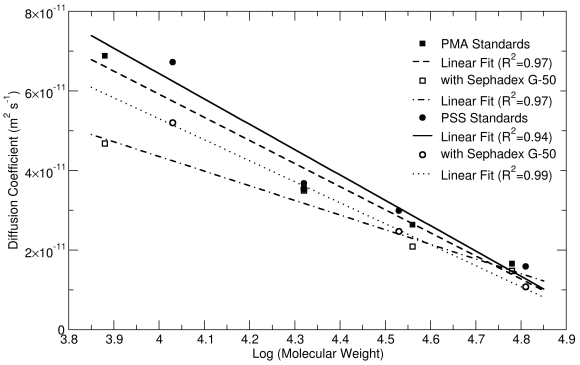
Figure Captions

Figure 1: Diffusion coefficients for some polymethacrylate molecular weight reference standards in the presence and absence of Sephadex G-50. The straight lines are the result of fitting Eqn. (1) to the experimental data, with parameters given in Table 1.

Figure 2: Diffusion coefficients for paired mixtures of polymethacrylate and poly(styrene sulfonate) in the presence and absence of Sephadex G-50. The straight lines are the result of fitting Eqn. (1) to the experimental data, with parameters given in Table 2.

Figure 3: (a) ^{1}H NMR spectra of a mixture of 20.3 kDa PMA and 63.9 kDa PSS in the absence and presence of the Sephadex G-50 stationary phase. (b) DOSY spectrum of the same mixture in the absence of the Sephadex G-50 stationary phase. (c) Diffusion coefficients for the polymer mixture in the presence and absence of Sephadex G-50.


8×10^-11
6×10^-11
4×10^-11
2×10^-11
0
Diffusion Coefficient (m^2 s^-1)
3.8
3.9
4
4.1
4.2
4.3
4.4
4.5
4.6
4.7
4.8
4.9
Log (Molecular Weight)
PMA Standards
Linear Fit (R^2=0.99)
with Sephadex G-50
Linear Fit (R^2=0.98)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4×10^-11
2×10^-11
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Diffusion Coefficient (m^2 s^-1)
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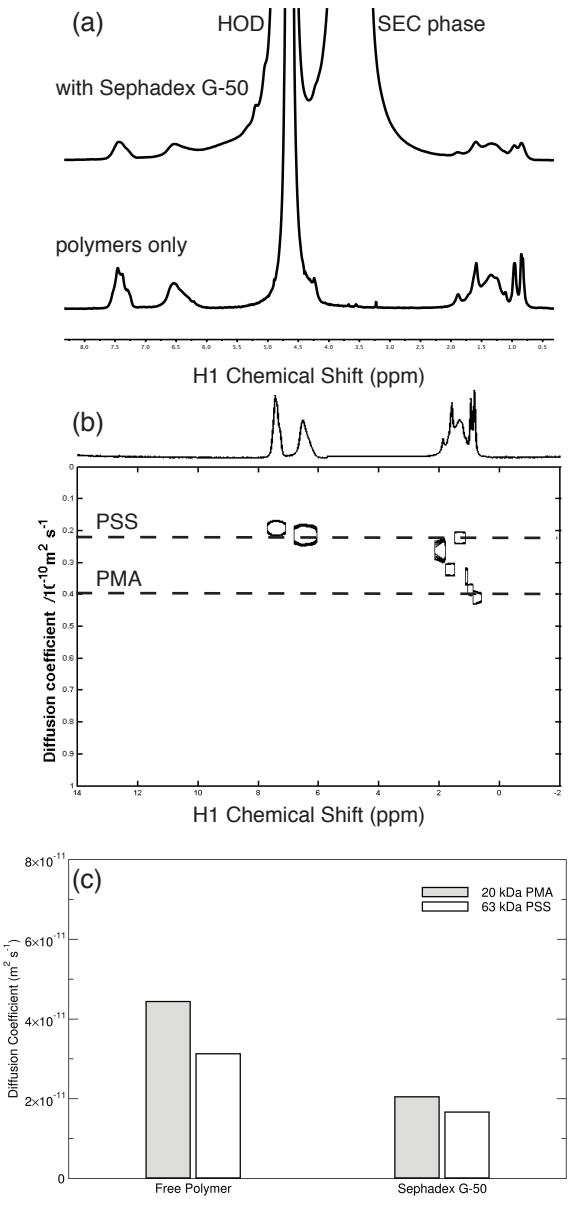
3.9
4
4.1
4.2
4.3
4.4
4.5
4.6
4.7
4.8
4.9
Log (Molecular Weight)
PMA Standards
Linear Fit (R^2=0.97)
with Sephadex G-50
Linear Fit (R^2=0.97)
PSS Standards
Linear Fit (R^2=0.94)
with Sephadex G-50
Linear Fit (R^2=0.99)


(a)
HOD
SEC phase
with Sephadex G-50
polymers only
H1 Chemical Shift (ppm)
(b)
PSS
PMA
Diffusion coefficient /(10^-10 m^2 s^-1
H1 Chemical Shift (ppm)
(c)
20 kDa PMA
63 kDa PSS
Diffusion Coefficient (m^2 s^-1)
8×10^-11
6×10^-11
4×10^-11
2×10^-11
0
Free Polymer
Sephadex G-50