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Review

Nuclear magnetic resonance chromatography: applications of pulse field gradient diffusion NMR to mixture analysis and ligand–receptor interactions

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Abstract

Pulse field gradient (PFG) diffusion NMR spectroscopy is a non-invasive method for the spectroscopic separation and identification of compounds of interest from a mixture. Because it relies on differences in translational diffusion rates to resolve NMR signals from individual components, pulse field gradient NMR is a unique method for analyzing complex mixtures and for detecting intermolecular interactions. A number of multidimensional pulse field gradient NMR experiments have been developed to alleviate the overlap of NMR signals arising from a complex mixture and facilitate component identification. The applications of pulse field gradient NMR for mixture analysis and for the direct identification of high affinity ligands are reviewed.

Keywords: Reviews; Pulse field gradient; Diffusion nuclear magnetic resonance; Mixture analysis

Contents

1. Introduction

The identification of compounds with a desired biological activity is of key importance to the drug discovery process. Over the past few years a great deal of investment has gone into technologies which promise to increase the number of chemical entities that can be generated and tested for biological activity. Part of this research effort has involved combinatorial chemistry techniques that create mix-

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tures as a final product, and high-capacity screening methods that assay mixtures for biological activity. Generating and testing compounds as mixtures offers increased efficiency and throughput relative to the making and testing of individual compounds.

Unfortunately, determining the composition of complex mixtures can often be challenging. Despite the necessity for analytical tools which could provide feedback to the chemist on the success of a synthetic process, or on the long-term chemical stability of a fabricated mixture, few are available. Without physical separation procedures, the majority of spectroscopic techniques do not lend themselves to the structural analysis of complex mixtures of unknown composition.

NMR spectroscopy, which has become an indispensable tool for elucidating structure and evaluating sample purity, has been successfully employed for the analysis of relatively simple mixtures. The power of methods that combine NMR and chromatography has been amply demonstrated [1–3]. These techniques depend on the physical separation of compounds and therefore may not be desirable for all situations. A recent attempt to extend NMR as a non-invasive tool for mixture analysis has involved the combined use of TOCSY and HMBC experiments to identify the structures of a mixture of six esters [4]. In this study, ^{1}H spin–spin coupling networks were identified from TOCSY data and the HMBC experiment allowed spin–spin coupling networks that were isolated by certain functional groups, such as ethers and esters, to be correlated. Although appealing, the utility of this method for more complex mixtures appears, at present, to be limited to approximately ten compounds.

An alternate approach for the non-invasive analysis of mixtures is the use of pulse field gradient (PFG) diffusion NMR. This method relies on the attenuation behavior of individual resonances under the influence of linear field gradients to measure translational diffusion [5]. Since diffusion is a property of a molecule as a whole, PFG diffusion NMR allows the spectroscopic resolution of individual mixture components based on the variance of their diffusion rates. By virtue of its ability to resolve complex mixtures, and allow component identification, PFG diffusion NMR can be thought of as a method for ''NMR chromatography''.

The applicability of PFG–NMR methodology for mixture analysis has been demonstrated on a number of complex mixture systems, including mixtures of small organic molecules [6,7], tissue extracts [8], and biofluids [9,10]. Because diffusion coefficients are related to the effective hydrodynamic radius of a molecule or of a molecular aggregate [11], the PFG diffusion ordered NMR experiment provides a unique means of establishing the occurrence of intermolecular interaction. To this end, PFG diffusion NMR has been extensively used to study molecular self-aggregation [12,13], macrocycle-guest interactions [14–16], and the partitioning of molecules into micelles [17–19]. The basis of these studies is the premise that diffusion rates are correlated with molecular size and thus the diffusion rate of a molecule would be significantly reduced upon complexation with a binding partner.

In affinity NMR, the alteration in a ligand's diffusion coefficient due to its complexation with a receptor is used to spectroscopically isolate the ligand from a pool of non-interacting compounds [20,21]. The concept of separating compounds by receptor affinity utilized in affinity NMR is reminiscent of affinity chromatography methods.

Currently, affinity NMR is being developed as a tool to aid the interpretation of high-throughput screening results [22,23]. Occasionally, a mixture has sufficient biological activity in a high capacity assay, but upon deconvolution, no active components can be identified. Such ''false positives'' can be due to the cumulative action of several compounds on the receptor or by the components of the mixture triggering an artifact in the assay detection scheme [22,23]. A number of approaches have been put forward to deconvolute mixtures and identify the components responsible for the desired activity, such as iterative rescreening of mixture subsets, recursive deconvolution, direct deduction of the active components by re-synthesis of the mixture in pools, and tagging [24–27].

The affinity NMR method, which allows direct identification of high affinity ligands, could minimize false positives and greatly reduce the effort required for determining the components responsible for the desired effect. affinity capillary electrophoresis [28,29] and affinity mass spectroscopy [30–32] are two other methods being investigated for this pur-

pose. However, both of these methodologies are invasive and require the physical separation of the mixture components.

Recently, Shuker et al. [33] have proposed an NMR method, which has generated a great deal of interest, for the identification of compounds that exhibit specific receptor binding from a pool of potential ligands. This method, termed ''SAR by NMR'', requires the use of an ^{15}N-labeled protein receptor with a molecular weight no greater than approximately 30 kDa. The binding affinity of the ligand is evaluated by the changes in the ^{15}N or ^{1}H chemical shifts of the protein upon binding of the ligand. This method promises to become a valuable tool in the drug discovery process as it allows the binding site on the receptor protein to be mapped. In addition, ligands that have activities below the biological assay's detection set point can still be identified and used as synthetic precursors for higher affinity ligands [34,35].

Like the SAR by NMR method, affinity NMR allows the identification of low affinity ligands for use as synthetic precursors. In addition, the affinity NMR method offers several advantages over the SAR by NMR method. Neither labeled receptor nor the chemical shift assignments for the receptor are required and, in principle, receptors much larger than 30 kDa can be used. Since the diffusion method does not rely on the comparison of two different data sets, small changes in sample conditions (i.e., pH, salt concentrations, etc.), that may effect chemical shifts, do not need to be taken into account. Potentially the most important advantage of affinity NMR is that the binding ligands are identified directly from the mixture based on diffusion, without the necessity of deconvolution.

2. Principles of PFG diffusion ordered NMR

2.1. Theory

In NMR experiments, nuclear spins precess about the magnetic field at a frequency defined by their chemical identity and local electronic environment. Provided that field inhomogeneity can be ignored, all spins experience an identical magnetic field despite being dispersed throughout the sample. The application of a field gradient has the effect of making the magnetic field strength linearly dependent on position.

Prior to the application of a gradient pulse, all the spins have a coherent phase. Under the influence of a gradient pulse, the phase of the individual spins becomes dependent on their transverse position and the spins are therefore spatially phase encoded. Provided that translational diffusion does not occur, this spatial phase encoding is fully reversible by the application of a second gradient of inverse polarity and no loss of NMR signal will occur. However, the second gradient pulse will not be able to realign phases of the spins that have undergone translational diffusion and the resulting NMR signal will appear attenuated. The intensity of the NMR signal in the PFG diffusion ordered experiment is described by:

$$I = I_0 \exp[-D\,(\Delta - \delta/3)\,\gamma^2 g^2 \delta^2] \quad (1)$$

where I and I_0 are, respectively, the intensity of the NMR signal in the presence and absence of external gradient pulses, D is the diffusion coefficient, Δ is the time period over which translational diffusion is allowed to occur, γ is the nuclear gyromagnetic ratio, g and δ are, respectively, the amplitude and duration of the gradient pulse [5].

For non-exchanging resonances, the diffusion coefficient D can be obtained directly by an exponential fit of the signal intensity I to Eq. (1). If chemical exchange is occurring at a rate that is fast relative to Δ, the observed diffusion coefficient (D_{obs}) will reflect a population weighted average of the diffusion coefficients of the exchanging species according to:

$$D_{obs} = D_{bound} f_{bound} + D_{free} f_{free} \quad (2)$$

Here it is assumed that exchange is between a free and bound state and the fraction of ligand in either the bound or free states is represented by f_{bound} and f_{free}, respectively [36]. The fraction of bound ligand (f_{bound}) and the binding constant (K) can be calculated from Eqs. (3) and (4), respectively.

$$f_{bound} = (D_{obs} - D_{free})/(D_{bound} - D_{free}) \quad (3)$$

$$K = (D_{obs} - D_{free})/((D_{bound} - D_{obs})[R]) \quad (4)$$

were [R] is the concentration of the unbound receptor.

2.2. Acquiring Data/Pulse Sequences

The two most commonly used PFG diffusion NMR sequences are the longitudinal eddy-current delay (LED) [37] and the bipolar pulse longitudinal eddy-current delay (BPP–LED) [38], shown in Fig. 1. As their names indicate, both sequences store magnetization along the longitudinal or *z*-axis during the delays T and T_e in order to minimize the deleterious effects of T_2 relaxation and *J*-modulation. The delay (T_e) provides a time interval for the gradient-induced eddy-currents to settle prior to acquisition. A series of gradient prepulses are applied prior to the first 90° rf pulse, in the LED sequence, in order to equalize the effects of the gradient-induced eddy currents on the magnetization during the execution of the sequence. Due to the self compensating nature of the bipolar pulse pairs used in the BPP–LED sequence, the need for the gradient prepulses is removed and the time required for T_e is considerably shortened. The bipolar pulse pairs have also been shown to be useful for eliminating the modulation introduced by chemical exchange [39].

The PFG–NMR methodology can be extended into a multidimensional format by incrementing either the gradient strength g and/or its duration δ [19,40,41]. The resulting data set can be processed and displayed as a two- or three-dimensional matrix with diffusion coefficients plotted along one axis. Chemical shift dimensions are obtained by Fourier transformation of the time domain data and, following this, the diffusion dimension is obtained by an approximate inverse Laplace transformation of the

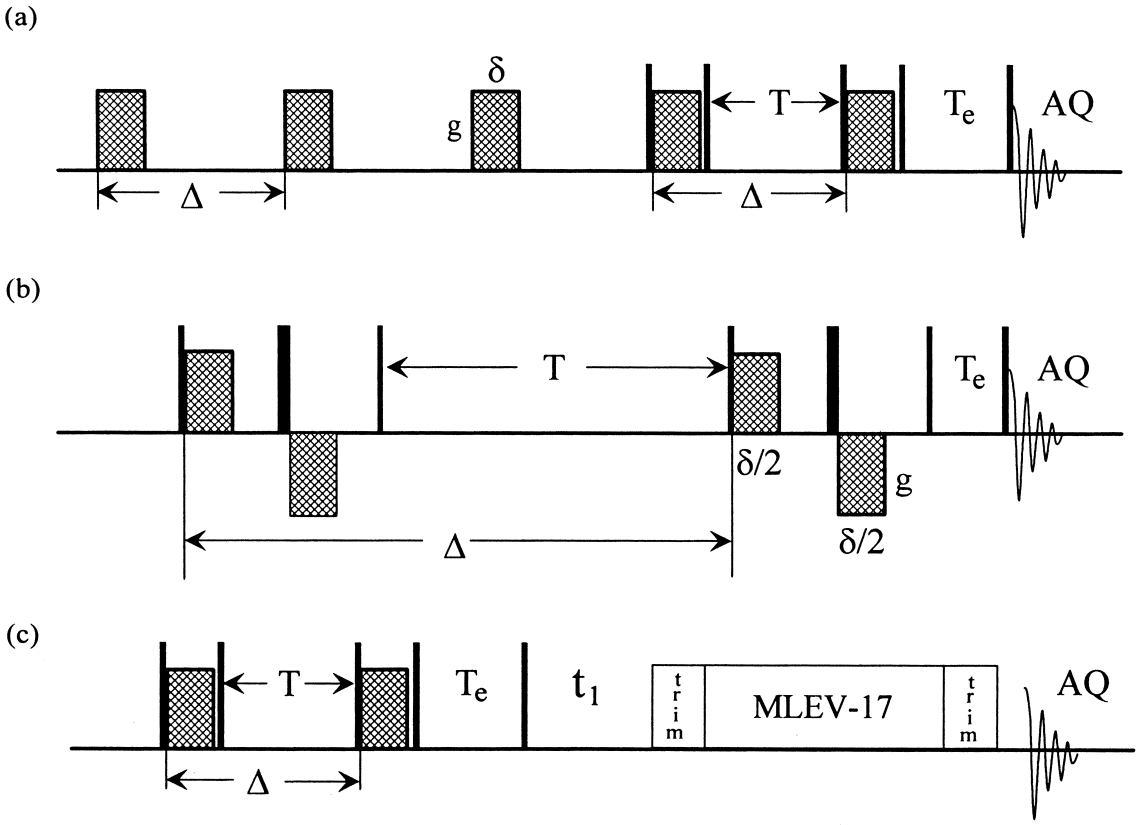

Fig. 1. Pulse sequences used for PFG diffusion NMR; A) The longitudinal eddy-current delay (LED) sequence. B) The bipolar pulse longitudinal eddy-current delay (LED) sequence. C) The LED–TOCSY (DECODES) sequence.

frequency domain data. In keeping with the traditions of NMR nomenclature, the PFG experiments which result in a two-dimensional data set, with chemical shifts plotted along one axis and diffusion coefficients along the other, have been termed Diffusion-Ordered SpectroscopY (DOSY)

3. Mixture analysis

In the DOSY spectrum, the NMR signals arising from discrete components are resolved based on the variance of their diffusion rates and chemical shifts. Resonances associated with individual molecules are expected to align exactly along the diffusion dimension. The DOSY experiment is capable of resolving components whose diffusion coefficients differ by only a few percent [8], making it of great interest as a tool for analyzing mixtures. However, if chemical shift overlap exists between different components and, the diffusion coefficients of these molecules differ by less than a factor of two, only a single peak with an averaged diffusion coefficient will appear in the DOSY spectrum [19,42].

Mixtures resulting from combinatorial chemistry synthesis and/or used in high-capacity screening assays are often composed of structurally related molecules of similar molecular weight. The challenge posed by such mixtures has motivated the development of DOSY experiments which reduce the probability of signal overlap in the chemical shift dimension.

To this end, Wu et al. [42] have proposed a series of DOSY experiments which combine the LED sequence with heteronuclear coherence transfer steps of the well known INEPT and DEPT–NMR experiments. These experiments directly detect ^{13}C resonances, taking advantage of the wider chemical shift range and much better chemical shift resolution relative to ^{1}H-NMR. The INEPT and DEPT coherence transfer steps help alleviate the problems associated with the low sensitivity of the ^{13}C nucleus. In addition, both INEPT and DEPT sequences have the rather convenient property of allowing the spectral discrimination of CH, CH_2, and CH_3 moieties. This spectral editing capability reduces the possibility of peak overlap as well as provides additional structural information.

It is possible to combine PFG methods with multidimensional NMR techniques. Like their conventional counterparts, these experiments reduce the probability of spectral overlap by spreading NMR signals over an entire two-dimensional plane rather than simply along a single chemical shift axis. The first experiment to combine PFG diffusion NMR with multidimensional NMR was reported by Gozansky and Gorenstein [43]. Their experiment merged the LED sequence with the two dimensional NOESY. Using the DOSY–NOESY, Gozansky and Gorenstein were able to resolve overlapping resonances from a 14-mer DNA duplex $d(ACAATATATATTGT)_2$ and a dinucleotide d(pAG). Despite this successful application of the DOSY–NOESY experiment, the strong dependence of the NOE on molecular tumbling and internuclear distance limits the general applicability of the DOSY–NOESY experiment for all types of mixture systems.

Lin and Shapiro [6] have described a DOSY–TOCSY (DECODES) experiment which combines the LED sequence with the TOCSY–MLEV-17 spin lock (Fig. 1). The TOCSY sequence correlates all ^{1}H resonances that are part of a *J*-coupling network (see Fig. 2). This is a particularly useful feature, as resonances that suffer from spectral overlap can be correlated to resonances that are fully resolved. This not only augments the potential for resolving mixture components, it also simplifies their identification.

For the DECODES experiment, and for other similar experiments like the DOSY–NOESY, a series of two-dimensional spectra are collected at incremented values of the gradient pulse strength. The intensity of each cross-peak, in each spectrum, is attenuated due to diffusion and gradient strength according to Eq. (1). Diffusion coefficients are obtained from a plot of the natural logarithm of the cross-peak intensities vs. the square of the gradient strength. As long as a component gives rise to a resolved cross-peak, the diffusion coefficient and molecular identity can be obtained. The use of the TOCSY spin lock aids this possibility by providing multiple opportunities for such resolution to occur. The utility of the DOSY–TOCSY (DECODES) sequence for the analysis of complex mixtures has been tested in two investigations on whole blood plasma by Liu et al. [8,9]

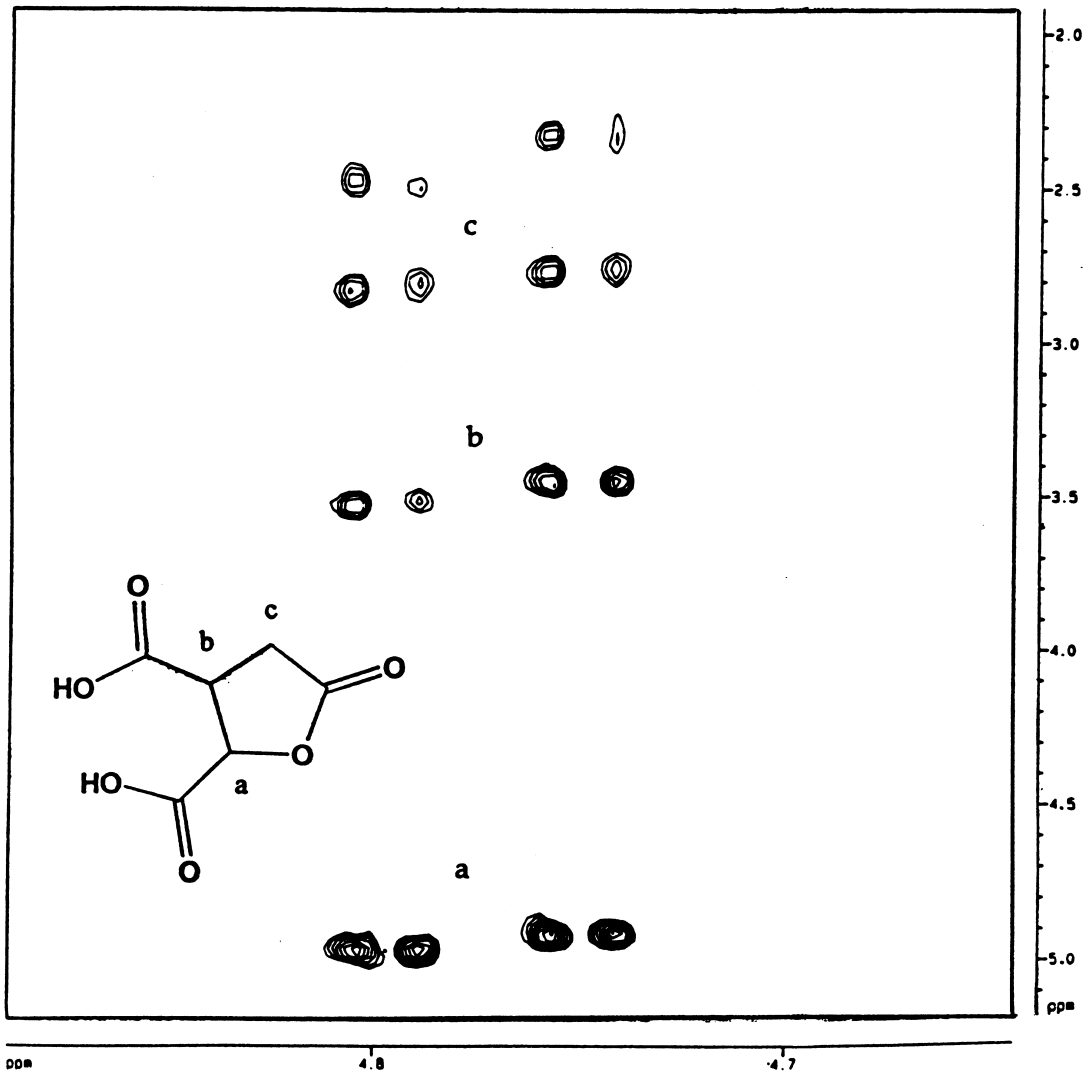

Fig. 2. DECODES spectrum of DL-isocitric lactone showing the multiple correlations achievable using this pulse sequence.

Wu et al. [44] have reported the development of fully automated software for the processing of three-dimensional DOSY data sets. The three-dimensional matrices generated form this processing protocol contain two chemical shift dimensions and a diffusion coefficient dimension. This processing procedure was demonstrated using a DOSY–COSY sequence. In this experiment, the two-dimensional COSY spectrum of individual mixture components was resolved along the diffusion axis. The identification of the spectroscopically separated mixture component was achieved by direct analysis of the two-dimensional COSY planes. Because NMR signals are spread over a three-dimensional volume, the three-dimensional technique offers better resolution relative to two-dimensional approaches.

Other three dimensional PFG–NMR sequences that have been reported include a three-dimensional DOSY–TOCSY by Jerschow and Müller [45] and a three-dimensional DOSY–HMQC from Barjat et al. [7] These experiments have been designed to decrease the potential for spectral overlap and facilitate

compound identification. The most significant limitations of the three-dimensional DOSY experiments are their lengthy experimental time and large data storage requirements.

4. Affinity NMR

PFG diffusion NMR has been extensively used to study molecular interactions in a number of systems [12–19]. The goal of the affinity NMR experiment is to spectroscopically separate and identify ligands that have binding affinity to a relevant receptor molecule. The fundamental premise of the affinity NMR approach is that, upon binding to its receptor, the diffusion rate of a low molecular weight ligand would become sufficiently distinct, as to allow the ligand to be spectroscopically separated, using PFG diffusion NMR, from a pool of non-binding molecules.

It is a prerequisite of the affinity NMR experiment that conditions (i.e., gradient strength and duration, and delay Δ) are determined for which no NMR signals are observed for the components of the mixture in the absence of receptor. Provided these conditions are used after the addition of the receptor, non-interacting compounds will be absent from the affinity NMR experiment (see Fig. 3). Due to their reduced translational diffusion rates, ligands that do bind to the receptor are expected to appear in the affinity NMR spectrum.

The first reported example of affinity NMR was on a model system consisting of hydroquinine 9-phenanthryl ether as the receptor and a mixture of eight potential ligands [20] (see Fig. 3). Using affinity NMR, it was determined that two of the mixture components were binding to the hydroquinine 9-phenanthryl ether. These two compounds DL-isocitric lactone and (*S*)-(+)-*O*-acetylmadelic acid, were resolved and identified using the DECODES experiment acquired under the same conditions as the affinity NMR experiment.

When multiple ligands exhibit binding affinity to a receptor, they can be resolved either through use of the DECODES experiment or by ''tuning'' (i.e., adjusting) the relative receptor–ligand concentrations to match the binding affinity of the ligand. An example of ''tuning'' the affinity NMR experiment to binding affinity has been reported by Lin et al. [21] In this study, hydroquinine 9-phenanthryl ether was added to a mixture of four organic acids. By adjusting the concentration of the hydroquinine 9-phenanthryl ether receptor relative to the four acids, each acid was made to sequentially appear in the affinity NMR spectrum according to their binding affinity (Fig. 4).

In an attempt to establish the applicability of affinity NMR to more biologically relevant systems, studies were performed using a DNA–drug system [46] and using the glycopeptide vancomycin in a mixture with ten oligopeptides [47]. Using the affinity NMR–DECODES methodology, the compound Hoechst 33342 was identified as binding to the Drew–Dickerson dodecamer $d(CGCGAATTCGCG)_2$ in the presence of the non-binding molecules adenine, adenosine and thiamine. One advantage of the DECODES method for studying DNA fragments is that the aromatic region of the DNA spectrum is devoid of cross peaks. This greatly facilitates the identification and interpretation signals from the ligands which have aromatic rings.

In the second study the interactions of the glycopeptide vancomycin with a pool of ten oligopeptides was examined using affinity NMR. Vancomycin is an important antibiotic agent whose binding interaction of with D-Ala–D-Ala containing peptides has been studied extensively [48–50]. Two oligopeptides, the all D-residue containing DDFA and DDFS, were identified as binding to vancomycin. As with the study involving hydroquinine 9-phenanthryl ether discussed above, the two oligopeptide ligands were identified through the use of the DECODES experiment. The ability of the DECODES experiment to correlate all ^1H within a spin system was crucial to this study, as there were significant chemical shift changes in both the vancomycin and the peptides upon binding.

A potential problem with affinity NMR is that the signals of the receptor are always present in the diffusion-edited spectrum. It is possible that signals from the ligand could become obscured by those of the receptor, particularly if the receptor is a large protein. While DECODES is an effective tool for systems involving small molecular receptors, the method may not be suitable for protein receptor systems.

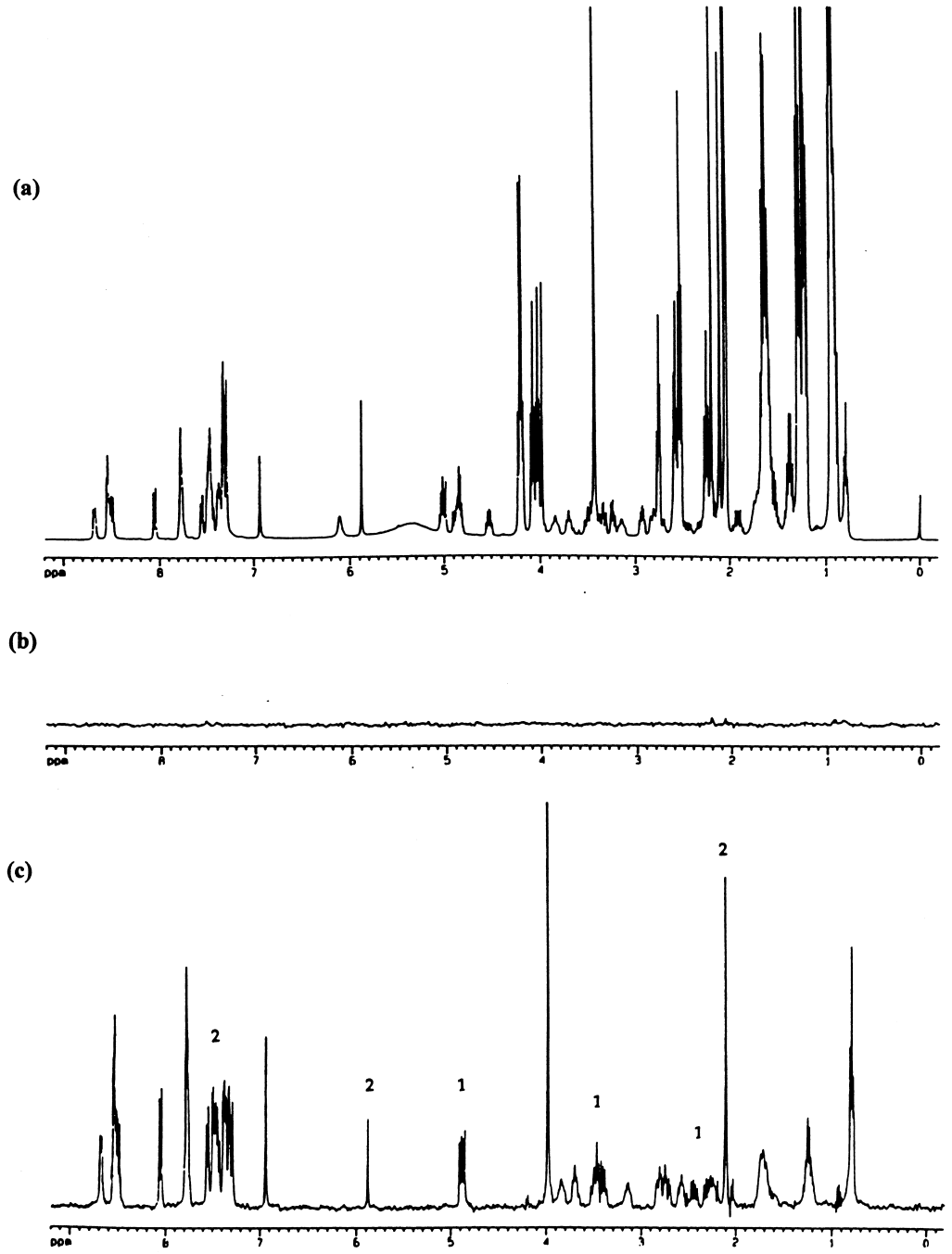

Fig. 3. (a) 1D 400 MHz ^{1}H-NMR spectrum of a nine-component mixture in $CDCl_3$. The concentration of each component is 10 mM. Components: **1** DL-isocitric lactone, **2** (*S*)-(+)-*O*-acetylmandelic acid, **3** DL-*N*-acetylhomocysteine thiolactone, **4** (±)-*sec*-butyl acetate, **5** propyl acetate, **6** isopropyl butyrate, **7** ethyl butyrylacetate, **8** butyl levulinate, **9** hydroquinine 9-phenanthryl ether. (b) 1D PFG-^{1}H-NMR spectrum of the mixture without hydroquinine 9-phenanthryl ether, using the LED sequence. (c) 1D PFG-^{1}H-NMR spectrum of the nine-component mixture. Resonances arising from DL-isocitric lactone (**1**) and (*S*)-(+)-*O*-acetylmandelic acid (**2**) are labeled. All other resonances are from hydroquinine 9-phenanthryl ether. The PFG conditions used were the same as in spectrum (b).

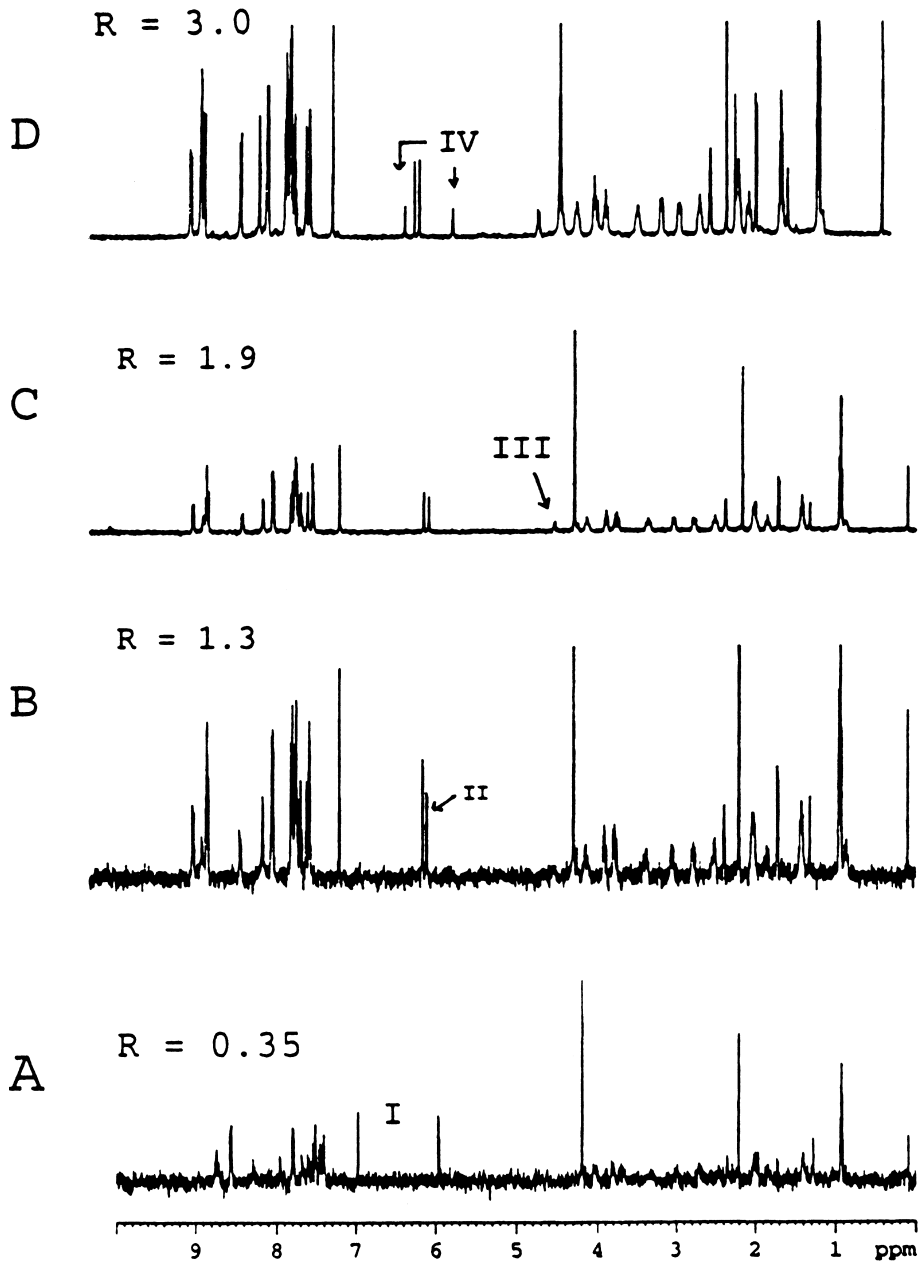

Fig. 4. Titration of four carboxylic acid mixture with hydroquinine 9-phenanthryl ether. *R* is the ratio of hydroquinine to the total equimolar mixture of acids. Key resonances arising from compounds **I–IV** are shown: (A) **I**, dichloroacetic acid, pK_a 1.26, K_d 0.9×10^{-5} *M*; (B) **II**, (*S*)-(+)-*O*-acetylmandelic acid, pK_a 2.09, K_d 1.3×10^{-5} *M*; (C) **III**, 2-chloropropionic acid, pK_a 2.84, K_d 5.0×10^{-5} *M*; and (D) **IV**, methacrylic acid, pK_a 4.46, K_d 45×10^{-5} *M*.

To extend the applicability of affinity NMR for the study of protein–ligand interactions, an isotope-filtered affinity NMR was developed [22]. This experiment, which was designed for use with ^{13}C-labeled proteins, combines the LED sequence with a ^{13}C filtering step. As can be seen in Fig. 5, this sequence

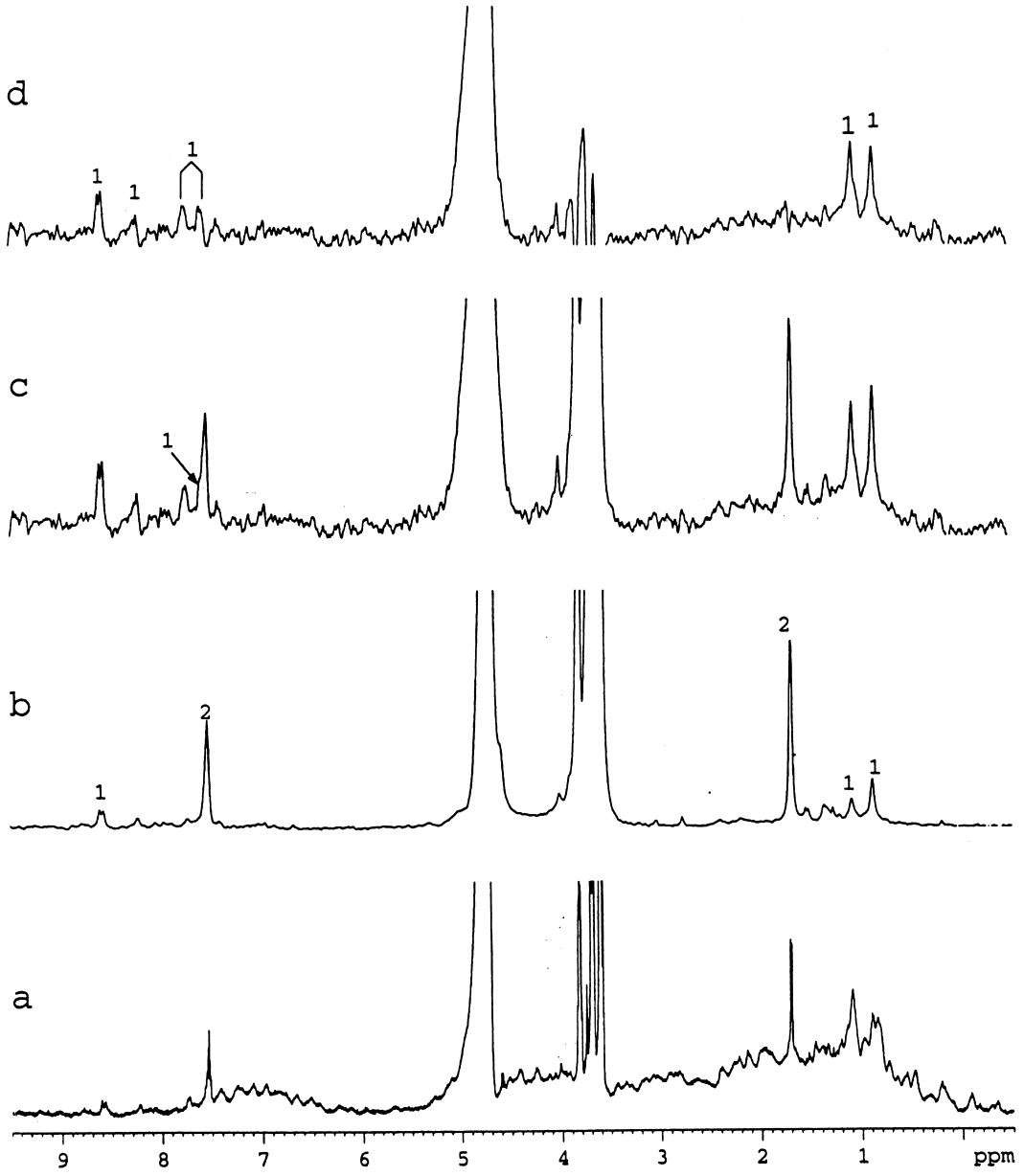

Fig. 5. a) The ^{1}H-NMR spectrum of the ^{13}C/^{15}N stromelysin with 0.3 m*M* of a known inhibitor **1** (*K*i 13 n*M*) and 1 m*M* of an inert compound **2**; (b) the ^{1}H-NMR spectrum with ^{13}C-isotope editing and a weak gradient; (c) the ^{1}H-NMR spectrum with ^{13}C-isotope editing and a strong gradient; (d) the difference spectrum resulting from subtracting spectrum (b) from (c). Resonances arising from **1** and **2** are indicated.

allows the simultaneous elimination of the signals from both non-binding compounds and the protein receptor.

A second approach, which does not require labeled protein, has been put forward by Hajduk et al. [23] Their method relies on the spectral subtraction of a series of data sets acquired at low and high gradient strengths and in the presence or absence of

the receptor protein. The resulting difference spectrum contains signals only from the binding ligands. Both the isotope-filtered PFG diffusion NMR experiment and spectral subtraction method have been demonstrated on the catalytic domain of the matrix metalloproteinase stromelysin, a protein of approximately 20 kDa [34,51].

5. Conclusions

A synopsis on the use of PFG diffusion NMR for mixture analysis and the identification of high-affinity ligands has been presented. The goal of these experiments is to spectroscopically separate and identify molecules of interest from a mixture of similar compounds. In this respect, the use of PFG diffusion NMR is reminiscent of the physical separation of compounds by chromatographic means. Because of this, we have termed the use of PFG diffusion NMR in this context as ''NMR Chromatography''.

The development of PFG experiments which reduce the opportunity for chemical shift overlap, through either heteronuclear coherence transfer steps or by spreading the NMR signals out over a two- or three-dimensional matrix has greatly augmented the capability to analyze complex mixtures. Sequences, such as DECODES, which utilize both molecular diffusion rates and magnetization transfer steps to assign mixture components, promise to become valuable tools for providing information on the success of a synthetic process or on the stability of a mixture. These methods are of greatest value in situations where, as is often expected for mixtures generated by combinatorial chemistry, there is severe spectral overlap between components and the variance in molecular size, and therefore diffusion rates, are small.

Because it allows the direct identification of high affinity ligands, affinity NMR promises to greatly reduce the effort necessary for deconvoluting biologically active mixtures. An additional advantage of the technique is that weakly binding ligands, which would often be missed by high-throughput screening, can be identified. Such low affinity ligands could potentially, serve as synthetic precursors for higher affinity ligands and provide valuable structure–activity information.

Compared to other methods, such as affinity capillary electrophoresis or affinity mass spectroscopy, NMR methods for screening mixtures are limited by their relative insensitivity. Simply raising the concentration of the mixture components and the receptor is not in general judicious, due to issues of compound solubility, protein precipitation, and potential non-specific binding. Despite these obstacles, affinity NMR remains an exciting area of research. Several laboratories, including our own, are currently investigating methods of increasing the applicability of affinity NMR toward the screening of larger and more complex mixtures.

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