Journal of Magnetic Resonance **147,** 361–365 (2000)
doi:10.1006/jmre.2000.2204, available online at http://www.idealibrary.com on IDEAL®

Three-Dimensional Electrophoretic NMR Correlation Spectroscopy[1]

Qiuhong He,[2] Wei Lin,* Yumin Liu,† and Ercheng Li

*Department of Chemistry, *Department of Computer Science and Engineering, and †Institute of Materials Science, University of Connecticut, Storrs, Connecticut 06269*

Received May 4, 2000; revised August 22, 2000

A novel method of three-dimensional electrophoretic NMR correlation spectroscopy (3D EP-COSY) has been proposed, developed, and implemented. It has a demonstrated potential of facilitating simultaneous structural assignments of multiple proteins in mixtures. The principle is to add a pulsed DC electric field that introduces a new dimension of electrophoretic flow, in which resonances of different molecules can be separated by their electrophoretic migration rates without physical separation. As a result, two COSY spectra were simultaneously obtained in a single 3D EP-COSY experiment from a mixture of 150 mM L-aspartic acid and 148 mM 4,9-dioxa-1,12-dodecanediamine with concurrent resolution of their chemical shifts and *J*-coupling constants. This approach creates a new horizon of multidimensional electrophoretic NMR. The technical advance opens doors for structure characterization of complex protein systems and protein interactions, which are at the basis of biochemical mechanisms and the phenomena of living systems. © 2000 Academic Press

The multidimensional nuclear magnetic resonance (NMR) of correlation spectroscopy (COSY) (*1–4*) and nuclear Overhauser spectroscopy (NOESY) (*5–9*) are extremely powerful in sequential and stereospecific structural assignments of proteins (*10–13*). These NMR spectroscopy methods can be used not only to determine protein structures at a resolution comparable to X-ray crystallography, but also to provide information on protein dynamics in aqueous environments. However, due to severe signal overlap, the conventional NMR methods have difficulties in characterizing structures or structural changes of multiple protein components in biochemical reactions. To solve this problem, we have proposed the multidimensional electrophoretic NMR (ENMR) spectroscopy (*14*), which separates NMR resonances of mixed proteins by electrophoretic mobilities. Thus, NMR spectra of different proteins can be simultaneously obtained in an isotropic mixture without physical separation of the proteins. The method was validated in a 2D ENMR experiment (*15–34*), which produced 1D NMR spectra of bovine serum albumin (BSA, 66 kDa) and ubiquitin (8.6 kDa) mixed in D_2O (*14*). In this paper, we report the results of a three-dimensional ENMR study, using 3D electrophoretic correlation spectroscopy (EP-COSY) as an example. COSY spectra of L-aspartic acid and 4,9-dioxa-1,12-dodecanediamine were simultaneously obtained, permitting concurrent measurement of their structural parameters in the form of chemical shifts and *J*-coupling constants.

The 3D EP-COSY method was developed based on a 2D COSY pulse sequence (Fig. 1). An electric field pulse drove the electrophoretic flow of ionic species, and a pair of pulsed magnetic field gradients labeled the spin locations and selected the spin coherence transfer pathways. Spin chemical shift evolutions and *J*-couplings in the evolution period (t_1) and detection period (t_2) generated the COSY type of chemical shift correlations in the first two dimensions. The electrophoretic motion of molecules modulated the COSY resonances as the electric field was stepwise increased in the third dimension. In the spin density matrix calculation, the electrophoretic migration of molecules can be treated as an independent event, sequential to the RF spin rotations and the spin evolutions under chemical shift and spin coupling terms, since the Hamiltonian of molecular motion commutes with the Hamiltonian of internal spin dynamics. In the rotating frame, the latter can be expressed as $H = (\omega_1 + \gamma gz)I_{1z} + (\omega_2 + \gamma gz)I_{2z} + 2\pi J I_{1z}I_{2z}$. Hence, for a weakly coupled two-spin system ($I_1 = I_2 = \frac{1}{2}$ and $|\omega_1 - \omega_2| \geq |2\pi J|$), the final spin density matrix (σ) of the 3D EP-COSY sequence are the product of the spin density matrix of the conventional 2D COSY experiment and a cosine factor, $\cos(K\mu E_{dc}\Delta)$, that describes the electrophoretic modulation of the COSY resonances:

$$\begin{aligned}\sigma(t_1, t_2) = i/4\Big\{ & I_1^- \exp[i\omega_1 t_2 - i\omega_1(t_1 + \Delta)] \\ & \times \cos(\pi J t_2)\cos[\pi J(t_1 + \Delta)] \\ & + \frac{1}{2} I_2^- \exp[i\omega_2 t_2 - i\omega_1(t_1 + \Delta)] \\ & \times \sin(\pi J t_2)\sin[\pi J(t_1 + \Delta)]\Big\} \\ & \times \exp[-i(\phi_1 - 2\phi_2)] \\ & \times \exp\left[-DK^2\Delta - \frac{t_1 + \Delta}{T_2}\right]\cos(K\mu E_{dc}\Delta),^3 \quad [1]\end{aligned}$$

[1] Some of the preliminary results were reported in the 41th Experimental Nuclear Magnetic Resonance Conference (ENC), Asilomar, CA, April 9–14, 2000.

[2] To whom correspondence should be addressed at: Sloan-Kettering Institute for Cancer Research, Medical Physics Department, 1275 York Avenue, New York, NY 10021. Fax: (212) 717-3676.

1090-7807/00 $35.00
Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

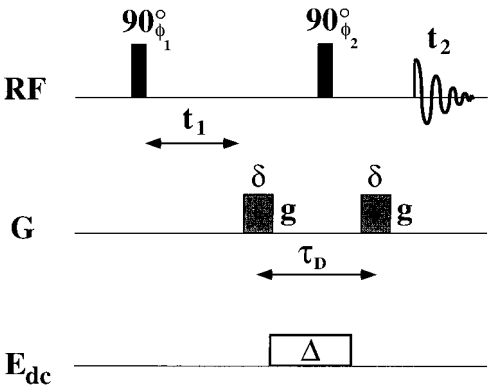

FIG. 1. The 3D EP-COSY pulse sequence and phase cycling procedures.

where parameter $K = \gamma g \delta$ is a function of the gyromagnetic ratio of the nucleus (γ), the amplitude (g), and duration (δ) of the magnetic field gradient pulses. The amplitude of the electric field, $E_{dc} = I_e/(\kappa A)$, is determined by the electric current (I_e), the solution conductivity (κ), and the cross-sectional area (A) of the U-shape ENMR sample cell. D is the molecular diffusion coefficient and T_2 the spin–spin relaxation time. The electrophoretic cosinusoidal modulation frequency is a function of the electric field, the magnetic field gradients, and the electrophoretic mobility (μ). Since the ENMR signal vs current curves was truncated, a direct Fourier transformation is not appropriate. Fittings to the cosine curves were performed to obtain the electrophoretic oscillation frequencies for different molecules. The truncated cosine curves were subsequently extended to have 256 data points using these frequencies before the third Fourier transformation. In this way, the COSY resonances of molecules $\{i, i = 1, 2, 3 \ldots\}$ migrating at different electrophoretic mobilities were distinguished and displayed at different frequencies $\{\nu_i\}$ in the third dimension of electrophoretic flow velocity, where $\nu_i = \pm(KI_\delta\Delta/2\pi\kappa A)\mu_i$ and I_δ is the increment of the applied DC electric current. Note that increasing the electric field at the constant gradient amplitude and interpulse delays avoided signal decays due to molecular diffusion and spin relaxation (*18*). The linewidth in the third dimension was determined by a 90° phase shifted sinebell squared window function applied to the extended cosine curves before Fourier transformation.

To demonstrate the concept of 3D ENMR, we acquired a 3D EP-COSY data matrix (256 × 156 × 28) from a solution mixture containing 150 mM L-aspartic acid and 148 mM 4,9-dioxa-1,12-dodecanediamine in D_2O. The experiment was performed on a Bruker AM 500 NMR spectrometer equipped with an actively shielded magnetic field gradient in the z-axis. The electric field was generated from an electric field/gradient driver from Digital Specialties (maximum output voltage = 1 kV). Capillary array ENMR (CA-ENMR) sample cells (*35*) were used uncoated after treatment with 1 M HCl, deionized water, and 1 M NaOH. The detected migration rates of L-aspartic acid and 4,9-dioxa-1,12-dodecanediamine were 1.6×10^{-4} and 2.0×10^{-4} cm$^2 \cdot$ V$^{-1} \cdot$ s^{-1}, respectively, resulting from their electrophoretic motion and the bulk electroosmotic flow of the solution. Because of the small capillary diameter, the electroosmotic velocity distribution caused no resolution reduction in the flow dimension. Twenty-eight COSY spectra, each containing resonances from both molecules, were obtained at different E_{dc} incrementing from 0 to 24.5 V $\cdot$ cm^{-1}. Two electrophoretic cosinusoidal oscillation

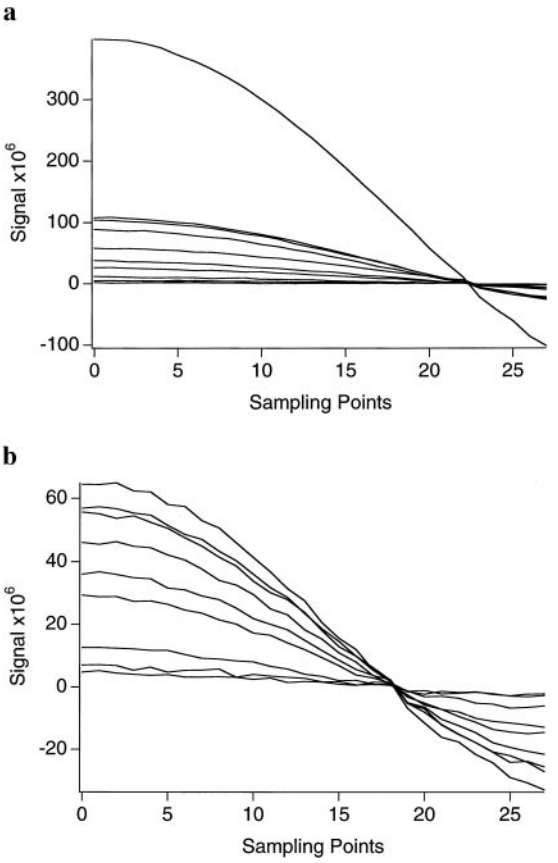

FIG. 2. Two electrophoretic oscillation frequencies from the 3D EP-COSY resonances of (a) L-aspartic acid (150 mM) and (b) 4,9-dioxa-1,12-dodecanediamine (148 mM) in D_2O. The solution conductivity (κ) was 5.82 mS $\cdot$ cm^{-1}. The data matrix was acquired using an eight-bundle CA-ENMR sample cell (250 μm). The electric current was increased from 0 to 0.56 mA in 28 steps (I_δ = 0.02 mA). The two gradient pulses had the same amplitude (g = 304.5 mT $\cdot$ m^{-1}) and duration (δ = 1 ms). Other parameters were: τ_d = 601.5 ms, Δ = 600.0 ms, NS = 16, T_R = 2s, t_1 = 5 to 30.264 ms with Δt_1 = 0.194 ms, and T = 25°C.

[3] Only terms from spin 1 are included; terms from the second spin can be obtained by exchanging subscripts 1 and 2.

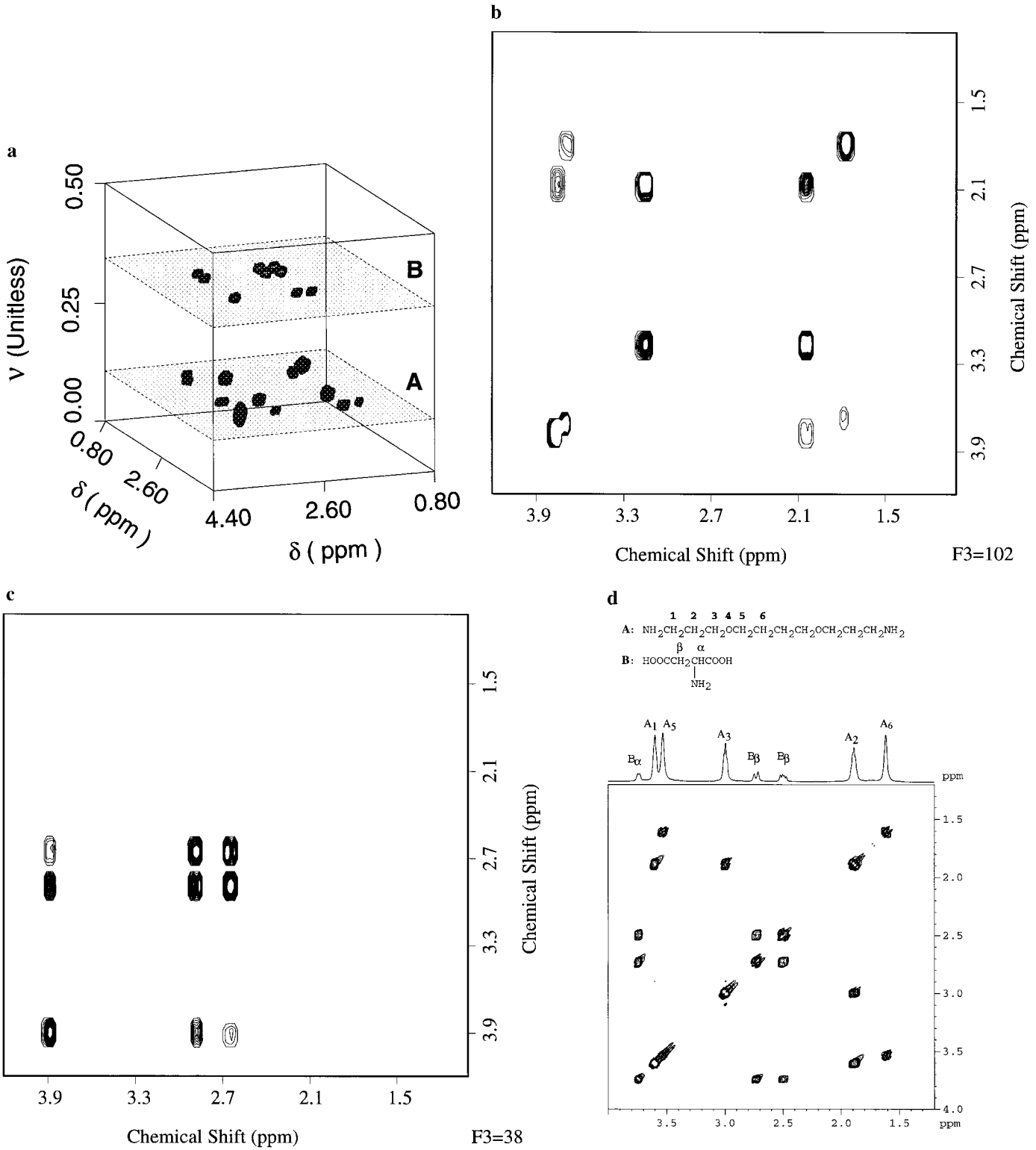

FIG. 3. (a) Spectroscopic separation of COSY planes of (A) 4,9-dioxa-1,12-dodecanediamine (148 mM) and (B) L-aspartic acid (150 mM) in a 3D EP-COSY matrix. Using the cosinusoidal oscillation frequencies that were scaled up 100 times, the truncated oscillation curves were extended from 28 to 256 data points before the third Fourier transformation. (Signals were folded in the flow dimension.) Superposition of the component 2D-COSY spectra (b and c) gave the same spectral resonance pattern of a double-quantum-filtered (DQF) COSY experiment (*2*, *37*) (d). The DQF-COSY data was acquired from a mixture solution of L-aspartic acid (100 mM) and 4,9-dioxa-1,12-dodecanediamine (100 mM) in D_2O on a Bruker DMX 500 spectrometer.

frequencies were observed that differentiated the COSY resonances of L-aspartic acid and 4,9-dioxa-1,12-dodecanediamine (Figs. 2a and 2b). In the 3D EP-COSY matrix (Fig. 3a), the chemical shifts and *J*-coupling constants can be measured for the two molecules in the separated COSY planes (Figs. 3b and 3c). Note that the electrophoretic mobilities of L-aspartic acid and 4,9-dioxa-1,12-dodecanediamine are opposite in sign but numerically close in magnitude, as measured with methylcellulose-coated CA-ENMR tubes (*36*). Satisfactory signal resolution was achieved in the flow dimension due to the electroosmotic effect. Obviously, the 3D EP-COSY can be used to sort out individual COSY components of mixed molecules for simultaneous structural assignments, a formidable task for protein mixtures using conventional COSY methods because of severe signal overlap (Fig. 3d). ENMR data of higher resolution have been obtained with our newly developed ENMR system. The results will be published elsewhere.

In conclusion, we have separated homonuclear COSY spectra of two molecules in their isotropic solution mixture using the demonstrated 3D EP-COSY method. This principle is applicable to other types of *n*D-ENMR for simultaneous structure determination of multiple proteins or protein conformations. Solutions of high ionic strength were examined using CA-ENMR sample cells, permitting ENMR experiments of proteins in high-salt biological buffer solutions (*35*). Thus, new investigations of protein interactions are possible using *n*D-ENMR to obtain detailed three-dimensional structure mapping of the interacting proteins. This structural information is mostly missing in the literature because of difficulties in distinguishing signals from different macromolecules coexisting in solution. Our new technology combines two powerful techniques—electrophoresis and NMR—to permit structural investigations, among others, of protein folding and aggregations in solution, as well as biological signaling processes that consist of essential mechanisms of living organisms.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Science Foundation (NSF MCB-9707550), the National Institutes of Health (NIH RR12774-01), the American Chemical Society Petroleum Research Funds (ACS-PRF No. 32308-G4), and the University of Connecticut Research Foundation (Grant 441833). We thank Dr. Douglas L. Rothman in the Magnetic Resonance Center of Yale University School of Medicine for use of the Bruker AM 500 NMR spectrometer and Drs. D. C. Shungu (Columbia University) and T. C. Ting (University of Connecticut) for discussions.

REFERENCES

1. K. Nagayama, A. Kumar, K. Wuthrich, and R. R. Ernst, Experimental techniques of two-dimensional correlated spectroscopy, *J. Magn. Reson.* **40,** 321 (1980).
2. M. Rance, O. W. Sorensen, G. Bodenhausen, G. Wagner, R. R. Ernst, and K. Wuthrich, Improved spectral resolution in COSY ^{1}H NMR spectra of proteins via double quantum filtering, *Biochem. Biophys. Res. Commun.* **117,** 479 (1983).
3. W. P. Aue, E. Bartholdi, and R. R. Ernst, Two-dimensional spectroscopy. Application to nuclear magnetic resonance, *J. Chem. Phys.* **64,** 2229 (1976).
4. J. Jeneer, "Ampere International Summer School," Unpublished lecture, 1971.
5. J. H. Noggle and R. E. Schirmer, "The Nuclear Overhauser Effect, Chemical Applications," Academic Press, New York, 1971.
6. I. Solomon, Relaxation processes in a system of two spins, *Phys. Rev.* **99,** 559 (1955).
7. G. M. Clore, L. E. Kay, A. Bax, and A. M. Gronenborn, Four-dimensional ^{13}C/^{13}C-edited nuclear Overhauser enhancement spectroscopy of a protein in solution: Application to interleukin 1β, *Biochemistry* **30,** 12 (1991).
8. A. Kumar, R. R. Ernst, and K. Wuthrich, A two-dimensional nuclear Overhauser enhancement (2D NOE) experiment for the elucidation of complete proton–proton cross relaxation networks in biological macromolecules, *Biochem. Biophys. Res. Commun.* **95,** 1 (1980).
9. A. W. Overhauser, Polarization of nuclei in metals, *Phys. Rev.* **92,** 411 (1953).
10. L. E. Kay, G. M. Clore, A. Bax, and A. M. Gronenborn, Four-dimensional heteronuclear triple-resonance NMR spectroscopy of interleukin-1β in solution, *Science* **262,** 411 (1990).
11. J. Cavanagh, W. J. Fairbrother, A. G. Palmer, III, and N. J. Skelton, "Protein NMR Spectroscopy: Principles and Practice," Academic Press, San Diego, 1996.
12. K. Wuthrich, "NMR of Proteins and Nucleic Acids," Wiley, New York, 1986.
13. R. R. Ernst, G. Bodenhausen, and A. Wokaun, "Principles of Nuclear Magnetic Resonance in One and Two Dimensions," Oxford Sci., Oxford, 1987.
14. Q. He, Y. Liu, and T. Nixon, High-field electrophoretic NMR of mixed proteins in solution, *J. Am. Chem. Soc.* **120,** 1341 (1998).
15. Q. He and C. S. Johnson, Jr., Two-dimensional electrophoretic NMR for the measurement of mobilities and diffusion in mixtures, *J. Magn. Reson.* **81,** 435 (1989).
16. C. S. Johnson, Jr. and Q. He, Electrophoretic NMR, *in* "Advances in Magnetic Resonance" (W. S. Warren, Ed.), p. 131, Academic Press, San Diego, 1989.
17. Q. He and C. S. Johnson, Jr., Stimulated echo electrophoretic NMR, *J. Magn. Reson.* **85,** 181 (1989).
18. Q. He, D. P. Hinton, and C. S. Johnson, Jr., Measurement of mobility distributions for vesicles by electrophoretic NMR, *J. Magn. Reson.* **91,** 654 (1991).
19. F. M. Coveney, J. H. Strange, A. L. Smith, and E. G. Smith, NMR studies of electrophoretic mobility in surfactant systems, *Colloid Surf.* **36,** 193 (1989).
20. Q. He, "Electrophoretic Nuclear Magnetic Resonance," Ph.D. thesis, University of North Carolina at Chapel Hill, Chapel Hill, NC, 1990.
21. S. R. Heil and M. Holz, Electrical transport in a disordered medium: NMR measurement of diffusivity and electrical mobility of ionic charge carriers, *J. Magn. Reson.* **135,** 17 (1998).
22. T. R. Saarinen and C. S. Johnson, Jr., High-resolution electrophoretic NMR, *J. Am. Chem. Soc.* **110,** 3332 (1988).
23. M. Holz, D. Seiferling, and X. Mao, Design of a new electrophoretic NMR probe and its application to $^{7}Li^{+}$ and $^{133}Cs^{+}$ mobility studies, *J. Magn. Reson. A* **105,** 90 (1993).
24. M. Holz, Electrophoretic NMR, *Chem. Soc. Rev.* **23,** 165 (1994).

25. S. R. Heil and M. Holz, A mobility filter for the detection and identification of charged species in complex liquid mixtures by ENMR phase difference spectroscopy, *Angew. Chem. Int. Ed. Engl.* **35,** 1717 (1996).
26. H. Holz and C. Muller, NMR measurement of internal magnetic field gradients caused by the presence of an electric current in electrolyte solutions, *J. Magn. Reson.* **40,** 595 (1980).
27. M. Holz and C. Muller, Direct measurement of single ionic drift velocities in electrolyte solutions. An NMR method, *Ber. Bunsenges. Phys. Chem.* **86,** 141 (1982).
28. M. Holz, O. Lucas, and C. Muller, NMR in the presence of an electric current. Simultaneous measurements of ionic mobilities, transfer numbers, and self-diffusion coefficients using an NMR pulsed-gradient experiment, *J. Magn. Reson.* **58,** 294 (1984).
29. M. Holz, C. Muller, and A. M. Wachter, Modification of the pulsed magnetic field gradient method for the determination of low velocities by NMR, *J. Magn. Reson.* **69,** 108 (1986).
30. C. S. Johnson, Jr., Electrophoretic NMR, *in* "Encyclopedia of NMR" (D. Grant, Ed.), p. 1886, Wiley, New York, 1995.
31. K. F. Morris and C. S. Johnson, Jr., Mobility-ordered 2D NMR spectroscopy for the analysis of ionic mixtures, *J. Magn. Reson. A* **100,** 67 (1993).
32. K. F. Morris and C. S. Johnson, Jr., Mobility-ordered two-dimensional nuclear magnetic resonance spectroscopy, *J. Am. Chem. Soc.* **114,** 776 (1992).
33. J. H. Strange, E. G. Smith, and F. M. Coveney, NMR apparatus and method for electrophoretic mobility, European Patent 0 361 942 A2 (1989).
34. H. Dai and T. A. Zawodzinski, Jr., Determination of lithium ion transference numbers by electrophoretic nuclear magnetic resonance, *J. Electrochem. Soc.* **143,** L107 (1996).
35. Q. He, Y. Liu, H. Sun, and E. Li, Capillary array electrophoretic NMR of proteins in biological buffer solutions, *J. Magn. Reson.* **141,** 355 (1999).
36. Q. He and Z. Wei, Convection compensated electrophoretic NMR for co-existing proteins in biological buffer solutions, submitted for publication.
37. U. Piantini, O. W. Sorensen, and R. R. Ernst, Multiple quantum filters for elucidating NMR coupling networks, *J. Am. Chem. Soc.* **104,** 6800 (1982).