High-Field Electrophoretic NMR of Protein Mixtures in Solution†

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Despite numerous successes of NMR in structural biology, the simultaneous structure determination of multiple proteins and the studies of protein interactions, especially weak interactions, remain a formidable task. Most NMR investigations of protein mixtures are limited to systems containing a single protein and small molecules with resolvable chemical shifts.[1−6] Coexisting protein conformations have been identified, for example, for well-characterized small protein, basic pancreatic trypsin inhibitor (BPTI),[7] and the three-dimensional NMR structures have been solved for the two conformations of the N-terminal zinc binding domain of HIV-1 integrase.[8] However, in most experimental conditions, severe signal overlap prevents full structural characterization of the multiple conformations of proteins. In this communication, we show experimental evidence that electrophoretic NMR[9−17] can resolve overlapping NMR resonances of mixed proteins based on different electrophoretic mobilities of the protein components without physical separation. When extended to multi-dimensions, the subspectrum of each protein component sorted in the dimension of electrophoretic flow will contain structural parameters (i.e., chemical shifts, *J*-coupling constants, or NOE correlations) for simultaneous sequential and stereospecific assignments (Figure 1).

The electrophoretic NMR experiments of proteins were carried out with a modified stimulated echo sequence (Figure 2) on a Bruker AMX 500 spectrometer, with a commercial NMR probe equipped with a single axial *z*-gradient. U-shaped electrophoretic cells (OD = 2.5 mm, length = 10 cm, and A = 3.758 mm^2) were coated with methylcellulose to reduce the electroosmosis effect.[21] Cylindrical tubes are also available for this experiment where the electrophoretic motion is detected by the phase rather than the amplitude modulation of the NMR signals.[12,18−20] Electric field pulses, generated by an electric field/gradient driver, were applied to the two platinum electrodes inserted into the protein solution, introducing a cosinusoidal amplitude modulation of the stimulated echo,[11,14]

$$M(E_{dc}) = \frac{M(0)}{2}\exp\left[-DK^2\left(\tau_D - \frac{\delta_1}{3}\right) - \frac{2\tau}{T_2} - \frac{\Delta}{T_1}\right]\cos[(KE_{dc}\Delta)\mu] \quad (1)$$

The modulation frequency depends on the gradient strength ($g_1\delta_1$), the amplitude (E_{dc}) and the duration (Δ) of the electric field, and the electrophoretic mobility (μ) of the protein. The parameter $K = \gamma g_1\delta_1$ characterizes the tightness of the magnetization helix, and τ_D designates self-diffusion time. Electric current (I_e), solution conductivity (κ), and the cross area (A) of the electrophoretic cell determine the strength of the electric field, $E_{dc} = I_e/(\kappa A)$.

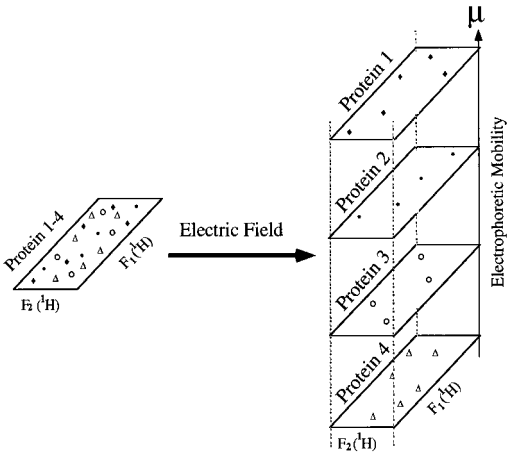
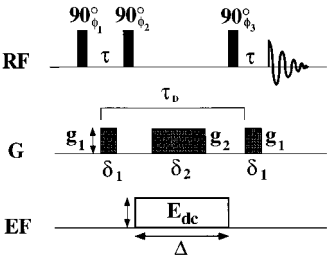


Figure 1. Four two-dimensional NMR spectra of different protein components in a mixture (represented by ♦, ●, ○, and △, respectively) can be sorted by electrophoretic mobilities (μ).



Phase cycling procedures:

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f1: +x +x +x +x +y +y +y +y -x -x -x -x -y -y -y -y
f2: -x -x -x -x -y -y -y -y +x +x +x +x +y +y +y +y
f3: +x +y -x -y +y -x -y +x -x -y +x +y -y +x +y -x
ACQ: +x +y -x -y +y -x -y +x -x -y +x +y -y +x +y -x
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Figure 2. The stimulated echo electrophoretic NMR pulse sequence with phase cycling procedures.

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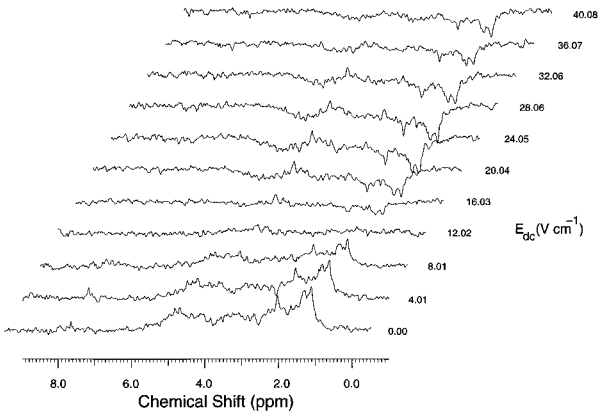

Figure 3. Electrophoretic interferograms of 0.5 mM ubiquitin and 10 mM ethylenediamine in D_2O (25 °C). Parameters: pH = 10.4, $\kappa = 0.332$ mS cm^{-1}, $\Delta = 0.6085$ s, $\tau = 1.535$ ms, $\tau_D = 0.6102$ s, NS = 128, T_R = 3 s, $g_1 = 426.3$ mT·m^{-1}, $g_2 = 487.2$ mT·m^{-1}, $\delta_1 = 1$ ms, $\delta_2 = 8$ ms, E_{dc} = 0 to 40.08 V cm^{-1}, and $\mu = 7.7 \times 10^{-5}$ cm^2 V^{-1} s^{-1}.

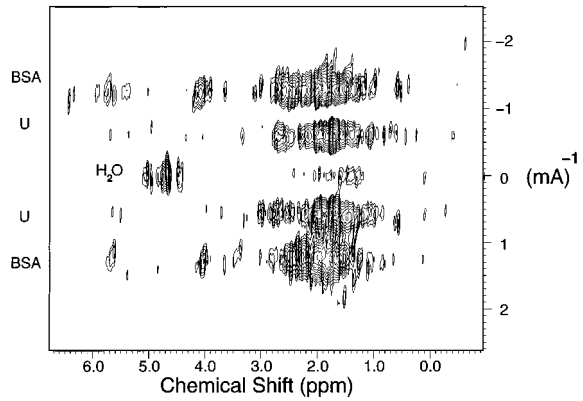

Figure 4. Signal separation of proteins in a D_2O solution of 0.15 mM BSA and 1 mM ubiquitin with 26 mM ethylenediamine. Parameters: pH = 10.37, $\kappa = 0.692$ mS cm^{-1}, $\Delta = 0.4085$ s, $\tau = 2.535$ ms, $\tau_D = 0.4111$ s, NS = 128, $T_R = 1.5$ s, $g_1 = g_2 = 487.2$ mT·m^{-1}, $\delta_1 = 2$ ms, $\delta_2 = 8$ ms, and E_{dc} = 0 to 38.45 V cm^{-1} with 21 increments.

Molecular diffusion (D) and spin T_1 and T_2 relaxation processes attribute to an exponential signal decay. A gradient with amplitude g_2 and duration δ_2 crushes the remaining xy-magnetization after the second rf pulse and eliminates secondary and primary echoes ($g_2\delta_2 \geq 4g_1\delta_1$).

Depending on isoelectric points (pI) and solution pH, proteins may have different electrophoretic mobilities.[22] The net charges of proteins, therefore, can be tuned with ethylenediamine (pK_{a1} = 10.712 and pK_{a2} = 7.564). For instance, the 8.6 kDa ubiquitin (1.09 mM) is close to neutral in D_2O (pH = 6.52); with 10 mM ethylenediamine treatment (pH = 10.4), however, negatively charged ubiquitin molecules move in the electric field, producing a cosinusoidal oscillating electrophoretic interferogram (Figure 3). Several other proteins were studied to determine the size and concentration limits of the technique. Lysozyme (14.3 KDa, pI = 11), which carries positive charges in D_2O (3.0 mM, pH = 8.37), migrates in the electric field; similar migration was observed for the negatively charged 1.0 mM pepsin (34.4 kDa, pI = 2.86 at 4 °C) in D_2O (pH = 4.19). Bovine serum albumin (BSA, 66 kDa, and pI = 5.85) moves slowly in D_2O in the electric field ($\mu = 2.6 \times 10^{-5}$ cm^2 V^{-1} s^{-1});[21] with ethylenediamine treatment (pH = 9.72), the negatively charged BSA molecules migrate more than six times faster ($\mu = 1.6 \times 10^{-4}$ cm^2 V^{-1} s^{-1}). Stimulated echo electrophoretic NMR may detect proteins of relatively large molecular weight—e.g., we have observed the electrophoretic motion of a 20 μM solution of 480 kDa urease with 1 mM ethylenediamine in D_2O.

Similarly in a solution of mixed proteins, each component produces its characteristic cosinusoidal oscillation as a function of electric field, gradient strength, and electrophoretic mobility. The sum of contributions from all components produces the observed electrophoretic interferogram,

$$S(E_{dc},\omega_2) = S_0 \int_{-\infty}^{\infty} g(\nu) \cos(K\mu E_{dc}\Delta) \exp\left[-DK^2\left(\tau_D - \frac{\delta_1}{3}\right) - \frac{2\tau}{T_2} - \frac{\Delta}{T_1}\right] d\nu \quad (2)$$

where the electrophoretic flow velocity $\nu = \mu E_{dc}$.[11,15] The velocity distribution function, $g(\nu)$, is either discrete when several proteins coexist in solution or continuous as demonstrated for polydispersed phospholipid vesicles.[15,23] After inverse Fourier transformation of $S(E_{dc},\omega_2)$ vs E_{dc}, $g(\nu)$ appears as paired resonances at frequencies $\pm(K\mu_i\Delta)/2\pi$ in the dimension of electrophoretic flow. Each pair originates from one protein component with signal intensity weighted by diffusion and relaxation decay. The spectroscopic separation of mixed proteins was demonstrated with a mixture of 0.15 mM BSA and 1 mM ubiquitin in D_2O with 26 mM ethylenediamine. A contour plot of the two-dimensional stimulated echo electrophoretic NMR spectrum is displayed after double Fourier transformation with respect to acquisition time, t_2, and electric current, I_e (Figure 4). Linear prediction programs are also available to analyze data in the electrophoretic flow dimension.[19,20] The resonances of the two proteins overlapping in the chemical shift dimension were separated by electrophoretic flow. In the flow dimension, the protein component resonates at $\nu_i = \pm[(K\Delta\mu)/(2\pi\kappa A)]$, proportional to the electrophoretic mobilities of the protein. The resolution in this dimension, limited by the data truncation effect with the Fourier transformation approach, increases with stronger gradients and longer electric field pulses. The electrophoretic mobilities of BSA and ubiquitin are 2.0×10^{-4} and 9.6×10^{-5} cm^2 V^{-1} s^{-1}, respectively, similar to those measured in pure protein solutions.

In conclusion, clear signal separation of two stable proteins in solution demonstrates the feasibility of simultaneous structure determination of multiple protein components by electrophoretic NMR. The methods may be useful, for example, to characterize transient protein folding intermediates without disturbing biochemical equilibrium, or to study regulatory protein binding to DNA molecules as a mechanism of genetic control. Work is in progress to characterize chemical exchange processes in protein−ligand, protein−protein interactions. Recently, we have successfully constructed cylindrical sample tubes for commercial NMR probes to improve signal sensitivity and to distinguish resonances from cations and anions (to be published elsewhere). Signal loss due to molecular diffusion minimizes in experiments of large proteins—which explains the sensitivity in urease detection (480 kD) at micromolar concentrations. As compared to diffusion ordered spectroscopy that offers similar potential in analyzing multiple protein components,[24,25] electrophoretic NMR provides a tunable parameter—the net charges of peptides, proteins and nucleic acids—to control the spectroscopic separation of the mixed components. With the simple setup using commercial gradient probes, the technique is now available to all NMR laboratories.

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