Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1053 (2004) 253–262
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Capillary isoelectric focusing and affinity capillary electrophoresis approaches for the determination of binding constants for antibodies to the prion protein

Guodong Li, Xiaojuan Zhou, Yonghui Wang, Ahmed El-Shafey[1], Norman H. L. Chiu, Ira S. Krull*

Department of Chemistry and Chemical Biology, Northeastern University, 102 Hurtig Building, 360 Huntington Avenue, Boston, MA 02115, USA

Abstract

For the development of specific immunological assays, the binding of a specific antibody (Ab) to the target antigen (Ag) has to be relatively strong. In this study, we have utilized affinity capillary electrophoresis (ACE), a form of capillary zone electrophoresis (CZE) to determine the binding constant (K_b) of specific Abs against bovine serum albumin (BSA) and the healthy prion protein (PrPc), in buffer solutions at fixed pHs, approximating in vivo conditions. We have also utilized capillary isoelectric focusing (cIEF) to determine the complexity and recognition of the various isoforms of PrPc Abs towards their Ag, PrPc. Only ACE and CZE have been used to derive K_b values. The selected Abs for the prion protein can recognize both healthy and diseased states of the protein and are commercially available. The K_b values of PrPc Abs appear to be as strong as the anti-BSA (Ab to BSA) and other reported K_b values for proteins of similar size to PrPc. This appears to be one of the few reports on K_b values for any PrPc Abs, and their applications for in vitro immunoassays (e.g., enzyme-linked immunosorbent assays (ELISAs)). Such assays are being used to detect the infectious agent, PrPres, in brain and related matter/tissues.

Keywords: cIEF; ACE; CE; Prion protein; PrPc; Binding constants; Antibodies; ELISAs

1. Introduction

Within the past few decades, there has arisen a significant interest in the development of new and sensitive assays for the presence of infectious prion proteins, termed PrPres (enzyme resistant form of the healthy, PrPc (cellular or native form)) [1–10]. PrPres signifies the infectious or diseased agent, that species of the prion protein responsible for all tissue spongiform encephalopathies (TSEs). It should be indicated that PrP is an ambiguous term, it does not really define if one is working with PrPc or PrPres forms. Hence, throughout this paper, we have used PrPc to describe the actual protein being studied, the native or healthy species. None of this work was done using the infectious species, PrPres.

Numerous analytical methods, mainly enzyme-linked immunosorbent assays (ELISAs), have been developed. Some of these assays are commercially available and applied daily around the world [11–17]. Virtually all of these are post-mortem assays, requiring the death of the animal or person and removal of brain, spinal column, pituitary gland, and/or related tissues for analysis of PrPres. There are some newer assays, recently described, that appear successful, ante-mortem. The term ante-mortem relates to tests performed on a subject's biofluids (urine, blood, tears, etc.) while the animal/human is still alive, hence before-death. However, none of these are applied on a routine basis to large numbers of animals [18–42]. Perhaps only the Gabizon and Schmerr approaches will be applicable ante-mortem, with any degree of specificity for PrPres, in biofluids [18,19,23–29]. Those approaches recently described by Saborio and co-workers

* Corresponding author. Tel.: +1 617 373 2862; fax: +1 617 373 8795.
E-mail address: irask@aol.com (I.S. Krull).
[1] Present address: Pacific Northwest Laboratories, Biological Sciences, P.O. Box 999, 902 Battelle Boulevard, Richland, WA 99352, USA.

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.08.138

[20,21] and Prusiner's group [32], as well as the method of Cashman's group [36], may utilize more PrPres specific Abs, but it is not yet clear they will be applicable ante-mortem [16,17].

In all of the above approaches, the preferred mode of detection is based on the ELISA format, using various Abs for PrPres. There are numerous other immunological approaches on the market today [15–17], but none of these appear usable, ante-mortem in simple biofluids. They are used post-mortem, on the brain matter from dead animals or people, as the preferred source of analyte. There are, at least, two crucial features of Abs that relate to their utility in any ELISA, one being their antigen (Ag) specificity and the other, their binding properties for the PrPres protein. Ideally, one wants an Ab that will only recognize the PrPres species, and totally ignore the healthy, PrPc form. It would appear that only the Prusiner and Cashman groups have such potentially, very useful Abs. However, those Abs only improve specificity and reduce sample preparation requirements (obviating the need to use proteinase K to eliminate PrPc from an infected sample, leaving only PrPres to be detected). Such highly specific Abs do not automatically lower limits of detection (LODs), they just reduce the complexity of sample preparation. Improved LODs can be realized by changing the nature of the ELISA format (e.g., immuno-polymerase chain reaction or i-PCR) [43] or by improving the K_b values for existing Abs. Using those Abs that already have strong K_b values could also lower LODs. It appears to be difficult to improve K_b values for existing Abs, and thus efforts are needed to generate Abs with native, higher K_b values, if possible.

Though there are numerous publications that deal with the determination of K_b values, very few of these relate to prion Abs [44]. And, even fewer of these deal with Ab–Ag K_b values, similar to the work being presented here [45]. We have been interested in determining the K_b values for existing, commercial Abs, so that future immunoassays would utilize such Abs, perhaps to the exclusion of others. It is possible that one of the Abs being described herein has already been used to develop a commercialized assay for PrPres. It would appear that there is very little in the literature describing absolute K_b values for any prion Abs, other than for Prusiner's work [32–35]. We have recently described, along with others, the application of ACE for the determination of K_b values for small drugs and large proteins (e.g., BSA) [48–50]. The application of ACE methods for Ab–protein binding has been described much less than for Ab–small molecules. In these approaches, one can introduce either the Ab or its Ag into the buffer, at varying concentrations, and inject the same concentration of the corresponding partner (Ag or Ab).

There are numerous approaches for the determination of K_b values, such as frontal chromatography, frontal CE, ultracentrifugation, surface plasmon resonance spectroscopy (Biacore), affinity stoichiometry, and others [46–50,57–58]. There are several ways by which ACE can be configured to provide binding or association constants (K_b) for proteins and their Abs. ACE has also, at times, been termed immunoaffinity CE (IACE), wherein an Ab and its Ag are involved [46–50]. Most ACE methods rely on changes in the apparent mobility of either the Ab or the Ag, when one or the other is added to the CE buffer in varying concentrations. As the partner being injected (at constant concentration) sees or recognizes increasing concentrations of its binding partner in the buffer, the original injected partner's mobility will change. And, it can change in either direction, towards longer or shorter migration times [versus an internal standard marker, often dimethyl sulfoxide (DMSO) or dimethyl formamide (DMF)], depending on how the charge/mass ratio of the Ab–Ag complex differs from the charge/mass of the injected species alone (Ab or Ag). The K_b determined should be independent of which partner is injected and which is contained in the run buffer. The theory of ACE for determining K_b values has been described in numerous publications, and rather than repeat what is already in the literature, we will summarize the salient points of the technique and theory, as below (Sections 2 and 3). It is also possible to first mix Ab and Ag outside of the capillary, as a function of time, until a true equilibrium is reached, and then inject that mixture into the ACE system. Changes in the peak heights/areas for either Ab or Ag partner or the complex(es) formed (Ab–Ag) can then be used to derive K_b values via the usual Scatchard plots [47–50]. This is really a capillary zone electrophoresis (CZE) method and not a form of ACE, and the parameter for quantitation is peak height or area, but not changes in migration times.

We describe here the application of an ACE method to determine K_b values for several commercial Abs for PrPc protein and BSA, in the hope of discovering Abs that may have stronger K_b values than otherwise. Stronger K_b values should then lead to improved (stronger) binding (large on and small off rates) and lower LODs, in the ELISA format. The combination of improved specificity for only the PrPres species and improved K_b values, in newer forms of ELISAs (e.g., i-PCR), should ultimately, lead to successful ante-mortem assays in biofluids. At least, that is the ultimate goal for many trying to develop any ante-mortem prion assays today.

In comparing cIEF with ACE, these are two quite different CE approaches for protein analysis and/or characterization. ACE is really used only to determine K_b values, it is not used to demonstrate purity or identity, but rather it is a kinetic method for measuring equilibrium constants. cIEF, on the other hand, is a true CE protein identification mode or technique, it does not provide K_b values, but rather it provides p*I* information and a demonstration of the complexity of a protein sample [number and isoelectric points (p*I* values) of all isoforms separable by cIEF]. It can also be used to demonstrate protein purity, and, as here, affinity or recognition of an Ab towards its Ag. It can also be used to show shifts in migration times and mobilities as an Ab complexes with one or more of the possible Ags present in the sample. cIEF can also be used to show the recognition of an Ab for its Ag, and even to show the formation of the various Ab–Ag complexes possible (1:1 and 2:1, etc.). Both cIEF and ACE

have found widespread applicability in protein analysis and characterization.

2. Experimental

2.1. Chemicals and reagents

The BSA and goat BSA Ab (polyclonal) were from Sigma (St. Louis, MO, USA). The recombinant prion protein was obtained from Calbiochem (EMD Biosciences, La Jolla, CA, USA). The Rubenstein Ab (01-16/6BIO) was obtained from Dr. Richard Rubenstein and Dr. Richard Kascsak at the NYS Institute of Basic Biomedical Research (Staten Island, NY, USA). The VMRD Ab was obtained from VMRD (Pullman, WA, USA), and the PolyLC prion Ab was from PolyLC Co. (Columbia, MD, USA), through the kind assistance of Dr. A. Alpert. The p*I* markers (proteins) and the mobilizer buffer were obtained from Bio-Rad (Hercules, CA, USA). The Z1-CH3 reagent was obtained from Waters (Milford, MA, USA). The ImmunoPure Plus Immobilized Protein G packing and the ImmunoPure binding/elution buffer systems were obtained from Pierce (Rockford, IL, USA). The concentrating and desalting steps were performed with a Millipore Microcon centrifugal filter and microcentrifuge (Millipore, Bedford, MA, USA).

2.2. Apparatus

All of the CE experiments were carried out with a Waters Quanta 4000 instrument equipped with a UV detector. The Beckman eCAP cIEF 3–10 kit (Beckman–Coulter, Fullerton, CA, USA) was used for the cIEF experiments. The bare fused silica capillary was obtained from Polymicro Technologies (Phoenix, AZ, USA).

2.3. Purification of the antibodies

All of the Abs being studied were purified after receipt from the supplier, using standard, immunoaffinity methods and filtration procedures, as provided by the vendors (Pierce) or available literature [59–65]. These involved the use of commercial Protein G immunoaffinity columns, as well as commercial binding and elution buffers (Pierce). Initial filtration or desalting of all Abs, as received or after immunoaffinity purification, was performed using centrifugal filters of regenerated cellulose [molecular mass cut-off (MWCO = 100,000)], designed for Ab isolation (Millipore). Specific protocols were provided by the vendors [62].

2.4. Buffer and sample preparation

For the cIEF work, a 4 μl eCAP cIEF 3–10 Ampholyte was put into 200 μl of the cIEF gel along with the p*I* markers, and the components were fully homogenized. The anolyte was 91 mM phosphoric acid in cIEF gel and the catholyte was 20 mM sodium hydroxide in water. A neutral, coated capillary from Beckman–Coulter, cut to 32 cm (24.5 cm to detector window) × 50 μm i.d., was used as the separation column. The detection wavelength was 280 nm. The analyte was focused at 15 kV for 6 min, and then mobilized at 22 kV with a cathodic mobilizer (Bio-Rad).

The ACE experiments were performed with a bare, fused silica capillary, 50 cm (43 cm to detector) × 50 μm i.d. The running buffer consisted of 50 mM of sodium phosphate and 1.0 M of Z1-CH3 zwitterion, that was used for eliminating capillary wall adsorption of proteins. Various concentrations of Ab (receptor) or protein (ligand, L) were added into the running buffer when desired. The monitored protein/Ab injection solution contained 0.001% DMF, which was used as a neutral EOF marker (DMSO and mesityl oxide are other possible markers). The samples were hydrostatically injected for 10 s. Ten kilowatts of high voltage was applied for the electrophoresis, and detection was set at 214 nm UV.

2.5. Determination of K_b values by ACE

Basically, the change in mobilities of the receptor (A) (species injected into the running buffer) is a function of the ligand (B) concentration in the sample. With reference to the internal standard, the changes in the electrophoretic mobility ($d\mu_A$) of the receptor are measured, in the absence and then presence of the ligand (B) at various concentrations. Depending on the strength of K_b, the $d\mu_A$ can be large or small. Hence, there is a direct relationship between $d\mu_A$ and K_b, which is the basic premise of using ACE to measure K_b values. In order to remove the effect of changes in electroosmotic flow (EOF), resulting from changes in the receptor concentration added to the running buffer, the relative mobility change should be used to replace simple mobility changes. The relative mobility change is slightly different from the absolute mobility change, in that it utilizes the EOF in the presence and absence of various concentrations of the binding species added to the running buffer. The relative mobility change, $D\mu_A$, should thus be used to replace $d\mu_A$. $D\mu_A$ is defined as:

$$D\mu_A = \frac{\mu_{AB}}{\mu_{eof,AB}} - \frac{\mu_A}{\mu_{eof,A}} \quad (\mu = \text{mobility})$$

where μ_{AB} and μ_A are the mobilities of the receptor–ligand complex and receptor alone, respectively, and $\mu_{eof,A}$ and $\mu_{eof,B}$ are the EOFs in the absence and presence of various concentrations of that species added to the running buffer, respectively.

According to the Scatchard theory:

$$\frac{D\mu_A}{[B]} = K_b\, D\mu_{A_{max}} - K_b\, D\mu_A \quad (1)$$

where [B] is the concentration of the ligand (B), and $D\mu_{A_{max}}$ is the relative mobility change of receptor when it is saturated with the ligand.

3. Results and discussion

3.1. cIEF of antibodies and their complexes

We have now used cIEF in order to discern the heterogeneity of isoforms present in various prion Abs [51–56]. This use of cIEF has been reported before for other Abs, though apparently never for any prion related species. A typical electropherogram for the BSA Ab is given in Fig. 1, with a final concentration in the cIEF buffer of 0.5 mg/mL. Fig. 1A is the electropherogram of the intact BSA Ab, polyclonal, which shows the presence of numerous isoforms, which could not be better separated under any cIEF conditions we evaluated. In Fig. 1B, we mixed BSA and its Ab, using 30 μl of an Ab concentration of 0.5 mg/mL and 10 μl of 1 mg/mL BSA solution. The resulting mixture was then mixed with 60 μl of the Beckman ampholyte kit (Section 2). The molar ratio of Ab:Ag in Fig. 1B was roughly 2:3, with an excess of the Ag, BSA. The BSA peak appears to the right of the complex peaks (not the spike peak), both in Fig. 1B and C. The multiple peaks that corresponded to the immunocomplexes in Fig. 1B and C were presumably derived from the isoforms of the Ab combining with one or two molecules of the BSA Ag. Other complexes are also possible. There did not appear to be any remaining isoforms of the Ab in Fig. 1B or C, suggesting

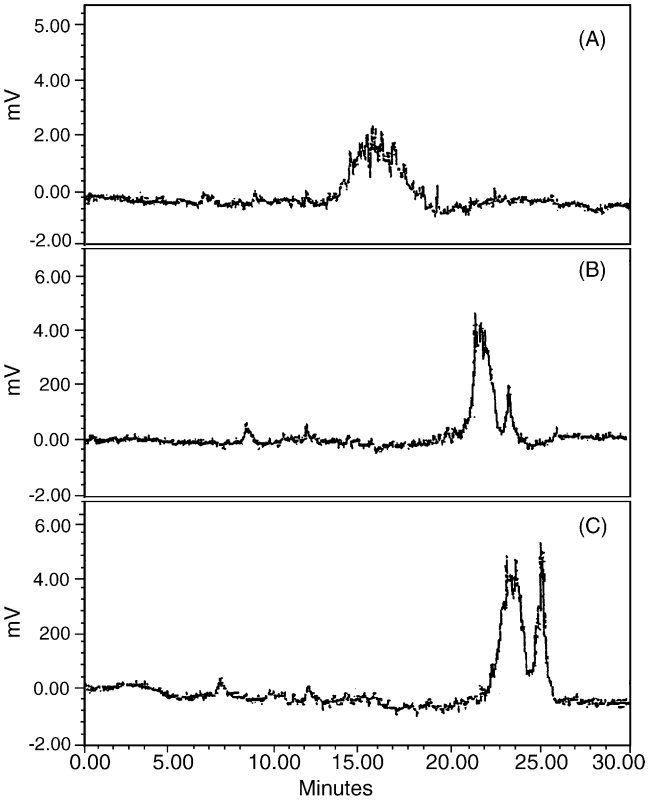

Fig. 1. cIEF electropherograms of goat anti-BSA Ab and its BSA complexes. (A) goat anti-BSA Ab alone; (B) Ab and BSA mixture, molar ratio of 2:3; (C) Ab and BSA mixture, molar ratio of 1:3. Specific conditions indicated in Section 2.

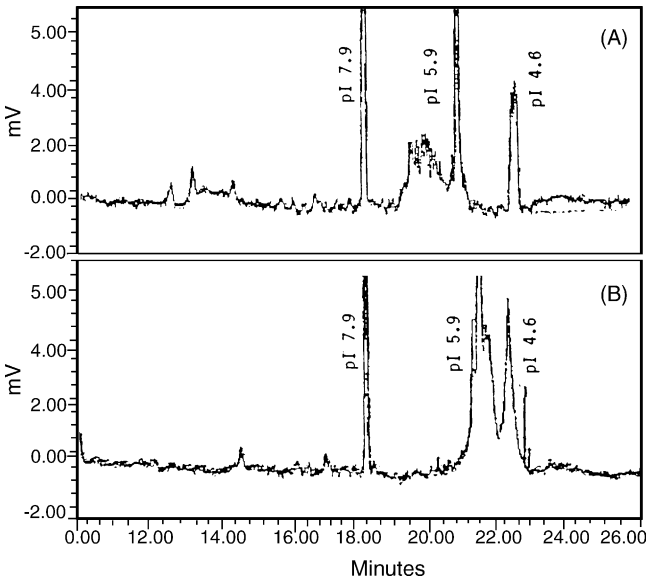

Fig. 2. cIEF of goat anti-BSA Ab and its BSA complexes, together with p*I* markers. (A) Ab and markers of p*I* values, 7.9, 5.9, and 4.6; (B) Ab, BSA, and p*I* markers, as in (A). The p*I* range of the Ab was 5.85–7.00, and the p*I* range of the complexes was 5.24–5.75. Specific conditions indicated in Section 2.

that all were active and bound to the Ag. In Fig. 1C, 30 μl of a 0.5 mg/mL Ab and 20 μl of a 1 mg/mL BSA were mixed with 60 μl of the ampholyte gel. In this case, the molar ratio was about 1:3 for Ab:BSA species. We can observe that the excess, uncomplexed BSA peak (far right peak) has become larger than in Fig. 1B. The pattern of peaks for the complexes has changed somewhat, perhaps because more 2:1 species are present in Fig. 1C. It would appear that the immunocomplex peaks having lower p*I* values (more acidic) have become dominant herein. This is reasonable, since BSA is an acidic protein and its Ab is more basic. As more and more BSA became bound to its Ab, the complexes should have smaller p*I* values and the immunocomplexes would become more acidic.

Fig. 2A is the electropherogram of the BSA Ab with p*I* markers of 7.9, 5.9, and 4.6, and Fig. 2B is the electropherogram of free BSA and its immunocomplexes with the same p*I* markers. The p*I* values of the BSA Ab and its immunocomplexes could be determined from these electropherograms, by using a standard calibration plot for the three p*I* markers [51]. For the BSA Ab, its p*I* values were from 5.9 to 7.0, and for the BSA immunocomplexes, the p*I* values were from 5.2 to 5.8. When BSA alone was injected with these same p*I* markers, its p*I* was found to be about 4.6. This further confirmed the above assertion that the complexes should have lower p*I* values and be more acidic than the free Ab isoforms (Fig. 1A).

However, the main thrust of this paper is to describe the cIEF and ACE of commercially available prion Abs. Thus, Fig. 3A illustrates the cIEF electropherogram of a polyclonal, PolyLC prion Ab. There are, of course, numerous Abs to the prion protein, many of which are not commercially available.

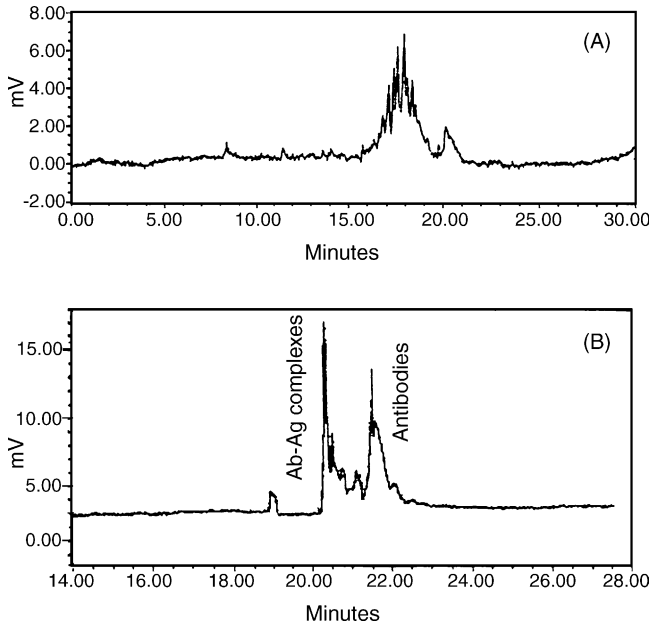

Fig. 3. (A) cIEF electropherogram of Poly LC anti-prion protein, specific conditions as indicated elsewhere (Section 2); (B) cIEF electropherogram of a mixture of Poly LC anti-prion Ab and the prion protein. The first set of peaks, those having shorter migration times, corresponded to the complexes. Specific conditions indicated in Section 2.

The cIEF pattern for these isoforms is very similar to that for the BSA Ab, but the p*I* values here are somewhat lower. Using again three internal standard proteins, as in Fig. 2B, we could determine the p*I* range in Fig. 3A to be from 5.2 to 6.9. When this PolyLC Ab was mixed with PrPc, a mixture of immunocomplexes was formed, Fig. 3B. The first set of peaks, those having shorter migration times, were due to the newly formed Ab–prion complexes. The second set of peaks, those with longer migration times, arose from the excess prion Ab present. The p*I* values of the complexes shifted to a more basic region (shorter migration times), since the prion protein was a basic protein, having a higher p*I* value than its Ab. The p*I* values for this commercial, recombinant prion protein were determined, Fig. 4, but now using cIEF with a different set of p*I* marker proteins. There were several major peaks for this recombinant prion protein (Calbiochem), with the two largest peaks (i.e., 2 and 3) having p*I* values of 8.4 and 8.6. We believe that the recombinantly formed PrPc has more than one isoform, and that these may well be post-translational modifications arising from the cells used to express the protein. Presumably, all of these isoforms represent Ab active species, though we have not conclusively demonstrated this. That could also be done by cIEF methods. The MALDI (matrix-assisted laser desorption/ionization)-TOF-MS (time-of-flight mass spectrometry) mass spectrum for this sample showed only a single peak, at the correct molecular mass (M_r) of the known prion protein structure (data not shown).

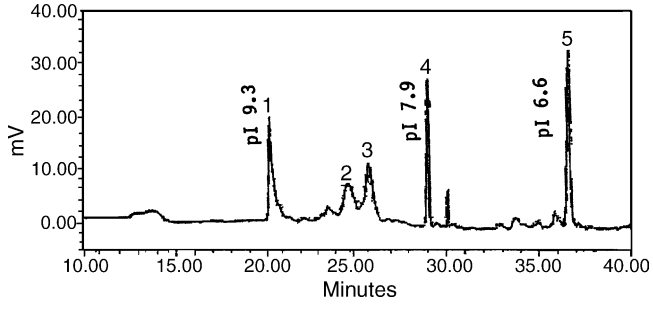

Fig. 4. cIEF electropherogram of Calbiochem prion protein, with p*I* markers of 9.3, 7.9, 6.6; peaks 2–3 were from the prion protein. p*I* values for peaks 2 and 3 were 8.6 and 8.4, respectively. Specific conditions indicated in Section 2.

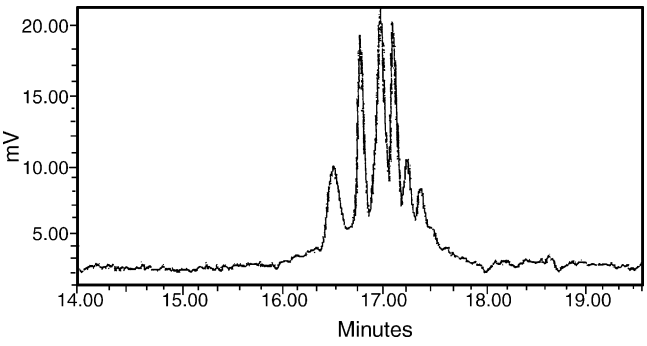

Fig. 5. cIEF of the Rubenstein (NYS-IBR) 01-16/6BIO monoclonal anti-prion Ab, with p*I* values in the range of 6.3–6.6. Specific conditions indicated in Section 2.

The cIEF isoform pattern for a monoclonal Ab should be much simpler than for polyclonal species [51]. Thus, Fig. 5 illustrates the electropherogram of a noncommercial, monoclonal prion Ab, termed here 6BIO by the Rubenstein/Kascsak group at NYS-IBR. Six isoforms were well separated under these cIEF conditions, with a p*I* range from 6.3 to 6.6, much narrower than for the polyclonal Abs above (BSA and prion). Fig. 6 is the cIEF electropherogram of a VMRD prion Ab, also a monoclonal Ab. Two major peaks (numbers 2 and 3) were observed, with p*I*s determined as 7.19 and 7.13, again using three internal standard, protein markers, as indicated in Fig. 6. This prion Ab was slightly more basic

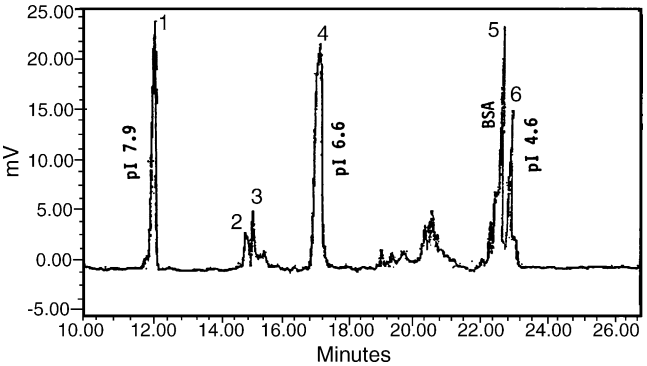

Fig. 6. cIEF electropherogram of the VMRD anti-prion Ab, peaks 1, 4, and 6 were markers of p*I* values 7.9, 6.6, and 4.6, respectively; peak 5 was BSA, and peaks 2–3 were the Ab peaks. Specific conditions indicated in Section 2.

than the other two prion Abs studied. The presence of the Ab peaks in this sample was determined by injecting a blank mixture of just the three internal standard, protein markers in the absence of the Ab. The only difference here was the lack of appearance of peaks 2 and 3 in Fig. 6. The other peaks at migration times 18.0–21.0 min were presumably due to impurities in the internal standard proteins.

3.2. ACE Determination of K_b values for antibodies (Abs) and protein antigens (Ags)

We are measuring K_b at equilibrium, using ACE or other analytical methods, which is really equal to the equilibrium association constant, K_a. There is general consensus that K_b is equal to K_a, at equilibrium, which is not the same thing as the association rate constant, k_a. In general, the larger the K_a, the stronger the K_b, and the smaller the K_d (equilibrium dissociation constant), the stronger K_b. The larger K_b corresponds to a stronger binding effect. Dissociation constants, K_d, are the reciprocal of association constants, and the smaller number is the stronger binding effect. From Eq. (1), the plot of $D\mu_A/[B]$ versus $D\mu_A$ should be linear, and K_b can be obtained from its slope. Numerous review articles have appeared in the past decade to describe ACE for the determination of K_b, and the excellent, pioneering work of Whitesides, Chu, Walsh, Karger, and others, should be studied [47–50].

Our purpose in these studies has been to estimate the K_b values for various Abs (commercial and private) towards the prion protein, PrPc, of a commercial source. There is, of course, a fundamental interest in knowing the K_b values for prion Abs, since the stronger the K_b, then presumably the better ELISA or other immunoassays will perform and the lower the limit of detection (LOD). It is useful to compare K_b values for the prion Abs, though few of these have already appeared in the literature [32–35]. Ideally, in the future, all immunoassays for prion proteins will use the very best Abs, those having the strongest K_b values. That is the main reason for determining K_b values for these or other Abs.

Prior to measuring the K_b for a prion Ab, we first validated the basic ACE method by using a model system, BSA and anti-BSA Ab. In this first study, goat anti-BSA Ab was added in varying concentrations into the running buffer, and a fixed concentration of BSA was injected into the capillary. The changes in the migration times of BSA was measured. In order to monitor the EOF in each run, DMF was coinjected with BSA, as a neutral marker. A high concentration of a zwitterionic compound, Z1-CH3 was added to the running buffer, in order to eliminate interactions between the capillary walls and the proteins present (injected or in the buffer). ACE can be performed at various pH values, depending on how the injected protein migrates. The ideal pH, where the injected protein is well resolved from the neutral marker peak and elutes within a reasonable timeframe, must be experimentally derived. In the case of BSA, the ideal pH was about 6.8, where BSA was negatively charged and the Ab was almost neutral. Relative migration time or mobility is again

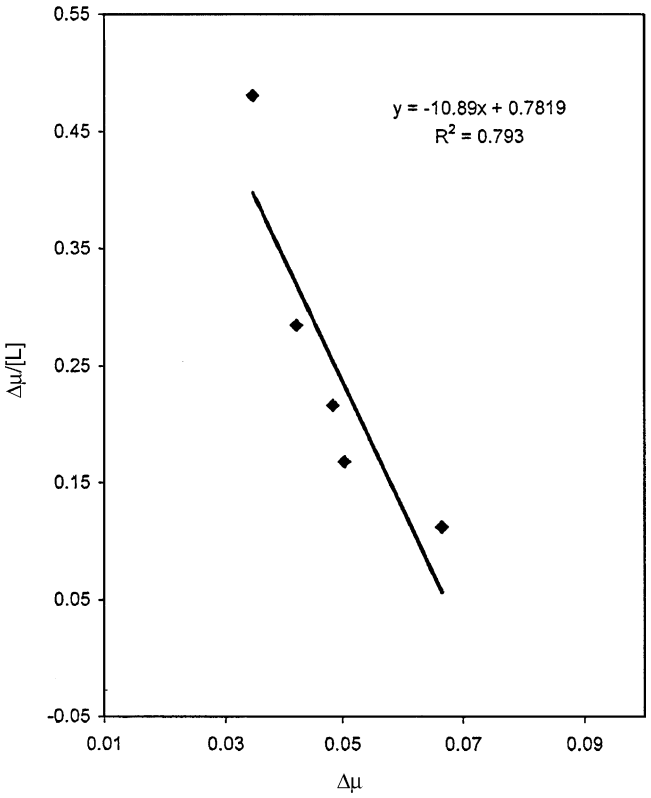

Fig. 7. A Scatchard plot of BSA with goat anti-BSA Ab, using ACE techniques to derive K_b. Specific conditions indicated in Section 2.

defined as the difference between that for BSA and DMF. Of course, the faster the mobility, the shorter the overall migration times. The migration time for BSA should be larger than its complex, since the charge/mass ratio of the complex (AB) increased. Absolute values of migration time changes could not be used, since the EOF was not constant, as the Ab concentrations in the buffer changed. However, the relative migration changes (from one Ab concentration to the next) were comparable. That data showed that the relative migration time differences decreased with an increase of Ab concentration in the buffer. Using the plot of $D\mu_A/[B]$ versus $D\mu_A$, a linear relationship was obtained, Fig. 7. From this plot, it was possible to derive the K_b for BSA, against this polyclonal Ab, goat anti-BSA, and this was $1.1 \times 10^7\ M^{-1}$ at pH 6.8.

In order to confirm this K_b for the BSA–Ab pair by the ACE method, we used an alternative approach, sometimes termed CZE stoichiometry [45,57]. In this approach, varying molar ratios of Ab to BSA were mixed pre-injection, to form the usual complexes. The mixture was then analyzed by direct CZE. K_b was then calculated from the peak areas observed. For the very same Ab and BSA, we determined the K_b values to be $K_{b0} = 3.58 \times 10^7\ M^{-1}$, $K_{b1} = 3.26 \times 10^7\ M^{-1}$, $K_{b2} = 2.13 \times 10^7\ M^{-1}$. Several different complexes are possible for most Ags and their Abs, depending on the type of Ab studied. With polyclonal Abs, several binding sites on the same Ag molecule are possible, and thus several K_b values can be

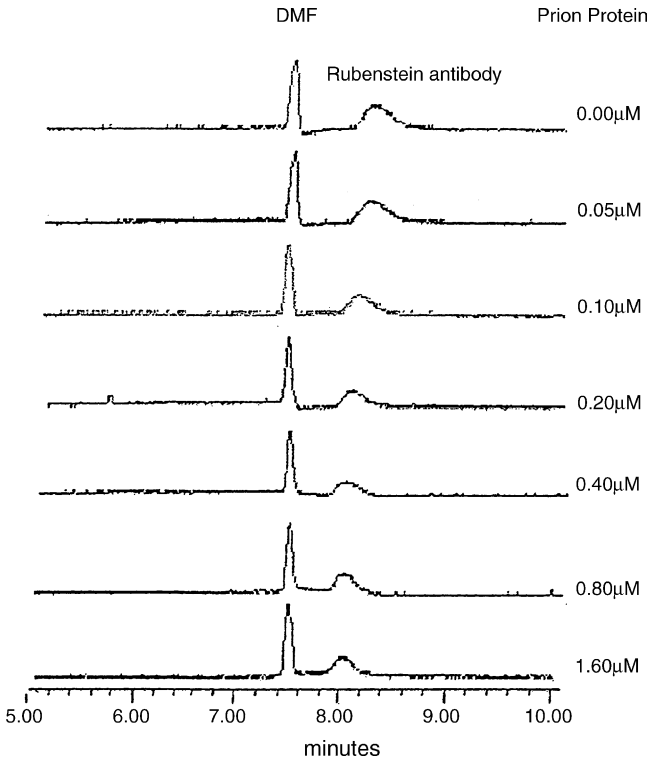

Fig. 8. A series of ACE electropherograms for the Rubenstein Ab. The running buffer consisted of 50 mM sodium phosphate and various concentrations of the prion protein, PrPc, pH 8.5. The sample of Ab was 0.4 μM with 0.005% DMF in water. Other experimental conditions are described in Section 2.

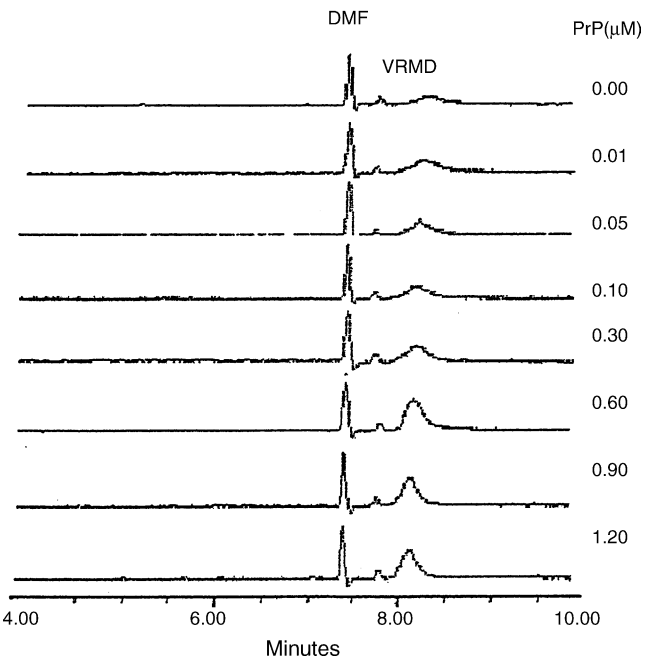

Fig. 9. A series of ACE electropherograms for the VMRD Ab. Experimental conditions as in Fig. 8.

derived, as in this instance. However, ACE only provides a single K_b, since only one peak in the electropherogram is being measured as it changes its mobility and migration times with changes in Ab concentrations in the buffer. Nevertheless, there was a good agreement between the K_b found here via ACE and that using CZE stoichiometry. These numbers for K_b were also consistent with those in the literature (ca. $10^7\ M^{-1}$), that have been determined by immunoprecipitation methods [57]. Thus, it was clear, as others had shown for Ab–Ag pairs using microchip ACE methods, that the basic ACE approach, based on mobility changes, was suitable to derive K_b values for Ab–Ag pairs, despite the fact that most previous applications of ACE methods have not been applied to Ab–Ag systems [47–50].

We next applied the same ACE method to derive the K_b values for the prion protein and various Abs, mainly commercial in nature. Fig. 8 illustrates the various electropherograms realized for a Rubenstein Ab (O1-16/6BIO anti-prion Ab) injected into varying concentrations of the prion protein. We have now reversed the protein injected versus that in the running buffer, mainly for the purpose to discern the peak changes. In some instances, the Ab or its Ag migrated too close to the EOF marker (DMF), making it unusable to derive K_b. In that instance, reversal of the injection protein can lead to valid K_b measurements. There is no absolute requirement as to which protein should be injected and which should be varied in the buffer. A main requirement for the ACE method to function, is that the CE peak shifts be separated from the EOF marker peaks, as suggested above. In this instance, it was not possible to inject the prion protein, but injecting the Ab proved to be entirely viable. When attempting to use the prion protein for injections, it always migrated (at any pH) too close to the EOF marker to observe changes in mobility as the [Ab] in the buffer was varied. Fig. 9 (VMRD-F99/97.6.1 anti-prion Ab) is a similar pattern of changing Ab migration times versus DMF, again as a function of changes in the prion concentration in the running buffer.

In these two studies, with two different prion Abs, the running buffer contained differing concentrations of the prion protein, and the Ab was injected with a neutral (DMF) marker. However, in order to discern the Ab peak at all, the pH had to be set at 8.5, very close to the prion protein's p*I*. If the pH was lowered, to say 7.8 or even 7.4, then the Ab peak migrated too close to that of DMF (or any other species having just EOF), and made it impossible to measure the changes in migration times. At the basic pH of 8.5, the Ab was negatively charged and the prion protein was close to neutral. Thus, as compared to the BSA case, at this pH, the mobility of the Ab was decreased (shorter migration times) with an increase of the prion protein concentration in the running buffer. Using the same Scatchard plots as for BSA, Figs. 10 and 11, we derived K_b values as being $1.8 \times 10^7\ M^{-1}$ for the Rubenstein Ab and $1.9 \times 10^7\ M^{-1}$ for the VMRD Ab. These were quite similar to the K_b values reported for BSA, but somewhat different from those already reported for different Abs towards the prion protein [32–35]. However, the studies by

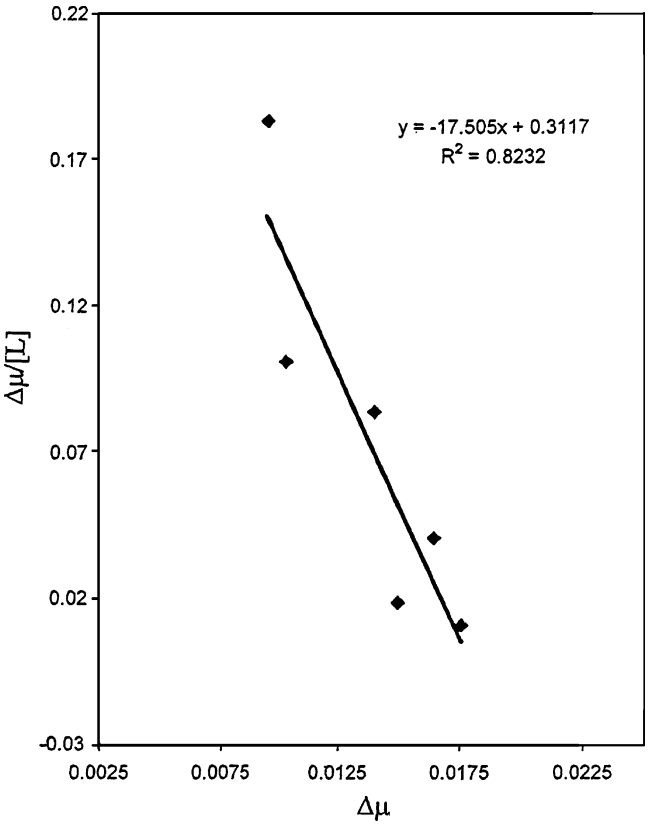

Fig. 10. Scatchard plot for the Rubenstein Ab, using the data from Fig. 8.

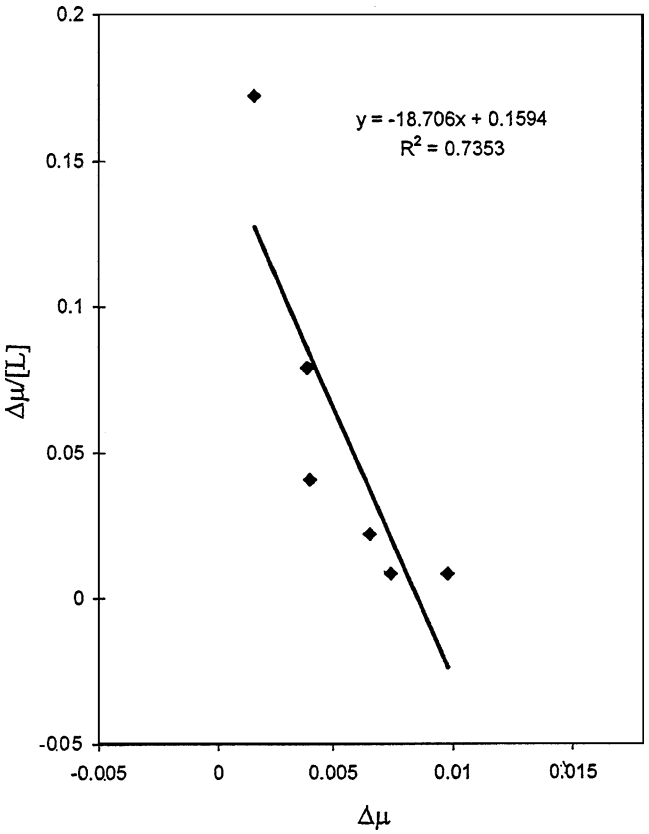

Fig. 11. Scatchard plot for the VMRD Ab, using the data from Fig. 9.

Safar et al. were done at pH 7.4 in TBS or MES buffered saline.

It has been pointed out already that the prion protein (PrPc) undergoes pH-induced conformational transitions and aggregation at other pH values (above 8.0), thus changing its affinity and K_b for any Abs [58]. Our K_b values derived, at a more basic pH, are thus not directly comparable with any derived at a more neutral pH, such as 7.4–7.7. Indeed, it could be argued that K_b values measured at a pH far away from that used for immunoassays (typically, neutral, 7.4–7.7), will not be comparable to those determined at neutral pH values. And, K_b values determined at pH values away from those used for immunoassays, cannot be directly used to predict LODs or efficiencies in such immunoassays. Then, why determine K_b values at any pH away from neutral values? In the case of ACE, this is a very real drawback, because not all pH values can be employed, as above for the prion Abs, but not for the BSA case. Migration times change with pH in ACE as in CZE, and if changes in migration times are needed to derive K_b, then without the ability to measure migration time changes at a given pH, nothing further can be derived. And, if a neutral pH is needed to derive K_b values that relate to immunoassay applications, that pH must permit for determination of migration time changes and thus, K_b derived. Again, ACE cannot function in all Ab–Ag instances at all pH values, and one must take this into consideration when attempting to derive the K_b at a specific pH value.

4. Conclusions

In this study, we have attempted to utilize both cIEF and ACE to derive information about prion Abs. The cIEF, as expected, has shown us the complexity of certain Ab species, the number of isoforms present, their p*I* values, and the homogeneity of such species against a target Ag. cIEF has also allowed us to determine which isoforms in a mixture were active towards the same Ag, using mobility changes and shifts, as in flat-bed gel electrophoresis. This can also be used to demonstrate Ab–Ag activity and recognition, by changes in p*I* values for the complexes. cIEF can also show approximately how many complex peaks are formed for any given Ab–Ag pair. Thus, cIEF, as shown by others for commercial Abs, can be quite useful in studying Ab–Ag interactions, recognition, and complex formation.

In the case of ACE, we have utilized this basic approach to determine K_b values for BSA and two prion Abs. With smaller and smaller K_d values, one obtains larger and larger K_b values. And, with larger K_a values, one also obtains larger K_b. The value of K_b is really determined by the two, opposing equilibrium constants, association and dissociation, of the complex between Ab and Ag. Binding equilibria are determined by the relative strengths of the complex formed (Ab–Ag) versus the free Ab and Ag. Factors such as size of the Ab and Ag, ionic bonds possible, pH, organic content of the buffer, and even temperature, can all affect the final K_b.

It would be ideal to control the K_b for any given Ab–Ag pair, but other than for experimental conditions, this has yet to be realized.

There are inherent limitations in all analytical methods, and ACE is not an exception. ACE can indeed provide K_b values for all/any Abs, but at times there are limitations in what pH values can be used with any given Ab–Ag pair. It is possible to reverse the placement of the Ab–Ag pair, that being injected or that having varying concentrations in the running buffer. Valid K_b values can be derived for the prion Abs, at certain pHs, but if those pH values are far away from the pH needed to perform immunoassays, then the K_b cannot be readily used to predict efficiency of operation or LODs in such immunoassays, when performed at a different pH. And, that is the crux of the ACE matter for Ab–Ag pairs. The greatest value, in our opinion, is to derive a K_b at a neutral pH commonly used for immunoassays, for only then can K_b values be compared from study-to-study, when they are derived at the same or a close pH value.

5. Nomenclature

Ab	antibody
Abs	antibodies
Ag	antigen
Ab–Ag	antibody–antigen complex(es)
ACE	affinity capillary electrophoresis
anti-BSA	antibody to BSA
[B]	concentration of binding ligand in ACE, B (sometimes termed L)
BSA	bovine serum albumin
CZE	capillary zone electrophoresis
CE	capillary electrophoresis
cIEF	capillary isoelectric focusing
dt	change in migration times (d = delta)
$D\mu_{A,B}$	differences in migration times for A in presence of varying concentrations of B (binding ligand) (D = delta)
EPF	electrophoretic flow or force
EOF	electroosmotic flow
EPF	electrophoretic migration force
ELISA	enzyme-linked immunosorbent assay
FL	fluorescence detection
IACE	immunoaffinity ACE
i-PCR	immuno-polymerase chain reaction
K_a	affinity constant
K_b	binding constant
L	ligand binding to receptor, A
[L]	concentration of ligand binding to receptor, A
LOD	limit of detection
log % (w/w)	natural logarithm of mass percent
M_r	molecular mass
M^{-1}	1/molar concentration or 1/molarity
MWCO	molecular mass cut-off (microfiltration)
NYS-IBR	New York State Institute for Basic Biomedical Research (Staten Island, NY, USA)
PCR	polymerase chain reaction
pH	−log [hydronium ion]
%RE	percent relative error
RE	relative error
t_R	retention or migration time in ACE
SDS	sodium dodecyl sulfate
SPR	surface plasmon resonance
TOF-MS	time-of-flight mass spectrmetry
% (w/w)	mass percent

Acknowledgements

We are very appreciative for all the assistance that we have received over the years from numerous sources in pursuit of these studies. Thus, Dr. Rubenstein and Dr. Kascsak at the NYS-IBR have provided us with various samples of their prion antibodies, as has Dr. K. O'Rourke of the USDA Laboratories in Pullman, WA, USA. Dr. A. Alpert of PolyLC was very helpful at the outset of our prion related studies, in providing us with a large amount of information, directions, and technical assistance in starting our first ELISA studies. This work was funded by a grant (2003) from the National Cattlemen's Beef Association (NCBA) of Denver, CO, USA.

References

[1] P. Yam, The Pathological Protein: Mad Cow, Chronic Wasting, and Other Deadly Prion Diseases, Copernicus Books, Spring, New York, 2003.
[2] S.B. Prusiner (Ed.), Prion Biology and Disease, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1999.
[3] www.madcow.org and www.madelk.org (websites).
[4] T. Chea, Staff Writer, Washington Post, Tuesday 29 May 2001, p. E014.
[5] http://more.abcnews.go.com/sections/living/yourhealth0515_cjd/index.html.
[6] S.B. Prusiner, The Prion Diseases. http://www.nmia.com/~mdibble/prion.html.
[7] R.M. Ridley, H.F. Baker, Fatal Protein, the Story of CJD, BSE and Other Prion Diseases, Oxford University Press, Oxford, UK, 1998.
[8] J. Collinge, M.S. Palmer (Eds.), Prion Diseases, Oxford University Press, Oxford, UK, 1997.
[9] H.F. Baker (Ed.), Molecular Pathology of the Prions, Humana Press, Totowa, NJ, 2001.
[10] H.F. Baker, R.M. Ridley (Eds.), Prion Diseases, Humana Press, Totowa, NJ, 1996.
[11] Chem. Eng. News (29 April 2002) 27.
[12] Prions: Mayhem and Management, Supplement to BioPharm Magazine, May 2002.
[13] J. Fricker, Drug Discovery Today 7 (2002) 537.
[14] B.K. Nunnally, Trends Anal. Chem. 21 (2002) 82.
[15] B. Nunnally, I.S. Krull (Eds.), Prions and Mad Cow Disease, Marcel Dekker, New York, 2003.
[16] Proceedings of the Symposium on Detection and Quantitation of Prions, Pittsburgh Conference, 17–22 March 2002.
[17] Proceedings of the Symposium on Analytical Chemistry of Prion Proteins, Fall National Meeting, American Chemical Society, New York, 7 September 2003.

[18] Chem. Eng. News (16 July 2001) 10.
[19] G.M. Shaked, Y. Shaked, Z. Kariva, M. Halimi, I. Avraham, R. Gabizon, J. Biol. Chem. 276 (2001) 31479.
[20] Chem. Eng. News (18 June 2001) 9.
[21] G.P. Saborio, B. Permanne, C. Soto, Nature 411 (2001) 810.
[22] D. Volkel, K. Zimmerman, I. Zerr, M. Bodemer, T. Lindner, P.L. Turecek, S. Poser, H.P. Schwarz, H.Y. Baxter, Transfusion 41 (2001) 441.
[23] M.J. Schmerr, A.L. Jenny, M.S. Bulgin, J.M. Miller, A.N. Hamir, R.C. Cutlip, K.R. Goodwin, J. Chromatogr. A 853 (1999) 207.
[24] M.J. Schmerr, K.R. Goodwin, R.C. Cutlip, A.L. Jenny, J. Chromatogr. B 681 (1996) 29.
[25] M.J. Schmerr, K.R. Goodwin, R.C. Cutlip, A.L. Jenny, J. Microcolumn Sep. 7 (1995) 521.
[26] M.J. Schmerr, A. Jenny, Electrophoresis 19 (1998) 409.
[27] M.J. Schmerr, R.C. Cutlip, A. Jenny, J. Chromatogr. A 802 (1998) 135.
[28] M.J. Schmerr, A. Jenny, R.C. Cutlip, J. Chromatogr. B 697 (1997) 223.
[29] A. Alpert, Technical Bulletin—We do Prions, PolyLC, Columbia, MD, January 2001.
[30] Anon., Anal. Chem. 73 (2001) 252A.
[31] M.J. Schmerr, A.J. Alpert, US Patent no. 61,50,172 (21 November 2000).
[32] J.G. Safar, M. Scott, J. Monaghan, C. Deering, S. Didorenko, J. Vergara, H. Ball, G. Legname, E. Leclerc, L. Solforosi, H. Serban, D. Groth, D.R. Burton, S.B. Prusiner, R.A. Williamson, Nature Biotechnol. 20 (2002) 1147.
[33] E. Leclerc, D. Peretz, H. Ball, H. Sakurai, G. Legname, A. Serban, S.B. Prusiner, D.R. Burton, R.A. Williamson, EMBO J. 20 (2001) 1547.
[34] R.A. Williamson, D. Peretz, C. Pinilla, H. Ball, R.B. Bastidas, R. Rozenshetyn, R.A. Houghten, S.B. Prusiner, D.R. Burton, J. Virol. 72 (1998) 9413.
[35] E. Leclerc, D. Peretz, H. Ball, L. Solforosi, G. Legname, J. Safar, A. Serban, S.B. Prusiner, D.R. Burton, R.A. Williamson, J. Mol. Biol. 326 (2003) 475.
[36] E. Paramithiotis, M. Pinard, T. Lawton, S. LaBoissiere, V.L. Leathers, W.-Q. Zou, L.A. Estey, J. Lamontagne, M.T. Lehto, L.H. Kondejeski, G.P. Francoeur, M. Papadopoulos, A. Haghighat, S.J. Spatz, M. Head, R. Will, J. Ironside, K. O'Rourke, Q. Tonelli, H.C. Ledebur, A. Chakrabartty, N.R. Cashman, Nature Med. 9 (2003) 893.
[37] B. Caughey, Nature Med. 9 (2003) 819.
[38] N. Cashman, personal correspondence, Summer 2003.
[39] C.M. Niemeyer, M. Adler, D. Blohm, Nunc Brand Products, Technical Note 5, No. 35 (2003).
[40] J.-M. Nam, C.S. Thaxton, C.A. Mirkin, Science 301 (2003) 1884.
[41] Chem. Eng. News Mag. 6 (19 September 2003).
[42] J. Schmitt, M. Beekes, A. Brauer, T. Udelhoven, P. Lasch, D. Naumann, Anal. Chem. 74 (2002) 3865.
[43] S. Schiavo, A. El-Shafey, N. Jordan, C. Orazine, Y. Wang, X. Zhou, N.H. Chiu, I.S. Krull, PharmaGenomics Mag. 36 (January 2004).
[44] R.H.H. Neubert, H.-H. Ruttinger, Affinity Capillary Electrophoresis in Pharmaceutics and Biopharmaceutics, Marcel Dekker, New York, 2003.
[45] N.H. Chiem, D.J. Harrison, Electrophoresis 19 (1998) 3040.
[46] L.C. Santora, P. Sakorafas, Z. Kaymakcalan, I.S. Krull, K. Grant, Anal. Biochem. 299 (2001) 119.
[47] I.S. Krull, C. Gendreau, H.J. Dai, in: M.E. Swartz (Ed.), Analytical Techniques in Combinatorial Chemistry, Marcel Dekker, New York, 2000. Chapter 6.
[48] A. El Shafey, G. Jones, H. Zhong, I.S. Krull, Electrophoresis 23 (2002) 945.
[49] N. Heegaard, S. Nilsson, N. Guzman, J. Chromatogr. B 715 (1998) 29.
[50] X. He, Y. Ding, D. Li, B. Lin, Electrophoresis 25 (2004) 697.
[51] L.C. Santora, I.S. Krull, K. Grant, Anal. Biochem. 275 (1999) 98.
[52] I.S. Krull, J. Dai, C. Gendreau, G. Li, J. Pharm. Biomed. Anal. 16 (1997) 377.
[53] X. Liu, Z. Sosic, I.S. Krull, J. Chromatogr. B 735 (1996) 165.
[54] I.S. Krull, S. Kazmi, H. Zhong, L.C. Santora, in: P. Thibault, S. Honda (Eds.), Methods in Molecular Biology, vol. 213, Capillary Electrophoresis Separation of Carbohydrates, Humana Press, Totowa, NJ, 2002. Chapter 12.
[55] R. Strong, H.-J. Liu, B.Y. Cho, I.S. Krull, J. Liq. Chromatogr. Related Technol. 23 (2000) 1775.
[56] T. Wehr, R. Rodriguez-Diaz, M. Zhu, Capillary Electrophoresis of Proteins, Marcel Dekker, New York, 1999.
[57] M.W. Steward, J. Steensgard, Antibody Affinity, Thermodynamic Aspects and Biological Significance, CRC Press, Boca Raton, FL, 1983.
[58] J.G. Safar, personal communication, 9 January 2004.
[59] I.S. Krull, B.-Y. Cho, R. Strong, M. Vanderlaan, LC GC Mag. 15 (1997) 620.
[60] B.-Y. Cho, R. Strong, H. Zou, D.H. Fisher, J. Nappier, I.S. Krull, J. Chromatogr. A 743 (1996) 181.
[61] R.E. Strong, B.-Y. Cho, D. Fisher, J.L. Nappier, I.S. Krull, Biomed. Chromatogr. 10 (1996) 337.
[62] Catalog and Handbook of Life Science and Analytical Research Products, Pierce, Rockford, IL, 2003–2004.
[63] J.-C. Janson, L. Ryden, Protein Purification: Principles, High Resolution Methods and Applications, VCH, New York, 1989.
[64] J.M. Walker (Ed.), The Protein Protocols Handbook, Humana Press, Totowa, NJ, 1996.
[65] E. Harlow, D. Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988.