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

By: Guodong Li, Xiaojuan Zhou, Yonghui Wang, Ahmed El-Shafey, Norman H. L. Chiu, Ira S. Krull

Li G, Zhou X, Wang Y, El-Shafey A, Chiu NH, and Krull IS. Capillary isoelectric focusing and
affinity capillary electrophoresis approaches for the determination of binding constants for

Made available courtesy of Elsevier: http://www.elsevier.com

***Reprinted with permission. No further reproduction is authorized without written
permission from Elsevier. This version of the document is not the version of record. Figures
and/or pictures may be missing from this format of the document. ***

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 (Kb) 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 Kb values. The selected Abs for the prion protein can recognize both healthy and diseased
states of the protein and are commercially available. The Kb values of PrPc Abs appear to be as
strong as the anti-BSA (Ab to BSA) and other reported Kb values for proteins of similar size to
PrPc. This appears to be one of the few reports on Kb 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 |
chemistry | biochemistry

Article:
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], [2], [3], [4], [5], [6], [7], [8], [9] and [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], [12],[13], [14], [15], [16] and [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], [19], [20], [21], [22], [23], [24], [25], [26], [27], [28], [29], [30], [31], [32], [33], [34], [35], [36],[37], [38], [39], [40], [41] and [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], [24], [25], [26], [27],[28] and [29]. Those approaches recently described by Saborio and co-workers [20] and [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] and [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],[16] and [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], [33], [34] and [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],[49] and [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], [47], [48], [49], [50], [57] and [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], [47], [48], [49] and [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], [48], [49] and [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 Kascak 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], [60], [61][62], [63], [64] and [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 pH 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:
\[
D\mu_A = \frac{\mu_{AB}}{\mu_{\text{eof,AB}}} - \frac{\mu_A}{\mu_{\text{eof,A}}} \quad (\mu = \text{mobility})
\]

where \(\mu_{AB}\) and \(\mu_A\) are the mobilities of the receptor–ligand complex and receptor alone, respectively, and \(\mu_{\text{eof,A}}\) and \(\mu_{\text{eof,B}}\) 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:

\[
\frac{D\mu_A}{[B]} = K_b D\mu_{A_{\text{mix}}} - K_b D\mu_A
\]

where \([B]\) is the concentration of the ligand (B), and \(D\mu\) 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],[52], \[53], [54], [55] \text{ and } [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 \(\mu\) l of an Ab concentration of 0.5 mg/mL and 10 \(\mu\) l of 1 mg/mL BSA solution. 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 that all were active and bound to the Ag. In Fig. 1C, 30 \(\mu\) l of a 0.5 mg/mL Ab and 20 \(\mu\) l of a 1 mg/mL BSA were mixed with 60 \(\mu\) l 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 immuno complexes peaks having lower pI 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 pI values and the immunocomplexes would become more acidic.
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. 2A is the electropherogram of the BSA Ab with pI markers of 7.9, 5.9, and 4.6, and Fig. 2B is the electropherogram of free BSA and its immunocomplexes with the same pI markers. The pI values of the BSA Ab and its immunocomplexes could be determined from these electropherograms, by using a standard calibration plot for the three pI markers [51]. For the BSA Ab, its pI values were from 5.9 to 7.0, and for the BSA immunocomplexes, the pI values were from 5.2 to 5.8. When BSA alone was injected with these same pI markers, its pI was found to be about 4.6. This further confirmed the above assertion that the complexes should have lower pI values and be more acidic than the free Ab isoforms (Fig. 1A).
Fig. 2. cIEF of goat anti-BSA Ab and its BSA complexes, together with pI markers. (A) Ab and markers of pI values, 7.9, 5.9, and 4.6; (B) Ab, BSA, and pI markers, as in (A). The pI range of the Ab was 5.85–7.00, and the pI range of the complexes was 5.24–5.75. Specific conditions indicated in Section 2.

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. The cIEF pattern for these isoforms is very similar to that for the BSA Ab, but the pI values here are somewhat lower. Using again three internal standard proteins, as in Fig. 2B, we could determine the pI 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 pI values of the complexes shifted to a more basic region (shorter migration times), since the prion protein was a basic protein, having a higher pI value than its Ab. The pI values for this commercial, recombinant prion protein were determined, Fig. 4, but now using cIEF with a different set of pI marker proteins. There were several major peaks for this recombinant prion protein (Calbiochem), with the two largest peaks (i.e., 2 and 3) having pI 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. 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.

Fig. 4. cIEF electropherogram of Calbiochem prion protein, with $pI$ markers of 9.3, 7.9, 6.6; peaks 2–3 were from the prion protein. $pI$ values for peaks 2 and 3 were 8.6 and 8.4, respectively. 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 pI 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 pIs 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 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.

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

Fig. 6. cIEF electropherogram of the VMRD anti-prion Ab, peaks 1, 4, and 6 were markers of pI 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.

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], [48], [49] and [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], [33], [34] and [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 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] and [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 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], [48], [49] and [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.
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.

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 pI. 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, Fig. 10 and Fig. 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], [33], [34] and [35]. However, the studies by Safar et al. were done at pH 7.4 in TBS or MES buffered saline.
Fig. 10. Scatchard plot for the Rubenstein Ab, using the data from Fig. 8.
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 pI 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 pI 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 $aK_b$ 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
tᵣ  retention or migration time in ACE
SDS  sodium dodecyl sulfate
SPR  surface plasmon resonance
TOF-MS  time-of-flight mass spectrometry
% (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. Kacsak 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:
R.M. Ridley, H.F. Baker. Fatal Protein, the Story of CJD, BSE and Other Prion Diseases
J. Collinge, M.S. Palmer (Eds.), Prion Diseases, Oxford University Press, Oxford, UK (1997)
B. Nunnally, I.S. Krull (Eds.), Prions and Mad Cow Disease, Marcel Dekker, New York (2003)
Proceedings of the Symposium on Detection and Quantitation of Prions, Pittsburgh Conference
    (17–22 March 2002)
Proceedings of the Symposium on Analytical Chemistry of Prion Proteins, Fall National
    Meeting, American Chemical Society, New York (7 September 2003)
    (2001), p. 31479
D. Volkel, K. Zimmerman, I. Zerr, M. Bodemer, T. Lindner, P.L. Turecek, S. Poser, H.P.


N. Cashman, personal correspondence, Summer 2003.


X. He, Y. Ding, D. Li, B. Lin. Electrophoresis, 25 (2004), p. 697


Catalog and Handbook of Life Science and Analytical Research Products
Pierce, Rockford, IL (2003–2004)

J.-C. Janson, L. Ryden. Protein Purification: Principles, High Resolution Methods and Applications
