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Journal of Chromatography B, 768 (2002) 93–103

JOURNAL OF CHROMATOGRAPHY B

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Review

# Antigen–antibody interactions in capillary electrophoresis

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**Abstract**

Immunoreactions in combination with separations by capillary electrophoresis (CE) are increasingly being used to quantitate specific analytes in biological fluids. Both competitive and non-competitive approaches have been used for the purpose and, in selected cases, now compare favorably with conventional quantitative immunoassays with respect to concentration limits of detection. CE is also a useful method to evaluate antigen–antibody binding on-line and offers unique possibilities for binding constant estimates, also for weakly binding antibodies and antibody fragments. In this review we cover recent developments in the use of antigen–antibody interactions in conjunction with CE and conclude that continued development of miniaturization, on-line preconcentration and more sensitive detection schemes will contribute to the further dissemination of CE-based immunoassays building on already established affinity CE approaches. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Reviews; Antigens; Antibodies

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PII: S0378-4347(01)00489-3

## 1. Introduction

The combination of immunoreactions and electrophoresis has a long history. In fact, electrophoresis has been used to characterize antigen–antibody interactions since the days of moving boundary electrophoresis in Tiselius cells [1] (Fig. 1). The advent of modern capillary zone electrophoresis (CE for short) has spurred the development of micro-analytical affinity electrophoresis methods (see, for example, the paper by K. Shimura in the present issue) [2–11] and an important subgroup of these methods with antigen–antibody interactions as the affinity system has emerged. After the initial demonstration in 1989–1991 of the separation of antibody–antigen complexes by CE [12,13], immuno-CE or CE immunoassays were developed for various purposes during the mid-1990s [14–19] and several recent reviews cover applications and methods [3,19–21].

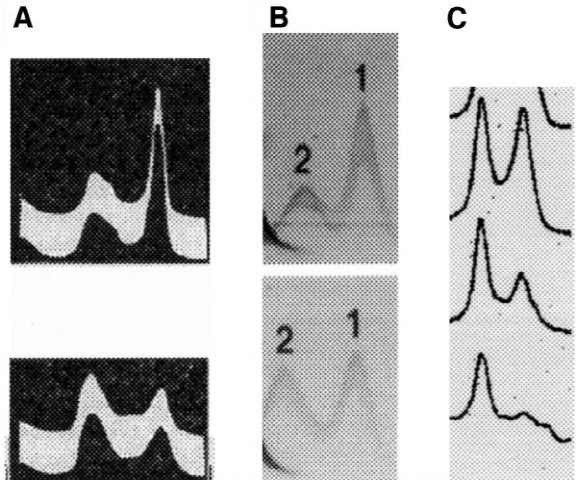


Fig. 1. Antigen–antibody interactions analyzed by electrophoresis — before and now. (A) Moving boundary electrophoresis analysis of antigen–antibody complexes of varying stoichiometry (modified from Fig. 3 of Ref. [1]). (B) Crossed affinity immunoelectrophoresis of $\alpha_1$-fetoprotein–anti-$\alpha_1$-fetoprotein complexes of different stoichiometry in agarose gels. The front complex (labeled 1) represents bivalent antigen–antibody complexes, while the trailing complex (labeled 2) represents univalent complexes (modified from Ref. [105]). (C) (Reprinted, with permission, from part of Fig. 2 of Ref. [79], ©1994, American Chemical Society.) CE of mixtures of a monoclonal antibody against human serum albumin (HSA) preincubated with decreasing concentrations of HSA (from top to bottom) resulting in a gradual decrease of the amount of bivalent antigen–antibody complexes.

The unifying feature of the CE applications that are reviewed here is that the analyte or part of the analyte mixture is involved in immunoreactions prior to or during CE. The term immunoelectrophoresis has been used for many years especially to describe agarose gel separations integrated with immunoprecipitation where antibodies are used as specific titrating tools to characterize, identify, and quantitate analytes even in complex mixtures [22–24]. In the capillary format the multi-dimensionality of these gel immunoelectrophoretic approaches is lost and precipitating antibodies are rarely used, but the quantitative precision, selectivity, speed, applicability to small molecules, low sample consumption and compatibility with automation of CE cannot be matched by gel immunoelectrophoresis methods. In comparison with conventional solid-phase assays, however, CE-based immunoassays do not usually achieve the same concentration limits of detection.

## 2. Principles of operation

The selectivity of non-affinity CE methods can be increased by introducing analyte-interacting molecules such as antibodies provided the interactions are not inhibited by the separation conditions and provided the complexed molecules are distinguishable from free molecules in the separation profile [2,25,26]. Any complexation that changes molecular size, shape, and/or charge will change the separation pattern. The high and reproducible separation efficiencies typical of CE ensure that interactions that lead to even quite small changes are precisely detected. Antibodies may interact with analyte molecules before and/or during electrophoresis. The change of analyte mobility caused by the interaction with antibodies combined with the characteristics of on-line detection make it possible to use CE for quantitative immunoassays [19,21,27] (cf. below — CE immunoassays). CE analysis of preincubated analyte–antibody mixtures is, in practice, the only approach used to quantitate analytes with high-affinity antibodies that form stable complexes.

The classical affinity electrophoresis approach (which is analogous to affinity chromatographic

methods) uses antibodies present at a constant concentration (e.g. in the electrophoresis buffer) during electrophoresis. Accordingly, in this type of affinity CE, migration shifts are indicative of interactions [28]. The antibodies here are used either in free solution or immobilized in the capillary and the analytes thus move through a constant concentration of ligand. This approach is especially well suited for determining binding constants of low-to-intermediate affinity interactions.

## 3. Antigen–antibody reactions used as tools in CE: CE immunoassays and immunoaffinity CE

### *3.1. Peak identification*

Antibodies may be used as peak identification tools in CE. The position of a specific analyte in a separation profile can be a major issue when analyzing mixtures because CE is not easily combined with a second dimension immunodetection step as used, for example, in immunoblotting of gel electrophoresis separations. Immunoidentification can be performed off-line with, for example, protein G-Sepharose bound antibody added to the sample followed by centrifugation prior to injecting the analyte supernatant [29,30]. Peaks that disappear — and do not disappear in control experiments using no or an irrelevant antibody — represent the specificity of the antibody. An on-line example of using antibodies for identification of a specific analyte is shown in Fig. 2, where a monoclonal antibody raised against $\beta_2$-microglobulin is added in free solution to the electrophoresis buffer to identify the protein peak. The analyte is an RPLC fraction of fluoresceinylated $\beta_2$-microglobulin. This fraction is a complex mixture of components as assessed by laser-induced fluorescence detection (Fig. 2A). When running the analysis in the presence of the antibody (Fig. 2B) a specific component (arrow) in the separation profile is affected in comparison with the control experiment. This component has a shorter peak appearance time, presumably because of complexation with the faster moving antibody, and is thus identified as $\beta_2$-microglobulin. The antibody itself is not seen in the electropherogram because only fluoresceinylated analytes are detected. Experiments such as these are

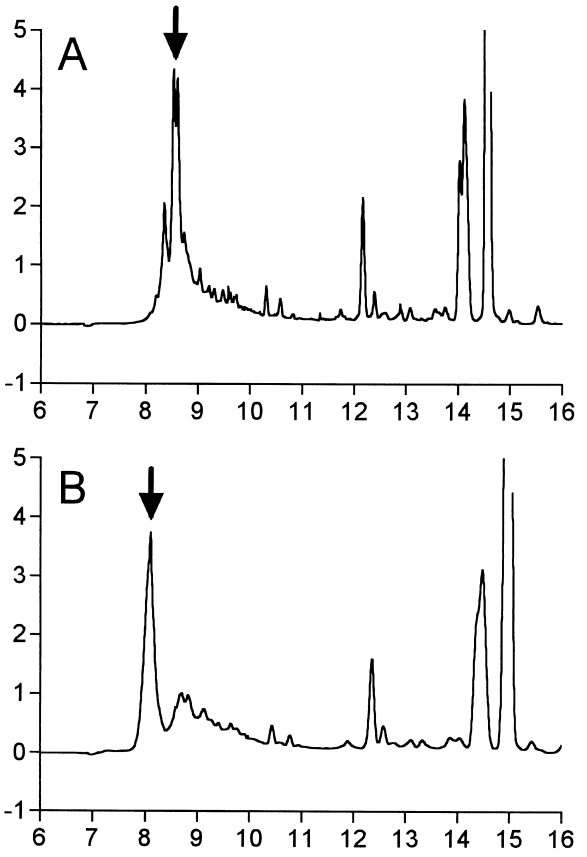


Fig. 2. Antibody-mediated identification of a specific peak in an analyte mixture. The analyte is a 5-carboxyfluoresceinylated $\beta_2$-microglobulin fraction collected from a reversed-phase preparative HPLC separation of a reaction mixture of $\beta_2$-microglobulin and 5-carboxyfluorescein. The sample is analyzed by capillary electrophoresis (15 kV in 50 m*M* phosphate, pH 7.36) in a 50 μm inner diameter capillary using laser-induced fluorescence detection (excitation at 488 nm, emission at 520 nm). In (B) a monoclonal antibody against $\beta_2$-microglobulin was added to the electrophoresis buffer at 35 μg/ml (approximately 0.2 μ*M*). This affects the appearance time of the peak marked with an arrow while the remainder of the separation profile is largely unaffected.

quick and easily performed, but will not always give clear answers. A typical problem is antibody adsorption to the capillary wall, which influences the electroosmotic flow and thereby the appearance time of all analyte molecules. This will often cause an even slower or the disappearance of a specific analyte because it is bound to the immobilized antibody. As a rule it is always necessary to include non-interacting marker compounds in estimates of

binding constants to eliminate mobility changes that are not due to specific antigen–antibody interactions [3,16]

### 3.2. Analyte quantitation

In any immunoassay it is necessary to separate bound from free analyte and thereby quantitate the reactants. This may be done by capillary electrophoresis as first demonstrated in Refs. [12,13]. The inherent instrumental and quantitative nature of CE has made on-line quantitative assays feasible (Table 1). These immunoassays are based on separating antigen–antibody complexes from free analyte and free receptor molecules by CE. Two distinct modes of CE-based immunoassays have been described [21]: ''direct'' or non-competitive assay and competitive assay. In the direct assay, also called affinity probe capillary electrophoresis (APCE) [31], the affinity reagent (the antibody) is labeled with fluorophore and added at a constant concentration to the sample for binding to the target (the antigen/hapten). Detection and quantitation of the complex peak is a direct measure of the target in solution. This type of assay may be combined with charged ligands to effect the separation of free from complexed analyte molecules [32,33]. In the competitive assay, a fluorescently labeled target (antigen or hapten) and limiting amount of antibody are added to the sample allowing the labeled target and the target in the sample to compete for binding to the antibody. The resulting bound and free labeled target are separated and quantitated by CE. The relative amounts of bound and free labeled target molecules are dependent upon the starting concentration of target in the analyte solution. Nearly all of the CE-based immunoassays used so far have relied on laser-induced fluorescence detection because of the high sensitivity and high selectivity for detection. This has necessitated labeling of either the affinity probe or the tracer antigen with a fluorophore, which can be problematic as discussed below. Recently, fluorescence polarization has also been implemented which has certain advantages such as confirmation of the complexed species [34].

Table 1
Important issues characteristic of contemporary quantitative CE immunoassays. Partly based on Refs. [32,103]

| |
|---|
| *Advantages* |
| Solution (homogeneous) assays (faster binding kinetics and no immobilization of reactants) |
| Both complexed and non-complexed species are visualized |
| Discrimination between specific and non-specific binding may be possible |
| Compatibility with automation |
| Miniaturization possible |
| Low sample consumption |
| Speed, may allow time-resolved immuno-monitoring |
| Wide analyte applicability |
| *Challenges* |
| Protein adsorption onto capillary walls |
| Useful detection limits normally require special reagents (fluorophore-labeled antibodies or antigens/probes) |
| Detection limits (CLODs) generally higher than those of solid-phase immunoassays |
| Each analyte requires new antibody or antigen analogues developed |
| Prior knowledge of the approximate level of the analyte is required |
| Parallel analysis as in ELISA not readily possible |
| Instrumentation relatively expensive in comparison with ELISA |

CE immunoassays have proven to be versatile both in the analytes and the sample matrices analyzed (for reviews, see Refs. [19,21,35,36]) (Table 2). Small molecules such as therapeutic and abused drugs [27,34,37–39], peptide [40–42] and steroid hormones [17,43], toxins [44], and proteins [45–47] have all been measured. In addition, analytes have been determined directly in serum [34,48,49], urine [39], food [44], and cell and tissue samples [18,46] with little or no sample preparation. The minute sample requirements allow multiple analyses to be performed on the same sample. An exciting recent application of CE immunoassay is the first assay for the abnormal prion species associated with scrapie, the prototype of transmissible spongiform encephalopathy [46,50]. With the recent examples of the analyses of other infectious agents such as viruses [30,51] and even bacteria [52] it is likely that CE immunoassays of such supramolecular assemblies will also be developed [53]. Thus, continued work with CE-based immunoassays is revealing potential applications in the clinic as well as for research purposes.

Most CE immunoassays have been restricted to one analyte, but the possibility of multi-analyte immunoassays by the electrophoretic separation of

Table 2
Representative examples of CE immunoassays of biomolecules and drugs

| Immunoassay | Analyte(s) | Sample matrix | Assay mode | Detection | Ab | Ag/Ab label | Detection limit | Ref. |
|---|---|---|---|---|---|---|---|---|
| Illicit drugs | Multiple (4) | Urine | Competitive | LIF | Wh | Fl–Ag | ~80 n*M* | [37] |
| | Multiple (2) | Urine | Competitive | LIF | Wh | Cy5 and Cy5.5 | 4–40 n*M* | [27] |
| Drugs | Theophylline | Dil. serum | Competitive on chip | LIF | Wh | Fl–Ag | 1.4 μ*M* | [48,59] |
| | Digoxin | Serum/urine with SPE | Direct | LIF | scFv | Fl–scFv | 200/300 p*M* 0.4 p*M* | [33] |
| | Digoxin | Serum | Competitive | LIF | Fab | PE–Ag | 0.5 n*M* | [104] |
| | Cyclosporine | Blood | Competitive | LIFP | | Fl–Ag | 0.9 n*M* | [34] |
| | Cortisol | Serum | Competitive | LIF | Fab/Wh | Fl–Ag | ~50 n*M* | [17,43] |
| | Cortisol | Serum | Competitive on chip | LIF | Wh | Fl–Ag | ~50 n*M* | [58] |
| Hormones | Glucagon and insulin | Islet cell secretions | Competitive | LIF | | Fl–Ag | 760 p*M* | [42] |
| | Insulin | Islet cell secretions | Competitive | LIF | | Fl–Ag | 3 n*M* | [18,41] |
| | Glucagon | RPLC-separated islet cell secretions | Competitive | LIF | | Fl–Ag | 20 p*M* | [62] |
| | Human growth hormone | Standards | Direct | LIF | Fab | TMR–Fab | ~5 p*M* | [31] |
| | Thyroxine | Serum | Competitive on microship arrays | LIF | Wh | Fl–Ag | ~40 n*M* | [49] |

Abbreviations: Ab, antibody; Ag, antigen/hapten; Cy, cyanine dye; Fab, antibody Fab fragment; Fl, fluorescein; LIF, laser-induced fluorescence; LIFP, LIF-polarization; PE, phycoerythrin; RPLC, reversed-phase liquid chromatography; scFV, single-chain antibody variable region fragment; SPE, solid-phase extraction; TMR, tetramethylrhodamine; Wh, whole antibody.

individual antigens, either sufficiently different in mobility, unmodified [42] or differentiated through labeling with different charge-modified fluorophores [27], has been demonstrated. This is an important and unique possibility compared with conventional immunoassays, as illustrated by the applications of CE immunoassays to drug screening where entire drug panels are determined in one assay [27,37,38]. The CE format is also useful when it is necessary to confirm peak identification as direct interfaces to mass spectrometry are possible [39].

CE-based immunoassays may have certain advantages, as illustrated by the large number of applications (reviewed, e.g., by Refs. [3,20,21]), but also some important problems (Table 1). The most important limitation regards sensitivity. Conventional quantitative immunoassays utilize much larger sample volumes than CE and analytes are effectively concentrated on the solid phase. Correspondingly low detection limits (often $<1$ p*M*) are achieved. Only the direct type of CE-based immunoassays have approached this detection limit at present. Another important limitation of CE immunoassays is the lack of demonstrated capability to run parallel samples (e.g. the throughput).

### *3.3. Assay development*

The need to label antigens to obtain sufficient detection limits is a definite drawback. Otherwise, however, the development of assays, especially the competitive assays, has proven to be remarkably straightforward [36]. A successful assay requires: (1) antibody with a good binding constant, (2) labeled target, and (3) separation conditions for resolving bound and free labeled target. Fragments of antibodies produce narrower complex peaks than whole antibodies [17], but the use of whole antibodies gives adequate performance in many cases (Table 2) and is much simpler to implement. Indeed, the antibody does not need to be especially pure and can be used

directly from ascites or serum if necessary. Preparation of labeled targets can sometimes be problematic. For small targets, it is necessary to identify a labeling site that does not interfere with binding of the target to antibody. However, this issue is not different from any other competitive immunoassay format. For larger targets, labeling is compounded by the need to prepare a single homogenous product so that a single peak is obtained in the electropherogram. In the case of insulin, for example, the presence of multiple primary amines for labeling necessitated chromatographic purification of the labeled target to generate a single peak in the CE analysis [41]. Another successful approach for obtaining a homogeneously labeled reactant is illustrated by an affinity-CE assay of the glaucoma drug dorzolamide, which interacts with the enzyme carbonic anhydrase II [32]. In that study, advantage was taken of the fact that the enzyme contains only one cysteine residue and thus could be homogeneously labeled with a thiol-specific fluorescein label. The drug-labeled enzyme complex was measured in an affinity probe setup to detect the drug in biological matrices at $<$100 p*M* detection levels in 10 min total analysis time. The study also serves to illustrate the possibility of exploiting other biomolecular affinity interactions than antigen–antibody interactions for the development of quantitative CE assays.

The final issue is the determination of good separation conditions. Achieving conditions in which only the free labeled target is monitored is trivial and will allow quantitation. However, developing conditions where both the complex and free labeled target can be detected is more difficult. Direct detection of the complex is advantageous for confirming complex formation and antibody saturation (cf. Fig. 1) and necessary for direct binding assays. When the recovery of an antigen–antibody complex turns out to be difficult it may be due to dissociation of the non-covalent complex during separation and/or to adsorption of the complex, for example through binding of the antibody, to the capillary wall. Common approaches to reducing protein adsorption such as pH manipulation, buffer choice, high salt concentration, zwitterionic buffer additives [26] and coated capillaries [33] are usually successful. Even micellar solutions have been used for CE immunoassays [54]. The only caveat is that the conditions used must not dissociate the complex too quickly. Success depends on having a separation time short relative to the dissociation time and this situation will be furthered by the use of short capillaries [55] and high electric fields. Separations as short as 1 s have been achieved and the amount of complex detected in that study was quantitative, indicating that insignificant amounts of complex dissociated during the separation [41]. Achieving such rapid separations requires instrumentation that is not presently commercially available, such as microchips [49,56–59], or flow-gating [41,60].

Development of direct assays is more difficult because an antibody or an antibody fragment must be labeled. One elegant approach has been to tag at the free thiol groups found in Fab fragments of antibodies [40]. In another approach, single chain antibody variable region fragments (scFv) were utilized as direct affinity probes. In this case, site-directed mutagenesis made it possible to introduce a unique cysteine residue which allowed uniform fluorescent labeling of the probe. The assay yielded low p*M* detection limits for aqueous digoxin standards in a direct ''mix and inject'' format and f*M* detection limits with off-column preconcentration by solid-phase extraction [33]. An alternative, which has yet to be used for CE, is to label antibodies by preparing fusions with green fluorescent protein. Alternatively, smaller affinity ligands that are easier to manipulate could be used instead of whole antibodies or antibody fragments. One example of a group of small (a few kDa) designer ligands is the aptamers, which are selectively binding oligonucleotides identified from combinatorial libraries [61]. Such aptamers can be readily synthesized and labeled with fluorophores and used in direct assays. In one example, IgE was determined in serum with detection limits of ~50 p*M* [61]. In some cases the separation of the free receptor molecule (e.g. antibody) from its complex with ligand will be a problem. One approach to remedy the lack of resolution is to add a charged competitive ligand to the electrophoresis buffer. This ligand (also called a shift ligand) will bind free receptor molecules that are injected from the sample mixture and carry them away from the blocked receptor in complex with ligand and thus achieve the

necessary resolution in the non-competitive assay format [32,33].

### 3.4. CE immunoassays versus conventional immunoassays

An important question is how does CE compare to established immunoassay techniques such as ELISA? (Table 1). Important advantages of the CE approach relative to ELISA are: (1) faster analysis for single runs, (2) multi-analyte analysis capabilities because a separation step is integral to the technique, and (3) compatibility with automation and on-line analysis. The compatibility with automation has been made apparent in both capillary and microchip systems where the reagents and sample can be mixed on-line and then separated by CE all in a matter of seconds [41,48,49,57–60]. These advantages suggest that CE would be useful for sample-limited analysis, process monitoring, rapid diagnostics, high-throughput screening, and ''immunosensing''. Published examples of such applications are, at present, rare. In one example of chemical monitoring on the microscale, insulin secreted from a single islet of Langerhans was monitored by performing CE immunoassays on-line every 3 s [41]. This application allowed the temporal pattern of secretion to be determined as a function of different stimulants. Other applications can be expected as the instrumentation develops.

The main disadvantages of CE systems at present relative to ELISA include: (1) poorer concentration sensitivity in many cases, (2) lack of proven methods for parallel operation and high-throughput analysis, (3) sample losses due to adsorption phenomena, and (4) more complicated and expensive instrumentation. The detection limits of competitive CE immunoassays are typically in the 0.1–1 n*M* range [14,27,34] (Table 2), whereas ELISA can have detection limits of 1 p*M* or better [21,35,36]. The concentration sensitivity issue is related to the fact that the CE immunoassays are performed on homogenous solutions where no preconcentration from a given sample volume can take place. This is in contrast to ELISA, which involves a surface reaction where analytes can preconcentrate. Application of preconcentration techniques in combination with the competitive assays should alleviate this situation. In a recent example, capillary LC was interfaced with a post-column, on-line CE immunoassay. The LC column allowed extensive preconcentration (and separation of cross-reactive species) prior to the competitive immunoassay. Concentration detection limits in the low p*M* range were reported; however, the sample usage was less efficient, resulting in higher mass detection limits [62]. Also, preconcentration of samples using solid-phase extraction devices to reduce sample volume can generate much lower detection limits (Table 2) [33]. In general, however, automated preconcentration that is compatible with the speed of CE is not yet routinely implemented.

The discrepancy in detection limits is less apparent for non-competitive assays. For example, when IEF was used for the separation of bound and free analyte in a non-competitive assay, detection limits in the low p*M* range were obtained because of the preconcentration involved in the IEF step [31]. This range was also achieved in a CE immunoassay for DNA damage using monoclonal antibodies against bromodeoxyuridine in combination with tetramethylrhodamine labeled secondary antibodies [63] and in the digoxin study mentioned above [33]. Another example of using secondary reagents is a study measuring $IgG_1$ in serum by fluorescein–protein G-tagged anti-$IgG_1$ antibodies, but this was less attractive because of a very slow binding kinetics [64]. While CE immunoassays can be performed quickly, the parallel nature of ELISAs performed in 96-well microtiter plates allows simple high-throughput measurements. A CE-based system with equally high throughput has yet to be demonstrated; however, the advent of capillary bundles and parallel separations on a chip suggests that it is quite feasible to have a parallel CE immunoassay system [49]. While still not rugged and simple enough for routine application, such a system would compete favorably with an ELISA in terms of throughput with the added advantages of minimal reagent consumption, fast turnaround, and multi-analyte capability.

### 3.5. Analyte enrichment by immunoaffinity CE

The small volumes that can be injected onto a common capillary (typically 1–20 nl sample volumes) together with the low sensitivity of UV-based

detection over a typical capillary diameter of 50 μm result in high analyte concentration detection limits, e.g. μ*M* for peptides. This has led to the development of a number of low specificity on-line preconcentration methods [65–70] and more selective methods that include immunoaffinity CE, where a pre-capillary immunoadsorbent with immobilized antibodies [71,72] (or antibody-binding molecules such as protein G [73]) captures and retains specific analyte molecules from large volume samples that may then be eluted in smaller volumes resulting in a selective 50–100-fold preconcentration. The different chemistries and materials available for immobilization and integration into the capillary line include functionalized glass beads, derivatized capillary inner surfaces, activated polymers, imprinted polymers, magnetic beads, and capillary bundles [66,69,74–76]. This approach makes possible the UV detection of analytes at otherwise too low concentration in complex sample matrices such as serum or urine and has also been successfully used for on-line mass spectrometry detection [77]. The approach, however, is not easily used for quantitative purposes because the efficiency of antigen capture and antigen elution is difficult to control. An example of a quantitative approach was presented in an application where the immunoadsorbent (sheep anti-mouse antibodies) was immobilized on magnetic beads kept in place inside the capillary by an external magnetic field. After desorption of bound mouse antibody isotachophoretic concentration enabled quantitative measurement of the unlabelled analyte [76] even though there were problems with keeping the magnetic bead suspensions uniform prior to injection and thereby ensuring a constant concentration of beads.

Immunoaffinity CE using immobilized antibodies may also be used as an approach to increase the selectivity of CE separations [75,78] in the same way as when antibodies in solution are used in affinity CE methods. The immobilization guarantees that interactions will be revealed to a maximum effect also when working with weakly binding antibodies [78]. However, immobilization may be detrimental to antibody binding and is not suited when quantitative binding parameters are going to be estimated since the actual active and accessible immobilized antibody concentration is not precisely known.

## 4. Immuno-CE: CE used to characterize antigen–antibody reactions

The fundamental parameters describing binding interactions: equilibrium binding constants and interaction stoichiometries, can be estimated by CE [5,79], especially in the migration shift approach where antibody is added directly to the electrophoresis buffer. Antigen–antibody interactions have been analyzed in this way in a number of publications [15,16,26,55,79,80]. A number of other analytical methods exist for binding studies [81], but the advantages (to which there are exceptions [28]) of using CE is the wide range of analytes that can be analyzed, the lack of need for labeling, the low sample consumption, and the speed of analysis.

### *4.1. Antibody specificity*

In much the same way as antibodies can be used as tools in CE to identify analyte peaks, antibodies themselves can be characterized with panels of known antigens separated and quantitated by CE. With labeled antigens it is possible to characterize the specificity of an antibody population in heterogeneous mixtures. This was done in a screen of anti-morphine antibodies using cyanine dye-labeled morphine [82] and this is a necessary test to determine if the antibody-binding capability of a labeled antigen is preserved. Once a stable complex was formed it was straightforward to characterize the antibody specificity by screening for the competitive ability of a number of antigen analogues [82]. The concept was also demonstrated using magnetic beads with immobilized antibodies [76]. Antibody immobilization to a solid phase, for example through protein G on beads, would seem promising for screening, for example, the antigen-binding activity of supernatants or ascites fluids in the course of production of monoclonal antibodies and this should also be feasible in solution [59].

### *4.2. Antibody rate and binding constants*

CE is useful for studies of immunoaffinity constants and binding kinetics because mono- and bivalent complexes as well as free antigen and antibody may be separated and quantitated in one

operation [79]. Uniquely for CE compared with other methods for binding studies the method is also well suited for the characterization of weakly binding antibodies [15,16] where complexes are less stable. Affinity CE with addition of ligand (antibody or antigen) to the electrophoresis buffer is used in this situation. Separation time and estimated complex stability should determine the design of quantitative binding experiments in CE. In practical terms, intermediate-to-low affinity interactions cause clean migration shifts when ligand is added to the electrophoresis buffer (i.e., there is no peak broadening, tailing, splitting, or disappearance) and can therefore be analyzed by migration shift affinity CE [83–85]. When the complex dissociation half-time, $\ln 2/k_{off}$ ($k_{off}$ is the dissociation rate constant), is equal to or less than 1% of the time required to separate free from bound molecules the equilibrium will be sufficiently fast to give clean shifts in peak positions [26,86,87]. Very long runs would, in theory, permit the analysis of more slowly dissociating interactions by migration shift affinity CE [86]. Conversely, if the separation (including sample introduction) is very fast (as in chip-based analysis [58] or takes place in very short capillaries [55]) peak broadening may occur even with fast interactions and a pre-equilibration approach will be better suited for the estimation of binding constants [88] much in the same manner as the quantitative CE immunoassays are designed. The interplay between $k_{off}$ and the speed of separation (for intermediate-affinity binding) and the lower limit of detection (for high-affinity binding) thus determines the experimental approach when using CE for immunochemical binding studies.

In high-affinity cases where samples are pre-equilibrated, interference from dissociating molecules during sample introduction and electrophoresis into an empty electrophoresis buffer is generally avoided if the complex dissociation rate constant is less than $0.105/t$, where $t$ is the time required to separate peaks. This will ensure that no more than 10% of the specifically bound ligand dissociates during the separation [89]. The analyte does not necessarily have to be pure because it is electrophoretically separated from other components of the sample. However, the concentration of the analyte must be known. In contrast, an advantage of migration shift affinity CE is that knowledge of the exact concentration of analyte is not needed even though a number of other requirements must be met for the quantitative uses of this approach (cf. Refs. [5,28,83–86,90–95]).

The importance of the value of the rate constants for compatibility with a given set of CE separation parameters is illustrated by Refs. [26,96]. Useful modelling results regarding the best way to fit binding curves of relevance for CE are covered in Refs. [97,98].

Antigen–antibody interactions have been characterized quantitatively by affinity CE in a number of cases, for example the interaction of anti-dinitrophenyl (DNP) monoclonal antibodies with DNP-containing antigens [26], the interaction of anti-DNA monoclonal antibody from a lupus mouse with double- and single-stranded DNA of different size [16], binding of monoclonal anti-phosphotyrosine antibodies to phosphotyrosine [15], monoclonal antibodies to phosphoserine interacting with phosphovitin [80], interaction of a monoclonal antibody with insulin [55], and characterization of a monoclonal antibody against human serum albumin [79]. In some cases the results have been validated using independent methods [16]. Even though the possibility of visualizing both singly and doubly occupied antibody molecules by electrophoresis has existed for at least 50 years (see Fig. 1) [1] the great majority of studies are not concerned with the binding constants of each antigen-binding site but calculate a single binding constant based on the assumption that the sites are independent and identical. A rigorous treatment of this issue is found in Ref. [26].

## 5. Conclusions

The need for labeled reagents to obtain acceptable detection levels continues to be an obstacle for the wide implementation of CE-based immunoassays. However, the continued efforts within multi-dimensional techniques such as combinations with other separation techniques (e.g. reversed-phase liquid chromatography [62,99]) or developments on the detector side, for example in the use of information-rich detectors (mass spectrometry [100], nuclear magnetic resonance [101]) promise even more rewards from, and potential uses of, antigen–antibody

experiments in capillaries. At the same time the miniaturization of the electrophoretic separation instruments together with improved detectors built with microfabricated devices in mind [102] are also promising for the utility of microelectrophoretic immunoassays in routine clinical chemistry.

## Acknowledgements

This work was partly supported by Lundbeckfonden, The Danish Medical Research Council, Fonden til Lægevidenskabens Fremme, M.L. Jørgensen og Gunnar Hansens Fond, and Apotekerfonden af 1991 (NH).

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