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Review

Capillary electrophoretic immunoassays

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Abstract

Capillary electrophoretic immunoassay (CEIA) has recently emerged as a new analytical technique. CEIA, when combined with sensitive detection methods such as laser induced fluorescence (LIF), offers several advantages over conventional immunoassays. CEIA can perform rapid separations with high mass sensitivity, simultaneously determine multiple analytes and is compatible with automation. The objective of this review is to describe the applications of CE in antibody related studies, focussing especially on recent developments of CEIA technique. The principles for competitive and non-competitive CEIA are described with examples. Several detection methods and various applications are summarized and future developments in CEIA are speculated. CEIA has many potential applications, especially if the throughput is improved by using either multicapillary array or microchips with multiple channels. © 1997 Elsevier Science B.V.

Keywords: Reviews; Antibodies; Antigens

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1. Introduction

Immunoassays are analytical techniques based on the specific immuno-reaction between an antibody (Ab) ad an antigen (Ag), for the determination of either reactant in solution. Immunoassays have become very powerful techniques in clinical, biopharmaceutical, and environmental chemistry because of their unique selectivity, extremely low limits of detection, and applicability to a wide variety of compounds of interest [1]. The unique selectivity of antibodies has enabled immunoassays to measure compounds in trace levels in the presence of potential structurally or chemically similar compounds at much higher concentrations. The high selectivity results from various labelling techniques such as radioactivity, fluorescence, chemiluminescence (CL), or enzyme amplification. Therefore, immunoassay techniques are especially suited for the analysis of analytes at low concentration in complex samples, such as urine or blood with little or no sample preparation. The most common format of conventional immunoassays is to immobilize the Ab (Ag) onto the solid surface of microtiter plates, glass fibers or plastic tubes. Samples containing the analyte (Ag, if Ab is immobilized or vice versa) are made in contact with the immobilized agent. After the separation of bound and free forms of Ab or Ag, quantitation is achieved by measuring the activity of an enzyme conjugated to either Ab or Ag, as in enzyme-linked immunosorbent assay (ELISA) or by measuring the radioactivity as in radioimmunoassay (RIA). Most conventional immunoassays are carried out manually. Tedious processes, slow reactions, poor reproducibility in attaching the reagent to the solid surface and non-specific binding of the reactant to the supporting surface are major problems associated with conventional immunoassays.

Capillary electrophoresis (CE) has proven to be a powerful separation technique for the separation of macromolecules, such as proteins [2]. In conjunction with laser induced fluorescence (LIF) detection, CE can easily determine analytes at 10^{-11} M concentration level, which is comparable to the detection limit of most conventional immunoassays. With the recently developed new technology in CE based enzyme assays, CE can measure enzyme and enzyme labelled analytes at extremely low concentrations [3–6]. Even single enzyme molecule can be detected when LIF detection and enzyme amplification power are combined together [7]. With both superior separation power and high detection sensitivity, CE can separate free Ab and Ag from bounded Ab and Ag rapidly and is especially suitable for immunoassays [8]. CE can, in principle, directly combine immunologic recognition with on-line quantitation, microscale analysis, and automatic instrumentation to offer unique advantages for immunoassays. Actually, CE based immunoassay, called capillary electrophoretic immunoassays (CEIA) or immunocapillary electrophoresis (ICE), has emerged as a new technique carrying out immunologic reactions involving anti-

bodies in CE. In addition to the rapidity and sensitivity, CEIA has other advantages over conventional immunoassays. (1) CEIA requires much less sample and consumes less reagents. (2) CEIA has a simplified procedure and is ready for automation. (3) CEIA allows simultaneous determination of multiple analytes. (4) The fact that, unlike other immunoassay methods, CEIA permits direct visualization of immunocomplex formation and dissociation and simplifies the interpretation of the test results. (5) CEIA can use literally all of the existing CE detection techniques from UV, LIF, to mass spectrometry (MS). (6) In enzyme labelled CEIA, it is also possible to determine the analyte of interest by separating and detecting the substrate and the product of the labelled enzymes. This characteristics offers another choice of selection in addition to the direct separation and detection of the labelled Ab or Ag. For example, in a glucose-6-phosphatase (G-6-PDH) labelled Ab system, it is possible either to separate the free Ab and Ab–Ag conjugate directly or to separate and detect NADH and NAD^+, one of the substrates and products of G-6-PDH, at unique wavelengths. This flexibility provides more choices in selecting different separation conditions for a specific system.

However, there are some limitations to CEIA. The most apparent one is that the current CE system is a serial technique. It can analyze only one sample at a time. This low throughput needs to be overcome before CEIA can be broadly accepted.

This review starts with the analysis of Abs by CE and follows with an in-depth discussion on the research, development and applications of CEIA techniques. An outlook to the future development of CEIA is provided at the end.

2. Analysis of antibodies by CE

CE has demonstrated tremendous power in the characterization of antibodies. CE has been involved in separating antibodies from its fragments and other impurities, in monitoring the process of Ab production, and in determining the binding constant between Ab and Ag. By taking advantage of the specific recognition between Ab and Ag, it is also possible to use Ab as a sample treatment tool for enhancing the sensitivity of analysis.

2.1. Characterizing antibodies by CE

CE is an excellent tool for the separation of biological macromolecules, such as antibodies, for two reasons: the near flat plug flow profile and the small diffusion constant of the antibodies. Both of these characteristics eliminate band broadening. This is because the efficiency of CE separations is inversely proportional to the diffusion constant of the analytes. Thus, CE has high efficiency in the separation of biopolymers, such as Ab, with small diffusion constants. Different separation modes such as capillary zone electrophoresis (CZE), capillary isotechophoresis (CITP), capillary gel electrophoresis (CGE), micellar electrokinetic capillary chromatography (MECC), and capillary isoelectrofocusing (CIEF) have all been used for the separation of antibodies.

In 1989, Takeo et al. separated 1–2 μg of anti-dinitrophenyl antibodies into several hundred homogeneous immunoglobulin G (IgG) spots in two-dimensional electrophoresis using CIEF as the first and slab gel affinity electrophoresis as the second dimensions [9]. These IgGs were grouped into a number of families which are composed of several IgG spots of the same affinity to the hapten but of a different p*I*. It is suggested that each individual family is derived from one monoclonal antibody (MAb) producing cell line. Later, similar work was done with MAb from antihapten antisera with polyacrylamide gel in both the capillary and the slab gel electrophoresis [10]. These researchers demonstrated that CE, along with slab gel electrophoresis is a powerful tool for solving fundamental problems in immunochemistry such as Ab heterogeneity, its hapten binding specificity and Ag-dependent somatic mutation.

Izumi et al. used a flattened perfluorinated poly(ethylene-proplene) tubing (0.2×0.8 mm I.D.) with a hydrophilic coating for the separation of IgG molecules from six different IgG preparations [11]. Bennett et al. analyzed bovine IgG and its light and

heavy chain subunits by SDS-CGE [12]. Martin investigated methods of improving the separation and identification of an IgA and its pepsin fragments by CZE [13]. CZE separation of peptides and small proteins is orthogonal to the traditional reversed-phase HPLC method. Purification of Ab fragments (Fabs) and the Ab light chain requires accurate and informative analysis of highly hydrophobic proteins, which can polymerize and fold to form secondary structures. To facilitate the biological activity of Ab, the buffer composition was kept simple and proper coating of the capillaries was used. It was found that phosphate binds to the Ag-binding site of the IgA with low affinity, and thus phosphate buffer has special effects on the separation.

In addition, CE can determine the formation of hapten–protein antigens for the production of Ab. Frokiær et al. used CE to determine the epitope density and the degree of coupling of Soyasaponin I to Kunitz soybean trypsin inhibitor and bovine serum albumin [14].

2.2. Monitoring Ab–ligand reaction by CE

Lausch et al. used a CE-based affinity assay method for the rapid and sensitive determination of IgG in cultivation media [15]. Protein A conjugated with a fluorescent dye such as fluorescein diisothiocyanate (FITC) or dichlorotriazinyl-amino-fluorescein was used as an affinity ligand. The ligand formed a fluorescent complex with IgG in the sample and rapid separation of the complex from excess protein A was performed by CZE. However, only partial resolution of the zones was achieved when protein A as a whole molecule was utilized. In contrast, baseline resolution of the zones was obtained when recombinant fragments of protein A were used as affinity ligands. IgG concentrations in the range of two orders of magnitude were determined. Due to the specificity of protein A for IgG, analysis can be carried out even in the presence of high concentrations of other components in cultivation media. Thus, the CE affinity assay was successfully applied to monitor MAbs in a cultivation process. Similarly, Reif et al. [16] and Freitag [17] analyzed human IgG in CZE with a 1-min separation time using FITC-marked protein G as affinity probe. The separation is fast enough to prevent the dissociation of the protein G–Ab complex and thus allowed the optimization of the conditions for the affinity reaction and the CE analysis independently. The results of the analysis of real sera containing 0–1000 $\mu g\ ml^{-1}$ IgG were confirmed by the conventional single radial immunodiffusion (SRID) method.

Harrington et al. [18] used CE to characterize enzyme–Ab conjugates. The enzyme (AP), the immunoglobulin G (IgG), and their conjugate (AP–IgG) were separated in less than 5 min.

Heegaard determined the immuno-affinity of Ag–Ab interaction by CE using MAbs against phosphotyrosine as a model system [19]. He evaluated the influence of the interaction kinetics on the peak profiles with addition of phosphotyrosine to the electrophoretic buffer. One of the two Abs that were tested exhibited peak broadening while the other showed no change in peak shape but had a decreased mobility proportional to the amount of phosphotyrosine present. The migration shifts were a consequence of the Ab–Ag complexes having a slower mobility than the non-complexed Ab. On the basis of measuring migration shifts at different Ag concentrations, dissociation constants were estimated and shown to be independent on the applied field strength.

Similarly, Mammen et al. [20] determined the two dissociation constants of the complex between the bivalent anti-DNP rat MAb IgG2b and charged ligands that contained a N-dinitrophenyl group by affinity CE. The influence of the charge on the mobility of the complex between IgG and its ligand(s) was established with singly and multiply charged ligands. The dissociation of monovalent ligands from the Ab occurred non-cooperatively. For certain multivalent ligands, cooperative binding was observed. The charge on the Ab can be obtained by analysis of the electrophoretic mobilities of complexes IgG2bL2, where the ligands L are structurally similar but have different charges (the charges on the ligands were also determined by CE).

2.3. Studying Ab–Ag interaction by CE

In order to understand the required sequences for Ab–Ag interaction, Martin synthesized VH fragments of the α-chain from myeloma McPC6O3, an IgA MAb and studied the folding of IgA variable

regions [13]. Using these fragments and natural light chains isolated from mouse, Martin explored domain association, folding, and stoichiometry by affinity chromatography and CE. It was found that correct folding of the synthetic VH proteins relied on the adoption of a stable secondary structure such as a β-barrel, that formation of a disulfide bond may be necessary, and that correct folding is facilitated by renaturation in the presence of a template.

Zou et al. characterized the immunochemical reaction of human growth hormone (hGH) with its MAb by both perfusion protein G affinity chromatography and CZE [21]. CE confirmed the validity of the affinity chromatographic method that can determine both the biological activity and the stoichiometry of immunological reactions between the Ab and Ag when a stable complex of them can be formed.

2.4. Using Ab for sample processing in CE

Cole and Kennedy used a protein G immunoaffinity capillary chromatography for selective pre-concentration of samples for CZE [22]. Ab was first loaded onto capillaries packed with protein G support to form an immunoaffinity stationary phase. Ag samples were injected onto the column and were selectively retained and pre-concentrated. The Ab and Ag were eluted out and then separated by CZE. Both on-line and off-line combinations were used and a more than 1000-fold pre-concentration (from 1 ml to 1 μl) was achieved. Serum samples spiked with insulin were studied.

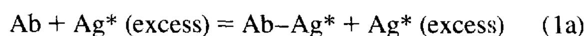
3. Capillary electrophoretic immunoassay

CEIA is a new technique which uses CE to perform the separation steps required in conventional immunoassays. Like conventional immunoassays, CEIA can be performed in either the non-competitive or the competitive format.

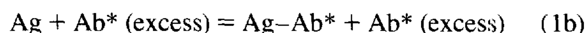
3.1. Non-competitive CEIA

3.1.1. Principle

Non-competitive CEIA is based on the separation of Ab–Ag complexes from free Ab and/or Ag. If the analytes can be directly analyzed by UV or the native fluorescence detection, the amount of the complex formed can be directly quantitated. However, in most non-competitive CEIA, one of the reactant, either Ab or Ag needs to be labelled with a fluorescent tag to enhance the detection sensitivity. An excess amount of the labelled reactant is added to the system to ensure the completion of the reaction. In cases where a label is needed for detection purpose, either Ag or Ab can be labelled. Thus, there are two different formats of non-competitive CEIA depending on which reagent, Ab or Ag is labelled.



$$\text{Ab} + \text{Ag}^* \text{ (excess)} = \text{Ab–Ag}^* + \text{Ag}^* \text{ (excess)} \quad (1a)$$



$$\text{Ag} + \text{Ab}^* \text{ (excess)} = \text{Ag–Ab}^* + \text{Ab}^* \text{ (excess)} \quad (1b)$$

where Ag* and Ab* are Ag and Ab labelled with a fluorescent tag. For the analysis of Ab (or fragment of Ab) in a sample, an excess amount of fluorescently labelled Ag could be added to the sample to form complexes with the Abs present in the sample. The formation of complex should be quantitative and directly dependent on the amount of Ab present in the sample. CE–LIF should in principle reveal two distinctive zones after the separation of the labelled Ag and the Ag–Ab complex. If the Ag–Ab complex is stable on the time scale of the separation, it is possible to quantify the amount of Ab in the mixture based on the amount of complex formed and/or the decrease in the amount of free fluorescently labelled Ag.

3.1.2. Examples of non-competitive CEIA

CE is becoming a widely accepted complementary technique for the characterization of Ab–Ag complexes as well as a good candidate for in-process evaluation of conjugates for immunoassays. Various CE modes, such as CZE, CIEF, CGE and MECC have all been used for these assays. Chen et al. first reported the separation of Ab–Ag using CIEF [23]. Nielsen et al. separated hGH, anti-hGH (IgG) and the Ab–Ag complex in 10 min by CZE [24]. Arentoft et al. and Steinmann et al. used MECC to separate immuno-complexes [25,26]. Liu et al. applied CGE with SDS to separate and analyze MAb chimeric BR96 and the corresponding immunoconjugate with doxrubincin [27].

By using LIF detection with appropriate labelling schemes, Schulzt and Kennedy directly linked together the separation power of CE, the high sensitivity of LIF and the high selectivity of immunoassay [28]. They took advantage of the proven power of LIF capable of detecting low-picomolar concentration of compounds and used the fluorescently labelled immunochemicals as highly selective tagging reagents to ameliorate the problems commonly associated with tagging reactions for analytes at low concentrations. Fig. 1A shows the power of CE separation for the analysis of insulin and Fig. 1B demonstrates the feasibility for the analysis of Ab–insulin complex resulted from a non-competitive CEIA. One of the major advantages of CEIA is the speed. Fig. 1C indicates that similar analysis can be accomplished within 1 min if the potential is increased to 1000 V cm^{-1}. The second advantage of this LIF based method is high mass sensitivity. In the determination of Fab by non-competitive CEIA, a detection limit of 280 zmol (0.14 nl of 2 n*M* sample) Fab was achieved.

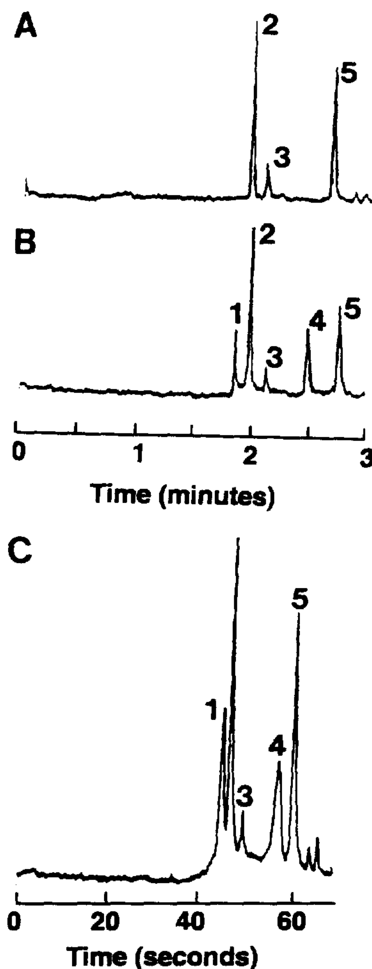


Fig. 1. Electropherograms of (A) 100 n*M* FITC–insulin, (B) 100 n*M* FITC–insulin and 50 n*M* Fab and (C) a rapid separation of mixture containing 50 n*M* FITC–insulin and 25 n*M* Fab at 1000 V/cm. Peaks 2, 3 and 5 are FITC–insulin. Peaks 1 and 4 are due to the formation of complex of Fab with FITC–insulin in peaks 2 and 5, respectively. Reproduced with permission from Ref. [28].

Evangelista and Chen used CEIA with LIF detection to screen Abs for binding to a particular compound (Ag) and then to study the cross-reactivity of different compounds of similar structures towards a specific Ab [29]. A fixed amount of morphine–cyanine dye (Cy5) conjugate was titrated with serial dilution of the Ab and the mixtures were separated and detected by CE–LIF. Fig. 2A shows that among the four commercial Abs screened for binding to morphine–Cy5 the Biodesign morphine N Ab was best suited for the immunochemical binding assays. The other three Abs showed poor or no affinity and/or Ab heterogeneity. Using the Ab selected from the above screening, the cross-reactivity of nine opiate compounds was evaluated. It was found that four morphine based opiates had different affinity towards the Biodesign morphine N Ab while the codeine derivatives and levorphanol did not show any significant cross-reactivity (Fig. 2B).

The above non-competitive CEIA was based on labelled Ags. Karger and co-workers reported a CEIA for Ag using fluorescently labelled Ab, or Ab fragment [30,31]. They quantified hGH by a non-competitive assay using tetramethylrhodamine (TMR)-iodoacetamide labelled anti-hGH Fab′ fragment (TR–Fab′) to react with hGH followed by CIEF to separate different compounds. Following mixing hGH containing samples with excess amount of the purified fluorescently labelled Ab fragment, hGH was analyzed by CEIA after the immunoreaction as depicted in Eq. (1b). The resulting Ab–Ag complex was quantitated after the separation of the excess free Ab by CIEF. As in the above Ag labelled cases, very high sensitivity was obtained in this assay. A $5\cdot10^{-12}$ *M* methionyl recombinant hGH (met-rhGH) sample was determined and a calibration curve covering four orders of magnitude (0.1–1000 ng ml^{-1}) was constructed. Here, electro-

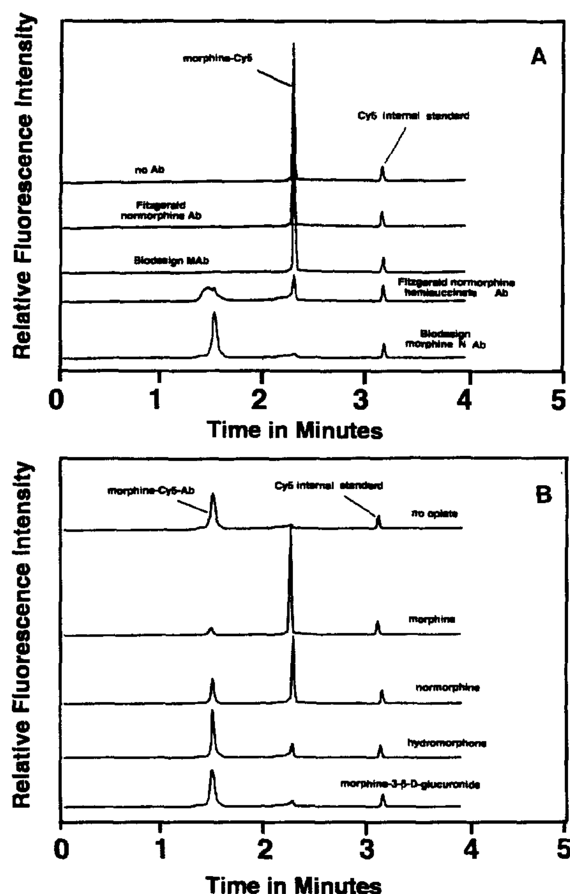


Fig. 2. (A) Screening of four commercial Abs for binding to morphine–Cy5. Mixtures containing 50 μl human IgG diluent, 10 μl $1\cdot10^{-7}$ M morphine–Cy5 and 10 μl 1:50 dilution of morphine Ab. (B) Cross-reactivity of opiate compounds towards morphine N Ab. Mixtures contained 50 μl $1\cdot10^{-5}$ M opiate or blank diluent, 10 μl $1\cdot10^{-7}$ M morphine–Cy5 and 10 μl 1:50 dilution of morphine N Ab. Adapted from Ref. [29].

phoretically different forms of Ag, even when they have the same epitope, can be quantitated simultaneously. Mono- and di-deaminated variants of met-rhGH were detected simultaneously by CEIA with the non-deamidated form of the Ag as separated peaks (Fig. 3). This is another advantage of CEIA over conventional immunoassays, such as ELISA, which cannot discriminate specific and non-specific bindings easily and cannot distinguish the reaction of different Ags sharing the same epitope (cross-reactivity).

Similarly, Chen also used fluorescein-labelled Ab

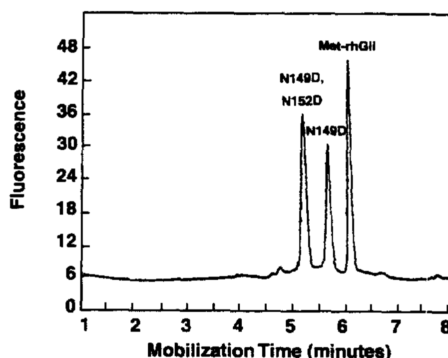


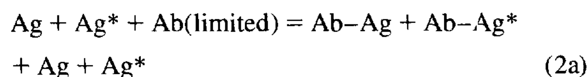
Fig. 3. Simultaneous detection of rhGH variants at 500 V cm^{-1}. An artificial mixture of met-rhGH, the monodeaminated variant (N149D), and the dideaminated variant (N149D, N152D) each at a concentration of 10 ng ml^{-1} was subjected to CIEF with Pharmalyte 3–10 and using TR–Fab'2 as an affinity probe. Each peak corresponds, from left to right, the dideaminated variant, the monodeaminated variant, and met-rhGH. Reproduced with permission from Ref. [31].

for CEIA of low level IgA with LIF detection [32]. In addition to the fluorescent label, he added charge-modification to the label to facilitate the separation of the Ag-bound and free labelled Ab. Both free and Ag-bound labelled Ab species were analyzed simultaneously by CE with LIF detection.

3.2. Competitive CEIA

3.2.1. Principle

Like competitive conventional immunoassays, at least one reagent is limited in competitive CEIA. The labelled reagent (Ag* or Ab*) competes with the analyte (Ag or Ab) binding the limited reagent (Ab or Ag). In the case where a known amount of the labelled Ag* and a limited amount of Ab are mixed with the analyte (Ag), the reaction scheme is:

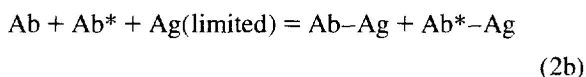


$$\mathrm{Ag} + \mathrm{Ag}^* + \mathrm{Ab(limited)} = \mathrm{Ab{-}Ag} + \mathrm{Ab{-}Ag}^* + \mathrm{Ag} + \mathrm{Ag}^* \quad (2a)$$

The Ag in a sample competes with fluorescently labelled Ag* to form complex with the limited amount of Ab (or fragment of Ab). CE–LIF will separate the mixture and give two distinctive peaks corresponding to the free Ag* (if any free Ag* exist) and the Ab–Ag* complex. The more Ag is present in the sample, the more free fluorescently labelled Ag

will remain and the less complex will be formed between the Ab and the Ag*. Therefore, Ag in the sample can be determined based on comparison of the two peaks with standards. Quantitation can be achieved following the same procedures adopted in conventional competitive immununoassays.

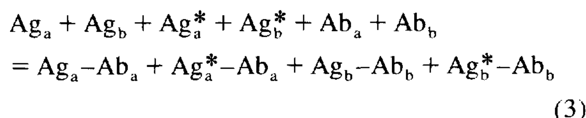
In the case where a known amount of the labelled Ab* and a limited amount of Ag are mixed with the analyte (Ab), the reaction scheme is:



$$Ab + Ab^* + Ag(limited) = Ab–Ag + Ab^*–Ag \quad (2b)$$

The Ab in a sample competes with the labelled Ab* to form a complex with the limited amount of Ag. CE–LIF will separate the mixture and give two distinctive peaks corresponding to the free Ab* (if free Ab* exists) and the Ab*–Ag complex. The more Ab is present in the sample, the more free Ab* will remain and the less complex will be formed between the Ab* and the Ag. Thus, Ab can be determined based on comparison of the two peaks with standards.

As mentioned earlier, CEIA is capable of detecting multiple analytes simultaneously. For example, when two structurally similar Ags are labelled and mixed together with samples containing these two Ags, they will react with the Abs according to:



$$Ag_a + Ag_b + Ag_a^* + Ag_b^* + Ab_a + Ab_b = Ag_a–Ab_a + Ag_a^*–Ab_a + Ag_b–Ab_b + Ag_b^*–Ab_b \quad (3)$$

where Ag_a, Ag_b, Ag_a^* and Ag_b^* are free and the labelled Ags. Ab_a and Ab_b, are Abs to Ag_a and Ag_b, respectively. The above form of reaction assumes no cross-reactivity between the two different antibodies and two different Ags. CEIA–LIF will reveal, in theory, up to four peaks corresponding to free Ag_a^* and Ag_b^* and complexes $Ag_a^*–Ab_a$ and $Ag_b^*–Ab_b$.

3.2.2. Examples of competitive CEIA

In addition to their work in non-competitive CEIA, Schulzt and Kennedy also pioneered the competitive CEIA [28]. As in non-competitive CEIA, speed and sensitivity are the major advantages of competitive CEIA. For example, in the competitive CEIA of insulin, a detection limit of 420 zmol insulin was easily obtained with LIF detector when a 3 n*M* sample was used. When an Ab with higher affinity, a more sensitive detector (1 p*M* detection limit for FITC–Arg), a shorter separation path (2 cm), a smaller I.D. capillary (10 μm) and a higher electric field strength (3 kV cm^{-1}) were used, insulin solution as low as 100 p*M* can be analyzed within 6 s [33]. They also demonstrated that insulin tagged with FITC has very little non-specific adsorption to IgG. This specificity is another advantage of utilizing Ab–Ag interaction for tagging.

Competitive CEIA has the potential for determining multiple Ags simultaneously. Chen and Evangelista developed a competitive CEIA for morphine using cyanine dye, Cy5 labelled morphine as the labelled Ag [35]. Using the same strategy, they extended this assay to simultaneously determining two drugs according to Eq. (3) [36]. In addition to the Cy5 labelled morphine, they labelled phencyclidine (PCP) with another dye, Cy5.5. The high resolving power of CE separated the individual labelled drugs and the Ab–Ag complexes, which were detected simultaneously by LIF. It was found that the free labelled Ags, Cy5–morphine and Cy5.5–PCP were proportional to the amount of each drug present in the urine samples (Fig. 4). Therefore, a reproducible method with a detection limit of 4 n*M* for PCP and 40 n*M* for morphine can be routinely run within 5 min.

Chen and coworkers also developed a competitive CEIA for digoxin [35–38]. In this assay, an oligonucleotide of $(dT)_{10}$ was selected as the charge modulator for to separation purpose [35]. Using serum-based digoxin calibrators, this CEIA method was able to measure digoxin at 0.42 ng ml^{-1} concentration. Alternatively, they used digoxigenin-labelled B-phycoerythrin as the labelled antigen to compete with digoxin and was able to determine digoxin in serum at the clinically useful concentration range of 10^{-9}–10^{-10} *M* by competitive CEIA [38].

Steinmann et al. developed an MECC based competitive CEIA for the analysis of theophylline in serum [26]. Quantitation of CEIA based upon multilevel calibration using the height of the peak produced by the free tracer provided theophylline serum levels in agreement with those obtained by another method.

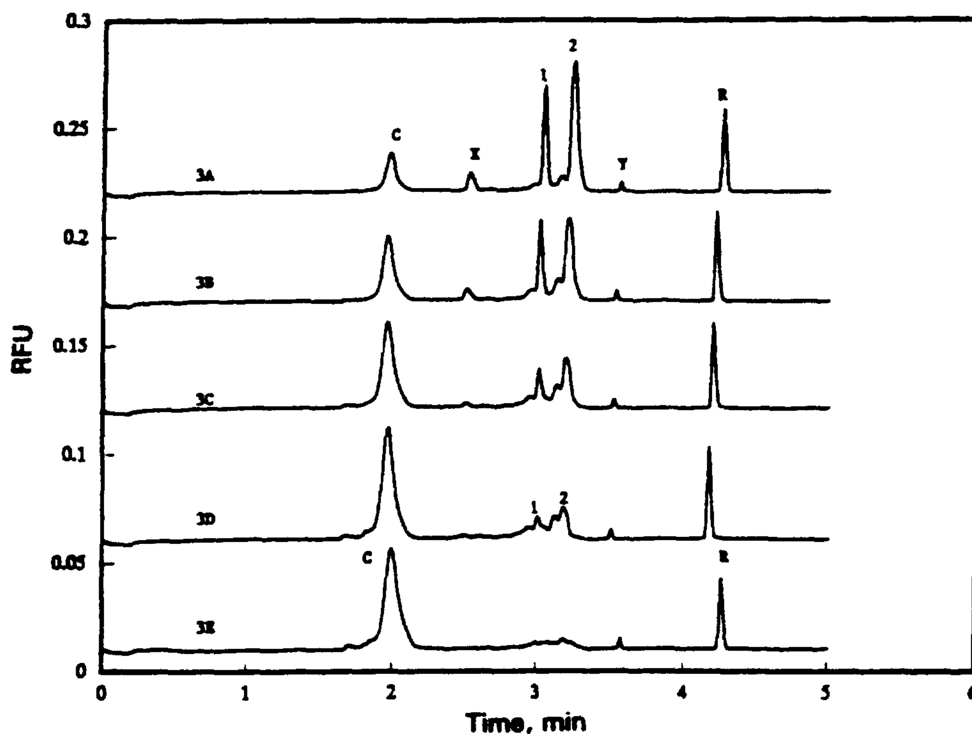


Fig. 4. Simultaneous assay of morphine and PCP in urine by CE–LIF: (A) PCP 24.3 μg ml^{-1}, morphine 285 μg ml^{-1}; (B) PCP 4.9 μg ml^{-1}, morphine 57 μg ml^{-1}; (C) PCP 1.9 μg ml^{-1}, morphine 23 μg ml^{-1}; (D) PCP 0.98 μg ml^{-1}, morphine 11 μg ml^{-1}; and (E) drug free urine. Peaks: 1, Cy5.5–PCP; 2, Cy5–morphine; c, Ab-labeled Ag complexes; R, Cy5 diacid (internal standard); X, immunoreactive impurity associated with Cy5.5–PCP; Y, non-immuno reactive impurity. Reproduced with permission from Ref. [36].

3.3. On-line immunoassay

In most of the above CEIA, CE functioned primarily as a separation tool to separate the Ab–Ag complexes from free Ag and/or Ab after the immuno-reaction was carried out off-line. In order to automate CEIA, it is desirable to have the immunoreaction and sample analysis on-line. None of the existing reports on CEIA has both the reaction and the analysis done in a single capillary. However, there are some interesting on-line related work.

3.3.1. On-line affinity capture

Phillips and Chmielinska immobilized MAb fragments directly onto the internal surface of the capillary [39]. Only a small segment of the capillary was immobilized with the Ab. The Ag, cyclosporin A and its metabolites are retained by the immobilized Ab when they pass through the capillary during initial incubation and are detected during the electrophoresis process. However, even though it involves on-line immuno-reaction of Ab and Ag, this work is solely utilizing the binding specificity of Ab to pick up the analyte of interest from a complex sample.

3.3.2. On-line Ab and ligand binding

Reif et al. reported using FITC-labelled protein G as an affinity ligand in affinity CE with LIF detection [16]. Abs from human sera (h-IgG) reacted with FITC-labelled protein G through the Fc fragment. CE was able to separate and quantify the complexes after the affinity reaction. This analysis was fast enough to prevent their dissociation during the measurement and thus allowed the optimization of experimental conditions for the affinity reaction and the CE analysis independently. Alternatively, h-IgG

containing serum samples and the FITC-labelled protein G were simply injected in consecutive zones into the CE capillary. Within 2 min, the affinity complexes were resolved and the IgG content of the serum quantified. The injection sequence was of no consequence and the measurements agreed well with those found in a SRID assay. In addition, FITC-labelled protein G-tagged anti-h-IgG1 antibodies were used to detect the specific Ag of the involved Ab, namely, h-IgG1, in human sera.

3.3.3. On-line immunoassays

Tao et al. used a system as shown in Fig. 5a to carry out on-line CEIA [33,34]. A capillary mixing chamber and the CE capillary are linked together with the interface controlled by a 'gating flow'. Ab and labelled Ag along with Ag samples mix together and react before CE injection. The reaction mixture is carried away by a constant gating flow except during injection time when the gating flow is stopped and the sample is pushed into the CE capillary. The Ag (insulin) samples can be analyzed every 5 s with this on-line system (Fig. 5b). Although the immuno-reaction and the analysis steps are carried out independently as in other CEIA, there are several major differences between this on-line system and other CEIA. First, the Ag and Ab are pumped in continuously and the mixing and reaction are continuous as the pumps are on. Second, the on-line injection is almost instantaneous which makes it feasible to link it with fast analysis techniques. Third, this system can be automated. However, one limitation of this system is that it is suited primarily to high affinity Abs with fast reaction kinetics. Abs with slow reaction kinetics tend to give more broad and less distinctive peaks [19]. These less reactive Abs need more time to form complexes with the Ag and thus cannot be assayed in this way unless more reaction time is allowed before the reaction mixture is introduced into the capillary. Another drawback of this on-line technique is the loss of sensitivity. The limit of detection (LOD) in this system is at least three time higher than that obtainable from the off-line CEIA. This sensitivity loss results from the dilution in the mixing reagents [33,34]. Therefore, the convenience of the sample injection in this system is obtained at the price of sensitivity. It is more advantageous to have the immuno-reaction and the CE analysis in the same capillary.

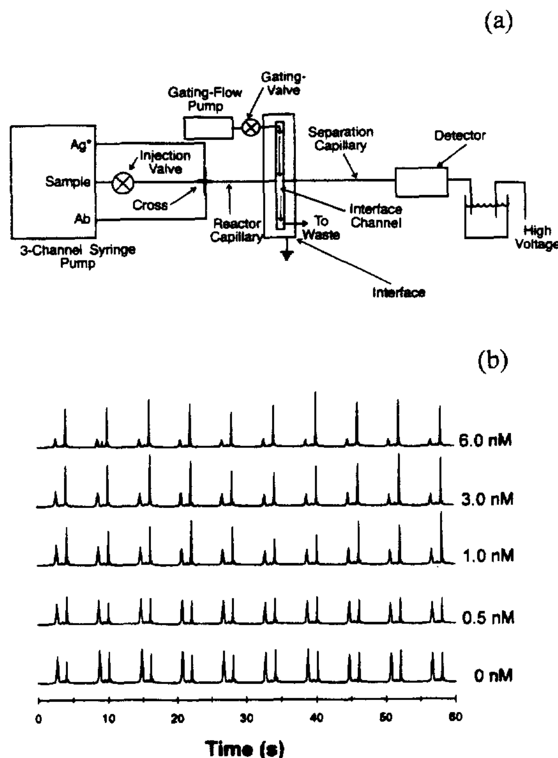


Fig. 5. (a) A system for on-line CEIA and (b) the results from this on-line immunoassay. Reproduced with permission from Ref. [34].

Rosenzweig and Yeung reported another on-line CEIA between G-6-PDH and Ab coated particles based on the agglutination of Ab-coated particles in the presence of macro-molecular Ag (usually a protein) [40]. G-6-PDH was injected and migrated in a buffer containing Ab-coated particles, with which G-6-PDH started to form Ab–Ag complex immediately after the injection. The Ag–Ab complex was accumulated when the potential was turned to zero, which allowed the analysis of Abs with slow reaction kinetics. The accumulated complex migrated electrophoretically towards the detection window and was measured by a particle counting technique after the power was resumed. An LOD of 620 molecules of G-6-PDH (1 zmol) was obtained in this on-line assay.

Liu et al. reported an alternative approach to

conducting on-line CEIA [41]. The Ab and the fluorescently labelled Ag solutions are placed in individual vials and injected into the capillary separately. Both the reaction between the injected Ab and Ag and the separation of the free fluorescently labelled Ag and the Ag–Ab complex occur in the capillary due to the mobility differences between Ab and Ag and between the free Ag and the complex. There are several advantages in this on-line CEIA. (1) The reaction, separation and detection are all carried out in a single capillary. (2) Ab and Ag are in separate vials and each of the reagents can be used for multiple samples. Each assay consumes only nano-liters of Ab and Ag solutions. (3) There is little waste and no dilution effect in the reaction mixture. (4) The automatic injection by the instrument increases the precision of sample injection and avoids the error in manual pipetting. (5) The automation of the CE instrument has the potential to allow the on-line CEIA to become an automatic immuno-analyzer for clinical diagnosis.

3.4. Multi-capillary and microfabricated immunoassay systems

As demonstrated in the above examples, CEIA has great potential to be fully automated. However, it is compulsory to increase the throughput of this method before CEIA can be widely accepted by the immunoassay community. Two major approaches have been conducted to improve the throughput by using either multi-array capillaries or microfabricated multi-channel devices. Yang et al. employed a multi-capillary scanner system in which the individual members of a capillary array are stepwise illuminated by a diode laser reflected from a galvometric scan mirror [42]. Competitive immunoassays were developed on this system for several analytes at clinically useful levels. Koutny et al. recently reported an CEIA on a microchip with a single channel micromachined into a fused-silica substrate [43]. Fig. 6 illustrates the channel geometry on the microchip and the injection process for the offset pinched injector. This miniaturization allowed rapid separations (15 s) and quick determinations of cortisol in blood serum over the range of clinical interest without sample preparation steps. Such microchips are attractive because of their small size, ruggedness and amenability to automation. Arrays of channels can be easily fabricated on a single substrate and operation of multiple separation systems can be conducted in parallel. For CEIA, this offers the potential for high throughput and multiple assay capability. Results from CEIA on multichannel microchips have been reported [44].

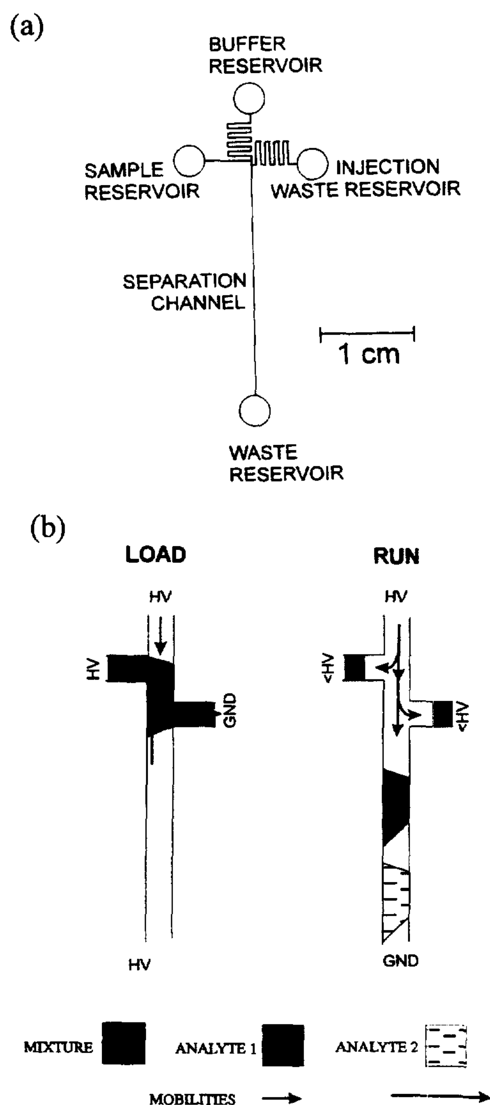


Fig. 6. (a) Scale drawing of the channel geometry on the microchip and (b) schematic drawing of the injection process for the offset pinched injector. Reproduced with permission from Ref. [43].

4. Detection methods in CEIA

Immunoassay detection can be roughly classified, depending on whether a label is used, into two groups: labelled and non-labelled immunoassays. Even though most common immunoassays involve labels, non-labelled detection techniques such as UV–Vis are still useful. Among the labelled immunoassays, radioimmunoassay (RIA), enzyme immunoassay, such as the enzyme-linked immunosorbent assay (ELISA), the enzyme-monitored immunotest (EMIT), fluorescence immunoassay, CL immunoassay, and electrochemical immunoassay are the most common ones. In CEIA, the primary detection methods used so far are UV–Vis and LIF. Particle counting is another unique detection method used for CEIA.

4.1. UV absorbance detection

In early CEIA work, UV absorbance detection was used for detecting the Ab and Ag interaction because of its simplicity, economy and availability. However, the major disadvantage of the UV detector is the lack of sensitivity. The minimum detectable concentration by UV absorbance is around 10^{-6} *M* [45]. For many immunoassays this is several orders of magnitude above the required sensitivity levels. Only those interactions involving two macromolecules can be monitored with this detection method. For example, Neilson et al. monitored the immunological reaction between hGH (hGH ca. 22 124 u) and its Ab, anti-hGH (IgG, ca. 150 000 u) at 200 nm [24]. Harrington et al. monitored the conjugation between enzyme, alkaline phosphatase (AP, ca. 140 000 u) and IgG (160 000 u) at 280 nm [18].

However, many Ags are small molecules without strong UV absorbance. In these cases, some alternatives have to be considered, such as using sample pretreatment or indirect reaction. For example, by using MAbs coated capillary, Phillips et al. were able to pre-concentrate Ag in the capillary and achieve a detection limit of 5 ng ml^{-1} cyclosporin in tears with simple UV detection at 214 nm [39]. Liu et al. detected the substrate and product (NAD^{+}/NADH) of the enzyme (G-6-PDH) label for CEIA determination of digoxin [46]. Since these species do not interfere with the immunoreaction directly, they can be present at relatively high concentrations to be monitored by UV absorbance.

A more general approach to improve sensitivity is to select a more sensitive detection method such as LIF.

4.2. LIF detection

CE with LIF detection may detect fluorescently labelled analytes in concentrations as low as 10^{-11}–10^{-12} *M* [47]. However, direct analysis of real samples at these concentration levels requires derivatization of the analyte with fluorescent tags. This can be problematic because of non-quantitative derivatization in complex sample matrices, as well as detector interferences resulting from an excess of the labelling reagent. In contrast to covalent derivatization, complex formation based on biospecific recognition has traditionally been used in complex systems. Fluorescent labelling of one of the interacting molecules in a biospecific complex can provide a sensitive probe for trace determination of an analyte.

Schultz and Kennedy were the first to combine together CE (high resolving power), LIF (high sensitivity detection) and immunoassay (high selectivity) [28]. Since then, most CEIA were conducted using this detection method (Table 1).

However, there are some challenges associated with LIF detection method. For example, the heterogeneity of the labelled Ag is one potential problem. If the label is a macromolecule, such as B-phycoerythrin, it has multiple sites for digoxigenin to bind and can result in multiple bindings. This heterogeneity will generate multiple peaks when the Ab is at high concentration and has the tendency to bind multiple Abs. Fig. 7 shows the separation of the labelled Ag, digoxigenin–B-phycoerythrin (D–BP) and the immuno-conjugate prepared with fixed concentration of D–BP and an increasing amount of Ab. The existence of heterogeneity in D–BP, which is about 5 fold the size of the Ab (Fab, goat antiserum to digoxin) becomes apparent when an excess amount of Ab is added to the reaction mixture [38].

Another challenge is the separation of the labelled Ag or Ab from the Ag–Ab complex. In case the separation is not straight forward, a charge

Table 1
Examples of LIF detection in CEIA

Label	Antigen	Antibody	Labeled	Assay format	Detection $\lambda_{exc}/\lambda_{em}$ (nm)	LOD	Ref.
FITC	Insulin	Mouse IgG, Fab	Ag	Competitive and non-competitive	442/510[a]	$3\cdot10^{-9}$ *M*	[28,33] [34,52]
Fluorescein	Chloramphenicol	Rabbit anti-chloramphenicol antiserum	Ag	Competitive	488/560	0.1 ng ml^{-}	[51]
Fluorescein	Cortisol	Mouse monoclonal anti-cortisol, Fab	Ag	Competitive	488/520	10^{-8} *M*	[49]
Fluorescein	Cortisol	Rabbit polyclonal anti-cortisol	Ag	Competitive solution phase	488/520	10^{-8} *M*	[50]
FITC	IgG	Anti-human sera (anti-h-IgG)	Ab and Ag	Non-competitive	488/520	10^{-9} *M*	[16]
TR-IAM	hGH	Mouse Mab (anti-hGH), Fab′	Ab	Non-competitive	488/580	$5\cdot10^{-10}$ *M*	[31]
Fluorescein	Theophylline	Mouse MAb theophylline antiserum	Ag	Competitive	488/520	<10 μg ml^{-1}	[26]
Cyanine dye, Cy5	Morphine	Biodesign morphine n	Ag	Non-competitive and competitive	632.8/665	$5\cdot10^{-8}$ *M* 14 ng ml^{-1}	[35]
Cy5 and Cy5.5	Morphine and phencyclidine	Human antisera, IgG	Ag	Competitive	632.8/690	$4\cdot10^{-9}$ *M*/ $4\cdot10^{-8}$ *M*[b]	[36]
B-phycoerythrin	Digoxin	Goat antiserum to digoxin, Fab	Ag	Competitive	543.5/580	10^{-10} *M*	[38]
TMR	Digoxin	Anti-digoxin, Fab	Ag	Competitive	543.5/590	10^{-10} *M*	[35]
fluorescein[c]	IgA	Goat antiserum to IgA, F(ab′)$_2$	Ab	Non-competitive	488/520	$7\cdot10^{-10}$ *M*	[32]

[a] Also used was a 50-W Hg–Xe arc lamp using a 470-nm interference filter.
[b] For phencyclidine and morphine, respectively.
[c] Fluorescein label was modified by reacting with succinic anhydride to form succinyl FITC–F(ab′)$_2$.

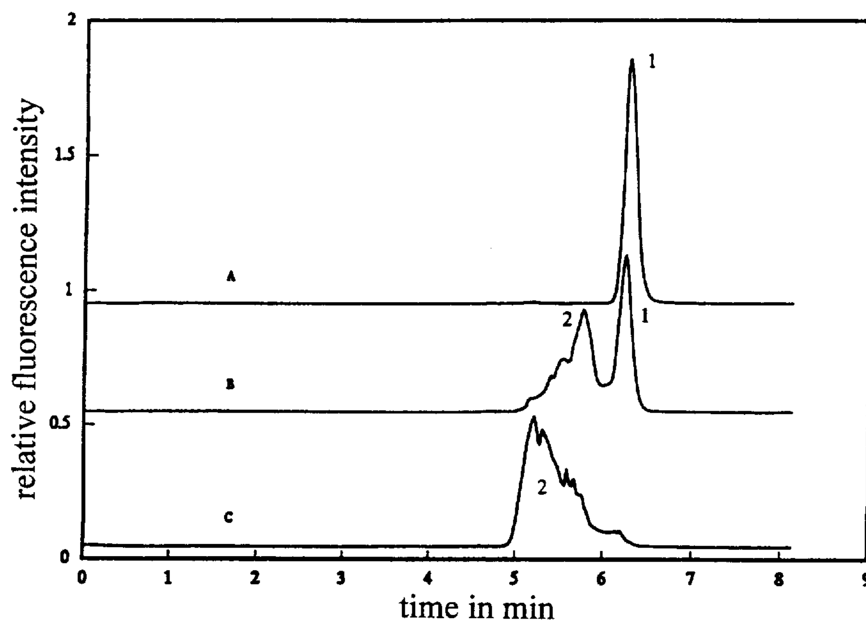


Fig. 7. Electropherogram of CE–LIF of digoxigenin–B-phycoerythrin (D–BP) and its reaction with Ab. Peaks: 1 = Ag–Ab complex of the Fab and D–BP; 2 = D–BP. (A) D–BP: 0.5 μg ml^{-1}; (B) reaction mixture between D–BP (0.5 μg ml^{-1}, 100 μl) and Fab Ab to digoxin (5 μg ml^{-1}, 10 μl); (C) reaction mixture between D–BP (0.5 μg ml^{-1}, 100 μl) and Fab Ab to digoxin (10 μg ml^{-1}, 10 μl). Adapted from Ref. [38].

modulator is needed to facilitate the separation. The charge modulator has unique charge characteristics and may alter the mobility of the compound to which it is bound. Both Ag and Ab can be modified with a charge modulator. For example, Chen and Sternberg used a conjugated oligonucleotide as a spacer between the label, TMR and digoxigenin to modulate the electrophoretic mobilities of the Ag in the CEIA of digoxin to ensured the separation of the Ag and the Ab–Ag complex [35]. For CEIA in the presence of potential interfering substances, a unique and easy-to-detect label on the immunochemical reactants, such as Ab, is required. For many protein Ags, the Ab–Ag complex may not be easily separated from the free Ab, or Ag. It is necessary to modulate the charge and electrical mobility on the Ab to facilitate the separation. Chen modified a fluorescein-labelled Ab with succinyl groups, each of which resulted in a net gain of two charges at neutral pH for every lysine residual group (ε–NH_2) on the Ab [32]. The functional activity of the resulting modified and fluorescently labelled Ab was preserved in CEIA.

Recently, Nashabeh et al. reported a CEIA in sandwich format to both solve the heterogeneity problem of Ab and Ag and provide charge modulation [48]. A pair of MAbs that are directed toward different sites of the Ag (i.e. matched pair) were used simultaneously. One of these Abs carries a detectable label while the other Ab is modified to be highly charged. When both Abs are added in excess to a matrix containing the Ag, a sandwich complex is formed and carries both a distinctive label for detection and a unique mobility for separation.

4.3. Particle counting

A laser-based particle counting micro-immunoassay system was developed for ultra sensitive analysis of proteins in a capillary by Rosenzweig and Yeung [40]. This technique is based on the agglutination of Ab-coated particles in the presence of an Ag (usually a protein). The particles generate scattering lights when they migrate past the detection window, where a laser beam irradiates continuously. The light scattering events generated by these particles will be counted where those produced by unreacted particles are electronically rejected. Using this method, they were able to analyze intracellular components at the zeptomolar (10^{-21} mol) level. An LOD of 620 G-6-PDH molecules (1 zmol) was achieved from the on-column assay of single human erythrocytes.

Both off-column and on-column assays were tested with this detection method. For the off column particle counting assay, a linear dynamic range of two orders of magnitude was obtained. With on-column particle counting CEIA, extremely small amounts of analytes can be detected. The LOD in the on-column assay is four times lower than that of the off-column assay and 20 times lower than that of the corresponding ELISA method.

5. Applications of CEIA

Immediately after Schultz and Kennedy's work on CEIA in a relatively 'clean' model system in 1993 [28], several groups further developed this technique and applied it to complex biological samples.

5.1. Trace analysis in biological samples

5.1.1. Analytes in urine

Chen and Evangelista analyzed morphine and other opiates in urine with CEIA in 1994 [35,36]. The concentrations of most of the frequently abused drugs and their metabolites in urine are in the range of 1 μM to 1 nM. The drugs and their metabolites can be functionalized and labelled with cyanine dye as the competing species for immunoassays. The fluoro-labelled drug species, along with an internal standard, should make possible simultaneous screening of several drugs in a single sample. For example, morphine and PCP in urine can be analyzed by CE–LIF simultaneously (Fig. 4).

5.1.2. Analytes in serum

Chen et al. determined nanomolar levels of digoxin at clinically useful concentration levels of 10^{-9}–10^{-10} M in human serum by competitive CEIA using fluorescently labelled Ag, D–BP [37,38]. Liu, et al. also analyzed digoxin in human serum by CE after an EMIT reaction [46]. CE separated the enzymatic reaction product (NADH) and remaining substrate (NAD^+), together with internal standard,

p-nitrophenol. The digoxin level in human serum was determined by comparing the peak area ratio of NADH and p-nitrophenol to the ratios established by digoxin standards. Both an internal calibration plot for NADH and a dose–response curve for digoxin in serum were constructed. It was demonstrated that this method was very specific and had little interference.

Steinmann et al. studied the feasibility of using MECC based CEIA with LIF detection [26]. They constructed a calibration curve covering the therapeutical range (10–20 $\mu g\ ml^{-1}$) and quantitatively determined the concentration of theophylline in serum using SDS as the surfactant to form micelles. Their data demonstrated that the Ab–Ag complex was sufficiently stable at alkaline pH in the presence of dodecyl sulfate, which is known not to favor immuno-reactions. This stability is the pre-requisition for differentiation between different drug levels in this free-solution competitive immunoassay.

Schmalzing et al. analyzed cortisol in serum using competitive CEIA [49,50]. At first, Fab fragments from mouse anti-cortisol MAb were used to form a complex with cortisol in serum samples without prior sample clean-up [49]. CE separated the free and bound labelled Ag and quantitated cortisol in serum. Later, polyclonal Ab was used for the same assay and similar results were obtained. The results are both accurate and precise in the clinically significant concentration range [50].

5.1.3. Analytes in milk

Blais et al. reported the determination of chloramphenicol at the level of 0.1 $ng\ ml^{-1}$ in milk using competitive CEIA [51]. A pre-treatment of the chloramphenicol spiked samples involved first extracting the milk with ethyl acetate then drying the organic phase under nitrogen and finally reconstituting the sample in buffer before the immuno-reaction. The analyte was then allowed to compete with a chloramphenicol–fluorescein conjugate for binding to polyclonal anti-chloramphenicol Ab. The bound and unbound forms of Ag were separated and quantitated by CE. They also confirmed the specificity of the reaction between chloramphenicol and Ab by adding structural analogs of chloramphenicol into the system.

5.1.4. Analytes in tears

In comparison with other body fluid such as urine and blood, tears are much more difficult to analyze because of their small volume and complexity. CE technique is advantageous for this analysis because it can analyze nano-liter samples. Phillips and Chmielinska reported the analysis of Cyclosporin A in tears by CEIA and demonstrated the advantage of CE technique over HPLC for simultaneously detecting toxic metabolites of cyclosporin in the same sample [39].

5.1.5. Insulin secretion from single islets of Langerhans

Schultz et al. used competitive CEIA to determine insulin secretion from single islets of Langerhans [52]. The assay had an LOD of about 6 fg (3 nM). The insulin content of single islets of Langerhans determined by this method was in good agreement with those studied in the literature. In addition, Schultz et al. quantitated the glucose-stimulated insulin release from single islets and the results also agreed with those studied in the literature.

5.2. Drug screening

Evangelista and Chen developed a competitive CEIA technique to determine morphine. The LOD was $5 \cdot 10^{-8}\ M$ (14 $ng\ ml^{-1}$) morphine and was well below the established cut-off concentration of $1 \cdot 10^{-6}\ M$ (300 $ng\ ml^{-1}$) for positive results in forensic urine drug testing [35,36].

5.3. Characterizing Ab–Ag interaction

Liu et al. used CE to study the characteristics of the Ab–Ag immuno-reaction between an MAb BR96 and the Ag doxorubincin with both UV and LIF detection [26]. Both the MAb and the immuno-conjugate were studied in their native, denatured and reduced states. Separation of the denatured Ab–Ag complex revealed six peaks which were confirmed to correspond to all the possible conjugated species as expected. Analysis of the resulting 'fingerprint' maps indicated that the light, heavy, and light–heavy chain conjugates were the predominant species. Thus, SDS based CGE offers an alternative approach to the conventional slab gel electrophoresis techniques with

rapid, efficient, sensitive, and accurate information for the analysis of Ab and its conjugates.

6. Future and conclusions

6.1. On-line CEIA

To fully take advantage of the small sample requirement of CE, it is desirable to carry out CEIA on-line, which means both the immuno-reaction and the assay are conducted inside the same capillary. For example, Halsall et al. reported an enzyme-linked capillary immunoassay with an LOD of 2800 molecules [53]. With electrochemical detection and sequential saturation technique, Kaneki, et al. analyzed digoxin at 10 pg ml^{-1} level and constructed a calibration curve up to 1000 pg ml^{-1} [54]. Jiang et al. further extended this work for the determination of atrazine in water at 0.1 $\mu g\, l^{-1}$ level with a calibration curve linear to 10 $\mu g\, l^{-1}$ [55]. de Frutos et al. reported an HPLC based enzyme-amplified capillary immunoassay with zeptomole detection [56].

All of the above work in capillaries has established the basis for on-line CEIA. More research is expected in this area.

6.2. More detection methods

LIF is by far the primary detection method in CEIA. It is expected that other sensitive detection methods will be used for CEIA. For example, electrochemical detection has been used for capillary immunoassays and can be readily used for CEIA. MS is another detection candidate for CEIA. With the amplification power of enzymes, even simple UV detection can be used in CEIA. Liu et al. recently reported their work in conducting enzyme based CEIA for the analysis of proteins and Abs [57]. The Ab–enzyme conjugates were used to recognize the antigenic proteins in order to improve detection and identification of individual proteins in complex sample matrices, such as biofluids. They demonstrated the feasibility of conducting CEIA in different CE modes, such as CZE, CIEF, and CGE with UV and CL detection. This method demonstrated high selectivity in recognition (from Ab), high sensitivity detection (from enzyme amplification), and unique detection (from the selection of enzyme substrate).

6.3. Multicapillary arrays and microchip based CEIA

The major drawback of CEIA is its inability to run multiple samples simultaneously. It is expected that research will be focused on developing more economic and more convenient CEIA systems which can handle multiple samples simultaneously. A multiple capillary system has already been marketed by Beckman for clinical diagnosis. The microchip with multiple microfabricated channels will improve the throughput of CEIA significantly [58].

7. Abbreviations

Ab/Abs	Antibody/antibodies
ACE	Affinity capillary electrophoresis
Ag/Ags	Antigen/antigens
AP	Alkaline phosphatase
CE	Capillary electrophoresis
CEIA	Capillary electrophoretic immunoassays
CGE	Capillary gel electrophoresis
CIEF	Capillary isoelectric focusing
CL	Chemiluminescence
CITP	Capillary isotechophoresis
CZE	Capillary zone electrophoresis
D–BP	Digoxigenin–B-phycoerythrin
ELISA	Enzyme-linked immunosorbent assay
EMIT	Enzyme-monitored immunotest
FIA	Flow injection analysis
FITC	Fluorescein isothiocyanate
G-6-PDH	Glucose-6-phosphatase dehydrogenase
hGH	Human growth hormone
HMPC	Hydroxypropylmethyl cellulose
IgG	Immunoglobulin G
IA	Immunoassay
LIF	Laser induced fluorescence
LOD	Limit of detection
MAb/MAbs	Monoclonal antibody/antibodies
MS	Mass spectrometry
NAD^+	β-Nicotinamide adenine dinucleotide

NADH	Reduced β-nicotinamide adenine dinucleotide
PCP	Phencyclidine
RIA	Radioimmunoassay
SDS	Sodium dodecyl sulfate
SRID	Single radial immunodiffusion
TMR	Tetramethylrhodamine
TR-IAM	Tetramethylrhodamine-iodoacetamide

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