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Review

# Capillary electrophoresis-based immunoassay

W.S.B. Yeung[a,*], G.A. Luo[b], Q.G. Wang[b,1], J.P. Ou[a,2]

[a] *Department of Obstetrics and Gynaecology, The University of Hong Kong, Queen Mary Hospital, Pokfulam Road, Hong Kong, PR China*
[b] *Department of Chemistry, Tsinghua University, Beijing 100084, PR China*

**Abstract**

Capillary electrophoresis-based immunoassay (CEIA) is a developing analytical technique with a number of advantages over conventional immunoassay, such as reduced sample consumption, simpler procedure, easy simultaneous determination of multiple analytes, and short analysis time. However, there are still a number of technical issues that researchers on CEIA have to solve before the assay can be more widely used. These issues include method to improve the concentration sensitivity of the assay, requirement for robust separation strategy for different analytes, and method to increase the throughput of the assay. The approaches to solve these issues are reviewed. Several studies have been devoted to develop general separation strategies for CEIA, and to enhance the sensitivity of detection. The recent development of microchip-based CEIA is encouraging and is likely to address more drawbacks of CEIA, particularly on the throughput issue.


*Keywords:* Review; Capillary electrophoresis; Immunoassays

**Contents**



## 1. Introduction

Capillary electrophoresis-based immunoassay (CEIA) is an analytical technique that combines the separation power of CE and the ligand specificity of antibody for detection of analyte in complex biological samples. Nielsen et al. [1] first used CE to separate human growth hormone (hGH) from its immunocomplexes formed with a monoclonal anti-hGH antibody. Subsequently, Schultz and Kennedy [2] demonstrated the concept of competitive and non-competitive CEIA. Since then, many reports described the optimization and use of this technique to determine the concentration of different antigens in various samples. Results obtained from CEIA correlate well with conventional immunoassay [3].

Compared with conventional immunoassays, CEIA offers a number of advantages. The main advantage is that CEIA is

* Corresponding author. Fax: +852-28-550-947.
*E-mail address:* wsbyeung@hkucc.hku.hk (W.S.B. Yeung).
[1] Present address: Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602-5700, USA.
[2] Present address: Department of Obstetrics and Gynaecology, First Affiliated Hospital of Sun Yat Sen University of Medical Sciences, Guangzhou, PR China.

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much simpler than the conventional manual immunoassay, yet it allows flexibility in developing simple, custom-made single-analyte and multi-analyte immunoassay in various formats; such flexibility is unavailable to conventional immunoassay using automatic immunoassay machine available in the market. This flexibility is attributed to the ability of CE to separate antigen/antibody from their immunocomplex using a wide varieties of mechanisms (see below). The other advantages include: (1) CEIA requires less sample and consumes less reagents; (2) CEIA allows relatively easy simultaneous determination of multiple analytes [4]; (3) the assay time per sample is faster because the immuoreaction occurs in solution, which allows rapid reaction by solution phase kinetics; and (4) CEIA permits direct visualization of immunocomplex formation and dissociation and simplifies the interpretation of the test results [5–7].

This review intends to update the development of CEIA, with particular attention to the various approaches for improving the performance of the technique. As there is an excellent review in this journal previously [7], our focus will be mainly on references published after 1997. Interested readers are also referred to more recent review by Schmalzing et al. [8].

## 2. Principle of CEIA

Like immunoassay, CEIA utilizes the specific binding of an antibody to a particular antigen to be analyzed. CE is then used to separate the free antigen/antibody from the immunocomplex. CEIA can be performed in either competitive or noncompetitive manner. In the early CEIA studies, the competitive format was more often studied because of easiness in preparing a uniformly labeled antigen. Noncompetitive format are now more commonly used due its larger linear dynamic ranges [9] and higher sensitivity [10] than the competitive format.

Non-competitive CEIA or affinity probe capillary electrophoresis (APCE) involves the quantification of the immunocomplex, which is directly proportional to the amount of analyte in the reaction mixture. Usually the antibody is labeled [4,11]. Labeling can be done before or after the immuno-reaction. An excess amount of the labeled antibody is added to the system to ensure the completion of the reaction.

$$\text{antigen} + \text{antibody} * (\text{excess}) \leftrightarrow \text{immuno-complex}*$$
$$+ \text{antibody} * (\text{excess})$$

where antibody* is the labeled antibody. With detector specific for the label used, CE should in principle reveals two peaks, corresponding to the excess free antibody and the immunocomplex. Similarly the quantity of an antibody can be determined using labeled antigen.

In competitive CEIA, the concentration of one of the reagents is limited. Usually a labeled antigen or analogue competes with the unlabeled antigen (analyte) in the sample for binding to a limited amount of the corresponding antibody.

$$\text{antigen} + \text{antigen}^* + \text{antibody(limited)}$$
$$\rightarrow \text{immunocomplex} + \text{immunocomplex}^*$$
$$+ \text{antigen} + \text{antigen}^*$$

The concentration of antigen in the sample is directly proportional to that of the free antigen* but inversely proportional to that of the immunocomplex*. The concentration of the antigen in the sample can be determined by comparing the intensity of the signal from the antigen*, immunocomplex* peak or ratio of the two with that from the antigen standards of known concentration. Taylor et al. [12] reported that the detection limit of a competitive CEIA for estradiol was 310 p*M*, which is less sensitive than that in the conventional assay. They concluded that for assays requiring low detection limit, a preconcentration of labeled tracer is necessary before detection in CEIA.

## 3. Antibody in CEIA

Antibody is an important component in CEIA. For noncompetitive CEIA with antibody labeled as tracer, electrophoretically homogeneous tracer is usually required. Heterogeneous antibody would lead to broad or multiple peaks in CE separation, and make separation of immunocomplexes from free tracer and, thus, quantification difficult. The heterogeneity of antibody comes from two sources. First the number of immunocomplexes in CEIA increases with the number of antibody species present in the reaction mixture [11]. Therefore, monoclonal antibody with only one antibody species is preferred to polyclonal antibody with a number of antibody species. Despite this, each antibody produces two immunocomplexes, one binds to one molecule of antigen and the other to two molecules of antigen. Second, each antibody molecule usually contains more than one sites that can be labeled with a probe. Different antibody molecules may contain different numbers of label.

To eliminate the first source of microheterogeneity of antibody, Fab and Fab′ fragment of monoclonal antibodies have been used for CEIA and produced results that were superior to those obtained using whole antibody [11,13,14]. Antibody fragments, Fab [2] and Fab′ [14] can be generated by papain and pepsin digestion of the intact antibody, respectively (Fig. 1). Each molecule of these antibody fragments binds to only one molecule of antigen, simplifying the electropherogram and resulting in sharper peaks. Their usage allows analysis of multiple analytes within one separation [15].

DNA technology has been used to limit the number of label in each antibody fragment. Hafner et al. [16] described a method to prepare a uniformly labeled probe for APCE using digoxin as a model analyte. In this method, the smallest construction of an antibody that retains the complete

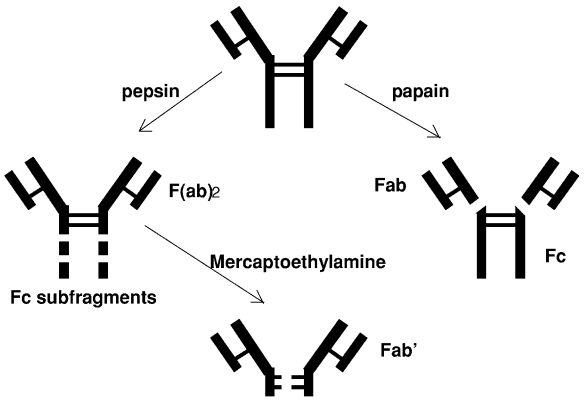


Fig. 1. Schematic diagram of preparation of antibody fragments.

binding site, i.e., single-chain antibody variable-region fragment (scFv) against digoxin, was produced by recombinant protein technology (Fig. 2). The recombinant scFv was constructed to contain a C-terminal 6-histidine sequence for purification of the protein by immobilized metal affinity chromatography and a unique cysteine residue for uniform labeling with a thiol-reactive fluorochrome, 5-iodoacetamido-fluorescein. The latter is important to avoid multiple labeling of the fragment. The labeled scFv showed a single sharp peak in capillary electrophoresis-laser-induced fluorescence (CE-LIF) analysis. The probe was successfully applied to the determination of digoxin by APCE, which in combination with solid-phase extraction could detect 400 f*M* digoxin in 1 ml of serum with a variation of 2.5% and a linear range of 3 orders of magnitude. Shimura et al. [17] used a similar approach to produce Fab′ fragment of mouse immunoglobulin G1 against human $\alpha_1$-antitrypsin. The Fab′ was labeled with tetramethylrhodamine on the single cysteine residue at the hinge region. A single p*I* isomer of the labeled Fab′ was purified by isoelectric focusing.

Despite the complicity of the immunocomplex signals when whole antibody or polyclonal antibody is used, quantification of the antigen is possible based on the change in the signal of the free labeled tracer in competitive CEIA [2,14,18], which is unaffected by the type of antibody used. Optimization of the CE condition in these cases can then be based on the separation of the small, well-characterized labeled antigens from all the immunocomplexes [19]. This approach also eliminates the needs for the digestion and purification procedure in producing the antibody fragments.

## 4. Alternative selectors for APCE

Besides antibodies, the CEIA strategy can be used to quantify molecules using other selectors that bind specifically to the analyte of interest. German et al. [20] demonstrated the use of a 5′-fluorescein isothiocyanate (FITC) labeled DNA aptamer against IgE to quantify IgE in a

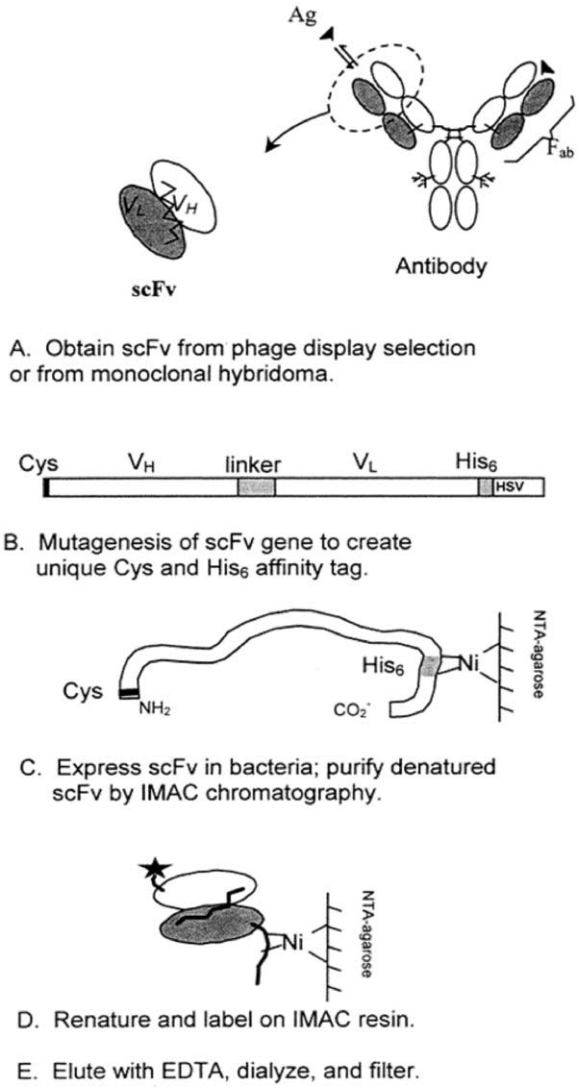


Fig. 2. Schematic diagram of preparation of uniform reagent from single-chain antibody variable-region fragment (scFv) gene. Reprint with permission from Ref. [16].

non-competitive manner with CE separating the free from the IgE bound aptamer peak. Aptamers are single-stranded DNA or RNA oligonucleotides that specifically bind to target molecules with high affinity. The specificity of the binding was shown by the inability of the aptamer against IgE to bind IgG. This method has the advantages that the DNA oligonucleotides are inexpensive to synthesize, highly stable and that the free and bound aptamers can be easily separated from each other in CE. In this report, a linear dynamic range of $10^5$ and a detection limit of 46 p*M* were found for IgE. Single-stranded DNA aptamer has also been used to determine the reverse transcriptase (RT) of the type 1 human immunodeficiency virus (HIV-1) in a noncompetitive fashion [21]. The aptamer was specific for HIV-1 RT, and had no cross-reactivity with other RT from avian myeloblastosis virus and Moloney murine leukemia virus, or denatured HIV-1 RT. Both the free probe and the

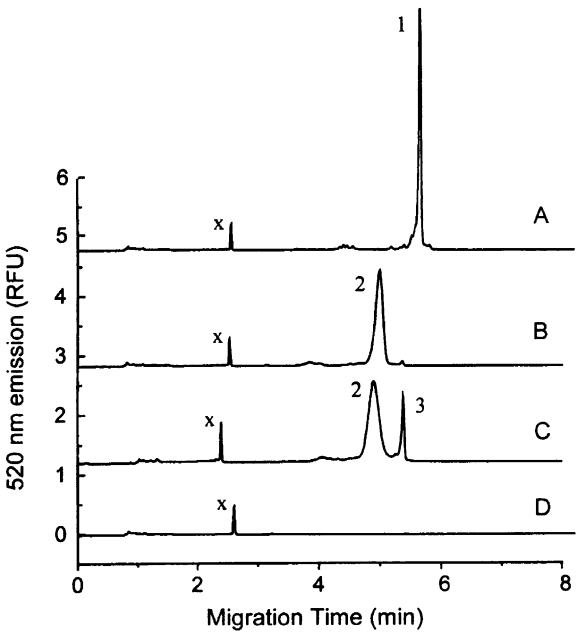


Fig. 3. Capillary electrophoresis–laser-induced fluorescence separation of 9 n*M* fluorescein-labelled human carbonic anhydrase II (HCAII) with (A) no ligands; (B) with 5 μ*M* *para*-carboxybenzenesulfonamide (*p*-CBS) in the background electrolyte; (C) preincubated with 2 n*M* dorzolamide (Dz) and 5 μ*M* *p*-CBS; (D) injection of sample buffer with no HCAII or ligands. Peaks: (1) HCAII; (2) HCAII shifted by *p*-CBS; (3) HCAII-Dz complex; ×, BSA in sample buffer. Fluorescence excitation at 488 nm and emission at 520 nm. Reprint with permission from Ref. [22].

complex peaks could be used for analytical quantification. The calibration curve was linear up to 50 n*M*.

Tim et al. [22] utilized the specific enzyme-inhibitor interaction for quantifying the glaucoma drug dorzolamide (Dz) with an enzyme, human carbonic anhydrase II (HCAII) by APCE. HCAII binds to Dz with high affinity (Fig. 3). The enzyme has another advantage as a probe in APCE in that it has only a single cysteine residue in its sequence, which is remote from the active site. This allowed covalent linkage to the thiol specific reagent, 5-iodoacetamidofluorescein, resulting in a uniform labeling of a single dye per enzyme molecule. The detection limit for Dz is in the low picomolar concentration.

## 5. Labeling of antigen and antibody as tracer

The method for obtaining uniformly labeled antibody has been discussed above. Apart from regular small fluorochrome, green fluorescent protein (GFP), an acidic, globular protein with native fluorescence, could be also used to produce tracer in CEIA [23] using DNA recombinant technology. GFP can be fused to a genetically engineered fragment of an antibody to produce a stable fluorescent antibody. In labeling the antibody, a search for a labeling site far from the antigen-binding site is advisable [22] as labels attached to the amino acids in the epitope regions may reduce the antibody affinity for the tracer, and lead to a loss in the sensitivity of the immunoassay.

Attiya et al. [24] found that CEIA was more sensitive to the antibody quality than were the immunosorbent methods such as enzyme-linked immunosorbent assay (ELISA). This had been attributed to the inability of the former to separate inactive antibody, an undesirable by-product of antibody labeling, from the active form, whereas the wash cycle in ELISA remove inactive material from the sorbent phase. Cyanine dye, Cy5, labeled monoclonal anti-ovalbumin antibody was not useful for CEIA but was functional in ELISA of ovalbumin. Therefore, an affinity protection chromatography (APC) procedure was developed to avoid degradation of the antibody binding sites during labeling. In this procedure, the binding sites of anti-ovalbumin antibody were protected by binding to ovalbumin-coupled sepharose beads before labeling (Fig. 4). The noncompetitive CEIA detection limit of ovalbumin using the 163 n*M* APC prepared labeled antibody was 170 n*M*.

In competitive CEIA, the analyte standard or its analogue is labeled as tracer. Large analytes are usually labeled with fluorescent probe and purified by chromatographic method aiming to produce labeled product with single peak in CE-LIF analysis [25,26]. Similar to labeling antibody discussed above, multi-labeling of the analytes also leads to loss in resolution and decrease in sensitivity.

Labeling condition may influence the affinity of the tracer to the antibody. Staphylococcal enterotoxin B (SEB) labeled with tetramethylrhodamine isothiocyanate (TRITC) at pH 7 had a higher affinity for the antibody than that produced at pH 8.0 and 9.0 [27]. In another publication, the same group [28] established a competitive CEIA for staphylococcal enterotoxin A (SEA) with FITC labeled SEA as tracer. Multi-labeling led to multiple peaks for the labeled SEA in CE and might contribute to the relatively small dynamic range (0.3–6.5 n*M*) of the assay. Recently, the same group compared the labeling conditions of SEB with TRITC. Although TRITC could potentially react with the N-terminal amines and lysines in SEB, a selectively labeling of the N-terminal amino groups over labeling of lysine residues was possible by keeping the labeling pH at 7.0 and reaction for 36 h with a 10-fold excess of TRITC [27]. When TRITC-labeled SEB was used as tracer in competitive CEIA, the linear dynamic range is larger with 2 orders of magnitude.

## 6. CE separation

CEIA analysis depends on CE separation of the free and bound tracers. Several CE separation modes, including capillary zone electrophoresis (CZE), micellar electrokinetic capillary electrophoresis (MEKC), capillary isoelectric focusing (CIEF) and capillary gel electrophoresis (CGE) have been successfully applied for this purpose. For competitive CEIA of small analytes, the binding with large antibodies

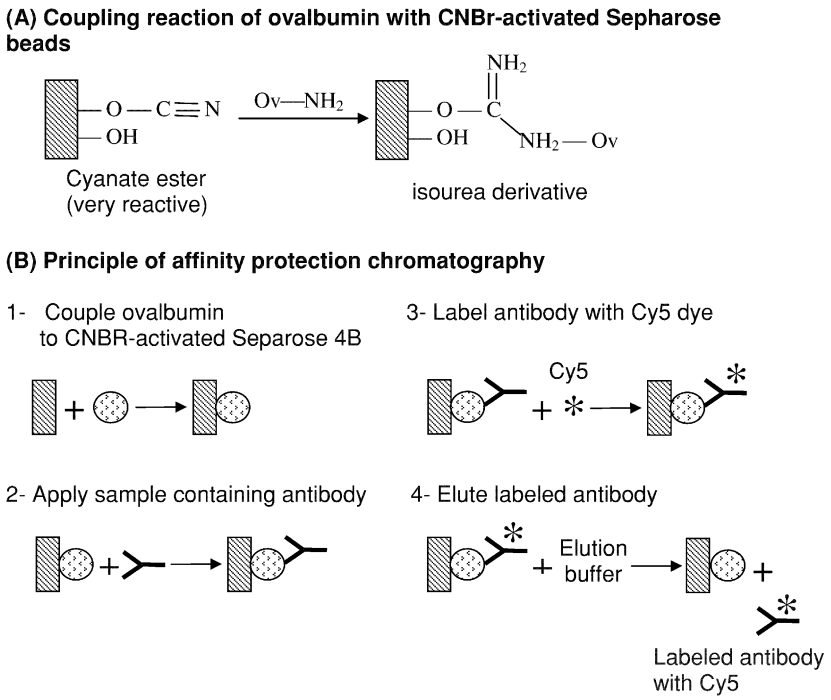


Fig. 4. Schematic diagram of affinity protection procedure for labeling antibody. (A) Activation chemistry on solid support for binding antigen, ovalbumin (Ov), to create an immunosorbent phase, (B) Series of steps for capturing, labeling and releasing antibody from the solid support. Reproduced with permission from Ref. [24].

significantly changes the electrophoretic mobilities of the tracers and, thus, separation is relatively easy. For such analyses, CZE and MEKC were mostly employed, depending on the properties of the analytes [29–35]. Shimura et al. [17] use CIEF to separate the immunocomplex from the free labeled recombinant Fab′ fragment in a noncompetitive CEIA for human $\alpha_1$-antitrypsin. For some unknown mechanism, carbamylation of the antigen sample by heat treatment with urea made the immunocomplex peaks appeared reproducible and more distinct. The method provided a linear response to a pure $\alpha_1$-antitrypsin over a concentration range of 1–1000 ng/ml, and a detection limit of around 2 ng/ml.

Competitive CEIA for some large analytes [5,26–28,36–38] has two problems associated with CE separation. First, the heterogeneity of the large antigen will generate multiple peaks when the antibody is at high concentration [7]. Second, the charge to mass ratios of the antibody and the immunocomplex are similar making CE separation difficult. These result in poor resolution in the electropherogram of CEIA for large-sized proteins, such as between bovine serum albumin (BSA) and monoclonal anti-BSA antibody [5], between human serum immunoglobulin-G (IgG) and anti-IgG antibody [39,40], and between human serum immunoglobulin-A (IgA) and anti-IgA antibody [4]. Although CZE could not resolve labeled anti-BSA antibody from the immunocomplexes, Ou et al. [5] could separate FITC-labeled BSA (FITC-BSA) from the immunocomplex by systematic optimization of the CZE separation conditions of CE, such as voltage, pH and ion strength of the background electrolytes and capillary coating. The optimized condition allowed the use of FITC-BSA as a tracer to quantify BSA in a competitive CEIA.

One of difficulties in CEIA is the need to optimize the separation protocol for different analytes. Very often, much effort has to be used to test the suitability of various separation strategies, e.g., CZE, MEKC. This contrasts greatly from ELISA, which can be used for most analytes with minor modification of a standard protocol. In order to make CEIA user-friendly and more widely used, a number of studies have addressed this issue on CEIA, aiming to develop “universal” approach for easy separation of the free and bound tracer for most analytes.

One of the approaches to address the problem is to increase the differences in electrophoretic mobility between the immunocomplex and the labeled tracer. In a competitive CEIA for methyltestosterone (MTS) with BSA-conjugated MTS as tracer, Zhang et al. [41] used a thermally reversible hydrogel cross-linked to a polyclonal anti-MTS antibody to improve the resolution between the large sized free and bound tracer. This method also eliminated off-line incubation. Compared with free-solution CE without the hydrogel, the resolution of CGE for free and bound MTS improved with increase in the gel concentration (Fig. 5). A detection limit of less than 50 ng/ml for MTS was reported. The improved resolution could be attributed to the large size of the gel bound antibody that significantly affected the mobility of the bound tracer but not that of free tracer [41], and to the ability of the hydrogel to reduce adsorption of the protein

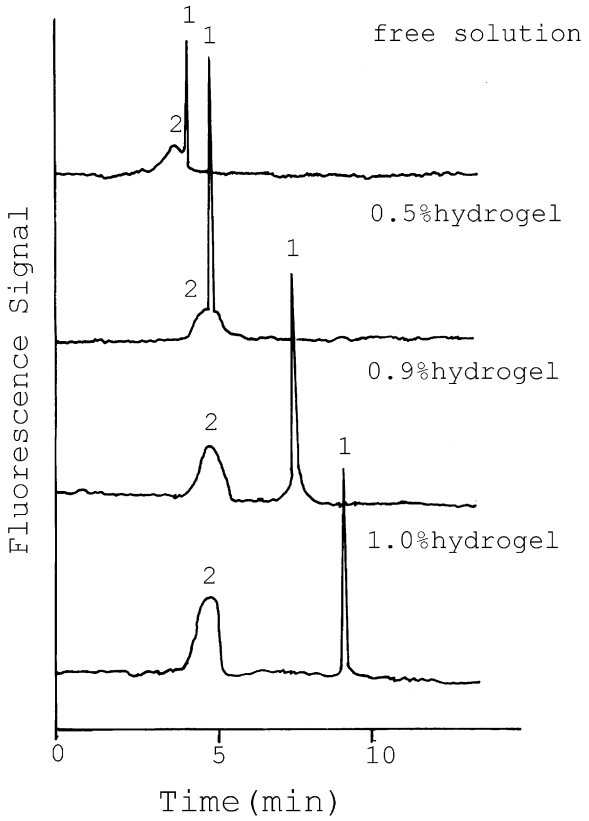


Fig. 5. Capillary electrophoresis separation of bound antigen and free antigen using different replaceable hydrogel at 363 V/cm and 100 m*M* Tris–borate buffer; (1) free antigen; (2) bound antigen. Reprint with permission from Ref. [41].

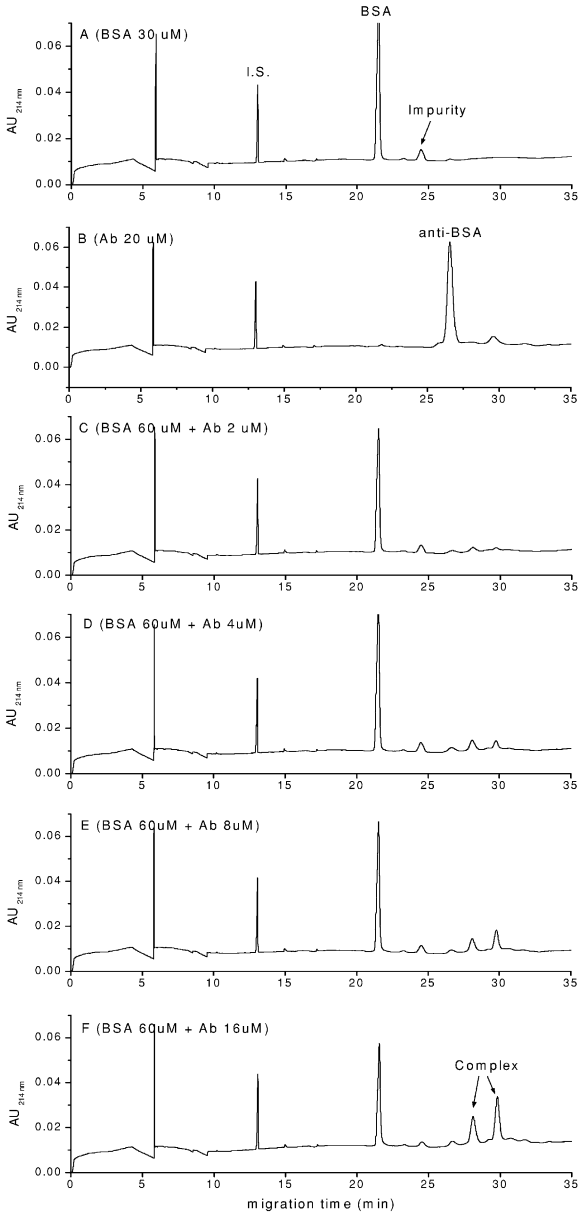


Fig. 6. Capillary gel electrophoresis of (A) bovine serum albumin (BSA) (30 μ*M*); (B) anti-BSA (20 μ*M*); and the mixtures of BSA (60 μ*M*) with different concentrations of monoclonal anti-BSA (C) 2 μ*M*; (D) 4 μ*M*; (E) 8 μ*M*; and (F) 16 μ*M*. Reprint with permission from Ref. [6].

tracer to the capillary wall [42]. The same methodology was applied to the quantification of morphine and estradiol (E2) with detection limits of 8.5 ng/ml and 30 pg/ml, respectively, [43,44].

Fuchs et al. [M. Fuchs, W. Nashabeh, D. Schmaizing US patent 5,630,924] used a similar strategy to drastically change the electrophoretic mobility of the immunocomplex in noncompetitive CEIA for large molecules. In this method, excess amount of a matched pair of antibodies was used: one was labeled as the tracer, while the other was modified to carry high charge. The analyte was sandwiched between the two antibodies in the immunocomplex. The charge-modified antibody significant influenced the mobility of the sandwiched immunocomplex and allowed separation from the free-labeled antibody. The feasibility of this approach was demonstrated in an assay for human chorionic gonadotrophin. Technically, this approach is more demanding, as it requires the use of two analyte-specific antibodies, with one having high charge.

One universal difference between the immunocomplex and the tracer in CEIA is the difference in size. This difference increases with the size of analyte. For large analytes, the difference in size between free and bounded antibodies allows their separation by CGE. Ou et al. [6] used SDS in non-denaturing conditions to equalize the charge of all proteins in the CEIA mixture and separated BSA, monoclonal anti-BSA antibody and their immunocomplexes by CGE according to their sizes only (Fig. 6). In fact, the method could separate antibody that binds to one and two molecules of BSA. For small analytes, this approach may be less useful because of the small size difference. The usefulness of this approach in future depends on the ability to shorten the separation time.

The third approach to a universal separation strategy is to use sorbent phase as in ELISA. Wang and co-workers [45] used this approach to separate the free and labeled tracer in a

competitive CEIA using FITC-labeled BSA. In this method, immunoreaction was allowed to occur off-line. The reaction mixture was pressure injected into a capillary containing protein-G beads. The capillary retained the antibody and the immunocomplex, while allowing the free FITC-labeled BSA to pass through. The immunocomplex was then eluted for detection by laser-induced fluorescence detector. A detection limit of 8 n*M* was reported. The detection limit was reduced to 1.2 n*M* with the use of another fluorescent dye, Cy5, and a diode laser [46].

For the quantification of small analytes using noncompetitive CEIA, the binding of the antigen usually cannot not change the size, charge or p*I* points of the labeled antibodies significantly. Tim et al. [22] developed a general noncompetitive APCE method for small (even neutral) analytes using a charged ligand (shift ligand), and demonstrated the method by determining the concentration of the drug, Dz, which binds to the enzyme, HCAII to form a tight-binding complex. Since Dz is a small and neutral drug, the complex co-migrated with the free-labeled HCAII. In separating the complex from the free-labeled HCAII, the authors added *para*-carboxybenzensulfonamide (*p*-CBS) to the electrolyte. *p*-CBS carries negative charge and binds to the same binding site on HCAII as Dz, but at much lower affinity (low μ*M* compared to 0.6 n*M* for Dz). In this assay, Dz was preincubated with labeled HCAII before CE separation. Excessive HCAII bound to the negatively charged *p*-CBS resulting in a shift in mobility (Fig. 3). The detection limits for Dz were 16.5 p*M* in aqueous solution and 62.5 p*M* in urine and plasma. This approach was also applied in the assay for digoxin where a digoxigenin–DNA 30-mer conjugate was used as a shift ligand [16].

## 7. Detection methods

LIF detection is still the usual choice in CEIA because of its high sensitivity and the ease in the preparation of fluorescent-labeled tracer. Compared with gas-phase laser, the use of semiconductor lasers is preferred due to its lower cost and smaller size. Wang et al. [46] use semiconductor laser-induced fluorescence detection to quantify Cy5-labeled BSA. The 635-nm line of the laser was used as the excitation source for LIF detection. Compared with a similar previous assay using FITC-labeled BSA as tracer and excitation with the 488-nm line of an argon-ion laser [45], the detection limit was improved from 8 to 1.2 n*M* due to significant reduction in the background fluorescence with the semiconductor laser. Sowell et al. [47] described a noncompetitive CEIA for insulin antibodies using a near-infrared dye, NN382 and the 787-nm line of a GaAlAs diode laser as excitation. The fluorescence was determined at 820 nm. A detection limit of 1.1 n*M* was achieved.

Several studies had been conducted to enhance the fluorescent signal in order to increase the sensitivity of the CEIA. There are three approaches. The first approach is to increase the number of fluorescent dye bound to the primary antibody by using a FITC-labeled second antibody that binds to the primary antibody multivalently [42]. The detection limit of detecting E2 using such approach was 9 pg/ml.

The second approach is to use enzyme amplification. Koizumi et al. [48] demonstrated this approach in CEIA for rat IgG using alkaline phosphatase-conjugated anti-rat IgG. In this assay, the enzyme substrate, fluorescein diphosphate (FDP), was added to the running buffer. The mixture of alkaline phosphate-conjugated anti-rat IgG and rat IgG was then injected into the capillary, and high voltage was applied for 1 min to separate the free and bound enzyme conjugated antibody. The voltage was shut off for 2 min to allow time for enzyme reaction, and was re-applied to separate the enzymatic product of FDP, fluorescein for detection by LIF. The future use of this approach depends on optimization to increase the signal-to-noise ratio and to improve the reproducibility of the method. A similar method using enzyme amplification with the use of electrochemical detection (CE-EIA-ED) has recently been described [49]. In this method, thyroxine competes with horseradish peroxidase (HRP)-labeled thyroxin (HRP–T) for a limited number of antibody binding sites. The free and bound HRP–T are separated by CE. The subsequent oxidation of the substrate 3,3′,5,5′-tetramethylbenzide with $H_2O_2$ allowed amperometric determination of the concentration of thyroxin.

The third approach is to concentrate the sample before CE separation or detection. To improve the concentration detection limit, German and Kennedy [50] used capillary reversed-phase liquid chromatography (RPLC) to preconcentrate the samples before coupling reversed-phase capillary liquid chromatography (RPLC) on-line to CE for competitive CEIA (Fig. 7). Five-microliter samples were injected onto the capillary LC column, desorbed by gradient elution, mixed on-line with fluorescent-labeled glucagon and anti-glucagon antibody, and analyzed by CE every 1.5 s with flow-gated injection and LIF detection. With this approach, the detection limit of the system was improved 45-fold to 20 p*M*. In a subsequent report, the detection limit of neuropeptide Y was improved from 850 to 40 p*M* by the coupled system [51], and simultaneous detection of glucagon and neuropeptide Y was also demonstrated. The incorporation of a stacking procedure had also been shown to improve the sensitivity of a newly designed CE-chemiluminescence detector and an immunoassay using horseradish peroxidase-labeled anti-mouse IgG with this technique had been developed [52]. Wang et al. [45] used a protein G column to concentrate the immunocomplex of BSA before detection by LIF.

Apart from LIF detection, other detection systems had been used for CEIA. A laser-induced fluorescence polarization detection (LIFP) system had been described in a competitive CEIA method for the immunosuppressive drug cyclosporin A in human blood [53]. The system could detect

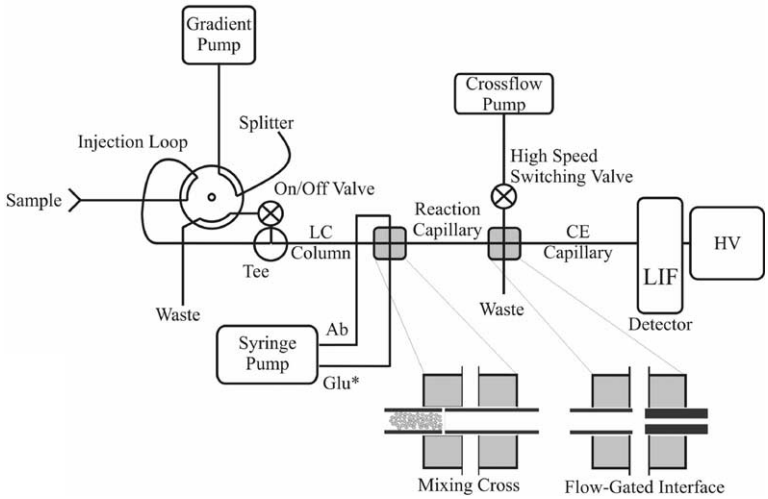


Fig. 7. Schematic diagram of reversed-phase capillary liquid chromatography–capillary electrophoresis immunoassay system. Reprint with permission from Ref. [50].

horizontally and vertically polarized fluorescence simultaneously. As the small antigen had shorter relaxation time, it did not exhibit significant fluorescence polarization when compared with the large immunocomplex. This difference in fluorescence polarization properties allowed direct determination of the free and the antibody bound fluorescent-labeled cyclosporine A by the system. The use of LIFP detection had an excellent detection limit, typically 0.9 n*M*, which was about 20 times lower compared with the conventional fluorescence polarization immunoassay. In a subsequent report, the LIFP detection was used in a homogeneous immunoassay for cyclosporin A without separating the free and bound tracer [54].

Tsukagoshi et al. [52] designed a simple and compact chemiluminescence detection cell for CE. The cell was tested with a mouse IgG/horseradish-peroxidase (HRP)-labeled anti-mouse IgG model. Luminol was used as substrate of the peroxidase and *p*-iodophenol was used as an enhancer of the luminal signal. CE separated the HRP-labeled anti-mouse IgG from its immunocomplex. A detection limit of 2 n*M* was observed.

Ma et al. [55] coupled an intensified CCD camera to a fluorescence microscope as a detector of CE system. A laser was used to induce fluorescence of the tracer molecules. Each fluorescent spot in an image represented one molecule. Images were taken with a frame rate of 11 Hz. The consecutive images were examined manually and the movements were tracked by counting the number of pixels traveled by each molecule over a known time interval. The authors demonstrated the ability of this system to record directly the differential electrophoretic migration of one molecule of β-phycoerythrin labeled digoxigenin and its immunocomplex in a capillary (Fig. 8). The results correlated well with that of CE. Although this detector system is still at its early stage of development, the authors expect that it can be used to detect infections at single-virus level.

## 8. Application of CEIA

The use of CEIA in the quantification of various analytes has been mentioned above. Some of the antigens recently measured by CEIA are listed in Table 1. A number of studies have adopted the use of reagents from commercial fluorescence polarization immunoassays kits for CEIA (e.g., Refs. [30,56–58]. Capillary electrophoresis–ion trap mass spectrometry has been used to confirm the presence of the analytes in the biological samples determined by CEIA [57].

This section concentrates on a few studies highlighting the unique advantage of CEIA. Due to the high resolving power of CE, simultaneous analysis of multiple analytes is more easily accomplished with CEIA methods. Caslavska et al. [59] described a competitive CEIA method for simultaneous analysis of four urinary drugs of abuse, including methadone, opiates, benzoylecgonine and amphetamines. All immunoreagents were obtained from commercial fluorescence polarization immunoassay (FPIA) kits, off-line mixed and incubated together with urine sample before CE analysis. The four free tracers and the internal standard were well separated (Fig. 9), and quantifications were achieved by multilevel internal calibration using ratios of free tracer peak heights with the peak height of the internal standard. This multianalyte CEIA method detected urinary drug with concentrations $\geq$30 ng/ml. Its data compared well with those obtained by routine screening methods based on conventional enzyme immunoassay techniques and FPIAs. German and Kennedy [60] also described a similar competitive CEIA method for the simultaneous determination of glucagons and insulin.

Multiple analytes can also be determined simultaneously with different fluorescent dyes. Chen and Evangelista [4] used Cy5-labeled morphine and Cy5.5-labeled phencyclidine (PCP) in a competitive CEIA to quantify these two drugs simultaneously in urine samples within 5 min. The

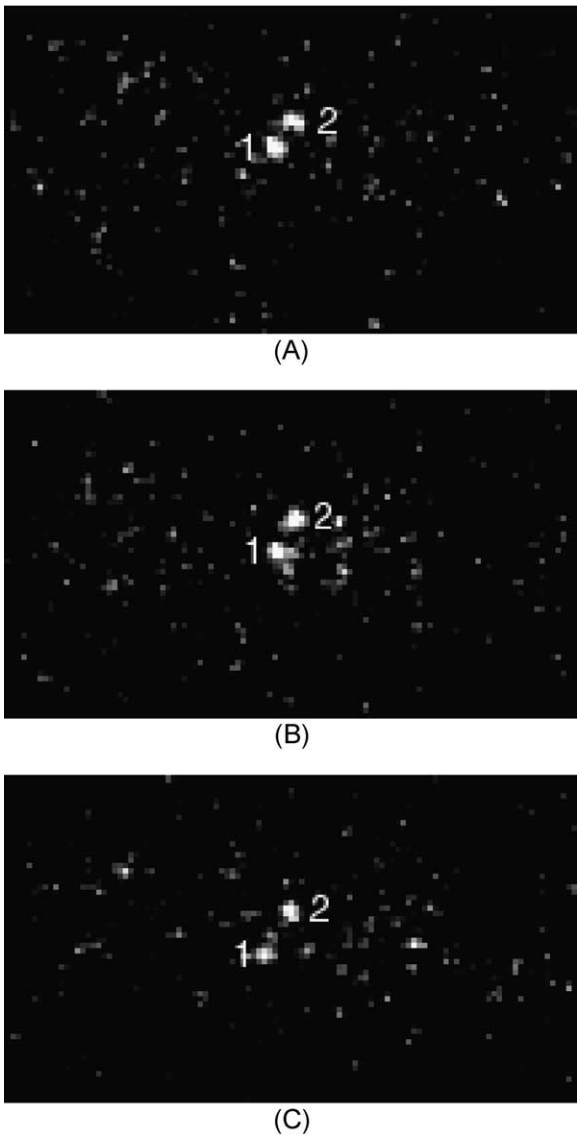


Fig. 8. Single molecule immunoassay showing B-phycoerythrin-labeled digoxigenin (1) moving faster downwards than its immunocomplex (2) in a series of three images. Exposure time, 15 ms; frame rate, 11 Hz; objective, ×20 Zeiss plan apochromat (0.75 n.a.); and electric field strength in the vertical direction 1125 V/16 cm. Reprint with permission from Ref. [55].

different antibodies did not show cross-reactivity with each other. With this approach, the detection limit of PCP and morphine was 4 and 40 n*M*, respectively. The same group subsequently demonstrated simultaneous quantification of four drugs of abuse (morphine, PCP, tetrahydrocannabinol, and benzoylecgonine) using the same strategy [18].

One advantage of CEIA over conventional immunoassay is the high speed of CE separation. Fully automated fast CEIA needs to combine with on-line reagent mixing. The first on-line CEIA was developed by Tao and Kennedy [61] to monitor insulin concentration competitively in a flowing stream. Subsequently, the authors extended this work to monitor insulin secretion from single islets of Langerhans with time-resolve ability [62]. In this system, perfusate from single rat islets was on-line mixed with fluorescent-labeled insulin and anti-insulin antibody, and was injected every 3 s for CE separation through a flow-gated interface. CE separation was achieved in only 1 s, and the detection limit was 0.3 n*M* with a sampling rate of 0.1 Hz. Such fast on-line CEIA system can function as biosensor to monitor important biological events in real time.

## 9. Microchip-based CEIA

A new development in CEIA is the miniaturization of the system on microchip. Both conventional CE and microchip device use minimal amount of reagents and sample and have short duration of analysis. Microchip device offers further advantage in being more flexible in adapting from homogeneous [63,64] to heterogeneous [65–67] immunoassays, in integrating multiple steps in CEIA, e.g., on-line pre- and post-column reactions [68] and in conducting massive parallel analysis.

In a microchip, sample loading and dispensing is controlled using external electrodes to generate electrical fields in a microchannel [69]. Applying voltages to the

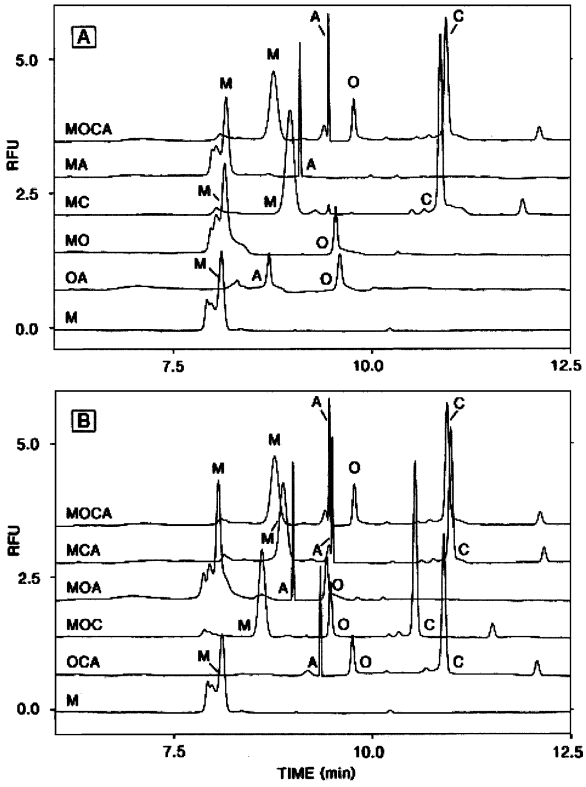


Fig. 9. Electrokinetic multianalyte immunoassay data of (A) double-analyte systems and (B) triple-analyte systems in comparison to the quadruple-analyte configuration (top electropherogram) and to methadone alone (bottom electropherogram). Labels M, O, C and A refer to the free tracers of methadone, morphine, benzoylecgonine and D-amphetamine, respectively. The *y*-scale offset is 0.7 RFU units and there is no *x*-axis shift. Reprint with permission from Ref. [59].

Table 1
Examples of CEIA

| Antigen | Antibody | Labeling dye | Labeled | LOD | Ref. |
|---|---|---|---|---|---|
| Theophylline | Monoclonal | Fluorescein | Ag | 0.26 μg/ml | [64] |
| Methamphetamine | Polyclonal | Fluorescein isothiocyanate | Ag | 20 ng/ml | [31] |
| Scrapie isoform of prion protein | Polyclonal | Fluorescein | Ag | 135 pg | [36] |
| Cyclosporin | Monoclonal | Fluorescein | Ag | 0.9 n*M* | [53] |
| Amphetamine | Polyclonal | Fluorescein | Ag | 80 ng/ml | [30] |
| Methadone | Polyclonal | Fluorescein | Ag | 10 ng/ml | [56] |
| Methyltestosterone | Polyclonal | Fluorescein isothiocyanate | Ag | 50 ng/ml | [41] |
| Staphylococcal enterotoxin A | Polyclonal | Fluorescein isothiocyanate | Ag | 0.3 n*M* | [28] |
| Digoxin | Polyclonal | Fluorescein | Ag | 26 p*M* | [78] |
| Gentamicin | Polyclonal | Fluorescein | Ag | 52 n*M* | |
| Phenobarbital | Polyclonal | Alkaline phosphatase | Ag | 30 μg/l | [79] |
| Glycoalkaloids | Polyclonal | 4′-(Aminomethyl) fluorescein | Ag | 50 n*M* | [35] |
| BSA | Monoclonal | Fluorescein isothiocyanate | Ag | 47 n*M* (CZE) | [5] |
| | | | | 100 n*M* (CGE) | [6] |
| | | Cy5 | | 8 n*M* (affinity column) | [45] |
| | | | | 1.2 n*M* (affinity column) | [46] |
| Glucagon | Monoclonal | Fluorescein | Ag | 17 p*M* (RPLC) | [50] |
| | Monoclonal | Fluorescein | Ag | 760 p*M* using (CZE) | [60] |
| Codein | Polyclonal | Fluorescein | Ag | 10 ng/ml | [57] |
| Morphine | | | | 10 ng/ml | |
| Dihydrocodine | | | | 40 ng/ml | |
| Ethylmorphine | | | | 40 ng/ml | |
| Digoxin | Single-chain Fv fragment | 5-Iodo-acetamido-fluorescein | Ab | 10 p*M* | [16] |
| Morphine | Polyclonal | Fluorescein isothiocyanate | Ag | 8.5 ng/ml | [43] |
| Quinidine | Polyclonal | Fluorescein | Ag | 0.5 μg/ml | [58] |
| Hirudin | Polyclonal | Fluorescein isothiocyanate | Ag | 20 n*M* | [26] |
| Neuropeptide Y | Polyclonal | Fluorescein | Ag | 40 p*M* | [51] |
| Human serum albumin | Monoclonal | Cy5 | Ag | 0.02 mg/ml | [80] |
| Estradiol | Monoclonal | Fluorescein isothiocyanate | 2nd Ab | 9 pg/ml | [42] |
| Insulin | Mouse IgG, | Fluorescein isothiocyanate NN382 | Ag | 3 n*M* | [62] |
| | Fab | | | 1.1 n*M* | [47] |
| Cortisol | Polyclonal | Horseradish peroxidase | Ag | 1.7 n*M* | [81] |
| Vancomycin | Polyclonal | Fluorescein | Ag | 0.98 ng/ml | [3] |
| Ovalbumin | Monoclonal | Cy5 | Ab | 173 n*M* | [24] |
| Human $\alpha_1$-antitrypsin | Recombinant Fab′ of mouse IgG1 | Tetramethyl-rhodamine | Ab | 2 ng/ml | [17] |
| Thyroxine | Polyclonal | Fluorescein | Ag | 3 μg $dl^{-1}$ | [82] |
| | Monoclonal | Horseradish peroxidase | Ag | 3.8 n*M* | [49] |
| DNA adduct of benzo[*a*]pyrenediol epoxide | Monoclonal | Tetramethyl rhodamine | Ag | 2 μ*M* | [83] |

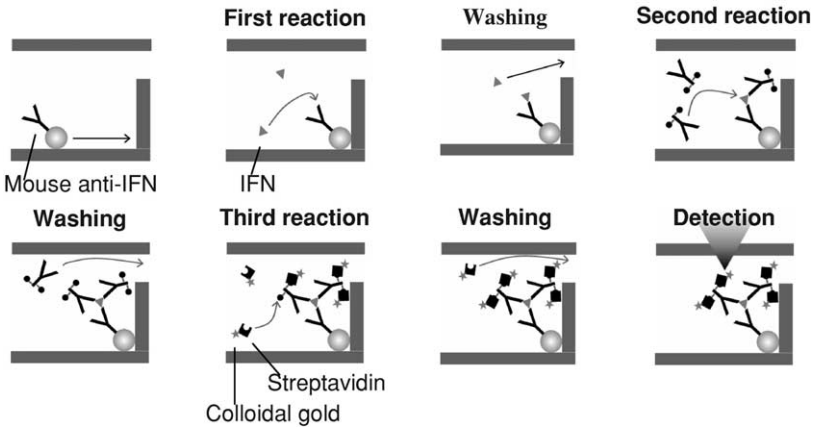


Fig. 10. Concept of microchip-based immunosorbent assay. Reproduced with permission from Ref. [74].

microchannels in a specific manner can easily control the flow-rate and flow direction in a microchip. Temperature dissipation on chips is more efficient than that in conventional capillaries because of the flat cross-section of the microchannels and the large thermal mass of the glass chip. This allows the use of higher electric field and thus faster separation is possible. Free and bound tracer can be separated electrophoretically in the microchannels of microchips. Microchip-based amperometric immunoassays (competitive and noncompetitive) using redox tracers can be accomplished in a few minutes [70]. Weiler et al. [71] compared conventional CE and microchip CE in separating lipoproteins and found that the latter had shorter analysis time and higher resolution. Fluorescence [63,72], electrochemical [68,70], post-separation chemiluminescence detector [73] and a thermal lens microscope [74] had been used to quantify the tracer in microchip-based CEIA.

The success of CE immunoassay systems is based on separation of the free and bound tracers, and is easily affected by sample composition and quality of the tracer and antibodies. Therefore, CE separation for each analyte has to be vigorously optimized. ELISA has effectively reduced this requirement with the use sorbent phase to capture the immunocomplex and separate it from the free antigen. The same approach has been reported in CE [45,75], though it is not commonly used. Microchip format can readily be adapted to immunosorbent assay [65,66,74] (Fig. 10). Method has been developed to reduce the non-specific binding to the microfluidic channels in heterogeneous immunoreaction [76] and applied to measure immunoglobulin [77]. Compared with ELISA in microtiter plates that usually takes hours to complete each assay, the high surface-to-volume ratio of the microchip format allows rapid diffusion between the solution bulk and the channel surface, thereby, shortens the equilibration time and enables the assays to be completed in 5–15 min.

One of the disadvantages of CEIA using conventional CE is the low throughput when compared with ELISA. Microchip-based CEIA has the potential to address this drawback. A six-channel microfluidic device has been developed to perform simultaneous direct immunoassay for ovalbumin and for anti-estradiol antibody [63]. In order to increase the throughput of microchip assay, Sato et al. [74] developed a microchip with branching multichannels for simultaneous assay of multiple samples by a bead-bed sandwich immunoassay. The assay time for four samples was 50 min instead of 35 min for one sample in the single-channel assay.

## 10. Conclusion

CEIA is a developing analytical technique. It possesses a number of advantages over conventional immunoassay. In particular, CEIA can be custom-made for single-analyte and multi-analyte in various formats. However, CEIA also has shortcomings when comparing with conventional immunoassay, including lower concentration sensitivity, requirement for robust optimization of CE separation strategy for different analytes, and low throughput. Some of these issues have recently been addressed with some success. Several studies have been devoted to develop general separation strategies for CEIA, and to enhance the sensitivity of detection. Though the development of microchip-based CEIA is still at its very early stage, available data are encouraging. The technique is likely to address more drawbacks of CEIA, particularly on the throughput issue.

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