Recent Developments in Affinity Capillary Electrophoresis: A Review

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Abstract: Affinity capillary electrophoresis (ACE) is a versatile analytical technique that has been shown to be an efficient and accurate tool to probe non-covalent interactions and to determine binding and dissociation constants between receptors and ligands. ACE uses as its basis the change in migration time of a receptor upon binding to a ligand generally found in the electrophoresis buffer. Subsequent analysis using non-interacting standards realizes values for the binding constant. The technique has a number of advantages over other binding assay methods in that binding parameters can be obtained expeditiously, reproducibly, and with minimal sample quantity requirements and preparation. This review focuses on the literature describing the use of ACE from January 2003 to July 2004.

Keywords: Affinity Capillary Electrophoresis, Binding Constants, Receptor-Ligand Interactions, Binding Assays

INTRODUCTION

Since the pioneering work of Hjerten [1] and of Jorgenson and Lukacs [2], capillary electrophoresis (CE) has become an indispensable analytical tool in molecular separations and analysis. The availability and power of commercial CE instruments has led to an exponential growth in publications fueled by the design of useful applications for a broad spectrum of the sciences. Of particular interest has been the use of CE in the pharmaceutical and biomedical sectors where high-throughput and analysis of target compounds and biological species is a major goal.

During the past decade, advances in molecular biology have yielded great insight into interactions between a myriad of biologically relevant molecules. Such interactions are critical in understanding the functions and molecular mechanisms of biological systems and the roles these interactions play in health and human disease.

Currently, there are a variety of techniques that can be used to estimate affinities between biological species including size exclusion, equilibrium dialysis, sedimentation, slab gel electrophoresis, and fluorescence quenching. Many of these techniques frequently require the separation and quantification of free and/or complexed molecules in an equilibrium mixture. If the amount of free and bound ligands can be distinguished, these techniques can provide reasonable estimates of binding and dissociation constants between biological species. Unfortunately, it is not always possible to accurately differentiate between complexed and uncomplexed molecules making the binding assay problematic.

Affinity CE (ACE) is a versatile analytical technique to study a variety of bimolecular noncovalent interactions and in determining binding and dissociation constants of formed complexes. The technique uses the resolving power of CE to distinguish between free and bound forms of a receptor as a function of the concentration of free ligand. Since the initial reports in 1992 documenting the use of CE to study receptor-ligand interactions ACE has been successfully used to examine a wide array of interactions including protein-drug, protein-DNA, peptide-carbohydrate, peptide-peptide, DNA-dye, carbohydrate-drug, and antigen-antibody [3-7]. A number of reviews on ACE have been published over the past two years [8-13]. Two other reviews touched on the use of ACE as it related to drugs-bio-polymer interactions and peptides [14,15]. Finally, Neubert and Ruttinger edited a book focusing on ACE in pharmaceutics and biopharmaceutics [16]. In a standard form of ACE a sample of receptor and non-interacting standard(s) is exposed to an increasing concentration of ligand in the electrophoresis buffer thereby causing a shift in the migration time of the receptor relative to the standard(s) Fig. (1). Subsequent Scatchard analysis yields a value for the binding constant. In ACE the amount of free and bound receptor (or ligand) need not be known since changes in the migration time of the receptor (or ligand) are used in the estimation of a binding constant. Among the many forms of analysis used in ACE that based on measuring changes in electrophoretic mobilities (µ) has found the most use. This review will not focus on the different forms of analysis but will point out those papers where novel forms of Scatchard have been utilized.

ACE benefits from a number of advantages over complimentary analytical techniques: 1) only minute quantities of material are required; 2) sample need not be pure provided CE can distinguish the analyte of interest from the impurities; 3) radiolabeling of molecules is not necessary; 4) automated CE instrumentation is available, and; 5) molecular interactions can be characterized in free solution.

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Herein, we describe the recent advances in ACE and its use in estimating binding constants between receptors and ligands. This review covers the literature on ACE from January 2003 through June 2004.

**Receptor-Ligand Interactions Using ACE**

The ever-growing wealth of information on receptor-ligand interactions made possible by the large array of analytical methods currently available has had a profound effect in many areas of science. Table 1 lists the wide spectrum of interactions examined by ACE during the past eighteen months.

**Protein-Small Molecule**

Kaddis et al. recently determined binding constants for the activator fructose-6-phosphate (F6P) and substrate ATP to the recombinant wild-type (WT) *Rhodobacter sphaeroides* adenosine 5'-diphosphate-(ADP)-glucose pyrophosphorylase (ADPGlc PPase) using ACE [17]. In these studies, the capillary was initially injected with a plug of sample containing ADPGlc PPase and non-interacting standards. The sample was subsequently subjected to increasing concentrations of F6P or ATP in the running buffer and electrophoresed. Analysis of the change in the migration times of ADPGlc PPase, relative to the non-interacting standards, as a function of the varying concentration of F6P or ATP yielded values for the binding constants. Fig. (2) shows a representative series of electropherograms for ATP and WT ADPGlc PPase. The protein peak shifts from left to right on increasing concentrations of ATP in the buffer. Fig. (3) is a Scatchard plot of the data. A $K_b$ of 5100 M$^{-1}$ was obtained for the interaction of ATP and enzyme. The values obtained by ACE were in good agreement with kinetic parameters obtained from steady-state activity assays. This method demonstrated the quantitative ability of ACE to

![Diagram of receptor-ligand interaction](image)

Fig. (1). Schematic of a typical receptor-ligand interaction and its relationship to charge, mass, and electrophoretic mobility.

**Table 1. Interactions measured by affinity capillary electrophoresis.**

<table>
<thead>
<tr>
<th>Type of Interaction</th>
<th>References</th>
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<tbody>
<tr>
<td>Protein-phosphates</td>
<td>17,18</td>
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<tr>
<td>Protein-sugar</td>
<td>19,20</td>
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<tr>
<td>Protein-proteoglycans</td>
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<td>Protein-carbohydrates</td>
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<tr>
<td>Protein-oligo/polysaccharides</td>
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<tr>
<td>Protein-metal</td>
<td>25</td>
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<tr>
<td>Protein-organic modiers</td>
<td>26</td>
</tr>
<tr>
<td>Protein-drug</td>
<td>27-31</td>
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<tr>
<td>Protein-DNA</td>
<td>32-36</td>
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<tr>
<td>DNA-DNA</td>
<td>37</td>
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<tr>
<td>DNA-small molecule</td>
<td>38</td>
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<tr>
<td>Metal-antibiotics</td>
<td>39,40</td>
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<tr>
<td>Metal-small molecule</td>
<td>41</td>
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<tr>
<td>Antigen-antibody</td>
<td>42-44</td>
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<tr>
<td>Antibiotics-ligands</td>
<td>45-47</td>
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<tr>
<td>Oligosaccharides-drugs</td>
<td>48</td>
</tr>
<tr>
<td>Cyclodextrins-drugs</td>
<td>49-58</td>
</tr>
<tr>
<td>Cyclodextrins-other molecules</td>
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<tr>
<td>Enantiomeric separations (non-cyclodextrin interactions)</td>
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<tr>
<td>Other interactions</td>
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Kennedy et al. developed an affinity probe CE (APCE) assay for guanine-nucleotide-binding proteins (G proteins) that used a fluorescently labeled guanosine 5'-triphosphate (GTP) analogue as the affinity probe [18]. Here, the labeled analogue was incubated with samples containing G proteins and the resulting complexes and free analogue separated by CE using laser-induced fluorescence (LIF). The half-life of the complex was $3060 \pm 240$ s and the binding constant was $1.16 \times 10^8 \text{M}^{-1}$. This technique is much faster than traditional gel electrophoretic assays and should be amenable for studying a host of biological systems involved in, for example, signal transduction pathways and other biochemical interactions involving proteins and ligands.

The binding between disaccharides and carbohydrate-binding proteins in human serum was examined [19,20]. Binding constants in the $10^6 \text{M}^{-1}$ range for the binding of bis-mercaptoethanesulfonate gentiobiose (MerES-Gen) to $\alpha$- and $\beta$-globulin, and $\alpha$-acid glycoprotein were obtained.

Dimitrellos et al. used CE to examine the binding between a heparin-bovine serum albumin (BSA) conjugate with basic fibroblast growth factor (bFGF) [21]. A complementary study using an enzyme solid phase assay was also conducted. ACE and LIF was used to classify a mixture of carbohydrate chains labeled with 8-aminopyrene-1,3,6-trisulfonate (APTS) [22]. The chains were classified on the basis of their migration behaviors in the presence of lectins.

ACE was used to examine the binding of fucoidan, an anticoagulant polysaccharide of marine origin, to antithrombin [23]. The results showed that fucoidan binds to antithrombin in a 1:1 stoichiometry with an affinity dependent on the molecular weight of the polysaccharide. Heparin binding was also examined. Militsopoulou et al. used CE to examine the interaction between heparin sulfate (HS) and bFGF [24]. In these studies HS was degraded to di- and oligosaccharides and then incubated with bFGF to determine the interaction between these species and bFGF.

A method was developed for determining stability constants of $\text{Ca}^{2+}$ and $\text{Na}^{+}$ to proteinase K and -lactalbumin [25]. Affinities as high as $10^7 \text{M}^{-1}$ were measured between $\text{Ca}^{2+}$ and -lactalbumin. Brenner-Weiss et al. demonstrated the use of CE-electrospray ionization time-of-flight mass spectrometry (CE-ESI-TOF-MS) for analyzing the interaction between myoglobin and organic modifiers [26]. The effect on complex stability caused by organic modifiers added to the sheath liquid was examined.

**Protein-Drug**

We evaluated the use of a competitive binding assay using flow-through partial-filling ACE (FTP FACE) to estimate binding constants of neutral arylsulfonamides to carbonic anhydrase B (CAB, EC 4.2.1.1) [27]. In this technique the capillary was first partially-filled with a negatively charged ligand, a sample containing CAB and two non-interacting standards, and a neutral ligand, then electrophoresed. Upon application of a voltage the sample plug migrated into the plug of negatively charged ligand resulting in the formation of a CAB-L complex. Continued electrophoresis resulted in mixing between the neutral ligand...
(L₀) and the CAB-L complex. L₀ successfully competed out L to form the new CAB-L₀ complex. Analysis of the change in the relative migration time ratio (RMTR) of CAB relative to the non-interacting standards, as a function of neutral ligand concentration, yielded values of $K_b$ in agreement with those estimated using other binding and ACE techniques. In the RMTR form of analysis two markers are used in the Scatchard analysis to estimate a value for $K_b$ (Fig. (4)).

Kuroda et al. studied the binding between (S)-verapamil and (S)-propranolol and phospholipid liposomes by frontal analysis CE [28]. They showed that electrostatic interactions have a significant effect on drug-liposome binding and that the contribution of hydrophobic interaction to drug-binding affinity varies among basic drugs. The effect of dextran molecular weight and concentration on mobility and separation of low molecular weight anionic ligands and human serum albumin (HSA) in frontal analysis CE was examined [29]. It was found that separation of ligand and protein increased with concentration of dextran (0-7.5% (w/w)) while molecular weight (70,000-2,000,000 g/mol) had minimal effect. Of equal importance was the finding that the use of dextran had no effect on drug-HAS interactions. Ostergaard and Heegaard reviewed frontal analysis (FA) CE and examined the advantages and limitations of FA-CE in comparison to both conventional and other CE based methods for quantifying binding interactions between species [30]. This review focused primarily on the binding of drugs to lipoproteins, HSA, and α1-acid glycoprotein. The binding of donepezil to serum albumin using CE and circular dichroism was described [31].

Protein-DNA

Biochemical and structural studies on proteins have generated a wealth of information on protein-DNA interactions. These interactions play a critical role in gene expression, DNA replication and DNA damage repair. Hence, the development of techniques that can provide quantitative information on the extent of binding are warranted.

Using a new method called nonequilibrium CE of equilibrium mixtures (NECEEM) Berezovski et al. used low-affinity aptamers as affinity probes to assess their binding to proteins [32]. In NECEEM, a small plug of the equilibrium mixture is injected into the capillary and electrophoresed under nonequilibrium conditions. Two peaks and an exponential curve are generated corresponding to the equilibrium amount of free aptamer in the equilibrium mixture, the amount of protein-aptamer complex intact at the point of detection, and the complex decay profile generated during the separation. Using an *E. coli* single-stranded DNA binding (SSB) protein and a fluorescently labeled 15-mer oligonucleotide they were able to measure a binding constant of $3.6 \times 10^6$ M⁻¹, the monomolecular rate constant of complex decay ($k_1$), and the bimolecular rate constant of complex formation ($k_2$) all in the same experiment. Following up on his earlier work Berezovski et al. used a single-stranded DNA binding protein (SSB) to separate a ssDNA probe from the probe (P)-target (T) complex in free-solution CE [33]. This work was novel in that it demonstrated for the first time that a mediator (SSB) could separate P and P-T efficiently and reproducibly.

![Fig. (4). Principle of the relative migration time ratio (RMTR) and its use in estimating binding constants between receptors and ligands in ACE.](image-url)
Fraga et al. developed a rapid capillary electrophoretic mobility shift assay (CEMSA) using LIF to examine the affinities of histones H2B and H4 for a double-stranded synthetic oligo [34]. A neutral polyacrylamide coated capillary column was used to reduce the electrostatic interactions between protein and the capillary walls. Binding constants in the micromolar range were obtained. Following up on this work the same group used reverse CEMSA (RCEMSA) to calculate the binding of methyl-CpG binding domain (MBD) to DNA [35]. MBD proteins were assayed on pairs of methylated and unmethylated duplex oligos corresponding to the promoter regions of the BRCA1, MLH1, GSTP1, and p16(INK4a) genes and binding affinities measured by Scatchard analyses. They found that small sequence differences in the MBD domain are manifested in large changes in DNA recognition properties. A review focusing on protein-DNA binding assays using CE was published [36].

**DNA-DNA and Other DNA Interactions**

Anada et al. devised a technique whereby a methacryloyl-modified oligodeoxyxynucleotide (ODN), complementary to the target DNA, was copolymerized with a polyacrylamide monomer to produce an affinity ligand [37]. This immobilized ODN served as an affinity ligand for sequence-based DNA separations. Complete separation of ODN’s with a single-base difference was observed. Six and 12-mer ODN with a sequence complementary to one of the c-K-ras genes was used as the immobilized ligand. This work outlines a simple process using CE to detect and differentiate known DNA mutations. Anthony et al. examined the binding of lexitropsin to DNA [38].

**Metal-Small Molecule**

Castagnola et al. studied the binding of metal ions (Ni$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$) to the dodecapeptide bacitracin by ACE [39,40]. In these studies the peptide effective mobility was measured at varying pH in the presence of increasing concentration of metal ion. They found the affinity to follow the order Ni$^{2+} >$ Cu$^{2+} >$ Zn$^{2+}$ with association constants of 2.3, 0.49, and 0.15 X 10$^3$ M$^{-1}$, respectively. A new method that combined complex formation and on-line sample preconcentration by a sweeping technique was described by Isoo and Terabe [41]. In this technique EDTA was used as a carrier to form a UV-absorbing chelate with nine metal ions (Pd$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Pb$^{2+}$, Ni$^{2+}$, Co$^{2+}$, Cd$^{2+}$, Ni$^{3+}$, Fe$^{3+}$). The limits of detection were in the range of 1.8 – 23.4 X 10$^{-5}$ M. Although this work is not a traditional ACE study, principles of ACE are utilized in the complexation mechanism.

**Antigen-Antibody**

Guzman designed a solid-phase microextraction device for use in on-line immunoaffinity CE [42]. Fab’ fragments derived from a purified immunoglobulin G (IgG) antibody were covalently bound to glass and used as constituents of the analyte-microreactor device. The antibodies were tested against ibuprofen, naproxen, angiotensin II, and neurotensin. These species were captured and then eluted from the analyte concentrator-microreactor. The microreactor device described herein has several advantages over previous systems including smaller sample volumes, reproducibility, and little sample preparation required. Buchanan et al. examined the effect of buffer, electric field, and separation time on the detection of aptamer-thrombin and aptamer-immunoglobulin D (IgD) using ACE and LIF [43]. It was concluded that the optimum conditions for detection of non-covalent complexes involved the use of a minimal column length and electric field. The use of tris(hydroxymino)-methane-glycine-potassium (TGK) provided the most stable complexes for analysis by CE. Another immunoaffinity study examined the binding of fluorescence-labeled antigen-binding fragments (Fab) to human erythropoietin [44].

**Antibiotics-Ligands**

Silverio et al. examined the binding between the glycopeptides teicoplanin (Teic) and ristocetin (Rist) and their derivatives to D-Ala-D-Ala peptides using on-column receptor derivatization coupled to partial-filling ACE (PFACE) and standard ACE techniques [45]. In these techniques, the capillary column is first partially filled with increasing concentrations of a D-Ala-D-Ala peptide. Plugs of buffer, antibiotic and non-interacting standards, and acetic and/or succinic anhydride are then injected. Upon electrophoresis, the antibiotic reacts with the anhydride yielding a derivative of of Teic or Rist. Continued electrophoresis results in the overlap of the antibiotic and the plug of peptide. Analysis of the change in the relative migration time ratio (RMTTR) of the glycopeptide relative to the standards, as a function of ligand yields a value for K$\alpha$. Binding of derivatized ligands to vancomycin (Van) was also examined. This work demonstrated for the first time the coupling of on-column molecular derivatization of receptors and ACE and highlights the potential for ACE in the field of organic synthesis and high-throughput screening of drug targets. Two later publications expanded the use of PFACE [46,47].

**Oligosaccharide-Drugs**

Hoffmann et al. examined the complexation trends and measured the binding between dextrin oligomers and drugs [48]. Using frontal analysis (FA) CE experimental conditions were adjusted and modified for each dextrin-drug pair. Binding constants between 10$^{2}$-10$^{3}$ M$^{-1}$ were obtained for the dextrin-drug interactions compared to a separate NMR binding assay.

**CDs-Drug Enantiomers**

During the past few years CE has become the technique of choice to examine chiral compounds. During the past eighteen months a number of papers have been published examining the enantioseparation of compounds using cyclodextrins [49-58]. For example, Gomez-Gomar et al. separated a novel thienylpyrzolylethanamine-based antidepressant using sulfated cyclodextrins [52]. Chankvetadze et al. examined the separation of basic chiral drugs with –CD, heptakis-(2,3-di-acetyl) –CD, and cetyl–CD (53). Finally, Abushhoffa et al. studied the separation of nonsteroidal anti-inflammatory drugs using charged cyclodextrins [55]. The binding constants between –CD and the psychopharmaceutical drugs tiapride, imipramine, chlorimipramine, amitripty-
line, trifluoperzaine, perphenazine, and carbamazepine were estimated using ACE [58]. Karakasyan et al. studied the formation of inclusion complexes between derivatized methoxypoly(ethylene glycol)s (MPEG)s and -CD by ACE [59]. It was shown that the complexation between MPEG and -CD involved a 1:1 stoichiometry. The binding constants, although weak (400 M⁻¹), are in good agreement with previous HPLC work with similar compounds.

**Other Enantiomeric Separations**

Schaeper et al. used DNA to separate transition metal complexes of the type [M(diimine)]⁺ into their isomers [60]. Equilibrium dialysis was used to verify the results obtained by CE. Propranolol enantiomers were separated using ACE using human serum albumin (HSA) as chiral selector [61]. The effect of HSA concentration, temperature, chiral selector plug length, and addition of organic modifiers was evaluated. Complete separation of R- and S-propranolol in less than five minutes was observed. A method to calculate binding constants between -acid glycoprotein (AGP) or cellulose (Cel 7A) and R- and S-propranolol was demonstrated [62]. Using migration time data, radius of capillary column, and amount of protein injected, the authors showed that the use of partial-filling techniques is a viable method to estimate receptor-ligand interactions. Factors such as field strength and electroosmotic flow (EOF) do not negatively effect the estimation of a Kₐ. Millot separated drug enantiomers by CE using immobilized proteins as chiral selectors [63]. Bossi et al. developed special capillary coatings for the separation of diastereoisomers and glycoproteins [64].

**Other Interactions**

ACE was used to determine the binding between heparin-like glycosaminoglycans and the (96-110) heparin-binding domain of amyloid precursor protein (APP) [65]. Using a non-linear regression form of analysis a binding constant of 2.6 X 10⁵ M⁻¹ was obtained for the peptide to the low-molecular weight heparin derivative. Breyer et al. developed vesicle ACE (VCE) to examine the interaction between apolipoprotein (apoCIII) and lipoproteins in a hydrodynamic system simulating physiological conditions [66]. Their vesicle model provided reasonable values for Kₐ and protein-lipid binding ratios using uniformly size unilamellar vesicles.

**ACE Analysis**

Fang et al. provided a detailed description of how migration behaviors of solutes can yield peak shapes that are characteristic of the mobilities of the additive, analyte, and the formed complex [67]. A computer program was designed that predicted the migration time of the peak complex maximums. This work has great bearing on the ability to predict representative complex peak migration times in the formulation of binding isotherms. Okhoniin et al. examined the theoretical basis of NECCEEM by developing a mathematical model for the method [68]. Binding parameters between a protein and DNA were obtained through nonlinear regression of the experimental data.

**CONCLUSION**

The use of ACE to examine biological interactions will continue to grow as more and more receptor-ligand combinations are discovered and as long as variations in the technique are developed. The past decade has shown ACE to be a complimentary technique to other traditional assays and, in some cases, a more robust method than previous techniques. Unlike other methods ACE has the potential to be the technique of choice mainly due to the small sample volumes employed and because of its natural relationship to current microfluidic technologies. It is not too difficult to state that ACE is the next technique to be fully developed on a “lab-on-a-chip” format. The future holds much in store for the use of ACE.

**ACKNOWLEDGMENTS**

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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ACE</td>
<td>Affinity capillary electrophoresis</td>
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<tr>
<td>ADPGlc</td>
<td>Adenosine 5'-diphosphate-(ADP)-glucose pyrophosphorylase</td>
</tr>
<tr>
<td>PPase</td>
<td>Adenosine triphosphatase</td>
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<tr>
<td>AGP</td>
<td>-acid glycoprotein</td>
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<tr>
<td>ApoCIII</td>
<td>Apolipoprotein CIII</td>
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<td>APP</td>
<td>Amyloid precursor protein</td>
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<td>APTS</td>
<td>8-aminopyrene-1,3,6-trisulfonate</td>
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<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
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<td>Carbonic anhydrase B</td>
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<td>CE-ESI-TOF-MS</td>
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<td>FA CE</td>
<td>Frontal analysis capillary electrophoresis</td>
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<tr>
<td>FTPFACE</td>
<td>Flow-through partial-filling ACE</td>
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<tr>
<td>G proteins</td>
<td>Guanine-nucleotide-binding proteins</td>
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<tr>
<td>GTP</td>
<td>Guanosine 5'-triphosphate</td>
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<td>HAS</td>
<td>Human serum albumin</td>
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IgD = Aptamer-immunoglobulin D
IgG = Immunoglobulin G antibody
LIF = Laser-induced fluorescence
MBD = Methyl-CpG binding domain
MerES-Gen = Bis-mercaptopentanesulphonate gentiobiose
MPEGs = Methoxypoly(ethylene glycol)s
NECEEM = Nonequilibrium CE of equilibrium mixtures
ODN = Methacryloyl-modified oligodeoxynucleotide
R-CEMSA = Reverse CEMSA
Rist = Ristocetin
RMTR = Relative migration time ratio (RMTR)
SSB protein = Single-stranded DNA binding protein
Teic = Teicoplanin
TGK = Tris(hydroxymino)-methane-glycine-potassium
PFACE = Partial-filling ACE
Van = Vancomycin
VCE = Vesicle ACE

REFERENCES


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