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Review

Affinity capillary electrophoresis in biomolecular recognition

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Abstract. Affinity capillary electrophoresis is a new method for studies of biomolecular recognition. Applications reported in the literature include chiral separation of racemic biomolecules, measurement of binding constants, estimation of kinetic on- and off-rate constants, determination of binding stoichiometries (a useful tool in examining electrostatic interactions), estimation of effective charges and molecular weights of proteins, characterization of enzymatic activities and library screening for tight-binding drug candidates in solution. This technique demands only small amounts of sample (nanolitre injection volumes, picograms of proteins), involves no radiolabelled materials or chemically immobilized ligands, and does not require changes in spectroscopic characteristics upon binding. This paper reviews the most recent applications of affinity capillary electrophoresis and its use in the analysis of biomolecules.

Key words. Affinity capillary electrophoresis; receptor-ligand binding interaction; molecular recognition; combinatorial library screening; protein.

Biomolecular recognition

Most, if not all, biological events are triggered by receptor-ligand interactions such as protein-protein recognition involved in signal transduction pathways and protein-carbohydrate binding interactions used by influenza virus to infect host cells. Therefore, the determination of equilibrium binding constants, binding stoichiometries and kinetic rate constants would greatly contribute to the understanding of mechanisms and specificities of receptor proteins.

The equilibrium binding constants for biomolecular interactions have been measured using a variety of experimental approaches including reaction kinetics [1, 2], calorimetry [3–6], spectroscopy [7–10], potentiometry [11, 12] and separations [13–15]. Most of the experimental methods involve a measured response at various ligand concentrations and fixed concentration of the receptor protein. Generally, the measured response can be related to the relative concentrations of free and bound ligand and, subsequently, to the binding constant; each of the different experimental methods can be related mathematically [16]. Binding assays such as equilibrium dialysis, membrane/gel filtration and ultracentrifugation often involve premixing the receptor protein and ligand, and subsequent separation of the receptor-ligand complex from the free ligand molecules [17]. The simple method of equilibrium dialysis measures the binding of ligand to a receptor, so long as the ligand is of small molecular weight, dialysable with an available assay for the free ligand [18]. The relatively rapid and sensitive method of membrane filtration is applicable to the binding of ligand molecules to nucleic acids, proteins and large particles [18]. Binding measurements made by this method can be obtained by filtering a solution containing both receptors and ligands, and measuring the amount of ligand associated

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with the receptor and retained on the membrane filter. Ultracentrifugation would be favourable for the detection of binding in the case where the receptor-ligand complex has a sufficiently high sedimentation coefficient and its dissociation is slow relative to analysis tiem [18]. Other methods of binding involve observation of change such as spectral properties induced upon binding of either the receptor or the ligand to its counterpart. These changes can be monitored using, for example, ultraviolet (UV)-visible absorbance, fluorescence polarization [19], nuclear magnetic resonance (NMR) [20] and surface plasmon resonance (SPR) [21, 22] by analysing the difference in the absorption intensity or wavelength, fluorescence intensity, band-width or chemical shift, and refractive index, respectively. A classical example is the measurement of oxygen binding to hemoglobin at 577 nm, where the absorbance of the heme group on each subunit changes upon oxygenation [23]. A great strength of spectrophotometric methods is the ability to obtain results rapidly. Additionally, the ligand and protein are allowed to be equilibrated and measured in one cuvette, so that problems of membrane permeability or rapid separations of the free and bound ligand are avoided. Recent advances in the scintillation proximity assay [24, 25] have proved its usefulness in receptor-binding studies. This method entails the covalent coupling of the receptor protein onto the surface of microbeads containing a scintillator. Radiolabelled ligands that become bound to the immobilized receptor are sufficiently close to the fluor-containing bead to cause light emission, while the energy of free ligand is absorbed by the solution medium, resulting in no light generated. Its disadvantage, however, involves the covalent immobilization of a pure receptor protein, without loss or modification of biological activity. Other methods of binding have also been developed using tube and slab gel electrophoresis [26] which are currently being adopted to capillary electrophoresis methodology. Although there are numerous other variations of the above methods for measuring binding between receptor and ligand(s), the most common and general methods have been discussed. This paper will review affinity capillary electrophoresis (ACE) as a new, alternative binding assay method for the study of receptor-ligand interactions. The stoichiometry of binding, affinity of the binding sites for the ligand, and kinetics of association and dissociation are discussed in depth in this paper. Using ACE for the screening of combinatorial libraries in solution will also be extensively covered along with a brief perspective on the future of ACE.

Capillary electrophoresis: an introduction

Capillary electrophoresis (CE) has emerged as a novel, high-resolution separation technique that has most of the advantages of modern automated technology [27, 28]. The technique measures the electrophoretic mobility of a charged species in the presence of an electric field gradient (typically ~ 300 V cm^{-1}). The value of the electrophoretic mobility, μ (cm V^{-1} s^{-1}), of a given species is directly proportional to its net charge and inversely proportional to its hydrodynamic drag. Equation 1 is an approximate expression of this relationship

$$\mu = C_{\mathrm{P}}(Z/M^{2/3}) \quad (1)$$

where C_{P} is an empirical constant for a protein (P) of charge Z and mass M. CE allows for rapid (typically minutes), efficient ($\sim 10^6$ theoretical plates) separation of minute quantities (picograms of proteins, nanolitre injection volumes) of analytes.

The basic instrumentation includes a separation capillary column dipped into the background electrolyte reservoirs equipped with platinum electrodes for the attachment of a high-voltage power source. Injection of samples onto the capillary column can be accomplished either by pressure drop to deliver a fixed volume or electromigration in which the applied electric field gradient transports the charged species onto the capillary column. After the introduction of high voltage to the ends of the bare fused silica capillary column filled with electrophoresis buffer at physiological pH, the electroosmotic flow (EOF) transports the sample along the capillary column while maintaining a plug-flow geometry [29]. Monitoring of sample separation can be achieved utilizing a wide variety of detection methods (see below). The mechanism of high-resolution separation has been well established with the observed migration time of the species dependent on its electrophoretic mobility and the velocity of the EOF. Recently, many biochemical applications using CE have been reported, including (i) the discovery of dopamine in single lymphocytes of human cerebrospinal fluid implicating a role in immune activation [30]; (ii) DNA sequence determination of a template from the malaria genome [31]; (iii) therapeutic monitoring of drugs such as antiepileptics [32–34], antiasthmatics [35, 36], analgesics [37], antidepressants [38], benzodiazepines [39] and antitumour drugs [40–42]; and (iv) quantitative immunoassays by CE for antigens such as cortisol [43, 44], morphine [45], digoxin [46], chloramphenicol [47] and angiotensin [48]. Pioneering studies by Karger [29, 49], Grossman [50, 51], Regnier [52, 53], Novotny [54, 55], Jorgenson [56, 57] and others have shaped the rapid expansion of CE to commercialization where the development of versatile and inexpensive instrumentation makes CE routinely used.

Although CE emerged as a high-resolution separation technique, it is primary limited by the particular detection method used. The development of detection methods has, nevertheless, rapidly advanced due to the relative ease of adaptation of high-performance liquid

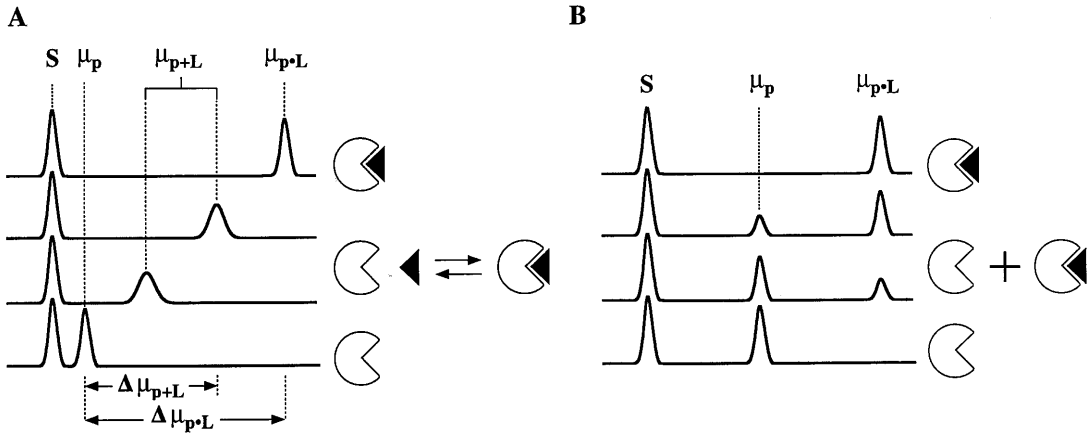


Figure 1. The principle of ACE. (*A*) For weak-to-moderate binding systems, the electrophoretic mobility of a receptor protein changes upon binding to the charged ligand present in the electrophoresis buffer, due to changes in its charge-to-mass ratio. The interaction of a protein and a ligand that induces a change in the electrophoretic mobility of a protein can also broaden the protein peak due to the protein in the region of concentrations corresponding to migration times intermediate between those of free and fully complexed protein. This peak broadening is most pronounced when the dissociation time ($1/k_{off}$) is of the same magnitude as the migration time of the protein. The protein peak sharpens and changes no more in its mobility ($\mu_{P\cdot L}$) at saturating concentrations of the charged ligand, despite having a different migration time. The binding constant is obtained using Scatchard analysis (eq. 4). (*B*) For tight-binding systems, the measurement of binding constant is straightforward; the protein of known concentration can be directly titrated with the ligand, since the complex once formed remains associated in electrophoresis. Direct integration of the peak areas of the free and bound protein measures the binding constant. S is the internal standard.

chromatography (HPLC) detectors to CE. The most widely employed detection methods include UV-visible (UV-VIS) absorbance [58–60], fluorescence [61–66] and mass spectrometry [67–73]. Since most of biologically active compounds absorb within the range of 200 to 400 nm, commercially available CE instruments are all equipped with a UV-VIS detector. With the important development of laser-induced fluorescence detection came the possibility of unprecedented low detection limits (typically 1 pM and as low as 1 fM) for compounds in trace quantities [74–77]. However, relatively few molecules fluoresce naturally upon excitation; therefore, suitable tags must be conjugated to the analyte molecule prior to analysis. In comparison, the coupling of CE with mass spectrometry (MS) via appropriate interfaces is a relatively new but rapidly developing technique [78]. The power of this technique has been demonstrated using a β-endorphin model system for the study of epitope mapping [79]. The transfer of sample from CE into MS is made possible by employing interfaces such as thermospray [80–81], plasma and matrix-assisted laser desorption [82–84], fast atom bombardment [85–87] and electrospray ionization [88, 89]. While separation by CE offers an efficient way to analyse samples, MS provides structural information such as molecular weight (see below). Less widely used detection methods include chemiluminescence [90–92], potentiometry [93–95], conductivity [96, 97], amperometry [98–100], thermooptics [101–104], radionuclide [105–107], Raman-based [108], refractive-index [109–111] and biosensors [112]. Because of the diversity (various degrees of sensitivity, selectivity, linear range, noise etc.) involved with CE detection, each detection method employed will be dependent on the specific application.

ACE

Among the available techniques for studying binding interactions, ACE has recently demonstrated its value in (i) the measurement of binding constants [113–115], (ii) estimation of kinetic rate constants [116], (iii) determination of binding stoichiometries of receptor-ligand interactions [117] and (iv) combinatorial library screening in biochemical systems [118–120]. The underlying principle of ACE to study weak-to-moderate binding systems is straightforward: the electrophoretic mobility (μ) of a receptor protein (P: charge Z, mass M) changes upon binding to the charged ligand (L: charge z, mass m) present in the electrophoresis buffer, due to changes in its charge-to-mass ratio (fig. 1A). If the protein binds a charged ligand of relatively small mass, the change in mobility due to the change in mass [from $M^{2/3}$ to $(M \pm m)^{2/3}$] is negligible relative to the change in mobility due to the change in charge [from Z to $(Z \pm z)$] (eq. 2). However, the experimentally observed difference, Δt, between the migration time (t_{P+L}) of a protein at a concentration of ligand and the migration time t_P at

$$\mu \cong C_P[(Z \pm z)/(M+m)^{2/3}] \cong C_P[(Z \pm z)/M^{2/3}] \quad (2)$$

[L] = 0 can be influenced by factors other than binding of ligand at the active site of the protein. The EOF is a particularly important contributor to the migration time of the protein. It is possible to correct values of t_{P+L} empirically for changes in EOF by observing the neutral internal standard; with this correction, it is possible to infer and analyse the contributions from biospecific interactions even in the presence of variable EOF [115]. Scatchard analysis of the electrophoretic mobility change of protein as a function of charged ligand concentration in the electrophoresis buffer allows the determination of the binding constant, K_b, with correction for EOF (eqs 3–4) [115]. The analysis initiates with eq. 3: l_c is the total length of the capillary and l_d is the length from the 'inlet' to the detector; V is the voltage across the capillary; t_P and t_N are the measured migration times of the sample peak and neutral marker, respectively; t_{P+L} and t_{N+L} at the concentration of ligand [L].

$$\Delta\mu_{P+L} = l_c l_d / V[(1/t_{P+L} - 1/t_{N+L}) - (1/t_P - 1/t_N)] \quad (3)$$

$$\Delta\mu_{P+L}/[L] = K_b \Delta\mu_{P\cdot L} - K_b \Delta\mu_{P+L} \quad (4)$$

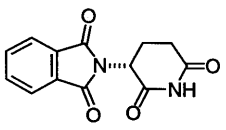
The change in mobility of the protein, $\Delta\mu_{P+L}$ as a function of ligand concentration is given by eq. 3 where a range of concentrations of L may be subsequently used for the Scatchard analysis (eq. 4). In eq. 4, $\Delta\mu_{P\cdot L}$ is the electrophoretic mobility of the receptor protein saturated with ligand. The overall attractive features of ACE include the following: (i) it provides an assessment of protein-ligand interactions using very small amounts of sample in a relatively short time; (ii) it does not necessitate high protein purity or an accurate value of its concentration, since the binding constant is based only on electrophoretic mobility and shape (but not the area) of the peak; (iii) it is applicable to the binding of several proteins to a given ligand in the same solution or vice versa (e.g. library screening); (iv) it does not require the synthesis of radioactive or chromophoric ligands, although it does require the synthesis of a charged analogue of the ligand, if the ligand itself is electrically neutral; (v) it is capable of differentiating between protein conformations that bind ligand from conformations of the same protein that are denatured and do not bind ligand; (vi) the commercial availability of automated instrumentation and the high reproducibility of data favour the use of this technique. A major limitation of ACE remains the tendency of proteins to adsorb onto the wall of uncoated capillary columns, which can lead to poor separations and loss of material. Although this limitation becomes more pronounced when the pH of the electrophoresis buffer lies close to or lower than the isoelectric point of the protein, the problem can be resolved by using coated capillaries [121, 122].

In binding systems where receptor proteins associate tightly with ligands (i.e. slow dissociation rates), the procedure of the measurement of binding constants is straightforward (fig. 1B). Direct integration of the peak areas of the free and bound receptor protein (or ligand) allows measurement of binding constants, provided that the free and bound species have different electrophoretic mobilities. Typically, sensitive detection of both free and complexed species at their low concentrations is important to achieve correct measurement of binding constants in these systems. For example, using laser-induced fluorescence detection can determine subnanomolar concentrations of dye-labelled oligonucleotides. This CE-based assay method should play an important role in rapid assessment of DNA-protein binding interactions.

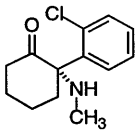
Applications of ACE

Qualitative biomolecular recognition: chiral separation

Enantiomers often do not possess the same physiological effects; therefore, analytical methods are required to discriminate between the two isomeric biomolecules. Thalidomide, for example, was widely prescribed (1950s to early 1960s) to pregnant women around the world as a treatment for morning sickness and banned in 1962 after some 12,000 babies were born with no limbs or tiny, flipperlike arms and legs, serious facial deformities and defective organs. It was later determined that the teratogenic effects were mainly caused by the *S*-enantiomer of thalidomide [123]. Also, the *S* isomer of an intravenous anaesthetic, ketamine, is known to be superior to its *R*-isomer in adequate anaesthesia. This *R*-ketamine has been shown to be the major cause of the postoperative side effects such as hallucinations and other transient psychotic sequelae.



(*R*)-Thalidomide



(*S*)-Ketamine

In 1982, there were 1675 pharmaceutical compounds on the market. Of the 1200 synthetic drugs, 58 were sold as a single isomer, and 422 were marketed as racemates. In 1992 the total number of drugs dropped to 668 with 521 of these being chiral, but only 140 being marketed as racemates [124]. Two significant trends can be inferred from these data: the total number of drugs on the market decreased, and the percentage sold in enantiopure form increased. Both of these were attributed to more stringent guidelines issued by the U.S. Food and Drug Administration (FDA) in response to the numer-

Table 1. Chiral separation studies using capillary electrophoresis reported in 1996–97.*

Applications	Chiral selectors	References
Chiral separation reviews		1–12
Acenocoumarol	monomethylamino-β-CD	13
Acidic drugs	maltodextrans	14
	native and modified CDs	15
Albuterol	CDs	16
Alloxydim	CDs	17
AMEBD	carboxymethyl-β-CD with various degree of substitution	18
Amines	heptakis (2,6-di-*O*-methyl)-β-CD	19
	β-, γ-CD and β-CD derivatives	20
Primary amines	18-crown-6-tetracarboxylic acid	21
Amino acids and amino compounds	neutral and charged CDs (e.g. sulphobutylether-derivatized β-CDs)	22
	teicoplanin	23
	histamine modified β-CDs	24
	β-CDs	25–27
	α-CDs	28
	native β-CD, 2-hydroxypropynl-β-CD, heptakis (2,6-di-*O*-methyl)-β-CD, heptakis(2,3,6-tri-*O*-methyl)-β-CD, β-CD polymer, and carboxymethyl-β-CD polymer (+)-18-crown-6 tetracarboxylic acid	29
	N-nonyl-β-D-glucopyranoside	30
	β-, γ-CDs	31
	syntheic cyclohexapeptide libraries	32, 33
	hydroxypropyl-β-CD and hydroxypropyl-γ-CD	34
	bovine serum albumin	35
	Cu(II) complexes of chiral proline and hydroxylproline	36
	β-CD/18-crown-6-tetracarboxylic acid (dual)	37
	vancomycin	38
	β-CD in polyacrylamide gel	39
	sulfobutyl ether γ-CD and γ-CD	40
Amino acid derivatives	6-amino-6-deoxy-β-CD and 6-deoxy-6-hexylamino-β-CD	41
Amino alcohols	chiral camphorsulphonates	43
N-Acylamino acid derivatives	surfactant derivatives of sodium *N*-undecylenyl amino acidates	43
Aminophosphornic acids	β-CD	44
Amphetamine	hydroxypropyl-β-CD	45
	heptakis (2,6,-di-*O*-methyl)-β-CD	46
Anaesthetics	sulphated CDs (degree of substitution 7–10)	47
Anisodamine	hydroxypropyl-β-CD	48
Antiarrhythmics	sulphated CDs (degree of substitution 7–10)	49
Anticoagulant drugs	heptamethylamino-β-CD	48
Anticonvulsants, antidepressants, antihypertensives, and antimalarials	sulphated CDs (degree of substitution 7–10)	13
		48
Antihistamines	sulphated CDs (degree of substitution 7–10)	48
	hydroxypropyl-γ-CDs	50
Aromatic compounds	α-, β-, γ-CDs and dimethyl-β-CD	51
Arylpropionic acids	heptamethylamino-β-CD	13
	mono(6-amino-6-deoxy)-β-CD/trimethyl-β-CD (dual)	52
Antropisomers ($\pm$)-1,1,′-bi-2-naphthol	poly(sodium *N*-undecylenyl amino L-valinate)	53
Atropisomers ($\pm$)-1,1′binaphthyl-2, 2′diamine	poly(sodium *N*-undecylenyl amino L-valinate	53
Atropisomeric binaphthyl derivatives	CD derivatives	54
3 Basic drugs and 2 carboxylic acids	CDs	55
7 Basic drugs	carboxymethyl-, dimethyl-, and hydroxypropyl-β-CD (e.g. carboxymethyl-β-CD)	56
29 Basic drugs	α1-acid glycoprotein	57
Weakly basic drugs	λ-carrageenan	58
Basic drugs	maltodextrans	14, 59
	native and modified CDs	15
	sulphobutyl ether γ-CD and γ-CD	41
	chiral camphorsulphonates	43
	neutral polysaccharides (e.g. dextran, dextrin) and ionic polysaccharides (e.g. chondrotin sulphate C)	60
	dextrain 10 sulphopropyl ether	61
Benzoin	sulphobutyl ether γ-CD and γ-CD	41
	human serum albumin	62
Benzoin derivatives	mono(6-amino-6-deoxy)-β-CD/trimethyl-β-CD (dual)	52
Binaphthyl compounds	sulphobutyl ether γ-CD and γ-CD	41
	dextrin 10 sulphopropyl ether	61
	DEAE-dextran hydrochloride, fradiomycin sulphate, kanamycin sulphate and streptomycin sulphate	63

Table 1. continued.

Applications	Chiral selectors	References
(*R*,*S*)-1,1′-Binaphthyl-2, 2′dihydrogenphosphate	β-CD in polyacrylamide gel	40
Bronchodilators	sulphated CDs (degree of substitution 7–10)	48
Bupivacine	human serum transferrin	64
Carnitine	CD and derivatives	65
Carprofen	zwitterionic mono(6-glutamylamino-6-deoxy)-β-CD/	66
	trimethyl-βCD (dual) β-CD; dimethyl-, trimethyl-	67
	and hydroxypropyl-β-CD	
Carvedilol	β-CD	68
Catecholamines and precursors	sulphated CDs	69
Cationic molecules	phosphate-substituted CDs	70
Chloramphenicol metabolite	2,6-di-*O*-carboxymethyl-β-CDs	71
Chloropheniramine	monomethylamino-β-CD	13
	β-CD	68, 73
	β-CD and derivatives	72
	2-monohydroxypropyl-β-CD	74
Chlorthalidone	zwitterionic mono(6-d-glutamylamino-6-deoxy)-β-CD and	66
	cationic mono(6-amino-6-deoxy)-β-CD	
8 Chromane compounds	heptakis(2,3,6-tri-*O*-methyl)-β-CD and γ-CD	75
Cisapride	chiral camphorsulphonates	43
Clidinium bromide	α-CD	76
Cloperastine	pepsin	77
Coumarinic anticoagulant drugs	poly(sodium *N*-undecylenyl amino L-valinate)	53
Mono- and tricyclic compounds	hydroxypropyl-γ-CDs	50
Denopamine	uncharged β-CDs (e.g. dimethyl-β-CD) and charged β-, γ-CDs	78
Dexfenfluramine	dimethyl-β-CD	79
Devrinol	CDs and chiral surfactants	17
Dichlorprop	β-CD derivatives	80
Diclofop	CDs and chiral surfactants	17
Diltiazem	neutral polysaccharides (e.g. dextran, dextrin) and ionic	60
	polysaccharides (e.g. chondroitin sulphate C)	
Synthetic intermediates of	DEAE-dextran hydrochloride, fradiomycin sulphate,	63
diltiazem analogues	kanamycin sulphate and streptomycin sulphate	
Dioxopromethazine	β-CD and derivatives	72
Dioxypromethazine	β-CD	81, 82
Disopyramide	15 different CDs and CD derivatives (e.g. carboxymethylated β-CDs)	83
Ephedrine	carboxymethyl-β-CD with various degrees of substitution	18
	2-monohydroxypropyl-β-CD	73
	(*S*)-, (*R*)-*N*-dodecoxycarbonylvaline	84
Ethofumesate	β-CDs derivatives (e.g. sulphobutylether β-CD)	85
Fenoprofen	dimethyl- or trimethyl-β-CD/sulphobutyl ether-β-CD (dual)	86
Fluazifop	CDs and chiral surfactants	17
Flurbiprofen	β-CD; dimethyl-, trimethyl- and hydroxypropyl-β-CD	67
	macrocyclic antibiotic (LY307599)	87
Folinic acid	bovine serum albumin	36
Glycopyrronium	hydroxypropyl-β-CD	49
Growth hormone secretagogue (MK-0677)	β-CDs	88
Reduced haloperidol	dimethyl-β-CD	89
	heptakis(2,6-di-*O*-methyl)-β-CD	90
Herbicides	β-CDs derivatives (e.g. sulphobutylether β-CD)	85
Phenoxy acid herbicides	octyl β-D-maltopyranoside chiral surfactant	91, 92
	N-octyl- and *N*-nonyl-β-D-maltopyranoside chiral surfactant	93
	native and modified CDs (e.g. 2,3,6-tri-*O*-methyl-β-CD);	
	β-CD/2,3,6-tri-*O*-methyl-β-CD (dual)	94
7 Chlorophenoxy acid herbicides	β-CD	95
Hexobarbital	dimethyl- or trimethyl-β-CD/sulphobutyl ether-β-CD (dual)	86
	7 different methylated-β-CD derivatives	96
Homatropine	β-CD	97
Hupivacine	dimethyl-β-CD	98
Hydrobenzoin	zwitterionic mono(6-δ-glutamylamino-6-deoxy)-β-CD and	66
	cationic mono(6-amino-6-deoxy)-β-CD	
o-, *m*- and *p*-Hydroxybenzoic acid	β-CD	40
Hydroxy organic acids	1-allyl derivatives of (5*R*, 8*S*, 10*R*)-terguride (ergot alkaloids)	99
Imazamethabenz	CDs	17
Imazaquin	CDs and chiral surfactants	17
Indoprofen	β-CD; dimethyl-, trimethyl- and hydroxypropyl-β-CD	67
Isoprenaline	β-CD	97
Isoproterenol	monomethylamino-β-CD	13
	methyl-*o*-β-CD	100

Table 1. continued.

Applications	Chiral selectors	References
Ketamine	monomethylamino-β-CD	13
	β-CD	68
	α-CD	76
Ketoprofen	β-CD; dimethyl-, trimethyl- and hydroxypropyl-β-CD	67
	dimethyl- or trimethyl-β-CD sulfobutyl ether-β-CD (dual)	86
Labetalol	human serum transferrin	64
Phenyllactic acid	monomethylamino-β-CD and heptamethylamino-β-CD	13
	bovine serum albumin	101
Lobeline	α-, β-, heptakis(2,6-di-*O*-methyl)-β-CD and hydroxypropyl-β-CD	102
Lobeline analogues	β-CD derivatives	103
LY213829 and isomeric sulphoxide derivatives	sulphobutylether-β-CD, trimethyl-β-CD, hydroxypropyl-β-CD	104
Mandelic acid	bovine serum albumin	36
Mianserin	β-CD	20
	sulphobutyl ether γ-CD and γ-CD	41
Mefloquine	2,6-di-*O*-methyl-β-CD	105
Melatonergic drugs	β-CD and heptakis(2,6-di-*O*-methyl)-β-CD	106
Mepivacine	dimethyl-β-CD	98
Methadome and primary metabolite	hydroxypropyl-β-CD	107
Methamphetamine	heptakis(2,6-di-*O*-methyl)-β-CD	108
Methoxamine	α-, β-, heptakis(2,6-di-*O*-methyl)-β-CD, hydroxypropyl-β-CD, γ-CD (e.g. α-, β-, heptakis(2,6-di-*O*-methyl)-β-CD)	102
3,4-Methylenedioxymethamphetamine and metabolites (MDMA or Ecstasy)	2-hydroxypropyl-β-CDs	109
Methyl phenidate	CDs	16
Metomidate	CD derivatives	110
Metoprolol	molecularly imprinted polymers	111
Mexiletine chloride	methyl-β-CDs	112
Muscarinic antagonists	α-, β-, γ-CDs	113
Nafronyl oxalate	2,6-di-*O*-carboxymethyl-β-CDs	114
Nadolol	β-CD	97
Napropamide	β-CDs derivatives (e.g. sulphobutylether β-CD)	85
Naproxen	β-CD; dimethyl-, triethyl- and hydroxypropyl-β-CD	67
Neutral molecules	phosphate-substituted CDs	70
Nicardipines	CDs	115
o-, *m*- and *p*-Nitrobenzoic acid	β-CD	40
Nitrophenols	α-CD	116
N-Methylephedrine	methylated-β-CD derivatives	96
Ofloxacin	β-CDs and derivatives	72
Omeprazole	bovine serum albumin	101
Orciprenaline	β-CD	68, 97
Orphenadrine	α-CD	76
Oxomemazine	α-CD	76
Oxprenolol	carboxymethyl-β-CD with various degree of substitution	18
	pepsin	77
Pantoprazole sodium	bovine serum albumin	101
Paveroline drugs	poly(sodium *N*-undecylenyl amino L-valinate)	53
Di- and tripeptides	β, γ-CDs	117
	vancomycin	118
Pesticides	CDs	119
Phenylalkylamine derivatives	2,6-di-*O*-methyl-β-CD	120
Phenylbutyric acids	α-CD	116
Phenylprine	hydroxypropyl-β-CD	46
Phenylephrine	β-CD	47
α-Phosphonosulfonic acids	β-CDs	121
Prilocaine	dimethyl-β-CD	98
Pinacidil	β, γ-CD and hydroxypropyl-β-CD (e.g. hydroxypropyl-β-CD)	122
Pindolol	pepsin	77
Pranoprofen	β-CD; dimethyl-, trimethyl- and hydroxypropyl-β-CD	67
Promethazine	β-CDs and derivatives	72
	pepsin	77
	human serum transferrin	64
Propiomazine	human serum albumin	62
Propranolol	monomethylamino-β-CD	13
	human serum transferrin	64
	2-monohydroxypropyl-β-CD	73
	pepsin	77
	β-CDs	97, 123–4
	molecularly imprinted polymers	111, 125
Quinolonecarboxylic acids and related compounds	vancomycin	126

Table 1. continued.

Applications	Chiral selectors	References
Relaxants	sulphated CDs (degree of substitution 7–10)	48
Ropivacaine	dimethyl-β-CD	98
	2,6-di-*O*-methyl-β-CD	127
Sulindac	dimethyl- or trimethyl-β-CD/sulphobutyl ether-β-CD (dual)	86
Anionic sulphonamides	native CD and derivatives	128
Sulpiride	α1-acid glycoprotein	57
Suprofen	β-Cd; dimethyl-, trimethyl- and hydroxypropyl-β-CD	67
Terbutaline	monomethylamino-β-CD	13
	7 different methylated-β-CD derivatives	96
Terpenes	sulphated β-CDs/α-CDs (dual)	129
Tetrahydrocarboline compounds	β-CD	130
Tetryzoline	β-CD	68
	α-CD	76
Thiinopyrroles	β, γ-CDs	131
Thiopyrans	β, γ-CDs	131
Thioridazine	β-CD	20
Tioconazole	β-CDs	132
o-, *m*- and *p*-Toluic acid	β-CD	40
Trimetoquinol	neutral polysaccharides (e.g. dextran, dextrin) and ionic polysaccharides (e.g. chondroitin sulphate C)	60
Trimipramine	β-CD	20
	pepsin	77
Troddoger's base	poly(sodium *N*-undecylenyl amino L-valinate)	53
Tropicamide	β-CD	68
	α-CD	76
Verapamil	pepsin	77
Warfarin	monomethylamino-β-CD	13
	dimethyl- or trimethyl-β-CD/sulphobutyl ether-β-CD (dual)	86
Zopiclone	β-CD	68
Anionic racemates	Teicoplanin	133
11 Chiral drugs	succinyl β-CD	134

*149 references were found on SciFinder (July 1997) containing the keywords 'capillary electrophoresis', and at least one of the following: 'chiral separation' or 'enantiomeric separation'.

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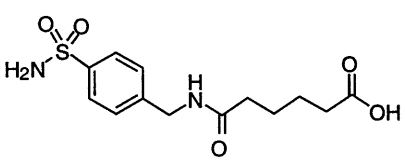
ous problems associated with racemic drugs such as thalidomide and ketamine. The FDA currently requires enantiomers to be considered as separate entities and that only one enantiomer of a chiral drug be marketed. This restriction creates an increasing demand for new and better enantioselective separation technologies.

Techniques typically employed for chiral separation include HPLC, thin-layer chromatography (TLC), gas chromatography (GC) and more recently CE. For example, protein-bound stationary phases, mobile phase additives, cavity phases and Pirkle-type phases are the common chiral stationary phases (CSPs) used in HPLC. In CE, the separation of enantiomers is usually performed by adding optically pure additives or chiral selectors (e.g. native and derivatized cyclodextrins, bile salts, proteins, carbohydrates, antibiotics) to the electrophoresis buffer (see refs 1–12 in table 1). The mechanism of separation is based on binding interactions of various affinities between the chiral selectors and the racemic molecules. Interactions such as hydrogen bonding, hydrophobic and dipole-dipole interactions, and steric hindrance often influence the extent of stability of complexes. Because the affinities between each of the enantiomers to the chiral selector differ, one enantiomer will migrate more slowly than the other upon binding to the chiral selector present in the electrophoresis buffer, thus resulting in chiral separation. Parameters such as chiral selectors, selector concentrations, pH of the electrophoresis buffer used, electric field strength, temperature, capillary length and the EOF all participate in the optimization of separation. Compared with HPLC, CE offers the advantages of low cost (e.g. the chiral selector is used as an additive and does not require immobilization as compared with the chiral stationary phase in HPLC), high efficiency, rapid method development and optimization, and easy application to chiral separation studies. For example, fig. 2 shows that all four possible stereoisomers of dansylated leucinylleucine dipeptides were separated to baseline in 21 min using γ-cyclodextrin as a chiral selector additive in the electrophoretic buffer. Table 1 provides a list of chiral separation studies using CE reported in 1996–97.

Measurement of binding constants

The development of ACE was initially demonstrated with a model system consisting of carbonic anhydrase (CA) as the receptor protein and arylsulphonamides as the binding ligands [113, 125, 126]. The protein was selected for its minute adsorption, if any, onto uncoated capillaries and specific recognition of arylsulphonamides as inhibitors. Also, it is widely available, structurally well characterized and catalyses a medicinally important reaction (i.e. hydration of carbon dioxide). In the study, binding constants for sulphonamide inhibitors to CA by ACE ($K_b = 0.48 \times 10^6$ M^{-1} for **1**, 0.22×10^6 M^{-1} for **2**) agreed well with those obtained



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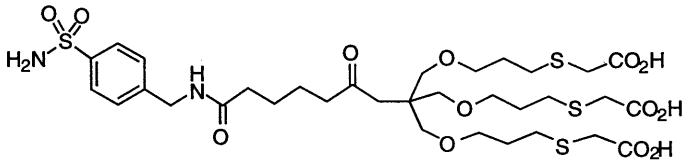

2

using a conventional competitive fluorescence assay ($K_b = 0.51 \times 10^6$ M^{-1} for **1**, 0.14×10^6 M^{-1} for **2**) [113]. Since then, numerous studies with other receptor proteins and ligands have established ACE as a general method for determining binding constants (table 2). Results shown in fig. 3, for example, demonstrate that calcium ion only binds the calcium-binding protein calmodulin (CaM) and does not recognize proteins such as haemoglobin, carbonic anhydrase, trypsin inhibitor and the neutral marker [113]. This ACE-binding study is rapid and highly efficient, since each CE experiment took less than 5 min to perform. Direct measurement of protein interaction with electrically neutral, small molecular weight ligands using ACE is not possible because the electrophoretic mobilities of the free and complexed proteins are often experimentally indistinguishable (eq. 2). The neutral ligand does not introduce a change in charge of the protein-ligand complex, and this complex formation results in an undetectable increase in the mass. One way of circumventing this limitation is to measure the binding constant of a neutral ligand by allowing it to compete with a charged ligand of known constant. Using this competitive ACE procedure, binding constants of CA to several neutral arylsulphonamides were measured, and their values agreed well with literature values, obtained by a fluorescence-based assay [116].

Besides protein-ligand interactions, ACE has also been extended to the study of biomolecular recognitions involving low molecular weight receptors. Using vancomycin and *N*-acyl-D-alanyl-D-alanines [114] as the model system, the binding constants of four compounds (two pairs of enantiomeric peptides) to vancomycin were measured. Two types of experiments, R_L [observe

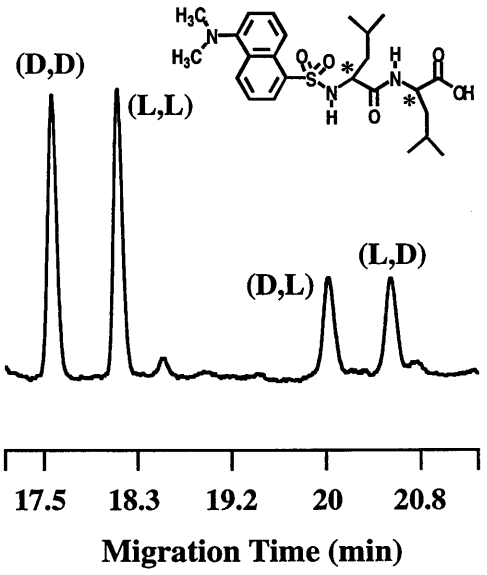

Figure 2. Chiral separation of racemic *N*-dansylated leucinylleucine dipeptides DNS-Leu-Leu (D,D; L,L; D,L; L,D) by γ-cyclodextrin. Buffer: 192 mM glycine, 25 mM Tris base, 100 mM sodium dodecylsulfate, 50 mM γ-cyclodextrin (pH 8.4); capillary: uncoated fused silica, 100 cm total length, 70 cm effective length, 50 μm inner diameter; CE: 30 kV, 38 μamp, 200 nm, 30 °C.

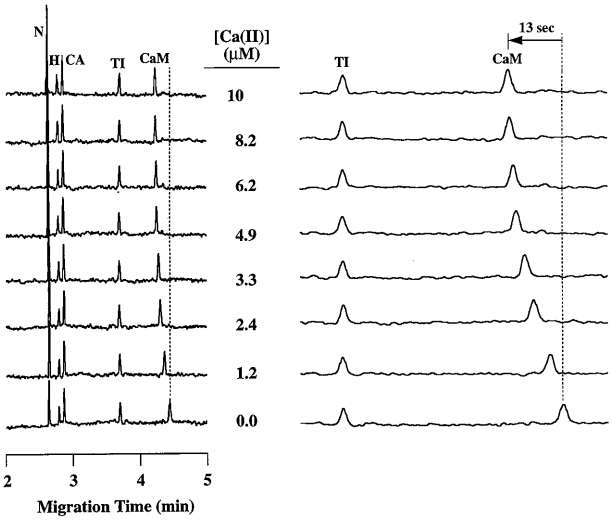

Figure 3. ACE of bovine testis calmodulin (CaM) in 0.192 M glycine-0.025 M Tris buffer (pH 8.4) containing various concentrations of calcium chloride. The electrophoresis time in each ACE experiment was ca. 4.5 min at 30 kV using a 70 cm (effective length), 50 mm uncoated fused silica capillary. Horse heart myoglobin (H), bovine carbonic anhydrase (CA), soybean trypsin inhibitor (TI) and mesityl oxide (N) were used as internal standards. Under the experimental conditions, the electrophoretic mobility of CaM decreased upon binding to calcium ion present in the electrophoresis buffer and the protein was thus detected earlier.

Table 2. Measurement of binding constants of receptor-ligand interactions using ACE.*

Examples	References
1. ACE reviews	1–15
2. Carbohydrate-peptide interactions	
mannose-1-phosphate and human serum amyloid P component	16
3. Guest-host interactions	
dansyl-amino acids and cyclodextrans	17, 18
isomeric methylbenzoats and cyclodextrans	19
melatonergic drug enantiomers and β-cyclodextrans	20
adamantane carboxylic acids and cyclodextrans	21, 22
anionic/neutral compounds and cyclodextrins/vancomycin	23
tryptophan and cyclodextrins	24
deprenyl (selegiline)/metabolites and cyclodextrans	25
derivatives of phenylalkylamine enantiomers and cyclodextrans	26
salicylates and bovine serum albumin	27
pharmaceutical amines and cyclodextrans	28
aminoquinolycarbamate derivatives of amino acids and vancomycin	29
salbutamol and cyclodextrans	30, 31
enantiomers of anaesthetics and cyclodextrans	32
isomeric dichlorophenols and chiral surfactants	33
antidepressant drugs/analogues (mianserine, Tolvin) and cyclodextrans	34
phenyl acetates and cyclodextrans	35
nitrophenolates and cyclodextrans	36
propranolol and cyclodextrins	18, 37
binaphtyl derivatives and cyclodextrans	38
leucovorin/5-methyltetrahydrofolate and cyclodextrins	39
tioconazole enantiomers and cyclodextrans	40, 41
4. Ion-ion/dye interactions	
acidic/basic/amphoteric pharmaceuticals and protonated forms	42
aromatic anions and quaternary ammonium ions	43, 44
peptides and different protonated forms	45
divalent anionic azo dyes and hydrophobic quanternary ammonium ions	46
5. Oligonucleotide-oligonucleotide interactions	
oligodeoxynucleotides and poly(9-vinyladenine)	47
hexopyranosyl analogues of thymidine and thymine	48
$d(A)_n/d(T)_n$	49–51
6. Oligonucleotide-peptide interactions	
oligonucleotides and human serum amyloid P component	52
7. Oligonucleotide-small molecules interactions	
cytidine and borate	53
human genomic DNA and DNA-based drug	54
thiazole gree (TAG) and DNA restriction fragments	55
8. Peptide-dye interactions	
synthetic peptide and Congo Red	56
9. Peptide-peptide interactions	
vancomycin and D-Ala-D-Ala-containing peptides	57
vancomycin and cytoplasmic peptidoglycan precursors	58
dimerization of vancomycin, ristocetin A, and LY264826 (A82846B)	59
LY191145 (glycopeptide antiobiotic) and peptides	60
vancomycin and peptide libraries	61–64
vancomycin and dipeptides/related dipeptides	65, 66
10. Protein-carbohydrate interactions	
lectins and charged polysaccharides	67
concanavalin A and monosaccarides	68
concanavalin A and rhodamine-labelled mannoside	69
Tetragonolobus purpureas lectin and fucose-1-phosphate	70
11. Protein-drug interactions	
bovine/human serum albumin and anti-inflammatory compounds	71
hsc70 and deoxyspergualin	72
haemoglobin/histone proteins and antiarrhythmic procainamide	73
bovine serum albumin and warfarin	74
bovine serum albumin and leucovorin	75
cellulase and β-blockers	76
bovine serum albumin, bacterial cellulase and Ca(II) tryptophan, benzoin, pindolol, promethazine, warfarin	77
human serum albumin and kynurenine, tryptophan, 3-indolelactic acid, 2,3-benzoyltartaric acid, *N*-2,4-dinotrophenylglutamate	78
12. Protein-ion interactions	
C-reactive protein and Ca(II)	55, 79, 80, 81
calmodulin, parvalbumin, thermolysin and Ca(II); carbonic anhydrase, thermolysin and Zn(II)	82–84

Table 2. continued.

Examples	References
13. Protein-ligand(s)/hapten(s) interactions	
Fab fragment of antibody McPC603 and antigen	85
rat monoclonal antibody and *N*-2,4-dinotrophenyl group-containing ligands	86–88
carbonic anhydrase and arylsulphonamides	14, 57, 82, 89–91
streptavidin and biotin derivatives	92
SH3 domains and peptide ligands	93
β-galactose-specific lectins and lactobionic acid	94
monoclonal antibody and fluorescein isothiocyanate-labelled insulin/unlabelled insulins	95
14. Protein-oligonucleotide interactions	
monoclonal antibody and 32-mer oligonucleotide	96
bovine serum albumin and phosphorothioate oligonucleotide	97
transcription factor (SpP3A2) and fluorescently-labelled DNA	98
15. Protein-protein interactions	
recombinant HIV-1 gp120 (rgp120) and sCD4	99
dimerization of insulin	100
human growth hormone (hGH) and anti-hGH (or its fragment)	101, 102
human serum albumin (HSA) and anti-HSA	92
methanol dehydrogenase and cytochrome c	103
concanavalin A and complexes of human serum proteins: α1-antitrypsin, orosomucoid (α1-acid glycoprotein), arylesterase/haptoglobin, α2-HS-glycoprotein, transferrin	104

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receptor and vary ligand (peptide) concentration] and L_R [observe ligand and vary receptor (vancomycin) concentration], were conducted. In L_R experiments, a single set of measurements can determine the values of K_b for a number of ligands simultaneously. The binding constants determined by ACE compared well with those obtained from other assays [114]. Table 2 provides an additional list of binding studies using ACE where some form of binding or dissociation constant was determined.

Estimation of kinetic rate constants

ACE is applicable for estimating the kinetic rate constants, k_{on} and k_{off}, for the interactions of proteins and ligands. Through the use of a computer-based simulation, information concerning the kinetics between protein and ligands in the electropherogram can be obtained by analysing the displacement and shape of the protein peak. Upon binding, the peak broadening observed around the intermediate migration times reflects the equilibration of species with different migration times. The migration times, in this case, of the species (free protein and protein-ligand complex) are comparable to the electrophoresis runtime. The binding of CA and its inhibitors **2** under conditions of the ACE experiments were used and studied as a model for the simulations [116]. Different combinations of k_{on} and k_{off} were used to approximate the experimental electropherograms; combinations of kinetic constants were limited to the binding constants that were equal to the experimentally determined binding constants. Based on the findings from these simulations, only one set of simulation-generated electrophoregrams approximated the peaks widths of the experimental electrophoregram. The kinetic constants obtained fell within the range of k_{off} ($0.05–0.5\ s^{-1}$) and k_{on} ($10^4–10^6\ M^{-1}\ s^{-1}$) previously reported for CA interaction with arylsulphonamides [127, 128].

Determination of binding stoichiometries

ACE, while primarily developed for measuring binding constants in biomolecular recognition, can easily be adapted to the determination of binding stoichiometries [117]. Information obtained from binding stoichiometries allows one to measure the concentration of biologically active proteins and to probe specificity. The method of ACE for stoichiometry determination is well suited for studying a wide range of binding interactions (weak and tight binding), including those that are difficult to study using other methods [117].

In the case of weak binding systems, in order to simplify the determination of binding stoichiometry, n, from an interaction (eq. 5), the protein P is maintained in its fully bound form

$$P \cdot L_n \rightleftharpoons P + nL \quad (5)$$

by using concentrations of ligand L much greater than the dissociation constant, both in the samples ($[L]_s$) and electrophoresis buffer ($[L]_b$). In the electropherograms, the peak integration at the migration time of ligand represents the free ligand concentration, $[L]_f$. Zero integration in the plot of the area of the free ligand peak vs. the total ligand concentration, $[L]_t$, present yields a value of ligand in the sample where the free ligand concentration equals the ligand concentration in the electrophoresis buffer, $[L]_f = [L]_b$. The difference between the total and free ligand concentrations is the amount of the ligand associated with the receptor ($[L]_{bound} = [L]_s - [L]_b$). The concentration ratio of bound ligand to the receptor yields the binding stoichiometry ($n = [L]_{bound}/[P]$).

A stoichiometry of 1:1 was obtained for CA binding to an arylsulphonamide ligand **1** using this methodology (fig. 4A). In figure 4A, the inflection point from the negative area of ligand to the positive area was between 69 and 73 μM. In a normalized plot of free ligand vs. total concentration, the zero area of free ligand was the stoichiometric titration point with a corresponding x-axis value of 71 μM. To obtain n, the difference of 71 and 67 μM (ligand concentration in buffer) was divided by the CA concentration (4.2 μM) to yield a stoichiometry of $n \approx 0.9$ [117].

In examples of tight-binding systems such as antibody-antigen and streptavidin-biotin interactions, for each mole of the protein in the sample solution, the addition of n mol of ligand results in the formation of exactly 1 mol of the complex. Samples containing a fixed receptor protein concentration and various ligand concentrations were prepared. In the electropherograms, as the concentration ratio $[L]_t/[P]$ increases, the complex peak concentration increases until $[L]_t/[P] = n$; beyond this point no additional complex can form. An abrupt slope

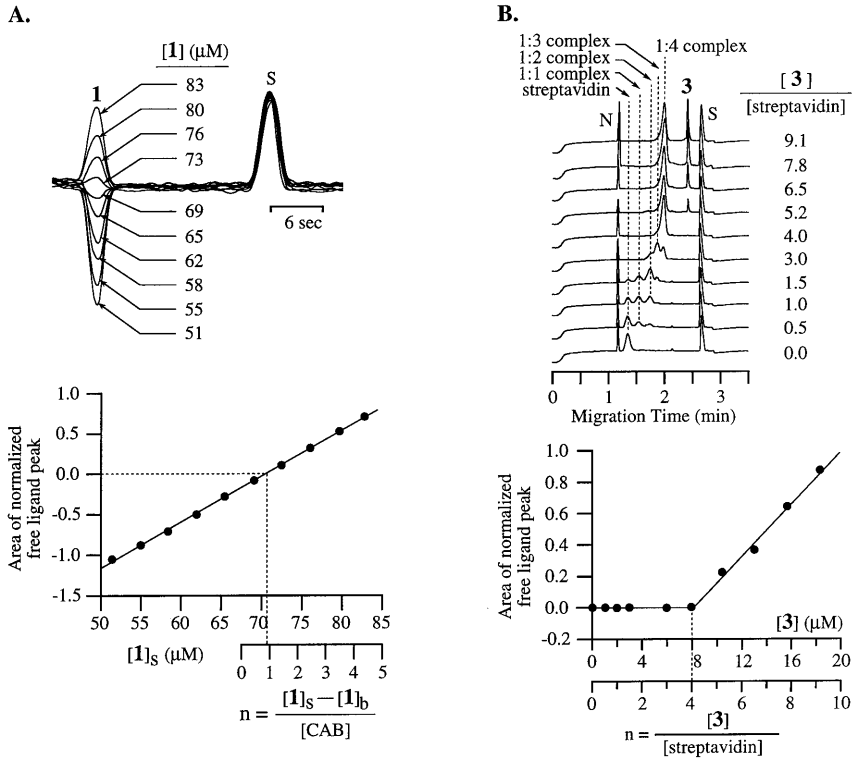

Figure 4. Determination of stoichiometries of (*A*) a weak-binding system: the carbonic anhydrase-arylsulphonamide **1** interaction, and (*B*) a tight-binding system: the streptavidin-biotin **3** interaction. (*A*) ACE of samples having a constant concentration of bovine carbonic anhydrase (CAB; pI 5.9, 4.2 μM) and the ligand **1** (67 μM) in a Tris-glycine buffer containing various concentrations of **1** (pH 8.3). The electrophoresis time in each experiment was 2.5 min at 12 kV using 200 nm as the detection. 2-Iodobenzoic acid (S) was used as the internal standard for peak areas. The graph summarizes experimental data and determines the stoichiometry of the binding interaction: $n = (71 - 67)/4.2 = 0.9$. (*B*) Determination of the stoichiometry of streptavidin (1.7 μM) binding to a biotin-oligonucleotide conjugate **3** of various concentrations using ACE in Tris-glycine buffer (pH 8.7). The total electrophoresis time in each experiment was 3.5 min at 15 kV using 214 nm as the detection. S was dinitrophenylaspartic acid used as the internal standard. A plot of the concentration of free ligand vs. the ratio [**3**]/[streptavidin] gives a sharp break at the stoichiometric point (4.0).

change in a plot of integrated free ligand vs. the ratio of $[L]_t/[P]$ in samples corresponds to the stoichiometry n of the systems studied (fig. 4B). Systems studied and reported were (i) the interaction of a monoclonal antibody to human serum albumin (anti-HSA) with its antigen HSA, and (ii) the binding of streptavidin to a biotin-oliogonucleotide conjugate (fig. 4B) to yield stoichiometries of 1:2 and 1:4, respectively [117].

The application of ACE to the determination of binding stoichiometry has demonstrative value for binding systems with both low- and high-affinity systems. For systems that have intermediate binding affinities, parameters (voltage, length of capillary, pH or concentration of electrophoresis buffer) can be manipulated so as to treat the system under the protocols of either the low- or the high-affinity system. The ability of rapid and high-resolution separation makes ACE more versatile than other methods that require changes in spectral characteristics and/or are limited to certain binding interactions

Combinatorial library screening in solution

Combinatorial library methods have been widely adopted by large and small drug discovery companies over the past few years as useful tools in identifying antigenic determinants, receptor-binding ligands, enzyme substrates and enzyme inhibitors [129, 130]. The idea of combinatorial biochemistry is to form large libraries of molecules, instead of synthesizing compounds one by one, and to identify the most promising lead pharmaceutical molecules. This successful identification of lead ligands from libraries requires efficient screening and binding assays to evaluate the extent of receptor-ligand interactions and rapid sequence determination or structural identification. ACE is valuable in

the screening of small soluble libraries. Two methods based on ACE have been developed and are reported to have been used successfully in searching for tight-binding ligands from small libraries of molecules in solution [118–120].

The first methodology was illustrated using vancomycin as the receptor and a library of 32 unlabelled peptides [118] and should be applicable, at least in principle, to larger libraries and to other receptor-ligand systems. In combination with the strategy of library deconvolution, the screening procedure involved the use of a known tight-binding ligand as the CE sample and the electrophoresis buffer containing vancomycin at limited concentration and a library of compounds of equal molar concentration. The change in the electrophoretic mobility of this tight-binding ligand indicated binding competition between the ligand and lead compounds present in the library for the binding site of vancomycin. This change of mobility was used as an indicator of lead compounds present in the library, new sublibraries were prepared from this library, the screening procedure was repeated and the libraries were further deconvoluted until the identities of all ligands were discovered.

This ACE method is useful in combinatorial library deconvolution and has the following advantages: (i) it can screen a number of compounds simultaneously and efficiently eliminate entire libraries of compounds that do not contain active lead molecules; (ii) it does not require derivatization of compounds in the library, nor does it require biological activity since it is a binding assay; and (iii) this screening assay is equally applicable to both peptide and nonpeptide ligands. The most attractive feature of this ACE-based screening technique is that it requires only small quantities of receptors and library compounds, all analyses are readily automated and require only short analysis times (typically minutes), and it is carried out in homogeneous solutions. The size of libraries (i.e. the number of compounds in the library that can be screened at one time) is, however, limited by the targeted dissociation/inhibition constant: the tighter the binding of lead compounds, the larger the number of molecules that can be screened [118].

The second library screening method integrates ACE with mass spectrometry (MS) [119, 120]. Since ACE does not provide any structural information for the selected ligands, the coupling of ACE with MS offers an attractive one-step screening: on-line separation and structure determination of ligands from combinatorial libraries that bind most tightly to a receptor. Initially, a binding system consisting of vancomycin and a small library of all-D tetrapeptides, Fmoc-DDXX ($10^2 = 100$), prepared by the method of split synthesis was used to demonstrate the usefulness of this ACE-MS method for library screening in solution [119]. In the ACE procedure, vancomycin was introduced in the electrophoretic buffer as a plug, either partially (10–50 s pressure injection) or completely filling the capillary, and the library was then sampled in a short time (3 s pressure injection) (fig. 5). Since vancomycin migrated away from and the whole library travelled towards the MS under the experimental conditions [119], ligands that bound tightly to the receptor were retained and thus appropriately separated from noninteracting species in the library (fig. 5). These interacting ligands were subsequently detected and their structures identified by on-line MS. This study resulted in the identification of three peptide ligands that bind more tightly than the Fmoc-DDAA to vancomycin [119]. By chemically synthesizing the ACE-MS-selected peptide ligands and evaluting their binding to vancomycin, it was confirmed that Fmoc-DDFA, Fmoc-DDYA and Fmoc-DDHA bind more tightly than Fmoc-DDAA (dissociation constants of 7.2, 7.8, 26 and 59 μM at pH = 8.1, respectively) [119]. This library screening revealed that vancomycin binds most tightly to ligands containing an alanine at the C-terminus and an aromatic amino acid at the penultimate position. Other active ligands obtained such as Fmoc-DDEA, -WA, -VA, -MA and -QA were also found to have affinities equal to or greater than Fmoc-DDAA [120].

This ACE-based approach for screening combinatorial libraries has been further extended to all-D libraries of Fmoc-EXX ($10^2 = 100$) and Fmoc-DDXX ($19^2 = 361$) and was successfully employed to determine interacting structural motifs. Results from these two libraries confirmed the binding motif found with the first library and indicated the ligand length (tri- vs. tetrapeptides) and proximity of the Fmoc group to the receptor had little or no importance in determining relative binding strength [120].

A 1000-member peptide library of the form Fmoc-DXXX ($10^3 = 1000$) was further synthesized to evaluate the performance of ACE-MS for larger libraries while determining the binding effect of the third amino acid from the C-terminus. The 1000-member library was shown to be at the limit of MS detection requirements for obtaining informative mass spectra. An affinity extraction with the receptor immobilized onto a solid support as a means of preselecting and preconcentrating the most active ligands was used to circumvent these requirements [120]. Preselection of the most active ligands from a large library of compounds can potentially extend the ACE-MS methodology to $>10{,}000$ species.

The ACE-MS methodology discussed in this review is a useful procedure for both separating and identifying the most tightly binding ligands from combinatorial libraries. Like ACE, this technique is economical, rapid (less than 4 min in fig. 5), experimentally straightforward and versatile, and most significantly it is performed in homogeneous solution, so that the non-

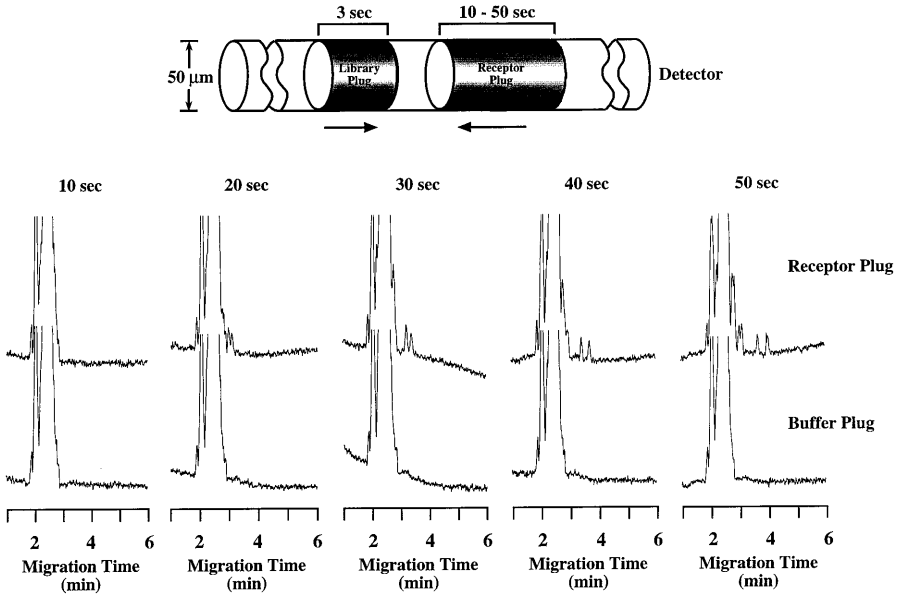

Figure 5. ACE of an all-D library of Fmoc-DDXX ($10^2 = 100$) tetrapeptides to search for ligands that bind tightly to vancomycin. In the procedure, vancomycin (70 μM) used as the receptor was first introduced into the electrophoresis buffer as a plug (10–50 s pressure injection), followed by a short plug of the library (3 s pressure injection), and the ACE experiment was carried out using a PVA coated capillary (27 cm total length, 20 cm effective length, 50 μm inner diameter) in 50 mM Tris-acetate buffer (pH 8.1) at 13 kV. Since vancomycin was slightly positively charged under experimental conditions, it migrated away from the detection. The whole library travelled towards the MS detector, and any ligands recognized by the receptor were retained and separated from noninteracting species in the library. This separation of ligands from the library can be readily manipulated by changing both the length of the receptor plug and the receptor concentration. Using 40-s injection of vancomycin (70 μM), three ligands in two peaks were detected and structurally identified by MS: Fmoc-DDFA, Fmoc-DDYA and Fmoc-DDHA. The electrophoresis buffer was used as the control to introduce into the capillary at corresponding plugs to ensure the specific binding of lead compounds to the receptor. The total electrophoresis time for each library screening was finished in less than 4 min.

specific interactions often observed in library screening on beads are minimized. Also, this ACE-MS can directly measure binding constants of mixtures of peptide ligands in libraries to a specific receptor [131] and can easily be applied to a wide variety of libraries of small organic molecules.

Other applications

Among the interactions involved in biomolecular recognition, electrostatic interactions often participate in the association between the charged groups of ligands and receptors. Although electrostatic interactions contribute energetically to many important biological systems, it has been difficult to evaluate these contributions quantitatively. Whitesides and co-workers at Harvard University [132–135] are the pioneers in this area of research and have recently illustrated that the combination of ACE and protein charge ladders provides a useful physical-organic tool for quantitatively examining these contributions. They described two related methods to estimate the effective charge of a protein in solution. The underlying strategy of the two methods was to generate and compare the electrophoretic mobilities of a protein with a series of derivatives of the same protein that differ in integral values of effective charges, but that differ minimally in hydrodynamic drag. The derivatized forms of the protein exhibited migration times in the electrophoregram as a set of evenly spaced-peaks referred to as a charge ladder. The intervals in electrophoretic mobilities established the influence of effective charge on mobility and allowed the effective charge of the native protein to be determined directly by extrapolation. The model system used involved carbonic anhydrase with arylsulphonamides substituted in the *para* position with charged and neutral groups. The set of derivatized proteins were generated by treatment of CA with acetic anhydride in which distributions of positively charged lysine side-chain NH_3^+ groups were changed to neutral *N*-acyl derivatives. Analysis of the data resulted in the measurement of the effective charge of -3.7 for CA (pI 5.9) and -5.0 for CA (pI 5.4). Vancomycin and D-Ala-D-Ala were also examined as a model system. It was found that the electrostatic interaction between the C-terminal carboxylate group of the

D-Ala-D-Ala moiety and the $-NH_2CH_3^+$ group of vancomycin contributes approximately 5.9 kJ mol^{-1} to the free energy of binding of these two species [136].

ACE can also be applied to enzyme-catalysed reactions taking place in an electrophoresis capillary under nondenaturing conditions [53, 137]. In these experiments, either enzymes and substrates were injected as separate plugs into the capillary or the enzyme was injected and the substrate was included in the electrophoresis buffer. The oxidation of glucose 6-phosphate to 6-phosphogluconate was demonstrated using 6-phosphate dehydrogenase with NAD^+ as a model system. Potential applications of this type of microreactor technique include analysing substrates for enzymatic activities or concentrations of substrates, evaluating molecules as substrates for enzyme-catalysed reactions, examining mixtures of proteins for activity against a particular substrate and studying enzyme-inhibitor interactions.

Future prospects

Only 17 years after its introduction [138, 139], CE is a well-established technique combining many of the advantages of HPLC with those of electrophoresis. CE offers rapid analysis of minute quantities of biomolecules with unprecedented high resolution, and ACE provides a new method for analysis of receptor-ligand interactions. ACE has been and will continue to be valuable in rapid assessment of receptor-ligand interactions; more than 100 peer-reviewed papers have reported the direct use of ACE or its related CE-based methods in quantitative measurements of binding constants (table 2).

Currently, the major limitation to CE and ACE is that many proteins, particularly those of opposite charge to the surface of the uncoated capillary, adsorb to the wall, resulting in either poor efficiency or loss of material. Much progress has been made in the past to reduce (but not always eliminate) protein adsorption on the wall of capillary. Since electrosatatic interaction is thought to be a primary source for protein adsorption to the wall of a capillary, most efforts have focused on eliminating this interaction. Among various methods used to achieve high resolution, three approaches are often used to minimize adsorption: manipulation of pH, dynamic coating of the capillary surface using additives to the electrophoresis buffer and chemical modification of the surface [29]. For example, the most popular coating today is the attachment of linear polyacrylamide, which gives very low adsorption of proteins. Although many attempts have been made to reduce active adsorption sites by derivatizing silanol groups, complete wall deactivation has not so far been successful. Also, it was suggested that ACE may prove valuable in quantitative analysis of ligand interaction with the binding domains of the receptor proteins (ref. 15 in table 2). Ligands are recognized by large proteins, but the actual sequence of amino acids responsible for the binding may be small, and these active fragments may be free of adsorption to the capillary wall.

In the life sciences, alternative and improved technologies are important for quantitative receptor-ligand binding studies. In terms of applications, ACE has demonstrated that it is useful in chiral separation of racemic biomolecules, measurement of binding constants, estimation of kinetic on- and off-rate constants, determination of binding stoichiometries, examination of electrostatic interactions, estimation of effective charges and molecular weights of proteins, characterization of enzymatic activities, and library screening for tight-binding drug candidates in solutions. Many other ACE applications are possible, including the analysis of protein-protein interactions often involved in signal transduction pathways and DNA-drug interactions. Although kinetic association and dissociation rate constants can be conveniently obtained (at least in principle) by surface plasmon resonance, useful information in binding kinetics of receptor-ligand interactions may be rapidly extracted from the peak shape and width in electropherograms, especially in cases of small molecule-small molecule binding interactions. In the future, ACE may find more uses in the determination of stoichiometries of receptor-ligand interactions. For example, using laser-induced fluorescence detection, ACE is well suited for studying of DNA-protein interactions. Because nucleic acids (oligonucleotides) and proteins (peptides) have exceedingly different electrophoretic mobilities at physiological pH and because once formed, their complexes often dissociate slowly, the binding measurement of labelled DNA to protein becomes straightforward (fig. 4B). This ACE method may have the potential to replace the conventional gel-shift assays which involve radiolabelled compounds.

ACE not only is valuable in studies of quantitative biomolecular recognition but can also be useful for solution immunoassays and clinical diagnosis [140]. In terms of soluble library screening, the integrated approach of ACE-MS should be directly applicable to the drug discovery programmes conducted in pharmaceutical and biotechnology companies, especially in the research of the lead compound discovery and optimization from 'focused' libraries. Finally, the new technology of CE-on-microchip will likely make ACE accessible to other unexplored areas in the life sciences.

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