Review

# Separation methods applicable to the evaluation of enzyme–inhibitor and enzyme–substrate interactions

Kristi L. Burns, Sheldon W. May*

*School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332, USA*

## Abstract

Enzymes catalyze a rich variety of metabolic transformations, and do so with very high catalytic rates under mild conditions, and with high reaction regioselectivity and stereospecificity. These characteristics make biocatalysis highly attractive from the perspectives of biotechnology, analytical chemistry, and organic synthesis. This review, containing 128 references, focuses on the use of separation techniques in the elucidation of enzyme–inhibitor and enzyme–substrate interactions. While coverage of the literature is selective, a broad perspective is maintained. Topics considered include chromatographic methods with soluble or immobilized enzymes, capillary electrophoresis, biomolecular interaction analysis tandem mass spectrometry (BIA–MS), phage and ribosomal display, and immobilized enzyme reactors (IMERs). Examples were selected to demonstrate the relevance and application of these methods for determining enzyme kinetic parameters, ranking of enzyme inhibitors, and stereoselective synthesis and separation of chiral entities.
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* Corresponding author. Tel.: +1-404-894-4052; fax: +1-404-894-2295.
*E-mail address:* sheldon.may@chemistry.gatech.edu (S.W. May).

# 1. Introduction

Enzymes are nature's catalysts. These proteins catalyze a rich variety of metabolic transformations, and do so with very high catalytic rates under mild conditions, and with high reaction regioselectivity and stereospecificity. These catalytic characteristics, which are obviously essential to the survival of all living cells, are also highly attractive from the perspectives of biotechnology, analytical chemistry, and organic synthesis. The regio- and stereospecificity of enzymes can be exploited for separations in cases where a high degree of discrimination between stereoisomers or structurally-similar compounds is required. Similarly, the high catalytic turnover rates of enzymes make these biocatalysts highly attractive for biosensor applications where rapid and selective signal generation is needed. Biocatalysis has always been a key focus area in biotechnology due to the ability of enzymes to carry out chemical reactions, which are difficult or impossible to accomplish using conventional chemical methods.

It is important to note that within the last few years, the horizons of biocatalysis have expanded greatly, to encompass both catalytic RNA molecules ("ribozymes") and catalytic antibodies ("abzymes"). Due to the emergence of these new classes of biocatalysts, the potential of biocatalysis for use in separations, detection or synthesis now extends well beyond the particular chemical reactions which normally occur within living cells.

This review focuses on separation methods related to the interactions of enzymes with their respective substrates and inhibitors. While coverage of the literature is highly selective, we have attempted to maintain a broad perspective. Thus, in addition to traditional topics such as vacancy techniques and affinity chromatography, we have included sections on the techniques of phage display, ribosome display, biomolecular interaction analysis (BIA) and immobilized enzyme reactors (IMERs). As detailed below, separation methodology is at the core of all four of these techniques when they are applied to the interactions of enzymes or abzymes with substrates or inhibitors.

Finally, we have made no attempt to introduce the reader to the basic concepts of enzyme catalysis, such as Michaelis–Menten kinetics, substrate binding, or reversible vs. irreversible inhibition. Applications of chromatography and other separation methods to biological systems is now very widespread, and in our opinion a basic introduction of this sort is no longer needed by the vast majority of active researchers. A recent review in this journal on utilization of enzyme–substrate interactions in analytical chemistry contains an introduction to some of the basic concepts in enzymology [1].

# 2. Chromatographic techniques

Chromatographic methods for protein–ligand interaction analysis, typically utilize either soluble protein–ligand pairs or immobilization techniques that create a stationary phase with bound protein or ligand. As technology advances, the latter approach is becoming increasingly popular. In immobilization techniques, either the ligand or the protein is attached to a solid support and some sort of capture of the free moiety enables analysis of protein–ligand interactions. However, chromatographic methods in which both the ligand and the protein are free in solution do offer some advantages over immobilized procedures. Firstly, these procedures typically employ traditional HPLC supports, thus obviating the need to purchase or synthesize specialized immobilized columns, and saving the time and effort needed to validate new immobilized supports for chromatography. Furthermore, these techniques avoid some of the complications inherent in immobilization, such as masking of enzyme activity which can lead to inactivation or degradation of the immobilized conjugate which can decrease signal detection and/or increase background noise.

Five major chromatographic techniques are currently available to analyze protein–ligand interactions using soluble reactants: the Hummel–Dreyer method, the vacancy peak method, zonal elution, frontal analysis, and retention analysis. Many variations of these techniques exist, such as adaptations for use with capillary electrophoresis and modifications for analysis with affinity columns. Perhaps the most widely used chromatographic method for analysis of the interactions of soluble ligands and proteins is the Hummel–Dreyer method. This method traditionally utilizes gel permeation chromatography to analyze the ligand–protein binding interactions that occur upon establishment of a dynamic equilibrium when a small quantity of a ligand–protein mixture is injected onto a column equilibrated in the presence of the ligand [1–5].

## 2.1. Hummel–Dreyer method

In the pioneering work by Hummel and Dreyer [4], the authors explored the interactions between the enzyme, RNAase, and the competitive inhibitor, 2′-cytidylic acid. In their experiments, a solution containing both enzyme and inhibitor was injected onto a gel permeation column that was equilibrated with an inhibitor-containing mobile phase. The elution profile exhibited two peaks; the first peak represented both free enzyme and enzyme–inhibitor complex; and the second peak, which determined the quantity of bound inhibitor, represented an inhibitor-depleted zone. Hummel and Dreyer performed these experiments using a mobile phase with a known inhibitor concentration. The enzyme sample solution was prepared in this same mobile phase, but rapid equilibration of the inhibitor with the enzyme resulted in a reduced free inhibitor concentration in this sample solution due to formation of the enzyme–inhibitor complex. Thus, the sample consisted of an equilibrated mixture of free inhibitor, free enzyme and enzyme–inhibitor complex, which was injected onto the column as a sample plug (or sample zone). During chro-

matography, since the pores in the gel filtration support excluded the free enzyme and the enzyme–inhibitor complex, these species migrated faster than the free inhibitor. Consequently, the sample zone became depleted in inhibitor as the enzyme–inhibitor complex migrated with the free enzyme rapidly away from this zone (Fig. 1). Given adequate time, the enzyme–inhibitor complex and the free enzyme completely separated from the sample plug, creating a second zone enriched in inhibitor bound to the enzyme. Thus, the characteristic elution profile in the Hummel–Dreyer method features two peaks. The first peak, corresponding to the zone formed by the free enzyme and the enzyme–inhibitor complex, is enriched in total inhibitor content with respect to the surrounding baseline of inhibitor-containing mobile phase; therefore, this peak is designated as "positive". The second peak, designated as "negative", corresponds to the zone that has become inhibitor-depleted as the inhibitor bound to the enzyme moves ahead, and this second peak exhibits the retention time characteristic of the free inhibitor.
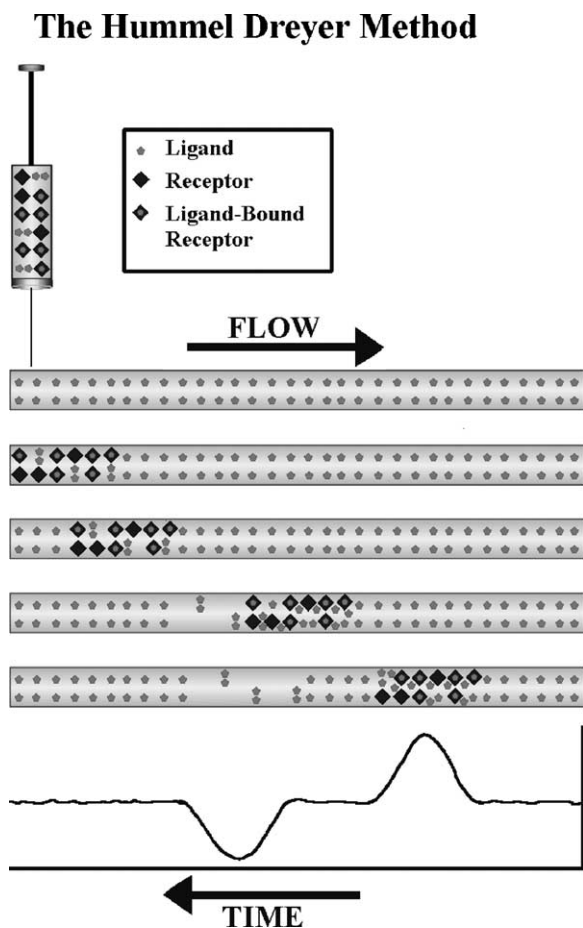


Fig. 1. The Hummel–Dreyer method: schematic of the Hummel–Dreyer method. A mixture of ligand and enzyme is introduced onto a gel filtration column equilibrated with ligand-containing buffer. As the substances travel down the column, the enzyme and enzyme–ligand complex migrate faster than the ligand thus allowing the protein to separate from the original sample plug. The elution profile characteristically features two peaks that result from the ligand-depleted and ligand-enriched areas.

It is important to note that in this method, accurate data analysis requires pure enzyme and inhibitor samples, rapid association and dissociation rates for the inhibitor–enzyme complex and good separations of well-shaped peaks with minimal tailing. Poor peak shape can indicate inadequate exchange kinetics or the presence of alternate binding sites which complex slowly with the enzyme. Binding constants are calculated by determining the quantity of bound inhibitor using either an internal or an external calibration of the vacancy peak [1–3,5]. In the internal calibration method, used initially by Hummel and Dreyer [4], increasing inhibitor concentrations are used in the inhibitor–enzyme sample to essentially fill the vacancy peak. A plot of absorbance of the vacancy peak versus the excess concentration of inhibitor added to the injection sample extrapolated to zero absorbance provides the quantity of bound inhibitor. When the vacancy peak absorbance is null, the enzyme has been completely titrated by the inhibitor and any additional inhibitor yields positive peaks [4–6]. In the external calibration method, the area of the vacancy peak is determined using a calibration curve constructed by injecting increasing inhibitor concentrations onto the column. Sun et al. [7] have validated the method of external calibration by comparison with the internal calibration method for warfarin binding to human or bovine serum albumin [3,5,7,8]. Pinkerton and Koeplinger [6] have formulated a modified Hummel–Dreyer method which requires only two injections to quantify the negative vacancy peaks. As in the traditional Hummel–Dreyer method, this technique utilizes inhibitor in the mobile phase; however, only enzyme without inhibitor is used in the injection sample.

Internal surface reversed-phase columns also known as "restricted access" columns are capable of separating compounds by both gel exclusion and bonded-phase partitioning. These columns are constructed by modification of spherical porous silica so that the outside of the silica particle is non-absorptive but contains a bonded reverse-phase inside the silica pores. Exclusion of large protein molecules from the pores occurs as is typical in gel permeation chromatography but smaller inhibitors are able to enter the pores and interact with the stationary phase thereby increasing retention times. These columns are very well suited for use in the Hummel–Dreyer method [6,9]. It has been demonstrated that the Hummel–Dreyer is sensitive enough to detect stereospecific binding differences between enantiomeric ligands [10].

Recently, Yoneyama and Hatakeyama successfully used the method of Hummel and Dreyer to study the regulation of stimulatory and inhibitory complexes formed between the GTP cyclohydrolase I feedback regulatory protein (GFRP) and GTP cyclohydrolase I [11]. GTP cyclohydrolase I catalyzes the first rate-limiting step in the synthesis of tetrahydrobiopterin ($BH_4$). $BH_4$ is the requisite coenzyme for enzymatic conversion of phenylalanine to tyrosine and then to DOPA in the pathway leading to the catecholamine neurotransmitters. $BH_4$ has also been implicated as a protecting factor against nitric oxide toxicity, and decreased levels of

$BH_4$ have been observed in several neurological diseases [12]. Using the Hummel–Dreyer method, these authors were able to observe formation of the inhibitor complex between two GFRP and one GTP cyclohydrolase I upon a rise in levels of $BH_4$. This regulation prevents $BH_4$ pathway synthesis.

Berger and Girault [13] examined the binding constants for nucleotide binding to chloroplast ATPase CF1 with the Hummel–Dreyer method. Utilizing gel filtration chromatography and the internal calibration method, the authors calculated a $K_D$ of 64 μM for magnesium-complexed ADP. The authors further investigated the interaction of nucleotides with ATPase CF1 by the simultaneous analysis of ATP and ADP. Since ATP and ADP co-elute on gel filtration supports, an anion-exchange column was chosen as the stationary phase to allow baseline separation of ATP, ADP, and any nucleotide–protein complexes [13]. Expanding the Hummel–Dreyer to include non-gel filtration supports is viable as long as good separation of the protein complexes from the free ligand and good peak shape is maintained [13].

Development of the Hummel–Dreyer method into a rapid, efficient method to investigate the binding of ligands to proteins has greatly increased the attractiveness of this approach. As long as the equilibrium between protein and ligand is rapidly established (as is normally the case for enzymes with their respective inhibitors or substrates), and provided a sufficient chromatographic separation of protein complexes from ligands is obtained, this method is an excellent technique for determining binding constants and stoichiometries of protein–ligand interactions.

## 2.2. Frontal analysis

In 1948, Tiselius was awarded the Nobel prize for his research on electrophoresis, delineating the complex nature of serum proteins. Among other techniques, Tiselius utilized frontal analysis for chromatographic separation of proteins [14]. Today, frontal analysis combined with chromatography is widely used for investigations of protein–ligand interactions in solution. Frontal analysis is implemented by injection of a solution containing both the protein and the ligand, typically onto a gel filtration column that has been pre-equilibrated with pure buffer. As long as the protein–ligand complex and the protein itself migrate differently, plateaus will be created corresponding to the protein itself, to the protein–ligand complex, and to the free ligand. Quantification of the relative amount of ligand bound per protein is conducted by comparing the heights of the free ligand zone with the known total concentration of the ligand in the applied mixture. Application of several different ligand concentrations permits calculation of the binding constants [1,3,5,15–18].

Shibukawa et al. recently applied frontal analysis to the determination of the binding affinities of enantiomers of N-desethyloxybutynin and oxybutynin with human serum albumin and α$_1$-acid glycoprotein [19,20]. Oxybutynin is

a therapeutic agent prescribed for the treatment of bladder spasms in patients with multiple sclerosis or for individuals with overactive bladder syndrome. N-Desethyloxybutynin, a major metabolite of oxybutynin, differs from the parent compound by removal of an N-terminal ethyl group. These authors employed an online switching system to collect, wash, and then analyze a predefined volume of the ligand plateau; in this manner they were able to quantify the amount of unbound drug without measuring the height of the free drug plateau. The results of their experiments eloquently established the enantioselectivity of oxybutynin and desethyloxybutynin binding to α$_1$-acid glycoprotein [19,20].

Morgunov and Srere [21] have used a modified version of frontal analysis to investigate interactions between two of the enzymes of the citric acid cycle. These authors utilized polyethylene glycol as a crowding agent to explore the formation of enzyme complexes between citrate synthase and malate dehydrogenase using gel filtration with frontal analysis. Since there is no "free ligand" in this experiment, the elution volumes, or protein fronts, were analyzed based on shifts in the retention time which were indicators of formation of enzyme–enzyme complexes. This approach enabled them to illustrate the formation of isozyme-specific complexes between malate dehydrogenase and citrate synthase.

## 2.3. Frontal affinity chromatography

In frontal affinity chromatography (FAC), the stationary phase of a packed column contains an immobilized enzyme (or other protein) and the mobile phase contains at least one potential inhibitor, substrate or ligand. The mobile phase flows through the column allowing increasing quantities of the inhibitor to bind the immobilized enzyme. When saturation of the immobilized enzyme is reached, no further binding can occur and the inhibitor elutes or "breaks through" the column. Inhibitor retention times are obviously dependent on binding affinity; the more potent the inhibitor, the later it elutes [22].

Since the inception of combinatorial methodology, large libraries can now be synthesized in relatively short time spans, creating a need for rapid screening of mixtures of potential inhibitors or substrate analogs. Several groups have reported utilizing frontal affinity chromatography to analyze the relative binding affinities of libraries of compounds [18,23–26]. A very nice illustration of this approach is the work of Schreimer et al. [27], who coupled an immobilized carbohydrate-binding antibody micro-scale column to electrospray mass spectroscopy for the analysis of a mixture of oligosaccharides. Miniature columns were utilized because of the advantage of minimizing valuable receptor and ligand expenditure. Detection by electrospray mass spectroscopy curtails false positives from impurities or reaction products since ion signature provides definitive identification of eluting moieties.
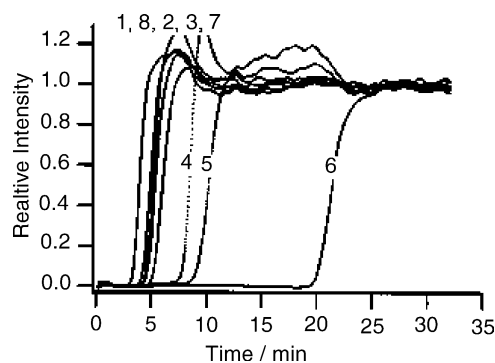
Fig. 2. Frontal affinity chromatography tandem mass spectrometry: frontal affinity chromatography–mass spectrometry (FAC/MS) screening of a mixture of eight trisaccharides flowing through a micro-scale GnT-V enzyme column, detected via ESI-MS with selected ion monitoring. Dissociation constants, $K_D$, for each ligand in the eight component mixture were estimated from a single FAC/MS run based on the equation $V_x - V_0 = B_t/(K_D + [X]_0)$ where $[X]_0$ is the ligand concentration, $V_x$ the elution volume the ligand, $V_0$ the void volume of the system, and $B_t$ is the column binding capacity. Note that compound 6 is retained much more strongly than the other compounds, hence it is the strongest inhibitor (reproduced from [28]).

Zhang et al. used the technique of frontal affinity chromatography–mass spectroscopy (FAC/MS) to determine the kinetic and binding constants of several inhibitors of $N$-acetylglucosaminyltransferase V (GnT-V) [28]. GnT-V is a rate-limiting membrane-bound enzyme that catalyzes the β-1-6 linking of $N$-acetylglucosamine to the α-1,6-mannoside arm of N-linked glycan acceptors. N-linked glycosylation is a known mechanism for cellular signaling, proliferation, and apoptosis, and a relationship between protein $N$-glycosylation and cancer has been established. Therefore, inhibition of a rate-limiting enzyme such as GnT-V has become an attractive target for chemotherapeutic agents [29–32]. This work utilized biotinylated-GnT-V bound to a streptavidin micro-scale column. Eight inhibitors were simultaneously injected onto the column, the elution profiles were analyzed using ESI-MS detection, and $K_D$ values were calculated from these elution profiles (Fig. 2). Very recently, frontal affinity chromatography with an immobilized beta-galactosidase affinity column was used for analysis of a library of 356 modified β-galactopyranosides. The library was constructed of 89 compounds, each of which consisted of four diastereomers. Using a de-convolution procedure in which the compounds were grouped into 10 mixtures of 24–40 members each, the authors identified 34 library entries which contained isomers exhibiting $K_D$ values below 10 μM [33].

## 3. Biomolecular interaction analysis tandem mass spectrometry

Biomolecular interaction analysis technology, based on the phenomena of surface plasmon resonance (SPR), allows real time determination of binding and equilibrium constants without analyte destruction. For enzymes or other proteins, this technique entails immobilization of the enzyme on a sensor chip. When a solution containing an inhibitor, substrate or other suitable ligand is allowed to flow over the chip, protein–ligand interactions alter the index of refraction of the sensor chip surface, and this change is detected and measured in arbitrary units known as refractive units (RU). Subsequent elution of the ligand likewise affects the refractive index, and analysis of these changes allows calculation of ligand association and dissociation rate constants and the equilibrium dissociation constant, $K_D$. Although many sensor chips are available, the basic sensor chip utilizes carboxymethylated (CM) dextrin covalently attached to a gold-coated glass plate. Among the advantages of biomolecular interaction analysis is that analyte modification is typically unnecessary, and that the technique is non-destructive. Furthermore, the technique is amenable to high throughput screening, and several groups have reported utilization of biomolecular interaction analysis to screen libraries of ligands [34–38].

Markgren et al. have reported on high throughput screening of HIV-1 protease inhibitors using biomolecular interaction analysis [39–41]. A critical step entailed optimizing the immobilization procedure and utilizing the Q7K mutant of HIV-1 protease, which exhibits enhanced stability, in order to create a suitably stable immobilized HIV-1 protease sensor chip. A library of 58 HIV-1 protease inhibitors was then screened at three different inhibitor concentrations to determine binding ability, estimate binding constants, and establish individual injection concentrations for each compound. Sensorgrams were evaluated with a kinetic model that the authors designed to account for the effects of mass transport. After identification of 39 inhibitors with significant binding activity, association and dissociation rate constants and the resulting equilibrium dissociation constants ($K_D = k_{off}/k_{on}$) were determined. A linear correlation between affinity constants derived with this technique and inhibition constants from steady-state kinetic studies was observed for all compounds except the most potent inhibitors. For unknown reasons, the affinity constants were typically 10-fold higher than inhibition constants. It was proposed that this discrepancy could be due to differences in experimental conditions.

It is important to note that a significant advantage of biomolecular interaction analysis is the ability to determine separately the association and dissociation rate constants for inhibitors. To illustrate this point, several compounds with low affinity were studied to determine if poor binding arose as a result of a slow association rate or because of fast dissociation rates. The authors found that association rates for most of the compounds were comparable to that of the prototypical HIV protease inhibitor, saquinavir, but dissociation rates were significantly faster. The authors therefore propose that future inhibitor design efforts should focus on lowering dissociation rates of inhibitor candidates. By compar-
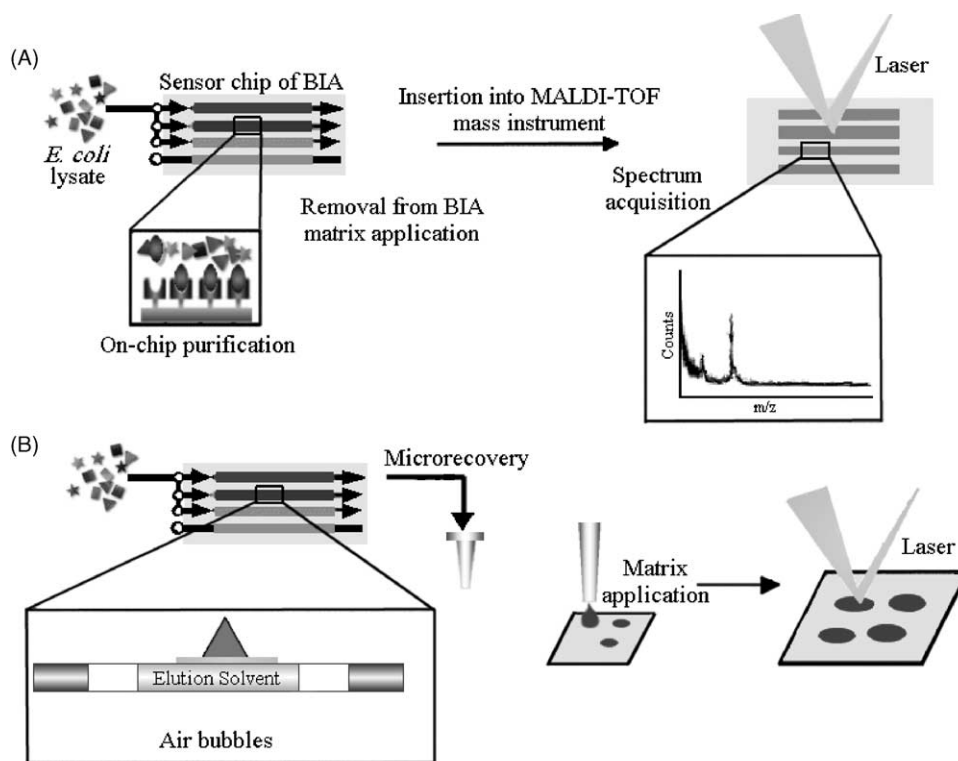
Fig. 3. The combination of BIA and MALDI–TOF–MS: (A) on-chip MALDI. The cell lysate expressing proteins of interest is injected onto the sensor chip. To characterize and evaluate bound proteins, the sensor chip is removed from the BIA instrument and inserted into a MALDI–TOF mass spectrometer following matrix application. Mass spectra are produced by individually targeting each flow cell with the laser. (B) Sandwich elution method. A simplified scheme of micro-recovery for subsequent mass spectrometric analysis. A small volume of elution buffer is delivered over the sensor chip, separated from the system buffer by two air bubbles to minimize dilution and dispersion of the molecules that were once bound to the chip (reproduced from [54]).

ing the $k_{on}$ and $k_{off}$ values of different classes of inhibitors the authors were also able to infer enzyme preference for certain structural groups and favored chiral conformations as well as enzymatic aversion for inhibitors containing certain functionalities. This type of information will likely be very useful in the future design efforts for HIV protease inhibitors.

Recently-developed multiple inhibitor analysis utilizing biomolecular interaction analysis mass spectrometry (BIA–MS) couples the ability of biomolecular interaction analysis to detect specific binding events with the ability of MS to explicitly identify bound compounds [42–45]. This coupling of BIA and MS is an especially powerful tool for analysis of combinatorial mixtures or complex biological systems [46–52]. The approaches used can be roughly classified into two categories (Fig. 3). The first approach is on-chip MALDI analysis. In this method the sensor chip is initially employed for biomolecular interaction analysis, an appropriate MALDI matrix is then applied and the chip is then inserted into a hand-crafted slot in a MALDI instrument for MS analysis. This technique has been used successfully for the detection of femtomole amounts of the peptide, myotoxin $a$ [47]. The second approach is known as the sandwich elution method. Biomolecular interaction analysis

is performed as usual, the chip is washed, and then elution is accomplished using a buffer which is flanked by small air bubbles which serve to mark the elution solvent front that contains the ligand of interest and which also prevent further sample dilution. This technique allows a very small volume (3 µl) of minimally diluted analyte to be collected and processed for MS analysis. After the eluent is collected, the sample may also be purified and/or concentrated using custom-made mini HPLC columns. The sandwich elution technique has been used to date with MALDI, ESI-MS/MS, and electrospray quadrupole–TOF MS [53–57].

Sonksen et al. [58–60] successfully utilized the sandwich elution technique with tandem nano-electrospray ionization ion trap MS and MALDI–TOF–MS for concurrent screening of mixtures of HIV-1 protease inhibitors. The authors successfully recovered minute quantities of inhibitors that specifically bound to immobilized HIV-1 protease. MALDI–TOF peak heights from eluted inhibitor mixtures showed a relative correlation to the quantity of inhibitor bound to the enzyme, thus providing data for inhibitor ranking. Once the most potent inhibitors in a mixture are thus identified, the individual inhibitors of interest can then be subjected to conventional BIA analysis in order to determine the kinetics of enzyme–inhibitor interactions.

## 4. Immobilized enzyme reactors

In the years since the debut of penicillin in the 1940s, semi-synthetic penicillins have been developed with an increased spectrum of activity, decreased acid lability, and β-lactamase resistance. 6-Aminopenicillanic (6-APA) acid is the β-lactam core of all semi-synthetic penicillins. While chemical methods for synthesis of 6-APA are well-established, the enzymatic synthesis of 6-APA from penicillin G via penicillin G acylase (PGA)-catalyzed hydrolysis is preferred, due to mild conditions, stereospecificity, and economic costs. Roughly 9000 t of 6-APA are produced annually in the US, and improved production methods are very much in demand [61,62].

Massolini et al. [63] have developed an immobilized enzyme reactor which consecutively synthesizes and chirally separates 6-APA and phenylacetic acid (PAA), the two products formed from the PGA-catalyzed hydrolysis of penicillin G. Development of the PGA–IMER was designed to optimize enzymatic activity, quantity of bound enzyme, product separation, and column chiral selectivity. Comparison of immobilization techniques, column packing materials, and regioselective formation, and separation of penicillin G hydrolysates revealed that amine attachment of the enzyme onto a pre-packed epoxyde-200 column gave rise to the most favorable characteristics. In related work, a PGA-chiral stationary phase was evaluated for enantiomeric resolution of 35 compounds, including several non-steroidal anti-inflammatory drugs. Chiral resolution was obtained for 27 of the 35 compounds [64]. Very recently, these investigators reported the use of new monolithic silica columns as an ideal support for their PGA–IMER [65]. Monolithic silica supports were developed using sol–gel technology, and incorporate a silica skeleton of mesopores and macropores. The large macropores allow analyte flow at low back-pressures while the smaller mesopores provide high surface area for separation. The low back-pressure and high mass transfer of monolithic silica columns is ideal for immobilization of enzymes and facile substrate reaction [66].

Markoglou et al. [67,68] recently developed an IMER based on immobilized dopamine β-monooxygenase (DBM), the enzyme which catalyzes the production of norepinephrine from dopamine. Inhibitors and substrate analogs of DBM, such as phenylaminoethyl selenides, have been recognized as mediators of adrenergic activity and potent anti-hypertensive agents [69]. Two different DBM–IMERs were constructed by linking DBM either to an immobilized artificial membrane or to glutaraldehyde-P. The immobilized artificial membrane support contains phosphatidylcholine head groups linked to the silica support via hydrophobic hydrocarbon side chains, with DBM being embedded in the immobilized artificial membrane therefore; the enzyme exhibits characteristics of the membrane-bound enzyme. In contrast, attachment of DBM to the glutaraldehyde-P membrane occurs via formation of an imino (Schiff's base) linkage, and in this support DBM behaves similarly to the soluble enzyme. Production and characterization of immobilized enzyme reactor is but the first step for utilization of these columns for design of new enzymatic inhibitors.

## 5. Display techniques

### 5.1. Phage display for identification of novel enzyme inhibitors

Phage display is a method which, in the most general sense, allows scientists to genetically manipulate the genome of a particular type of virus, known as a phage, in such a way that the phage will 'display' properly folded and functional peptide(s) on the surface of the phage coat protein. In this manner, a library of phages each displaying a different peptide variant can be created and assayed against bead-immobilized enzyme(s) to screen for peptides which bind to the immobilized enzyme. After selection for active phages is complete, in a process known as biopanning, the peptide is cleaved from the phage, and the phage is then amplified by infecting bacterial host cells. After repeated rounds of biopanning, phage-displayed libraries can become highly enriched for very potent enzyme inhibitors (Fig. 4) [70–74].

A protein produced by *Streptomyces clavuligerus* known as β-lactamase inhibitory protein (BLIP) is of considerable current interest, since widespread antibiotic resistance has lead to the situation where antibiotics must now be routinely co-administered with β-lactamase inhibitors, generating a pressing need for new classes of such inhibitors with broad efficacy. In this regard, Palzkill and coworkers have successfully expressed wild type BLIP an N-terminal fusion to the g3p coat protein of the filamentous M13 bacteriophage to create a BLIP–phage construct [75,76]. To ascertain whether the phage-displayed BLIP retained its activity as an enzyme inhibitor, the authors determined the concentration at which the BLIP–phage caused 50% inactivation ($IC_{50}$) of the lactamase enzyme. The measured $IC_{50}$ of 1 nM was comparable to the previously-reported $K_I$ of 0.6 nM, indicating that the expressed BLIP has folded properly and is capable of interacting normally with the lactamase enzyme. A library of BLIP–phage mutants was then constructed, so as to specifically mutate two small regions of BLIP that previous studies had implicated as being important to β-lactamase binding. After three rounds of biopanning and amplification, kinetic analysis detected a desirable decrease in the $K_I$ for two mutant BLIP–phages of 12- and 2.4-fold. A single phage constructed by incorporating the gene mutations from both of the two top phages showed inhibitory enhancement that was intermittent between 12 and 2.4, indicating that binding at one mutation site affects binding at the other site.

A complementary approach is to examine fragments of BLIP in order to obtain structural leads for the development of small molecule inhibitors. Accordingly, Rudgers and Palzkill [77] randomly fragmented the gene which encodes
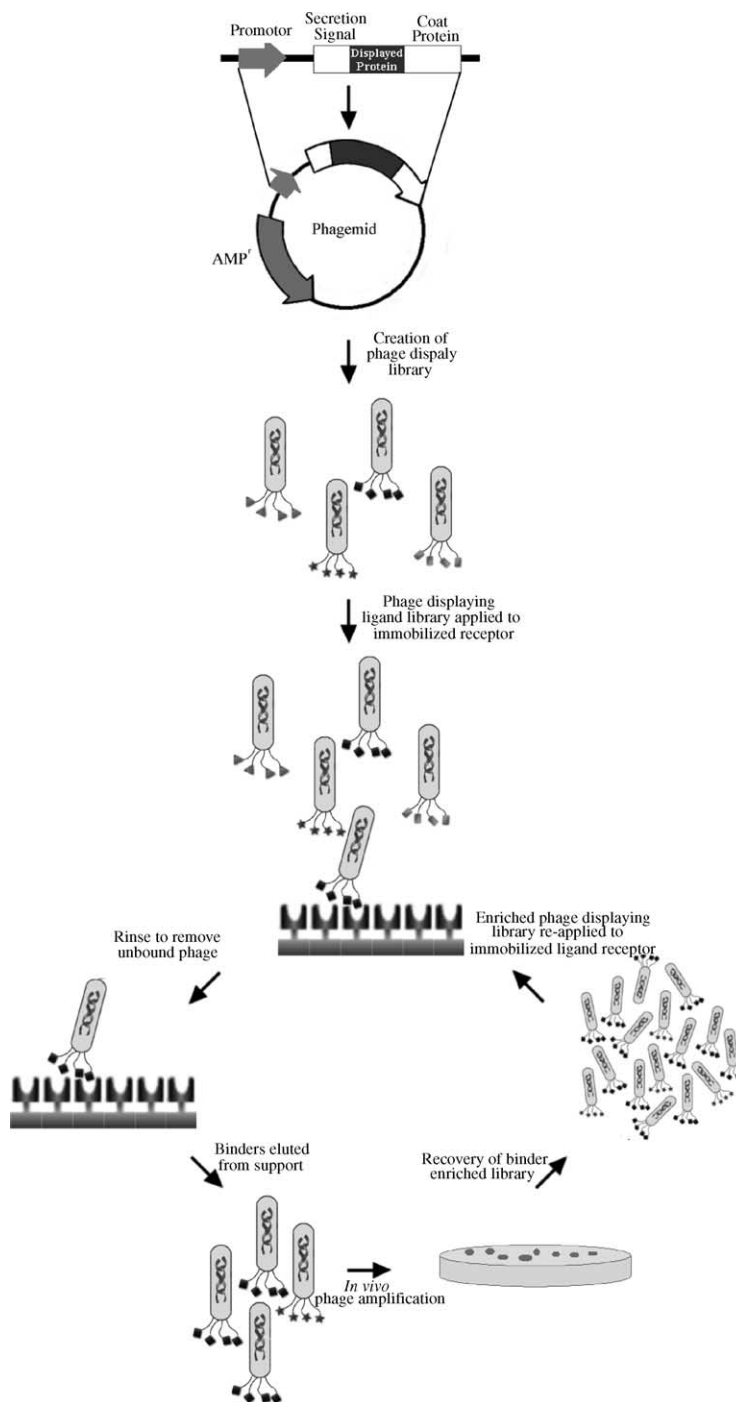
Fig. 4. Phage display: insertion of a gene into the phage genome yields a library of phage, displaying a protein of interest. Displaying phage are screened against immobilized receptors, washed, and amplified in vivo to produce an enriched library which can be further screened or sequenced.

BLIP and then inserted the fragments into the phage genome, thus creating a library of phage-displayed BLIP fragments. After several rounds of panning, eight unique fragments were identified, and sequencing revealed that six of these contained one section with identical amino acid residues, a conserved sequence. The authors synthesized a peptide with this conserved sequence, and kinetic analysis confirmed that it is indeed an effective β-lactamase inhibitor. Thus, by us-

ing phage display of BLIP fragments, this work successfully identified a small region of the 165-residue BLIP protein that is critically involved in enzyme inhibition.

### 5.2. Phage display for directed evolution of enzymes

Phage display is a very versatile technique that is tradi-tionally used to display relatively small peptides. However,

an interesting variant of this technique displays fully active enzymes on phage for use in "directed evolution" experiments aimed at creating novel enzymes. Active enzymes displayed on phage are subjected to the same biopanning and amplification process as is done in conventional phage display experiments, but phage capture is carried out using beads (typically streptavidin-coated) on which appropriate inhibitors have been immobilized.

When attempting to create new enzymes, proper inhibitor selection is crucial, since the selection process often results in enzymes that bind tightly to the immobilized inhibitor but are incapable of catalyzing a reaction to produce a meaningful product. To select specifically for catalytic activity, immobilized mechanism-based (i.e. "suicide") inhibitors are preferred, since these inhibitors function by undergoing catalytic turnover at the enzyme's active site, which in turn generates a reactive transient species that reacts covalently with the enzyme thereby irreversibly inactivating that enzyme molecule. By employing immobilized mechanism-based inhibitors, enzymes displayed on phage can therefore be selected based on the efficacy of enzyme catalysis.

Fastrez and coworkers [78] successfully constructed an active phage–β-lactamase conjugate. The authors performed biopanning and amplification of a solution containing active and inactive β-lactamase phages using a penicillin sulfone derivative as the mechanism-based inhibitor, immobilized on streptavidin-coated magnetic beads. After only one enrichment cycle, enzymatic activity increased by 50-fold. In a subsequent study [79], the activities of a mixture of wild type and four mutant β-lactamase enzyme–phage conjugates were compared to the relative recoveries of each conjugate after several rounds of enrichment. The relative quantities of each enzyme–phage conjugate correlated well with the known specific activities. This indicates that the more active enzymes bind more efficiently to the inhibitor for greater recovery. These experiments illustrate phage display is a viable system for selection of enzymes based on catalytic activity [79–81].

Fastrez and coworkers [82,83] have also reported on the application of variable selective pressures to a phage display library of mutants in order to ultimately produce enrichment in either β-lactamases or in penicillin binding proteins (PBPs). These latter proteins operate on β-lactam antibiotics via the initial steps of the lactamase-catalyzed hydrolysis mechanism, but then are unable to undergo deacylation and product release; consequently, PBPs become mechanistically-inactivated by β-lactam antibiotics. The library was designed by analyzing the genomes of PBP 4 from *E. coli* and of 20 β-lactamase enzymes and then selecting a nine residue region for mutation, culminating in the creation of a library containing $1.3 \times 10^6$ variants. To select for PBP activity, the authors utilized immobilized penicillin as the mechanism-based inhibitor. Phages displaying proteins exhibiting PBP activity are collected by the immobilized ligand, whereas phages displaying β-lactamase catalytic activity hydrolyze the immobilized

penicillin thus avoiding capture. On the other hand, to select for phage conjugates displaying β-lactamase activity, the authors utilized a counter-selection technique for affinity enrichment. The library was first pre-incubated with free penicillin G. Phages displaying PBP activity become mechanistically inactivated during this pre-incubation, whereas phages displaying β-lactamase activity simply react with and hydrolyze the penicillin G, leaving them free to bind another substrate. After this pre-incubation step, an immobilized mechanism-based inhibitor, penicillin sulfone, was added to the mixture, and this resulted in phages displaying β-lactamase activity binding to the immobilized inhibitor. Utilizing this technique, the authors achieved an impressive 40,000-fold enrichment of β-lactamase activity after four rounds of biopanning and amplification.

An exciting application for improved β-lactamase enzymes is in cancer therapy. Cephalosporins are a class of β-lactam antibiotics [84,85], and lactamase-catalyzed cleavage of cephalosporin-conjugated pro-drugs, such as C-Dox, results in the release of the active anticancer agent, doxorubicin [85]. Antibody-β-lactamase fusion proteins, designed to recognize proteins on the surface of a cancer cell permit the lactamase activity to be specifically targeted to these cancer cells. Upon administration of a β-lactam pro-drug conjugate such as C-Dox, this β-lactamase bound to the cancer cell hydrolyzes the pro-drug, thereby delivering the chemotherapeutic agent directly to the targeted cells. Genencor, in collaboration with Seattle Genetics, is currently developing this technology known as Antibody-Directed Enzyme Pro-drug Therapy (ADEPT) for the targeted treatment of melanoma [86]. In this regard, Seimers et al. [85] have constructed a β-lactamase phage display library to investigate the importance of a postulated cephalosporin-binding region for the design of enhanced β-lactamase enzymes.

### 5.3. Phage display techniques which utilize substrates

Two groups have independently developed related techniques which use enzyme substrates, as opposed to inhibitors, for selecting phage-displayed enzymes that exhibit desirable properties. In the approach developed by Schultz and coworkers [87] (Fig. 5), the substrate is covalently attached to the enzyme–phage conjugate, and, in addition, the substrate is also attached to a streptavidin-coated bead via a biotin linkage. Active enzymes will react with the substrate to which they are attached resulting in release of the enzyme–phage conjugate from the support. Therefore, all active enzyme–phage conjugates will be released from the column by cleaving their substrates, thus allowing collection for amplification. The authors have illustrated this technique with the enzyme staphylococcal nuclease. This enzyme is an extracellular nuclease which digests double and single stranded nucleic acids leading to cleavage of DNA or RNA in the presence of $Ca^{2+}$. The authors initially attached the enzyme–phage conjugate to the streptavidin support via the single stranded DNA substrate. This was performed in
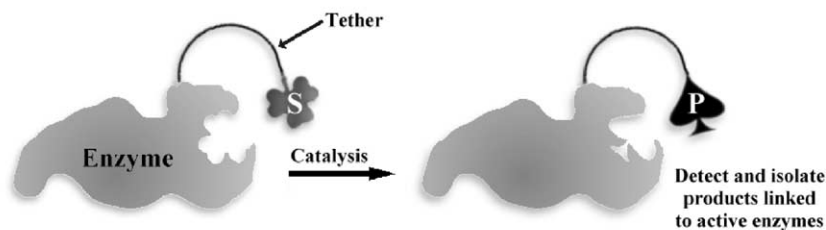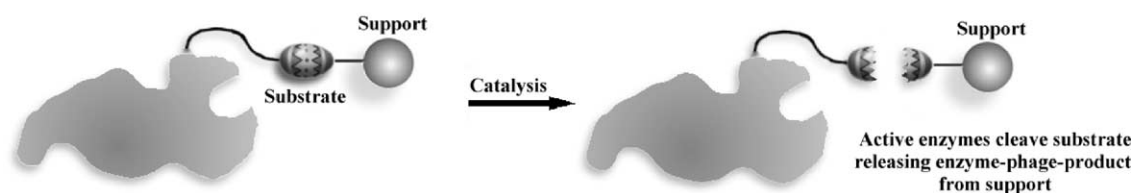
**General Approach:**



**Clevage Reaction:**



Fig. 5. General approach for utilizing phage display with a tethered substrate: in this example, DNA is bound to a support and to a phage-displayed enzyme. Active enzymes are able to cleave the DNA, resulting in release from the support for collection and analysis (modified from [87]).

absence of $Ca^{2+}$ to ensure that the enzyme remained inactive. After washing away unbound phage, the cleavage reaction was initiated by adding $Ca^{2+}$. Collection of the eluent yields only active phages that were able to cleave the DNA and thereby be released from the streptavidin support.

The second approach, dubbed proximity coupling, was developed by Jestin et al. [88] and is ideal for bi-substrate synthesis reactions. In this technique, the enzyme requires two substrates, A and B, which react by bond formation to create a single product P. In this procedure, one substrate is attached to a biotin tag and the other to a maleimidyl tag. Biotin tags specifically and strongly bind to streptavidin, whereas maleimides were chosen due to their binding promiscuity. When the enzyme–phage conjugate reacts with its two substrates, product will be formed and the maleimide tag will simultaneously react with the coat of the phage. Therefore, the product will be linked to the enzyme–phage conjugate whence it was synthesized. In addition, the product still possesses the biotin tag which has been present on the other substrate. After reaction, streptavidin-coated beads are applied to the solution to extract labeled enzyme–phage-product conjugates. Jestin et al. illustrated this approach using DNA polymerase–phage conjugates. A DNA primer was attached $5'$ to a maleimide tag and a biotin tag was introduced to the $3'$-end of dUTP. Upon addition of phage displaying active polymerase, biotin-tagged product attached to the polymerase–phage conjugate via the maleimide linker was formed, and subsequent application of the mixture to streptavidin-coated beads separated out enzymatically-active phages.

## 5.4. Ribosome display

A technique akin to phage display is known as ribosome display (Fig. 6). This technique may, in time, prove to be superior to phage display since shorter experimental times and utilization of larger libraries is possible. In ribosome display, the process is initiated by in vitro transcription of DNA encoding a library of interest, to produce mRNA lacking a stop codon. Subsequently, the mRNA undergoes in vitro translation to form a protein–ribosome–mRNA complex, which can be subjected to selection procedures by interaction with an immobilized binding partner. After active complexes are culled from the displayed library, application of EDTA dissociates the mRNA from the ribosome, and the harvested mRNA is then amplified by reverse transcription-polymerase chain reaction.

An additional feature of ribosome and related in vitro display techniques is the use of low-fidelity polymerases for the amplification. This results in the introduction of multiple new mutations in the freshly amplified DNA library. Consequently, this procedure can increase the genetic diversity of displayed libraries in hopes of creating new mutants with increased specificity for the target. After the library has been enriched to a satisfactory degree, a high-fidelity polymerase is employed to avoid introduction of additional mutants and the final library is amplified for further analysis. Multiple rounds of ribosome display utilizing error-prone polymerase followed by fixing of the library with high fidelity polymerase has yielded up to 40-fold increases in target affinities as compared to progenitor libraries [89].

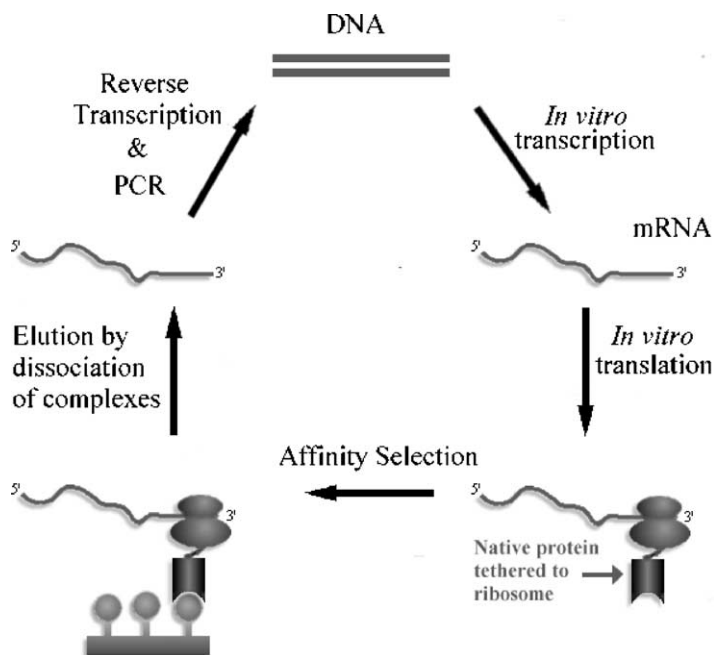A significant advantage of ribosome display is the elimination of the need for in vivo amplification. In phage dis-

Fig. 6. Ribosome display: the DNA encoding the library is transcribed in vitro. The resulting mRNA lacks a stop codon, giving rise, after translation, to linked mRNA–ribosome–protein complexes, which are then subjected to affinity selection (modified from [90]).

play, amplification of phage occurs via bacterial infection, which is necessarily time consuming. In ribosome display, amplification is accomplished completely in vitro. This can effectively decrease the length of each biopanning experiment. Furthermore, because no there is no in vivo transcription step, ribosome display can accommodate initial libraries containing $>10^{12}$ members, which is significantly greater than what is possible in phage display. Library size is technically limited only by the quantity of active ribosomes and the concentration of mRNA [90,91].

Recently, ribosome display of β-lactamases was accomplished by translating mRNA lacking a stop codon and containing a C-terminal spacer sequence to form a functional protein tethered to the ribosome via this spacer sequence. Retrieval of the complexes with the mechanism-based inhibitor, ampicillin sulfone, immobilized on magnetic beads provided a simple method to separate active conjugates. Testing ribosome display with an entire β-lactamase library is pending, but initial results of 100-fold per cycle enrichment factors indicate that this method may be an attractive system for future development of novel β-lactamase enzymes [91,92].

It has recently been reported that catalytic antibodies ("abzymes") exhibiting significant β-lactamase activity have been constructed using phage display [93,94]. Furthermore, testing of phage-displayed lipase libraries is underway with chiral suicide inhibitors to develop enzymes with unique enantioselectivities [95–100].

## 6. Capillary electrophoresis of soluble enzyme reactions

Capillary electrophoresis has traditionally been exploited for its ability to chromatograph compounds rapidly with ex-

cellent resolution using very small sample quantities. This technique separates compounds based on electrophoretic mobility (hydrodynamic radius and charge) providing an excellent alternative to traditional HPLC analysis. Consequently, capillary electrophoresis has lead to facile analysis of compounds that proved difficult if not impossible to separate via traditional HPLC. Furthermore, capillary electrophoresis obviates the need for organic modifiers in the mobile phase allowing analysis under near physiological conditions. A number of systems, such as capillary zone electrophoresis, have been developed which utilize capillary electrophoresis for the analysis of protein–ligand binding interactions.

### 6.1. Capillary zone electrophoresis

Capillary zone electrophoresis is perhaps the most straightforward and best-known method for analysis of enzyme–inhibitor interactions via capillary electrophoresis. In this method, the enzyme and inhibitor are pre-mixed to allow formation of the enzyme–inhibitor complex, prior to sample application as a small plug, or zone. Provided that the dissociation rate of the enzyme–inhibitor complex is small with respect to the separation time, and adequate electrophoretic separation is achieved, the enzyme–inhibitor complex will create a new peak amid the peaks of the free-analytes [101]. This technique has been utilized by Hu and Li [102] for analysis of microcystin binding to protein phosphatases.

Microcystins are toxic cyclic heptapeptides that inhibit protein phosphatases, enzymes that mediate phosphate group transfer between proteins. These potentially lethal
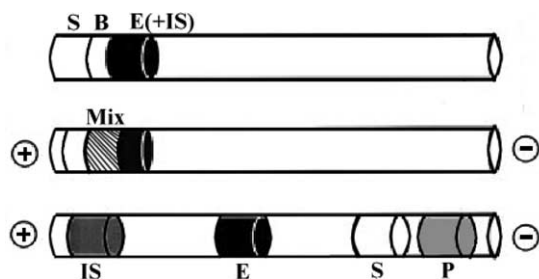
Fig. 7. Electrophoretically-mediated microanalysis: schematic illustrating electrophoretically-mediated microanalysis (EMMA). The enzyme, E, substrate, S, and an internal standard, IS, are introduced onto the column in defined zones separated by a plug of buffer, B. Upon application of a current, the rapidly-migrating substrate zone enters the sluggish enzyme zone, which initiates the enzymatic reaction. The internal standard, enzyme, substrate, and newly formed product then migrate along the column (reproduced from [117]).

toxins, produced by cyanobacteria, are considered a significant world wide public health threat [103]. Using capillary zone electrophoresis, Hu and Li [102] illustrate microcystin inhibition of protein phosphatases which supports the putative mechanism characterized by initial rapid enzyme inhibition followed by slow covalent-complex formation. Capillary electrophoregrams of mixtures of microcystins and protein phosphatases initially reveal the formation of the inhibition-complex, but as the incubation time increases, there is a decrease in the inhibition-complex with simultaneous increase in the covalent-complex. The authors report a highly efficient, baseline separated, method to clearly

delineate a multi-step inhibition mechanism with capillary zone electrophoresis.

## 6.2. Electrophoretically-mediated microanalysis (EMMA)

Capillary zone electrophoresis provides several advantages over HPLC with respect to sample size, analysis time, and distinctive chromatographic separations. Furthermore, the development of methodology for on-column enzyme reactions with ensuing separation and detection of substrates and products could further increase the efficiency of analyzing enzymatic systems. Consequently, the Regnier laboratory developed a technique in the early 1990s known as electrophoretically-mediated microanalysis [104–106]. EMMA is accomplished by applying reactants to a capillary column in separate zones. The reactants are introduced to the column in such a way that upon electrophoretic migration the separate zones merge; as the zones merge, a reaction occurs forming products (Fig. 7). The unreacted substrates and the resultant products are then electrophoretically transported along the column to the detector. Consequently, EMMA allows on-line detection of reaction-based chemical systems [107,108].

Enzyme–substrate reactions are model systems for analysis using EMMA [109–119]. For example, the Glatz group has used EMMA to analyze the activity of rhodanese, the enzyme which catalyzes the reaction of thiosulfate with cyanide to form thiocyanate and sulfite [120–122]. EMMA analysis of rhodanese was performed by sequential applica-
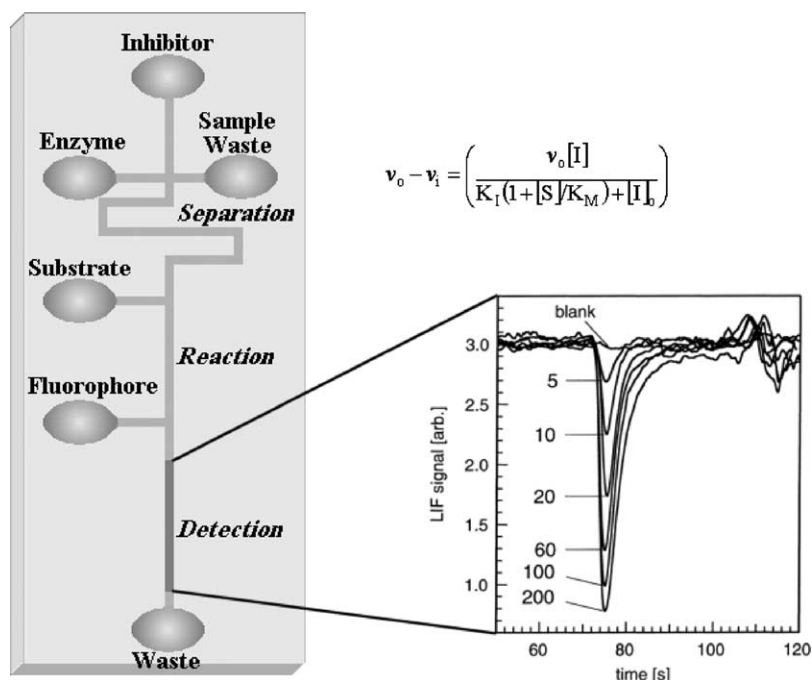


$$v_0 - v_i = \left( \frac{v_0 [I]}{K_I (1 + [S]/K_M) + [I]_0} \right)$$

Fig. 8. On-chip capillary electrophoresis: schematic of the "lab-on-a-chip" approach for assaying competitive inhibition of acetylcholinesterase. The inhibition constant, $K_I$, is determined from the equation shown by varying the concentration of inhibitor [I], at a constant substrate concentration [S], when the Michaelis–Menten constant, $K_M$, is known. As shown in the graph, a decrease in fluorescence is observed upon increasing inhibitor concentration (modified from [124]).

tion of the enzyme and then the substrates to the capillary column, such that during electrophoresis the faster-moving substrate zone merged with the more slowly-traveling rhodanese to allow product formation. Subsequently, the unreacted substrates and reaction products, thiocyanate and sulfite, electrophoretically travel along the column, and are separated, and detected. Utilizing this method, the authors succeeded in obtaining $K_M$ values for both substrates. Inhibition of rhodanese by 2-oxoglutarate was also examined using EMMA. The authors were able to demonstrate competitive inhibition with respect to cyanide with a $K_I$ of 0.362 mM, and uncompetitive inhibition with respect to thiosulfate with a $K_I$ of 1.4 mM [121].

### 6.3. On-chip enzymatic assays

The capacity of electrophoretically-mediated microanalysis to allow on-column enzymatic reaction and electrophoresis provides a distinct advantage in terms of time; furthermore, application of capillary columns to this technique significantly reduces sample expenditure. In this regard, researchers have been adapting capillary electrophoretic techniques to on-chip microanalysis. "Lab-on-a-chip" devices have been demonstrated to be exceptional with regard to utilization of sub-nanoliter analyte volumes, computer controlled electrokinetic migration of compounds, and a wide diversity of chip designs [123].

The Ramsey laboratory has developed a microchip for determination of the kinetic inhibition parameters of acetylcholinesterase (Fig. 8) [124]. The design of the chip was based on a fluorogenic assay in which the catalytic product of acetylcholinesterase, thiocholine, is detected by reaction with a thiol-reactive fluorophore. The microchip contained separate reservoirs for substrate, inhibitor, enzyme, fluorophore, and waste that were each connected to a central reaction channel leading to a detection channel. Analytes were mixed by electrokinetic flow at channel intersections; then migration through the channel separated reactants and products for detection at the terminus of the channel. Determination of kinetic parameters utilized laser-induced fluorescence detection to quantify the decrease in the peak height of the inhibited versus uninhibited enzymatic reaction, which was proportional to the initial reaction rate. The initial velocities were used to calculate a $K_M$ of $75 \pm 10\,\mu M$ and a $K_I$ of $1.5 \pm 0.2\,nM$ which correlated well with the bench top spectrophotometric analysis [124].

Microchip analysis of kinetic parameters is dependent on selection of a sensitive detection method, such as fluorescence, which can provide reasonable signal to noise ratios with very small sample sizes [123]. In addition, on-chip analysis may be constrained by the upper limits of detection; this can prevent enzymatic analysis under the entire range of desirable substrate concentrations (i.e. $5 \times K_M$) [124]. Furthermore, this technique typically utilizes 'end point' analysis for determination of the initial enzymatic rate; a method which assumes linearity over the sample acquisi-

tion time. Consequently, for accurate analysis, care must be taken to ensure linearity of the rate for the entire range of substrate concentrations.

The potential of on-chip electrophoresis has recently been illustrated by Wang et al. [125] for rapid quantitative analysis of four renal markers. The authors utilized an on-chip multiple enzyme reaction sequence to determine the concentration of $H_2O_2$ produced by enzymatic catalysis of the renal markers creatinine and creatine. Furthermore, the enzymatic system was performed, without interference, in the presence of two other known renal markers providing a method to quantitate a total of four different renal markers within 5 min. This technique clearly demonstrates the diversity and efficacy of on-chip capillary electrophoresis [125].

## 7. Resolution of tautomeric chiral enzyme inhibitors

A prime driving force behind many efforts to develop novel enzyme inhibitors or substrate analogs is the possible therapeutic potential of such compounds. In this regard, the enantiomeric purity of any new enzyme inhibitor has become a very critical issue. Indeed, while a 1994 study found that only 11.5% of the chiral synthetic drugs extant were offered as the individual enantiomers, more recent data confirms a marked surge in sales of single enantiomers of chiral drug molecules [126]. With the increased regulatory attention being given to stereoisomeric drugs, chiral synthesis and chiral resolution, techniques are increasingly in demand to obtain enantiomerically-pure drug candidates.

There are two general approaches for obtaining enantiomerically-pure substances; asymmetric synthesis of the desired isomer, and resolution of the racemic mixture into individual enantiomers. Synthesis of a racemic mixture is almost always the simpler approach, and after separation, both enantiomers are available for testing purposes. Indeed, a recent report on chiral drugs emphasizes that the dominant production method to introduce asymmetry into synthetic drugs remains the resolution of racemates [126]. High-performance liquid chromatography has been widely applied for this purpose, via the formation of diastereomers or via direct separation on chiral stationary phases (CSPs). Chromatography on chiral stationary phases allows direct resolution of the pure enantiomers on both an analytical and a preparative scale, and thus eliminates the recovery process needed when diastereomers are formed. Several categories of CSPs have been developed for general and specific separation purposes. Among these are: Pirkle-type CSPs; polysaccharide triesters or carbamates on silica; and columns which are based on inclusion complexes, ligand exchange or protein interactions [127]. CSPs based on polysaccharide derivatives have proven to be very effective in resolving a wide range of racemates, including aromatic hydrocarbons, amines, carboxylic acids, alcohols, amino acid derivatives, and many commercially available drugs.

Recently, this author's laboratory reported the successful enantiomeric separation using HPLC on CSPs of a chiral enzyme inhibitor which exists in solution in several tautomeric forms [128]. The compound 2,4-dioxo-5-acetamido-6-phenyl-hexanoic acid is the most potent inhibitor known for peptidylamidoglycolate lyase, an enzyme which plays an essential role in carboxyl-terminal amidation of many biological peptides. Synthesis of this inhibitor entails an alkaline hydrolysis step, under which condition the chiral center is racemized; thus, HPLC with CSP was employed to obtain the individual enantiomers of this inhibitor. Since 2,4-dioxo-5-acetamido-6-phenyl-hexanoic acid exists in solution in several tautomeric forms, the strategy of first converting this compound from its multiple enol forms into a single diketo tautomer, which was then applied to various CSPs, was employed. Successful preparative scale separation of this compound was achieved using a CHIRALPAK AD chiral stationary phase. Enantiomeric separation was also accomplished on a D-penicillamine column, but this CSP was found to be less satisfactory for preparative purposes. This work can be viewed as a model for other molecules which possess both a chiral center and a tautomeric moiety. Resolution of such tautomeric chiral molecules using chiral chromatography is an issue which has not been widely addressed in the literature, but will likely be of increasing concern in the years ahead.

## 8. Conclusions

The diversity and efficiency of enzymatic catalysis is truly remarkable. Enzymes catalyze processes ranging from the synthesis of stereoregular plastics to catalysis of complex organic reactions under mild conditions. The elucidation of enzyme–substrate and enzyme–inhibitor interactions is a crucial issue in fully exploiting the potential of biocatalysis for a variety of purposes.

Classical techniques, such as the Hummel–Dreyer method and frontal analysis, provide researchers with well-established approaches for investigating interactions between binding partners. The development of reliable technology for enzyme immobilization has now resulted in the evolution of classical chromatographic methodology into techniques such as frontal affinity chromatography, whereby libraries of compounds are simultaneously ranked for inhibitory potency. Immobilized enzyme reactors developed for simultaneous synthesis and chiral separation can provide useful information about enzyme–ligand binding interactions as well as enabling the enzymatic synthesis of highly desirable compounds.

The high resolution and miniscule sample requirements characteristic of capillary electrophoresis are ideal for enzymatic analysis. Techniques such as EMMA and on-chip electrophoresis combine reaction and electrophoretic steps into one convenient format thereby increasing efficiency. Furthermore, the convenience and portability of 'lab-on-a-chip' devices may eventually permit rapid point-of-care analysis of biological samples, thus improving patient care.

Biomolecular interaction analysis tandem mass spectrometry provides new opportunities for the rapid screening and ranking of compounds which interact specifically with an immobilized enzyme. This technology has great potential for the rapid screening of new drug candidates.

Finally, display techniques, which have traditionally been used to select leading inhibitors from large libraries, have recently been modified for expression of phage- or ribosome-displayed enzymes. This offers exciting future possibilities for the directed evolution of enzymes to produce improved biocatalysts exhibiting superior activity, increased stereospecificity, or altered substrate specificity.

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