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Review

Recent advances in CE-mediated microanalysis for enzyme study

This review gives an overview of the recent developments and applications in the use of CE-mediated microanalysis for enzyme studies. The period covers mid-2011 until mid-2013. Both off-line and in-line enzyme assays with their applications using CE are described in this article. For the in-capillary enzyme reaction, the techniques using electrophoretically mediated microanalysis (EMMA) as well as immobilized enzyme reactor (IMER) are discussed. The applications include the evaluation of enzyme activity, enzyme kinetics, enzyme inhibition, screening of enzyme inhibitors, and the study of enzyme-mediated drug metabolism.

Keywords:
CE / Electrophoretically mediated microanalysis / Enzyme study / In-line / Off-line
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1 Introduction

Enzymes are not only major drug targets in drug discovery processes in the pharmaceutical industry but also play an important role in clinical research as well as in diagnosis [1]. Therefore measurement of enzyme activity is clearly important for future diagnostics and for development of therapeutic products.

Absorption or fluorescence spectrometry are routinely used for enzyme assay because they can readily be automated and high sample throughput can be achieved by using commercial plate readers with 96-well plates. However, this approach can be applied only to substrates and products that have a significant difference in their spectrometric properties. In addition, a homogenous assay performs the quantification in a mixture without a separation step. In many situations, however, substrate and product of the enzymatic reaction have similar spectrometric properties. The fluorescence signal could also be influenced by background absorption and auto-fluorescence in biological samples [2]. Therefore, separation methods followed by spectrometric detection may be needed to perform enzyme assays in some cases.

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Abbreviations: FMO, flavin-containing monooxygenase; **GlcNAc**, *N*-acetyl-D-glucosamine; **GOx**, glucose oxidase; **Hex**, β-*N*-acetylhexosaminidase; **HS-β-CD**, highly sulfated β-CD; **IMER**, immobilized enzyme reactor; **LDH**, lactate dehydrogenase; **MB**, magnetic bead; **MMP**, matrix metalloproteinase; **PMMA**, pressure-mediated microanalysis; **TDLFP**, transverse diffusion of laminar flow profiles

Chromatographic methods allow the separation of the reaction mixture into its individual components. Due to the high selectivity and sensitivity, LC-MS is applicable to the assay of enzyme activity and the screening of the inhibitory activity of small molecules. Various LC-MS methods have been applied to enzyme assay and inhibitor screening [3–7]. However, separation optimization and long elution time limit sample throughput for complex library compounds. In addition, the consumption of large amounts of organic solvents increases the cost.

CE as an alternative tool to LC offers unique advantages in enzyme assay and inhibitor screening due to its short analysis time, low reagent cost, and minimal sample requirement as reported elsewhere [8, 9]. Miniaturization and/or multiplexing of electrophoretic separations in a microfluidic or capillary array format greatly increases sample throughput [10, 11].

Various detection formats can be coupled directly to CE, including UV absorbance, LIF, MS, and electrochemical detection [12]. CE-UV is the most widely used detection format with moderate sensitivity when using native substrates or their analogs that possess UV-active chromophores, whereas LIF detection enables ultra-sensitive detection of fluorescently labeled substrates when using an appropriate laser source for excitation. However, fluorescently labeled or fluorogenic substrates are not natural substrates of the enzyme. Further improvements in sensitivity and sample processing can also be achieved in CE when using on-line sample preconcentration in conjunction with in-capillary chemical derivatization [13]. CE-MS features high selectivity together with compound identification, yet is a relatively unexplored format for drug screening [14]. ESI is the most widely used interface in CE-MS [15], and requires a volatile BGE which limits selectivity while imposing compatibility issues with

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Table 1. Summary of precapillary enzyme assays

Enzyme	CE mode	Sample preparation	Incubation temperature	Detection	Application	Ref.
Hex	CZE	Direct injection	RT	UV 200 nm	Separation of saccharides and Hex assay	[24]
PNP and ADA	MEKC	REPSM	37°C	UV 260 nm	Enzyme kinetics and inhibition	[25]
AtIPT1	CZE	Centrifugation	25°C	UV 265 nm; ESI(−)	Enzyme activity	[29]
FM01	CD-MEKC	Centrifugation	37°C	UV 240 nm	Enzyme kinetics and inhibition, drug-drug interaction	[30]
Sirtuins	CZE	Centrifugation	37°C	UV 200 nm	Enzyme kinetics, substrate and inhibitor screening	[31]

REPSM, reversed electrode polarity switching mode; AtIPT1, isopentenyltransferase 1; PNP, purine nucleoside phosphorylase; ADA, adenosine deaminase.

respect to buffer conditions required for optimal separation. Atmospheric pressure chemical ionization (APCI) and Atmospheric pressure photoionization (APPI), as alternative ion sources for CE-MS, can expand the use of buffers and additives for improving separation performance without ion suppression, including phosphate salts and SDS micelles [16].

In general, three major formats have been used for enzyme assay in CE, where enzymatic reactions are performed either (i) off-line (precapillary), (ii) at the capillary inlet (at inlet), by diffusion-based mixing of reagents in free solution or via an immobilized enzyme microreactor (IMER) or (iii) in-capillary via electrophoretically mediated microanalysis (EMMA) [17].

Among the CE modes, CZE became a well-recognized technique for either off-line or in-line enzyme assays. Other CE modes like CEC and MEKC, CGE, and CIEF can also be applied to enzyme assay. These less common CE modes with applications in either off-line or in-line enzyme assay were discussed by Křížek and Kubíčková [18].

This review, as a continuation of a previous report [19], gives an overview of the recent developments and applications of these formats of enzyme assay, over the period from mid-2011 to mid-2013.

2 Precapillary enzyme assays

In precapillary enzyme assays, also termed off-line assays, the incubation of substrate, cofactor, and enzyme is carried out in a separate vial. Only the analysis of substrate and/or product is conducted in CE. Typically, all components are mixed and the reaction is initiated either by the addition of the enzyme, cofactor, or substrate. After incubation for a certain period of time, the reaction is stopped, and the sample is injected into the CE system for analysis.

The method development is relatively straightforward for off-line assays because enzyme reaction and CE separation can be performed under the respective optimal conditions. However, some problems may arise upon direct injection of the incubation mixture into the CE system. In order to avoid protein adsorption onto the capillary wall or even blocking of the capillary, deproteinization prior to sample injection can be achieved by addition of ACN in the quenching step of the reaction followed by centrifugation of the precipitated proteins. A stacking effect can be simply achieved in the precapillary assay by the injection of low ionic strength samples that can be obtained using ACN for quenching of the reaction [20]. Protein adsorption to the capillary wall may be reduced using permanently polymer-coated capillaries [21] or employing a dynamic coating of the capillary wall [22, 23]. Precapillary assays have been applied for the determination of enzyme activity and kinetics, substrate, and inhibitor screening as well as in vitro drug metabolism studies. Some examples of precapillary assays in the past 2 years are summarized in Table 1.

The assay of β-*N*-acetylhexosaminidase (Hex) involves the separation of chitotriose, chitobiose, and *N*-acetyl-D-glucosamine (GlcNAc). However, the analysis of saccharides is a challenge in CE separation due to the poor UV absorption and lack of electric charge. The high pH BGE used to enhance the UV absorption leads to excessive Joule heating. The derivatization of saccharides needs an extra step before the analysis with fluorescence detection. Křížek et al. developed a borate-based BGE with pH 10 for the separation of chitotriose, chitobiose, and GlcNAc [24]. The off-line setup proved to be convenient for Hex assay as well as for a small series of experiments. Although the optimized separation took 15 min, 10 measurements could be done in 1 h when the separation was shortly interrupted every 5 min and a new sample of the reaction mixture was injected into the running analysis. This allowed for automated monitoring of the reaction in 5-min intervals. Nevertheless, all reaction mixtures must be prepared separately and reactions must be timed by the experimenter.

There is a strong need for the development of a CE method for the determination of neutral compounds due to the fact that the products of some enzyme reactions are neutral analytes such as nucleosides and nucleobases. Iqbal et al. [25] developed a simple and sensitive MEKC method combined with on-line concentration for the characterization and inhibition studies of the nucleoside-metabolizing enzymes, purine nucleoside phosphorylase (PNP), and adenosine deaminase (ADA), present in membrane preparations of human 1539 melanoma cells. Hydrodynamic pressure injections were performed at 5 psi for 36 s, which corresponds to an injected volume of 91.0% of the capillary. Then a negative voltage (−20 kV) was applied and the large plug of sample matrix was electroosmotically pumped out of the capillary.

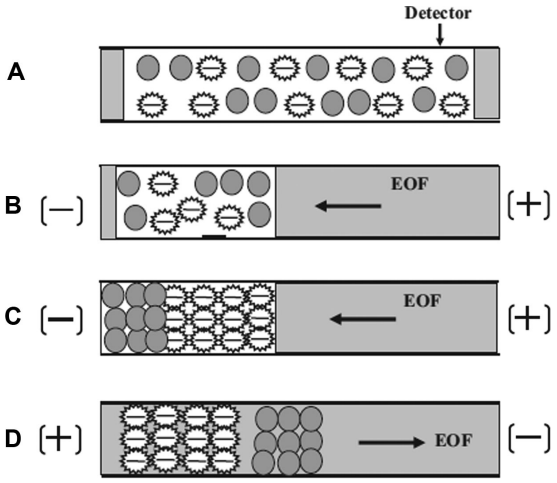

Figure 1. Steps of MEKC with reversed electrode polarity switching mode (REPSM) conditions. (A) A large-volume sample (prepared in water) was injected hydrodynamically and a small buffer plug was subsequently injected; (B) voltage was applied with reversed polarity (reversed EOF direction), the sample-matrix was pushed back into the inlet vial by the EOF; (C) neutral analytes were focused on passing through the concentration boundary; (D) optimal stacking was achieved, the polarity was switched to normal mode, and the separation voltage was reapplied for the analytes' separation and detection. Figure was adapted from [25] and reproduced with permission.

Since SDS was present in the BGE, the sample species stack at the boundary between the sample zone and the BGE when a negative voltage is applied. When the sample buffer is almost completely out of the column, which is monitored by the current (from lower to normal), the polarity is switched to the normal configuration, and the separation of the analytes can proceed. This is the so-called reversed electrode polarity switching mode (REPSM)-MEKC as shown in Fig. 1. Around tenfold improvement of sensitivity for the five investigated analytes (adenosine, inosine, adenine, hypoxanthine, xanthine) was achieved by large-volume stacking with polarity switching when compared with CE without stacking.

In contrast, nucleotides are easily charged over a wide pH range. They can be separated under acidic [26], neutral [27], or basic [28] conditions. Beres and Gemrotova [29] developed a CZE method with UV and MS detection to determine the activity of recombinant isopentenyltransferase 1 in *Arabidopsis thaliana* (AtIPT1). Simultaneous determination of the substrates/products (isopentenyladenosine 5′-monophosphate (iPMP), isopentenyladenosine 5′-diphosphate (iPDP), isopentenyladenosine 5′-triphosphate (iPTP), AMP, ADP, ATP) was achieved by CZE-UV using a 100 mM sarcosine/ammonia buffer at pH 10.0. Baseline separation of isopentenylated nucleotides was accomplished by CE-ESI-MS using a volatile buffer (30 mM ammonium formate, pH 10.0). The analytes were detected in negative ionization mode. A mixture of isopropanol and water in the ratio of 80:20 with addition of 0.4% ammonium hydroxide was selected as sheath liquid at a flow rate of 5 μL/min.

Although many types of enzymes have been characterized by in vitro drug metabolism studies, there is little attention for other drug-metabolizing enzymes such as flavin-containing monooxygenases (FMOs) in comparison with cytochromes P 450. Yeniceli et al. [30] developed an aqueous CD-MEKC method as an alternative to NACE methods to investigate FMO1-based drug metabolism. The method was applied to FMO1 kinetics and inhibition study using tamoxifen (TAM) as a probe substrate and nicotine as an inhibitor for the first time. No inhibition effect on TAM metabolism was observed.

In addition, there are other applications of CE-based enzyme assay beside enzyme kinetics and inhibition. Scriba et al. [31] developed a CE-based off-line assay for evaluation of substrates for human sirtuin. The study was conducted in order to further modify the structure of small molecule substrates as this may result in assays with simpler substrates compared to the published compounds. Field amplified sample injection (FASI) was employed to achieve sufficient assay sensitivity. The optimized sample injection was FASI at 5 kV for 30 s after a water plug injected hydrodynamically at 2.1 kPa for 2 s. The assay was subsequently validated according to ICH guideline Q2 (R1) with respect to linearity, LOD, LOQ, repeatability, interday precision, and recovery.

3 In-capillary assay

The capillary not only can serve as a separation tool but also as a reaction vessel. The in-capillary reaction of enzyme and substrate can be achieved either by electrophoresis or by diffusion. For in-capillary enzyme assay, it can be divided in two categories, namely at inlet or IMER and EMMA. The major advantage of in-capillary enzyme assay is that enzyme reaction, quenching, and analysis steps are fully integrated into a single instrument using small amounts of reagents.

3.1 At inlet and IMER

In the at inlet mode the reactants are mixed by diffusion and reacted for a given time at the inlet part of the capillary. No voltage is applied for mixing the reactants and no prior knowledge of enzyme and substrate mobility is needed to perform in-capillary enzyme assay and inhibitor screening.

In addition, another mode of mixing without voltage is transverse diffusion of laminar flow profiles (TDLFP), which allows for more effective mixing of multiple plugs of reactants at the inlet of the capillary [32–34]. In this case, solutions of reactants and buffer are consecutively injected by high pressure for a short time in the capillary inlet as narrow plugs that have parabolic profiles due to the laminar flow. Mixing of sample plugs is enhanced by transverse diffusion due to the greater contact area between elongated zones by using an additional buffer injection plug after the reagents. Some examples of at inlet or IMER are summarized in Table 2.

Table 2. Summary of in-capillary enzyme assays

Enzyme	CE mode	In-line mode	Cartridge temperature	Detection	Application	Ref.
β-Gal	CZE	At inlet/EMMA/PMMA	25°C	UV 405 nm	Enzyme kinetics and inhibition	[36]
GOx	CZE	IMER	25°C	UV 220 nm	Enzyme kinetics and inhibition	[43]
Trypsin	CZE	IMER	37°C	UV 214 nm	Enzyme kinetics and inhibition, IC_{50}	[44]
ADH and LDH	CZE	IMER	NA	UV 340 nm	Enzyme kinetics and determination of the acetaldehyde and pyruvate in beer	[45]
CYP3A4	CZE	EMMA partial filling	37°C	UV 195 nm	K_m, V_{max}, CL_{int}	[50]
CYP3A4	CZE	At inlet/partial filling	37°C	UV 200 nm	Enantioselective evaluation	[52]
CYP 2C9	CZE	TDLFP-based EMMA	37°C	UV 200 nm	Enzyme kinetics and inhibition	[53]
LDH	CZE	Plug-plug EMMA	22°C	UV 340 nm	L-/D-Lactate determination	[54]
LDH	CZE	Plug-plug EMMA	25°C	UV 214 nm	Protein conformation	[55]
GPT	CZE	Sequential online analysis	NA	UV 340 nm	Enzyme kinetics	[56]
Hex	CZE	TDLFP + EMMA	25°C	UV 200 nm	Enzyme inhibition	[24]
Tyrosinase	CZE	EMMA	37°C	UV 214 nm	Inhibitor screening	[57]
Aromatase	CZE	EMMA partial filling	37°C	UV 260 nm	Inhibitor screening	[58]
GK	CZE	plug-plug EMMA partial-filling	25°C	UV 254 nm	Enzyme inhibition	[60]
MMP-9	CZE	EMMA and at inlet PMMA	25°C	SRM(+)	Enzyme inhibitor screening	[62]

ADH, alcohol dehydrogenase; GPT, glutamate pyruvate transaminase; GK, glycerol kinase; β-Gal, β-galactosidase.

The capillary can serve as a reaction vessel, as an alternative to an incubation vial. An in-capillary reaction for CYP450 isoenzyme drug metabolism study was developed by Veuthey et al. [35]. The capillary was first filled with the incubation buffer suitable for the enzymatic reaction. Enzyme and its substrate were injected in the capillary in a sandwich mode. By applying a suitable pressure, the reactants are mixed by longitudinal and transversal diffusion. Then the capillary content was flushed with water into a vial containing an ACN-water mixture (1:1 v/v) to stop the enzymatic reaction. The reaction mixture was collected for subsequent off-line UPLC-MS/MS analysis. This technique was referred to as pressure-mediated microanalysis (PMMA) since the enzymatic reaction is mediated by pressure [36].

On-line immobilized capillary enzyme microreactors are a very useful tool for the study of enzyme reactions, inhibitor screening, and proteomics [37–39]. In this approach, the enzyme is covalently immobilized on either the surface of the capillary or a suitable carrier such as a monolith, sol-gel, controlled pore glass, silica, and polystyrene beads [40–42]. With the immobilized enzyme microreactor, the enzyme is reusable within a certain amount of experiments. However, this technique requires a long time to prepare the microreactor.

Glucose oxidase (GOx) coupled to a peroxidase reaction that visualizes colorimetrically the formed H_2O_2 is widely used for the determination of free glucose in plasma for diagnostics. By cross-linking, GOx was immobilized to the capillary and an on-line microreactor was developed based on an enzymatic redox reaction with 1,4-benzoquinone as an acceptor of electrons, replacing the molecular oxygen typically used in a GOx reaction to achieve direct UV detection without derivatization. The corresponding reduction product (hydroquinone) can be monitored without using peroxidase as a coupling enzyme. In addition, an on-line enzyme inhibition study was performed on the immobilized GOx microreactor with metal ions Ag^+ and Cu^{2+} used as model inhibitors [43]. The proposed IMER approach is expected to adapt to capillary array electrophoresis for high-throughput screening of enzyme reactions and inhibitors. Based on the same glutaraldehyde cross-linking technology, Min et al. [44] established an immobilized trypsin microreactor for the characterization of trypsin and it was successfully applied to the screening of trypsin inhibitors from 19 natural extracts.

In addition, magnetic beads (MBs) with micron or submicron size have been used as a solid support for IMER. Typically, MBs containing immobilized biomolecules were injected into the capillary and the short plug of the MBs can be fixed by a magnetic field at any place of the capillary. Yang et al. developed a novel method using MBs to form a dual-enzyme capillary microreactor by immobilizing alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) at desired positions of the capillary using two pairs of magnets [45]. The dual-enzyme assay was quantified by measuring the consumption of coenzyme NADH. The dual-enzyme capillary microreactor was constructed without any modification of the inner surface of the capillary. It showed great stability and reproducibility for different batches. The RSD of peak height is about 2.5 and 5.2%, and that of migration time is about 3.5 and 2.4%, for the alcohol dehydrogenase and LDH reaction, respectively. The proposed method was successfully applied to determine the acetaldehyde and pyruvate contents in real beer samples with recovery ranging from 87 to 116%. The combination of MBs with CE has great potential to create a multi-enzyme capillary microreactor for investigation of a series of enzyme reactions and screening of multisubstrates.

3.2 EMMA

In general, two major EMMA formats are used to load and mix reagents in a capillary under electrophoretic conditions, namely continuous engagement EMMA (long contact mode) and transient engagement EMMA (plug–plug format or short contact mode) [19]. In the long contact mode of EMMA, the capillary is initially completely filled with one of the reactants, whereas the second reactant is introduced as a plug (zonal sample introduction) or continuously from the inlet vial (moving boundary sample introduction) [46]. The product continuously forms during the electrophoretic mixing of enzyme and substrate. In contrast to this, in the plug–plug mode of EMMA, enzyme, and substrate are introduced in the capillary as distinct plugs; the first reactant injected being the one with the lower electrophoretic mobility [46, 47]. The enzymatic reaction is initiated by the application of an electric field since the zones interpenetrate due to the differences in their electrophoretic mobilities. Due to the high efficiency of electrophoretically driven separations, zonal EMMA shows advantages when monitoring multiple compounds simultaneously. The plug–plug mode has been the most commonly used EMMA mode due to the fact that less amount of reactants is needed because only a small plug of reactant is required instead of filling the whole capillary and buffer reservoirs. The classical plug–plug approach is limited when the separation buffer is not compatible with the enzyme reaction buffer. In this case, separation buffer and enzyme can be separated by injecting an additional plug of incubation buffer. This "partial filling mode" was applied to EMMA by Van Dyck et al. [48]. Some examples of EMMA in the past two years are summarized in Table 2.

Enzyme-mediated drug metabolism study is important in drug discovery, development, and optimization of therapy, including the in vitro characterization of drug metabolism pathways and kinetics. The on-line CE methods for investigation of in vitro drug metabolism implemented from 2002 to 2012 have been summarized by Nowak [49]. Recently, an EMMA method was developed to investigate the stereoselectivity of the CYP 3A4-mediated *N*-demethylation of ketamine [50]. Partial filling of two incubation buffer plugs was used to protect enzyme and substrates from the pH of the separation buffer as well as the chiral selector in it. Optimized performance was obtained with equal plug lengths and having an injection time of 4 s at 1 psi. In such a configuration, mixing of reactants is assumed to occur by both longitudinal diffusion and TDLFP generated during pressure injection as was described in detail by Krylova et al. [51]. The mixing was enhanced by applying voltage at −10 kV for 10 s and followed by 8 min zero-potential amplification at 37°C, then the capillary was cooled to 25°C within 3 min. A voltage of −10 kV and a positive pressure of 0.1 psi were applied during the separation of the formed enantiomers of norketamine. The proposed EMMA method was applied to estimate the intrinsic clearances CL_{int}.

For the in-line development of the enzymatic reaction a partial filling technique with highly sulfated β-CD (HS-β-CD) was applied for the separation of verapamil enantiomers and its metabolite norverapamil enantiomers. This was successfully employed for the evaluation of enantioselective metabolism of verapamil by CYP 3A4 [52]. The in-line reaction was performed in at inlet and partial filling mode for 5 min after the injection of verapamil, surrounded by two CYP 3A4 plugs. The NADPH regenerating system and in-line formation of NADPH were employed in order to protect NADPH from degradation. The separation of the enantiomers was carried out by partially filling the capillary with a 2.5% w/v HS-β-CD solution by applying a pressure of 10 psi for 2 min prior to the injection of the reagents. A little pressure of 0.2 psi was applied for the separation step, leading to migration times reduced by half. The consumption of chiral selector was kept low since no HS-β-CD was included in the BGE vials. A slight enantioselectivity was found for the CYP 3A4 metabolism of verapamil but a significant enantioselectivity cannot be concluded.

As we know, the TDLFP-based mixing is heterogeneous, and therefore the diffusion coefficients of all reactants have to be determined and their concentration profiles calculated in the reaction mixture to perform enzyme kinetics or inhibition study. A new on-line CE method for the direct determination of kinetics and inhibition parameters of CYP 450 was developed by adapting TDLFP and modifying the injection procedure [53]. The enzyme and substrate were injected by hydrodynamic pressure as a series of repeated consecutive plugs. The mathematic modeling showed that the reactants plugs lead to a practically homogeneous reaction mixture. In practice, the injection of three plugs of enzyme surrounded with plugs of substrate leads to homogeneity. Injection times of 3 and 4 s at a pressure of 15 bar (1.5 kPa) were applied for plugs of substrate and enzyme solution, respectively. The proposed method was validated by the study of CYP 2C9 with diclofenac as a probe substrate and sulfaphenazole as a potent inhibitor. The values determined were in agreement with those reported in literature using different techniques.

Chiral discrimination and determination of substrate enantiomers is an important aspect of enzyme assay. Yang et al. [54] developed a modified mode for plug−plug EMMA to achieve on-line chiral discrimination and determination of the pair of lactate enantiomers in one step, as shown in Fig. 2. In this procedure, the plugs of L-LDH, running buffer, and lactate sample (a mixture of D-/L-lactate) were injected first. Then L-lactate enantiomer in the sample is discriminated by L-LDH and is oxidized to produce NADH. After electrophoresis for 60 s to separate lactate (mixture of D-lactate and residual L-lactate), NADH (stoichiometric to L-lactate), and L-LDH (Fig. 2, step c), the high voltage is turned off, and D-LDH, running buffer, and the same lactate sample as in step (A) were injected into the capillary accordingly (Fig. 2, step d). This time, D-lactate is discriminated by D-LDH, and the product NADH stoichiometric to D-lactate is formed (Fig. 2, step e). Finally, six plugs, that is, two lactate sample plugs, two product NADH plugs, and two enzyme plugs, are separated and move to the detection window (Fig. 2, step f). According to

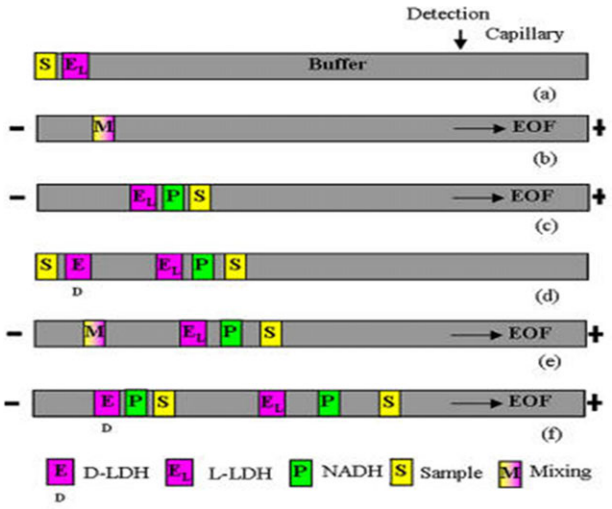

Figure 2. Schematic diagram of modified EMMA procedure for on-line chiral discrimination. Figure was adapted from [54] and reproduced with permission.

the absorbance of the NADH peaks, concentrations of L- and D-lactate are measured. The presented EMMA method was able to determine L- and D-lactate in three brands of yogurts and two brands of wines. The LOD of L-lactate and D-lactate in this method was obtained to be 49 and 26 mM, respectively. The sensitivity is expected to be improved using LIF detection.

The LDH/lactate system was also studied in another paper, but more specifically to answer the question whether protein conformers can be fractionated by crystallization [55]. LDH was crystallized from a variety of solutions and X-ray crystallography revealed the presence of several distinct conformations where the active site loop of any of the four LDH subunits can be in an open or a closed conformation. These microcrystals were separately dissolved and enzyme activities were determined by a plug–plug EMMA method in which the NAD^+ plug moves through the LDH zone during electrophoresis. The LDH zone was a dilution in order to keep the reaction in the linear region. Since lactate is present in the BGE, NADH can be quantified in the UV detector at 214 nm. While fragments from the same crystal showed identical enzyme activities, different crystals showed markedly different activities. It was thus confirmed that crystallization selects even small conformational variants of proteins.

For enzyme assays, on-line monitoring of the enzymatic reaction from beginning to end is important to fully understand the enzyme kinetics. Yang et al. [56] developed a novel method for the sequential on-line CE analysis of enzyme reactions by co-axially aligning two capillaries through a sample vial with a distance of 5 μm between the capillary ends. In this set up, the enzymatic reaction catalyzed by glutamate pyruvate transaminase (GPT) occurred in the sample vial. Direct on-line sample injection and sequential CE analysis were achieved by periodically switching the high-voltage power supply off (for sample injection by diffusion) and on (for CE separation). The sample was injected via concentration diffusion with in-capillary derivatization of the amino acids occurring at the interface of the capillaries, which greatly enhances the diffusion efficiency. Highly repeatable sequential injections were demonstrated by analyzing a standard mixture of the amino acids alanine and glutamate. The developed sequential on-line CE enzyme assay was successfully used for a GPT-catalyzed enzyme reaction by simultaneously monitoring the alanine consumption and the glutamate formation every 30 s from the beginning to the end of the reaction.

Instead of using derivatization reagent, a simple EMMA method with borate-based BGE at pH 10 was developed for the separation of saccharides such as chitotriose, chitobiose, and GlcNAc. The developed EMMA assay was applied to the inhibition study of Hex by DMF [24]. The combination of EMMA with TDLFP can enhance in-capillary mixing of substrate and enzyme plugs. According to the TDLFP model, the reagent plugs were injected using a pressure of 5 kPa for 3 s in the order of substrate-enzyme-substrate and a zone of the reaction buffer three times longer than the reagent plugs was injected after the last substrate plug to assure efficient mixing in this study.

The EMMA-based approach showed advantages for screening the extracts of traditional Chinese medicine. Zhang et al. [57] developed an EMMA method to screen tyrosinase inhibitors from 21 extracts of traditional Chinese medicine. The extract and L-tyrosine were mixed in the same plug and the enzyme activity was assayed by measuring the indole quinine at 214 nm and the method was validated using kojic acid, which is a commercially available tyrosinase inhibitor.

It has been demonstrated that the depletion of NADPH or production of $NADP^+$ may serve as a universal assay for NADPH-dependent enzyme activity. An EMMA method with partial filling technique was developed for screening aromatase inhibitors in traditional Chinese medicine [58]. The enzyme activity was determined by the measurement of $NADP^+$ production. The reaction inside the capillary was initiated with a mixing voltage of 5 kV for 40 s and 20 min of zero-potential amplification was applied to increase the product amount. The developed method was applied to the screening of aromatase inhibitors from 15 natural products and seven compounds were found to be positive for aromatase inhibition. The ranking order was in agreement with a structure–function relationship study [59].

Although it has been reported that mixing of more than two reactants by using the electrical field is difficult, in-capillary electrophoretic mixing of three reactants has been successfully developed [12]. Furthermore, in order to study the inhibition of glycerol kinase (GK), Nehmé et al. [60] developed an in-capillary mixing of four plugs (GK, its two substrates and the allosteric-inhibitor fructose 1,6-bisphosphate or FBP). This study indicates, for the first time, that at least four reactant plugs can be mixed in-capillary using the EMMA approach. They also compared precapillary and in-capillary electrophoresis techniques with both EMMA and PMMA

for the study of 4-nitro-phenyl-galactopyranoside (PNPG) hydrolysis by β-galactosidase (β-Gal) [36]. Unlike EMMA, the in-capillary reaction driven by pressure was referred to as PMMA. PMMA combined with UV detection was used for the first time in this study. In practice, an at-inlet incubation time (waiting period) of 2 min was needed for the inhibition study and a pressure of 25 mbar was applied to push the reaction mixture through the capillary until the UV-detection window. Only reaction product was detected at 405 nm. EMMA proved to be the best technique in terms of sample consumption and speed. Short-end injection was successfully used to speed up the electrophoretic analysis (<0.8 min). This work showed that the combination of at-inlet reaction with EMMA-CE allows enzyme inhibition to be realized without any prior mixing of the substrate and the inhibitor, which could be very interesting for rapid-inhibitor screening without excessive substrate consumption. In addition, the proposed PMMA method can be very powerful for screening inhibitors especially when combined with MS detection.

In some cases, the reaction mixture is complex and it is always hard to have a specific UV absorbance for the reaction product. The sensitivity for UV detection sometimes is not sufficient to have detectable product unless a high reactant concentration and a long incubation time are applied. Even if the detection sensitivity problem is solved by using fluorescence detection [61], the use of labeled compounds is still a considerable issue. It is also not applicable to optimize an EMMA procedure for each compound within a large library of compounds. Therefore, it is necessary to have a universal tool for all compounds in the screening process.

Therefore, a direct on-line CE-MS method is necessary for label free and specific detection of reaction product. Wang et al. [62] developed a sensitive and selective in-capillary assay method combined with MS detection for the on-line screening of several tetracycline antibiotics and natural products against matrix metalloproteinase (MMP)-9. Fast in-line reaction was achieved by highly sensitive SRM detection and EMMA was achieved in a single step by integrating in-line mixing, reaction, separation, and detection. The premixing of enzyme and inhibitor allows screening of MMP inhibitors using relatively low drug concentrations. This technique is very useful in the early stage of drug discovery when only a small amount of drug is available. The developed PMMA-based MS system is expected to become a universal tool for MMP inhibitor screening with the advantage of low consumption of enzyme and substrate. This approach can also be applied to other enzyme systems in inhibitor screening.

4 Conclusion

A wide variety of enzymes from different sources such as in vitro recombinant enzyme, cell membrane, and cell lysates have been studied. The targeted enzymes are selected from drug targets related to certain diseases (e.g. kinase and MMP) or enzymes with known metabolic function (e.g. CYP 450 and FMO1). The applications include enzyme activity assay, enzyme kinetics and inhibition study, inhibitor screening, CL_{int} estimation, enantioselective evaluation as well as drug metabolism study. Various detection modes including UV, fluorescence, and MS were utilized for both off-line and on-line studies. While numerous off-line assays were developed an increasing number of on-line assays were reported due to advantages such as further automation and the use of less enzyme and reagents compared to the off-line methods.

Most enzymes are proteins, and adsorption of the proteins to the capillary wall will result in low assay repeatability. A proper protein precipitation step is suggested in off-line assays and a suitable washing step is needed to perform in-line assays in order to have a repeatable assay [63]. A dynamic or permanent coating is also an option to prevent protein adsorption. Some enzymes need additional cofactors (e.g. metal ions). Coenzymes such as NAD and ATP are usually considered to be second substrates and their products namely NADP and ADP can be detected by UV detection. Currently, UV detection is still the most frequently used detector in CE-mediated enzyme assay both off-line and in-line. However, high concentration of reactants and longer incubation time might be needed to form a detectable amount of product. Fluorescence detection is sensitive but it needs labeled substrates that are not natural substrates of the enzyme. High-throughput systems for compounds screening can be achieved by combining optical detection with a capillary array assay. MS detection is not only a sensitive but also a label free technique. It has the advantage of selective detection and compound identification. However, the sample throughput is limited compared with optical detection. More recently, an eight-capillary CE apparatus in conjunction with an 8-inlet mass spectrometer has allowed eight CE-MS analyses to be performed at the same time, significantly increasing sample throughput [64].

Method development is relatively straightforward for off-line assay since the reaction and separation system can be dealt with separately. In the in-capillary formats assay, EMMA is the more frequently used approach compared with IMER due to its easy operation. The plug–plug mode is still the most frequently used mode in EMMA. Partial filling has been widely applied for cases where incubation buffer and separation buffer are different. The combination of EMMA and TDLFP or at inlet was used to enhance the mixing efficiency. Recently, PMMA combined with at inlet has been used in inhibitor screening, which is advantageous when coupling with selective MS detection. Instead of 37°C commonly used mostly in off-line incubation, most of the in-capillary incubations are performed at 25°C to simplify the procedure. The separation for both off-line and in-line are performed at 25°C. Based on different detection and enzyme systems, the time for zero potential amplification varied from 0 to 20 min. Highly sensitive detection and fast reaction are essential for short in-line incubation times. The off-line incubation typically takes about 15 to 20 min with a preincubation time of 5 to 10 min in some cases.

In summary, CE-mediated microanalysis is still actively used in enzyme study due to its advantages of high speed, low reagent consumption, and minimal sample requirement. The in-line CE method has the potential to become the method of choice for high-throughput screening when the single capillary-based approach is adapted to high-throughput capillary array formats.

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