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Review

Electrophoretically mediated microanalysis

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Abstract

This review describes the existing developments in the use of the capillary electrophoretic microanalytical technique for the in-line study of enzyme reaction, electrophoretically mediated microanalysis (EMMA). The article is divided into a number of parts. After an introduction, the different modes, basic principle, procedure, and some mathematical treatments of EMMA methodology are discussed and illustrated. The applications of EMMA for enzyme assay and for non-enzymatic determination are summarized into two tables. In addition to classical capillary electrophoresis (CE) instrument EMMA, special emphasis is given to a relatively new technique: EMMA on CE microchip. Finally, conclusions are drawn.

Keywords: Reviews; Electrophoretically mediated microanalysis; Chip technology; Enzymes

Contents

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1. Introduction

Enzymes are biological catalysts [1] that play an important role in biochemical reactions necessary for normal growth, maturation and reproduction through the whole living world. They catalyze virtually all chemical reactions in living systems and the assay of enzyme activity is probably one of the most frequently encountered procedures in biochemistry and molecular biology [2]. Due to their low concentrations in samples containing a large amount of other proteins, direct measurements of enzymes by mass are impossible. However, enzymes can be measured more easily by their catalytic activities, which are the most relevant properties of enzymes in the biochemical context.

There is no ideal assay for any enzyme and in general, appropriateness of an assay will depend on the nature of the enzyme, its purity and the purpose of the assay. To be acceptable an assay should be specific, sensitive, quantitative, simple and rapid. It should also be unaffected by side reactions and by presence of drugs or antimetabolites in both crude and purified samples. And just capillary electrophoresis (CE) with its different modes—capillary zone electrophoresis, micellar electrokinetic capillary chromatography, capillary isoelectric focusing, capillary electrochromatography, etc. fulfils almost all these demands.

CE is a powerful and relatively new analytical tool [3,4]. The small dimensions of the CE separation systems utilized are a primary advantage for the bio-research. A recent trend is the use of CE in the area of immunoassays and enzyme assays, due to its capabilities and advantages over other techniques. First, CE offers fast analysis time and requires extremely small amounts of sample. Second, CE provides the capability of highly efficient separations of the reaction products from the substrates. Third, several relatively sensitive detection methods, such as UV-Vis spectrophotometry, laser-induced fluorescence (LIF) and mass spectrometry, are available for CE. Therefore, detection can be accomplished without the use of radiolabeled materials. Currently, the availability of sophisticated and automated CE equipment makes the technique suitable to be implemented in routine laboratories.

CE enzyme assays can be divided into three distinct categories [5]:

(i) a “pre-capillary” assay which is performed by sampling from an assay solution and separating substrate(s) and product(s) by means of CE;
(iii) an “on-capillary” assay which is performed by electrophoretically mixing enzyme and substrate(s) within the capillary and consecutive on-capillary detection of the product(s);
(iii) a “post-capillary” assay which is performed by mixing substrate(s) with enzyme or vice versa, previously separated by CE.

The second alternative—the on-capillary assay—is of particular interest since the assay, and all its necessary operations, completely occurs within the capillary, thus reducing the volume of the assay from μl to nl.

2. Electrophoretically mediated microanalysis

In CE, the separations are generally based upon differences in electrophoretic mobility. The first work, in which the variability in electrophoretic mobilities among enzyme and its substrate(s) was used not only for the initiation of an enzymatic reaction inside the capillary but also for the separation and the detection of the reaction product(s), was published by Bao and Regnier in 1992 [6]. These authors initially named the new method “ultramicro enzyme assays in capillary electrophoretic system”. A year later, the article dedicated to mathematical treatment of this new concept in enzyme assay was published by the same group (Professor F.E. Regnier, Department of Chemistry, Purdue University, West Lafayette, IN, USA) and the methodology was denominated as electrophoretically mediated microanalysis (EMMA) [7]. Since this time, the term EMMA has been accepted by other authors and it has become “terminus technicus”. In relation with other methods applied for enzyme assay, Bao et al. classified the EMMA methodology as *homogeneous enzyme assay by CE* [8], whereas in *heterogeneous enzyme assay by CE* one of the reactants (most often the enzyme) is immobilized onto the wall of the capillary.

In general, enzyme assay requires a number of operations such as the mixing of reagents and the initiation of the reaction, the incubation of the reaction mixture, and consequently detection of the reaction product(s). The EMMA methodology couples all these operations in one integrated technique utilizing the different electrophoretic mobilities of enzyme and substrate(s) to initiate reaction inside the capillary and to separate the components of the reaction mixture from each other for the final on-capillary quantification [7].

There are basically two ways to mix the reaction components in a capillary under electrophoretic conditions [9–11]. In the continuous mode of EMMA (long contact mode), the capillary is initially completely filled with one of the reactants while the second reactant is introduced. In contrast, the plug–plug mode of EMMA (transient format or short contact mode) is based on a plug–plug interaction of reactants in the capillary.

2.1. Continuous modes of electrophoretically mediated microanalysis

The continuous mode of EMMA can be divided into two variants on the basis of the difference in introduction of the second reactant.

2.1.1. Zonal sample introduction method

In this variant, the capillary is initially filled with one of the reactants and upon injection of the second reactant,

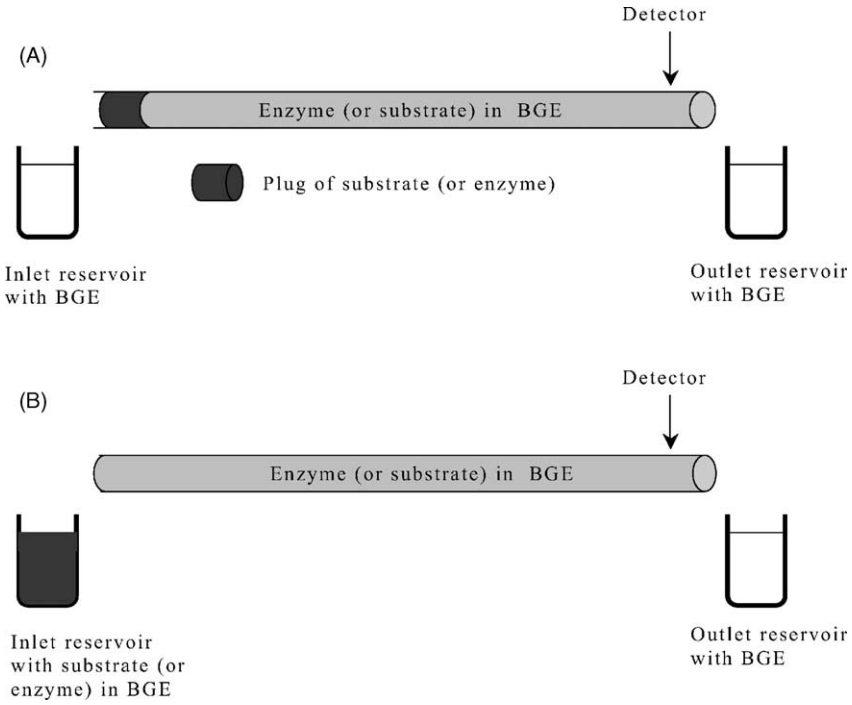

Fig. 1. Schematic illustration of the continuous modes of EMMA: (A) zonal sample introduction; (B) moving boundary sample introduction.

product(s) are formed during the electrophoretic mixing of the enzyme and the substrate(s) (Fig. 1A) [6,7,12–21]. This type of electrophoretically mixing of the reactants was used in the pioneering work of Bao and Regnier [6]. In their experiments, the running buffer (background electrolyte (BGE)) contained the substrate (glucose-6-phosphate) and coenzyme (NADP) required for the catalyzed reaction. The enzyme—glucose-6-phosphate dehydrogenase (G-6-PDH)—was injected into the capillary, and the formation of product (NADPH) was monitored at a downstream absorbance detector at 340 nm (Fig. 2).

2.1.2. Moving boundary introduction method

Although CE has traditionally employed zonal injection mode, moving boundary CE has been reported by Pawliszyn and Wu as an alternative sample introduction technique [22,23]. This alternative injection mode was first used in EMMA methodology by Harmon and co-workers for a microsomal leucine aminopeptidase assay [24,25]. In this variant, the capillary is initially filled with the slower migrating reactant (enzyme or substrate) while the faster migrating complementary reactant is maintained in the inlet buffer reservoir (Fig. 1B). Upon the application of an electric field, the faster migrating reactant electrophoretically enters the capillary from the inlet reservoir and interpenetrates the slower migrating species present in the capillary. This variant results in greater reactant overlap than with the zonal injection mode and, consequently, typically yields an order of magnitude higher concentration sensitivity, which can be seen from the electropherograms in Fig. 3.

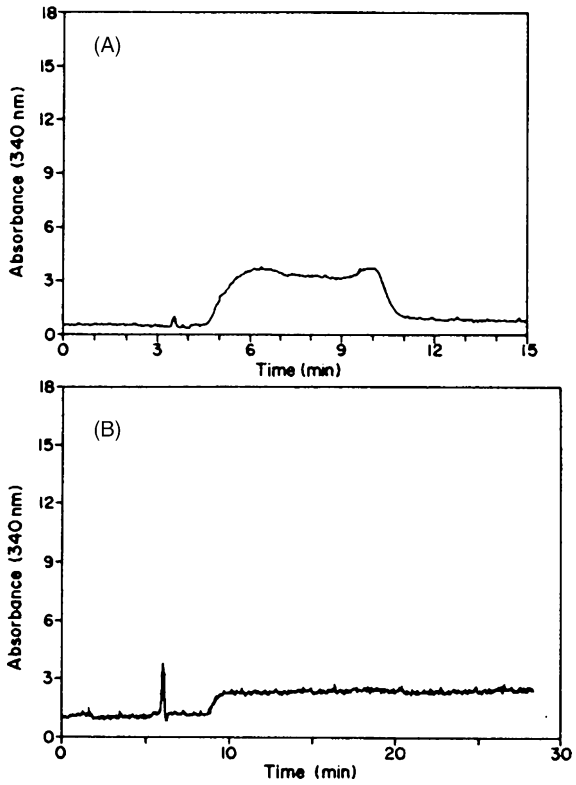

Fig. 2. Typical electropherograms, after zonal sample introduction, showing the formation of NADPH during the process of G-6-PDH migration through the capillary: (A) short separation length or high potential situation; (B) long separation length or low potential situation. From [6], with permission.

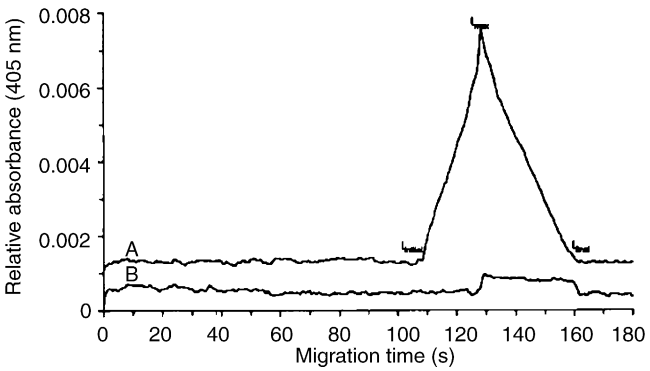

Fig. 3. Moving boundary (A) and 5 nl zonal injection kinetic EMMA (B) determinations of microsomal leucine aminopeptidase (LAP). LAP was assayed by hydrolysis of L-leucine–*p*-nitroanilide to L-leucine and *p*-nitroaniline, which was monitored on basis of unique absorbance at 405 nm. From [24], with permission.

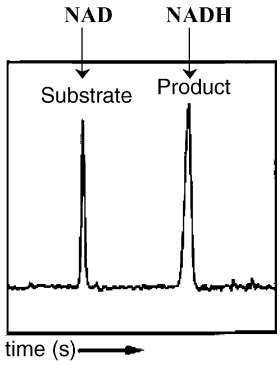

Fig. 5. Electropherogram of the classical plug–plug EMMA assay of G-6-PDH. This electropherogram shows a model system of the conversion of NAD to NADH, in the oxidation of glucose-6-phosphate to 6-phosphogluconate by G-6-PDH. From [5], with permission.

2.2. Plug–plug modes of electrophoretically mediated microanalysis

The plug–plug mode can be divided into four different variants.

2.2.1. Classical plug–plug mode

In this variant, enzyme and substrate(s) are introduced into the capillary as distinct plugs, the first reactant injected being the one with the lower electrophoretic mobility [5,26–39]. Upon the application of an electric field, the two zones interpenetrate due to differences in their electrophoretic mobilities. Enzymatic reaction takes place and the resultant reaction product(s) and the unreacted substrate(s) are electrophoretically transported towards the detector, where they are individually detected (Fig. 4). As an example, Kwak et al. applied the plug–plug mode of EMMA to almost the same model system as Bao and Regnier—the conversion of NAD to NADH in the oxidation of glucose-6-phosphate to 6-phosphogluconate by G-6-PDH (Fig. 5) [5].

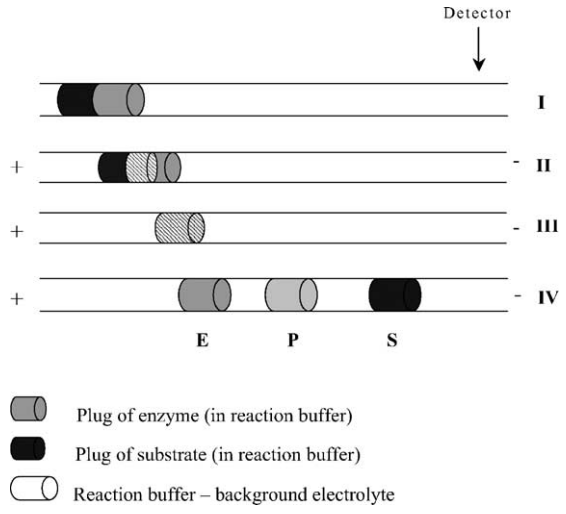

Fig. 4. Schematic illustration of the classical plug–plug mode of EMMA (E: enzyme; S: substrate; P: product of enzymatic reaction). (I) A plug of enzyme and substrate are introduced consecutively in the capillary. (II and III) Upon the application of an electric field the two zones interpenetrate due to differences in their electrophoretic mobilities, and enzymatic reaction takes place. (IV) The reaction product and the unreacted substrate are electrophoretically transported to the detector.

2.2.2. Partial filling technique

The most important factor for successful application of the EMMA methodology in the study of enzymes is, that the electrophoretic conditions, especially the composition and pH of the background electrolyte, are favorable for both the separation of substrate(s) and product(s), and the enzymatic reaction itself. Sometimes the conditions required for the enzymatic reaction and for the electrophoretic separation are totally different making it impossible to use the classical EMMA arrangement. Recently, Van Dyck et al. introduced the combination of the EMMA methodology with a partial filling technique [40]. In this set-up, part of the capillary is filled with the optimum buffer for the enzymatic reaction whereas the rest of the capillary is filled with the background electrolyte optimal for the separation of substrate(s) and product(s) (Fig. 6). Van Dyck et al. combined the EMMA methodology with micellar electrokinetic capillary chromatography for determining bovine plasma amine

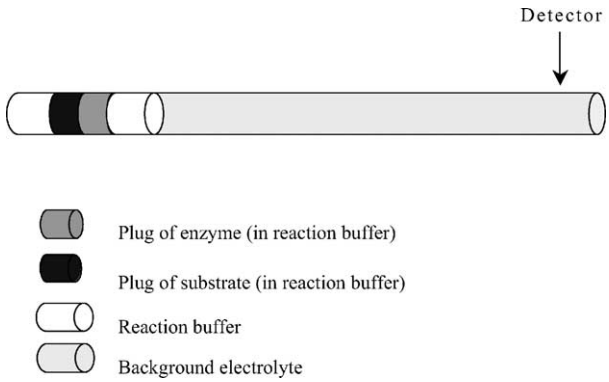

Fig. 6. Schematic illustration of plug–plug mode of EMMA with partial filling technique.

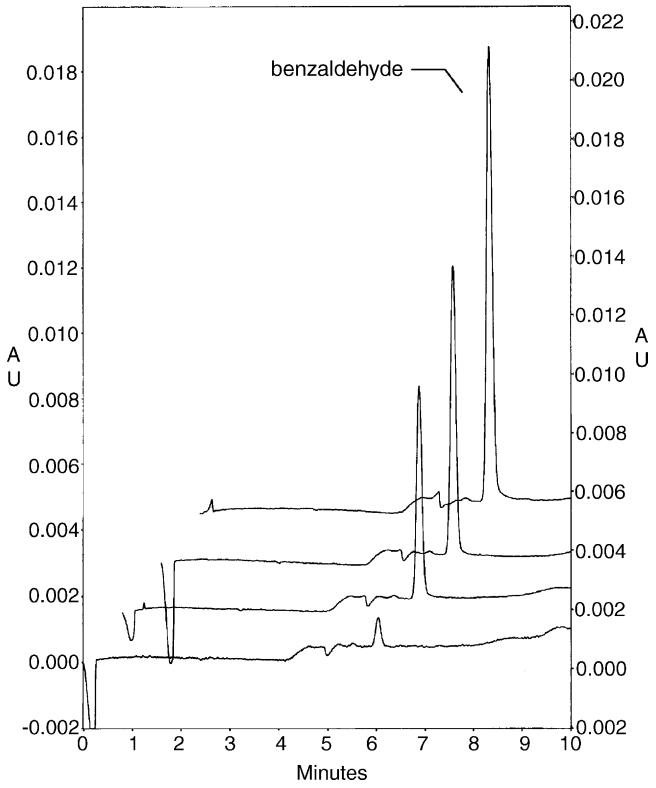

Fig. 7. Overlay of four electropherograms showing plasma amine oxidase assay with benzylamine as a substrate using partial filling EMMA combined with micellar electrokinetic chromatography. Two zones were used in this work: a zone filled with phosphate buffer that allows the reaction to proceed and a second zone, the separation zone, which separates the formed benzaldehyde from the other reaction products. From [40], with permission.

oxidase activity with benzylamine as substrate (Fig. 7). So far, several other background electrolytes have been applied in this innovative EMMA modification—low pH background electrolyte in combination with direct detection for adsorbing inorganic anions with high mobilities [41,42], 10 mM chromate–0.1 mM cetyltrimethylammonium bromide (CTAB) in combination with indirect detection for non-adsorbing inorganic anions with high mobilities [43] and 30 mM sorbic acid–0.1 mM CTAB in combination with indirect detection for non-adsorbing organic anions with moderate mobilities [11].

2.2.3. At-inlet reaction

The at-inlet technique has been described by Taga and Honda, who performed CE with derivatization reactions at the capillary inlet, using amino acids as model compounds [44]. Van Dyck et al. applied this technique on enzymatic reaction [45]. After a sandwich mode injection, enzyme–substrate(s)–enzyme, the overlayed plugs of enzyme and substrate(s) are allowed to react at the capillary inlet. Unlike in a typical EMMA analysis, enzyme and substrate(s) are not electrophoretically mixed prior to the reaction, their mixing is driven by simple diffusion. Subsequently, voltage is applied and the reaction compounds are separated and quantified (Fig. 8). This technique was used for kinetic study of angiotensin converting enzyme (Fig. 9). This variant is especially suitable for enzymes not resistant to an electric field.

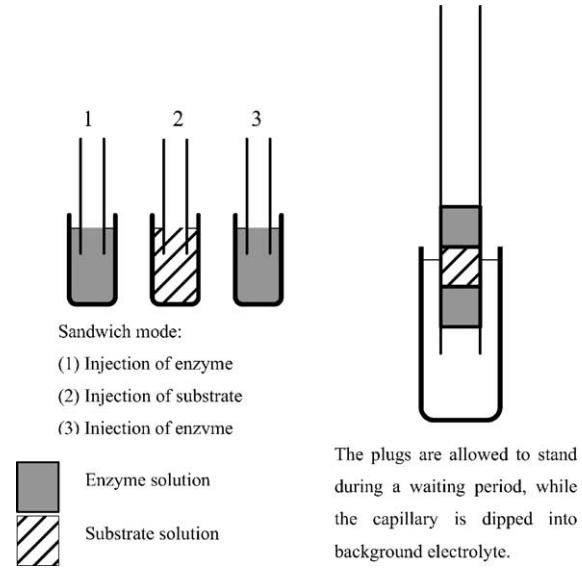

Fig. 8. Schematic illustration of the at-inlet reaction. After the injection of the individual reagents the overlayed plugs are allowed to stand during a predetermined waiting period; subsequently voltage is applied and the reaction compounds, together with the in-line generated reaction product, are transported to the detector.

2.2.4. Electroinjection analysis

The electroinjection analysis (EIA) was introduced by Andreev and co-workers as an alternative to the EMMA methodology [46–48]. In view of the fact that the principle is similar, EIA is covered in this review as a special variant of the plug–plug mode of EMMA.

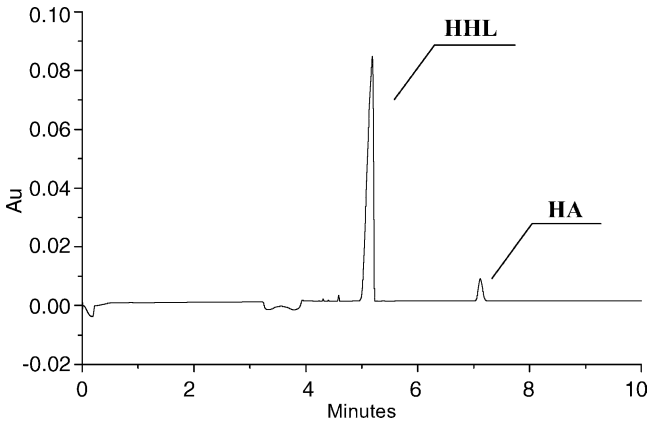

Fig. 9. Electropherogram after on-capillary reaction of angiotensin converting enzyme (ACE) at the capillary inlet. ACE activity was assayed by the conversion of hippuryl-L-histidyl-L-leucine (HHL) to hippuric acid (HA). From [45], with permission.

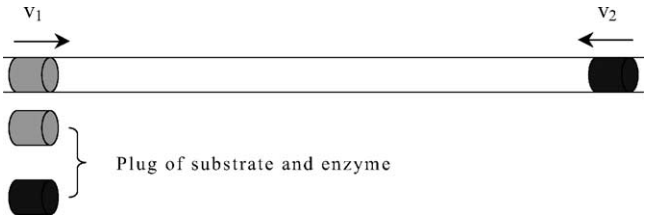

Fig. 10. Schematic illustration of electroinjection analysis (EIA), v_1 and v_2 are substrate and enzyme velocities, respectively.

In EIA, mixing of the reactants is also due to the difference of their electrophoretic mobilities in the applied electric field, but the reactants are simultaneously injected from the opposite ends of the capillary by using electrokinetic injection and they are moving in opposite directions (Fig. 10). Because of the presence of the electroosmotic flow, it is possible to mix not only oppositely charged reactants but even reactants with the same charge and different electrophoretic mobilities. EIA brought several advantages over classic EMMA methodology: (i) the possibility of using very short capillaries and consequently lower electric fields; and (ii) the prevention of cross-contamination of enzyme and its substrate(s) because they are injected through opposite ends of the capillary. Andreev et al. used this technique for non-enzymatic Cr(VI) determination as a red complex of Cr(III)–diphenylcarbazone (Fig. 11). This reaction was easily performed by EIA since Cr(VI) as CrO_4^{2-} has high mobility whereas the reagent—diphenylcarbazide is almost neutral [47].

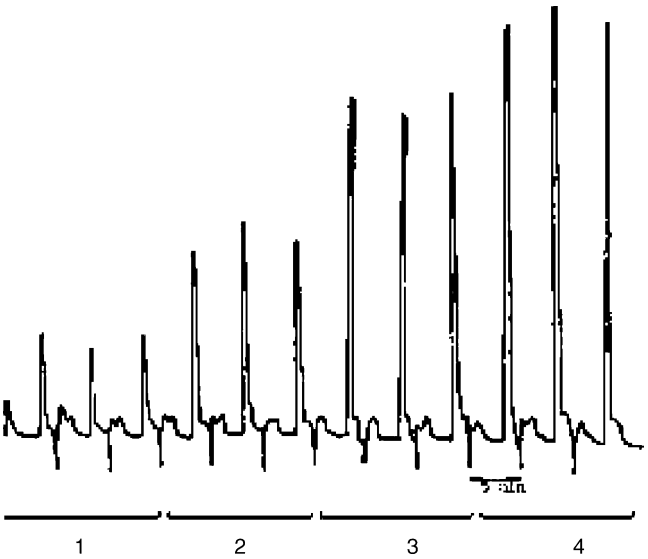

Fig. 11. Electropherogram of EIA determination of Cr(VI). This figure shows a series of product peaks in the determination of Cr(VI) with diphenylcarbazide corresponding to: (1) 50 ng/ml Cr; (2) 100 ng/ml Cr; (3) 150 ng/ml Cr; (4) 200 ng/ml Cr. From [47], with permission.

3. Electrophoretically mediated microanalysis methodology

At present, the plug–plug mode of EMMA is more advantageous compared to the continuous mode, owing to several reasons, e.g. less reagent is required for analysis since only small plugs of enzyme and substrate(s) are used in the experiment. Moreover, the electropherograms of all variants of the plug–plug mode are similar to that of conventional CE, in contrast with those of the continuous mode, and their evaluation can be performed by means of classic integration software. This is especially evident from the comparison of Figs. 2 and 5, in which the authors studied the same enzymatic system. For these reasons the following part, dedicated to the EMMA methodology, is oriented mainly on the plug–plug mode. Generally, the EMMA methodology includes three steps:

(i) reagent metering;
(ii) initiation and progress of reaction;
(iii) detection of reaction product.

3.1. Reagent metering

The EMMA methodology requires that the enzyme and its substrate(s) are sequentially introduced into the capillary. To preserve the efficient separation capabilities of CE, the injection system should not cause significant zone broadening. Therefore, it is important that the sample injection method employed is capable of delivering small volumes of sample efficiently and repeatedly into the capillary [3]. In EMMA, reactants are typically introduced as zones of finite width by traditional CE injection methods, such as hydrodynamic or electrokinetic injection. Hydrodynamic sample injection is the most widely used method, in which the volume of sample loaded is a function of the capillary dimensions, the viscosity of the buffer, the applied pressure, and the injection time. Electrokinetic, or electromigration, injection is performed by applying a voltage, which is typically three to five times lower than the voltage used for separation.

Injection volumes in CE are typically a few nl. These ultramicroinjection volumes allow the analysis of samples available in very small amounts, such as the interstitial and intercellular fluids, which is a very promising application field for EMMA methodology.

3.2. Initiation and progress of reaction

In traditional methods, chemical and enzymatic reactions are generally initiated by turbulent mixing of the solutions containing the reactants. However, in all EMMA modes, with exception of the at-inlet variant, the mixing of the enzyme and its substrate(s) is accomplished by exploiting the variability in transport velocity among the chemical species in the chosen electrophoretic medium [7]. Following the injection of the reactants into the capillary, electrophoretic

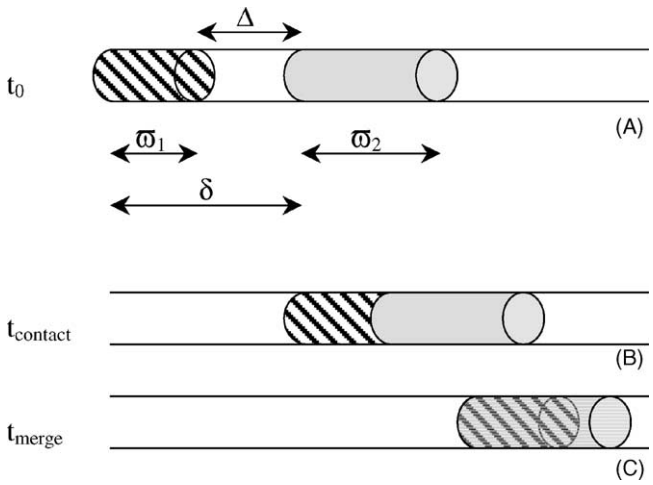

Fig. 12. An illustration of electrophoretic mixing of two zones: (A) spatially distinct zones; (B) beginning engagement; (C) completely mixed.

mixing is initiated by the application of an electric field. The reactant zones migrate at a differential velocity v_{DIFF} based on the difference in electrophoretic mobility between the two components of interest $\Delta\mu_{EP}$ and the applied electric field strength E:

$$v_{DIFF} = \Delta\mu_{EP}\, E \tag{1}$$

Knowledge of the difference in electrophoretic mobilities and distance between reactant zones allows to calculate the contact time $t_{contact}$ (Fig. 12B), neglecting the effect of longitudinal diffusion:

$$t_{contact} = \frac{\Delta}{\Delta\mu_{EP}\, E} \tag{2}$$

where Δ is the distance between the leading edge of the zone of greater transport velocity and trailing edge of the zone of lesser transport velocity (Fig. 12A). If the reactant zones are injected adjacently ($\Delta = 0$), interpenetration of the zones occurs immediately upon the application of an electric field. The interpenetration of the zones continues as the potential is maintained, and the reactant of greater transport velocity migrates through that of lower transport velocity. Furthermore, the total time of the enzymatic reaction $t_{reaction}$ can be estimated as:

$$t_{reaction} = \frac{\varpi_1 + \varpi_2}{\Delta\mu_{EP}\, E} \tag{3}$$

where ϖ_1 and ϖ_2 are the lengths of the reagent zones (Fig. 12A).

Prolonged on-capillary incubation of enzyme and substrate(s) can be achieved by turning off the voltage when the two zones are completely mixed. The time required for the two zones to completely merge t_{merge} (Fig. 12C), can be estimated from the formula:

$$t_{merge} = \frac{\delta}{\Delta\mu_{EP}\, E} \tag{4}$$

where δ is the distance between the trailing edge of the zone of greater transport velocity and the trailing edge of the zone of lesser transport velocity (Fig. 12A). Turning off the voltage at this point (t_{merge}) allows the reaction to continue in the absence of an electric field. The technique is called "zero potential amplification". This built-in step is usually necessary to accumulate enough product(s) for the spectrophotometric detection. Occasionally, the EMMA methodology without turning off the voltage is called "constant potential EMMA" and the one with turning off the voltage is called "zero potential EMMA".

3.3. Detection of reaction product

Detection in the EMMA methodology is generally performed by electrophoretically transporting the detectable species to the detection system. The small capillary dimensions and the tiny injection volumes present a real challenge to achieve sensitive detection without introducing zone dispersion. Zone broadening normally caused by joints, fittings and connectors as in LC is eliminated by on-capillary detection. The lower limit of detection in CE varies with the detection method. With the nl volumes used in CE, it is often possible for on-capillary UV-Vis absorbance detection to achieve a limit of detection of 10^{-7} M (i.e. 10^{-16} mol) [49]. Although UV-Vis spectrophotometry is probably the most used detection technique in EMMA methodology, several applications of LIF were also published. LIF detection can provide highly sensitive detection and allows a very low concentration detection limit of 10^{-12} M (i.e. 10^{-21} mol) [49]. For example, Lee et al. reported a method for the determination of picomolar concentration of proteins, in which EMMA methodology was used to label proteins on-capillary with a fluorogenic reagent [50]. In this particular case, the method was called EMMA–CE–LIF. Chemiluminescence is yet another detection technique that is highly sensitive and often highly specific. Regehr and Regnier described chemiluminescent detection in EMMA methodology for three different enzymes and demonstrated a limit of detection for catalase of 15 zmol (9300 molecules) [49]. However, LIF and chemiluminescence detections require expensive equipment and are rather difficult to operate. Electrochemical detection is another method, which can provide comparable limits at lower cost, but special equipment, not always familiar with commercial CE instruments is also necessary [17].

As mentioned earlier, the drop in the detection limits could be achieved by chemical amplification and zero potential modes.

4. Application of electrophoretically mediated microanalysis

Since its discovery by Bao and Regnier, EMMA methodology has been applied in a number of biochemical systems—for assays of enzyme activity, determination of substrates, Michaelis constants, inhibitors and inhibition constants. In addition, it has also been used in several

Table 1
Overview of the applications of the EMMA methodology on enzyme systems

Enzyme	Type of EMMA	Detection	Note	Reference
Glucose-6-phosphate dehydrogenase	Plug–plug	UV (260 nm)		[5]
Glucose-6-phosphate dehydrogenase	Zonal	UV (340 nm)	First work using EMMA	[6]
Alcohol dehydrogenase	Zonal	UV (340 nm)	Mathematical treatment of EMMA	[7]
Alkaline phosphatase	Combination zonal and plug–plug	LIF	Inhibition study	[9]
β-Glucosidase	Plug–plug	UV (214 nm)	EMMA in coated capillary with linear polyacrylamide	[10]
Fructose-biphosphate aldolase, fructose-1,6-diphosphatase	Partial filling mode	Indirect UV (254 nm)	30 mM sorbic acid–0.1 mM CTAB	[11]
Alcohol dehydrogenase	Zonal	UV (340 nm)	EMMA of ethanol	[12]
Leucine aminopeptidase	Zonal	LIF		[13]
Alkaline phosphatase, β-galactosidase	Zonal	Vis (405 nm)	Gel filled capillaries	[14]
Alcohol dehydrogenase, leucine aminopeptidase, alkaline phosphatase, β-galactosidase	Zonal	UV (340 nm) Vis (405 nm)	Selectivity in EMMA by control of product detection time	[16]
Alkaline phosphatase	Zonal	Electrochemical: carbon electrode held at +180 mV vs. Ag/AgCl, Vis (405 nm)	Electrochemical detection is more sensitive than spectrophotometric detection	[17]
Lactate dehydrogenase	Zonal	LIF	Detection limit: 1.3×10^{-21} mol of LDH	[18]
Hexokinase/apyrase, lactate dehydrogenase/glucose-6-phosphate dehydrogenase	Zonal	UV (260 nm)	Double enzyme-catalyzed microreactors using CE	[20]
Leucine aminopeptidase	Moving boundary	Vis (405 nm)		[24]
Leucine aminopeptidase	Zonal and moving boundary	Vis (405 nm)	Dynamic modeling of EMMA	[25]
Glucose-6-phosphate dehydrogenase, alcohol dehydrogenase	Zonal and plug–plug	UV (260 and 340 nm)	According to authors: an enzymatic CE microreactor	[26]
Pepsin	Plug–plug	LIF	Peptide mapping	[27]
Adenosine deaminase	Plug–plug	UV (254 nm)		[28]
Adenosine deaminase	Plug–plug	UV (254 nm)	Inhibition study	[29]
Phosphodiesterase I	Plug–plug	UV (260 nm)	Oligonucleotide analysis	[30]
Phosphodiesterase I	Plug–plug	UV (260 nm)	Degradation of oligonucleotides with capillary polymer sieving electrophoresis	[31]

Glucose-6-phosphate dehydrogenase, acid phosphatase	Plug–plug	UV (200 nm)		[32]
Alkaline phosphatase	Plug–plug	UV (230 nm)	Michaelis–Menten analysis of ALP	[33]
Creatine kinase	Plug–plug	UV (256 nm)		[34]
α-Amylase, glucoamylase	Plug–plug	UV (280 nm)	Measurement of α-amylase and glucoamylase activities in sake rice koji	[35]
Cyclophilin (peptidyl-prolyl-*cis*/*trans*-isomerase)	Plug–plug	UV (200 nm)	Combined with affinity capillary electrophoresis	[36]
α-Glucosidase, β-galactosidase, β-*N*-acetylglucosamidase	Plug–plug	UV (214 nm), Vis (405 nm)		[37]
Catechol-*O*-methyltransferase	Plug–plug	UV (200 nm)		[38]
γ-Glutamyltransferase	Plug–plug	UV (380 nm)	EMMA combined with MEKC	[39]
Amine oxidase	Partial filling mode	UV (254 nm)	EMMA combined with MEKC	[40]
Rhodanese	Partial filling mode	UV (200 nm)		[41]
Rhodanese	Partial filling mode	UV (200 nm)	Inhibition study	[42]
Haloalkane dehalogenase	Partial filling mode	Indirect UV (315 and 375 nm)	10 mM chromate–0.1 mM CTAB	[43]
Angiotensin converting enzyme	At-inlet reaction	UV (230 nm)		[45]
Glucose oxidase, galactose oxidase, catalase	Zonal	Chemiluminescent		[49]
Leucine aminopeptidase	Plug–plug	Two-photon excited fluorescence detection	EMMA on microchip	[51]
Alkaline phosphatase	Zonal	LIF	EMMA on microchip	[52]
β-Galactosidase	Plug–plug	Fluorescence	EMMA on microchip	[53]
β-Galactosidase	Zonal	LIF	EMMA on microchip-inhibition study	[54]
Protein kinase	Zonal	Fluorescence	EMMA on microchip-inhibition study	[55]
Acetylcholinesterase	Zonal	LIF	EMMA on microchip-inhibition study	[56]
Lactate dehydrogenase	Zonal	UV (280 and 340 nm)		[57]
Glucose oxidase	Zonal	LIF		[58]
Alkaline phosphatase	Combination zonal and plug–plug	LIF	Inhibition study of ALP by theophylline	[59]
Lactate dehydrogenase	Zonal	Electrochemical: carbon electrode held at +0.8 V vs. SCE		[60]
N-Acetylneuramidase	Zonal	UV (200 nm)	According to authors: throughout-capillary derivatization	[61]
Alkaline phosphatase, acid phosphatase	Plug–plug and partial filling mode	UV (270 nm)	Determination of water-soluble vitamins	[62]

MEKC: micellar electrokinetic chromatography; SCE: saturated calomel electrode.

Table 2
Overview of the applications of the EMMA methodology on non-enzymatic determinations.

Determination	Type of EMMA	Detection	Note	Reference
EMMA of calcium	Zonal	Vis (575 nm)	Complexometric reaction of calcium with *o*-cresolphtalein	[15]
Redox activities of microorganisms	Zonal	Vis (610 nm)	Characterization of microorganisms	[21]
Determination of Cr(VI) and Co(II)	EIA	Vis (500 and 540 nm)	Mathematical model	[47]
Analysis of proteins	Plug–plug	LIF	EMMA was used to label proteins on-capillary with fluorogenic reagent	[50]
Analysis of catalytic role of *Monascus* pigment	Zonal plug–plug	UV (264 nm)	On-capillary MEKC	[63]
Quality control of gentamicin	Plug–plug	UV (330 nm)	EMMA is used for on-capillary derivatization	[64]
Protein fingerprinting of six *Staphylococcus* species	Plug–plug	LIF	Manipulation of protein fingerprints during on-capillary fluorescent labeling	[65]
Jaffé reaction between creatinine and picrate	Plug–plug	Vis (485 nm)	EMMA with small molecules	[66]
Quality control of kanamycin	Plug–plug	UV (335 nm)	EMMA is used for on-capillary derivatization	[67]
Determination of glutathione, DTT, cysteine, homocysteine	Plug–plug	UV (200 and 343 nm)	Specific thiol determination by on-capillary reaction with 2,2′-dipyridyldisulfide	[68–71]

special non-enzymatic determinations. Most of the applications together with the type of EMMA, detection technique and special remarks are summarized in Tables 1 and 2.

5. Electrophoretically mediated microanalysis on microchip

Miniaturized CE systems for chemical, especially for biochemical and clinical analysis are attractive for a number of reasons: low sample and reagent consumption, decreased analysis times, automated control of dilution, mixing and separation and integration on a single device (Fig. 13). The EMMA methodology in a chip format easily combines the operations of reactants loading, merging and mixing, execution of enzymatic reactions for a fixed time, product(s) separation, and detection in a single channel. The fact that multiple operations are combined in one channel greatly simplifies device fabrication and operation. Given the high throughput of microfabricated systems and many advantages of the EMMA methodology, it seems highly advantageous to combine these two techniques. As a result, the EMMA methodology was transferred to different microchip formats. Zugel et al. reported determination of leucine aminopeptidase by means of two-photon excited fluorescence detection via the EMMA methodology on a microchip [51]. Murakami et al. described the EMMA assay of alkaline phosphatase on a glass chip [52], and Burke and Regnier published EMMA methodology of β-galactosidase on microchip [53] (Fig. 14). Microchip devices have also been used for inhibition studies of β-galactosidase [54], protein kinase A [55], and acetylcholinesterase [56].

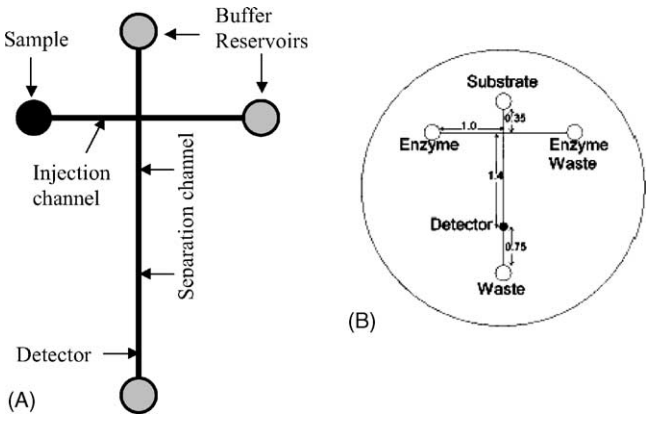

Fig. 13. Schematic illustration of simple CE microchip devices: (A) typical cross-channel topography of miniaturized CE chip; (B) schematic diagram of cross-shaped microchip, which was used for EMMA assay of leucine aminopeptidase. All dimensions are in cm. From [51], with permission.

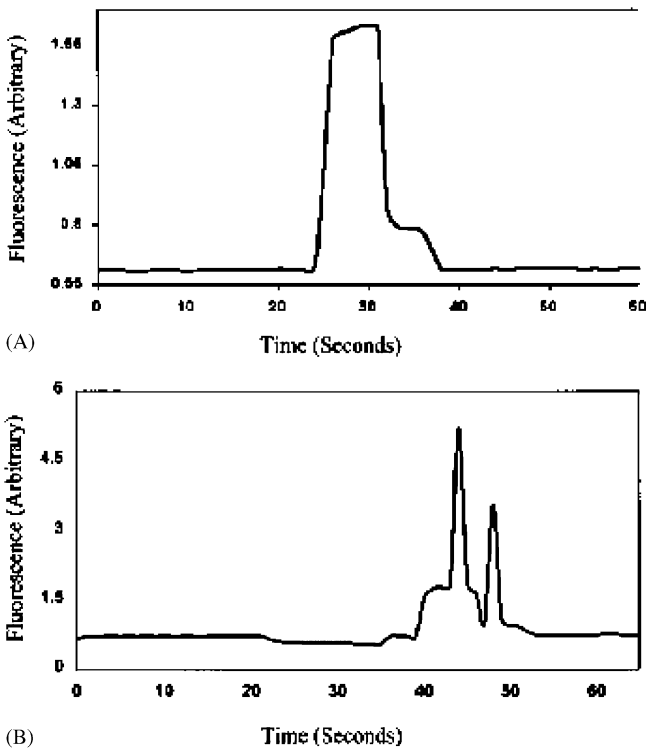

Fig. 14. The overlayed electropherograms of EMMA assay of β-galactosidase on borosilicate microchip: (A) constant potential EMMA of β-galactosidase; (B) zero potential EMMA of β-galactosidase. From [53], with permission.

6. Conclusion

The EMMA methodology offers numerous advantages over traditional enzymatic assays. The use of electrophoretic mixing allows reagent zones to be merged without the concurrent dilution experienced in bulk methods, does not cause turbulence and the resulting band spreading. Moreover, it allows homogeneous kinetic enzyme assays to be performed and detected entirely on-capillary with very high mass sensitivity due to the small dimension of CE separation systems and the amplifying nature of enzymatic reactions. Compared with spectrophotometric and other discontinuous assays, the method is rapid, can be automated, and requires only small amounts of reagents, which is especially important in the case of enzymes. EMMA could become a very powerful method when it is combined with the microchip capillary electrophoresis systems or the multichannel electrophoretic microchips.

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