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RECENT ADVANCEMENT IN THE ENZYME INHIBITORS SCREENING BY CAPILLARY ELECTROPHORESIS

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ABSTRACT
Capillary electrophoresis with many advantages plays an significant role in pharmaceutical analysis and drug screening. This review gives an outline on the recent advances in the developments and applications of capillary electrophoresis in the field of enzyme inhibitor screening. The period covers 2013 to 2017. Both the pre-capillary enzyme assays and in-capillary enzyme assays that include electrophoretically mediated microanalysis (EMMA) and immobilized enzyme microreactor (IMER) are summarized in this article.

KEYWORDS: Capillary electrophoresis, Enzyme inhibitor screening, Pre-capillary enzyme assays, Electrophoretically mediated microanalysis, Immobilized enzyme microreactor.

INTRODUCTION
Enzyme, a kind of biocatalyst which has high catalytic efficiency, is essential for maintaining normal life activities. Enzymes take part in the process of physiological metabolism including cells regulating homeostasis and reproduction of living organisms. So some human diseases often result from abnormal regulation of enzymes, like inflammation, tumor, cardiovascular disease and infectious disease. Therefore, we can treat these diseases with targeted therapy by intervening in enzyme pathway and pathologic expression.[1,2] It has been revealed that enzymes are potential drug targets.[3-9] One situation is that the enzyme expression level is higher than the expected value, which result in disturbed body functions. One of the therapeutic ideas is restricting the enzyme activity by enzyme inhibitors. So the development of enzyme inhibitors is one way to find new drugs. Therefore, it is essential to study effective methods to screen enzyme inhibitors for the treatment of human diseases.

Ultraviolet (UV) and fluorescence spectrophotometry, electrochemical method and high performance liquid chromatography (HPLC) are conventional methods, that are usually used as analytical tools to screen enzyme inhibitors. But these methods have some disadvantages. UV and fluorescence spectrophotometry are only suitable for the situation in which substrates and products have considerable difference in their spectrometric properties. The fluorescence signal can also be interfered by background absorption and auto-fluorescence in biological samples.[10] Electrochemical method requires that substrates and products have electrochemical activity. HPLC can avoid these defects from the above methods. However, long elution time in HPLC limits high efficiency of inhibitor screening and the consumption of large amounts of organic solvent increases the cost.

Capillary electrophoresis (CE) has been widely applied in various fields including enzyme inhibitor screening, as it has many advantages such as high separation efficiency, quick analysis, low sample consumption, low solvent volume[11,12], automation and ability to be combined with various detection techniques[13] including UV, laser induced fluorescence (LIF)[14,15], electrochemical detectors[16,17] and mass spectroscopy (MS).[18,19]

In general, the methods for screening enzyme inhibitors by CE can be divided in two categories: (i) pre-capillary enzyme assays in which the enzymatic reaction takes place off-line and the capillary is just used as the separation channel; (ii) in-capillary enzyme assays in which enzymatic reaction takes place on-line and sampling, reaction, separation, and detection can be integrated into a single capillary. Electrophoretically mediated microanalysis (EMMA) and immobilized enzyme microreactor (IMER) are included in in-capillary

enzyme assays. These methods are summarized in this review.

This review covers the literatures and gives an overview of the recent advances in the developments and applications of these methods in the field of enzyme inhibitor screening, over the period from 2013 to late 2017, which is a continuation of previous reports[20,21] and a supplement of recent reports.[22,23]

Capillary electrophoresis

Capillary electrophoresis is an analytical technique that separates ions based on their electrophoretic mobility with the use of an applied voltage. The electrophoretic mobility is dependent upon the charge of the molecule, the viscosity, and the atom's radius. The rate at which the particle moves is directly proportional to the applied electric field--the greater the field strength, the faster the mobility. Neutral species are not affected, only ions move with the electric field. If two ions are the same size, the one with greater charge will move the fastest. For ions of the same charge, the smaller particle has less friction and overall faster migration rate. Capillary electrophoresis is used most predominately because it gives faster results and provides high resolution separation. It is a useful technique because there is a large range of detection methods available.[24]

Introduction

Endeavors in capillary electrophoresis (CE) began as early as the late 1800's. Experiments began with the use of glass U tubes and trials of both gel and free solutions.[24] In 1930, Arnes Tiselius first showed the capability of electrophoresis in an experiment that showed the separation of proteins in free solutions.[25] His work had gone unnoticed until Hjerten introduced the use of capillaries in the 1960's. However, their establishments were not widely recognized until Jorgenson and Lukacs published papers showing the ability of capillary electrophoresis to perform separations that seemed unachievable. Employing a capillary in electrophoresis had solved some common problems in traditional electrophoresis. For example, the thin dimensions of the capillaries greatly increased the surface to volume ratio, which eliminated overheating by high voltages. The increased efficiency and the amazing separating capabilities of capillary electrophoresis spurred a growing interest among the scientific society to execute further developments in the technique.

Instrumental Setup

A typical capillary electrophoresis system consists of a high-voltage power supply, a sample introduction system, a capillary tube, a detector and an output device. Some instruments include a temperature control device to ensure reproducible results. This is because the separation of the sample depends on the electrophoretic mobility and the viscosity of the solutions decreases as the column temperature rises.[26] Each side of the high voltage power supply is connected to an electrode. These electrodes help to induce an electric field to initiate the migration of the sample from the anode to the cathode through the capillary tube. The capillary is made of fused silica and is sometimes coated with polyimide. Each side of the capillary tube is dipped in a vial containing the electrode and an electrolytic solution, or aqueous buffer. Before the sample is introduced to the column, the capillary must be flushed with the desired buffer solution. There is usually a small window near the cathodic end of the capillary which allows UV-VIS light to pass through the analyte and measure the absorbance. A photomultiplier tube is also connected at the cathodic end of the capillary, which enables the construction of a mass spectrum, providing information about the mass to charge ratio of the ionic species.

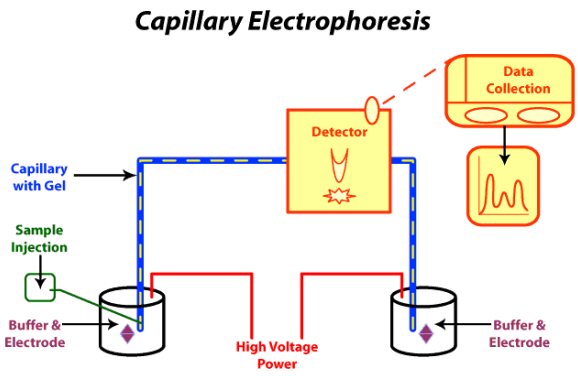

Figure: 1 Capillary electrophoresis setup.

THEORY

Electrophoretic Mobility

Electrophoresis is the process in which sample ions move under the influence of an applied voltage. The ion undergoes a force that is equal to the product of the net charge and the electric field strength. It is also affected by a drag force that is equal to the product of ff, the translational friction coefficient, and the velocity. This leads to the expression for *electrophoretic mobility*:

$$\mu EP=qf=q6\pi\eta r(1.1)(1.1)\mu EP=qf=q6\pi\eta r$$

Where f for a spherical particle is given by the Stokes' law; η is the viscosity of the solvent, and rr is the radius of the atom. The rate at which these ions migrate is dictated by the charge to mass ratio. The actual velocity of the ions is directly proportional to E, the magnitude of the electrical field and can be determined by the following equation[27]:

$$v=\mu EPE(1.2)(1.2)v=\mu EPE$$

This relationship shows that a greater voltage will quicken the migration of the ionic species.

Electroosmotic Flow

The electroosmotic flow (EOF) is caused by applying high-voltage to an electrolyte-filled capillary.[27] This flow occurs when the buffer running through the silica capillary has a pH greater than 3 and the SiOH groups lose a proton to become SiO^- ions. The capillary wall

then has a negative charge, which develops a double layer of cations attracted to it. The inner cation layer is stationary, while the outer layer is free to move along the capillary. The applied electric field causes the free cations to move toward the cathode creating a powerful bulk flow. The rate of the electroosmotic flow is governed by the following equation:

$$\mu EOF=\epsilon 4\pi\eta E\zeta(1.3)(1.3)\mu EOF=\epsilon 4\pi\eta E\zeta$$

Where ε is the dielectric constant of the solution, η is the viscosity of the solution, E is the field strength, and ζ is the zeta potential. Because the electrophoretic mobility is greater than the electroosmotic flow, negatively charged particles, which are naturally attracted to the positively charged anode, will separate out as well. The EOF works best with a large zeta potential between the cation layers, a large diffuse layer of cations to drag more molecules towards the cathode, low resistance from the surrounding solution, and buffer with pH of 9 so that all the SiOH groups are ionized.[24]

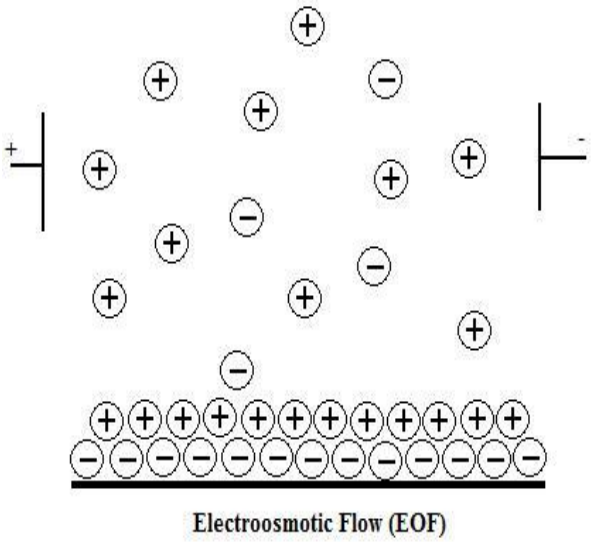

Figure 1.2: Electroosmotic Flow due to Applied Voltage.

Capillary Electroseparation Methods

There are six types of capillary electroseparation available: capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic capillary chromatography (MEKC), capillary electrochromatography (CEC), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP). They can be classified into continuous and discontinuous systems as shown in Figure 3. A continuous system has a background electrolyte acting throughout the capillary as a buffer. This can be broken down into kinetic (constant electrolyte composition) and steady state (varying electrolyte composition) processes. A discontinuous system keeps the sample in distinct zones separated by two different electrolytes.[29]

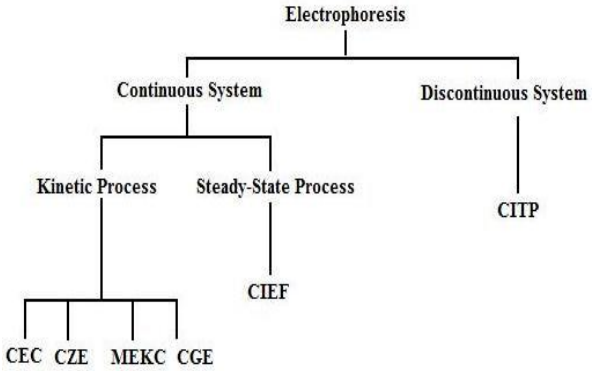

Figure 1.3: Categorization of Electrophoresis Techniques.

Capillary Zone Electrophoresis (CZE)

Capillary Zone Electrophoresis (CZE), also known as free solution capillary electrophoresis, it is the most commonly used technique of the six methods. A mixture in a solution can be separated into its individual components quickly and easily.The separation is based on the differences in electrophoretic mobility, which is directed proportional to the charge on the molecule, and inversely proportional to the viscosity of the solvent and radius of the atom.The velocity at which the ion moves is directly proportional to the electrophoretic mobility and the magnitude of the electric field.[24]

The fused silica capillaries have silanol groups that become ionized in the buffer. The negatively charged SiO^- ions attract positively charged cations, which form two layers—a stationary and diffuse cation layer. In the presence of an applied electric field, the diffuse layer migrates towards the negatively charged cathode creating an electrophoretic flow (μepμep) that drags bulk solvent along with it. Anions in solution are attracted to the positively charged anode, but get swept to the cathode as well. Cations with the largest charge-to-mass ratios separate out first, followed by cations with reduced ratios, neutral species, anions with smaller charge-to-mass ratios, and finally anions with greater ratios. The electroosmotic velocity can be adjusted by altering pH, the viscosity of the solvent, ionic strength, voltage, and the dielectric constant of the buffer.[24]

Capillary Gel Electrophoresis (CGE)

CGE uses separation based on the difference in solute size as the particles migrate through the gel. Gels are useful because they minimize solute diffusion that causes zone broadening, prevent the capillary walls from absorbing the solute, and limit the heat transfer by slowing down the molecules. A commonly used gel apparatus for the separation of proteins is capillary SDS PAGE. It is a highly sensitive system and only requires a small amount of sample.[24]

Micellar Electrokinetic Capillary Chromatography (MEKC)

MEKC is a separation technique that is based on solutes partitioning between micelles and the solvent. Micelles

are aggregates of surfactant molecules that form when a surfactant is added to a solution above the critical micelle concentration. The aggregates have polar negatively charged surfaces and are naturally attracted to the positively charged anode. Because of the electroosmotic flow toward the cathode, the micelles are pulled to the cathode as well, but at a slower rate. Hydrophobic molecules will spend the majority of their time in the micelle, while hydrophilic molecules will migrate quicker through the solvent. When micelles are not present, neutral molecules will migrate with the electroosmotic flow and no separation will occur. The presence of micelles results in a retention time to where the solute has little micelle interaction and retention time tmc where the solute strongly interacts. Neutral molecules will be separated at a time between to and tmc. Factors that affect the electroosmotic flow in MEKC are: pH, surfactant concentration, additives, and polymer coatings of the capillary wall.[24]

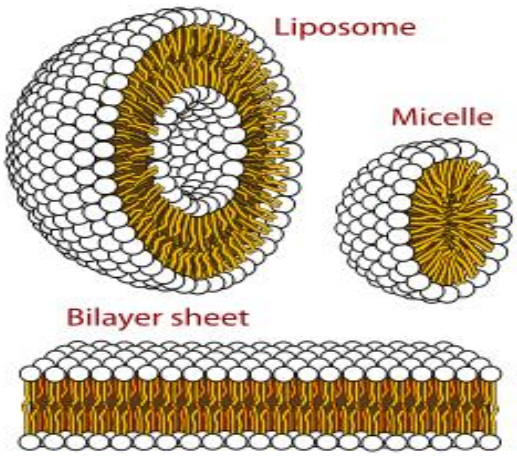

Figure 1.4: Micelles.

Capillary Electrochromatography (CEC)

The separation mechanism is a packed column similar to chromatography. The mobile liquid passes over the silica wall and the particles. An electroosmosis flow occurs because of the charges on the stationary surface. CEC is similar to CZE in that they both have a plug-type flow compared to the pumped parabolic flow that increases band broadening.[24]

Capillary Isoelectric Focusing (CIEF)

CIEF is a technique commonly used to separate peptides and proteins. These molecules are called zwitterionic compounds because they contain both positive and negative charges. The charge depends on the functional groups attached to the main chain and the surrounding pH of the environment. In addition, each molecule has a specific isoelectric point (pI). When the surrounding pH is equal to this pI, the molecule carries no net charge. To be clear, it is not the pH value where a protein has all bases deprotonated and all acids protonated, but rather the value where positive and negative charges cancel out to zero. At a pH below the pI, the molecule is positive, and then negative when the pH is above the pI. Because the charge changes with pH, a pH gradient can be used to separate molecules in a mixture. During a CIEF separation, the capillary is filled with the sample in solution and typically no EOF is used (EOF is removed by using a coated capillary). When the voltage is applied, the ions will migrate to a region where they become neutral (pH=pI). The anodic end of the capillary sits in acidic solution (low pH), while the cathodic end sits in basic solution (high pH). Compounds of equal isoelectric points are "focused" into sharp segments and remain in their specific zone, which allows for their distinct detection.[29]

APPLICATIONS

Capillary electrophoresis may be used for the simultaneous determination of the ions NH_4^{+}, Na^{+}, K^{+}, Mg^{2+} and Ca^{2+} in saliva.

One of the main application of CE in forensic science is the development of methods for amplification and detection of DNA fragments using (PCR) which has to lead to rapid and dramatic advances in DNA typing in forensic. DNA separations are carried out using thin CE, 50-mm, fused silica capillaries filled with a sieving buffer. These capillaries have excellent capabilities to dissipate heat, permitting much higher electric field strengths to be used than slab gel electrophoresis. Therefore separations in capillaries are rapid and efficient. Additionally, the capillaries can be easily refilled and changed for efficient and automated injections. Detection occurs via fluorescence through a window etched in the capillary. Both single-capillary and capillary-array instruments are available with array systems capable of running 16 or more samples simultaneously for increased throughput.

METHODS

1. Pre-capillary enzyme assays

In pre-capillary enzyme assays, incubation reaction and sample analysis are separate. The enzymatic reaction is initiated by mixing substrate, enzyme and cofactor, and off-line incubated for a specific time. After the incubation reaction is stopped, the reaction mixture is injected into the CE system for subsequent separation and detection. This method is easy to realize because enzymatic reaction is easy to carry out outside the CE system and there is no need to specifically modify separation capillary. And incubation reaction and sample analysis can be performed without mutual interference under respectively optimized conditions. Therefore, this method has been applied to enzyme inhibitor screening, determination of enzyme activity and kinetics, and drug metabolism studies for many years.

Iqbal et al. developed a pre-capillary enzyme assay for characterization and inhibition study of bovine carbonic anhydrase (CA) II. It was the first time to carry out CA enzyme assay by CE. The enzyme and substrate with or without inhibitors were added in a vial. After incubation

at 37 °C for 10 min, the reaction was terminated by freezing the reaction mixture. The reaction mixture was injected into the capillary by applying 0.5 psi for 5 s, followed by the application of 15 kV voltages for separation of substrate and product. The developed method was used to determine the Michaelis-Menten kinetics of CA and inhibition constant of furosemide, a standard inhibitor of CA. The result showed it was a fast and efficient pre-capillary CA inhibitor screening method.[30] Iqbal's group also applied a similar pre-capillary enzyme assay for the characterization and inhibition study of α-glucosidase. The results obtained with the established method were in excellent agreement with data from other literatures.[31] Zhang et al. combined high performance purification of HPLC with enzyme assay of CE to screen inhibitors of mammalian target of rapamycin from natural product extracts. This method facilitated in finding bioactive components among minor constituents of natural extracts.[32]

LIMITATIONS

There are some limitations to the pre-capillary mode. Firstly, the enzymatic reaction which is very fast must be terminated by adding quenching reagents or changing the reaction conditions before analysis by CE system. Secondly, although only nanoliter-scale sample is consumed by CE, the pre-capillary enzyme assay requires a large number of reactants to initiate the reaction, which would cause the waste of reagents especially for those expensive enzymes. Thirdly, enzymatic reaction and analysis by CE are separate in the pre-capillary mode which increases complexity of operation and is also time consuming.

2. In-capillary enzyme assays

In in-capillary enzyme assays, the capillary can be used not only as a separation channel but also as a microreactor. Injection of reactants, mixture, enzyme reaction, separation and detection of analytes can be integrated into a single capillary, which improves the analysis efficiency. The in-capillary enzyme assay realizes automatic operation, short analysis time, low consumption of sample and low cost.[33] It can be divided into two categories: electrophoretically mediated microanalysis (EMMA) and immobilized enzyme microreactor (IMER).

2.1. EMMA

Bao and Regnier[34] firstly reported the in-capillary enzyme assay known as electrophoretically mediated microanalysis (EMMA), in which the reactants were mixed and the enzymatic reaction was triggered by utilizing the difference of electrophoretic mobility of each reactant under electric field. EMMA was generally divided into two major modes according to the pattern of sampling and mixing: the continuous engagement EMMA (long contact mode) and transient engagement EMMA (plug-plug or short contact mode).

In the long contact mode, the capillary is initially completely filled with either substrate or enzyme and the second reactant is introduced as a plug (zonal sample introduction)[34] or in continuous flow (moving boundary sample introduction).[35] The product was continuously produced after applying the voltage which brings the electrophoretic mixing of enzyme and substrate.

In the plug-plug mode, plugs of enzyme and substrate are consecutively introduced into the capillary. The enzymatic reaction is initiated by the application of voltage, because the difference of their electrophoretic mobility under electric field brings interpenetration among zones. It has been the most commonly used EMMA mode because of less consumption of reactants compared with long contact mode. However, in classical plug–plug mode, the buffer is required to be suitable for enzymatic reaction and separation, which limits its application when the separation buffer is incompatible with the enzymatic reaction buffer. To solve this problem, additional plugs of incubation buffer were injected between reactants and running buffer. This approach is termed as “partial filling mode”, as shown in fig.2A[36]

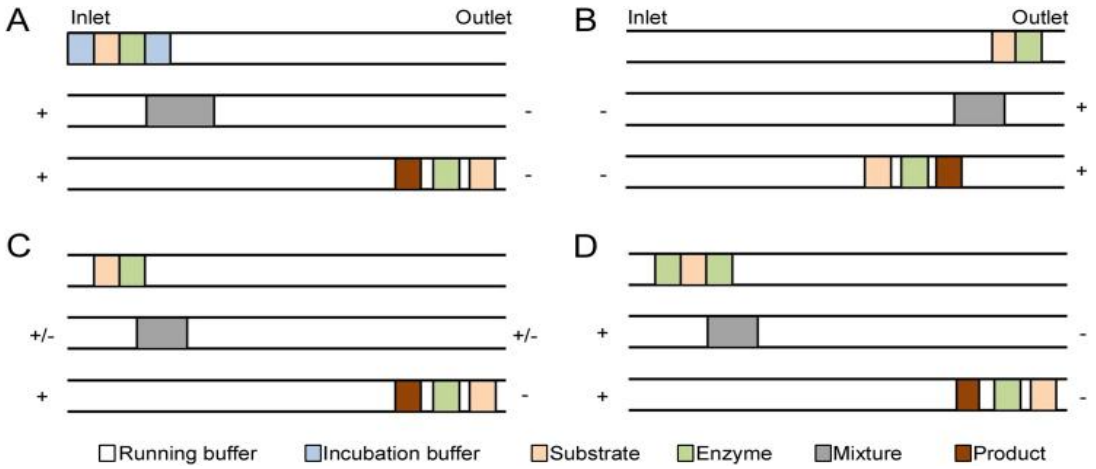

Fig.2: EMMA combined with partial filling mode (A), short-end injection (B), rapid polarity switching technology (C), and sandwich mode (D).[37]

As shown in fig.3, Tang et al. integrated EMMA with sandwich mode, partial filling, and rapid polarity switching technique to screen tyrosinase inhibitors from TCM.[37] Han and Chen developed a cathepsin B inhibitor screening method that integrated longitudinal diffusion and TDLFP to efficiently mix reactants. Twelve potential compounds were evaluated and dauricine showed inhibitory potential for cathepsin B. This work provides a new approach to screen cathepsin B inhibitors.[38]

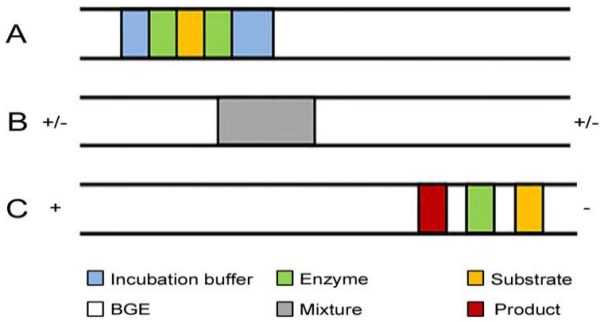

Fig.3: Schematic overview of EMMA. (A) Sequential injection of the incubation buffer, tyrosinase, L-DOPA, tyrosinase, incubation buffer, BGE. (B) Mixing by applying a voltage switch sequence. (C) Mixture separation and product detection.

Applications

Besides enzyme inhibitor screening, EMMA can also be used for the determination of enzyme activity and stereospecificity of enzymes, the understanding of enzyme-mediated metabolic reactions and other determination which is related to enzymatic reaction.

All recent studies related to EMMA are summarized in table.1

Enzyme	CE mode	Injection and mixing mode	Cartridge temperature	Detection
Thrombin	CZE	Short end injection + partial filling mode rapid polarity switching	25	uv
Human protein kinase	CZE	TDLFP	30	UV
Glycogen synthase kinase 3-β	CZE	TDLFP	30	UV
Protein kinase	CZE	Short end injection	25	LIF
Aromatase	CZE	Partial filling mode	25	UV
Tyrosinase	CZE	Sandwich mode + partial filling mode + rapid polarity switching	30	UV
Cathepsin B	CZE	Longitudinal diffusion + TDLFP	30	UV

2.2. IMER

IMERs mean that the enzyme is immobilized in the first part of the capillary for enzymatic reaction, while the remaining part of the capillary is used for separation of analytes. Compared with the enzymatic reaction in solution, the immobilized enzyme has some advantages. First, the immobilized enzyme is reusable which avoids the waste of enzyme and reduces the experimental cost. Second, the stability of the immobilized enzyme may be improved. Third, the efficiency of enzymatic reaction can be increased. Fourth, because the enzyme is immobilized, adsorption of the enzyme on the inner wall of capillary can be avoided, which is helpful to increase reproducibility of separation. But the IMERs are not suitable for enzymatic reaction system in which the incubation buffer and separation buffer are different. In addition, preparation of IMERs is complicated and different immobilization methods have their own defects. Generally, there are three methods for enzyme immobilization: physical adsorption, covalent attachment and encapsulation.[39] Physical adsorption has advantages of low cost, easy operation, mild immobilization conditions and moderate harm for the enzyme. And the capillary can be reused by rinsing with NaCl, HCl and NaOH. Enzymes immobilized by covalent attachment have good stability, but the activity of enzymes may decrease because of chemical bonding. Encapsulation brings large enzyme adsorption and keeps high enzyme activity[40,41] but the operation is complicated and hard.

IMERs are a good tool to study enzyme assays. Min et al. established an efficient trypsin IMER at the inlet of the capillary by glutaraldehyde cross-linking technology. The method was successfully used to study the enzyme kinetics of trypsin and on-line screen trypsin inhibitors from 19 kinds of natural extracts.[42]

Zhao and Chen fabricated a simple and effective neuraminidase IMER by glutaraldehyde cross-linking technology for screening neuraminidase inhibitors which are effective for influenza type A and B viruses in humans, avians and animals. With short-end injection mode, the substrate and product were separated by CE within 2 min. As shown in fig.4, six compounds were found as potent inhibitors.[43]

APPLICATIONS

Enzymatic reactions in IMERs are used not only for enzyme inhibitor screening but also for other applications. An IMER using graphene oxide as support was developed based on layer-by-layer electrostatic assembly by Yin et al. Angiotensin and bovine serum

albumin (BSA) were successfully digested by the CE-based IMER. Liu et al. fabricated a novel CE-based IMER by particle-packing technique. The IMER was accomplished by utilizing large-pore beads as the enzyme supports and perfusive silica single particles as the frits. The IMER was successfully applied for accurate analysis of trypsin inhibition and on-line digestion of standard proteins (myoglobin and BSA).[44]

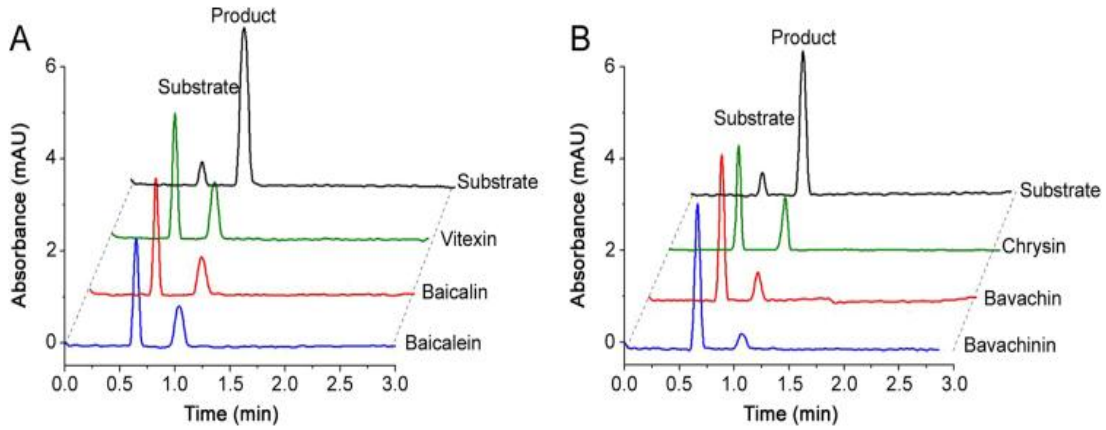

Fig.4: Overlaid electropherograms for neuraminidase inhibitor screening by on-line immobilized enzyme assay detected at 320 nm with reference wavelength of 260 nm (A) and at 320 nm without reference wavelength (B).

All recent studies about IMER mentioned above are summarized in table.2:

Enzyme	CE mode	Immobilization technique	Cartridge temperature	detection
Trypsin	CZE	Glutaraldehyde cross linking technology	37	UV
Tyrosinase	CZE	Ionic binding technique with cationic polyelectrolyte hexadimethrine bromide	37	UV
Glucose-6-phosphate dehydrogenase	CZE	Layer-by-layer electrostatic assembly	22	UV
Cytochrome P450	CZE	Magnetic SiMAG-carboxyl microparticles as a support	30	UV
Adenosine deaminase and xanthine oxidase	CZE	Gold nanoparticles as a support		UV

CONCLUSION

As can be concluded from the literature collected in the present review article, over past years huge advances have been made in the CE-based enzyme inhibitor screening. In pre-capillary assays, the enzymatic reaction and separation conditions can be optimized separately, which is universal for most enzymes. In the mode of in-capillary assays, the sampling, mixture, reaction, separation and detection are incorporated into one single capillary, which simplifies the operation, reduces reagent consumption, shortens analysis time, facilitates the automatization and miniaturization of enzymatic assays and realizes high-throughput enzyme inhibitor screening. With the speedy development of CE-based enzyme inhibitor screening, the CE will be more widely applied to enzyme assays for study of enzyme kinetics, understanding of enzyme-mediated metabolic reactions, study of rapid peptide mapping in proteomic and so on.

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