Monitoring of Enzymatic Reactions using Capillary Electrophoresis with Conductivity Detection

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He who knows others is learned; he who knows himself is wise.

- Lao-Tze

Summary

Capillary electrophoresis combined with contactless conductivity detection allows to separate and detect the ionic species, which are neither UV absorbing nor fluorescent. This thesis focuses on the applications of this method on enzymatic reactions in different analytical tasks.

First, the non-ionic species ethanol, glucose, ethyl acetate and ethyl butyrate were made accessible for analysis by capillary electrophoresis via charged products or byproducts obtained in enzymatic conversions using hexokinase, glucose oxidase, alcohol dehydrogenase and esterase. The conversion of glucose and that of ethylacetate were also successfully demonstrated on a microchip-device. Quantification of ethyl acetate, was found possible with a detection limit of approximately 7 μM. Then the model of urea catalyzed by urease was chosen for the study of the enzymatic mechanism, the effect of substrate concentration and pH value as well as the Michaelis-Menten constant. The determination of urea in human blood as clinical application of this enzymatic reaction was tested. 10 human blood samples were collected from a hospital and analyzed. The results were comparable with the established methods. The method was then extended to proteome analysis; identification of proteins is generally achieved through proteolytic digestion with enzymes such as pepsin and trypsin. Protein digestion with pepsin and trypsin was successfully monitored by capillary electrophoresis. Minigastrin I, myoglobin, cytochrome C, human serum albumin and bovine serum album were the model proteins digested by pepsin, cytochrome C and myoglobin were the model proteins digested by trypsin. Electrophoretically mediated micro-analysis (EMMA) technique is employed for the tryptic digestion of cytochrome C and apomyoglobin.

Finally, the enantioselective hydrolysis of esters of amino acids with lipase was monitored. Porcine pancreas lipase was found to have a better efficiency on hydrolysis and enantioselectivity than wheat germ lipase. L-threonine methyl ester demonstrated stronger enantioselectivity than L-serine methyl ester. Acetylcholinesterase inhibitors can be used as drug against Alzheimer disease or nerve agents. Three compounds, namely galantamine, paraoxon and Huperzine-A, were the model inhibitors to study the behavior and kinetics of the inhibitors. The values of IC_{50} were obtained through graphical plot. Their dependence on the time course was monitored and graphically illustrated.

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I. Introduction to CE-C^{4}D

1. Historical background

Electrophoresis is the movement of charged ions in a fluid or gel under the influence of an electric field, it is based on the principle that charged molecules will migrate toward the opposite pole and separate from each other based on physical characteristics. Electrophoresis was first introduced as an analytical technique by Arne Tiselius in 1930. When a layer of pure fluid without particles was placed over a quantity of the same fluid containing colloidal particles; he discovered the boundary between two layers of fluid was visible and moved at the speed of electrophoresis of the particles. From this observation, he originated the moving-boundary method, which was used to separate serum proteins in solution [1]. Later this method became also known as zone electrophoresis. Its separation mechanism is based on differences in the charge to mass ratio of the analytes. In the 1940's and 1950's, two other methods were also developed known as isoelectric focusing and isotachophoresis. Isoelectric focusing is a separation technique based on the different isoelectric points (pI) of the sample components, the pI is the point at which the sample has an overall net charge of zero [2]. Isotachophoresis uses a discontinuous electrical field to create sharp boundaries between the sample constituents, it is based on differences of migration velocities of the analytes [3].

These electrophoretic separation methods can also be described by the different matrices used, which give different advantages over different types of samples. Papers were used in the first electrophoretic techniques because of its simplicity; however gels were more often used. Smithies used starch gel as a supporting medium for electrophoresis in 1955 [4]. The method was developed for separation in a whole process of protein analysis using color forming reagents. The gel formed a solid, yet porous matrix. By placing the molecules in wells in the gel and applying an electric current, the molecules will move through the matrix at different rates, usually determined by their mass to charge ratio. It was also observed that the relative values of starch concentration and ionic strength were the fundamental criteria for the separation. The separation of proteins was largely depending on the effective pore size of the gels. The resolving power of the starch gels was found much superior in such cases in comparison with paper filter.

Buffer-filled capillaries instead of gel medium planar were first used by Jorgenson and Lukacs in the early 1980's [5, 6]. 75 μm open-tubular glass capillaries coupled with on-column fluorescence detector was first developed to perform zone electrophoresis, with such small inside diameter of these capillaries, the heat generated by the application of 30 kV high voltages was efficiently dissipated. Jorgensen also described the relationship between the operational parameters and separation quality, from which he declared the potential of high performance capillary electrophoresis (HPCE) as an analytical technique.

2. Basic principles of CE

1) Basic concepts

In a capillary electrophoresis system, the ends of a capillary are placed in buffer reservoirs, each containing a positive (anode) or negative (cathode) platinum electrode, the electrodes are connected to a high voltage power supply capable of delivering up to 30 kV. The capillary is filled with a buffer identical to that in the reservoirs. The sample is introduced into the capillary by replacing one of the buffer reservoirs with sample reservoirs containing ions (usually at the anode end); the sample can be injected either electrokinetically or hydraulically. When the electric field is applied across the electrodes, analytes ions of different charge will move through the solution towards the electrode of opposite charge, where they are visualized and the signal is recorded on the data acquisition system. A schematic diagram of capillary electrophoresis is illustrated in Figure 1. The basic components required for CE instrumentation includes a high-voltage power supply, a capillary, detector, data acquisition system, vials for buffer and sample.

2) Electrophoretic mobility

The separation of compounds by capillary electrophoresis is dependent on differential migration velocity of ions or solutes in an applied electric field. The migration velocity of an ion can be expressed as:

$$\upsilon = \mu_e E \qquad \text{(I.1)}$$

where υ is ion migration velocity ($m\ s^{-1}$), μ_e is electrophoretic mobility ($m^2V^{-1}s^{-1}$) and E is electric field strength (Vm^{-1}).

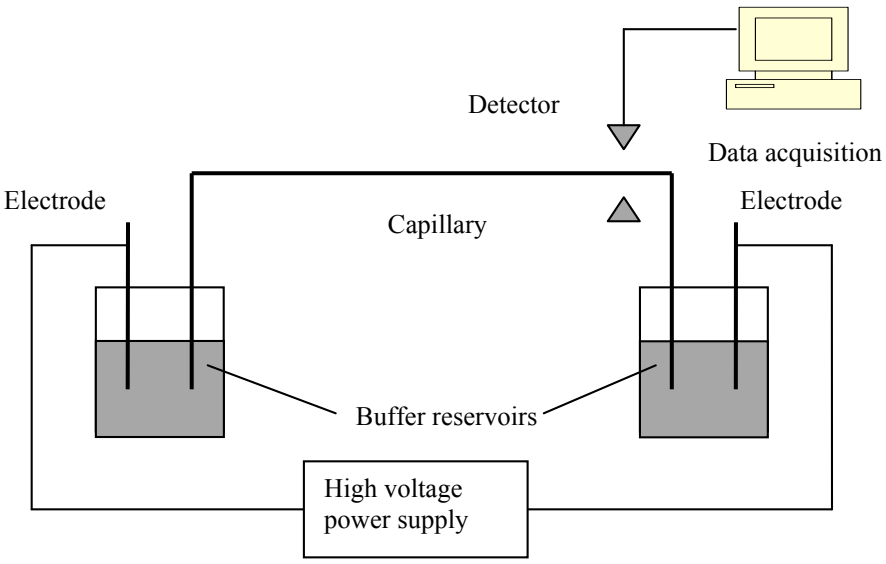

Fig. 1 A schematic representation of the main components of capillary electrophoresis

The electric field strength is a function of the applied voltage divided by the total capillary length. Electrophoretic mobility is a factor that indicates how fast a given ion or solute may move through a buffer solution. It is an expression of the balance of forces acting on each individual ion, the electrical force are in favor of motion and the frictional force are against motion, during electrophoresis, these forces reach a steady state. For a given ion under a given set of conditions, electrophoretic mobility is a constant and can be described as the following equation:

$$\mu_e = q/6\pi\eta r \qquad \text{(I.2)}$$

where q is the charge on the ion, η is the solution viscosity and r is the ion radius. The charge on the (q) can be affected by pH changes in the case of weak acids or bases. The ion radius (r) can be affected by counter-ion present or by any complexing agents used. Differences in the charge-to-size ratio of analyte ions, higher charge and smaller size confer greater mobility, whereas large minimally charged species have low mobilities.

Electrophoretic mobility is a characteristic property for any given ion or solute and decides migration velocities. Different ions and solutes have different electrophoretic mobilities so they have different migration velocities at the same electric field strength. The differences in electrophoretic mobility make it possible to separate

mixtures of different ions and solutes. However, the mobility is also dependent on the temperature and electroosmotic flow (EOF) of buffer solution.

3) Electroosmotic flow (EOF)

In a typical system, the electroosmotic flow is caused by an uncoated fused-silica capillary tube. The surface of the inside of the tube has ionisable silanol groups, which give the capillary wall a negative charge. The negatively charged capillary wall attracts positively charged ions from the buffer solution, generating an electrical double layer and a potential difference (zeta potential) close to the capillary wall. An electrical double layer includes a rigid layer of adsorbed ions and a diffuse layer. The zeta potential is the potential at any given point in the double layer and decreases exponentially with increasing distance from the capillary wall. Electroosmotic flow results from cations in the diffuse layer migrating towards the cathode, carrying the bulk solution with them. An electroosmotic flow velocity is proportional to the zeta potential and can be described as

$$v_{EOF} = (\varepsilon\zeta/\eta)\, E \qquad \text{(I.3)}$$

where ε is the dielectric constant of the buffer, ζ is the zeta potential, η is the viscosity of the buffer, E is the applied electric field. The dielectric constant and viscosity of the buffer and the size of the zeta potential are the main factors affecting EOF mobility.

Electroosmotic flow is important in CE separation. The pH values may be affecting the separation. At a high pH, the rapid EOF may result in the elution of the solute before separation taking place; at a low pH, the negatively charged wall can cause absorption of cationic solutes through coulombic interactions. Therefore, optimized EOF and solute mobility properties are critical to successful separations. The most frequently used methods to control electroosmotic flow are:

- Change electric field: increasing the electric field generates an increase in EOF
- Modify buffer pH: EOF decreased at low pH and increased at high pH
- Modify ionic strength of buffer concentration: zeta potential is decreased and EOF is lowered when the ionic strength of buffer is reduced.
- Temperature changes: high temperature leads to low buffer viscosity and high EOF.

- Adding organic modifier: change zeta potential and viscosity, usually decreases EOF.
- Covalent coating: chemical bonding to capillary wall.

4) Classification of electrophoresis modes

Ever since separation techniques have evolved. Use of even narrower bore fused-silica capillaries of typically 25-75 μm inner diameters for separation allowed more efficient dissipation of the heat. Sample introduction can be accomplished by immersing the end of the capillary into a sample vial and applying pressure, vacuum or voltage. Based on the types of capillary and electrolytes used, capillary electrophoresis can be briefly classified as follows:

- Capillary zone electrophoresis (CZE) is the simplest form of CE. The separation is realized based on the size of the species to charge ratio in the interior of a small capillary filled with an electrolyte. Homogeneity of buffer solution and constant field strength throughout the capillary are key to this technique.
- Capillary gel electrophoresis (CGE) is the adaptation of traditional gel electrophoresis into capillary. Polymers are used in solutions to create a molecular sieve known as replaceable physical gel, which allows analytes to be resolved by size.
- Capillary isoelectric focusing (CIEF) allows separation of amphoteric molecules in a pH gradient generated between the cathode and anode. A solute migrates to a point where its net charge is zero. At the solutes isoelectric point (pI), migration stops and the sample is focused into a tight zone. The zone will be mobilized past the detector by either pressure or chemical means when a solute focuses at its pI.
- Isotachophoresis (ITP) is a separation mode based on the migration of sample components between leading and terminating electrolytes, which stack into sharp, focused zones according to their mobilities.
- Micellar electrokinetic capillary chromatograph (MECC) is a mode of electrokinetic chromatography. Its principle is based on the differential partition between the micelle and the solvent. The micelle is formed by adding surfactants to the buffer solution. Both charged and neutral solutes can be separated involving either stationary or mobile micelles.

- Micro emulsion electrokinetic chromatography (MEEKC) employs moving oil droplets in buffer. The microemulsion droplets are formed by sonicating immicible heptane or octane with water. SDS is added to stabilize the emulsion. The separation of both aqueous and water-insoluble compounds is possible.
- Capillary electrochromatography (CEC) is a hybrid of CZE and HPLC. This technique uses an electric field rather than hydraulic pressure to propel the mobile phase through a packed bed. High efficiencies can be achieved by using small-diameter packing. It exploits the combined advantages of both capillary electrophoresis (high efficiencies) and HPLC (mobile and stationary phase selectivity).

5) Capillary electrophoresis on microfabricated device

In 1987, Manz et al. first integrated a liquid chromatographic chip containing a capillary column with an electrochemical detector, the work was first published in 1990 [7]. Since then, silicon and glass microstructures [8-10] have been used to integrate with capillary electrophoresis channels. When Soper et al. [11] integrated a miniaturized solid phase sequencing reactor with a capillary electrophoresis apparatus, this technology has become an attractive alternative for DNA sequencing, which previously was dominated by capillary gel electrophoresis. Lately, considerable interest has been focused on the area of micro total analysis systems, especially CE microchips.

In this microfabrication technology, a capillary column is replaced with a microchip with much smaller channel length and dimensions, which offers dramatic decrease in analysis times and reagent consumption. A typical microchip used for electrophoretic separations can be illustrated as in Figure 2.

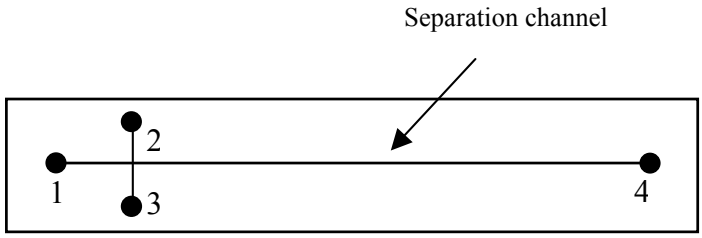

Fig. 2 A schematic layout of a typical microchip
1. Electrolyte inlet, 2. Sample inlet, 3. Sample outlet, 4. Outlet

Photolithographic and wet etching techniques are mainly used to produce these planar devices with micrometer dimensions, which are hence named as microchips. Non-insulating substrates glass, silicon and polymer are used. A typical fabrication process involves metal film deposition, photolithography and etching of a CE channel with desired dimensions on a bottom plate. A coverlid was bonded on top of that. Plastic vials used as fluids reservoirs are glued on to the chips. They are connected to the microchannel through holes drilled into the cover plate. Electrodes mainly made from gold, platinum or carbon are placed into these reservoirs and connected to a high voltage power supply via relays.

3. Detection in capillary and microchip electrophoresis

1) UV-Vis and fluorescence detection

Among the techniques listed above, detection methods rely mainly on UV-absorption and fluorescence detectors. Optical detection methods have proved to be valuable techniques; however, they have also limitations. Most components of samples separated with electrophoresis are not directly UV absorbent or fluorescent. Methods have been also developed to help visualize and quantify components of such sample mixtures. The short detection path length in narrow-bore capillaries results often in unfavorable detection limits for absorbance-based detection methods. Despite the fact that the adaptation of optical detectors into CE is fairly easy, the detection hardware involves a few elements such as a light source, monochromator, optical detectors and focusing optics, which are often expensive. Most of the detection methods applied to conventional CE are adopted in the microchip format, sensitive methods such as LIF are preferred, due to the significantly reduced sample volume detected [12]. However, most compounds do not naturally exhibit fluorescence, the instrument is large and expensive, and these facts limit the use of LIF.

2) Electrochemical detection

The call for alternative and robust detection methods for CE was then followed, during the 1980s and 1990s, electrochemical detection has gained acceptance as an analytical method. The principle methods of electrochemical detection for CE can be distinguished as amperometric, potentiometric and conductometric. Electrochemical detection became powerful detectors for microchip CE too, because of its high sensitivity, simplicity, low cost and eases of miniaturization.

Amperometric detection relies on oxidation or reduction of the analyte species on a working electrode. Amperometric detection in capillary electrophoresis was first introduced by Wallingford and Ewing in 1987 [13]. The ground electrode for the applied high voltage was positioned at a porous joint which was formed by fracturing a fused-silica capillary 5 cm from the detector end. A carbon fiber electrode as working electrode inserted into the end of the capillary. Together with a reference electrode and an auxiliary electrode, the working electrode was connected to a three-electrode potentiostat. A potential was applied across a supporting electrolyte between the working electrode and the reference electrode effecting solute oxidation or reduction. A potential difference was created between the working and reference electrodes. The current flowing through the working electrode is proportional to the number of electron transfers taking place and therefore to solute concentrations. Amperometric detection was also coupled to microchip electrophoresis. Schwarz et al. from Hauser's group analyzed biochemicals such as neurotransmitters [14, 15], amino acids [16] by employing amperometric detection on microchip. It is a detection method which offers more selectivity, however its applications are limited to electro-active species.

Potentiometric detection is to measure a potential developed on an ion-selective electrode or membrane in contact with an analyte ion. Detectability increases with lipophilicity of the ion, the larger the ion and the lower its charge, the greater the response of the electrode. Potentiometric method was first introduced in 1991 by Haber et al. [17] to detect cations with ion-selective microelectrodes (ISME). Nann and Simon [18, 19] developed this method further. Using ion-selective microelectrodes (ISME), they demonstrated sensitive determination of inorganic and

organic cations. Since then, the majority of work on potentiometric detection in CE has focused on the use of ion-selective electrodes in the form of ion-selective microelectrodes (ISME) or coated wire ion-selective electrodes. The ion-selective electrodes are difficult to construct and tend to be too fragile to be applicable to routine CE analysis. So far the adaptation of potentiometric detection on the microchip has not been reported.

Conductometric detection exploits the ability of ions to conduct charge in solution. Upon applying a voltage, the current produced between two electrodes is measured. As conductimetric detection is the method exclusively used for my work, comprehensive details are discussed in the next chapter.

4. The basic principle of conductivity detection

Conductivity detection can be considered as a universal detection method, it is possible to carry out a direct or an indirect measurement of the analyte's response signal. It is based on the change in bulk solution conductivity between two electrodes when an analyte band passes through the electrode gap. Any kind of molecules can be detected when it causes a change in the conductivity between the two electrodes. The response is proportional to the concentrations of analyte ions. Most analytes are determined in background electrolyte (BGE) solutions, in which they are fully ionized, and the detector response is essentially due to the difference in molar conductivity between the analyte ion and the ion of the same charge of the BGE. Both positive and negative going peaks from the baseline are therefore possible. Conductivity detection generally works by applying an AC voltage between two electrodes to produce a current. This current can be measured and yields the resistance and conductivity according to Ohm's law respectively. It is preferable to use an alternating current to a direct current (DC), the latter may cause electrochemical reaction at the electrode surfaces and polarization of the electrodes, and it may interfere with the detection electronics.

In conductivity detection, the solution resistance R (Ω) is calculated from its conductance G (S), defined as $G = 1/R$. G can also be denoted as $G = \kappa / k_{cell}$, where κ is the ratio of the specific conductance ($S \cdot cm^{-1}$) and k_{cell} the cell constant (cm^{-1}), which indicates the relationship between the surface area (A) and the distance

between the electrodes (l) in a detector (k_{cell} = l/A). The ratio of the specific conductance can be described as

$$\kappa = \Sigma\lambda_i \cdot c_i \quad (I.4)$$

where c_i indicates the molar concentration of the ionic species i in solution. The limiting equivalent conductivity λ results from μ the absolute mobility values for the respective ions by multiplication with the Faraday constant F.

$$\lambda_i = \mu_i F \quad (I.5)$$

Conductivity detection has been an attractive method for CE analysis. It requires no additional chemical properties for detection. The detector can be miniaturized to scale down with narrow bore capillaries and to the microchip format. Conductivity detection can be carried out either as contact or contactless mode. Although the integration of conductivity detection and CE was a challenge for mechanical grounds for a while, after minimizing the influence of capacitors in series or parallel with solution resistance during conductivity measurements, both contact conductivity detection and contactless conductivity detection were developed and made available commercially. However the commercial contact conductivity detector received a setback, while contactless conductivity detection enjoyed popular attention. A high-frequency contactless conductivity detector was first introduced by Gaš et al. for isotachophoresis (ITP) in the late 1970s [20]. In 1998, the contactless conductivity detector was introduced into CE by two independent research groups, Zemann et al. [21] and Fracassi da Silva and Do Lago [22]. In their approach, two cylindrical electrodes were placed around separation capillary. When applying an AC voltage to the input electrode, the conductivity of the liquid inside the capillary can be measured using capacitive coupling. Zemann [23] and Kuban et al. [24] attributed the popularity of contactless conductivity detection in capillary electrophoresis to the improvements made in the design of the detector for easier handling and higher sensitivity. Most of reported capillaries used were fused-silica with an inner diameter of 50 or 75 μm. Conductivity methods require only very small sample volumes, often in the microliter range, coupled with the low detection limits allowing analysis on subpicogram amounts of analyte. It offers an excellent selectivity in complex samples because fewer electroactive interferents are often encountered than spectroscopic interferents.

Contactless conductivity detection in microfluidics was also developed. In 2002, in the group of M. J. Vellekoop from Netherland, Berthold et al. [25] developed a detector consisting of four aluminum electrodes that were physically isolated and electrically insulated from separation channel by a 30 nm thick layer of silicon carbide. Laugere et al. [26-28] and Bastemeijer et al. [29] have continued to discuss the advantages of four – electrode conductivity detection over two-electrode measurements. Two of these four electrodes were replaced with platinum electrodes by Lichtenberg et al. [30]. A simpler and movable detector was developed by Tanyanyiwa in the group of Hauser [31], the detection electrodes were placed in the chip holder instead of on top or integrated into the microdevice. The microfabrication process was then simplified and the cost of device was reduced.

While the instrumental device has been improved, the range of applications has also been widened**.** The applications of CE with contactless conductivity detection and microchip include the detection and determination of amino acids [32-35], amine [36, 37], vitamin C and preservatives [38], glutamic acid [39], inorganic ions [40-42], metal ions [43-45] and drugs [46-48].

5. The principle of C^{4}D

Capacitively coupled contactless conductivity detection (C^{4}D) as a quantification method for capillary electrophoresis was introduced in 1998. It was a configuration made by the introduction of axial electrodes. Two metal tubular electrodes were positioned side by side along the capillary axes. The usual axial arrangement of two tubular electrodes used on a conventional capillary is illustrated in Figure 3. In capacitively coupled contactless conductivity detection method, the measurement is not fundamentally different to conventional conductivity measurements. An AC-voltage is applied to one of the galvanically isolated electrodes and the resulting AC-current is measured at the second electrode. The currents are only limited by the concentration and mobility of the ionic charge carriers in the solution. But the insulation layer present in the contactless arrangement the capacitances are much lower than the double-layer capacitances of non-isolated electrodes, so the working frequency has to be significantly higher. A simplified circuit diagram can be given as Figure 4.

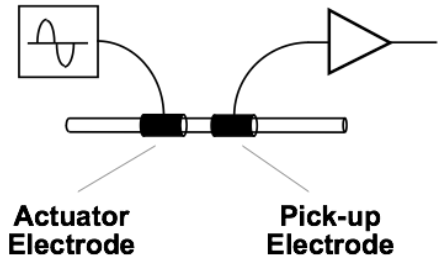

Fig.3 Axial arrangement

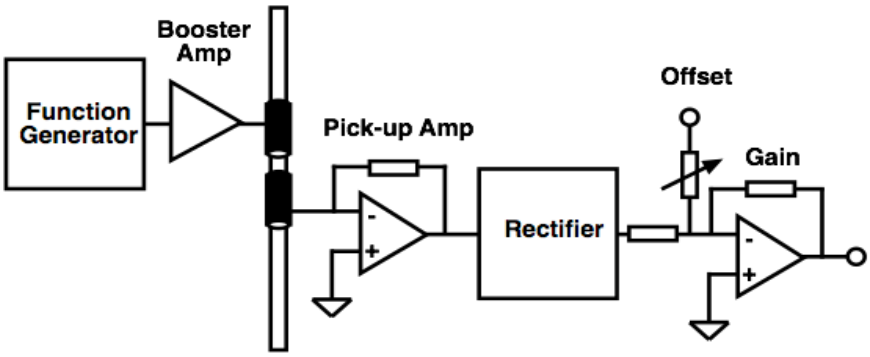

Fig. 4 Circuit diagram

A possible capacitively coupled contactless conductivity detection device can be demonstrated as in Figure 5.

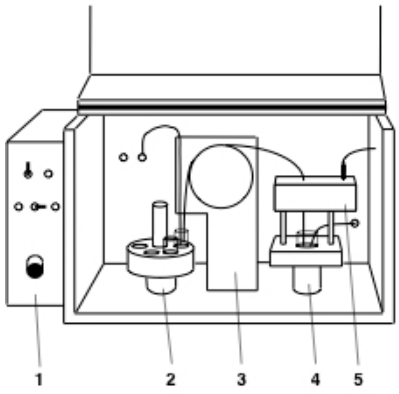

Fig. 5 Schematic drawing of the portable capillary electrophoresis system
1. Control electronics, 2. Sample tray, 3. Capillary holder, 4. Vial holder, 5. Detector cell

Figure 6 illustrates a possible electrode arrangement for measurements of the capacitively coupled contactless conductivity detection conductivity on a microfluidic chip. The electrodes consist of 2 mm wide strips of copper foil, placed across the microchannel and leaving a 1 mm wide gap in between for the shielding. The electrode may be embossed in the chip, and covered with an insulation layer, or placed either in the holder or on top of the device. Micro device made from glass may be used; the electrodes can be placed on the bottom of two trenches of 1-mm width milled across the top of the separation channel by using a high–frequency cutting wheel. A PMMA chip can also be used; they are mounted on the holder. The holder bears a Faraday shield to separate the excitation and pick-up electrodes. During the detection, the sinusoidal excitation signal is created by a function generator and boosted by a high-voltage amplification stage. The cell current is then converted to an AC voltage by a pick-up amplifier. The AC voltage is rectified and amplified.

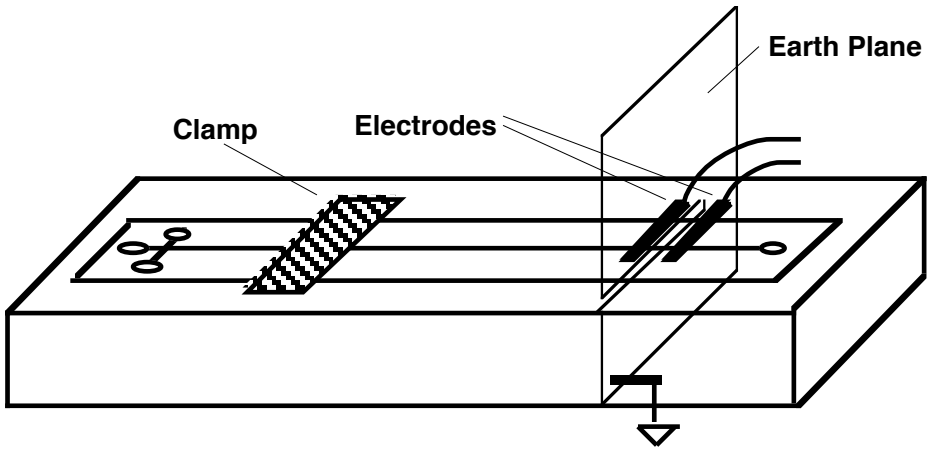

Fig. 6 Microchip

Microchip CE coupled with conductivity detection holds considerable analytical promise for applications such as determination of peptides [49], quantitative analysis of alcoholic and non-alcoholic beverages [50], and organic and inorganic acids expected in wine [51].

A review from Zemann [52] on capacitively coupled contactless conductivity detection for capillary electrophoresis is available. Another review from Kuban et al. [53] focusing on the development in capacitively coupled contactless conductivity detection appeared in 2008.

II. Introduction to Enzymology

1. Enzymes

1.1 Definition

Enzymes are giant biomolecules which catalyze biochemical reactions. Each enzyme catalyzes a single chemical reaction on a particular chemical substrate, converting it into a different molecule called the product. Enzymes are usually evolved in nature to speed up and co-ordinate the multitude of chemical reactions necessary to develop and maintain life. Chemical reactions are too slow to be effective under the conditions existing in normal living systems, in comparison enzymes achieve up to 10^7 fold faster reaction rate.

Most enzymes are proteins; they exist in living cells, in the cytoplasma of the cells, in the biomembrane, in the body cavities, such as the digestion enzymes in intestine. The first pure enzyme form urease was crystallized from Jack beans by James. B. Sumner in 1926 [54].

1.2 Structure and Mechanism

Enzymes are macromolecular proteins; they range from 62 amino acid residues to over 2500 residues. Their molecular weight varies from 5 000 to 5 000 000 Da, with typical values in the range 20 000 – 100 000 Da. The common feature of proteins is that they are polypeptides: their structure is made up of a linear sequence of α-amino acid building blocks joined together by amide linkages. This linear polypeptide chain then folds to give a unique three-dimensional structure. The activities of enzymes are determined by their three-dimensional structure. The part of enzyme tertiary structure that contains the catalytic residues, binds the substrate and then carries out the reaction is known as the active site.

Depending on complementary shape, charge and hydrophilic/hydrophobic characteristics of enzymes and substrates, enzymes are usually specific to reactions they catalyze and the involved substrates in these reactions. To explain the specificity, Emil Fischer suggested in 1894 [55] that this was because both the enzyme and substrate possess specific complementary geometric shapes that fit exactly into one another, it is referred as „the lock and key“ model, see Figure 6.

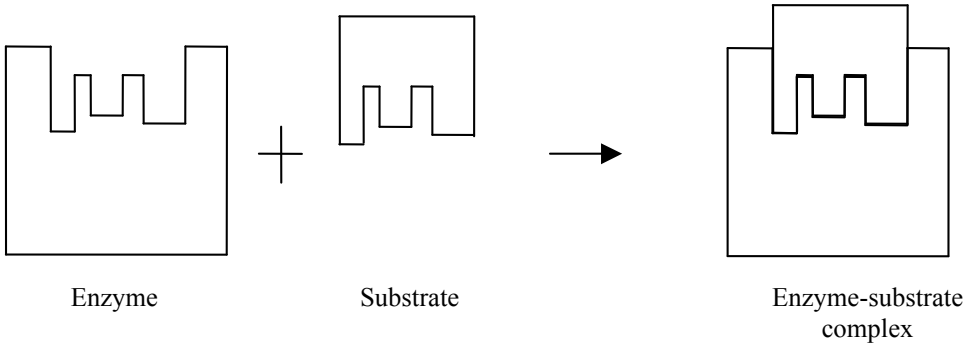

Fig. 6 Lock and key model

The induced fit model is a more popular model, which was introduced by Daniel Koshland in 1958 [56], it is a modification to the lock and key model. He explained: both enzymes and substrates have flexible structures, one protein has a cavity or indentation that another protein perfectly fits in. The interaction of an enzyme with its substrate resembles the fit of a hand in a glove; the amino acid side chains which contain the active site are moulded into the precise positions that enable the enzyme to perform its catalytic function. The final shape and charge is determined when the enzyme is completely bound to the substrate, see Figure 7.

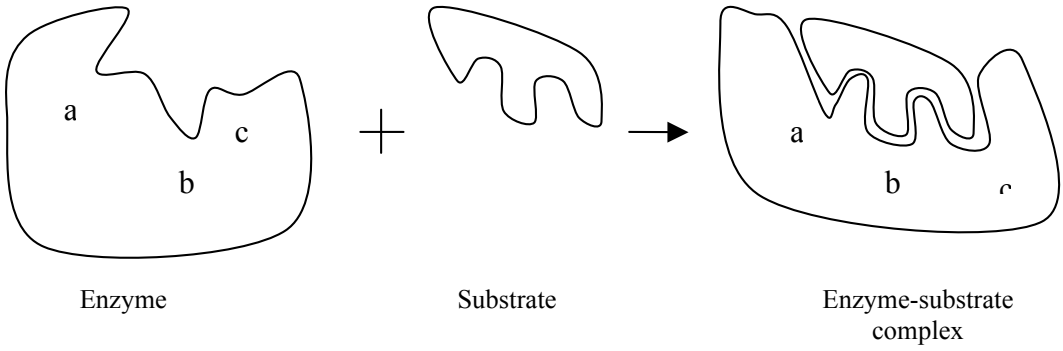

Fig. 7 Induced fit model

1.3 Cofactors and coenzymes

Some enzymatic reactions require additional components to show full activity. These compounds are usually non-protein molecules, either tightly or loosely bound to an enzyme and required for catalysis, they are called cofactor. Cofactor can be either

inorganic such as metal ions or iron-sulfur clusters or organic compounds. Cofactors can be divided into two broad groups: prosthetic groups and coenzymes. A prosthetic group is a non-protein (non- amino acid) component of a conjugated protein that is important in the protein's biological activity, it forms a permanent part of the protein structure. Coenzymes are those molecules which act to transfer chemical groups between enzymes and are released from the enzyme's active site during the reaction. Coenzymes make up a part of the active site. Nicotinamide adenine dinucleotide reduced form (NADH), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), adenosine-5'-triphosphate (ATP) and adenosine diphosphate (ADP) are the most commonly used coenzymes.

1.4 Kinetics

In 1902, a quantitative theory of enzyme kinetics was first proposed by Victor Henri [57], it was to investigate how enzymes bind substrates and turn them into products. Since then, various chemists have developed different kinetic theories, among which, Michaelis –Menten kinetics [58] is most widely used.

1) Reaction rate and enzyme activity

Chemical reactions can be classified as unimolecular, bimolecular and trimolecular according to the molecularity, which defines the number of molecules that are altered in a reaction. A unimolecular reaction is A → P; a bimolecular reaction is A+B → P; a trimolecuar reaction is A+B+C →P. An order of a reaction describes its kinetics that defines how many concentration terms must be multiplied together to get an expression for the rate of reaction. In a first-order reaction, the rate of a reaction is proportional to one concentration; in a second –order reaction, the rate is proportional to the product of two concentrations or to the square of one concentration. Many reactions consist of sequences of unimolecular and bimolecular steps, the molecularity of the complete reaction need not be the same as its order.

The rate ν of a first-order reaction A → P can be described as

$$v = \frac{dp}{dt} = -\frac{da}{dt} = ka = k(a_0 - p) \qquad \text{(II.1)}$$

in which a and p are the concentrations of A and P respectively at any time t, k is a first-order rate constant and a_0 is a constant. As in this equation, rate v is defined in

terms of the appearance of product P or disappearance of reactant A. However, in real experiments, the relative changes in p are much larger than those in a in the early stages of a reaction, it is usually more accurate to measure increases in p than decreases in a.

A first-order reaction is specified as the third equality sign in the equation, because it states that the rate is proportional to the concentration of reactant. The last equality in the equation describes, when the time zero is defined as $a=a_0$, $p=0$ when $t=0$, the values of a and p at any time can be related according to the equation $a + p = a_0$.

Principally, all the chemical reactions are reversible, the reversity must be considered in the rate equation when the reverse reactions are easily observable. The reaction can be described

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} P \qquad \text{(II.2)}$$

a_0-p $\qquad\qquad$ p

While II.1 can be changed to

$$v = \frac{dp}{dt} = k_1(a_0 - p) - k_{-1}p = k_1 a_0 - (k_1 + k_{-1})p \qquad \text{(II.3)}$$

2) Michaelis-Menten-theory

The Michaelis-Menten model for enzyme kinetics was based on the assumption on the steady state of an enzyme-catalysed reaction. The enzymatic reaction involves two steps: reversible formation of the enzyme-substrate (EA) complex and conversion to product.

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EA \xrightarrow{k_2} E + P \qquad \text{(II.4)}$$

e_0-x $\quad a$ $\qquad\qquad$ x $\qquad\qquad$ p

This model assumes: the enzyme binds only a single substrate, there is only one kinetically significant step between the EA complex and product formation, the product formation is irreversible, the enzymatic reaction reaches a situation of steady state in which the concentration of the intermediate species, EA, remains constant.

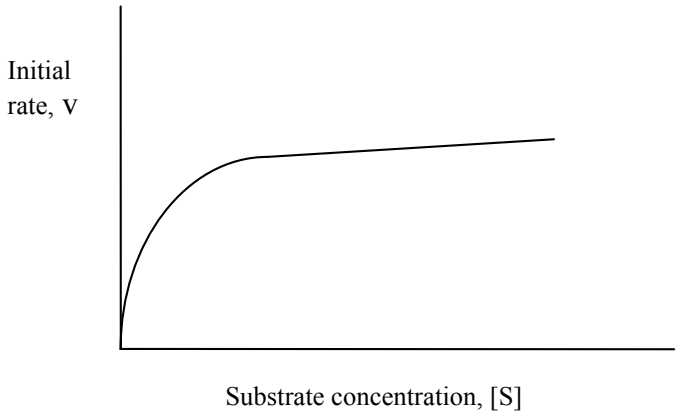

Fig. 8 Michaelis-Menten curve

In equation 8, *e* and *a* represents the instantaneous concentrations of free enzyme and substrate respectively, they are not directly measurable so they are expressed in terms of the initial concentrations e_0 and a_0, using the stoichiometric relationship $e_0 = e + x$ and $a_0 = a + x$.

The rate equation can be obtained as following,

$$\frac{dx}{dt} = k_1(e_0 - x)a - k_{-1}x - k_2x \tag{II.5}$$

when *a* steady state is reached, the $dx/dt = 0$, and $v=k_2x$, thus the Michaelis-Menten equation is obtained as

$$v = \frac{k_2 e_0 a}{\frac{k_{-1} + k_2}{k_1} + a} = \frac{k_{cat} e_0 a}{K_m + a} = \frac{V_{max}}{K_m + a} \tag{II.6}$$

in which k_2 has been written as k_{cat}, k_{cat} *a* as V_{max}, $(k_{-1}+k_1)/k_1$ as K_m, which is referred as Michaelis constant. The Michaelis-Menten curve can be illustrated as in Figure 8.

3) Catalytic efficiency—the importance of K_m and V_{max}

The Michaelis-Menten constant K_m has specific significance; it combines first-order rate constants k_{-1} and k_2 with a second-order rate constant k_1. Its units are mol^{-1} or M. In practice, the K_m is the concentration of substrate that provides a reaction velocity that is half of the maximal velocity obtained under saturating substrate conditions. It

can also be taken a rough indication of how tightly the enzyme binds its substrate. A substrate bound weakly by an enzyme will have a large K_m value, a substrate bound tightly will have a small K_m. K_m depends on the conditions of solution, such as temperature, the nature of the substrate, pH, ionic strength and other reaction conditions. Determination of K_m values serves to characterize a particular enzyme-substrate system under specific conditions. Values of K_m are typically in the range of 10^{-1} M – 10^{-7} M [59].

The maximum rate V_{max} represents the maximum rate attainable; it is the rate at which the total enzyme concentration is present as the enzyme-substrate complex. The catalytic constant k_2 is usually referred to as turnover number. The turnover number of an enzyme is the number of substrate molecules that are converted to product per unit time, when the enzyme is fully saturated with the substrate.

1.5 Graphical determination of K_m and V_{max}

The kinetic constants K_{max} and K_m are determined graphically with initial rate measurements obtained at varying substrate concentrations. Experimentally, prepare a stock solution of substrate at the highest concentration that is experimentally reasonable, before it is twofold diluted from this stock solution to produce a range of lower substrate concentrations. In principle, both K_m and V_{max} can be determined from a Michaelis-Menten plot such as previous drawing Figure 8. In reality, it is difficult to locate the asymptotic value V_{max} at very high substrate concentrations is often difficult; therefore the plot of v_0 versus a is not very useful in determining the value of V_{max}. Lineweaver–Burk plot is the most commonly used method for linearizing enzyme kinetics. The Michaelis-Menten equation can be rewritten as

$$\frac{1}{v_0} = \frac{K_M}{V_{max} a} + \frac{1}{V_{max}} \tag{II.7}$$

both K_m and V_{max} can be obtained from the slope and intercepts of the straight line, as shown in Figure 9.

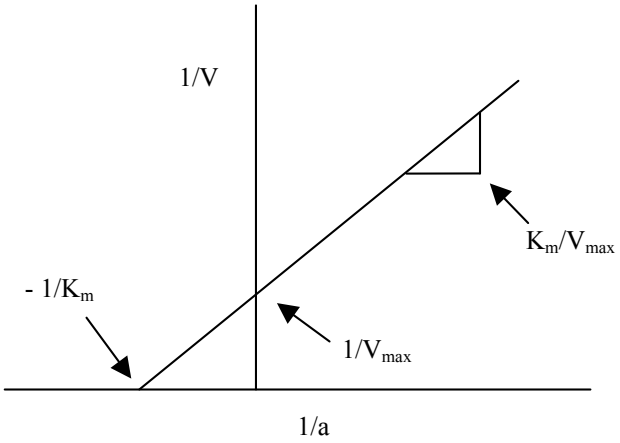

Fig. 9 Lineweaver –Burk plot

However, Lineweaver –Burk plot has disadvantage of compressing the data points at high substrate concentrations into a small region and emphasizing the points at lower substrate concentrations, which are sometimes not very accurate.

2 Enzyme inhibitors

2.1 Definition

An enzyme inhibitor reduces the effectiveness of enzyme in an enzyme-catalysed reaction; it decreases the rate or prevents a chemical reaction. Inhibitory molecules can bind to sites on the enzyme that interfere with proper turnover. This binding stops a substrate from entering the enzyme's active site or hinders the enzyme from catalysing its reaction. Inhibitor binding can be either reversible or irreversible. Reversible inhibitors generally bind to enzymes but do not undergo chemical reactions. Irreversible inhibitors usually react with the enzyme and change it chemically.

Reversible inhibitors bind to enzymes using weak bonds with non-covalent interactions. These bonds are usually hydrogen bonds, hydrophobic interactions and ionic bonds. They are formed rapidly and break easily, they do not undergo chemical reactions therefore do not permanently disable the enzyme; the inhibition can be easily removed by dilution or dialysis. The mechanism of this type of inhibition can be expressed as following, the inhibitor comes to equilibrium with the enzyme, to

form an enzyme-inhibitor complex, and the degree of inhibition depends on the position of equilibrium and the amount of enzyme which is bound to inhibitor:

$$E + I \Leftrightarrow EI \tag{II.8}$$

Usually reversible inhibitors can be classified as three types. When the substrate and inhibitor cannot bind to the enzyme at the same time, the substrate and inhibitor compete for access to the enzyme's active site, this form of inhibition is known as competitive inhibition. In practice, this type of inhibition can be overcome by high concentrations of substrate. K_m is an indication of enzyme-substrate affinity, in the presence of an enzyme inhibitor, enzymes will exist either as free enzymes or enzyme-inhibitor complex, so it reduces enzyme-substrate affinity, or increases K_m. Competitive inhibitors do not slow the reaction at high substrate concentrations and their is no change in V_{max}.

Sometimes the inhibitor can bind to enzyme at the same time as the substrate; the binding of the inhibitor to the enzyme does not affect the binding of substrate but reduce its activity by preventing the enzyme from converting the bound substrate to product. This type of inhibition can be reduced, but not overcome by increasing concentrations of substrate, and is known as non-competitive inhibition. A classical non-competitive inhibitor has no effect on substrate binding as well as the substrate-enzyme affinity, so the K_m is unchanged. However it inhibits at high concentrations so the V_{max} is decreased.

Another type of inhibitor can bind to the enzyme at the same time as the enzyme's substrate. However, the binding of the inhibitor affects the binding of the substrate, and vice versa. A change to the shape of the active site alters the ability of the substrate to bind. It doe not stop the enzyme activity all together but the affinity will be reduced. This type of inhibition is called as mixed inhibition; it has some properties of competitive and noncompetitive types. A mixed inhibitor allows the substrate to bind but reduce its affinity, so K_m is increased. It inhibits at high concentrations, so V_{max} is decreased.

Irreversible inhibitors usually modify the enzyme by forming a strong, covalent bond:

$$E + I \rightarrow EI \qquad \text{(II.9)}$$

The inhibition can not be reversed; the enzyme is effectively permanently disabled. They are also known as enzyme inactivator. Irreversible inhibitors take some time to react with the enzyme as covalent bonds are slower to form. They display time dependency, the degree of inhibition increasing with the time with which the enzyme is in contact with the inhibitor. Irreversible inhibitors are often electrophilic and contain reactive functional group such as nitrogen mustards, aldehydes, haloalkanes or alkenes.

2.2 Graphic determination of activities of inhibitors

There are a few graphic methods to determine the mode of inhibition of a particular molecular. The double reciprocal (Lineweaver-Burk) plot, Dixon plot, dose-response plot have been described to be used for the different type of inhibitors. The double reciprocal plot is the most straightforward means of diagnosing inhibitor modality. It graphs the value of reciprocal velocity as a function of reciprocal substrate concentration to yield a straight line, overlaying the double-reciprocal lines for an enzyme reaction carried out at several fixed inhibitor concentrations will yield a pattern of lines that is characteristic of a particular inhibitor type. The double-reciprocal plot serves a means of easily estimating the kinetic values K_m *and* V_{max} from the linear fits of the data in the plot.

K_i is known as inhibitor constant, it refers to the binding affinity of the inhibitor. The Dixon plot is a common method for determining K_i value of a competitive inhibitor. The initial velocity of the reaction is measured as a function of inhibitor concentration at two or more fixed concentrations of substrate. The data are then plotted as *1/v* as a function of [I] for each substrate concentration, and the value of $-K_i$ is determined from x-axis value at which the lines intersect.

A specific signal as a function of the concentration of some exogenous substance can be measured in many biological assays. A plot of the signal obtained as a function of the concentration of exogenous substance is referred to as a dose-response plot, and

the function that describes the change in signal with changing concentration of substance is called as dose-response curve, as seen in the Figure 10.

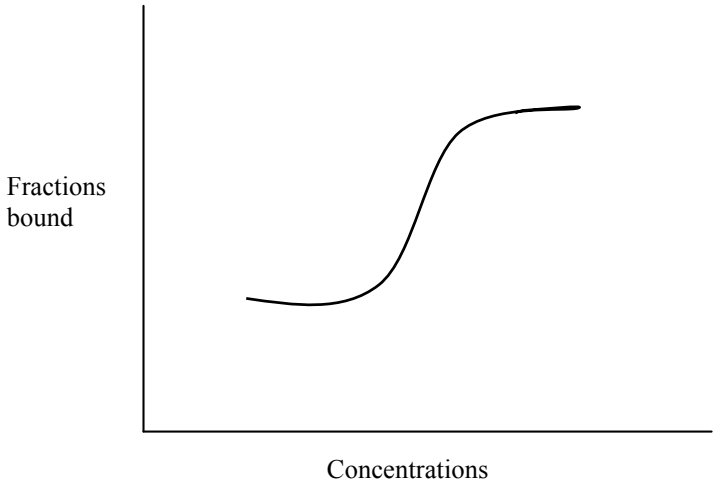

Fig. 10 Dose-response curve

2.3 IC_{50}

The concentration of inhibitor required to achieve a half-maximal degree of inhibition is referred to as the IC_{50} value, it indicates how much a particular inhibitor is needed to inhibit a given biological process by half, IC_{50} is commonly used as a measure of antagonist drug potency and its effectiveness in pharmacological research. IC_{50} values are dependent on conditions under which they are measured. Generally, the higher the concentration of inhibitor, the more will agonist activity be lowered. IC_{50} value increases as enzyme concentration increases. The following equation describes the effect of inhibitor concentration on reaction velocity:

$$\frac{v_i}{v_0} = \frac{1}{1 + \frac{I}{IC_{50}}} \tag{II.10}$$

where v_i is the initial velocity in the presence of inhibitor at concentration [I] and v_0 is the initial velocity in the absence of inhibitor. Dose-response plots are popularly used to determine IC_{50}, the plot is made by making measurements over a broad range of inhibitor concentrations at a single, fixed substrate concentration. A range of inhibitor concentrations spanning several orders of magnitude can be studied by means of the twofold serial dilution scheme. The method is convenient.

While the inhibitor constant K_i is an absolute value, IC_{50} is not a direct indicator of affinity of the inhibitor, it is the functional strength of the inhibitor and its value may vary between experiments depending on the concentration of enzymes. The relationship between IC_{50} and K_i is described by Cheng – Prusoff [60] as

$$K_i = \frac{IC_{50}}{1 + \frac{A}{K_m}} \tag{II.11}$$

where S is substrate concentration and K_m the affinity of the substrate for the enzyme.

3. The importance of enzymes in the life sciences

In clinical analysis, many species are not directly detectable, usually they need to be converted through enzymatic reactions to another species either using UV absorption or colorimetric. Typically, the detection of ammonia in blood sample uses the GLDH (Glutamatdehydrogenase) and cofactor NADPH [61],

$$2\,NH_4^+ + \alpha\text{-}Ketoglutarate + 2\,NADH \xrightarrow{GLDH} 2L-Glutamate + 2NAD^+ + 2H_2O \tag{II.12}$$

the amount of oxidized NADPH reflects the ammonia concentration, it can be determined through photometric detection.

In a clinical procedure to detect creatine in blood serum, the following steps using creatinase are involved [62],

$$Creatine + H_2O \xrightarrow{creatinase} Sarcosine + urea \tag{II.13}$$

$$Sarcosine + H_2O + O_2 \xrightarrow{Sarcosinoxidase} Glycin + HCHO + H_2O_2 \tag{II.14}$$

$$H_2O_2 + Phenolderivate + 4-aminophenazone \xrightarrow{Peroxidase} Benzochinonimine \tag{II.15}$$

The intensity of red benzochinonimine is then proportional to the creatine concentration.

Enzymatic reactions using lactatedehydrogenase (LDH) and alanine-amino-transferase (ALT) are employed to detect lactate in plasma [63],

$$L-lactate + NAD \xrightarrow{LDH} Pyruvate + NADH \tag{II.16}$$

$$Pyruvate + L-Glutamate \xrightarrow{ALT} L-Alanine + \alpha-Ketoglutarate \tag{II.17}$$

The detection of Pyruvate in plasma uses LDH and cofactor NADH [64].

$$Pyruvate + NADH + H^+ \xrightarrow{LDH} L-lactate + NAD^+ \quad \text{(II.18)}$$

Many plants and micro-organisms contain natural products that possess potent biological activities. To isolate these natural product is fundamental to the development of modern medicine, the discovery of quinine, morphine and penicillin was the benefit of this technique. Many of these natural products are structurally so complex that it is not practical to synthesise them in the laboratory at an affordable price. However, using enzyme-catalysed biosynthetic pathways, it is much easier to operate highly selective enzymatic reactions and biosynthesise these molecules. One specific application is to use enzyme in asymmetric organic synthesis. Enzymes are highly selective and enantiospecific, they can be highly efficient under mild reaction conditions and due to that they can save cost in energy and waste treatment, and are environmental friendly.

A selective inhibitor of an enzyme can be used for selective toxicity against the specific organism or cell type, which hosts the enzyme. This inhibitor can be developed for the bacterial enzyme, if the difference is significant comparing to a particular enzyme found in bacteria with the same enzyme in humans. If this inhibitor did not inhibit the human enzyme, then it could be used as an antibacterial agent, which leads to a new drug discovery.

III. Enzyme assays

Enzyme assays are laboratory analytical methods for measuring enzymatic activity, quantification and determination of an enzyme as an analyte, which can be achieved by determining the rate of product formation or substrate used during the enzyme-catalyzed reaction. There are several alternative assay procedures available and convenience, cost, the availability of appropriate equipment and reagents and the level of sensitivity are the grounds required for the choices to make.

1 Development of assay method

The determination of kinetic constants k_{cat} and K_m relies on the information obtained during the enzymatic reaction; one of the most important information is the initial velocity. The ability to measure accurately the initial velocity of an enzymatic reaction under well-controlled conditions is the key to the measurement of enzyme activity. In order to measure the velocity of a reaction, it is necessary to follow a signal that reports product formation or substrate depletion over the time.

According to their sampling method, the assays can be distinguished as:

- Continuous assays: the assays give a continuous reading of activities, it is usually most convenient.
- Discontinuous assays: the samples are taken when the reaction stopped and then the concentrations of substrates/products are determined.
- Coupled assays: some enzyme-catalyzed reactions do not result in changes in the properties of the reactants. It is necessary to use an indirect assay method which involves some further treatment of reaction mixture. These further treatment of reaction mixture will either produce a measurable product or to increase the sensitivity or convenience of the assay procedure. The reduction of $NAD(P)^+$ or the oxidation of the corresponding reduced coenzymes is often involved in a coupled assay.

It is possible to use indirect assays both continuously and discontinuously to monitor the progress of the reaction, which can be described as:

- Discontinuous indirect assays: the enzymatic reaction is stopped after a fixed time and the reaction mixture is treated to separate a product for

analysis or to produce a change in the properties of one of the substrates or products.

- Continuous indirect assays: the assays involve carrying out the manipulations necessary to detect product formation, or product remaining within the assay mixture, which will allow the change to be followed continuously as it occurs.

2 Detection methods

Depending on the changes in absorbance, fluoresces, pH, optical rotation, conductivity, enthalpy or viscosity or volume of the reaction mixture, various following detection and separation methods have been developed and used to assay the activities of individual enzymes and follow the course of the reaction:

- UV/VIS absorption detection is based on the changes in electronic configuration of molecules which result from their absorption of light energy of specific wavelength. This wavelength lies in the UV-visible region. Absorption measures transitions from the ground state to the excited state. It is convenient.
- Fluorescence detection uses a difference in the fluorescence of substrate from product to measure the enzyme reaction. A beam of light is used to excite the electrons in molecules of certain compounds. Emission of light of a lower energy is measured. In contrast to UV/VIS method, it measures transitions from the excited state to the ground state; it is more sensitive, permitting the detection of much lower concentration changes in substrate or product.
- Calorimetric detection measures the heat released or absorbed by enzymatic reactions.
- Chemiluminescent detection detects the emission of light by a chemical reaction.
- Radioisotopic detection measures the amount of radioactivity in the substrate and product fractions. A radioactive species is incorporated into the structure of the substrate, after separation, the substrate loss and product production can be quantified. It is accurate, specific, sensitive, quantitative, simple and rapid to perform.

- Immunologic detection detects antibodies raised or developed against a certain protein substrate. It can be used to follow proteolytic cleavage of a protein substrate by Western blotting and ELISA type assays.
- Polarographic detection measures the current as the potential is varied, therefore information about an analyte can be obtained. A dropping mercury electrode is used as working electrode; it is useful for its wide cathodic range and renewable surface.
- Conductivity detection measures the change in bulk solution conductivity between two electrodes when an analyte band passes through the electrode gap. It is a universal detection method.

The UV/visible type detector are most widely used, due to their versatility, high sensitivity, and wide dynamic range, relative insensitivity to temperature and flow variations. Fluorometric detection provides higher sensitivity than absorption. This method can be used to detect any compounds that can not be easily detected by other methods such as UV. This high sensitivity and selectivity have been extensively applied in biochemical systems since many biologically important compounds are strongly fluorecence, such as biogenic amines, amino acids, and drugs. Typically, fluorometric detection is used for enzyme-linked immunoassays [65].

3 Separation methods.

1) Chromatographic separation methods

Paper chromatography, thin-layer mode chromatography (TLC) and high performance liquid chromatography (HPLC) are the three most commonly used chromatographic separation methods.

Paper chromatography and TLC are commonly used to separate low molecular weight substrate and products of enzymatic reactions, the separation of these two methods is accomplished through the differential interactions of molecules in the sample with ion exchange or silica-based resins that are coated onto paper sheets or plastic or glass plates.

Liquid chromatography is a separation process to separate a mixture into its individual components followed by their detection with a suitable monitor. It uses gravity to pull

the solvent or mobile phase through a column packed with a stationary phase, a lower limit on the size of particles was eventually reached beyond which flow under gravity completely diminished. HPLC is the result of development on generating high pressure to meet this need. The introduction of low compressibility resins, typically based on silica, has made it possible to run liquid chromatography at high pressure. At this high pressure, the resolution is enhanced, much faster flow rate can be used, and the time for a chromatographic run is shortened.

A typical HPLC separation may take between 5 and 30 min compared to several hours in the case of conventional liquid chromatography. HPLC has been extensively used to separate low molecular weight substrates and products, the peptide-based substrates and products of proteolytic enzymes. Reversed phase [66-69], ion exchange [70-72] and size exclusion [73-75] HPLC are the three most commonly used separation techniques used in enzyme assays.

2) Electrophoretic methods

Electrophoresis is mostly used to separate macromolecules in hydrated gels of acrylamide or agarose. The most common electrophoretic technique used in enzyme assays is sodium dodeczl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). It serves to separate proteins and peptides on the basis of their molecular weights. In SDS-PAGE, the anionic detergent SDS is employed to coat samples of proteins or peptides to give the similar anionic charge densities, then such samples are applied to a gel, an electric field is applied across the gel, the negatively charged proteins will migrate toward the positively charged electrode. Depending on the molecular weight of the species undergoing electrophoresis, the polymer matrix of the gel causes different degree of retardation when molecules migrate toward the positive pole. The purpose of the electrophoresis in a protease assay is to separate the protein or peptide substrate of the enzymatic reaction from the products, Examples of SDS-PAGE used for enzymatic assays include proteolytic enzymes [76], kinases [77] and DNA–cleaving nucleases [78]. After electrophoresis, protein or peptide bands are visualized with a peptide-specific stain, Coomassie Brilliant Blue or silver staining [79] are often used, Wirth and Romano has published a review on staining methods in gel electrophoresis [80]. Reviews on the methods to detect enzymatic activity using gel

electrophoresis and after gel electrophoresis are available from Hames and Rickwood [81], Gabriel and Gersten [82].

4. Capillary electrophoresis (CE) in enzymatic assays

Recently, CE methods are reportedly used to monitor and investigate enzymatic activity, metabolism, production of reactive oxygen species and cellular function. CE separation is compatible with many detection methods, most popularly used are the fluorescence and UV absorption detections.

CE-CIF Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was one of the most frequently used methods on enzyme assay. LIF utilises superior properties of laser light, narrow laser beam is characterised by minimal divergence and focused between inner walls of capillary. Lee et al. [83] developed a quantitative assay relying on capillary electrophoresis with laser-induced fluorescence detection. The interconversion of sphingosine and sphingosine-1-phosphate by enzymatic reaction was followed and measured. This assay was also able to determine the in vitro activity of both kinase and phosphatase using purified enzymes. Another CE assay was described by Jameson and co-workers [84] to detect G protein-coupled receptor (GPCR)-stimulated G protein GTPase activity in cell membranes expressing alpha2A adrenoreceptor-Galphao1 wild-type (wt) or C351I mutant fusion proteins using a fluorescent, hydrolysable GTP analogue. There was no change in total fluorescence observed from substrate to product. CE was used to separate the fluorescent substrate (*GTP) from the fluorescent product (*GDP). Whitmore et al. [85] have reported an ultra sensitive method by using CE-LIF for the analysis of glycosphingolipid catabolism. CE was used to separate a cellular homogenate prepared from the cells. The transformation from fluorescently labelled substrate to fluorescently labelled product was monitored at the yoctomole level. CE-LIF was also used by Kim et al. [86] to measure nitric oxide in single neurons. Acorbate oxidase was used as an enzyme to catalyze the substrate ascorbic acid (AA) to the product dehydroascorbic acid (DHA), CE-CIF detection was used to distinguish the various reaction products. This specific, effective and simple method allowed nitric acid to be measured in single cells without detectable interference from other compounds. Eder et al. [87] adopted CE-CIF to CE-MEKC-CIF to separate and quantitate doxorubicin and doxorubicinol. Doxorubicinol is a human metabolite of the chemotherapy agent

doxorubicin and is associated with dose-dependent cardiotoxicity and decreased drug efficacy.

CE-UV method has been another one of mostly used analytical methods, especially in chiral analysis. Developed by Ha et al. [88], a chiral capillary electrophoresis with UV detection method was used for the analysis of verapamil. A method for CYP3A4 activity assay with verapamil as a substrate was proposed. Both R, S-verapamil and its metabolites R, S –norverapamil in the reaction mixture with cytochrome P450 were separated and determined. Meanwhile, Koval et al. [89] have reported a CE method using UV detection for the determination of D-serine in the presence of L-serine and the evaluation of serine racemase (SR) activity and SR's inhibitor screening. A good agreement was achieved between the developed CE method and the previously established HPLC method for determination of the inhibition constant, K(i), of a new SR inhibitor, L-erythro-3-hydroxyaspartate.

CE-MS was used to study proteins by analysis of metabolic changes [90]. Purified proteins were incubated with a pool of metabolite present in yeast extract. MS was used to detect the transformations in this pool of metabolites, therefore to discover the enzyme activities of proteins such as YbhA and YbiV.

5. EMMA

Electrophoretically mediated microanalysis (EMMA) uses the variability in electrophoretic mobilities among enzyme and substrates to initiate an enzymatic reaction inside the capillary, to separate the components of the reaction mixture from each other for the final quantification. It was first introduced by Bao and Regnier in 1992 [91] to achieve enzyme assays of glucose-6-phosphate dehydrogenase. Enzyme, substrates, coenzyme and running buffer were injected into a deactivated fused-silica capillary in a capillary zone electrophoresis apparatus and electrophoretically mixed. A UV-detector was used to detect the products. EMMA method is also classified as homogeneous enzyme assay by CE, while heterogeneous enzyme assay by CE refers to one of the reactants is immobilized onto the wall of the capillary.

Depending on the techniques on how to load and mix the enzyme and substrates in the capillary, a few approaches have been reported and classified as following:

- Zonal sample introduction (throughout-capillary reaction): the capillary is initially filled with one of reactants (enzyme or substrate) while the second reactant is introduced as a plug, the product is continuously formed during the electrophoretic mixing. See Figure 11(a).
- Moving boundary sample introduction (Figure 11(b)), the capillary is initially filled with one of reactants (enzyme or substrate) while the second reactant is introduced continuously from the inlet vial, the product is continuously formed during the electrophoretic mixing.
- Classical plug-plug mode (zone passing reaction), both enzyme and substrate are introduced in the capillary as distinct plugs, the reactant with lower electrophoretic mobilities is first injected, as in Figure 11(c).
- Plug-plug mode with partial filling (Figure 11(d)), an alternative to the classic plug-plug mode, part of the capillary is filled with the optimum buffer for the enzymatic reaction; the rest of the capillary is filled with the BGE optimal for the separation of substrate and product.
- At-inlet reaction, enzyme and substrate plugs are consecutively injected (Figure 11(e)); they are mixed by simple diffusion and reacted for a given time at the inlet part of the capillary.

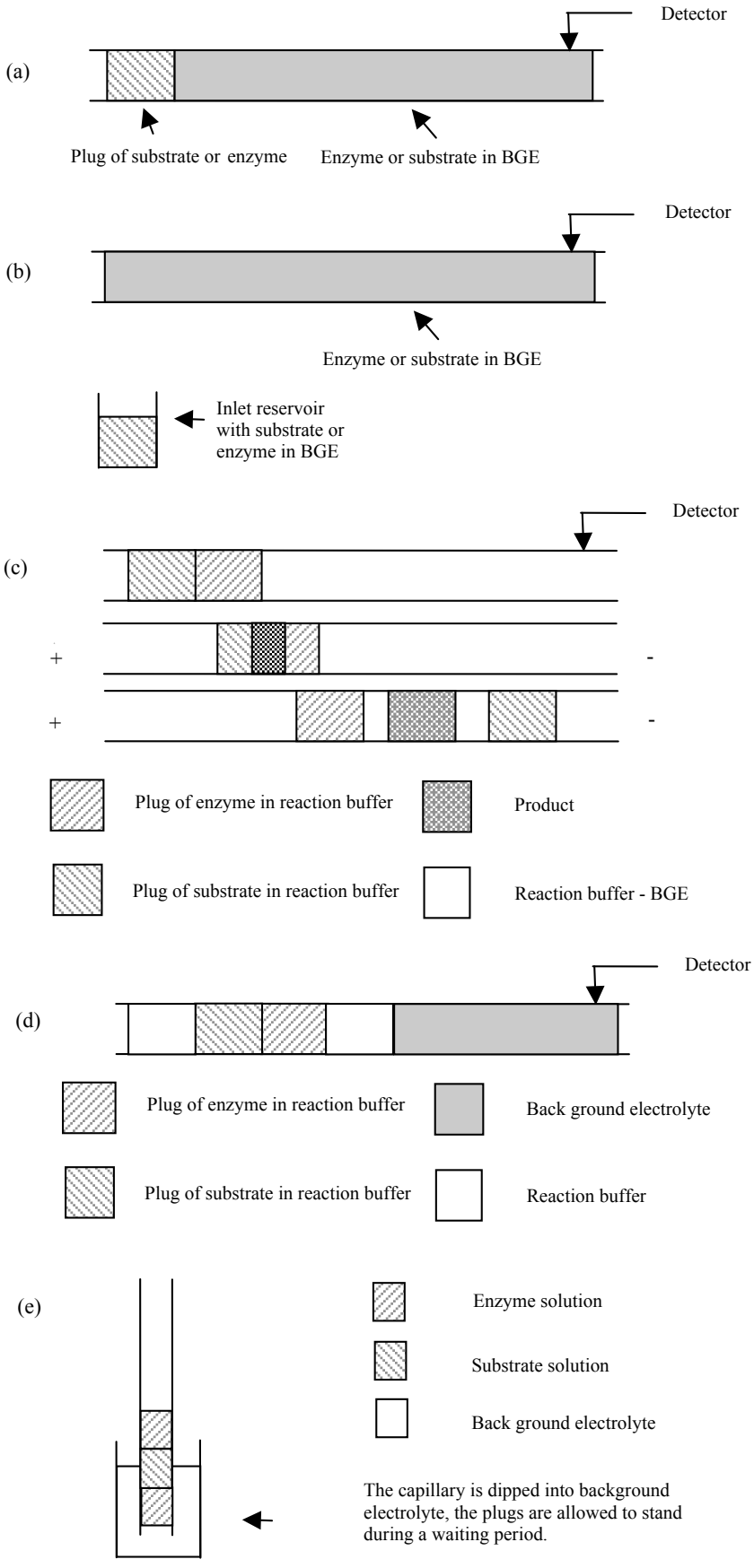

Fig. 11 Schematic illustration of EMMA sample introduction methods

The EMMA methodology has been successfully employed with different enzyme assays, kinetic studies, inhibitor study and screening, quantitative studies. Kim et al. [92] carried out an assay of uridine diphhosphate glucuronosyltransferases using plug-plug mode. Iqbal et al. [93] used plug-plug partial filling technique assayed adenosine kinase. Urban et al. [94] [95] [96] made quantitative studies of glucose oxidase, penicillinase and yeast alcohol dehydrogenase by plug-plug mode. An electrophoretically mediated microanalysis method with a partial filling technique was developed for flavin-containing monooxygenase assay in the group of A. Van Schepdael [97]. Among these assays, UV detectors were used. So far, no report has appeared using conductivity detection in EMMA methodology.

IV. Aims of this thesis

Since capacitively coupled contactless conductivity detection (C^4D) in the axial electrode configuration was introduced as a quantification method for capillary electrophoresis in 1998, the application of this detection was first used for inorganic ions and organic ions, it was then extended to the determination of biomolecules. Kuban et al. [53] has made a comprehensive review on the applications of CE-C^4D detection method. However, the use of this method for monitoring the enzymatic reactions and for the determination of non-ionic species converted to ionic species via enzymatic reactions has not been reported.

The main goal of this thesis is to explore applications of conductometric detection on enzyme assays. Analytical methods for clinical analysis and in the life sciences in general rely on optical detection by UV-Vis absorption or fluorescence. However, many species are not directly accessible optically. In enzyme assays, important methods in which the selectivity of the enzyme for the substrate is made use of, the analytical reaction therefore usually has to be coupled to a second reaction (often via H_2O_2 as intermediate [98] [99]) which leads to the formation of a colored product. However, conductometric detection is a universal detection method for ionic species which does not require derivatization, thus exploration of an indirect application of this technique remains as an interesting task.

First investigation involved the non-ionic species glucose, ethanol, ethyl acetate and ethyl butyrate, which can not be detected either by UV absorption or fluorescence. Through the conversion to ionic species via enzymatic reaction, these species can be selectively quantified via CE separation with C^4D. After this successful model, the investigation is then extended to urea, one of the species routinely determined in clinical samples (blood serum, urine). The suitability of conventional CE and microchip electrophoresis with C^4D for this purpose is further explored. This investigation involved detailed urease kinetic studies and the determination of urea in human blood serum. A common method for the identification of protein is to use enzymes for selective cleavage of amide bounds. This produces a mixture of peptides, each protein having its characteristic peptide pattern, which can be determined by capillary electrophoresis. In the third approach, peptic and tryptic

digestion of model proteins was examined; the suitability of online (EMMA) digestion was also investigated. The development of chiral synthesis using soft catalysts, such as enzymes, is currently an important trend. Expensive HPLC column is commonly used to analyze the enantioselectivity during the synthesis. By directly monitoring the enantioselective hydrolysis of amino acid ester, CE C^4D was exploited as an inexpensive alternative to HPLC. Furthermore, the investigation was applied on study of acetylcholinesterase inhibitors. Some of acetylcholinesterase inhibitors are used to treat Alzheimer's disease. Others are used as chemical weapons such as nerve agents and as pesticides. The antagonist drug potency can be demonstrated through the value of IC_{50}.

V. Results and discussion

The results and discussion chapter comprises reprints of three published paper in Electrophoresis and Analytical Biochemistry, one manuscript of a paper recently submitted for publication in Chirality, another manuscript is in preparation for submission. All the papers are contained in the subsections.

CE C^4D can separate and detect inorganic species, organic ions and biochemicals, their applications include clinical analysis, food analysis and enantiomeric separations. However, so far, the application of this method on enzymatic reactions has been unknown. The evaluation of this new approach started with glucose. The determination of glucose is of high interest because of clinical importance. When hexokinase [100] is used as a catalyst together with cofactors ATP, glucose is converted to glucose-6-phosphate (G6P) and ATP to ADP. Another enzyme glucose oxidase (GOX) catalyzes the oxidation of glucose to glucono-1,5-lactone which then hydrolyzes to gluconate. In both assays, the products from reactions, glucose-6-phosphate, ADP and gluconate are ionic, negatively charged, thus they were possible to be measurable in capillary electrophoresis with contactless conductivity detection. Ethanol is another substrate which is not ionic, using alcohol dehydrogenase as enzyme, together with cofactor NAD, ethanol is converted to acetaldehyde and NAD to NADH. Esterases hydrolyze esters into their corresponding acids and alcohols. Acetate and butyrate are the products converted from ethyl acetate and ethyl butyrate. NADH, ethyl acetate and ethyl butyrate were all detectable using CE C^4D. A microfluidic device with the standard channel configuration of an elongated cross was used to demonstrate the possibility of using this platform. The conversion of glucose to gluconate using GOX for catalysis and the enzymatic hydrolysis of ethyl acetate were both carried out on micro-chip. More detailed discussions can be found in subsection V.1.

With the successful detection of these three substrates via enzymatic reactions using CE C^4D, the method was then applied to more routinely determined species in clinical analysis. Urea is routinely examined in blood serum samples. The most common way to detect urea is to employ the enzyme urease to catalyze the hydrolysis of urea and to determine the products. In this urease enzymatic assay,

one of the products ammonia is positively charged, it is possible to use conductivity detection to determine ammonia, as well as directly quantify the ammonium. Being able to monitor the amount of ammonium produced as product of the enzymatic conversion allows the direct study of the enzyme kinetics. The efficiency of the enzymatic reaction is pH-dependent, a series of experiments were carried out at different pH-values ranging from 5.2 to 7.6 using constant concentrations of urea (20 mM) and urease (0.1 mg·mL), graphs were plotted in relation between v, substrate concentration and pH values, the maximum rate of reaction, V_{max}, was determined as 5.1 mmol·mL^{-1}·min^{-1}, and the Michaelis-Menten constant, K_M, was determined as 16 mM. The real blood samples were collected from a hospital. The determination of urea in human blood serum was investigated. The possibility of determining urea in serum samples was first investigated by examining the separation. Quantification of urea in 10 serum samples was subsequently carried out. The results were close in comparison to the values provided by hospital, which had been determined with a standard photometric method. Determination and quantification of urea in these 10 serum samples were also carried out on micro-chip device. Detailed work see subsection V.2.

The method was then further extended to study the proteolytic enzymes pepsin and trypsin, as discussed in the subsection V.3. These two enzymes are frequently used for *in-vitro* studies of proteins. Both enzymes occur in the digestive tract and break down proteins, but preferentially cleave the proteins only at specific peptide bonds. Amino acids are best separated and detected under acidic conditions in cationic form, peptides can also be determined under these conditions. Mass spectrometry is often used to determine the peptides and amino acids. The low pH-value assures a positive charge of all compounds and therefore fast migration and sensitive detection by conductometry. The peptic digestion and the separation of the products were carried out using a buffer consisting of 2.3 M acetic acid and 0.05% (v/v) Tween with a pH 2.1. Minigastrin I, myoglobin, cytochrome C, HSA and BSA were cleaved by pepsin, the peaks of peptides were detected. Tryptic digestion was carried out in an optimized buffer consisting 10 mM ammonium bicarbonate solution and 2 M urea with pH 7.8. For separation and detection the 2.3 M acetic acid buffer was again used in order to assure that all analytes are rendered in the protonated cationic form. Cytochrome C and myoglobin were the model proteins

cleaved by trypsin, and the peaks of their products were detected. Electrophoretically Mediated Micro-Analysis or EMMA [91] is a technique developed to carry out the assays in a real nanoliter scale. An adoption from partial filling technique [101] with some modifications was employed for tryptic online digestion. Cytochrome C and apomyoglobin were employed to be digested by trypsin, the peaks of peptides resulting from the digestion were detected.

Subsection V.4 focuses on the use of CE C^4D on monitoring enantioselective hydrolysis of amino acid esters. A buffer consisting of 2 M acetic acid and 5 mM the chiral crown ether $18C6H_4$ was used for detection of DL-esters and DL-amino acids. DL-serine methyl ester (DL-SME) and DL-threonine methyl ester (DL-TME) were the two model groups to be examined. Lipase is a water-soluble enzyme that catalyzes the hydrolysis of ester bonds in water-insoluble, lipid substrate. A reaction buffer with pH at 7.4 consisting 0.2 M $NaHCO_3$ was employed for the hydrolysis of ester to amino acid using lipase from porcine pancreas (PPL). Follow the course of the hydrolysis, the increase of the both D- and L-serine peaks was observed, as well as those of D- and L-threonine. The enantioselectivity ee and yield were studied through the model group DL-SME and DL-TME. Comparison was made on the selectivity of different lipases, namely between lipase from porcine pancreas (PPL) and lipase from wheat germ (WGL). The rate of reaction is slightly higher for the PPL than for the WGL. While the WGL also prefers the L-serine ester, a higher selectivity for the L-form is found for the PPL. L-SME and L-TME were selected together as starting substrates, the rate of reaction is higher for L-TME than for L-SME.

In the final section V.5, the behaviours of acetylcholinesterase (AChE) inhibitors are studied. Acetylcholinesterase inhibitors are chemicals that inhibit the cholinesterase enzyme from breaking down acetylcholine, as a result, increase both the level and duration of action of the neurotransmitter acetylcholine. AChE inhibitors are used as medicinally to treat neurodegenerative disorder such as Alzheimer's disease (AD) and as chemical weapons in the form of nerve agent and as pesticides. The enzymatic assay based on the reaction can be described as the following, Acetylthiocholine$^+$ $\rightarrow$ ✡cetate + thiocholine$^+$, in this reaction, AChE hydrolyzes acetylthiocholine (ATCh) and breaks it into acetate and thiochline.

Acetate is a negatively charged species, it is possible to be separated and detected by CE C^4D. The buffer consisting 15 mM Mes and 16 mM Arg with pH 8.0 was made for the purpose of hydrolysis, another buffer consisting 25 mM Mes, 5 mM Arg and 50 μM CTAB with pH 5.7 was used for the separation. The assays were first carried out with ACh and AChE. After a mixture of AChE and ACh was injected into capillary, the peak of acetate was detected; the size of the peak increased following the time course with incubation at 37°C. ACh is used for *in vivo* experiments; ATCh is more often used for *in vitro* experiments. The further experiments were carried out with ATCh using the same protocol. The kinetics from this reaction was monitored, 20 $U \cdot mL^{-1}$AChE was an optimized concentration used for monitoring the enzymatic reaction with the inhibitors. The rate of reaction is higher for ACh than ATCh. Galanthamine is a model of a reversible inhibitor. A range of concentration of galanthamine between 0.1 μM to 100 mM was mixed with ACh before the enzymatic reactions took place. IC_{50} for galanthamine was 39.8 μM at incubation time 5 min. Paraoxon is a model of a quasi-irreversible inhibitor, IC_{50} of paraoxon at interval time 2 hours was 89.12 μM. Huperzine A is a naturally occurring novel Lycopodium alkaloid found in the extracts of the firmoss Huperzia serrata and Chinese herb Qian Cheng Ta. At 5 min, IC_{50} was 31.62 μM. The model of galanthamine was also tested on electrophoretically-mediated microanalysis (EMMA), 3.89 μM was obtained for the IC_{50} of galanthamine.

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Research Article

Monitoring of enzymatic reactions using conventional and on-chip capillary electrophoresis with contactless conductivity detection

The use of CE with contactless conductivity detection was evaluated for monitoring enzymatic reactions. The nonionic species ethanol, glucose, ethyl acetate, and ethyl butyrate were made accessible for analysis by CE *via* charged products or by-products obtained in enzymatic conversions using hexokinase, glucose oxidase, alcohol dehydrogenase, and esterase. Two of the reactions, namely the conversion of glucose with glucose oxidase and that of ethylacetate with esterase, were also successfully demonstrated on a microchip device. Quantification for ethyl acetate, taken as an example, was found possible with a detection limit of approximately 7 μM.

Keywords:
Alcohol dehydrogenase / Capillary electrophoresis / Contactless conductivity detection / Enzymatic reactions / Esterase DOI 10.1002/elps.200700332

1 Introduction

Conductometric detection for CE has been known since the introduction of this ion separation technique, but has not been used widely. This is mainly due to the challenge in constructing measuring cells of the small dimensions necessary to match the internal diameters of the separation capillaries, and partly caused by the difficulty of carrying out electrochemical measurements in presence of an applied electrical field. Commercial CE instruments, in contrast to ion-chromatographs, are therefore fitted with UV-absorption or fluorescence detectors. The capacitively coupled contactless conductivity detector (C^4D) introduced in 1998 independently by two research groups [1, 2], elegantly overcomes the complications of the earlier conventional conductivity detectors. The cell is based on two short external tubular electrodes aligned with the axis of the capillary. An ac-signal is coupled through *via* the capacitances formed by the electrodes and the conductive solution, but the dc-voltage of the separation field is effectively blocked by the insulating capillary wall. Publications concerning the fundamental working principles [3–6] are available. Commercial contactless conductivity detectors based on this newer design have very recently become available and due to the small size of the measuring cells these can be retrofitted to existing CE-instruments.

Conductivity detection is universal for CE in that, at least in principle, any ion can be detected. The possible applications are therefore diverse and include the determination of inorganic anions and metal cations as well as small organic ions such as those contained in food or beverages. Species which are not accessible *via* UV-absorption or fluorescence, are of primary interest, but the universality of conductivity detection means that the method is not restricted to such analytes. Several reviews detailing reported uses have appeared [7–10]. Recently published applications indicate that contactless conductivity detection is also useful in biochemical and clinical analysis. The detection of peptides [11, 12], proteins [11], and even Ig's [13] was found to be possible. In the clinical field, the determination of inorganic blood and urine electrolytes [14, 15] has been reported, as has the determination of amino acids in biological fluids [16, 17]. The method also has promise for the therapeutic drug monitoring of pharmaceuticals, as demonstrated by the determination of the antibiotics fosfomycin [18] and tobramycin [19], as well as the anticonvulsant valproic acid [20].

Herein a study on the use of CE with C^4D for the monitoring of enzymatic reactions is presented. This task is important for the study of the enzymes themselves, but also

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Abbreviations: ADH, alcohol dehydrogenase; **ADP,** adenosine 5′-diphosphate; **ATP,** adenosine 5′-triphosphate; **C^4D,** capacitively coupled contactless conductivity detection; **G6P,** glucose-6-phosphate; **GOX,** glucose oxidase; **NAD,** nicotinamide adenine dinucleotide; **PMMA,** poly(methyl methacrylate)

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in the investigation of inhibitors. Furthermore, such reactions are frequently employed in clinical analysis to selectively convert an analyte to a species which can be quantified *via* a nonspecific colorimetric reaction. The approach suggested here is different. Electrically neutral analytes are enzymatically converted to yield charged species, which can then be selectively quantified *via* CE separation with C^4D. Glucose, ethanol, ethyl acetate, and ethyl butyrate were used as model substrates. Digestions were carried out with hexokinase (glucose), glucose oxidase (GOX; glucose), alcohol dehydrogenase (ADH; ethanol), and esterase (ethyl acetate and ethyl butyrate).

2 Materials and methods

2.1 Reagents

All chemicals were of reagent grade and deionized water (Millipore, Bedford, MA, USA) was used throughout. Hexokinase from yeast (as a suspension of 3.81 mg/mL (172 U/mg) in an aqueous ammonium sulfate solution), GOX from *Aspergillus niger* (205 U/mg) as well as MES, CHES, CTAB, ethanol, glucose, gluconic acid, and acetic acid were purchased from Fluka (Buchs, Switzerland). The enzymes ADH from equine liver (1.33 U/mg) and esterase from porcine liver (27 U/mg), as well as gluconolactone, NADH disodium salt, nicotinamide adenine dinucleotide (NAD) sodium salt, adenosine 5′-triphosphate (ATP) disodium salt, adenosine 5′-diphosphate (ADP) monopotassium salt dihydrate, and glucose 6-phosphate dipotassium were obtained from Sigma (Buchs, Switzerland). Ethyl acetate was bought from Aldrich (Buchs, Switzerland).

2.2 Conventional CE

Separations in conventional fused-silica capillaries were carried out on a purpose-made electrophoresis instrument. This is based on a case made from poly(methyl methacrylate) (PMMA), which is divided into injection and detection compartments. The separation voltage is provided by a high-voltage power supply from Start-Spellman (model CZE2000, Pulborough, UK). For safety, a microswitch is fitted to interrupt the high voltage power when opening the instrument to carry out manipulations. The detector was also constructed in-house, is based on the design reported in ref. [21, 22] and the cell used for conventional capillaries includes the modifications reported in ref. [23]. An excitation frequency of 300 kHz at a peak-to-peak amplitude of 300 V was used. Fused-silica capillaries of 75 μm id and 375 μm od and total and effective lengths of 50 and 45 cm respectively were used for the conventional separations. These were purchased from Polymicro Technologies (Phoenix, AZ, USA) and were preconditioned with 1 M sodium hydroxide solution, flushed with water and 1 M hydrochloric acid solution followed by further flushing with water. Before use the separation capillaries were flushed with the appropriate running buffer. Sample injection was carried out manually in a hydrodynamic manner by lifting the injection end of the capillary to a height of 15 cm for 10 s. All separations in standard fused-silica capillaries were carried out at −25 kV. Enzymes, substrates, and any cofactors were dissolved in the electrolyte solution used for the electrophoretic separation, which were prepared with pH-values appropriate for the enzymatic reactions. These were performed in 1.5 mL microvials. For the detection of ethanol following the digestion with ADH, a buffer solution containing 50 mM CHES, 30 mM Arg, and 50 μM CTAB with a pH 9.1 was used, the enzymatic reaction took place at a room temperature of approximately 20°C. The reaction mixture consisted of 1 mL of 20% ethanol, 0.4 mL 100 mM NAD solution, and 0.1 mL of a solution of 1 mg/mL of ADH. The same buffer was used for the enzymatic reaction of glucose with hexokinase. A mixture of 1 mL of a 1 mM glucose solution, 0.1 mL 10 mM ATP solution, and 0.1 mL hexokinase suspension (3.81 mg/mL) was prepared, the enzymatic reaction took place at a room temperature of approximately 20°C. A buffer containing 20 mM MES, 5 mM His, and 50 μM CTAB with pH 5.7 was used for the enzymatic reaction of glucose with GOX. To 1 mL 100 mM glucose solution which had been thermostatted at 35°C 0.1 mL of a solution of 0.17 mg/mL GOX, which had also been thermostatted to 35°C, was added and incubation was continued at 35°C in a water bath. A buffer solution containing 15 mM MES, 16 mM Arg, and 50 μM CTAB (pH 8.0) was used for the enzymatic reactions with esterase at a room temperature of approximately 20°C. A reaction solution consisting of 1 mL 0.2% v/v ethyl acetate or ethyl butyrate and 0.1 mL of 1 mg/mL esterase solution was used in these cases.

2.3 Microchip electrophoresis

Microchips made from PMMA were purchased from the Microfluidic Chip-Shop (Jena, Germany, part number: 10-02-0052-0013-01). The separation channel had a cross-section of $70 \times 42\ \mu m^2$ and a depth of 20 μm and was sealed with a PMMA cover with a thickness of 175 μm. The effective separation length from the injection cross to the detector was 75 mm. Details on the computer controlled high-voltage system used for injection and separation on the chip can be found in ref. [24]. The injection was carried out electrokinetically (7 s at 1 kV) and the separation was performed at −5 kV. The chip was mounted on a chip holder constructed from PMMA which was fitted with a pair of electrodes of 1 mm width, 1.4 cm length, and with a gap of 0.5 mm [25]. Except for the cell, the detector circuitry was identical to that used for conventional capillaries. Excitation was carried out at 500 kHz with a peak-to-peak amplitude of 25 V. The electropherograms were recorded on a Macintosh personal computer (Apple, Cupertino, CA) using a MacLab/4e data acquisition system (ADInstruments, Castle Hill, NSW, Australia). Before each use, the chip was flushed with the ap-

propriate buffer solution. For the experiments with glucose, a buffer solution consisting of 20 mM MES/5 mM His/ 50 μM CTAB (pH 5.7) was used and for the hydrolyses of the ethyl acetate ester the buffer solution consisted of 15 mM MES and 16 mM Arg and 50 μM CTAB (pH 8.0). The substrates glucose, and ethyl acetate, as well as the enzymes GOX and esterase were first dissolved individually in the same buffer solutions as used for the separations. The solutions of substrate and enzyme were then mixed and stirred directly in the sample hole on the chip; the reactions were carried out at a room temperature of ca. 20°C. To 0.06 mL of a 100 mM glucose solution was added 0.06 mL of a GOX solution of 3 mg/mL and to 0.1 mL 10% ethyl acetate were added 0.05 mL of a solution of 1 mg/mL esterase for the two reactions respectively.

3 Results and discussion

3.1 Standard capillaries

3.1.1 Glucose

The determination of glucose is of high interest because of its clinical importance. According to ref. [26] two different commercial enzymatic assays exist. In the first method, glucose is converted to glucose-6-phosphate (G6P) in a reaction catalyzed by hexokinase:

$$\text{Glucose} + \text{ATP}^{4-} \rightarrow \text{Glucose-6-Phosphate}^{2-} + \text{ADP}^{3-} + \text{H}^{+}$$

In this reaction, the enzyme hexokinase transfers phosphate groups from the cofactor ATP to glucose. In the common photometric method, a second auxiliary reaction, yielding NADH, which shows a pronounced optical absorption, is used for quantification. As G6P is negatively charged it was expected that this product of the first reaction should be directly measureable in CE with contactless conductivity detection, and thus the second step of the reaction would not be necessary.

In Fig. 1, electropherograms for repeated injections of a reaction mixture of glucose, the enzyme hexokinase and the cofactor ATP are shown. The first trace for an injection carried out 1 min after mixing of the compounds shows a large peak which is thought to be due to sulfate which is part of the enzyme preparation used, a second peak most likely due to carbonate adsorbed from air, and a peak due to ATP. Evident in the second trace, for an injection which took place 6 min after mixing the components, are G6P as well as the side product ADP at detectable levels. The identity of the peaks was confirmed by spiking with standards. The peak heights for both products, G6P and ADP, increased for the subsequent injections, but are very similar for the last two injections at 18 and 24 min, indicating that the reaction had nearly reached completion. Note that a decrease of the peak for the cofactor ATP could also be observed.

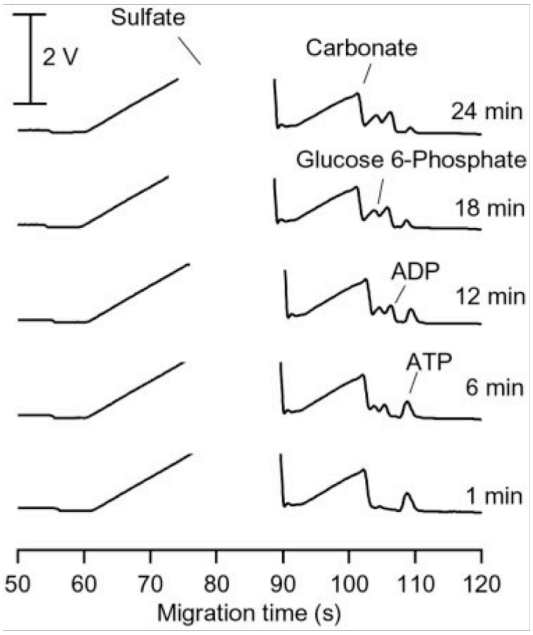

Figure 1. Electropherograms obtained with a standard capillary for the conversion of glucose with hexokinase. Reaction mixture: 1 mL 1 mM glucose, 0.1 mL 10 mM ATP, and 0.1 mL hexokinase solution (0.38 mg/mL), room temperature. Buffer: 50 mM CHES/ 20 mM Arg/50 μM CTAB, pH 9.1. Capillary: 75 μm id, total length 50 cm, effective length 45 cm. Injection: hydrodynamic, 15 cm height elevation for 10 s. Separation: −25 kV.

The second commonly used enzymatic assay for glucose is based on the enzyme GOX [26]. GOX, catalyzes the oxidation of glucose to glucono-1,5-lactone which then hydrolyzes to gluconate, as shown below:

$$\text{Glucose} + \text{O}_2 \rightarrow \text{Gluconolactone} + \text{H}_2\text{O}_2$$
$$\text{Gluconolactone} + \text{H}_2\text{O} \rightarrow \text{Gluconate}^{-} + \text{H}^{+}$$

As gluconate is not accessible photometrically, also in this method an additional auxiliary reaction is needed. Commonly, the by-product H_2O_2 is used to oxidize *o*-dianisidine to a colored product in a reaction, which is catalyzed by the enzyme peroxidase. The hydrolysis product of the first reaction sequence, gluconate, should be accessible by CE-C^4D, allowing the elimination of the color forming step.

Electropherograms for a reaction mixture incubated at 35°C which was injected after different time intervals are shown in Fig. 2 together with a standard solution of gluconate for illustration. For the first injection carried out 1 min after mixing, no gluconate could be detected. From 20 min on, a peak for the product gluconate could be observed. The analyte is the only anionic species present in the reaction mixture in this case. An increase of the peak height with time could again be followed and thus it is possible to use CE-C^4D

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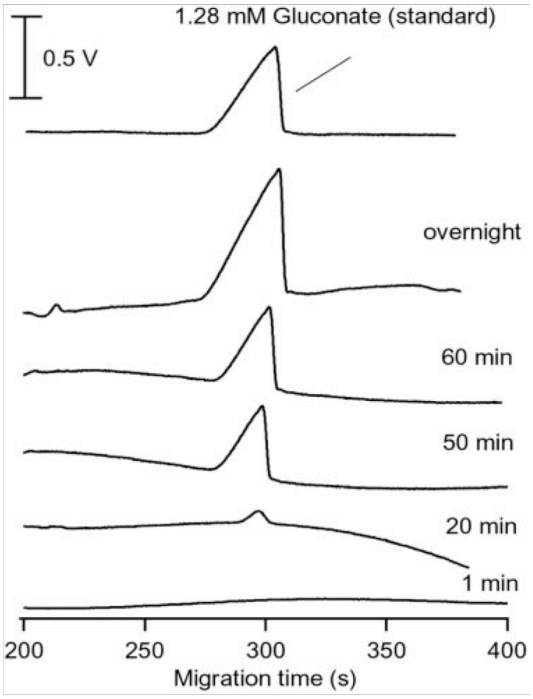

Figure 2. Electropherograms obtained with a standard capillary for the conversion of glucose with GOX. Reaction mixture: 1 mL 100 mM glucose was first incubated for 20 min at 35°C before it was mixed with 0.1 mL 0.16 mg/mL GOX, 35°C. Buffer: 20 mM MES/5 mM His/50 μM CTAB, pH 5.7. Other conditions as for Fig. 1.

also for direct monitoring of this reaction. Note that, at least for the conditions employed, this reaction was found to be significantly slower than the previous one, even though the reaction mixture was thermostatted to 35°C in this case. After incubation overnight the reaction was found to be complete.

3.1.2 Ethanol

ADH is the principle ethanol-metabolizing enzyme. Using the cofactor NAD^+, ADH catalyzes the following reaction:

$$\text{Ethanol} + NAD^+ \rightarrow \text{Acetaldehyde} + NADH + H^+$$

In this reaction not only the substrate is not charged, and therefore not detectable *via* direct conductivity measurements, but also the product, acetaldehyde is electrically neutral. However, the cofactor NAD^+ is reduced in the reaction to a negatively charged product, and thus an indirect monitoring of this enzymatic reaction with CE-C^4D was deemed possible. Note, that we have followed the usual denotations for both forms of the cofactor, which do not reflect the charges on the molecules as both species are negatively charged at neutral pH-values.

Repeated injections of a reaction mixture, containing ethanol, NAD^+, and ADH, were made in order to follow the course of the enzymatic reaction, and the results are shown in Fig. 3. Clearly, an increase of the NADH peak, commensurate with the enzymatic oxidation of ethanol could be observed.

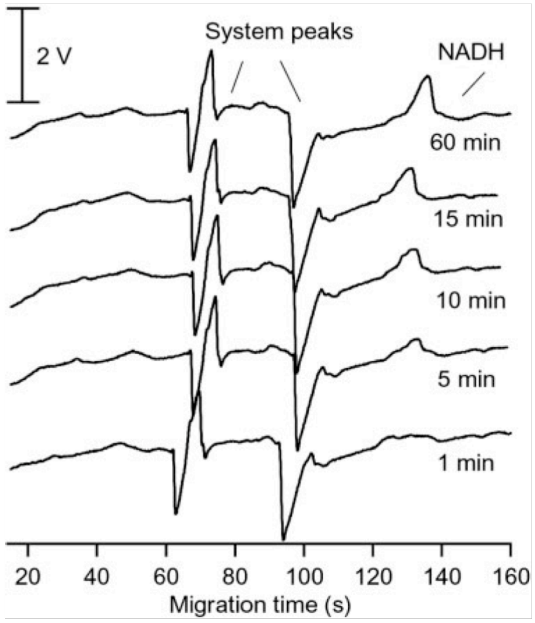

Figure 3. Electropherograms obtained with a standard capillary for the conversion of ethanol with ADH. Reaction mixture: 1 mL 20% v/v ethanol, 0.4 mL 100 mM NAD, and 0.1 mL 1 mg/mL ADH, room temperature. Buffer: 50 mM CHES/20 mM Arg/50 μM CTAB, pH 9.1. Other conditions as for Fig. 1.

3.1.3 Esters

Esterases hydrolyze esters into their corresponding acids and alcohols; they are widely distributed in organisms and play important roles in the metabolism of various compounds. Ethyl acetate was chosen as an initial model substrate to evaluate the suitability of CE with C^4D for the monitoring of this type of enzymatic conversion:

$$\text{Ethyl acetate} + H_2O \rightarrow \text{Acetate}^- + H^+ + \text{Ethanol}$$

The only ionic species in this reaction is the anion of the acetic acid produced. The determination of small carboxylates is readily possible in CE with conductivity detection, see for example ref. [27–30], so that the indirect determination of the ester *via* determination of acetate as the reaction product was expected to be feasible.

As seen in Fig. 4, a peak for acetate is visible a short time after initiating the reaction with porcine liver esterase and it is possible to monitor the gradual increase in its concentration as the conversion progresses. Peak identification is again simple in this case as acetate is the only anion in the reaction mixture.

Similarly, it was found possible to also follow the hydrolysis of ethyl butyrate:

$$\text{Ethyl butyrate} + H_2O \rightarrow \text{Butyrate}^- + H^+ + \text{Ethanol}$$

The same conditions as for the hydrolysis of ethyl acetate were employed. The results given in Fig. 5 are very similar, but it appears that, at least for the conditions employed, the hydrolysis of ethyl butyrate is slower than that of the acetate ester.

Among the five enzymatic reactions carried out, the hydrolysis of ethyl acetate was chosen as an example to investigate the possibility of carrying out quantitative measurements. The substrate ethyl acetate at concentrations between 80 and 4.1 mM was digested with esterase according to the conditions as given for Fig. 4. A reaction time of 2 h was allowed in each case before injection into the CE-system for quantification of the acetate produced. The resulting calibration curve is given in Fig. 6 along with a calibration curve obtained for pure acetate standards. A linear calibration curve was obtained for the acetate standards with the following regression equation: peak area = 5.305 conc. (mM) − 0.2283 (r^2 = 0.9999). The detection limit for acetate

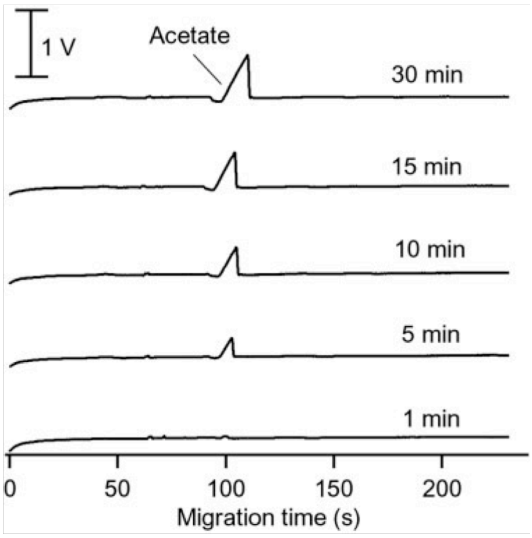

Figure 4. Electropherograms obtained with a standard capillary for the conversion of ethyl acetate with esterase. Reaction mixture: 1 mL 0.2% v/v ethyl acetate and 0.1 mL 1 mg/mL esterase, room temperature. Buffer: 15 mM MES/16 mM Arg/50 μM CTAB, pH 8.0. Other conditions as for Fig. 1.

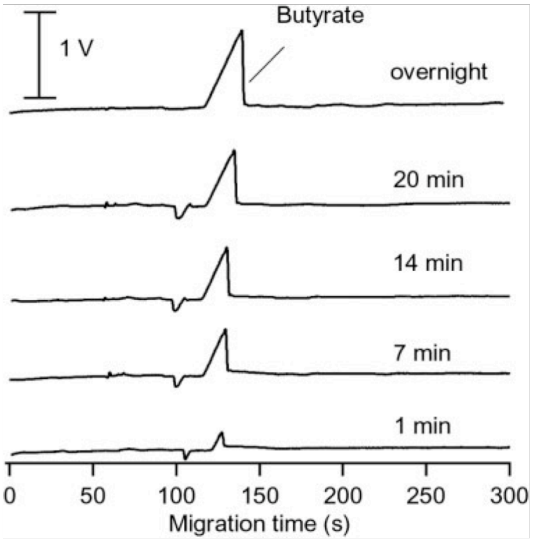

Figure 5. Electropherograms obtained with a standard capillary for the conversion of ethyl butyrate with esterase. Reaction mixture: 0.4 mL 1% v/v ethyl butyrate and 0.1 mL 1 mg/mL esterase, room temperature. Buffer: 15 mM MES/16 mM Arg/50 μM CTAB, pH 8.0. Other conditions as for Fig. 1.

was determined as 7.0 μM (estimated concentration giving a peak height corresponding to three times the baseline noise, obtained through an extrapolation of the calibration curve for the acetate standard of Fig. 6). The results for the acetate produced from ethyl acetate indicate that under the conditions used the conversion is not complete. As would be expected, the fraction of conversion is smaller for the higher concentrations and hence the calibration curve for acetate produced from ethyl acetate is not linear.

3.2 Microfluidic device

Two of the enzymatic conversions reported above were also carried out on an electrophoresis chip to demonstrate the possibility of using this platform as well. A microfluidic device with the standard channel configuration of an elongated cross was used, and the enzymatic digestion was carried out in the injection well on the chip itself.

The conversion of glucose to gluconate using GOX for catalysis is illustrated in Fig. 7. The buffer and reaction conditions were identical to those used with the standard capillary, but the reaction was carried out at room temperature (approximately 20°C) instead of 35°C owing to the fact that the chip station could not be thermostatted easily. As the electropherograms shown in Fig. 7 demonstrate, a clean peak of gluconate was observed and the increase of this peak was followed with the repeated injections at 0, 16, 40, 55, and 80 min. Note that the automated chip station allowed a first injection with virtually no delay after mixing of reagents.

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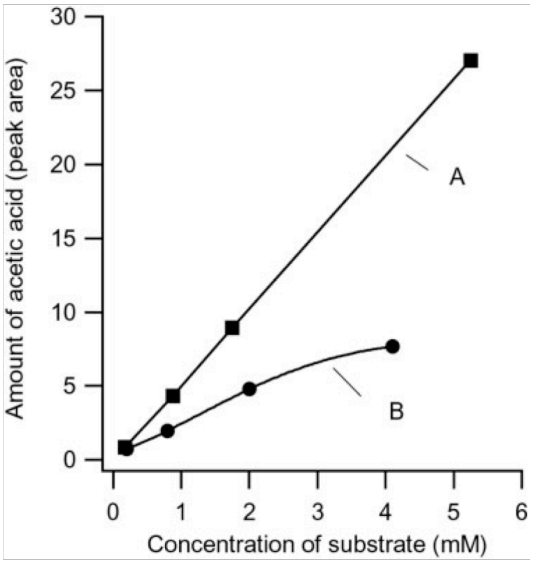

Figure 6. Calibration curves for acetate obtained in a standard capillary by injecting acetate standards (A) and the digestion product of ethylacetate with esterase (B). Conditions as for Fig. 4.

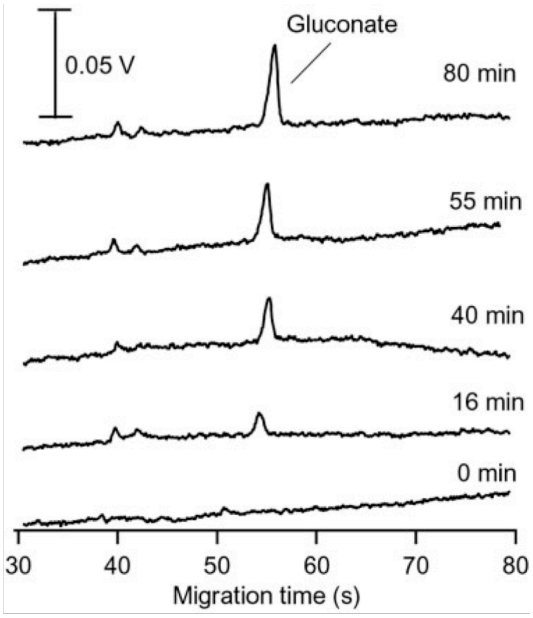

Figure 7. Electropherograms obtained on a microchip for the conversion of glucose with GOX. Reaction mixture: 0.06 mL 100 mM glucose and 0.06 mL 3 mg/mL GOX, room temperature. Buffer: 20 mM MES/5 mM His/50 μM CTAB, pH 5.7. Injection: electrokinetic injection for 7 s at 1 kV. Separation voltage: −5 kV.

The enzymatic hydrolysis of ethyl acetate was also successfully carried out on-chip as illustrated by the electropherograms shown in Fig. 8. For a complete set of data for the integrated peak areas, the plot of Fig. 9 is obtained. It is thus possible to use this method as an easy approach to monitor the reaction kinetics of this enzymatic conversion.

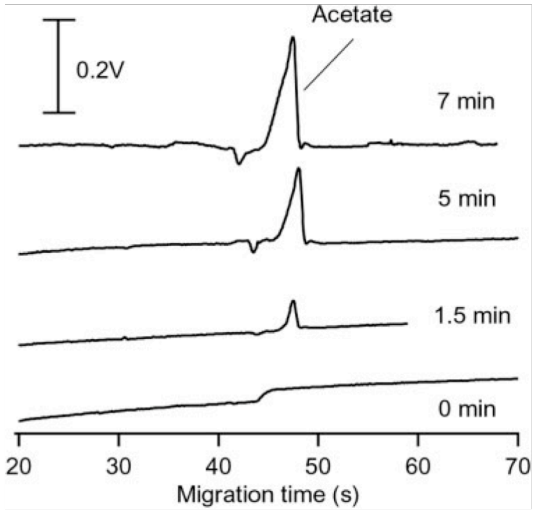

Figure 8. Electropherograms obtained on a microchip for the conversion of ethyl acetate with esterase. Reaction mixture: 0.1 mL 10% v/v ethyl acetate and 0.05 mL 1 mg/mL esterase, room temperature. Buffer: 15 mM MES/16 mM Arg/50 μM CTAB, pH 8.0. Injection: electrokinetic injection for 7 s at 1 kV. Separation voltage: −5 kV.

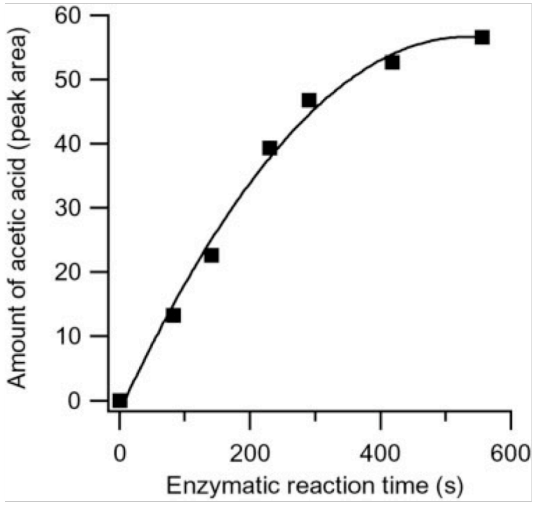

Figure 9. Increase of acetic acid concentration produced by hydrolysis of ethyl acetate with esterase on a microchip. Conditions as for Fig. 8.

4 Concluding remarks

It is thought that this is the first report on the use of CE with contactless conductivity detection for the monitoring of enzymatic reactions. Not only are the nonionic substrates not accessible to electrophoresis, but also the products are not detectable by direct UV-absorbance detection because of a lack of strong chromophores. The auxiliary, color-forming reactions needed in clinical analysis are not required when using CE-C^4D for quantification. The on-chip experiments indicate the possibility of using this platform for routine analysis and also demonstrate the potential of CE-C^4D for microprocess monitoring. On the other hand, when using external electrodes for conductivity detection, the sensitivity on the microchip cannot be expected to be as good as what is achieved on conventional capillaries [25]. Furthermore, when high selectivity is required, as for example in the determination of glucose with the hexokinase method, the separation efficiency obtained with a chip might not be adequate.

Funding for this work was provided by the Swiss National Science Foundation through Research grant no. 200020-105176/1 and 200020-113335/1 and a Marie Heim-Vögtlin Scholarship for A. Schuchert-Shi (grant no. PMCD2-110198/1).

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Monitoring the enzymatic conversion of urea to ammonium by conventional or microchip capillary electrophoresis with contactless conductivity detection

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ABSTRACT

Capillary electrophoresis with contactless conductivity detection was used to directly quantify the ammonium produced in the enzymatic conversion of urea with urease. This allowed the characterization of the reaction without having to use more elaborate indirect optical methods for quantification. The maximum rate of reaction, V_{max}, was determined as 5.1 mmol·mL^{-1}·min^{-1}, and the Michaelis–Menten constant, K_m, was determined as 16 mM. Furthermore, the method was successfully applied to the determination of urea in clinical samples of human blood by using a conventional capillary and a microchip device.

Conductometric detection can be regarded the most universal quantification method in capillary electrophoresis (CE)[1] because it is, at least in principle, applicable to all ions. The method is of primary interest for the analysis of species that do not absorb UV radiation nor fluoresce and therefore are not accessible by the standard detection methods in CE. Although conductivity detection has been used for electrophoresis since the introduction of modern capillary electrophoresis in the 1980s, it has, however, not been widely applied due to the difficulty of constructing measuring cells with the small dimensions needed and the necessity of decoupling the separation voltage from the electrochemical detector. A commercial conductivity detector available in the mid-1990s disappeared from the market after a short period. Only the description of a contactless arrangement (capacitively coupled contactless conductivity detection, C^{4}D) based on external tubular electrodes by two independent research groups in 1998 [1,2] opened the path to a wider adoption of conductivity detection. Following the conception, several research groups studied the fundamental properties over several years using purpose-built devices (see for example [3–7]) and a comprehensive elucidation and optimization was reached by about 2004. This then recently led to the availability of commercial detectors, which can be retrofitted to most existing equipment.

Capillary electrophoresis with contactless conductivity detection is relatively simple and therefore has also been adopted for lab-on-chip devices (see for example [8–11]). The use of C^{4}D on this platform is attractive because detection by UV absorption is difficult to implement on microfabricated devices and tends to be limited in sensitivity.

The first reported applications of C^{4}D for CE concerned inorganic species. More recently, the determinations of organic ions, for example carboxylic acids [12,13], sulfonic acids [14], quaternary amines [2,14,15], amino acids [16,17], catecholamines [18], basic drugs [19], saccharides [20], and human immunoglobulin [21], were also communicated. Recent reviews summarizing the fundamental developments and reported applications are available [22–26].

Urea is routinely determined in blood serum samples because it serves as a predictive index of renal failure and as a diagnostic aid in distinguishing between the various causes of renal insufficiency. The most common way to detect urea is to employ the enzyme urease to catalyze the hydrolysis of urea and to determine the products:

$$NH_2CONH_2 + 2H_2O + H^+ \rightarrow 2NH_4^+ + HCO_3^-.$$

Because the products, ammonium and bicarbonate, are not optically active, in clinical analysis this reaction is usually followed by a second enzymatically catalyzed step in which the conversion of the cofactor NADH is used for indirect quantification via molecular absorption photometry in the UV range:

$$2NH_4^+ + \alpha\text{-ketoglutarate} + 2NADH \rightarrow 2L\text{-glutamate} + 2NAD^+ + 2H_2O.$$

For studies of the enzyme kinetics of urease this two-step procedure is not suitable [27] and therefore alternative methods, using for

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[1] *Abbreviations used:* CE, capillary electrophoresis; C^{4}D, capacitively coupled contactless conductivity detection; PMMA, poly(methylmethacrylate); Mes, 2-morpholinoethanesulfonic acid monohydrate.

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example chemiluminescence [27], microcalorimetry [28] or ammonium-selective electrodes [29], have been developed for this purpose. Duffy et al. [30] described the batchwise use of a specially designed cell for measurement of the increase in bulk conductance due to the charged products obtained in the enzymatic conversion of urea. Thavarungkul et al. [31] used a flow-through system in which the urea was separated from the sample matrix by dialysis and then converted to the charged products in a reactor with immobilized urease. Quantification was again carried out by conductivity measurement.

The use of capillary electrophoresis with contactless conductivity detection for the monitoring of enzymatic reactions has recently been demonstrated for conversions of ethanol, glucose, ethyl acetate, and ethyl butyrate [32]. In the present project, the suitability of conventional CE and microchip electrophoresis with C^4D for application in urease enzymatic assays and therefore also in the determination of urea in human blood serum was investigated.

Material and methods

Instrumentation

Separations in capillaries and on microchips were performed on instruments built in-house. The conventional instrument was based on a power supply with adjustable high-voltage output up to ±30 kV (Model CZE2000; Start-Spellman, Pulborough, UK). The instrument features a safety interlock to prevent accidental exposure to the separation voltage. Fused-silica capillaries with 75 μm ID and 60 cm length from Polymicro (Phoenix, AZ, USA) were used for the separations. The detector was also built in-house and is based on two tubular electrodes of 4 mm length which are separated by a detection gap of 2 mm and a Faraday shield. A schematic sketch of the cell is given in Fig. 1, and more details on the contactless conductivity detector can be found in an earlier publication [33]. For microchip analysis, poly(methylmethacrylate) (PMMA) devices with a separation channel length of 80 mm were purchased from the Chip-Shop (Jena, Germany). The depth and width of the channel was 50 μm. The chips were mounted on a holder constructed from PMMA which was fitted with a pair of antiparallel-oriented electrodes of 1 mm width, 1.4 cm length and with a gap of 0.5 mm. This detector arrangement is also shown in Fig. 1.

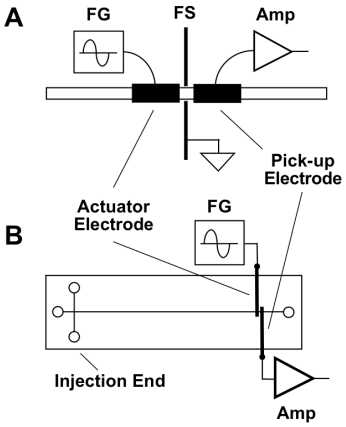

Fig. 1. Sketches of the detector assemblies for a conventional capillary (A) and an electrophoresis chip (B). FG, function generator; Amp, pick-up amplifier to convert the cell current to an AC voltage; FS, grounded Faraday shield.

Two high-voltage power supply units (Model CZE1000R; Start-Spellman) required for injection and separation were controlled with a LabVIEW (National Instruments, Austin, TX, USA) program via a multifunctional I/O card (Model PCI-MIO-16XE-50; National Instruments) located in the computer. All data acquisition and analysis were performed with a MacLab/4e system (ADinstruments, Hastings, UK) which was connected to a Macintosh personal computer (Apple, Cupertino, CA, USA). Note, that the output of the detectors is given in arbitrary voltage values; a calibration of the cells in terms of absolute conductivity is not needed, because only small relative changes from the baseline constitute the signal of interest.

Reagents and samples

All chemicals were of analytical-reagent grade and were used as received. 2-Morpholinoethanesulfonic acid monohydrate (Mes), maleic acid, urea, urease (from jack bean, 9.2 $U \cdot mg^{-1}$), and 18-crown-6 were purchased from Fluka (Buchs, Switzerland); L-arginine (Arg) was from Acros (Geel, Belgium), and ammonium chloride was from Merck (Darmstadt, Germany). Deionized water was used throughout. Solutions were passed through 0.2-μm nylon filters (BGB Analytik AG, Böckten, Switzerland) before analysis. The human blood serum samples were obtained from the Clinical Chemistry Laboratory of the University Hospital Basel and kept in a refrigerator until use. Reference measurements were performed at the Clinical Chemistry Laboratory of the University Hospital Basel using a standard photometric method based on a secondary enzymatic reaction using NADH as cofactor.

Enzymatic reactions and separations

All experiments were carried out at a room temperature of approximately 22 °C. The reactions were carried out in 1.5- mL vials. These were shaken manually for about 10 s after transfer of all solutions to ensure good mixing. For the kinetic studies the reaction mixture was composed of 0.75 mL urea of a chosen concentration and 0.75 mL of a 0.1 $mg \cdot mL^{-1}$ urease solution; both solutions were prepared in the Mes buffer. In the blood serum sample tests, the reaction mixture consisted of 0.75 mL of the sample solution diluted 1:100 and of a 0.15 $mg \cdot mL^{-1}$ urease solution, both contained in a buffer consisting of 5.5 mM maleic acid, 7.5 mM Arg, and 1.5 mM 18-crown-6, pH 5.5. All solutions were prepared freshly every day.

Separations were carried out in the same buffers as used for the enzymatic reactions. Capillaries used for CE separation were preconditioned with 1 M NaOH for 10 min, deionized water for 10 min, 1 M HCl for 10 min, and deionized water for 10 min before flushing with running buffer for 20 min. They were rinsed again with 0.1 M NaOH for 5 min and the buffer solution for 5 min when peak distortions were observed. Otherwise capillaries were flushed with the buffer solution after every 10 runs. Samples were hydrodynamically injected, directly from the vials in which the reaction was taking place, by siphoning at 15 cm height difference for 10 s. The separation voltage was +10 kV for the enzyme kinetic studies and +20 kV for the serum analysis. The chip was flushed first with deionized water for 10 min and then with the buffer solution for 10 min before the first analysis. Electrokinetic injections were performed at +1 kV for 7 s and the separations were carried out at +5 kV.

Results and discussion

Determination of ammonium by CE-C^4D

Electropherograms acquired at different time intervals following the mixing of a urea standard and urease in a buffer solution

of pH 7.0 are shown in Fig. 2. Clearly, the product of the reaction, the ammonium cation, can be detected readily using the conductometric method. The triangular shape of the peaks is a common feature of zone electrophoresis, and the slight distortions at the top of the peaks and their tail ends are artifacts of the contactless conductivity detector which are of no consequence for quantification [34]. A calibration curve for ammonium was acquired with ammonium chloride standards in the concentration range 2–50 mM. The following linear regression equation was obtained: y [mV·s] = 1.013 · conc [mM] + 1.016 (correlation coefficient, r, = 0.996).

Examination of the enzyme kinetics

Monitoring the amount of ammonium obtained as product of the enzymatic conversion allows the direct study of the enzyme kinetics. In Fig. 3, the concentrations of ammonium obtained for a fixed concentration of urease of 0.1 mg·mL^{-1} after different time intervals are given. Several starting concentrations of urea were investigated. It is evident that for the lower concentrations of the substrate (2, 5, and 10 mM) a decrease in ammonium production is reached after about 500 due to the near completion of the conversion. For the higher concentrations (20 and 30 mM) the reaction is not complete at the end of the time interval.

Because the efficiency of the enzymatic reaction is pH dependent, a series of experiments was carried out at pH values of 5.2–7.6 using constant concentrations of urea (20 mM) and urease (0.1 mg·mL). The data for three of the pH values investigated are shown in Fig. 4 in the form of plots of the ammonia concentrations produced after different time intervals. As can be seen, under the conditions employed, the initial rate of production of ammonium was maintained in each case up to the maximum monitored time of 750 s. The reaction rate, *v*, of an enzymatic reaction is given by $v = -d[S]/dt = d[P]/dt$, where [S] and [P] denote the concentrations of the substrate and product, respectively. Thus the initial rate of reaction is obtained from the slopes of the data as given in Fig. 4. Plotting v for all pH values investigated results in the graph of Fig. 5. The results correspond to the expected behavior with pH and the data agree with results published, for example, by Krajewska et al. [29], who studied urease kinetics with an ion-selective electrode for ammonium.

A plot of the initial reaction rates, v, vs substrate concentration obtained at a fixed pH-value of 7.0 is given in Fig. 6. This nonlinear

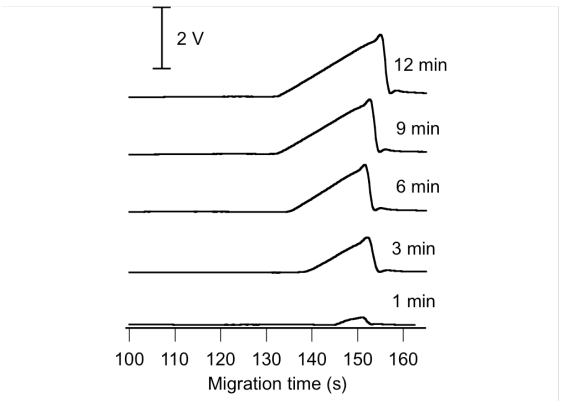

Fig. 2. Electropherograms for ammonium produced from a urea standard by enzymatic conversion measured at increasing time intervals. Buffer, 20 mM Mes, pH 7.0. Hydrodynamic injection, 10 s at 15 cm elevation. Separation voltage, +10 kV. Reaction mixture, 0.75 mL of 20 mM urea + 0.75 mL of 0.1 mg·mL^{-1} urease.

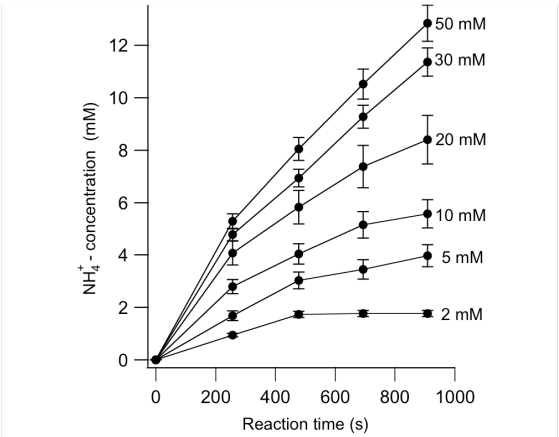

Fig. 3. Production of ammonium with time for different starting concentrations of urea. The data points are the averages of five measurements; error bars denote the relative standard deviations. Conditions as for Fig. 1.

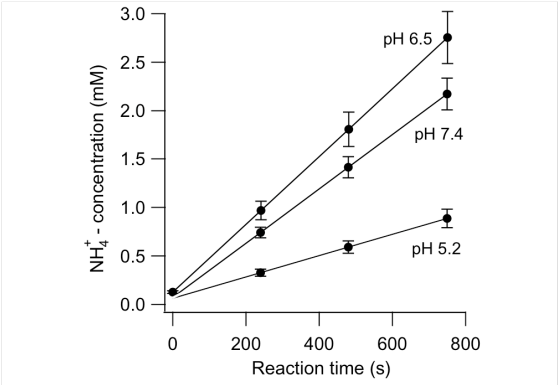

Fig. 4. Production of ammonium with time at different pH values. Concentration of urea, 20 mM. The data points are the averages of five measurements; error bars denote the relative standard deviations. Conditions as for Fig. 1.

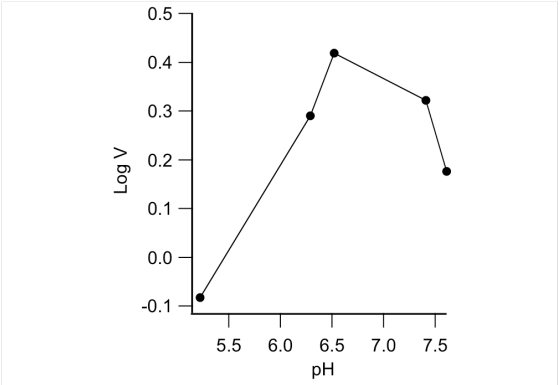

Fig. 5. Plot of the logarithm of the initial rates of reaction, *v*, against the pH value. Conditions as for Fig. 1.

behavior is generally expected for enzymatic reactions[35] and the data are further analyzed below.

In Fig. 7, the data are plotted according to Lineweaver–Burk:

$$\frac{1}{v} = \frac{K_M}{V_{\max}[S]} + \frac{1}{V_{\max}}.$$

K_m is the Michaelis–Menten constant, and $V_{\max}$ is the maximum rate of reaction.

Examination of Fig. 7 shows that the plot is linear, confirming that the reaction follows one-substrate enzyme kinetics. From the intercept of the Lineweaver–Burk plot, the maximum rate of reaction, $V_{\max}$, was found to be 5.1 mmol·mL^{-1}·min^{-1} and, from the slope of the linear fit of the data, K_m was determined as 16 mM. According to Mobely and Hausinger [36] the K_m values for ureases can vary widely between about 0.1 and 100 mM depending on the source of the enzyme. For jack bean urease, which was used in our study, Lynn [37] reported a value of 6.1–8.0 mM (pH 7.5, 21 °C), Petersen et al. [38] determined a value of 10 mM (pH 7.0, 25 °C), and Blakely and Zerner [39] quoted a value of 2.9 mM (pH 7.0, 38 °C).

Determination of urea in human serum samples by conventional capillary electrophoresis

The possibility of determining urea in serum samples was first investigated by examining the separation. The background electrolyte solution based on Mes is not ideal for the analysis of the inorganic cations in serum; in particular the separation of Na^+, Ca^{2+}, and Mg^{2+} is not perfect. For this reason a buffer based on maleic acid and arginine, which had been optimized for this task [40], was adopted because this would allow the concurrent determination of these cations also. This buffer solution contains the crown ether 18-crown-6 which also enables separation and hence selective determination of ammonium and potassium, two cations of similar size, by partial complexation. As illustrated by the electropherograms of a serum sample obtained after increasing time intervals after addition of the reagent urease (Fig. 8), the NH_4^+ produced from the hydrolysis of urea with urease can be easily detected in a serum sample. Along with the ammonium and potassium peaks, the metal ions Na^+, Ca^{2+}, and Mg^{2+} were also detected in the blood samples. Note that the native concentration of ammonium in blood serum is negligible (approximately 22 μM [41]) in comparison to the levels produced from urea.

Quantification of urea in 10 serum samples was subsequently conducted. To assure completeness of the enzymatic conversion,

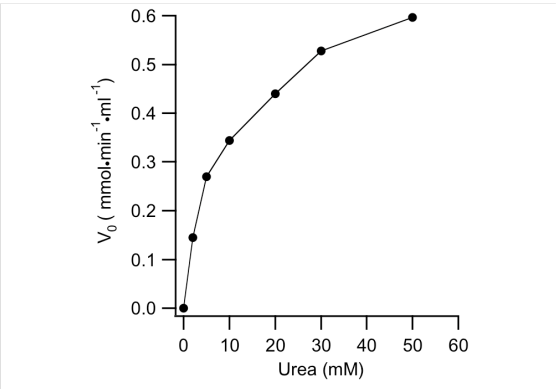

Fig. 6. Michaelis–Menten curve, a plot of the initial rates of reaction against the starting concentration of urea. Conditions as for Fig. 1.

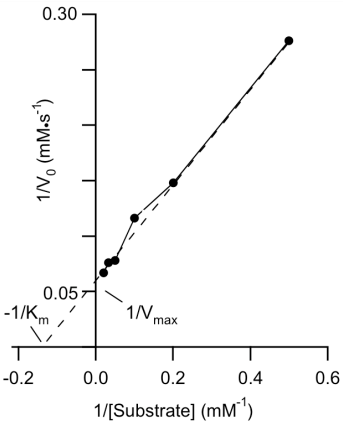

Fig. 7. Lineweaver–Burk plot. Conditions as for Fig. 1.

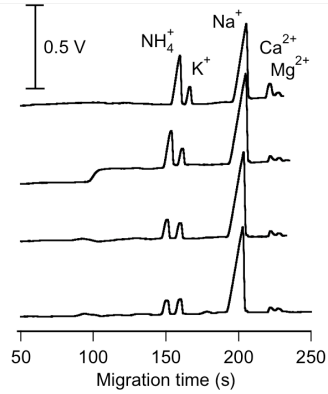

Fig. 8. Electropherograms showing the production of ammonium from urea after different time intervals for one of the serum samples (No. 5). Buffer, 5.5 mM maleic acid, 7.5 mM L-arginine, 1.5 mM 18-crown-6, pH 5.5. Separation voltage, +20 kV. Other conditions as for Fig. 1.

a relatively high concentration of urease (0.2 mg·mL^{-1}) in the reaction mixture was used, and a reaction time of 2 h was allowed. A recovery of 99.5% was confirmed for a standard solution of 20 mM urea against ammonium standards. A calibration was carried out in the maleic acid/arginine buffer for concentrations of 2– mM (four points, $n = 5$ for each concentration). The linear regression equation was obtained as y [mV·s] = 4.5833 · conc [mM] + 0.0178 ($r = 0.9999$). The urea values determined in the human blood serum samples are shown in Table 1. It was found that, with the exception of sample Nos. 8 and 9, the results were close to the values provided by the hospital, which had been determined with a standard photometric method. The reason for the bias of the two samples is not known. The correlation coefficient, r, was calculated as 0.961.

Determination of urea in human serum samples on a microchip device

Whether the indirect determination of urea is also possible on a microfabricated device was also investigated. An electrophoresis chip with a manifold in the often-used configuration of an elongated cross and a separation length of approximately 8 cm was em-

Table 1
Concentrations of urea in human blood samples determined by conventional and microchip CE

Sample	Conventional CE		Microchip CE		Reference (mM)
	(mM)	RSD (n = 5),%	(mM)	RSD (n = 5),%	
1	2.8	1	4.4	2	3.1
2	3.5	0.6	4.6	2	3.5
3	5.6	0.8	5.5	2	5.6
4	6.0	0.3	5.7	2	6.5
5	7.7	0.6	9.6	3	7.9
6	10.6	1.4	9.0	2	8.8
7	10.3	0.2	10.0	4	10.3
8	17.3	1	13.9	4	11.5
9	18.3	1.4	19.5	5	14.7
10	19.7	0.7	17.5	6	18.4

The reference values were determined at the University Hospital of Basel using a standard NADH-based photometric method.

ployed. The digestion was carried out in microvials before the mixture of the solution was injected into the microchip. As evidenced by the electropherograms for a serum sample after addition of urease (Fig. 9), the inorganic ions can also be determined on this platform, even though the separation of ammonium and potassium ions is only just possible. The same buffer as that used for the separation in the conventional capillary was employed. A calibration for urea for the range 2–30 mM (three points) carried out after a digestion period of 2 h gave a correlation coefficient for a linear regression of $r = 0.9999$. The urea concentrations in the human blood serum samples determined on chip are also given in Table 1. In comparison to the results obtained through conventional CE, the results from microchip were less close to the reference values from the hospital. The correlation coefficient, r, was calculated as 0.947.

Conclusions

Both conventional CE and microchip CE with contactless conductivity detection could be successfully used for the direct monitoring of the enzymatic conversion of urea to ammonium and for the determination of urea in clinical samples. The methods are a simpler alternative to the photometric methods which require an auxiliary reaction to enable quantification. It is hoped that the new approach will prove useful for the direct study of the mechanism of further enzymatic reactions which could otherwise be monitored only indirectly and for clinical applications.

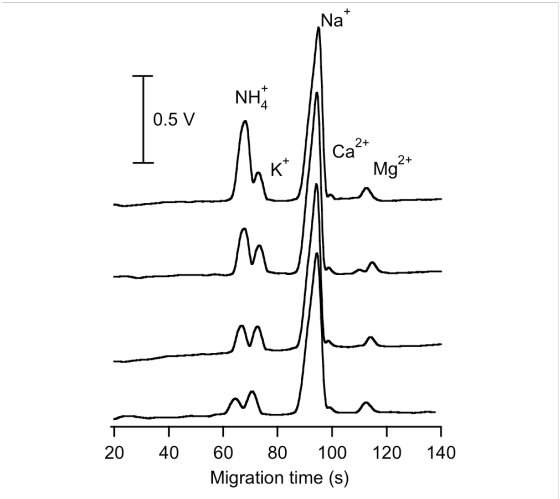

Fig. 9. Electropherograms obtained with an electrophoresis chip showing the production of ammonium from urea after different time intervals for one of the serum samples (No. 5). Buffer, 5.5 mM maleic acid, 7.5 mM L-arginine, 1.5 mM 18-crown-6. Electrokinetic injection, 7 s at 1 kV. Separation voltage; +5 kV.

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Peptic and tryptic digestion of peptides and proteins monitored by capillary electrophoresis with contactless conductivity detection

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ABSTRACT

The feasibility of monitoring the peptic and tryptic digestion of peptides and proteins with capillary electrophoresis using contactless conductivity detection was investigated. The peptide minigastrin I and the proteins cytochrome *c* from bovine heart, human serum albumin (HSA), myoglobin, and bovine serum albumin (BSA) were digested off-line with pepsin, and the resulting peptide and amino acid fragments were successfully separated and detected by conductivity measurement. Cytochrome *c* and myoglobin were also subjected to off-line cleavage with trypsin. On-line digestion using the electrophoretically mediated microanalysis (EMMA) approach was demonstrated with cytochrome *c* and apomyoglobin using trypsin.

The proteolytic enzymes pepsin and trypsin are frequently used for in vitro studies of proteins. Both enzymes occur in the digestive tract and break down proteins, but they preferentially cleave the proteins only at specific peptide bonds. Trypsin in particular, and pepsin to a lesser extent, is widely used in the identification of proteins via the determination of the resulting unique peptide patterns [1]. Pepsin digestion is often used in food chemistry to assess the digestibility of proteins. Wilfart and coworkers, for example, have studied the in vitro digestion of pig feeds [2]. Degradation of proteins by pepsin is also investigated in artificial stomach fluid as their digestibility relates to their allergenicity (see, e.g., Ref. [3]). Of further interest is the study of the decomposition products in view of their biological activities. Schmelzer and coworkers, for example, investigated the peptides obtained from β-casein of bovine milk by in vitro digestion with pepsin [4].

Following the tryptic or peptic digestion, the peptide fragments obtained are analyzed by mass spectrometry (MS)[1] or by a separation technique such as gel electrophoresis, high-performance liquid chromatography (HPLC), or capillary electrophoresis (CE) (see, e.g., the review in Ref. [5]). When column separation is employed, detection is often carried out again by MS, but other detection methods, in particular ultraviolet (UV) absorbance, have been used for HPLC separations of digestion products [6,7] and CE [8–10]. CE is often used for the study of enzymatic reactions due to the high separation efficiency and its suitability for small sample volumes. An overview of such applications can be found in the review by Glatz [11]. Liu and coworkers reported laser-induced fluorescence detection on an electrophoresis chip of tryptic digestion products of bovine serum albumin (BSA) that had been derivatized with fluorescein isothiocyanate (FITC) [12].

For CE, during recent years a new detection method, capacitively coupled contactless conductivity detection (C^4D), has been developed. It relies on external electrodes to affect the monitoring of the conductivity of the solution inside the capillary and has unprecedented simplicity (for reviews of the fundamental principles, see Refs. [13–19]). C^4D is often applied to the determination of inorganic and small organic ions [20,21], but its inherent universality also makes it well suited also for the determination of biochemical species.

Several research groups have reported the determination of amino acids by CE–C^4D [22–26]. Small peptides were determined by Gong and coworkers [27]. Abad-Villar and coworkers found that it was also possible to detect larger peptides, proteins, and immunoglobulins [28,29]. The analysis of DNA fragments was reported by Xu and Li [30]. The use of CE–C^4D in enzymatic methods has been reported previously. Schuchert-Shi and coworkers reported the monitoring of the enzymatic conversions of the neutral species ethanol by alcohol hydrogenase, of glucose by hexokinase and glucose oxidase, and of ethyl acetate and ethyl butyrate by esterase to charged products, which could be separated by CE but needed the

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[1] *Abbreviations used:* MS, mass spectrometry; HPLC, high-performance liquid chromatography; CE, capillary electrophoresis; UV, ultraviolet; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; C^4D, capacitively coupled contactless conductivity detection; His, histidine; Tris, tris-(hydroxymethyl)-aminomethane; HSA, human serum albumin; BSA, bovine serum albumin; HCl, hydrochloric acid; EMMA, electrophoretically mediated microanalysis.

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conductivity detector for quantification [31]. Urban and coworkers demonstrated the monitoring of the action of tyramine oxidase on tyramine by CE–C^4D [32]. Also successful was the indirect determination of urea in clinical samples via the determination of the enzymatic degradation product ammonium [33].

The work reported here is an extension of this previous work on enzymes. The possibility of employing CE–C^4D to monitor both peptic and tryptic digestion of peptides and proteins was investigated. Although C^4D does not allow an identification of the fragments, the method may allow the recognition of proteins by fingerprinting and should be suitable for the monitoring of the progress of a digestion reaction.

Materials and methods

Instrumentation

All digestions were carried out in 1.5-ml microvials. Separations were conducted in conventional fused-silica capillaries either on an electrophoresis instrument built in-house or on a commercial unit. The former includes a perspex box, a function generator, a C^4D cell, and detector electronics. The perspex box is subdivided into injection and detection compartments. A microswitch is fitted and interrupts the power supply on opening for safety. The separation voltage was provided by a high-voltage power supply from Spellman (model CZE2000, Pulborough, UK). The commercial system was a PrinCE instrument (600 series, Prince Technologies, Emmen, The Netherlands). Both instruments were fitted with a contactless conductivity detector constructed in-house. The details on the detector cell can be found in a previous publication [34]. The cell current was transformed into an AC voltage with an operational amplifier (OPA655, Texas Instruments, Dallas, TX, USA) in the current follower configuration (feedback resistor = 220 kΩ). The signal was then rectified and further amplified. The electropherograms were recorded on a MacLab/4e data acquisition system (AD Instruments, Castle Hill, Australia).

Reagents and methods

All chemicals used in this work were of analytical grade. Acetic acid, histidine (His), pepsin, trypsin, Tris (tris-(hydroxymethyl)-aminomethane), and human serum albumin (HSA) (fraction V, product no. 05430) were purchased from Fluka (Buchs, Switzerland). The proteins cytochrome *c* from bovine heart, myoglobin from horse heart, and bovine serum albumin (BSA) (fraction V, product no. A-3059) were obtained from Sigma (Buchs, Switzerland). The peptide minigastrin I (human, HG-13) was obtained from Bachem (Bubendorf, Switzerland). Ammonium bicarbonate and the surfactant Tween 20 (polyoxyethylene sorbitan monolaurate, 70% [m/m] in water) was also purchased from Sigma. Hydrochloric acid (HCl) was supplied by Fluka. Deionized water obtained from a Milli-Q Plus 185 system (Millipore, Bedford, MA, USA) was used for the preparation of the buffer solutions. Stock solutions were prepared and diluted in the running buffers. All peptide and protein solutions were frozen at −20 °C for storage except HSA and the pepsin and trypsin solutions, which were kept at 4 °C. Capillaries with 75-μm inner diameters and 365-μm outer diameters and a total length of 50 cm were used except in the experiments on the commercial instrument, for which capillaries of 70 cm length were used. These had been purchased from Polymicro Technologies (Phoenix, AZ, USA). The capillaries were preconditioned with sodium hydroxide and hydrochloric acid (0.1 M each) and prior to sample injection were thoroughly flushed with the background electrolyte. For the samples digested off-column, the separations were carried out on the instrument built in-house and the injections were performed hydrodynamically at 15 cm elevation for 10−s. The separation voltage was +25 kV. The electrophoretically mediated microanalysis (EMMA) experiments were carried out on the commercial instrument because this could be thermostatted and allowed the precise timing of the automated injection sequence. Injections were made by applying 250 mPa for 10 s for both samples and reagents. Mixing of the plugs was carried out by applying 5 kV for 5 s. These conditions were arrived at empirically, but a detailed optimization was not carried out. The separation voltage used for these experiments was +30 kV.

Results and discussion

Peptic digestion

Because pepsin is found in the stomach, it is most active in an acidic environment in the pH range between approximately 1.5 and 2.0 and a temperature of approximately 37 °C. Pepsin is also found to autocatalyze its own breakdown.

Peptide: Minigastrin I

Amino acids are best separated and detected under acidic conditions in cationic form [22,23], and peptides may also be determined under these conditions [29]. The low pH value ensures a positive charge of all compounds and, therefore, fast migration and sensitive detection by conductometry. Because peptic digestions need to be carried out in an acidic environment, both steps (the digestion and the separation of the products) can be performed under the same conditions. Thus, a background electrolyte consisting of 2.3 M acetic acid and 0.05% (v/v) Tween with a pH value of 2.1 was employed throughout the experiments. Tween is included to eliminate any possibility of wall adsorption of the analytes. Minigastrin I was chosen as the first model substance for the experiment. It is a commercially available peptide and has a molar mass of 1646 g mol^{-1}. Here 0.2 ml of a solution of 300 μM minigastrin I and 0.01 ml of 50 μM pepsin were prepared separately in the background electrolyte and first thermostatted at 37 °C in a water bath for 30 min before mixing together. The mixture was kept in the water bath throughout the experiment. The amino acid sequence of minigastrin I is as follows: H-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Try-Met-Asp-Phe-NH_2. Pepsin cleaves preferentially at the carboxyl side of phenylalanine, leucine, and glutamic acid residues, and it does not cleave at bonds connecting valine, alanine, or glycine. Therefore, in minigastrin I, any of the bonds that are underlined in the sequence given above may be cleaved by pepsin. With a complete digestion, six bonds might be broken, and after complete digestion, four fragments are expected. For intermediate digestion, up to seven different fragments may be present. Aliquots of the reaction mixture were analyzed at 0, 10, 20, and 60 min and after overnight reaction. The results are shown in Fig. 1. Note that the concentration of the enzyme pepsin in the final reaction mixture was kept low to minimize autocatalysis. A peak due to pepsin occurs before the time window shown in the figure, and the concentration of autocatalysis products is expected to be below the detection limits. As can be seen in Fig. 1, for the first three injections, only a peak for the substrate minigastrin I can be identified. Clearly discernible peaks due to digestion products were present for the injection 60 min after mixing substrate and enzyme. After the overnight digestion, the peak for minigastrin I had completely disappeared. The peak pattern had changed and more peaks occurred at earlier times, indicating smaller fragments (which have higher electrophoretic mobility). A total of 7 peaks can be clearly counted, indicating that the digestion is not quite complete, but the electropherogram possibly shows an unexpected 8th peak that is not completely re-

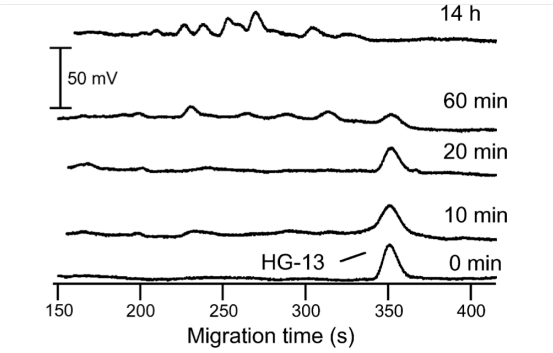

Fig. 1. Electropherograms for minigastrin I (HG-13) digested with pepsin. Off-line digestion was carried out at 37 °C. Digestion and separation were conducted in 2.3 M acetic acid/0.05% (v/v) Tween 20 (pH 2.1). Other details are given in the text.

solved. No attempt to identify the peak identities was made, but the results clearly demonstrate that it is possible to directly monitor the overall progress of the digestion reaction.

Proteins: Cytochrome c, myoglobin, BSA, and HSA

In this series, four proteins were chosen as model substances to be studied: myoglobin with a molar mass of 16,890 g mol^{-1}, cytochrome *c* with a molar mass of 12,327 g mol^{-1}, HSA with a molar mass of 68,500 g mol^{-1}, and BSA with a molar mass of 66,338 g mol^{-1}. Solutions of 100 μM myoglobin, 100 μM cytochrome *c*, 20 μM HSA, and 20 μM BSA, as well as of 50 μM pepsin, were prepared individually in the same acetic acid background electrolyte as used for minigastrin I. Here 0.2 ml of a solution of 100 μM myoglobin, 100 μM cytochrome *c*, 20 μM HSA, or 20 μM BSA and 0.01 ml of 50 μM pepsin were mixed after thermostatting at 37 °C in a water bath for 30 min. The mixtures were kept in the water bath throughout the experiments. Cytochrome *c* consists of 104 amino acids [35]. There are 25 bonds that can be cleaved. It was found that the digestion followed similar kinetics as had been observed for minigastrin I. After 30 min, 6 peaks were observed, and as shown in Fig. 2, after incubation for 2 h, a total of 15 fragment peaks were found. The position of the original peak for cytochrome *c* is indicated by an arrow in the figure, and as can be seen, in this case the protein could not be detected anymore after this digestion time. The reaction was continued overnight, and the number of peaks was found to have increased to 19.

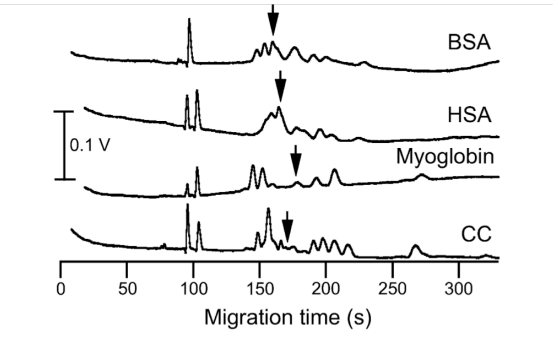

Fig. 2. Electropherograms for several proteins digested with pepsin. Off-line digestion was carried out at 37 °C for 2 h. Digestion and separation were conducted in 2.3 M acetic acid/0.05% (v/v) Tween 20 (pH 2.1). Other details are given in the text. The arrows indicate the positions of the original protein peaks. CC, cytochrome *c*.

The experiment was repeated with myoglobin, HSA, and BSA. After 20 min of digestion, the breaking down of these three proteins was already apparent; the heights of the peaks for the substrates were reduced, whereas additional peaks were observed. The number of peaks increased in the course of time. The electropherograms for the reaction mixtures of these three proteins after 2 h of digestion time are also shown in Fig. 2. The positions of the original protein peaks are again indicated by arrows, and traces of the original proteins were still present for these species after this period of time. A total of 9 peaks were observed for myoglobin, 10 peaks were observed for HSA, and 11 peaks were observed for BSA. The digestions of these three proteins were also continued through overnight incubation. After digestion overnight, myoglobin led to 11 fragments, HSA led to 21 fragments, and BSA led to 16 fragments. The protein peaks had eventually disappeared.

Tryptic digestion

Trypsin is known to cleave peptides on the C-terminal side of lysine and arginine amino acid residues. The rate of hydrolysis is expected to be slower if an acidic residue is on either side of the cleavage site, and the cleavage will not take place if a proline residue is on the carboxyl side of the cleavage site. A high concentration of trypsin is not preferred because the peaks of autocatalytic fragments of trypsin would overlap with the digested fragments of protein and, therefore, complicate the electropherogram. For this reason, the trypsin/substrate ratio should be very low (<1:50) [5]. The optimal pH for trypsin is in the range from 7.0 to 9.0.

Tryptic digestion off-line: Cytochrome c and myoglobin

To provide adequate conditions for both digestion and separation, the use of two separate buffer solutions is necessary. For separation and detection, the 2.3-M acetic acid background electrolyte was again used to ensure that all analytes are rendered in the protonated cationic form. Two buffer solutions at a pH value around 7.8 were first tested for the tryptic digestion: 0.2 M Tris–HCl with a pH value of 7.9 and 70 mM His with a pH value of 7.7. Here 0.2 ml of 500 μM cytochrome *c* and 0.01 ml of 1 mg ml^{-1} trypsin were mixed for the reaction. Both the Tris–HCl and His buffers were successfully used for carrying out the tryptic digestions. However, Tris and His are detectable in the separation background electrolyte and occur in the window for the analytes. Furthermore, His is an amino acid and, hence, a possible product of tryptic digestion. The resulting electropherogram from the Tris–HCl buffer digesting cytochrome *c* is shown in Fig. 3, where a huge peak from the high Tris concentration can be observed between 140 and 200 s. This peak covered some peaks of the digested peptide fragments. Apart from the Tris peak, a total of 31 peaks can be counted. Some of the peaks occurred between 80 and 120 s and can be attributed partly to trypsin autolysis.

A buffer based on ammonium bicarbonate was tested next. The 10-mM ammonium bicarbonate solution had a pH value of 7.8. In addition, 2 M urea was added to this buffer because it helps to denaturize the protein during the incubation so as to get a more complete digestion [7]. In this approach, 0.2 ml of a solution of 500 μM cytochrome *c* was mixed with 0.01 ml of a solution of 1 mg ml^{-1} trypsin. The microvial was placed in a water bath at 37 °C for incubation. After periods of 120 and 160 min and of 21 and 60 h, the sample was injected into the capillary and separated. From Fig. 4, it can be seen that the peak due to the buffer cation, ammonium, occurs very early and, therefore, is largely removed from the digestion products. A peak for cytochrome *c* could not be detected; it is thought that this is due to an overlap with the

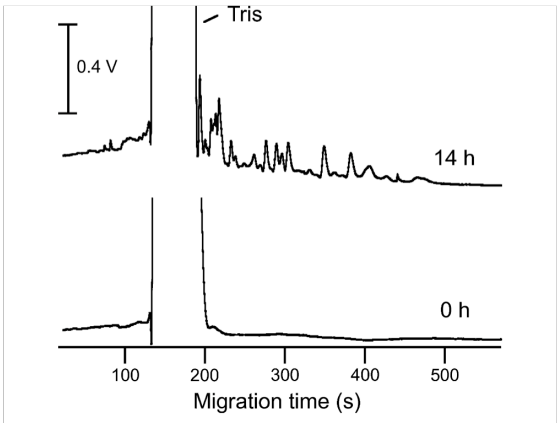

Fig. 3. Electropherogram for cytochrome *c* digested with trypsin. Off-line digestion was carried out at 37 °C in 0.2 M Tris–HCl (pH 7.9). Separation was conducted in 2.3 M acetic acid/0.05% (v/v) Tween 20 (pH 2.1). Other details are given in the text.

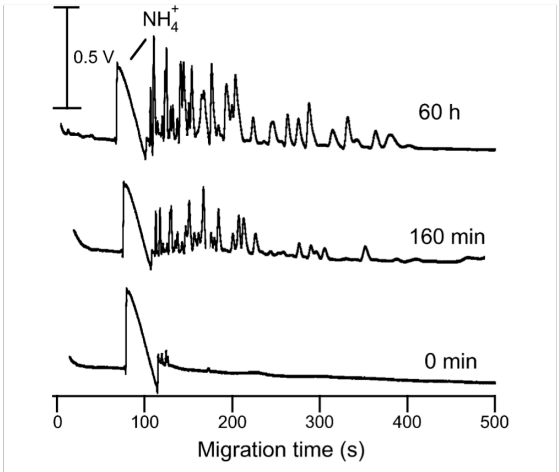

Fig. 4. Electropherogram for cytochrome *c* digested with trypsin. Off-line digestion was carried out at 37 °C in 0.2 M NH_4HCO_3 and 2 M urea (pH 7.9). Separation was conducted in 2.3 M acetic acid/0.05% (v/v) Tween 20 (pH 2.1). Other details are given in the text.

large peak for ammonium. After a digestion period of 2 h, 31 peaks were observed from the cleavage, and 40 min later, the number of peaks was increased to 34. Overnight digestion still led to a change in the peak pattern that was still evident for the electropherogram taken after 3 days. Approximately 34 peaks could be observed, as shown in Fig. 4. The fragmentation with trypsin is strongly dependent on the conditions used, with missed cleavages being common. For example, for cytochrome *c*, Craft and coworkers reported 26 fragments [36], Zhao and coworkers reported 17 fragments [37], and Al-Lawati and coworkers reported 8 peptide fragments [38], with all of these authors using MS quantification.

With this combination of the two electrolyte systems, the digestion of myoglobin was also successfully monitored, as shown in Fig. 5; however, baseline resolution was clearly not obtained. A total of 29 peaks were observed. Zhao and coworkers reported 18 fragments for this protein [37], whereas Al-Lawati and coworkers found 10 peptide fragments [38].

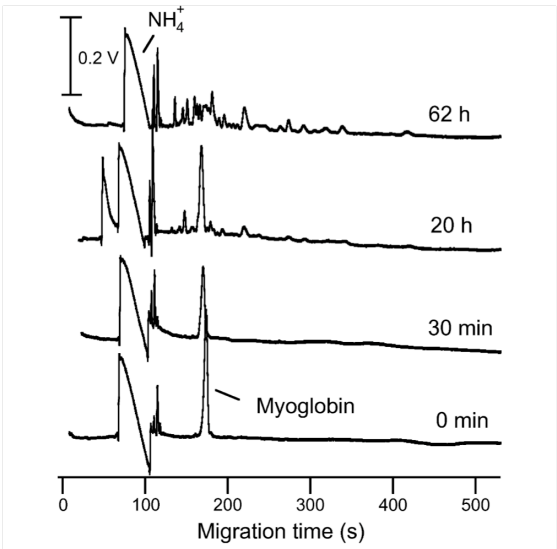

Fig. 5. Electropherogram for myoglobin digested with trypsin. Off-line digestion was carried out at 37 °C in 0.2 M NH_4HCO_3 and 2 M urea (pH 7.9). Separation was conducted in 2.3 M acetic acid/0.05% (v/v) Tween 20 (pH 2.1). Other details are given in the text.

Tryptic digestion on-line: Cytochrome c and apomyglobin

In the off-line enzyme assays, the reaction between enzyme and substrate occurs outside the capillary; the reaction mixture is then injected into the capillary. To be able to carry out the assays in a real nanoliter scale, efforts were made to carry out the reaction directly in the capillary. This approach has generally been referred to as electrophoretically mediated microanalysis. In EMMA, the capillary is used as a reaction vessel. The separation is conducted in the same capillary. The reagents (enzymes and substrates in this case) are introduced into the capillary as distinct zones. When a voltage is applied to the capillary, these zones merge due to their electrophoretic mobility differences. The reaction takes place after the merging of the reaction solutions. The products are subsequently transported to the detector under the influence of an applied voltage. (More details on the EMMA approach can be found in Refs. [39–41].) Two basic modes can be distinguished. In continuous engagement EMMA the separation buffer contains the reagent, whereas in transient engagement EMMA the reagent is contained in a distinct injected plug. Van Dyck and coworkers described a modified version of the latter approach in which a large segment of the capillary was filled with a distinct buffer solution (referred to as partial filling technique) [42]. This allows the creation of two environments that are optimized (mainly with regard to the pH value) for the enzymatic reaction and for the separation, respectively. Part of the capillary was filled with the reaction buffer that is optimal for the enzyme activity, and the rest of the capillary was filled with the separation buffer. As discussed above, tryptic digestion requires an environment with a pH value around 7.8, whereas the separation should be carried out at approximately pH 2.0. For this reason, an adapted transient engagement EMMA protocol, as illustrated in Fig. 6, was employed. First, a plug of substrate contained in the buffer appropriate for digestion was injected hydrodynamically into the capillary filled with the separation buffer. This was followed by a plug of enzyme, also contained in the digestion buffer, and then by a second plug of substrate in the digestion buffer. The bracketing ensures that the pH

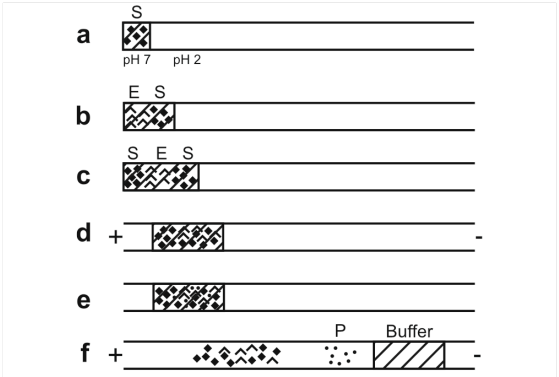

Fig. 6. Schematic illustration of the plug–plug EMMA mode employed for tryptic digestion. S, substrate; E, enzyme; P, products. Shown are hydrodynamic injection of buffer plug with substrate (a), hydrodynamic injection of buffer plug with enzyme (b), hydrodynamic injection of buffer plug with substrate (c), application of voltage to achieve mixing of substrate and enzyme (d), digestion (e), and electrophoretic separation (f).

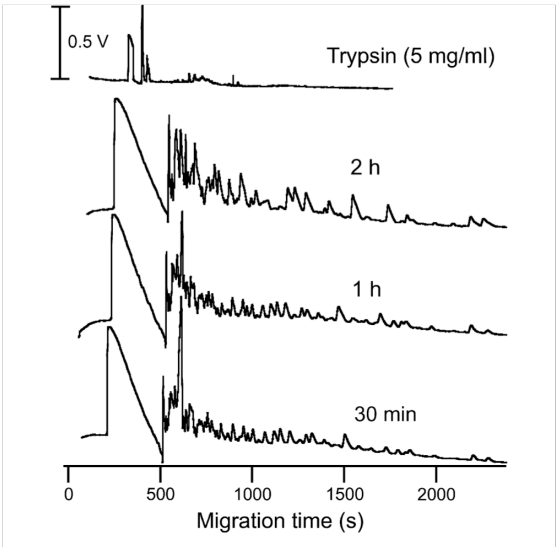

Fig. 7. Electropherograms for cytochrome *c* digested with trypsin. On-line digestion was carried out at 37 °C according to the EMMA scheme detailed in Fig. 6 in 0.2 M NH_4HCO_3 and 2 M urea (pH 7.9). Separation was conducted in 2.3 M acetic acid/0.05% (v/v) Tween 20 (pH 2.1). Other details are given in the text.

value required for digestion is maintained at the desired value. Substrate and enzyme were then merged by a brief application of the separation voltage. After allowing a predetermined time for digestion, the separation voltage was applied and the products were detected. Note that it is not essential to include the substrate in both of the bracketing plugs, but the approach used is convenient in that it does not require prior knowledge of the relative mobilities of enzyme and substrate. An equally valid approach would be to use the reverse procedure, that is, to bracket the substrate with two plugs containing the enzyme.

Thus, a solution of 500 μM cytochrome *c* in a buffer containing 0.2 M ammonium bicarbonate and 2 M urea was first injected at 250 mPa for 10 s, followed by a solution of 5 mg ml^{-1} trypsin at 250 mPa for 10 s. A second plug of buffer with the substrate cytochrome *c* was then injected at 250 mPa for 10 s. Then 5 kV was applied for 5 s to mix the compounds. After a period of 10 min to allow on-line digestion, a voltage of 30 kV was applied and the digestion products were observed and recorded, as seen in Fig. 7. The experiment was repeated, and the separation step was carried out after different time intervals. A substantial degree of digestion was already evident after 30 min. Note that a peak for cytochrome *c* again could not be detected, most likely due to an overlap with the large system peak caused by the discontinuity of the electrolytes. After 2 h of online digestion, the complete degradation of the protein cytochrome *c* was observed. Approximately 40 peaks can be counted. After extended periods of time, severe diffusion would occur; therefore, the online digestion was not performed at time periods longer than 2 h. The reason for the increased rate of digestion in comparison with the off-line procedure is the higher concentration of enzyme; instead of 1 mg ml^{-1} trypsin in the off-line mode, 5 mg ml^{-1} trypsin was used to speed up the digestion process. This means that after 2 h, some products from the autocatalytic digestion of trypsin are also obtained, as can also be seen in Fig. 7, but these peaks are minor in comparison with the products from the digestion of cytochrome *c*.

The protein apomyoglobin was another model compound investigated in the on-line digestion procedure. Pohlentz and coworkers reported that apomyoglobin digested by trypsin in the capillary in a molar ratio of 25:1 yielded complete degradation already after 15 min [43]. However, the experiments carried out with a high ratio of trypsin led to oversized peaks from autolysis products of trypsin that were overlapping the peptide peaks from apomyoglobin. Therefore, a ratio of 30:1 was used. Apomyoglobin digestion was monitored at 15, 30, and 50 min, and a gradual increase of the degree of digestion was observed. To assess how complete the apomyglobin was digested and degraded in the EMMA mode, an off-line digestion of apomyglobin was carried out overnight for a time period of 20 h. After 50 min of on-line digestion, a total of 41 peaks were observed; however, compared with the overnight off-line digestion that created 59 peaks, it was not digested completely.

Conclusion

Monitoring of peptic and tryptic digestion in CE with C^4D has been successfully demonstrated for the first time to our knowledge. The progress of the reaction could be followed for peptides and proteins, and detection of the resulting products was possible both off-line and on-line. It is a significantly less expensive detection option than a mass spectrometer, which is often used for this application. Although C^4D does not offer the detection limits of the mass spectrometer or give any information on peak identity, the sensitivity is comparable to UV detection and the method may find use in studies where the requirements on sensitivity are less demanding. These should include the task of monitoring the progress of digestion reactions as well as peptide and protein identification by fingerprinting.

Acknowledgments

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Chirality

Following the lipase catalyzed enantioselective hydrolysis of amino acid esters with capillary electrophoresis employing contactless conductivity detection

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Abbreviations

SME -- serine methyl ester

TME -- threonine methyl ester

18C6H$_4$ -- (+)-(18-Crown-6)-2,3,11,12-tetracarboxylic acid

PPL -- lipase from the porcine pancreas

WGL -- lipase from wheat germ

CE-C^{4}D -- capillary electrophoresis – capacitively coupled contactless conductivity detection

INTRODUCTION

Capillary electrophoresis (CE) is an attractive method for the separation of enantiomers as in contrast to HPLC it is not necessary to use a chiral column. Chiral reagents can be dissolved in the relatively low volumes of the separation buffer and do not have to be covalently bound to the packing material of separation columns. Optimization is possible by adjusting concentrations, pH-values or ratios of different dissolved reagents without having to resort to modifications of a stationary phase. This application has therefore become one of the major uses of capillary electrophoresis. For a recent review see for example.[1] A further advantage of CE is the fact that only small sample volumes are required as the separation is carried out in narrow capillaries.

A shortcoming of CE has long been the limitation of commercial instrumentation to UV-absorbance or fluorescence detection. Due to the short optical pathlengths available, this has meant that weakly absorbing species, such as most amino acids,

could only be detected following chemical derivatization in order to render them accessible by optical means. Often studies of amino acids by CE have been restricted to those few species which have an aromatic moiety and are thus easily detectable by UV-absorbance. See, for example, the recent review by Lindner and coworkers.[2] This shortcoming can, however, be overcome by employing conductivity detection, which is universal for all ionic species.

Conductivity detection has been known for electromigration separation techniques for a long time, but has played a marginal role until the introduction of the contactless approach relying on a pair of external tubular electrodes.[3,4] The method has been termed C^4D for 'capacitively coupled contactless conductivity detection'. Its fundamentals have been studied extensively [5-8] and at least two commercial devices are now available and can be retrofitted to existing CE-instrumentation. Recent reviews on applications of C^4D are also available.[9-11]

Several research groups have demonstrated the determination of amino acids at low pH in cationic form using contactless conductivity detection.[12-16] Gong et al. in 2005 demonstrated that the determination of the enantiomers of non-UV-absorbing amino acids is possible by CE-C^4D using a combination of the non-charged additives hydroxypropyl-β-cyclodextrin and (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid ($18C6H_4$) as the chiral separators.[17] It has furthermore been shown that CE-C^4D may be employed for the monitoring of enzymatic reactions when the species involved are not detectable by optical means. The non-ionic species glucose, ethanol, ethyl acetate and ethyl butyrate were made accessible for analysis by capillary electrophoresis via charged products or byproducts obtained in enzymatic conversions using hexokinase,

glucose oxidase, alcohol dehydrogenase and esterase respectively.[18]. Lately, also urea was determined by CE-C^{4}D in clinical samples via enzymatic conversion to ammonium with urease.[19]

The application of CE-C^{4}D to the monitoring of the lipase-catalyzed hydrolysis of amino acid esters is reported herein. Many workers have investigated the use of lipases for enantioselective synthesis, and in particular Reetz and coworkers have been studying the optimization of the enzyme structures in an approach named 'directed evolution'.[20-22] This reaction has also been investiged as a useful path to the resolution of racemates of the amino acids, work in this area has been reviewed by Miyzawa in 1998.[23] More recently, Malhotra et al.[24] have researched the enantioseparation of the esters of α-amino acids by lipase in an ionic liquid.

Materials and methods

Chemicals and procedures

All chemicals were of reagent grade and deionized water (Millipore, Bedford, MA, USA) was used throughout. All chemicals were purchased either from Sigma, Fluka, Aldrich (Buchs, Switzerland), or Acros Organics (Geel, Belgium). The details of the suppliers are as follows: DL-serine methyl ester hydrochloride (Sigma), L-serine methyl ester hydrochloride (Aldrich), DL-threonine methyl ester hydrochloride (Sigma), L-threonine methyl ester hydrochloride (Fluka), DL-serine (Fluka), L-serine (Sigma), DL-threonine (Fluka), L-threonine (Sigma). Acetic acid was purchased from Fluka. HPLC-grade acetonitrile was obtained from Acros. Sodium bicarbonate was purchased from Sigma. (+)-(18-(Crown-6)-2,3,11,12-tetracarboxylic acid was bought

from Fluka. The enzymes lipase from porcine pancreas, type II, as well as lipase from wheat germ, type I, were also purchased from Sigma. All stock solutions were prepared in deionized water. Fused-silica capillaries of 50 µm ID and 375 µm OD were used for the separation. These were purchased from Polymicro Technologies (Phoenix, AZ, USA) and were preconditioned with 1 mM sodium hydroxide solution and then flushed with water followed by 1 mM hydrochloride solution and again by water. Before use they were flushed with the running buffer. All capillaries had a total length of 50 cm and 45 cm effective length. Sample injection was carried out manually directly from the reaction vessels in a hydrodynamic manner at 10 cm elevation for 10 s. The separation voltage was +15 kV unless stated otherwise.

Instrumentation

Separations were carried out on a purpose-made electrophoretic instrument based on a high-voltage power supply from Spellman (model CZE 2000, Pulborough, UK). It comprises a Perspex box which is fitted with a microswitch to interrupt the power supply for safety reasons when opening. The contactless conductivity detector was also constructed in-house, details can be found elsewhere.[25,26] The cell design detailed in Zhang et al. was employed.[27] Data acquisition was carried out with a Macintosh personal computer (Apple, Cupertino, CA, USA) using a MacLab/4e analog-to-digital convertor system (AD Instruments, Castle Hill, Australia).

RESULTS AND DISCUSSION

Separation and detection of the enantiomers of serine and threonine

In order to monitor the process of the hydrolysis it is necessary to be able to separate and detect both enantiomers of the amino acids. DL-serine methyl ester (SME) and

DL-threonine methyl ester (TME) were chosen as model compounds. These compounds do not contain benzyl groups; therefore it would not be possible to detected them optically in CE. The choices were made partly also owing to the commercial availability of the esters. An electrolyte solution consisting of 2 M acetic acid in order to render the analytes in the protonated positively charged form and 5 mM of the chiral crown ether $18C6H_4$ ((+)-(18-(Crown-6)-2,3,11,12-tetracarboxylic acid) as selector was employed. This was a variation of a buffer found suitable by Gong et al.[17] for the separation of the enantiomers of amino acids.

As illustrated by electropherogram 1 of Fig. 1, which is the result of the injection of a mixture of 5 mM DL-SME and 5 mM DL-serine, the enantiomers of serine can be separated from the substrate, and the two enantiomers are well separated. Furthermore it is possible to also separate the enantiomers of the methyl esters using the electrolyte solution containing the chiral reagent. For the enantiomers of serine a high resolution, R_s, of 3.7 was obtained (the separation factor, α, is 1.2). The enantiomeric separation of DL-SME is also good with a resolution of 3.8 (α = 1.3). The result of the separation of a mixture of DL-TME and DL-threonine is shown in electropherogram 2 of Fig. 1. The separation is generally not as good as that of the serine species, but baseline resolution was still obtained for all compounds. The R_s values were calculated as 2.0 and 1.3 for the threonine and TME enantiomers respectively (α = 1.1 and 1.2). A calibration curve of L-serine was acquired for a concentration range from 0.5 to 25 mM. The following regression equation was obtained: peak area [mV·s] = -0.0616 + 0.9451 × concentration ^{2} (correlation coefficient, r = 0.9999). A calibration curve for L-threonine in the range from 0.5 to

10 mM resulted in the following regression equation: peak area [mV·s] = 0.012 + 0.8054 × concentration ^{2} (r = 0.9993).

Hydrolysis of the methyl esters of DL-serine with porcine lipase

The enzyme catalyzed hydrolysis requires a pH-value of around 7.4. The reaction could thus not be carried out in the electrolyte solution used for separation, which has to have a low pH-value in order to render the analytes in the protonated cationic form. Into a micro-vial of 1.5 mL total volume 1 mL of a $NaHCO_3$ aqueous buffer solution (0.2 M, pH 7.8) was placed, 10 mg of a racemic mixture of DL-serine methyl ester hydrochloride was added as solid, and the enzyme porcine pancreas lipase (PPL) (2.5 mg) was added dissolved in 0.5 mL of 15% acetonitrile in water. After mixing, the vial was placed in a water bath of 37°C and the reaction monitored for two days. Analysis of the reaction mixture was performed immediately after mixing, and after 20 min, 2 hrs, 19 hrs and 43 hrs. The separation was carried out in the acetic acid electrolyte containing the chiral crown ether as described above. The concentrations of D- and L-serine were then determined from the calibration curve. Plots of enantioselectivity expressed as the enantiomeric excess (ee) value and the yield for L-serine (fraction of the L-serine methyl ester converted to L-serine) against time calculated from these values are given in Fig. 2. Clearly, at the beginning of the hydrolysis, a high selectivity for L-serine was obtained, which then decreased with time as the yield increased.

Hydrolysis of the methyl esters of DL-threonine with porcine lipase

5 mg of a racemic mixture of DL-threonine methyl ester hydrochloride and 2.5 mg of the porcine pancreas lipase were mixed in the same fashion as described above for

DL-SME and the mixture incubated again at 37°C. The reaction mixture was analysed immediately after mixing, and then after 15 min, 2 hrs, 5 hrs and 21 hrs. The results are shown in Fig. 3. The pattern is similar to the one obtained for serine, with the most pronounced enantiomeric excess obtained early in the reaction. However, the selectivity of this reaction is generally less pronounced then for the hydrolysis of the serine ester.

Comparison between porcine lipase and wheat germ lipase

Next a comparative study of the selectivity of two different lipases was carried out using the hydrolysis of DL-serine methyl ester. 2.5 mg of porcine lipase and of the same amount of wheat germ lipase where added respectively to two batches of 10 mg of the racemic mixture of the substrate. The same protocol as outlined above was used. As in the previous experiments the most pronounced enantiomeric selectivity was obtained at early times, the analysis of the reaction mixture was carried out at short intervals of 15 min for the first hour of the reaction. In Fig. 4, the yield of the reaction in terms of production of L-serine is shown. It is evident that for the conditions used, the rate of reaction is slighty higher for the porcine lipase than for the wheat germ lipase. The results for the enantiomeric excess given in Fig. 5, indicate that while the wheat germ lipase also prefers the L-serine ester, a higher selectivity for the L-form is found for the porcine lipase. No attempt was made to correct the data for differences in activity, but it appears that the difference is partially due to the initially higher rate of reaction for the wheat germ lipase (Fig. 4).

The selectivity between the methyl esters of L-serine and L-threonine

In the final experiment L-SME and L-TME were selected to react together to investigate the preference of the porcine lipase for the two substrates. 5 mg L-SME and 5 mg L-TME were mixed with 2.5 mg of the porcine pancreas lipase, using the protocol used thus far. The progress of hydrolysis was monitored over the period of 24 hours and the results in terms of the yield for the two amino acids are given in Fig. 6. Clearly, in the competitive situation the rate of reaction is higher for the threonine ester, the compound for which the discrimination between the two enantiomers is less proncounced.

CONCLUSIONS

It has been demonstrated that the CE-C^4D method is well suited for the monitoring of an enantioselective reaction involving compounds which are not readily detectable by UV-absorption measurement. The fact that only low volumes are consumed for each analysis enabled the repeated sampling from a very small reaction volume to observe the progress of the conversion, which would not have been possible by HPLC.

Acknowledgements

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Figure captions

Fig. 1 Electropherograms of the concurrent separation of the enantiomers of (1) DL-SME and DL-serine, and (2) DL-TME and DL-threonine. Buffer: 2 M acetic acid / 5 mM 18C6H$_4$. Capillary: 50 µm i.d., total length 50 cm, effective length 45 cm. Injection: hydrodynamic, 15 cm height elevation for 10 s. Separation voltage: +15 kV.

Fig. 2 Plot of the ee of DL-serine and the yield of L-serine resulting from the hydrolysis of DL-SME to DL-serine by PPL vs. reaction time. Conditions for the reaction are given in the text. Separation as for Fig. 1.

Fig. 3 Plot of the ee of DL-threonine and the yield of L-threonine resulting from the hydrolysis of DL-TME to DL-threonine by PPL vs. reaction time. Conditions for the reaction are given in the text. Separation as for Fig. 1.

Fig. 4 Plot of the yield of L-serine produced from the hydrolysis of DL-SME by (1) PPL and (2) WGL vs. reaction time. Conditions for the reaction are given in the text. Separation as for Fig. 1.

Fig. 5 Plot of the ee of DL-serine produced from the hydrolysis of DL-SME by (1) PPL and (2) WGL vs. reaction time. Conditions for the reaction are given in the text. Separation as for Fig. 1.

Fig. 6 Plot of the yields of L-threonine and L-serine resulting from the competitive hydrolysis between L-TME and L-SME by PPL vs. reaction time. Conditions for the reaction are given in the text. Separation as for Fig. 1.

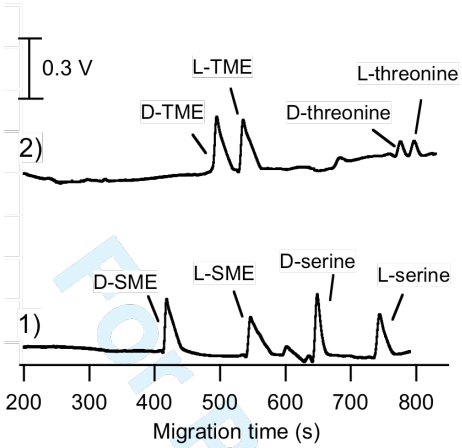

Fig. 1

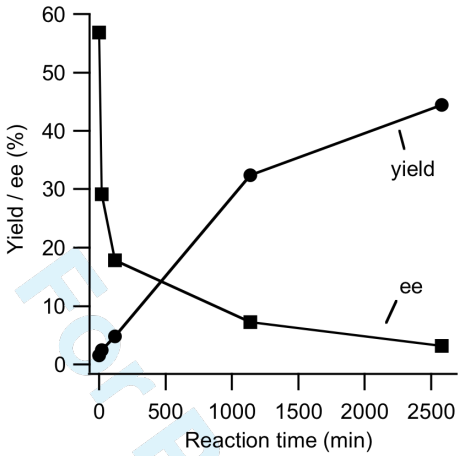

Fig. 2

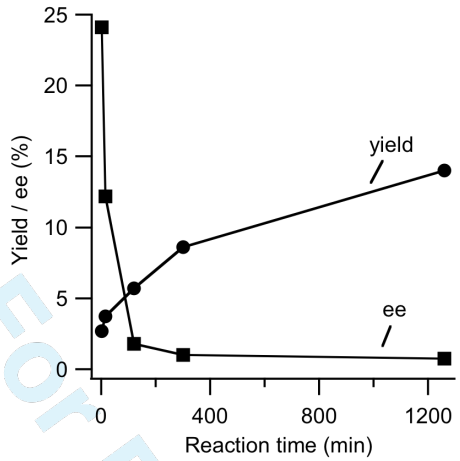

Fig. 3

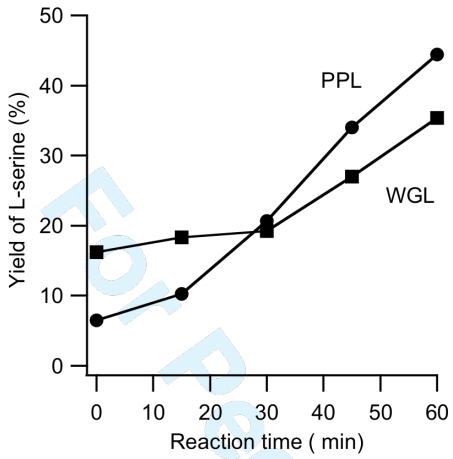

Fig. 4

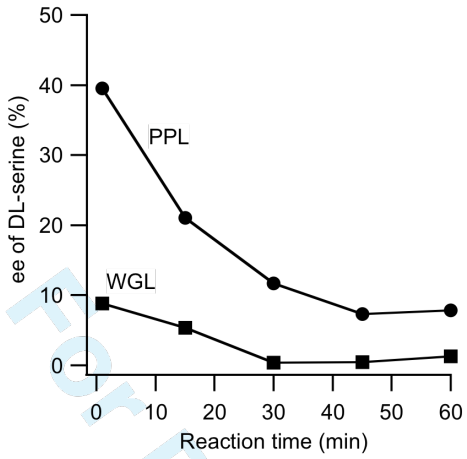

Fig. 5

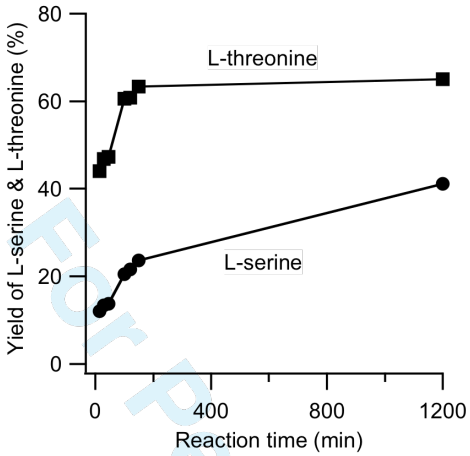

Fig. 6

Study of acetylcholinesterase inhibitors using capillary electrophoresis with contactless conductivity detection

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Keywords: Acetylcholinesterase, acetylcholine, acetylthiocholine, inhibitor, capillary electrophoresis, conductivity detection, electrophoretically mediated microanalysis (EMMA), galanthamine, huperzine A, paraoxon

Abbreviations

C^4D	Capacitively Coupled Contactless Conductivity Detection
EMMA	Electrophoretically Mediated Microanalysis
MES	2-Morpholinoethanesulfonic acid
Arg	Arginine
CTAB	Cetyltrimethylammonium bromide
ACh	Acetylcholine
ATCh	Acetylthiocholine
AChE	Acetylcholinesterase

Abstract

The hydrolysis of acetylcholine and acetylthiocholine as catalyzed by the enzyme acetylcholineesterase was monitored by capillary electrophoresis with contactless conductivity detection by determining the acetate produced in the reaction. This approach eliminates the need for a colour forming derivatization procedure. The effects of the three inhibitors galanthamine, hyperzine A and paraoxon on the enzyme kinetics could also be investigated by the new procedure and the IC_{50}-values were determined. The contactless conductivity detection was also found to be compatible with the electrophoretically mediated microanalyis approach (EMMA), in which the enzymatic reaction is carried out directly inside the capillary prior to separation and quantification.

Introduction

Acetylcholinesterase inhibitors are substances which inhibit the enzyme from breaking down acetylcholine, increasing both the level and duration of action of the neurotransmitter acetylcholine. Such substances occur naturally as venoms and poisons. Importantly, synthetic inhibitors are used as medicines to treat neurodegenerative disorders such as Alzheimer's disease. Some pesticides and chemical weapons compounds also act as acetylcholine inhibitors.

Alzheimer's disease is the most common cause of dementia. It is affecting 22 million people worldwide. It usually occurs in people over 65 years old, and has become one of the major diseases threatening the life of old people. According to one of three competing hypotheses, Alzheimer's disease is due to the reduced biosynthesis of the neurotransmitter acetylcholine. Based on this cholinergic hypothesis, many drugs have been developed to treat acetylcholine deficiency by using acetylcholinesterase (AChE) inhibitors to increase the levels of acetylcholine, such as tacrine, donepezil, T AK147, physostigmine, galanthamine and huperzine A. Since the current AChE inhibitors may also be useful for the treatment of other diseases such as Parkinson's disease [1], the search for new AChE inhibitors is receiving much attention. On the other hand, the inhibition of acetylcholine breakdown is a detrimental effect of pesticides on humans and of nerve agents used as chemical weapons. When AChE is not effective in hydrolyzing the breakdown of the neurotransmitter acetylcholine, coordination within the nervous system will break down due to a buildup of neurotransmitter within synaptic nerve junctions. This causes respiratory failure, and eventually death. Nerve agents and pesticides often belong to the organophosphorous compounds and their extreme toxicity has

been know since the 1930s. VX, sarin and soman are important examples of organophosphorous nerve agents. Organophosphorous esters with less toxicity such as parathion are employed as pesticides.

The most commonly used methods for assaying AChE activity are based on a spectrometric method developed by Ellman *et al.* in 1960 [2]. The enzyme activity is measured by following the increase of a yellow colour produced from the product thiocholine when it reacts with dithiobisnitrobenzoate. In order to enable this colorimetric reaction, acetylthiocholine is used commonly as substrate for *in-vitro* studies, rather then acetylcholine, the substance present in living organisms. A chemiluminescent procedure to determine acetylcholine was described by Israël and Lesbats [3, 4]. The specificity of the chemiluminescent method depended on the fact that choline oxidase (which was used as a secondary enzyme) received its substrate only when acetylcholine was hydrolyzed by acetylcholinesterase. Rhee *et al.* [5] developed a high sensitivity fluorometric assay of AChE inhibitory activity in a flow system using a fluorogenic substrate. Hai *et al.* [6] designed a bioelectronic hybrid system for the detection of acetylcholine and acetylcholineesterase inhibitors; the enzyme was immobilized on the surface of the gate of an ion-sensitive field-effect transistor (ISFET).

Capillary electrophoresis may also be used as an analytical technique for enzyme assays, it offers the advantage of specificity through separation, *i.e.* colour forming reagents are not necessarily required for selective quantification. A further advantage is the ability to work with small sample volumes. CE systems have for example been successfully applied to assaying enzymatic activity [7] and [8],

including the determination of Michaelis-Menten constants (K_m values) [9, 10] and inhibition constants [11, 12]. CE is useful for investigating enzyme reactions involving charged substrates or products, *e.g.* for monitoring phosphorylation or dephosphorylation reactions [13].

Enzyme assays may also be wholly carried out inside an electrophoresis capillary [14]; this microanalysis technique is based on the fact that transport velocities of the enzyme, reagents and products are different under applied potential and this may be used both to mix the reactants and, after a reaction period without applied voltage, separate the product from the enzyme and remaining substrate. This technique has come to much attention as electrophoretically mediated microanalysis (EMMA). Van Schepdael and coworkers recently published an updated review on CE-mediated microanalysis [15]. Heleg-Shabtai *et al.* [16] presented a sensitive on-chip AChE assay, in which the resulting labelled product was detected by laser-induced fluorescence. An EMMA technique using UV detection was reported by Tang *et al.* [17] to screen AChE inhibitors in natural extracts. Thiocholine, as product from enzymatic reaction, was measured directly as a peak in the electrophoretic separation by its UV-absorbance at 230 nm.

Capacitively coupled contactless conductivity detection (C^4D) is an alternative to UV-detection, also suitable for only weakly or not absorbing species, which has been explored in past years (see for example the recent reviews [18-22]). CE-C^4D has been applied to the monitoring of enzymatic reactions in which the five non–ionic substrates ethanol, glucose, ethyl acetate, and ethyl butyrate were converted to charged products or by-products by alcohol dehydrogenase, hexokinase, glucose

oxidase, or esterase [23]. The method was also used to directly quantify the ammonium produced in the enzymatic conversion of urea with urease and this was applied to the analysis of clinical samples [24]. An investigation of the use of CE-C^4D for further enzymatic assays, namely for acetylcholine inhibitor studies, is presented herein.

2 Materials and methods

2.1 Instrumentation

Separations were conducted in conventional fused-silica capillaries either on an electrophoresis instrument built in-house or a commercial unit. The former is similar to a design detailed elsewhere [25], but uses a high-voltage power supply from Spellman (model CZE2000, Pulborough, UK) capable of delivering up to 30 kV. The commercial system was a PrinCE instrument (600 series, Prince Technologies, Emmen, The Netherlands). Both instruments were fitted with a contactless conductivity detector constructed in-house. The mechanical details of the detector cell can be found previous publications [26, 27]. The cell current was transformed into an AC voltage with an operational amplifier (OPA655, Texas Instruments, Dallas, TX, USA) in the current follower configuration (feedback resistor: 220 kΩ). The signal was then rectified and further amplified. The electropherograms were recorded on a MacLab/4e data acquisition system (AD Instruments, Castle Hill, Australia) which was connected to a computer.

Fused-silica capillaries of 50 μm id and 375 μm od (Polymicro Technologies, Phoenix, AZ, USA) were used for all the separations. Two buffers were prepared and used throughout the experiments. A buffer consisting of 15 mM 2-

morpholinoethanesulfonic acid (MES) and 16 mM Arginine (Arg) with pH 8.0 was used for all enzymatic reactions with and without enzyme inhibitors. Another buffer containing 25 mM MES, 5 mM Arg and 50 μM cetyltrimethylammonium bromide (CTAB) with pH 5.7 was used for separation and detection. The enzymatic reactions took place at a temperature of 37°C in a water bath.

2.2 Reagents and methods

All chemicals were of reagent grade and deionized water (Millipore, Bedford, MA, USA) was used throughout. Acetylthiocholine chloride (minimum 99% TLC), acetylcholine chloride (minimum 99% TLC), and acetylcholinesterase from electric eel (1052 Units/mg solid) were purchased from Sigma (Buchs, Switzerland). The inhibitors galanthamine hydrobromide from *lycoris sp.*, and (-)–huperzine A were also obtained from Sigma. The inhibitor paraoxon was bought from Supelco (Buchs, Switzerland). MES, CTAB and acetic acid were purchased from Fluka (Buchs, Switzerland). L-(+)-Arginine was bought from Acros Organics (Geel, Belgium). Enzymes and substrates were dissolved in the corresponding buffers which have the suitable pH-environment for the enzymatic reactions. Stock solutions of the inhibitors were prepared in deionized water, before dilution in the reaction buffer to the final concentrations. All enzymatic reactions were performed in 1.5 ml-microvials, except for the EMMA measurements. All capillaries were preconditioned with 1 M sodium hydroxide solution, flushed with water and 1 M hydrochloric acid solution followed by further flushing with water. Before use the separation capillaries were flushed with the appropriate running buffer. Except for the EMMA procedures, measurements were carried out on the instrument constructed in-house. The enzymatic reaction was studied by mixing a solution of 1

mL 1 mM acetylthiocholine or acetylcholine and of 0.05 mL 10 μM acetylcholinesterase. For the investigation of the inhibitor activity, a solution of 0.05 mL containing the inhibitor at different concentrations was mixed with 1 mL of the 1 mM acetylthiocholine solution before adding the enzyme solution. Sample injection was carried out manually in a hydrodynamic manner by lifting the injection end of the capillary to a height of 15 cm for 10 s and separations were carried out at -25 kV. In the EMMA mode, each injection was made automatically by applying 250 mPa pressure for 10 s. This was followed by the application of 5 kV for 5 s to mix the compounds, an incubation time of 5 min and finally separation at –30 kV.

3 Results and discussion

3.1 Conditions for acetylcholinesterase assays

Acetylcholinesterase breaks down acetylcholine into positively charged choline and negatively charged acetate. In the common Ellman procedure [2], acetylthiocholine is used instead of the native acetylcholine as the product thiocholine allows the colour forming coupling reaction used for quantification. When using CE-C^4D, quantification of either of the ionic species produced is possible directly without an auxiliary reaction. As in CE the concurrent determination of cations and anions is only possible in special cases, a decision had to be made to either monitor the choline (or thiocholine) cation or the anionic acetate. It was chosen to quantify the acetate produced, rather then the choline cation, but similar results would be expected for the alternative procedure.

A buffer consisting 25 mM MES, 5 mM Arg of pH 5.7 and containing 50 µM CTAB was deemed suitable for the determination of acetate. As the optimal pH-value for acetylcholinesterase is between 7.0 and 9.0 [28], a MES / Arg buffer of slightly different composition, namely 15 mM MES and 16 mM Arg, with a pH-value of 8.0 was chosen to investigate the reaction of acetylcholine with acetylcholineesterase. Solutions of acetylcholine and acetylcholineesterase were prepared separately in the buffer and then mixed in a microvial. Upon mixing, a first injection into capillary was made, before placing the vial into a 37ºC of water bath for incubation. The reaction mixture was analysed again after 4 min, 8 min, 12 min and 30 min. The resulting electropherograms are shown in Fig. 1. Clean peaks for acetate were obtained. The chloride peaks are due to the fact that acetylcholine was added as the hydrochloride salt. The results clearly illustrate the gradual increase of the acetate concentration as the enzymatic reaction progresses.

The reaction was repeated for different concentrations of the enzyme added (between 1 and 20 $U \cdot mL^{-1}$ AChE in the 0.05 mL solution added to 1 mL of the 1 mM ACh solution) and the resulting reaction kinetics are shown in Fig. 2A as plots of peaks areas for acetate obtained vs. reaction time. Clearly, for the conditions used, a strong dependence of the reaction kinetics on the amount of enzyme added was obtained. As the use of acetylthiocholine rather than acetylcholine has become standard for *in vitro* studies of AChE activities, the reaction was also performed with this substrate. All reaction conditions and detection procedures were identical to those used for ACh. The kinetics for the reaction of ATCh catalyzed by different concentrations of AChE is illustrated in Fig 2B. Similar results were obtained,

albeit, the reaction kinetics were somewhat slower for the sulfur containing compound. ATCh was adopted as substrate for the subsequent investigations.

In order to study the effect of a potential enzyme inhibitor a suitable concentration of the enzyme had to be chosen. By adding an inhibitor to an enzymatic reaction, the enzyme reaction will be significantly slowed down. For 1 $U \cdot mL^{-1}$ AChE, the reaction was already too slow without inhibitor, within the time scale of first 30 min, it was not possible to detect any peaks. For concentrations between 5 and 15 $U \cdot mL^{-1}$ AChE, a inhibitory effect would be noted, but for strong effects quantification might not be possible. A concentration of 20 $U \cdot mL^{-1}$ AChE was thus used for the inhibitor studies reported below.

3.2 Acetylcholinestrase assay with inhibitors

Galanthamine

Galanthamine is a naturally occurring alkaloid and has recently been approved as a drug for the treatment of mild to moderate Alzheimer's disease [29]. It is a competitive and reversible cholinesterase inhibitor. It binds to enzymes with non-covalent interactions such as hydrogen bonds, hydrophobic interactions and ionic bonds.

The inhibitory effect of galanthamine was tested using CE-C^4D by mixing 0.05 mL of solutions of galanthamine at different concentrations between 10 and 50 µM with 1 mL of a solution of 1 mM ATCh before adding 0.05 mL of 20 $U \cdot mL^{-1}$ AChE. All solutions were prepared in a buffer of 15 mM MES and 16 mM Arg (pH 8.0). The

reaction mixture, which was thermostatted at 37°C, was then analysed by injection of aliquots into the capillary after different time intervals of up to 120 min. The effect of the inhibitor galanthamine on the enzyme kinetics is shown in Fig. 3. Clearly with increasing concentration of galanthamine a more pronounced effect is observed.

The effectiveness of a particular drug or a inhibitor is usually quantified by determining the so-called IC_{50}-value, indicating the inhibitor concentration which reduces the effectiveness of the enzyme by 50%. For the purpose of determining this value, a range of concentrations of galanthamine between 0.1 μM to 100 mM was mixed with ATCh using the conditions as detailed above. A dose-response curve for the relative inhibition of the reaction after 5 min incubation time is shown in Fig. 4. 50% inhibition was obtained at a concentration of 40 μM (or $10^{-4.4}$ M) in the added solution. This corresponds to 1.8 μM in the final solution. The IC_{50}-values are strongly dependent on the conditions used for their determination. But for comparison, de Jong *et al.* reported a value of 0.38 μM at 0.75 $U \cdot mL^{-1}$ AChE [30]. Dose-response curves were also constructed for the incubation times of 15 min, 30 min, 45 min and 2 hours, and the resulting IC_{50}-values are plotted against reaction time in Fig. 5. The fact that the values were relatively independent of the reaction time is an indication for the reversable nature of inhibitor.

Huperzine A

Huperzine A is being investigated as a possible new treatment for diseases characterized by neurodegeneration, particularly Alzheimer's disease [31, 32]. It is a naturally occurring *Lycopodium* alkaloid found in the extracts of the moss

Huperzia serrata and the Chinese herb Qian Cheng Ta [33]. Huperzine A is a reversible AChE inhibitor.

Solutions of huperzine A with concentrations of 4.1 nM, 41 nM, 410 nM, 4.1 μM, 41 μM, 410 μM and 4.1 mM were prepared. 0.05 mL of these solutions were mixed with ATCh and AChE as described above. At 0 min, 5 min, 15 min, 30 min, 45 min and 2 hours incubation time, samples were injected and the acetate peaks were determined on application of the separation voltage. From a dose-response curve for the incubation time of 5 min an IC_{50}-value of $10^{-4.5}$ M of the added solution was obtained. This corresponds to an IC_{50}-value of 1.4 μM in the final solution. Some variation of this value for the other reaction times was obtained as is also shown in Fig. 5. The data again matches that of a reversible inhibitor. An IC_{50}-value of 3.2 μM was reported for (-)-huperzine A by de Jong *et al.* [30].

Paraoxon

Paraoxon, is an organophosphate compound and the active degradation product of the insecticide parathion. It is known to be highly toxic and a quasi-irreversible acetylcholinesterase inhibitor [34]. The IC_{50}-values were determined as described above for the other two inhibitors by using a series of 8 concentrations of paraoxon from 3.6 μM to 36 mM. The values obtained after incubation times of 5 min, 15 min, 30 min, 45 min and 2 hours, are plotted again in Fig. 5. The strong dependence of the IC_{50}-value is a result of the irreversable nature of this inhibitor. It is noted that the difference of IC_{50}-values between the incubation times of 5 min

and 2 hours is almost 20 fold. The lowest value, obtained after 2 hours is about 4 μM. Lessire *et al.* reported a value of 0.2 μM obtained by *in vivo* studies [34].

3.3 EMMA

As different buffers are used for the enzymatic reaction and the subsequent separation, the on-capillary EMMA-procedure has to be tailored to accomodate this situation. A partial filling technique combined with the EMMA plug-plug mode was therefore used for the on-line reaction and separation. This technique was first introduced by Van Dyck *et al.* [9] and has been adopted widely since [35]. The detailed plug introduction scheme is seen in Fig 6. The capillary is rinsed and filled with the separation buffer (buffer 1), which consists of 25 mM MES / 5 mM Arg / 50 μM CTAB at pH 5.7. The first plug of buffer 2, which consists of 15 mM MES / 16 mM Arg with pH 8.0, is to isolate the reaction medium from buffer 1 and create a suitable pH-environment for the enzymatic reaction. AChE has a larger mass compared to ATCh, therefore the injection of the enzyme solution (20 $U \cdot mL^{-1}$ AChE) is followed by the 1 mM ATCh-solution. Another plug of buffer 2 is again used to isolate the reaction medium from the separation buffer. After 5 kV was applied for 5 s to achieve mixing, 5 min of incubation time was allowed to let the enzymatic reaction take place. The production of acetate can be observed in the resulting electropherograms as illustrated in trace 1 of Fig 7. Following the addition of 0.01 mL 100 μM of galanthamine solution to 1 mL of 1 mM ATCh solution, the online procedure was repeated, and electropherogram 2 of Fig. 7 was obtained. The inhibitor effect of galanthamine could thus also be monitored by using the EMMA procedure. The experiment was thus repeated for galanthamine concentrations (in

the 0.01 mL aliquot mixed to the substrated solution) of 0.01 μM, 0.1 μM, 1 μM, 10 μM, 100 μM, 1 mM, 10 mM and 100 mM. A dose-response curve for the inhibition activity of galanthamine was plotted and the resulting IC_{50}-value determined. This came to a value of 36 μM for the undiluted solution, or 0.36 μM for the substrate/inhibitor solution before injection.

4. Conclusions

The use of CE-C^4D as analytical method was found to be useful for the facile study of acetylcholineesterase inhibitors, without having to employ colour forming reagents, or the thiocholine variant as substrate. To our knowledge, this is the first report employing this technique for this purpose. The sampling of small aliquots for the CE-step allowed an easy determination of kinetic data from small samples volumes. The EMMA-method is an elegant on-line method, which allows to work with even smaller volumes. However, the acquisition of time dependent data by this means is more elaborate as the reaction mixture is available always for only a single measurement. There is also a question related to numerial data, due to non-quantifiable possible dilution effects taking place inside the capillary. This may partly account for the differences in the IC_{50}-values obtained for the off-line and on-line procedures (1.8 μM vs. 0.36 μM).

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Figure captions

Fig. 1 Electropherograms showing the acetate produced after different intervals from acetylcholine by acetylcholineesterase catalyzed hydrolysis as well as the concomitant chloride. Reaction mixture: 1 mL of 1 mM ACh in 15 mM MES / 16 mM Arg, at pH 8.0 and 37°C, to which was added 0.05 mL of 10 $U \cdot mL^{-1}$ AChE-solution. Separation buffer: 25 mM MES / 5 mM Arg / 50 µM CTAB, pH 5.9. Injection: hydrodynamic, 10 s at 15 cm elevation. Capillary length: 50 cm. Separation: -25 kV.

Fig. 2 Production of acetate from the hydrolysis of (A) ACh and (B) ATCh by different concentrations of AChE. AChE concentrations in the aliquot: ♦ 1 $U \cdot mL^{-1}$, ▲ 5 $U \cdot mL^{-1}$, ▼ 10 $U \cdot mL^{-1}$, ■ 15 $U \cdot mL^{-1}$ and ● 20 $U \cdot mL^{-1}$. Other conditions as for Fig. 1.

Fig. 3 Production of acetate from the enzymatic reaction inhibited by galanthamine at different concentrations in an aliquot of 0.05 mL added to the reaction mixture: ■ no galanthamine, ▲ 10 µM, ▼ 20 µM, ● 40 µM and ♦ 50 µM. A concentration of 20 $U \cdot mL^{-1}$ of AChE in the aliquot was used for all reactions. Other conditions as for Fig. 1.

Fig. 4 Dose-response curve for the inhibition activity of galanthamine for the determination of the IC_{50}-value. Range of galanthamine concentrations used: 0.1 µM, 1 µM, 10 µM, 100 µM, 750 µM, 1 mM, 10 mM and 100 mM in the aliquot added to the reaction mixture. Incubation time: 5 min. Other conditions as for Fig. 1.

Fig. 5 Time dependence of the IC_{50}-values for (A) the inhibitors galanthamine, huperzine A and (B) paraoxon. Experimental conditions as for Fig. 4.

Fig. 6 Schematic illustration of the EMMA methodology with a partial filling technique. Buffer 1: 25 mM MES / 5 mM Arg / 50 μM CTAB, pH 5.9. Buffer 2: 15 mM MES / 16 mM Arg, pH 8.0. Substrate solution: 1 mM ATCh in buffer 2. Enzyme solution: 20 $U \cdot mL^{-1}$ AChE in buffer 2. Injection order: 1) Injection of buffer 2 for 10 s at 250 mPa; 2) Injection of the enzyme solution for 10 s at 250 mPa; 3) Injection of substrate and inhibitor solution for 10 s at 250 mPa; 4) Injection of buffer 2 for 10 s at 250 mPa; 5) Application of -5 kV for 5 s to mix the substrate and inhibitor with the enzyme; 5) Electrophoretic separation after high voltage -30 kV is applied.

Fig. 7 Electropherograms of ATCh catalyzed by AChE with the EMMA mode. Electropherogram 1: production of acetate without galanthamine. Electropherogram 2: reduced production of acetate on adding galanthamine in the ATCh solution. Online incubation time: 5 min. Other conditions as for Fig 6. Capillary length: 70 cm.

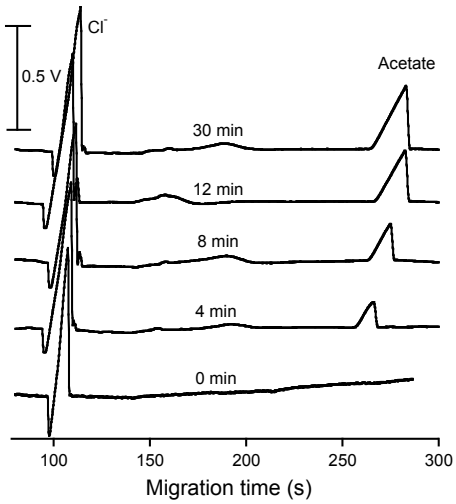
Cl-
0.5 V
Acetate
30 min
12 min
8 min
4 min
0 min
100
150
200
250
300
Migration time (s)

Fig. 1

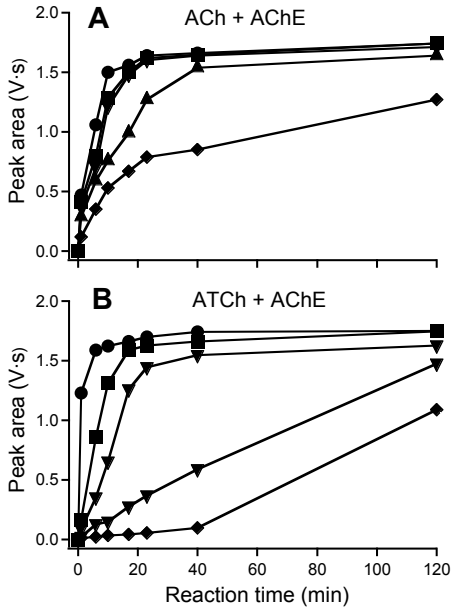
A
ACh + AChE
Peak area (V·s)
2.0
1.5
1.0
0.5
0.0
B
ATCh + AChE
Peak area (V·s)
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
Reaction time (min)

Fig. 2

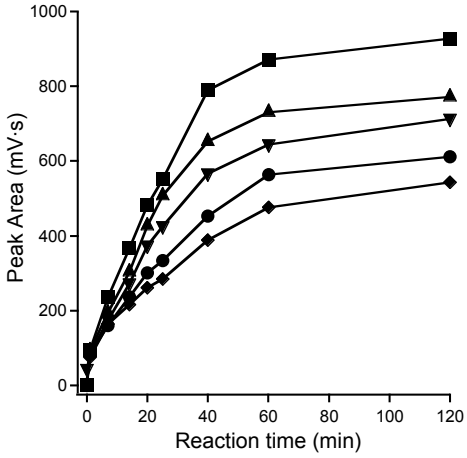
1000
800
600
400
200
0
Peak Area (mV·s)
0
20
40
60
80
100
120
Reaction time (min)

Fig. 3

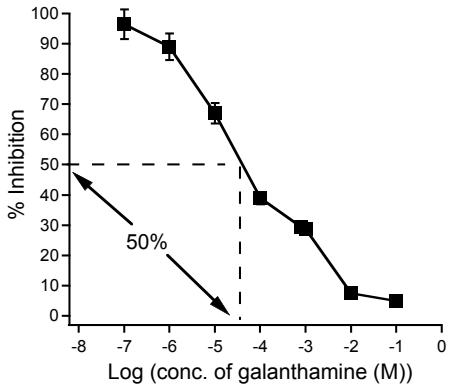
100
90
80
70
60
50
40
30
20
10
0
% Inhibition
50%
-8
-7
-6
-5
-4
-3
-2
-1
0
Log (conc. of galanthamine (M))

Fig. 4

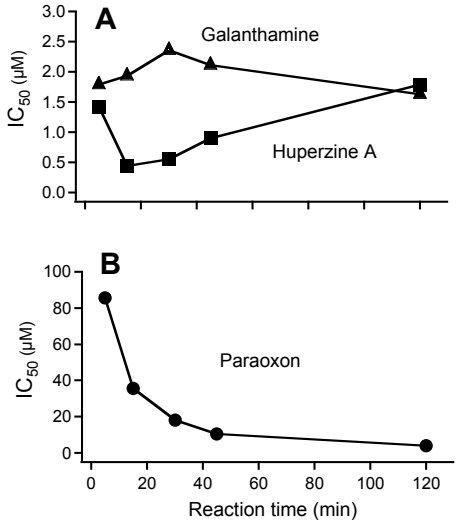
A
Galanthamine
Huperzine A
IC50 (μM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
B
Paraoxon
IC50 (μM)
100
80
60
40
20
0
0
20
40
60
80
100
120
Reaction time (min)

Fig. 5

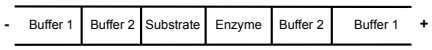

Fig. 6

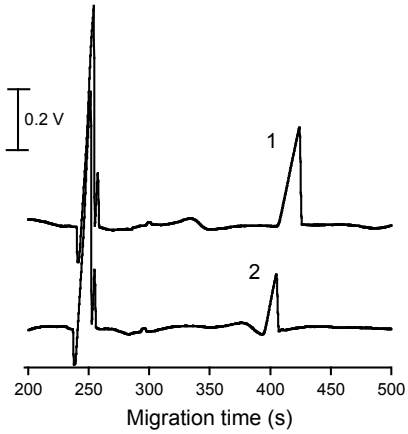

Fig. 7

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VII. Curriculum Vitae

Personal Information

Surname:	**Schuchert-Shi**
First name:	Aiping
Date of Birth:	24. December 1968
Nationality:	Swiss
Gender:	Female
Marital Status:	Married
Children:	2 daughters
Address:	Münchensteinerweg 68 4153 Reinach Tel: 0041-61-7110929

Education

2005 – present **PhD study in Analytical Chemistry,** University of Basel, Switzerland. Doctoral thesis: Monitoring of Enzymatic Reactions Using Capillary Electrophoresis with Conductivity Detection.

1990 – 1993 **MSc in Organic Chemistry,** Shanghai University, China**.** Master thesis: Synthesis & Characterization of Poly(t-BOC-Styrenesulfone)- Resists for Lithography.

1988 – 1990 **BSc in Business management,** Shanghai University, China. Thesis: Functional demands for the person who is in charge of R&D department.

1986 – 1990 **BSc in Fine Chemical Engineering,** Shanghai University, China. Thesis: Formulation & development of a new plotter ink.

Work Experience

1993 – 1994	**Shanghai Institute of Lighting Technology,** Shanghai, China. Project Manager: Researched on a specific adhesive used in the lighting industry**.**
1994 – 1996	**Syngenta (SuZhou) Crop Protection Limited,** Shanghai, China, R. Nobs (Supervisor). Process Engineer: Planned, designed and commissioned new agrochemical manufacturing facility, coordinated between contractors and designing institute.Responsible for project budgeting and monthly report.
1996 – 1997	**Syngenta Crop Protection Limited,** Shanghai, China, S. Poupon (Supervisor). Supply chain management: Sourced local suppliers of raw materials and exported raw materials and finished products to other countries, contributed to the set-up of a new office.

Major programs and trainings:

10/1995	**HS&E (Health, Safety & Ecology):** Ciba Global HS&E, Shanghai
1998 – 1999	**Graphic Design** , St. John's College, Cork, Ireland
1999 – 2000	**European Union Business Study**, St. John's College, Cork, Ireland

Language skills

Chinese:	mother tongue
English:	fluent
German:	intermediate
French:	beginner

Hobbies

English literature, international travel, meditation, tennis, photography, graphic design, snooker

VIII. List of Publications and Posters

Publications

1. Monitoring of enzymatic reactions using conventional and on-chip capillary electrophoresis with contactless conductivity detection
   Electrophoresis 2007, 28, 4690-4696

2. Monitoring the enzymatic conversion of urea to ammonium by conventional or microchip capillary electrophoresis with contactless conductivity detection
   Analytical Biochemistry 376 (2008) 262-267

3. Peptic and tryptic digestion monitored by capillary electrophoresis with contactless conductivity detection
   Analytical Biochemistry 387 (2009) 202-207

4. Following the lipase catalyzed enantioselective hydrolysis of amino acid esters with capillary electrophoresis employing contactless conductivity detection
   Submitted to Chirality

5. Study of acetylcholinestrase inhibitors using capillary electrophoresis with contactless conductivity detection
   Submitted to Electrophoresis

Participations on the following conferences with Posters:

1. 22nd International Symposium on Microscale bioseparations and methods for systems biology, March 9-13, 2008, Berlin, Germany

2. 16th International Symposium on Capillary Electroseparation Techniques, Aug. 31-Sept. 3, *2008*, Catania, Italy

3. Swiss Chemical Society – Fall Meeting, University of Zurich, September 11, 2008, Zurich, Switzerland