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STUDY OF BIOLOGICAL MEMBRANES AND THEIR COMPONENTS BY CAPILLARY ELECTROPHORETIC AND MASS SPECTROMETRIC METHODS

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Institute of Mathematics and Natural Sciences, Tallinn University, Tallinn, Estonia

The dissertation was accepted for the defence of the degree of Doctor Philosophiae in Analytical Biochemistry by the Doctoral Committee of Natural Sciences of Tallinn University on October 24th, 2013

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The defence will take place on December 13th, 2013 at 12 o'clock at Tallinn University lecture hall M-213, Uus-Sadama st 5, Tallinn

CONTENTS

LIST OF PUBLICATIONS

This dissertation is based on the following publications, which are referred by the Roman numerals:

I. K. Martma, S. Z. Fernaeus, T. Land, R. Shimmo 2010. Study of cell membrane based coatings in capillary electrochromatography – *Procedia Chemistry*, 2, 26-33.

II. K. Martma, S. Z. Fernaeus, T. Land, R. Shimmo 2010. New capillary coatings in open tubular CEC as models for biological membranes – *Electrophoresis*, 31, 1586-1589.

III. K. Martma, K.-L. Habicht, X. M. Ramirez, K. Tepp,T. Käämbre, O. Volobujeva, R. Shimmo 2011. Polydopamine as an adhesive coating for open tubular capillary electrochromatography – *Electrophoresis*, 32, 1054-1060.

IV. K. Martma, P. W. Lindenburg, K.-L. Habicht, K. Vulla, K. Resik, G. Kuut, R. Shimmo 2013. Potential of PolyE-323 coated capillaries for capillary electrophoresis of lipids – *Journal of Chromatography A,* 1317, 193-198.

Author's contribution

Publication I: The author performed the cell membrane extractions and sample preparations, capillary electrochromatographical analyses and inverted light microscope studies. He interpreted the data, wrote part of the manuscript and presented the results at the *5th Conference by Nordic Separation Science Society*, *NOSSS 2009* (26-29 August 2009 Tallinn, Estonia).

Publication II: The author performed the cell membrane extractions and sample preparations, capillary electrochromatographical and Western blot analyses. He interpreted the data, wrote part of the manuscript and presented the results at the *17th International Symposium on Electro- and Liquid Phase-separation Techniques, ITP 2010* (29 August-01 September 2010 Baltimore, USA).

Publication III: The author performed the cell membrane extractions and SEM studies. He also performed part of the mitochondria extractions, sample preparations and capillary electrochromatographical analyses. He interpreted the data, wrote part of the manuscript and presented the results at the *IUPAC International Congress on Analytical Sciences, ICAS 2011* (22-26 May 2011 Kyoto, Japan).

Publication IV: The author performed part of sample preparations, capillary electro-chromatographical analyses and interpreted the data and contributed to the writing of the manuscript.

ABSTRACT

STUDY OF BIOLOGICAL MEMBRANES AND THEIR COMPONENTS BY CAPILLARY ELECTROPHORETIC AND MASS SPECTROMETRIC METHODS

Biological membranes represent complicated and essential structures in living organisms. Investigating their natures and interactions is important for studying molecular basis behind several diseases. The aim of the current work is to study important biological entities – cell membranes – as a whole but also on a molecular basis. The main object of this work is to develop novel models which describe cell membranes.

In creation the new cell membrane models the main emphasis has been to evaluate their stability. The major part of the work describes the development of cell membrane fragment based stationary phases (membrane models) in capillary electrochromatography. These membrane models have been based on lipid mixtures, cell membrane fragments and mitochondria. Direct cell membrane immobilization onto the capillary wall has been compared to the immobilization over different capillary coatings. The bonding of membrane fragments to the capillary wall involved electrostatic interaction, physical adsorption and covalent bonding. The effect of different coating parameters (coating time, pH of the environment, coating temperature etc) on the stability and repeatability of these stationary phases has been monitored.

In addition to the membrane fragment studies, single lipid components (cardiolipin, sphingomyelin, phosphatidylcholines) were studied by capillary electrophoresis, mass spectrometry or by the combination of the two methods. In capillary electrophoresis the aim has been to develop a reliable method for the analysis of membrane lipid components. Mass spectrometry was used to follow cardiolipin oxidation and to support the experimental data derived from capillary electrophoresis experiments.

The main results of this study are different membrane models based on human neuroblastoma SH-SY5Y, murine microglia BV-2, human glioma U-87 MG cell lines as well as on lipid mixtures and mitochondria from Wistar rat liver. Construction of the membrane models included the development of coating protocols. The experiments demonstrated that the presence of PolyD coated layer in fused silica capillaries improves the performance of lipid and membrane fragment based membrane model analysis in capillaries.

The work also demonstrated that the polyamine-based coating PolyE-323 can be used in highly organic media for the analysis of markedly hydrophobic lipids.

PREFACE

Biomembranes are essential components of living cells and cell organelles, keeping them intact and controlling the transport of biomolecules in and out of the cell and its organelles. Biomembranes have many functions and their importance is hard to overestimate. Changes in biological membranes are often related to changes in the whole body. Knowing more about the membrane or its components opens new perspectives to study diseases and to discover their molecular mechanisms.

In the current work, capillary electrophoresis (CE), capillary electrochromatography (CEC) and mass spectrometry (MS) have been used to study several cell membranes and their chosen lipid components. The main aim has been to develop new capillary electromigration (CEM) methods for studying biomembranes. In CE, the fragments of the studied cell membranes have been immobilized onto fused silica capillary walls. The membrane models have been based on lipid mixtures, cell membrane fragments and mitochondria. The immobilization methods involve electrostatic interaction, physical adsorption and covalent bonding.

In CE, the aim has been to develop reliable methods for the analysis of membrane lipid (cardiolipin, sphingomyelin, phosphatidylcholines) components. MS has been used both as an independent method to study different possibilities for lipid ionization (atmospheric ionization methods) or as the detector for CE experiments.

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LIST OF ABBREVIATIONS

BGE – background electrolyte
CE – capillary electrophoresis
CEC – capillary electrochromatography
CEM – capillary electromigration
DESI – desorption electrospray ionization
EOF – electroosmotic flow
ESI – electrospray ionization
HPLC – high-performance liquid chromatography
LC – liquid chromatography
MS – mass spectrometry
SEM – scanning electron microscopy

INTRODUCTION

It is well known that complex systems – like biological membranes – are more than the sum of their component parts, meaning that oversimplifying of the study object may result in false results. On the other hand, a substantial part of biology related to basic research is done based on simplifications – biological models. The model systems would enable easier and faster monitoring of the behavior of a single component (or class of compounds) and its interactions with analytes of interest within the changing environment.

In the current work we aim to create models for cell membranes. One way to investigate these models is to greate stationary phase in flow systems. Several interesting studies have been made by Prof. Wainer's group where different biological membranes were used in open tubular columns with LC-MS system (Moaddel, Wainer 2009; Sanghvi et al. 2010, 2011; Moaddel et al. 2011; Temporini et al. 2013). In field of CEM methods a number of works describing the optimization of membrane models has been published by Prof. Riekkola's and Dr. Wiedmer's group where synthetical lipid mixtures were used as membrane materials (Hautala et al. 2007; Muhonen et al. 2008; Linden et al. 2008; Wiedmer, Shimmo 2009; Lokajova et al. 2011; Banos et al. 2012; Wiedmer, Lokajova 2013).

In the present research the models and coating material are based on fragments of cell membranes, or lipid extracts of different neuronal cell lines, or mitochondria. The model materials are separated from their natural environment (cell culture) and then immobilized onto fused silica capillary walls where they serve as stationary phases for CEC. CEC belongs to a group of separation methods, which is combined by CE and high-performance liquid chromatography (HPLC): the separation takes place in narrow capillaries – like in

CE but in the presence of a stationary phase. The stationary phase interacts with the sample components and contributes to the separation. The separation mechanism is therefore combined by electroseparation and mass distribution between different phases.

Important part of the work is devoted to the optimization of the attachment procedures of the membrane models and to the evaluation of their stability. The stability of the coating was estimated by monitoring the mobility of electroosmotic flow over successive runs. The effects of pH, storage time and temperature on the coating stability were studied as well.

The main aim of this study is to demonstrate that natural membrane pieces can be reproducibly immobilized to silica capillaries and used as new coatings for open tubular CEC (OT-CEC).

An additional goal of the work was to develop CE, CE-MS and MS methods to study selected lipid components of biological membranes: cardiolipin, sphingomyelin and phosphatidylcholines – CE method development involved a throughout study of a hydrophilic polymeric capillary coating – PolyE-323 – in organic environment.

OBJECTIVES

The general aim of this work is to describe the development of biological membrane based stationary phases (membrane models) in CEC, concentrating on the development of different immobilization protocols.

The objectives of this dissertation are following:

- To develop and investigate new biological membrane based membrane models in CEC and to compare those with synthetical lipid mixture models which have been tested in literature. The membrane models were based either on cell membrane fragments from neuronal cell lines (U-87 MG, BV-2 and SH-SY5Y), whole mitochondria from the liver of Wistar rat or lipid extracts. In order to find the most reliable membrane models, the stability and coverage of the immobilized membrane materials were tested and optimized under different conditions.

- To find the most effective approach for membrane models immobilization (physical adsorption, electrostatic interaction and covalent bonding were compared).

- To develop reliable CE methods for the analysis of the biological membrane lipids (cardiolipin, sphingomyelin, phosphatidylcholines).

- To investigate the suitability of desorption electrospray ionization mass spectrometry (DESI-MS) for the analysis of lipids and to optimize the ionization parameters.

1. BIOLOGICAL MEMBRANES

All living cells and their organelles are surrounded by membranes that can be considered as selective and protective wall for cells (Standring 2008). These membranes consist of a lipid bilayer, transmembrane proteins, interior protein network and cell surface markers (Standring 2008; Tortora, Derrickson 2009). The key functions of common membrane proteins are transport, enzymatic activities, reception and identification activities, cell-to-cell adhesion and attachment to the cytoskeleton.

1.1. Lipids in membranes and their importance

Lipid bilayers in cell membranes contain different lipids, such as glycolipids, phospholipids and cholesterol. The lipid membrane protects the cell and provides a permeability barrier matrix for membrane proteins. In addition, lipids are responsible for various important biological activities like signalling, mediating chemical messages, maintaining the body temperature etc. Modifications of lipids (i.e. oxidation by reactive oxygen species or pathogens) have been related to many different pathologies including neurodegenerative diseases and cancer. (Wenk 2005; Orešic et al. 2008; Uttara et al. 2009)

1.1.1. Phospholipids

Phospholipids are composed of a glycerol unit linked to two fatty acids and a phosphate group. The phosphate group can have additional molecules attached, such as the positively charged choline. Phosphatidylcholines are the most common phospholipids in membranes. The phosphate group of molecule is hydrophilic, and fatty acid tails are hydrophobic. Such structure allows the lipids to associate into bilayers, with the hydrophobic tails in the

middle and polar heads outside facing watery environment (Figure 1). (Nelson, Cox 2008; Losos et al. 2008; Garret et al. 2013)
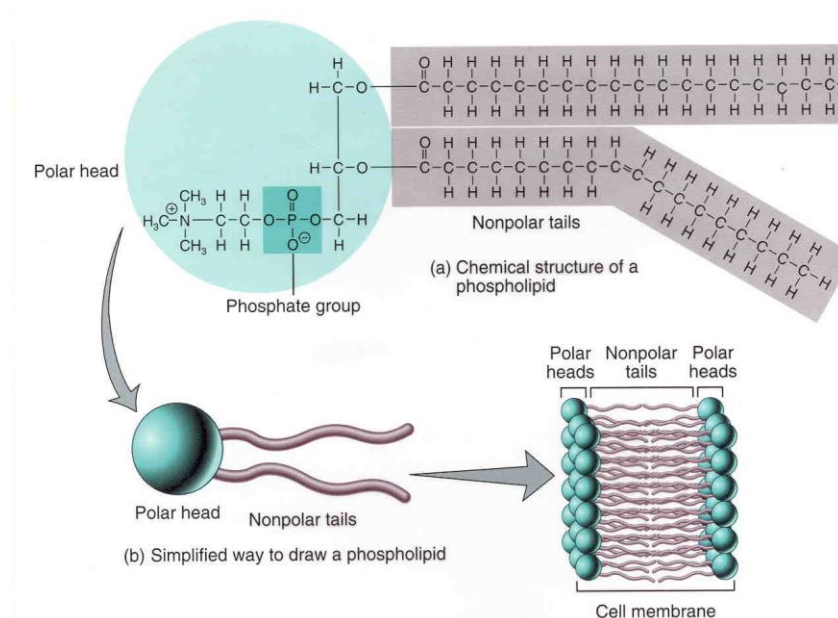
Figure 1. *Illustrative sample of phospholipid as a compound of membranes.* (Wissmann 2013)

Phospholipids have many several important functions, e.g. in cell protection and signal transductions (Nelson, Cox 2008; Garret et al. 2013).

1.1.1.1. Sphingolipids

Sphingolipids are a large class of membrane lipids; they have a polar head group and two nonpolar tails. Sphingolipids are composed of a molecule of the long-chain amino alcohol sphingosine or one of its derivates, the long-chain fatty acid molecules and the polar head group are joined either by a glycosidic linkage or by a phosphodiester bond. (Nelson, Cox 2008)

Ceramide-based sphingolipids like the sphingomyelins (Figure 2), are important components of muscle and nerve membranes in animals (Garrett, Grisham 2013). They

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have a number of important cellular functions, despite the fact that they are present only in small amounts in most membranes. For example cell-cell recognition and tissue immunity depend on sphingolipids called glycosphingolipids (Garrett, Grisham 2013).
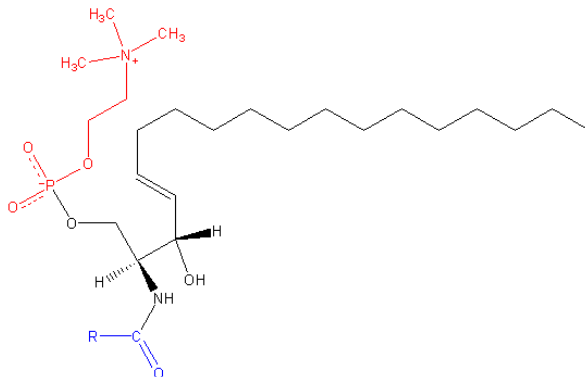
Figure 2. Sphingomyelin structure. Blue – fatty acid residue, red – phosphocholine group, black/blue – a ceramide.

1.1.1.2. Cardiolipin

Cardiolipin is a major component of the inner mitochondrial membranes but not of the plasma membrane (Nelson, Cox 2008). Cardiolipin constitutes about 20% of the total lipid composition of inner mitochondrial membranes and it was first found in animal heart (Pangborn 1942). Cardiolipin has several functions, for instance it is essential for the optimal function of numerous enzymes that are involved in mitochondrial energy metabolism, it activates mitochondrial cholesterol side-chain cleavage and it is responsible for cholesterol translocation from the outer to the inner membrane of mitochondria and has also an anticoagulant function (Nelson, Cox 2008; Garret et al. 2013).

Cardiolipin is a diphosphatidyl glycerol which is esterified through the C-1 hydroxyl group of the glycerol moiety of the head group to the phosphoryl group of another phosphatidic acid molecule (Figure 3) (Garret et al. 2013).
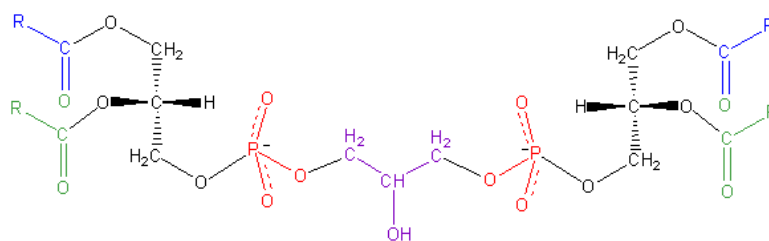
Figure 3. Cardiolipin structure. Blue, green – fatty acid residues, red – phosphate groups, lilac – glycerol backbone.

Cardiolipin is also reported to form complexes with cytochrome C and might function as a trigger of apoptosis. Oxidated cardiolipin may also move from inner- to outer mitochondrial membrane functioning as a signal to recruit apoptotic proteins Bax and truncated Bid. It has also been established that the activities and normal function of essentially all respiratory chain and ATP synthasome complexes depend directly on the presence of cardiolipin. Several studies state that cardiolipin (or its changes) could serve as a marker for numerous diseases (Hauff, Hatch 2006; Chicco, Sparagna 2007; Chacinska et al. 2010).

1.2. Lipid peroxidation and connection with diseases

Lipid peroxidation is the process in which free radicals take electrons from the lipids in cell or cell organelle membranes, resulting in cell damage. This proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds. (Ostrea et al. 1985)

The overall mechanism of lipid oxidation consists of three phases:

    1) initiation, a process during which the free radicals are formed,

    2) propagation, the free-radical chain reactions occur,

3) termination, nonradical products are created.

Lipid hydroperoxides – the products of oxidation are non-radical intermediates derived from different lipids and fatty acids (Wenk 2005). Their formation is a result of enzymatic or non-enzymatic reactions that in turn are activated by chemical species known as "reactive oxygen species" (ROS) which are responsible for toxic effects in the body via various tissue damages which connects with different diseases (Wenk 2005).

ROS mediated lipid peroxidation damages biological membranes and generates a number of secondary products that possess neurotoxic activity. Oxidative changes in lipids are suspected to be in direct connection with many diseases including neurodegenerative and infectious diseases, cancer and diabetes. (Uttara et al. 2010)

2. CAPILLARY ELECTROMIGRATION METHODS

CEM methods are a group of attractive separation techniques where all the analysis is driven by an electric field and carried out in narrow tubes. The methods are versatile, have high separation power, need tiny amounts of sample and solvents and are easily automated. CEM can be divided into CE techniques and CEC.

2.1. Capillary electrophoresis

In CE the separation is based on the electrophoretic mobility differences of analytes. The separation medium depends on the analytes to be separated and can range from purely aqueous to so-called non-aqueous environment.

2.1.1. CE system and electroosmotic volume flow

CE is one of the most common (Kuhn, Hoffstetter-Kuhn 1993; Altria 2010) instrumental analytical chemistry separation methods and uses a capillary tube (typically 25-100 μm inner diameter) filled with an electrolyte solution called background electrolyte (BGE) (Baker 1995; Riekkola et al. 2004).

The principal components are: high-voltage power supply, a capillary that passes through the detection system which is connected to a data acquisition device, a sample introduction system, and an autosampler (Figure 4).
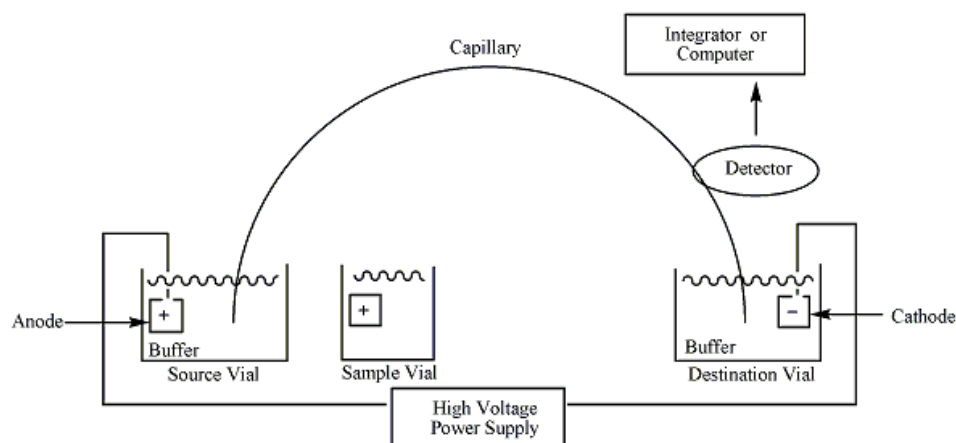
Figure 4. *Scheme of CE system.* (Precissi 2013)

In the CE process, the sample ions move under the influence of an applied voltage towards the electrode and pass through the detector (Kuhn, Hoffstetter-Kuhn 1993). The speed of movement of an ion is determined by its size and charges. For instance, (if no other force is present) a smaller ion will move faster than a larger ion with the same number of charges. The electrophoretic mobility (μ_{ep}) is therefore related to the charge/mass ratio (Equation 1). (Kuhn, Hoffstetter-Kuhn 1993; Baker 1995; Altria 2010)

$$\mu_{ep} = \frac{z}{6\pi\eta r},\tag{1}$$

where μ_{ep} is electrophoretic mobility, z is number of charges, η is solution viscosity and r is radius of the ion.

The actual electrophoretic velocity of the solute ions is related to their mobilities and the magnitude of the applied voltage (Equation 2). (Kuhn, Hoffstetter-Kuhn 1993; Baker 1995; Altria 2010)

$$v_{ep} = \mu_{ep} \cdot E,\tag{2}$$

where v is velocity of the ion and E is applied voltage (v/cm).

If the electrophoretic mobility described by Equation 1 would be the only moving force in capillary then the solute ions would move with their mobilities towards the electrodes of opposite charges. However in fused silica capillaries another moving force is often present – called electroosmotic flow (EOF). EOF occurs because the capillary wall material contains numerous silanol groups which start to deprotonate already at pH 2. The higher the pH the more of the silanol groups dissociate and the more negatively charged groups are on the capillary wall (Baker 1995; Altria 2010). To maintain electroneutrality, cations build up near the surface and when the electric field is created they migrate towards the cathode (Hoffstetter-Kuhn 1993). The movement of the solvated cations drag the bulk of the solution towards the cathode, causing a net solution flow along the capillary (Figure 5) (Kuhn, Hoffstetter-Kuhn 1993; Baker 1995; Altria 2010).
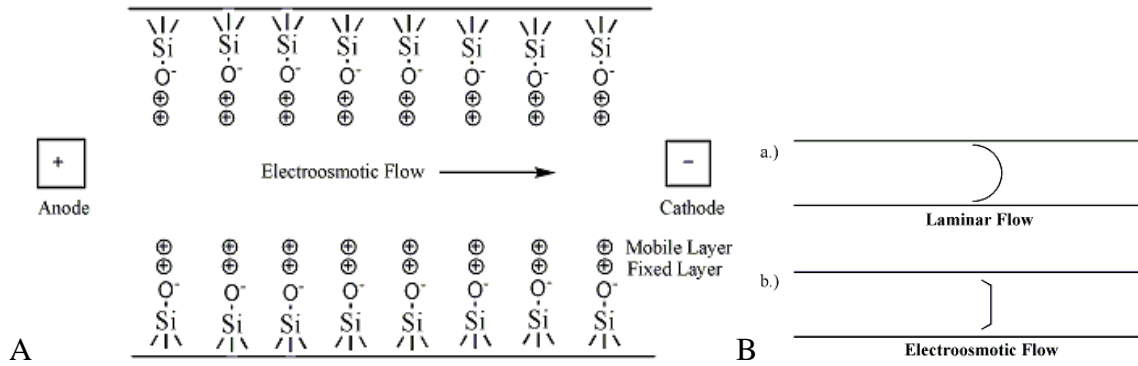
Figure 5. A: Schematic of electroosmotic flow – EOF. B: Laminar (a) and electroosmotic (b) flow profiles. (Precissi 2013)

The extent of the EOF is related to the charge on the capillary wall, the buffer viscosity and dielectric constant of the buffer (Equation 3).

$$\mu_{EOF} = \frac{\varepsilon \zeta}{\eta},$$

(3)

where μ_{EOF} is EOF mobility, η is viscosity and ζ is zeta potential.

In practice, the level of EOF is highly dependent on electrolyte pH, since the ζ potential is largely governed by the ionization of the acidic silanol. The presence of EOF allows the detection of both cations and anions within a single analysis. The presence of EOF can be both desired and undesired. EOF usually speeds up the analysis and allows to analyse cations and anions during a single run. However, it is also a source of uncertainty contributing to unrepeatability of the analysis. Especially when highly hydrophobic analytes are analysed as the uncoated silica wall attracts strongly hydrophobic compounds (Kuhn, Hoffstetter-Kuhn 1993; Baker 1995; Altria 2010).

To overcome these problems, the capillary wall can be coated with hydrophilic coatings. Also, when the target analytes are anionic it is sometimes beneficial to coat the capillary with a positively charged polymer to create anodic, i.e. reversed, EOF (Kuhn, Hoffstetter-Kuhn 1993; Baker 1995; Altria 2010).

2.1.2. CE coatings

As already mentioned – the modification of CE capillary wall is needed to achieve reduction or elimination of analyte-wall interactions (e.g. fat-dissolving samples like lipids), for alternation of EOF to achieve more rapid separation and also improved reproducibility or resolution of difficult separation problems (Horvath, Dolník 2001). For those reasons different capillary coatings are used.

The most commonly used coatings are neutral and charged organic polymers like polyvinyl alcohol (PVA), poly(n-undecyl-α-D-glucopyranoside) (PUG), polysaccharides, polyacrylic acid (PAA), polyarginine (PA), polyethyleneimine (PEI), polyalkylene glycol, polyethylene oxide (PEO), polyvinylpyrrolidone (PVP) (Horvath, Dolník 2001).

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2.2. Capillary electrochromatography

The origin of the use of an EOF in chromatography was already suggested in 1939 by Strain, who used a combination of electrophoretic and chromatographic adsorption methods for the separation of a number of organic dyes on Tswett adsorption columns (Tswett[2] 1906). However, this suggestion did not develop any further until 1974 when Pretorius and co-workers (Pretorius et al. 1974) applied this concept for the use of an EOF in liquid chromatography (LC) (Pretorius et al. 1974; Jiskra 2002). At the beginning of 1980s, technical developments in the field of the manufacturing capillaries pushed electroseparation techniques forward to practical laboratory use (Pretorius et al. 1974; Jiskra 2002).

CEC is a technique, which can be regarded as a hybrid of HPLC and CE. CEC offers a unique mode of separation by combining the chromatographic retention with electrophoretic mobility differences. Due to its high separation efficiency CE has developed into a powerful analytical tool for the separation of charged compounds.

2.2.1. Stationary phases in CEC

Chromatography (Tswett[1] 1906) is one of the most used separation methods where a mixture is separated into compounds in a column, which always consists of two phases: a stationary phase and a mobile phase (Tswett[1] 1906). The compounds of a mixture separate because (or if) each compound has different affinity towards mobile and stationary phase. The compounds that have greater affinity towards the stationary phase are delayed more than those that have lesser affinity. Figure 6 demonstrates a simple separation process of 2-compound mixture. The red compound interacts less with the stationary phase than the blue compound and exits the column faster. The stationary phase or adsorbent in column

chromatography is usually solid. The most important role of stationary phase is to separate compounds from a mixture or sample (Figure 6). The most common stationary phase for column chromatography is silica gel (Mohrig et al. 2006, 2010).
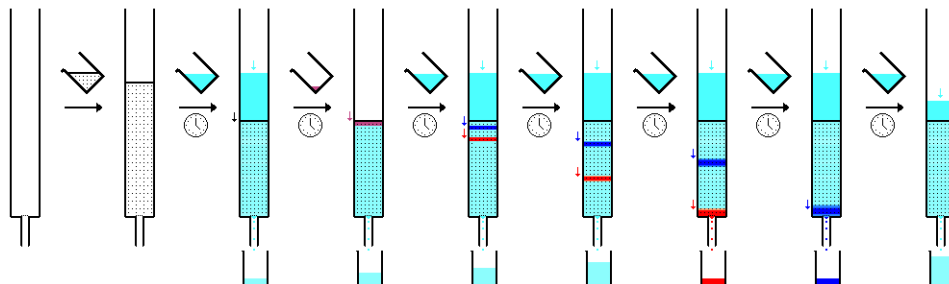
Figure 6. *Scheme of column chromatography separation with stationary phase filling.*

Most of the stationary phases, which have been used in LC, have been also transferred to the CEC format. In CEC the main division is made between OT-CEC and packed columns. The packed columns represent the classical column chromatography, where the stationary phase is filling the whole column. In OT-CEC the stationary phase is attached to the inner wall of the capillary. OT capillaries have several advantages over packed columns. The variety of possible stationary phases is extensive. Filters and frits are not needed and the air bubble formation often occurring close to the border between the packing bed and the outlet frit is eliminated (Wang et al. 2005). The easier preparation of columns avoids the imprecision and containment problems of the stationary phase (Wang et al. 2005; Mohrig et al. 2006, 2010). However, the sample capacity in OT-CEC is less than in packed CEC capillaries. According to Crego et al. 1993 a phase ratio of dynamically coated OT-CEC columns is around 350 times lower than that in packed columns packed with commercial reversed phase HPLC packing materials.

Stationary phases (either for CEC or OT-CEC) could be classified based on the stationary phase material as follows (Horvath, Dolník 2001; Jiskra 2002):

- Silica based

    - Normal phase

    - Reversed phase: conventional (ex. C8, C18, C30, polymeric phases), phases with enhanced EOF (ex. Hypersil C18), phases with charged groups (ex. cation- or anion-exchangers), special phases (ex. fluorocarbon coated; chiral stationary phases).

- Affinity interactions with specific ligand phases

    - Amino acids and polyamines

    - Porphyrin macrocycles

    - Calixarene-based stationary phases

- Aptameric stationary phases

- Polymer brushes and dendrimers

- Sol-gel derived stationary phases

- Molecularly imprinted polymers

- Polymer-coated capillaries

- Nanoparticle coatings

- Phospholipid coatings

- Biological materials: proteins, peptides, biological nanoparticles (e.g. lipoproteins), lipid mixtures.

The numerous stationary phases listed above demonstrate that there is a wide range of possibilities and long list of samples to be analysed by CEC. On the other hand, the creation

of the stationary phases, especially of those based on biomaterial, needs careful development of coating protocols. (Horvath, Dolník 2001)

2.2.2. Membrane models as stationary phases

Among the various possible column stationary phases are also biological materials, which are derived from or resemble biological membranes. Such materials could be used (along with all the materials listed above) to enhance separation of complex mixtures. But these materials could also be viewed as models for biological membranes and used to learn more about these objects. Among the research groups dealing with membrane-resembling stationary phases the work of Prof. Wainer's group from US National Institute of Aging and Prof. Marja-Liisa Riekkola's and Dr. Susanne Wiedmer's groups from University of Helsinki should clearly be mentioned. The work of Prof. Wainer's group describes affinity studies between numerous cell membrane proteins and selected drug candidates (Moaddel, Wainer 2006; Moaddel, Wainer 2009). These stationary phases contain not only the target proteins but also many other components naturally present in the cell line under study and present therefore well-presentative membrane models (Figure 7).
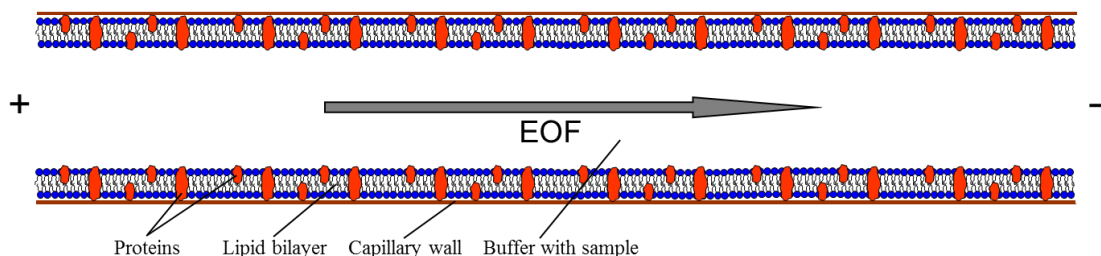
Figure 7. *Illustrative sample of membrane model on CE capillary wall.*

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The advantages of the membrane models in CEC format are that the analysis of biological processes is very flexible. One can easily start and stop reactions in capillary; rinse different solvents through the capillary and out again and change an experimental condition fast and easily. In addition the amount of sample and reagents is small.

3. MASS SPECTROMETRIC IONIZATION METHODS FOR LIPIDS

The progress in lipid analysis during recent years has been greatly due to the rapid development of analytical instruments, especially mass spectrometers (Hong et al. 2007; Domingues et al. 2008; Masoodi et al. 2008; Tyurin et al. 2009). However, the analysis of lipids in complex biological samples (e.g. in tissue samples) is still challenging (Spickett et al. 1998). This is partly because of the complexity of the samples, which contain a huge number of different compounds, and partly because the ionization of lipids is not a trivial task.

Mass spectrometry is rapidly developing into a powerful analytical method, which can be used either alone or in combination with other instrumental methods (including chromatography and CE). The ionization method of a chosen MS technique determines the range of suitable analytes. The most popular ionization methods for biological samples have been electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). Recently, new atmospheric pressure desorption ionization MS techniques, often referred to as ambient MS (Takáts et al. 2004), have been developed, which allow the direct analysis of analytes from surfaces, often without any sample preparation (Cooks et al. 2006; Haapala et al. 2007). The development of MS ionization methods is continuously ongoing and a wide selection of methods is available. In this chapter only the ionization methods used in current study are described.

3.1. Electrospray ionization – ESI

ESI is a well-known ionization method in MS which can be applied to wide selection of analytes including lipids (Fenn et al. 1989). ESI might be the most common ionization

technique and it can easily be combined with some other analysis method (e.g. chromatography)**.** The main solvents for ESI are prepared by mixing water with volatile organic compounds. ESI is the „soft" ionization method with low fragmentation (Ho et al. 2003).

In ESI ions also can be produced from macromolecules and thats why it is suitable for several bioorganic compounds like lipids analysis (Milne et al. 2006).

3.2. Desorption electrospray ionization – DESI

Desorption electrospray ionization (DESI) is an atmospheric pressure ion source which ionizes gases, liquids and solids in open air under ambient conditions. DESI takes place by directing an electrically charged mist to the sample surface that is a few millimeters away. The electrospray mist is attracted to the surface by applying a voltage on the sample holder. After ionization, the ions travel through air into the atmospheric pressure interface which is connected to the mass spectrometer (Takáts et al. 2005). It was developed in 2004 by Professor Graham Cooks et al. from Purdue University (Lafayette, Indiana) and is now commercially available.

Exclusion of sample preparation does not only save time and material but helps to avoid the modifications of the biological sample during the procedures. Carrying out the analysis in ambient atmosphere enables high-throughput analyses of untraditional samples (e.g. cells and tissues).

DESI is a similar ionization technique to direct analysis in real time (DART) in its versatility, applications and analysis time (Takáts et al. 2004).

4. EXPERIMENTAL

4.1. Chemicals and materials

Chloroform (CHL), ethylenediaminetetraacetic acid (EDTA) and tris(hydroxymethyl)-aminomethane (Tris) were purchased from AppliChem (Darmstadt, Germany). Dimethyl sulfoxide (DMSO), acetic acid (99.7%), leupeptin, dopamine hydrochloride, 1,2-diacyl-sn-glycero-3-phospho-L-serine, L-a-phosphatidylcholine, sucrose, testosterone, rutin, quercetin, caffeic acid, salicylic acid, propylene carbonate (PropCarb), 1,2-bis(3-aminopropylamino)ethane, epichlorohydrine, ferric ammonium citrate (FAC), cardiolipin (sodium salt from bovine heart), sphingomyelin, ammonium acetate and sodium dodecyl sulfate (SDS) were from Sigma (St Louis, Mo, USA). Ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate (BMI-PF$_6$) from (Sigma-Aldrich, Germany). Phosphate buffered saline (PBS) from Naxo OÜ (Tartu, Estonia). Methanol (MeOH), isopropanol (IPA) and acetonitrile (ACN) from Rathburn (Scotland), 1 M sodium hydroxide from Agilent Technologies (Germany). Sodium hydroxide, ammonium hydroxide, phosphoric acid, hydrochloric acid and from Lach-Ner (Czech Republic). Fetal bovine serum "GOLD" (FBS), d-glucose, penicillin, Dulbecco's modified Eagle's medium, streptomycin, trypsin EDTA (TE), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *N*-cyclohexy-l-3-aminopropanesulfonic acid (CAPS) and phenylmethylsulfonyl fluoride (PMSF) were from PAA The Cell Culture Company (Pasching, Austria). The murine microglial BV-2 and SH-SY5Y cells were from Dr. Katarina Bedecs and human glioma U-87 MG cells were generously provided by Dr. Kerstin Iverfelt from Stockholm University. Bidestilled water (made in Tallinn University lab) was used for all the solutions.

4.2. Isolation buffers and background electrolyte preparations

PBS buffer buffer consisted of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH was 7.4. TE buffer contained 10 mM Tris and 0.1 mM EDTA (**I**). The lysis buffer contained leupeptine 0.5 μg/mL, PMSF 1 mM in TE buffer (**I**). Western Blotting Detection Reagents (GE Healthcare, Germany) for Western Blotting (**I**). DMEM with Glutamax II and 4.5 g/L d-glucose supplemented with 5% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin for cell cultivation (**I**). The ionic strength of the BGE solutions (sodium phosphate, ammonium acetate, CAPS, EDTA, Tris buffers) was 10-20 mM with pH adjusted to 4.5-10.8 with 1.0 M sodium or ammonium hydroxide (**I**). EOF measurements were carried out with 0.1% DMSO aqueous solution as sample (**I**). The isolating solution for mitochondria extraction contained 250 mM sucrose, 10 mM HEPES and 0.2 mM EDTA, pH was 7.2 (**III**). Different organic BGE systems like ACN/IPA/aqueous buffer, ACN/MeOH/aqueous buffer (listed in Table 4) were used for cardiolipin analysis (**IV**). The % ratios of those BGE components were 56.5/38.0/5.5 respectively (**IV**). The aqueous buffer was either 20 mM sodium phosphate or 20 mM Tris buffer at pH 7.4 unless stated otherwise (**IV**). 30 μL/20mL of ionic liquid BMI-PF$_6$ was added to BGE used for analysis to increase the conductivity of the environment (**IV**).

For better stability and repeatability of experiments BGE vials were changed after every 5 runs.

4.3. Methods

4.3.1. Cell cultivation and cell line materials

BV-2, SH-SY5Y and U-87 MG cells were generated as previously described by Pontén and Macentyre 1968, Biedler et al. 1973 and Blasi et al. 1990 respectively. Cells were

maintained at 37 °C under an atmosphere of 5% CO_2 in DMEM with Glutamax II and 4.5 g/L d-glucose supplemented with 5% fetal bovine serum "GOLD" (FCS), 100 U/mL penicillin and 100 mg/mL streptomycin (**I-II**). Cells were sub-cultivated (1:10) once per week, and medium was changed 3-4 days after sub-cultivation (**I-II**). For cell membrane extracts, two 75 cm^2 cell culture flasks were seeded (1:10), seven days before the preparation (**I-II**).

For membrane coating solution cell membrane suspensions were prepared by a modified procedure as described by Karelson et al. 2005. Shortly, the complete culture medium was removed from 250 mL CELLSTAR Tissue Culture Flask and the cells were washed with ice cold PBS two times (10 mL + 10 mL). Then 1.5 mL of lysis buffer was added. After that the cells were scraped out of the flask with plastic cell scraper, collected into a tube and centrifuged at 1000 rpm for 5 min at 4 °C. The pellet was resuspended in 1 mL of lysis buffer and vortexed. Cell were then homogenized with 15 strokes in Dounce cell homogenizer on ice. After that the solution was centrifuged again at 1600 rpm for 10 min at 4 °C to remove cell nucleus. The supernatant was saved and centrifuged again at 16000 rpm for 20 min at 4 °C to remove mitochondria. The supernatant was sonicated for 3x15 min. After that the solution was ready to use for coating. (**I-II**)

4.3.2. Preparation of lipid solutions

Extraction of lipids was done by the method of Bligh and Dyer (Bligh, Dyer 1959) which is widely used for lipid extraction and purification from biological samples (Xiaoa 2012). The cells were first treated the same way as in the membrane suspension procedure. After breaking the cells and removing larger pieces of membrane by centrifugation the membrane suspension was mixed with methanol/chloroform solution. The lipid containing

bottom layer was removed and the solvent was evaporated to dryness under a stream of nitrogen. The solid residue was either redissolved in chloroform and stored at −18 °C or directly dissolved in the BGE.

4.3.3. Extractions and coating of mitochondria

The mitochondria were isolated from adult white Wistar rats of 300 g body weight, as described by Saks et al. 1975. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Wolff, Smith 1994). The rat liver was washed with isolation medium and put into the vial at +4°C. Then livers were collected on a Petri dish and pressed through the stainless steel net. Crushed livers were homogenized in two passages at 200 rpm. After that the livers were incubated with trypsin (0.5 mg/mL trypsin for 15 min at +4°C) and then 20 mL of isolating solution with 10 mg STI and BSA (1 mg/mL) was added. After that the mixture was homogenized with three passages at 250 rpm followed by another three passages at 300 rpm. After homogenization the liver mass was filtrated through the crude net. Mitochondria were extracted by centrifugation for 10 min at 4000 rpm. The supernatant was then separated and centrifuged again at 6000 rpm for 20 min. Then the mitochondrial pellet was resuspended into a 15 mL ice-cold extraction medium, supplemented with 0.1% BSA (fatty acid free), and washed three times by differential centrifugation (for 10 min at 6000 rpm each time). All the procedures were carried out at +4 °C. Isolated mitochondria were then dissolved in 1 mL isolation medium. (**III**)

Mitochondia solution was applied by rinsing the capillary for 40 min at 50 mbar with the solution. The coating solution was then kept in the capillary for 15 min (unless stated otherwise in the text). Finally, the capillary was flushed for 10 min with BGE to remove

unbound coating material. After the coating procedure EOF measurements were made using the DMSO solution. (**III**)

4.3.4. Preparation of polydopamine coated capillaries

Polydopamine-coated capillaries were prepared by using dopamine in 0.1 M phosphate buffer (pH 8.5) concentration of 6 mg/mL (unless stated otherwise). Capillaries were flushed with dopamine solution for 10 min at 50 mbar pressure. The dopamine solution was kept in capillary for 1-20 h. Then the second layer material (lipid, membrane or mitochondrion solution) was applied. (**III**)

4.3.5. Preparation of membrane fragments coated capillary for SEM

In order to make the study of membrane coating structure by SEM easier the SEM photos were taken of the capillaries were the membrane solution was immobilized onto the outside wall of the capillary. First the 75 μm inner diameter (i.d.) silica capillary was thermally cleaned from outside and then washed with methanol. Capillary was coated onto the outer side to place capillary into the vial with polydopamine (PolyD) solution. The coating procedure (in terms of the composition of the coating solution and coating time) is carried out similarly to the capillary coating described in 6.3.4. Experiments were made both with uncoated capillaries, with the capillaries where membranes attached directly to the capillary wall and with capillaries which were previously coated with PolyD. (**III**)

4.3.6. Preparation of PolyE-323 coated capillaries

The PolyE-323 coating solution was prepared according to Hardenborg et al. 2003. Shortly, 17.65 g 1.2-bis(3-aminoprophylamino)ethane was diluted in 20 g bidestilled water. Then

9.3 g of ephichlorohydrine was added and the mixture was gently stirred at room temperature during 48 hours. After that an additional 100 g of bidestilled water was added and the mixture was kept 2 weeks at +8 °C until the polymerization reaction was complete. Then uncoated capillary was flushed (at 900 mbar) 30 min with 1M NaOH and 1M HCl, then 15 min with bidestilled water. After that capillary was rinsed with 7.5% PolyE-323 coating solution at 45 min (pressure 50 mbar). Then capillary was flushed 10 min with BGE solution. All coating procedures were performed at 25 °C (set as cassete temperature).

4.3.7. Western blot analysis

In order to confirm the presence of membrane proteins in the membrane suspension 7-10 µg of total cell extracts prepared in TE buffer were analyzed by Western blotting (Burnette 1981). Briefly, the cell extract was separated on a 10% SDS-PAGE before transfer onto a nitrocellulose membrane. Proteins were detected with primary antibodies against c-terminal fragment of amyloid precursor protein (APP), followed by secondary horse radish peroxidase (HRP) labeled goat anti-mouse antibodies. Membranes were developed using ECL Plus Western Blotting Detection Reagents.

4.3.8. Sample preparation for DESI

The samples were prepared and diluted in water/methanol (50v/50v) or methanol/chloroform (50v/50v). The final concentration of the samples was 10-100 µM. Four 2 µL spots of each sample were applied on the sample plates for measurements. DESI and ion trap parameters were optimized by using $H_2O/CH_3OH/HCOOH$ (50v/50v/0.1%) solvent with verapamil. These optimized conditions were used for all solvents and analytes.

The tested solvents are shown in Table 1. The solvents were chosen to test the effect of solvent polarity, different organic modifiers, and different acidic, basic or salt additives.

4.4. Instrumentation

4.4.1. CE

Fused silica capillaries of 50, 75 and 100 µm i.d. total length of 40 and 60 cm, length to detector 31.5 and 51.5 cm were from Composite Metal Services (Worchestershire, UK). A Hewlett-Packard 3DCE system (Agilent, Waldbronn, Germany) with Agilent Chemstation software was used for the CE and CEC experiments.

4.4.2. SEM

SEM photos of membrane coatings were made with Hitachi Tabletop Microscope TM-1000 with EDS detector Oxford Swift-ED was used. The operating voltage was 15 kV.

4.4.3. MS

Agilent 6330 Ion Trap equipped with ESI and homemade DESI ionization module was used for the analyses. Spray solvents were pumped using a Harvard PHP 2000 infusion pump. Fused silica capillary (i.d. 50 µm) was used to direct the spray solvent into the DESI system.

Agilent TOF 6230 mass spectrometer was used for CE-MS measurements. Dry gas was set to 5 L/min at 325 $^{\circ}$C, nebulizer pressure was set to 5 psig. All MS voltage parameters had same absolute value in both negative and positive mode: 1) MS heated capillary 5000 V 2) skimmer 65 V 3) fragmentor 140 4) RF hexapole 800 V.

4.4.4. Other equipment

Inverted Microscope AE 21 was used to get cell images. Ultrasonic bath J.P.Selecta Ultrasons Medi-II was used to produce small unilamellar vesicles and also to hydrate liposomes into the buffer solution after chloroform extraction. Karl Hecht GmbH & Co. KG vortexer was used to accelerate the hydration. Eppendorf Centrifuge 5804R (rotor Eppendorf GL 089 F-34-6-38) was used after cell homogenization for fractionation. Nitrogen gas (99.996%) from Elme Messer Gaas (Tallinn, Estonia) was used to evaporate chloroform from lipid extractions. The gel electrophoresis systems used for Western blotting were Bio-Rad Mini-Protean Tetra Cell and Bio-Rad Trans-Blot SD, Semi-Dry Transfer Cell.

4.5. Data analysis

Data analysis and interpretation performed by using Microsoft Excel 2013, OriginLab Origin Pro 9.0, DataAnalysis for 6300 Series Ion Trap LC/MS Version 3.4 and 3D-CE ChemStation Version B.03.02 from Agilent Technologies.

Standard deviation was calculated by using Microsoft Excel function „STDEV" which based on following formula (Equation 4):

$$\sigma = \sqrt{\frac{\sum(x_i - \mu)^2}{n}}, \tag{4}$$

where σ is standard deviation mobility, x_i is each individual number, μ is mean and n is quantity of numbers in the group.

5. RESULTS AND DISCUSSION

In the current work new biological membrane models were created and tested in CEC format. The performance of different models was compared by EOF evaluation and by the analysis of test compounds. In addition, single components of membrane models were analysed by analytical and bioanalytical methods including CE and MS.

5.1. Development of membrane models

The first aim of this work was to develop and investigate new biological membrane based membrane models and to compare those with synthetical lipid mixture models which were already tested in literature (Lindén et al. 2004; Varjo et al. 2005; Hautala et al. 2007). The membrane models were based either on cell membrane fragments consisting of both cell membrane proteins and lipids (all cell membrane fragments were derived from neuronal cell lines: U-87 MG, BV-2 and SH-SY5Y), on lipid mixtures extracted from the cell lines or on intact mitochondria. In order to find the most reliable membrane models the stability and coverage of these membranes based coatings in CEC needed to be tested and optimized under different conditions. In current work the development of coating procedure included variation of several experimental conditions like pH, coating time, the concentration and composition of coating solution, the BGE used, temperature and capillary pre-treatment. The OT-CEC format was used throughout the CEC studies.

5.1.1. Membrane models as single capillary coatings

The first part of the membrane model optimization involved a study of membrane fragments derived from the cell lines listed in the previous subchapter (4.1). The cell lines

chosen for the current research are important study objects as they represent neuronal, astrocytic and microglial phenotype. The human neuroblastoma SH-SY5Y has been demonstrated to have biochemical properties of neuronal cells (Biedler et al. 1973). U-87 MG phenotype is the representative of astrocytes which shape the blood-brain-barrier (Liu et al. 2004). The murine microglial BV-2 cell line has microglial properties (Blasi et al. 1990).

In the following chapters (5.1.1.1.-5.1.1.4.) the influence of coating parameters on the immobilization is discussed. However, the binding mechanism itself − physical adsorption − will be kept the same. During the immobilization no chemical mediator will be used − the solution of membrane fragments will be simply inserted into an uncoated fused silica capillary. In order to avoid confusion with the membrane model studies described in chapter 5.1.2 the coatings discussed in current chapter (5.1.1.1.-5.1.1.4.) will be denoted as single capillary coatings.

5.1.1.1. Influence of coating parameters on model coated capillaries

In order to find the best conditions for membrane model immobilization different parameters like capillary inner diameter, capillary length, and capillary storage conditions were varied and their influence was evaluated by monitoring the EOF in coated capillaries. Capillary inner diameter has been one of the factors influencing the performance of capillary coatings in CE as well as the success of analyte separation in those capillaries (Hautala et al. 2003).

The length of capillary influences the total amount of stationary phase and therefore should also have impact on the later analysis. In current work we tested three different capillary i.d.'s: 50, 75 and 100 μm and two capillary lengths (60 and 40 cm total). Diameters of 50

and 75 µm and the different capillary lengths gave quite similar effects (Figure 8) in terms of the coating coverage (evaluated by the speed of EOF) and the stability (evaluated by the variation of EOF). This indicates that up to 1.5 times variations in the capillary dimensions do not affect the performance of the coating significantly. The effect of the amount of the coating on analytes separation was not tested at this stage. For the 100 µm i.d. capillary the change in EOF mobility was more pronounced (Figure 8). However, even this change was not dramatic. The 100 µm capillary caused irregularity in capillaries current and therefore the use of 100 µm capillary was excluded from further experiments.
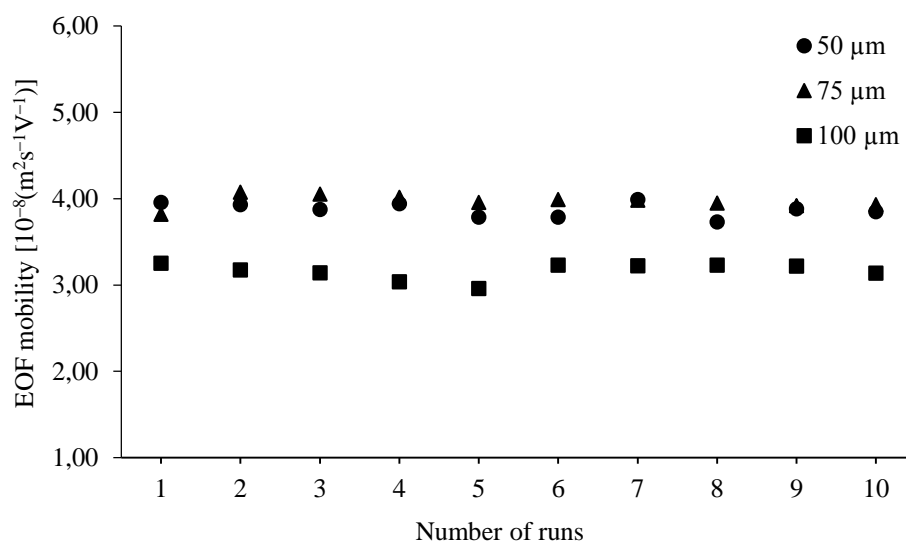
Figure 8. *Comparision of different inner diameters with lipid coated capillaries over PolyD. BGE: TE buffer, pH 7.4. 0.1% DMSO was used as neutral marker. Running conditions: temperature of cassette 25 °C, voltage 20 kV, injection 8 s at 20 mbar, detection 210 nm, capillary length 31.5/40 cm, i.d. 75 µm.*

It is known from the literature that the temperature influences the spatial regulation of biological membranes (Pabst et al. 2003) and may also influence the coating process when

an artificial lipid mixture is used as a capillary coating. Therefore as the next step the storage temperature of the membrane fragments was studied. The membrane fragment solutions were stored at +25, +4 or −18 °C and then used for capillary coating (**II**). The performance of the coating was evaluated by measuring EOF in the freshly coated capillary (data not shown) (**II**). The results demonstrated that there was no notable difference between the coatings of solutions stored at +4 or −18 °C (**II**). The only difference was that the coating stored at −18 °C yielded slightly more variability in the EOF times of successive experiments (**II**). The solution kept at +25 °C yielded somewhat more negative coating than the other membrane solutions (judged by faster EOF times) (**II**). The result is in accordance with Kuldvee et al. 2004, where the similar phenomenon was noted with egg-phosphatidylcholine coating.

As the coating solution stored at +4 °C gave the most repeatable results it was selected for further use. The stability of the coating was tested over 3-day period (39 runs). The difference between the average EOF mobility on the first and third day was less than 2% The coating could be used for 3 week without notable deteriorations in performance. (**II**)

5.1.1.2. Coating stability of different cell lines

It is known that the cells membranes of different cells vary, therefore our goal was to test if the differences show itself also in terms of coating performance. For this BV-2, U-87 MG and SH-SY5Y cells were selected. These cells are quite different in their appearance, origin and functions.

Cell membrane fragments from the above mentioned cell lines were treated according to the same protocol (described in section 4.3.1) and used for capillary coating. In order to correlate the coating performance with their phospholipid concentrations the phosphorus

content of the membrane solution (which is in direct correlation with the concentration of phospholipids in the cell membrane) was measured according to the procedure of Murphy and Riley 1962 modified by Koroleff et al. 1983. The results (Figure 9) demonstrated that different cell lines yield very similar coating in terms of the charge (evaluated by the EOF mobility) and stability of the coating (**I**).
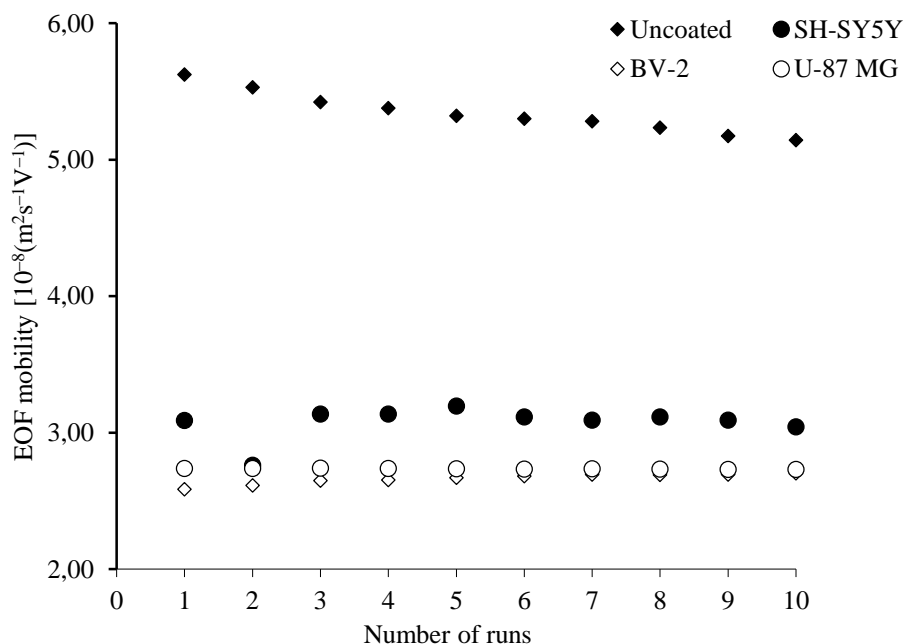
Figure 9. Repeatability of the EOF in uncoated capillary and with different membrane based solutions coated capillaries. BGE: TE buffer, pH 7.4. About 0.1% DMSO was used as neutral marker. Running conditions as in Fig. 8.

Also, up to 25% variation in coating solutions phosphorus content of the membrane-based coating solutions did not cause marked differences in the coating performance (**I**).

Figure 9 demonstrates also that the wall charge of the membrane fragments coated capillaries is less dependant of small variations in environment than the uncoated silica wall. As seen from the figure the EOF mobility in an uncoated capillary is slowly

decreasing during successive runs while the mobilities are rather stable towards the average in membrane coated capillary. (**I**)

5.1.1.3. Influence of the pH

It has shown in the literature that lipid based capillary coatings are stable in a relatively wide pH range and that pH of the BGE influences the EOF in capillary. In our work we wished to compare the membrane fragment based capillaries to the lipid based coatings and to evaluate the pH range where the membrane fragment based coatings would be stable (Hautala et al. 2003).

For the study of the pH influence, two different approaches were used: the pH of the BGE (TE buffer) solution was either changed in the same coated capillary (Figure 10A) or the capillary was coated freshly for every pH series (Figure 10B). For the comparison, the pH values (4.5, 6.5, 7.4, 8.5 and 10.8) were chosen according to Hautala et al. 2003, where the influence of pH on the formation and stability of PC/PS coatings was thoroughly studied (Hautala et al. 2005). Note that only the experimental pH was varied and the coating solutions pH was kept the same – the capillary was always coated at pH 7.4 (**I**). Directly after the coating, the EOF mobility was measured and after that the BGE with desired pH value was introduced (**I**). Figure 10A demonstrates that the stability of the membrane coating is good over the chosen pH range – the RSDs for successive runs were less than 3 %. However, comparison of Figure 10A and 10B shows that basic pH values introduce irreversible changes into the membrane coating. The EOF mobility is steadily increasing with increasing pH when fresh coatings were used for every pH (Figure 10B). However the EOF mobility is not changing with changing pH when the same coating was in contact with the BGEs at different pH value. In Figure 10B it can be seen that the stability of

coating was the poorest at most acidic pH in freshly coated capillary (RSD 25% during 10 runs). At the most basic pH value, however, the stability was quite good (RSD 6.5% during 10 runs).
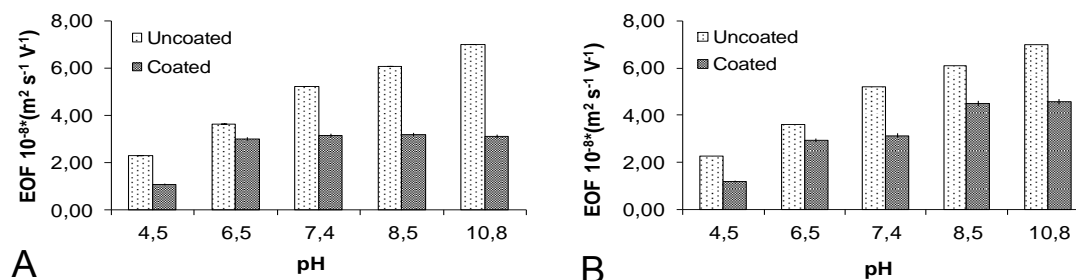
Figure 10. Average EOF mobility in uncoated and coated capillaries at different pH values. The coating pH was 7.4. A – the pH change was carried out on the same coating; B – the capillary was freshly coated for every pH study. Running conditions as in Fig. 8. (**I**)

Hautala et al. 2003 has also demonstrated that the use of HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid), has a significant effect on the success of the phosphatidyl choline/phosphatidyl serine (PC/PS) 80:20 mol % coating (Hautala et al. 2003). In the present work, we studied the influence of HEPES on the membrane fragment based coating. The results showed that the presence of HEPES in the coating solution did not improve the performance of membrane fragment based coatings (**I**). The stability of the coating as well as the EOF mobility was in the same range as with TE buffer or inferior. The result supports our hypothesis that the coating mechanisms are different for cell membrane fragments based coatings and for the artificial lipid mixture based coatings (**I**).

5.1.1.4. Comparison of membrane suspension and membrane lipid extraction

The composition of a desired membrane model depends (among other factors) on the purpose of the study. If membrane proteins would be the targets then membrane fragments based coatings would be the choice. However, if the purpose of the investigation is to follow lipid oxidation in membranes it may be beneficial to simplify the model and have it based on lipid mixture only. Therefore, in addition to membrane fragment based models the work involves the study of membrane models which are based on lipid mixtures. The lipid mixtures are not composed of commercial lipids mixtures but based on U-87 MG cell line membrane lipids. Here the preparation of the coating solution involved an extra step where lipids were extracted from the cell membrane fragments solution (the extraction protocol is described in Experimental, chapter 4.3.1).

The membrane model based on lipid mixture from U-87 MG cell membranes was compared to the cell membrane fragments from the same cell lines. Figure 11 demonstrates that the cell membrane fragments based coating and the lipid mixture based coating yield similar initial EOF mobility ($2.3 \cdot 10^{-8}$ $m^2 s^{-1} V^{-1}$).
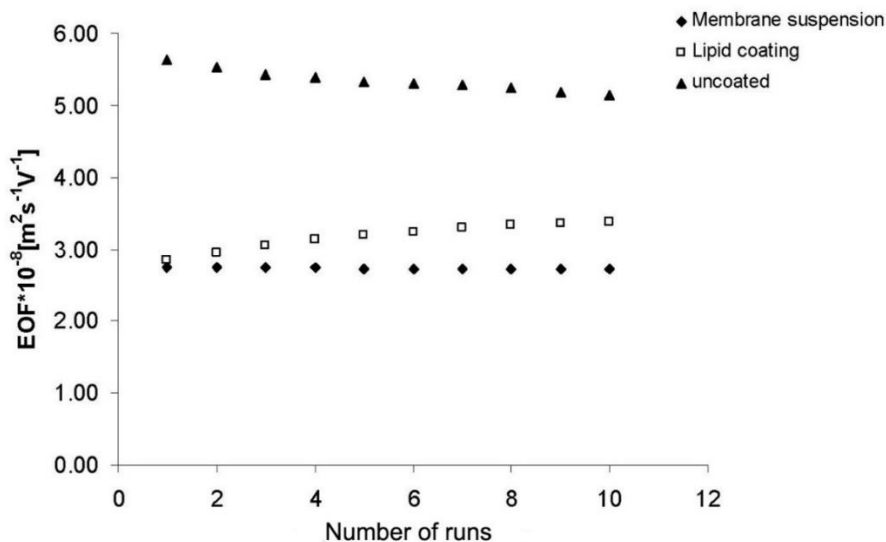
Figure 11. *Repeatability of the EOF mobility in uncoated capillary and in columns coated with membrane suspension or lipid extraction of U-87 MG cell based solutions. Running conditions as in Fig. 8.* (**I**)

However, the stability over 10 successive runs is higher for the membrane fragments based model (**I**). This is in accordance with the studies of Wiedmer and Riekkola (Hautala et al. 2003) showing that lipid mixtures based coatings often need re-coating during the runs. Nevertheless, the results show that it is possible to use lipid extracts from biological membranes as a membrane model material (**I**).

5.1.2. Capillary coating with mitochondria

Mitochondria (approximately 0.5-1 µm in size) are well defined biological structures and could possibly be useful as a membrane model in the study of lipid peroxidation or lipid-drug interactions under various environmental conditions. In our knowledge, mitochondrial material has not been used as an OT-CEC stationary phase. Therefore, it was of interest to investigate how well mitochondria could be attached to the capillary by physical adsorption. Simple rinse of the capillary with mitochondrial solution (described in detail in 4.3.3.) introduced a marked decrease in EOF mobility. The coating was rather stable and the resulting EOF in the capillary was comparable to membrane fragments based coating (**III**). The RSD of 20 successive runs was 5% for a mitochondria based coating (**III**).

5.1.3. Membrane models as double capillary coatings

To further investigate the possibilities of immobilization membrane models into CEC capillaries and to improve the coating protocol, additional approaches for the interactions

between the membrane models and capillary wall were searched. In previous chapter the physical adsorption of the membrane model onto capillary walls was the interaction mechanism. As no chemical mediator was involved the resulting models were denoted as single coatings. In present chapter electrostatic and covalent bonding over selected polymeric capillary coatings are examined. The membrane model immobilization procedure involves two coating events (coating the capillary with PolyE-323 or PolyD layer followed by coating with membrane model solution) and the resulting coatings are denoted as double layer membrane coatings. The performance of the double coating and single coating models will be compared below. The study involves optimization of the first layer (when PolyD is used) but no further optimization of the coating parameters for the second layer (except the contact time with the first layer). The experimental conditions (capillary length, inner diameter, pH of the BGE, composition of the coating solution, contact time with coating solution and coating temperature) for the latter are based on the study described in chapters 5.1.1.1.-5.1.1.3.

5.1.3.1. Double coating membrane models based on electrostatic interaction

It is known from literature that the net charge of biological membranes at physiological pH is slightly negative (Jones 1995; Radko, Chrambach 2002). Also a successful immobilization of negatively charged liposomes onto a capillary with a positively charged polymeric coating has been demonstrated (Örnskov et al.2002). Based on that knowledge an hypothesis that cell membrane models could be immobilized into silica capillaries through electrostatic interaction was tested. For the positively charged polymer a polyamine denoted as PolyE-323 was used. Since PolyE-323 was developed, it has been reported as coating in several studies, employing microfluidic devices (Hua 2013; Gao

2013) and capillaries (Zuberovic 2008, Zuberovic 2009). PolyE-323 has proved to create a stable positively charged capillary coating (**II**). However, the results were not confirming the hypothesis. Figure 12 demonstrates that there is certain interaction between the PolyE-323 coating and the membrane fragments as the EOF of the fresh coating is very similar to the EOF of physically adsorbed membrane fragment coatings. However, it is also clear from Figure 12 that the membrane model coating which is introduced into PolyE-323 coated capillary starts to leak out already after few successive runs.
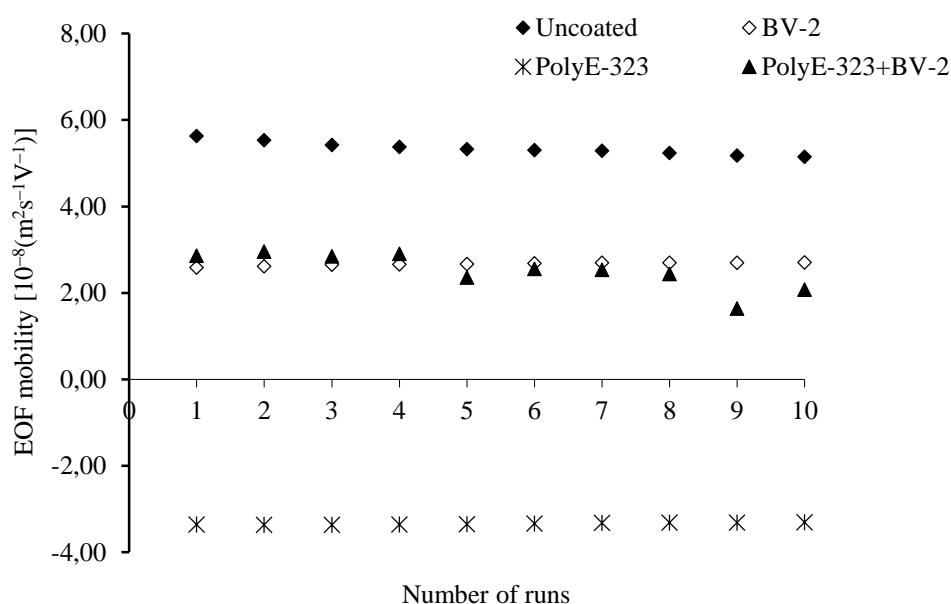
Figure 12. Repeatability of the EOF in uncoated capillary, in PolyE-323-coated capillary and in columns coated with BV-2 membrane based solutions. BGE: TE buffer, pH 7.4. About 0.1% DMSO was used as neutral marker. Running conditions as in Fig. 8. (**II**)

After 10 successive experiments the change in EOF mobility is already quite significant. The conclusion is that the created double coating is less stable than the membrane coating attached directly to uncoated capillary wall. These results indicate that the physical adsorption forces are stronger between the membrane fragments and capillary wall than

the electrostatic interactions between the PolyE-323 and the fragments (**II**). Based on the results PolyE-323 coating was no further tested as the coating for the membrane model study (**II**).

5.1.3.2. Double coating membrane models based on covalent bonding

The next step in membrane model studies was to investigate the possibilities of covalent bonding. PolyD is a polymer which rapidly forms in aqueous alkaline solutions of dopamine and creates a sticky polymer which can be attached to many different materials (Waite 2008). PolyD coating has been successfully tested for different open surfaces. In narrow capillaries the polymerization process appears to be different and not so straightforward. However Yin and Liu have demonstrated that PolyD can be used in CEC as a stationary phase to separate auxins (Yin, Liu 2008). The work showed that the capacity of the stationary phase depended on the thickness of the PolyD layer which in turn was increasing with coating time.

In the current work we aimed to use PolyD not as a stationary phase itself but as an adhesive layer for membrane models in capillaries. Different experimental conditions like the freshness and concentration of dopamine solution as well as coating time and temperature were tested to find out the fastest and most stable way for PolyD coating process. The performance of the PolyD coating was monitored by measuring the EOF in coated capillaries. After coating the capillary with PolyD layer the secondary layer material (e.g. cell membrane solutions, phospholipid mixtures or mitochondria) was introduced into the capillary. The performance of the resulting double-coated capillaries was compared with capillaries containing the respective biological material directly attached to the capillary wall (single coating membrane models).

5.1.3.3. Influence of different coating parameters on the PolyD coatings

According to the literature there is a correlation between the surface charge of capillary wall and the thickness of PolyD layer (Yin, Liu 2008). As the charge of capillary wall is in direct correlation with the EOF mobility in capillary the EOF mobility was used as the primary indicator for monitoring the thickness of the coating.

As discussed above, oxygen is needed for the PolyD formation. However, it is not clear if PolyD needs to form directly onto solid surface to provide a reliable coating or would it be possible to create the stable PolyD layer by bringing already formed PolyD into contact with solid surface. To address this question the coatings formed either via rinsing the capillary with 20 h old PolyD solution or with a fresh PolyD solution were compared. For both occasions the capillary coating consisted five times 5-hour-coatings. After every 5 hour coating the charge of the capillary wall was evaluated by measuring the EOF mobility in the capillary. The results (Figure 13) demonstrate slightly more pronounced EOF change for old PolyD solution based coating compared to the fresh PolyD solution based coating.

(**III**)
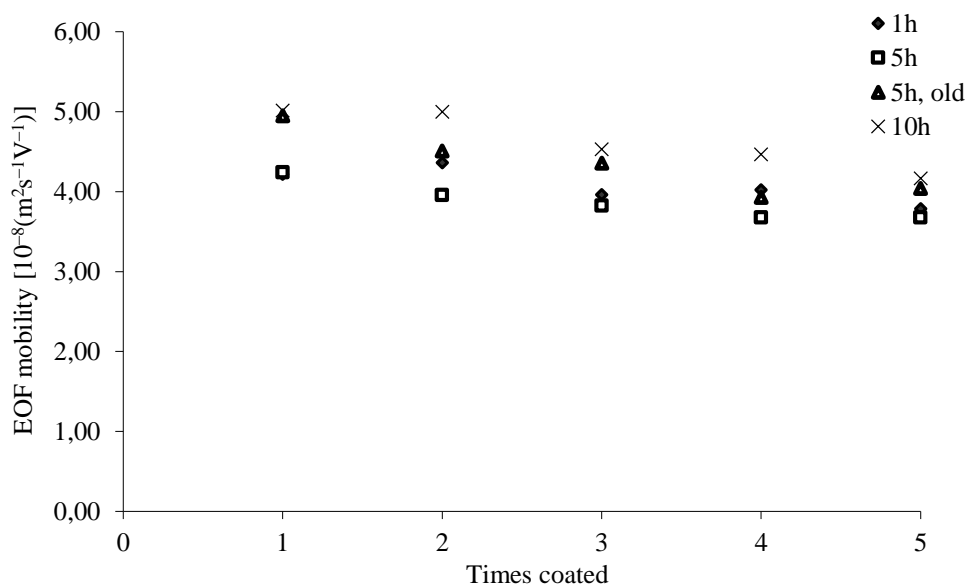
Figure 13. *The impact of the coating time duration on the EOF mobility in PolyD-coated capillaries. BGE: TE buffer, pH 7.4. 0.1% DMSO was used as the neutral marker. Running conditions: capillary length 51.5/60 cm, i.d. 50 μm. Other conditions as in Fig. 8.* (**III**)

Yet the difference is not dramatic. The same figure shows the results when the contact time between PolyD solution and the capillary was varied (1 h and 10 hour events). 10 hour contact time yields slightly increased EOF mobility but the overall conclusion is that no dramatic change in PolyD layers performance is introduced by aging the dopamine solution or prolonging the contact time over 5 hour. After 1 hour coating events the EOF change was not steady but rather fluctuating. This indicates that 1 h coating time may not be sufficient for PolyD coating. Based on the result, the 5 h coating time was used in further studies. The 5 h coatings appeared to be fairly reproducible varying only 5% over five repetitions. (**III**)

As the next step the impact of PolyD concentration (Figure 14A), coating temperature (Figure 14B) and impact of capillary inner diameter (Figure 14C) was studied.
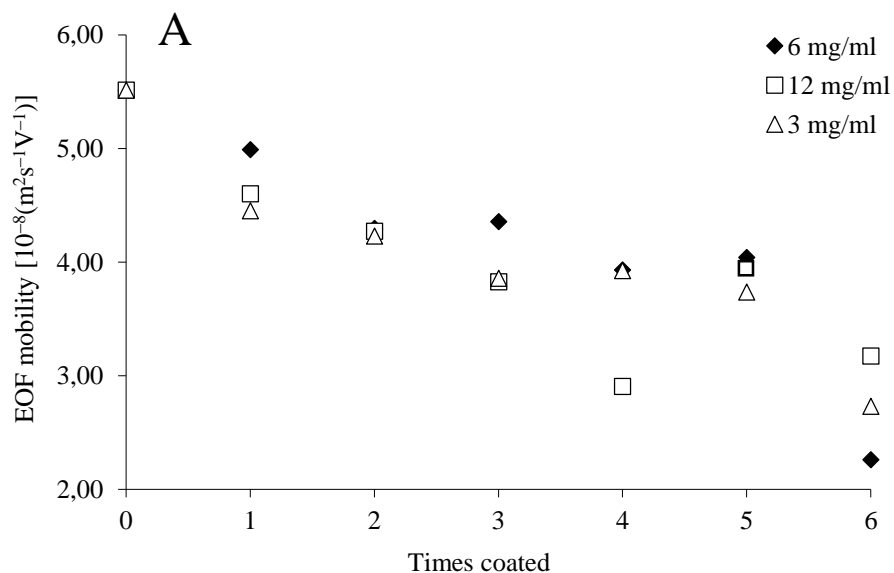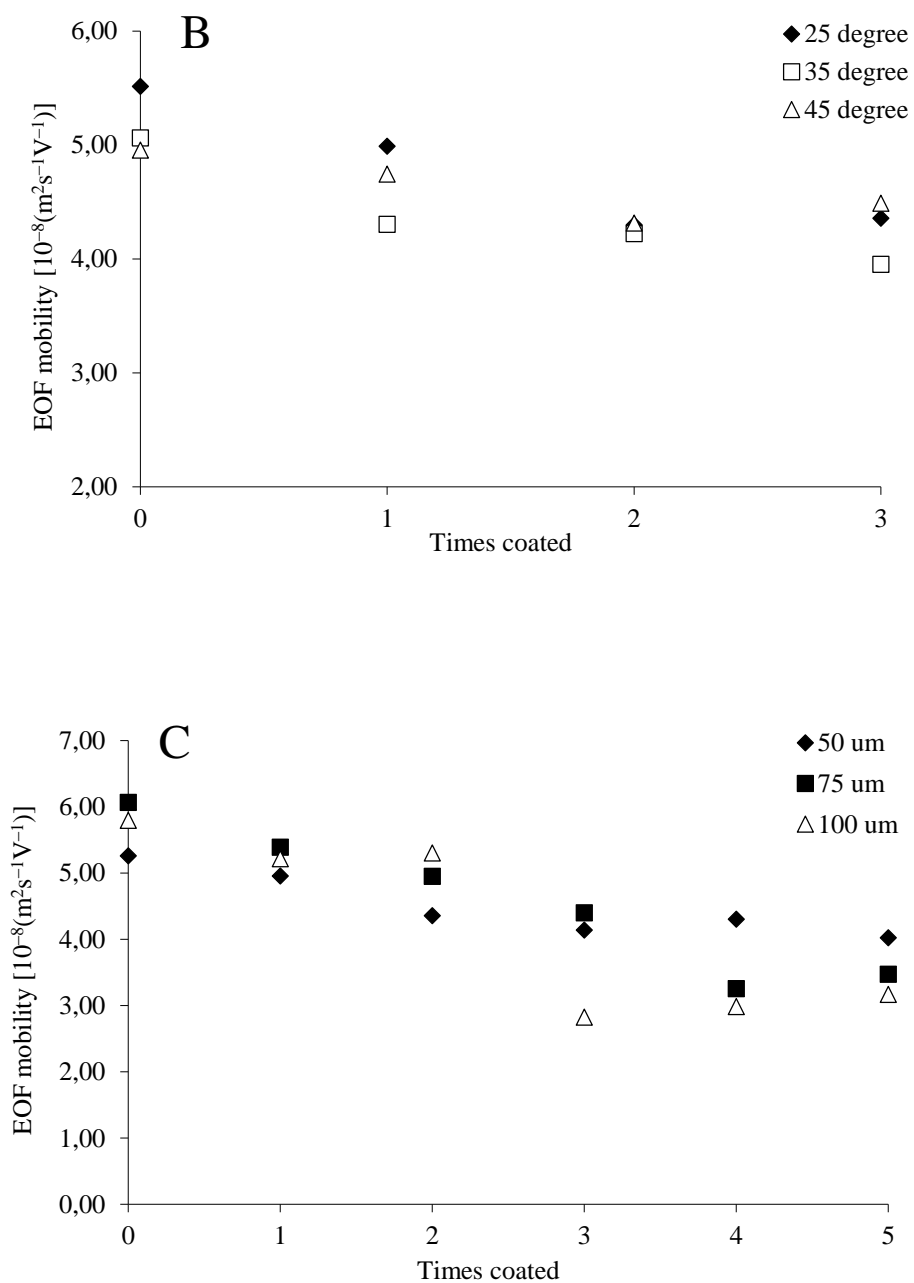
Figure 14. The impact of the PolyD concentration (A), temperature (B) and capillary inner diameter (μm) (C) on the EOF mobility in PolyD-coated capillaries. BGE: TE buffer, pH 7.4. 0.1% DMSO was used as the neutral marker. Running conditions: capillary length 51.5/60 cm, i.d. 50 μm (except in Figure C). Other conditions as in Fig. 8.

The results indicate that four-fold change in dopamine concentration and two-fold change in temperature had only marginal effect on the coating performance. A likely reason is that in the capillary the amount of available oxygen for the polymerization reaction is the limiting factor. Therefore, the repetitive coatings with fresh portions of dopamine solutions have more effect than the increase in reaction temperature or dopamine concentration. (**III**) The influence of i.d. of the capillary column has been demonstrated to affect coating performance at several occasions (Hautala et al. 2003). In order to evaluate the influence of inner diameter on PolyD formation, three different capillary diameters were tested: 50, 75 and 100 μm. The results demonstrate that 50 and 75 μm i.d. capillaries yield relatively similar coatings with respect to capillary (coating) coverage, the 100 μm capillary the change in EOF mobility is somewhat more pronounced. However, variations in EOF mobility during the repetitions indicate that the formation of the polymer layer is more random for the wider, 100 μm capillary. The 50 and 75 μm capillaries were used in further studies. (**III**)

Based on the experiments described in this chapter it is possible to conclude that repeating the coating events influenced the EOF mobility in coated capillary the most – more than any other variable. Yet after the fourth coating the change was not any more noticeable. A possible reason is the complete shielding of the capillary wall by PolyD. (**III**)

As mentioned before, the PolyD layer was not meant to use for separation but as an adhesive layer for the membrane models. Therefore the success of the PolyD coating could not be properly evaluated without the second coatings. At first simple phospholipid based coatings (containing POPC/PS 1:1) were introduced into PolyD-coated capillaries (the PolyD coating time was varied). The stability and coverage of the resulting double coatings were compared to the single coatings of the same biological material (**III**). As seen from

Figure 15 the test experiments with phospholipids indicate that the presence of the PolyD coating improves the performance of the secondary phospholipid coating.
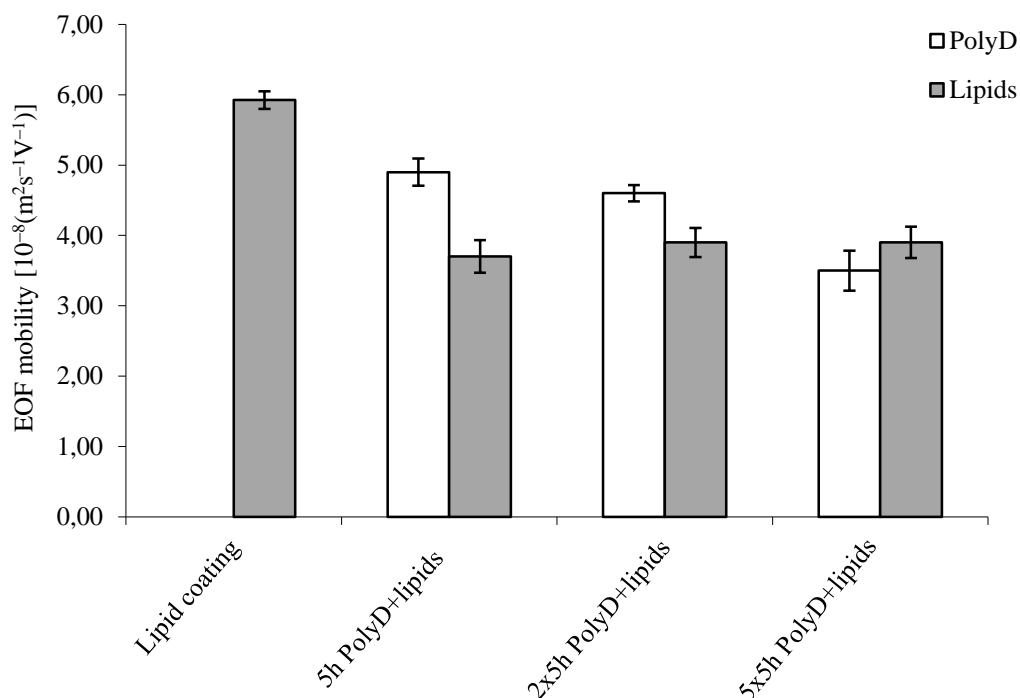
Figure 15. The effect of PolyD coatings. Lipid coatings with and without PolyD coating(s). The first bar corresponds to the lipid coating on bare fused silica capillary. Transparant bars indicate the EOF mobilities in capillaries coated 1, 2 and 5 times with PolyD. Grey bars show the EOF mobility of the lipid coatings. Error bars represent standard deviation which based on EOF mobility of 5 successive runs. Running conditions as in Fig. 8. (**III**)

The figure also demonstrates that the thickness of the PolyD coating layer does not have significant effect on the performance of the second layer. The EOF mobility for PolyD coatings decreases as expected with increased numbers of coating cycles and is clearly lower for capillaries coated five times compared with capillaries coated only one or two times. In the case of PolyD capillaries coated five times, EOF mobility was actually lower

for the final PolyD layer than for the lipid-coated capillary, adding to the reliability of our results and indicating that the lipid coating reproducibly covers the capillary wall. In addition, these results show that there is no need to keep the PolyD solution in the capillary for more than 5 h for it to be used effectively as an adhesive for biomaterial-based coatings. Coating experiments using cell membrane fragments were also more successful in PolyD coated capillaries compared with bare, fused silica capillaries. The effect of PolyD for membrane fragment based coatings is quite visible in Figure 16 where pherograms of differently immobilized membrane fragment coated capillaries are presented. It is obvious that the coating shown on Figure 16A does not contain as much of biomaterial than the coating presented on Figure 16B as the analytes interacting with the coating have retained much less. The most retarded analytes – caffeic acid and salicylic acid interact so strongly with the membrane fragment double coating that they did not appear during 1 h analysis. These results demonstrate that PolyD coatings can be effectively used as an adhesive layer in OT-CEC to enhance and improve the attachment of biological materials to silica capillaries. (**III**)
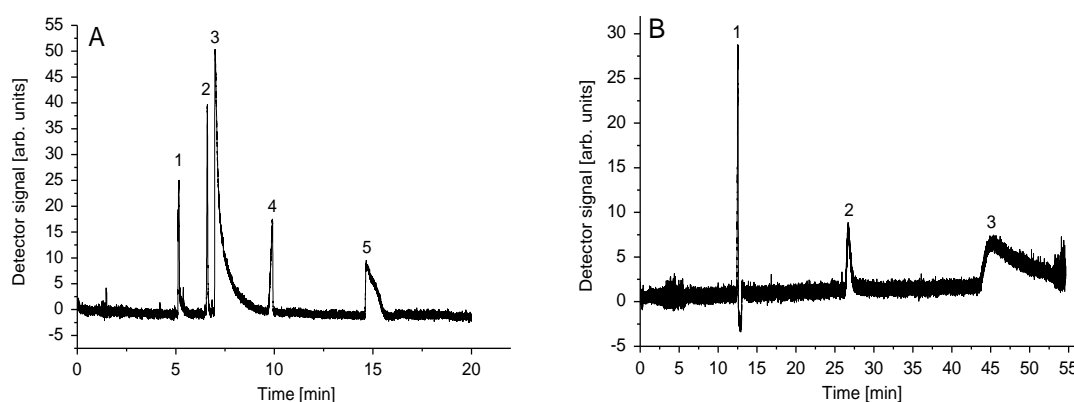
Figure 16. Separation and interaction of test analytes in (A) PolyD/phospholipid-coated and (B) PolyD/membrane fragments-coated capillary. Peaks: 1, EOF marker, 2, rutin, 3,

56

quercetin, 4, caffeic acid, 5, salicylic acid. BGE: 40 mM phosphate buffer, pH 7.4, detection 192 nm, injection 5 s at 50 mbar. Other conditions as in Fig. 8. (**III**)

Mitochondria were the only biomaterial tested which behaved similarly both in uncoated capillaries and PolyD coated capillaries: the EOF in capillary was the same in terms of mobility and repeatability (**III**).

5.1.3.4. The supporting SEM studies

SEM studies done for membrane fragment based coatings to visualize that the membrane fragments were covering PolyD-coated capillaries with bigger density (Figure 17). The results are in agreement with the EOF measurements described in previous chapter. Both SEM and CEC experiments support each other and give a ground to conclude that the coating stability was better for the PolyD/membrane coatings (**III**).
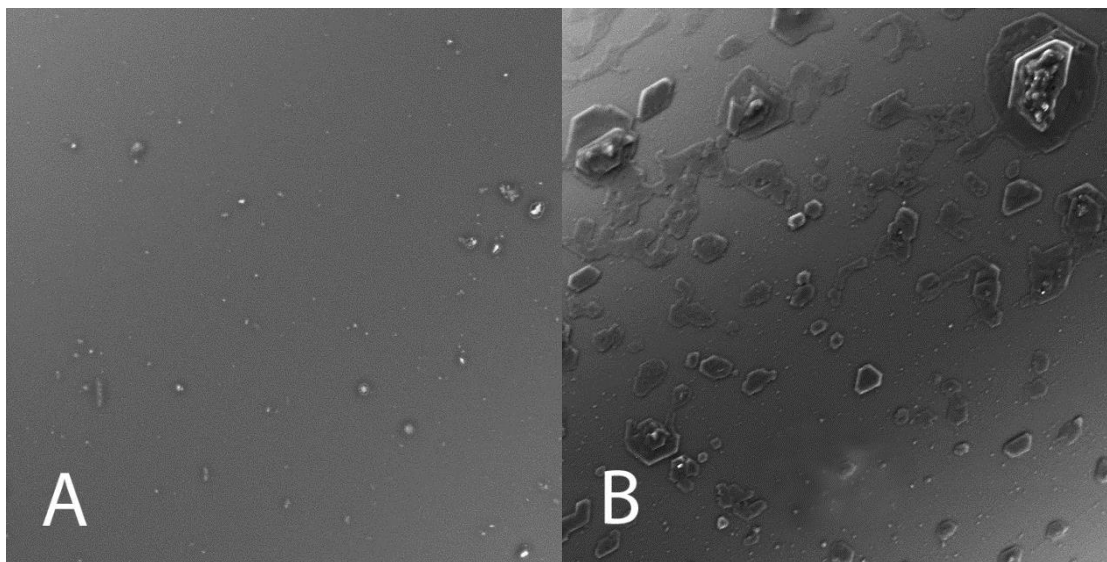
Figure 17. *Illustrative SEM photos of membrane coating on bare fused silica capillary (A) and on PolyD coated capillary (B). The cut-out image dimensions are 100x100 μm.* (**III**)

5.2. Lipid analysis by CE and MS

In current study the membrane models were also aimed to investigate on molecular level. The target membrane lipids were chosen and MS as well as CE methods were developed for the study. Most of the studies were done by both methods separately but at the final stage a CE-ESI-MS analysis was carried out for selected lipid samples.

As target molecules cardiolipin and cellular membrane lipids were chosen. The first part of this chapter deals with optimization of atmospheric pressure MS ionization method for lipid analysis.

5.2.1. Optimization of the lipid analysis with DESI-MS

Among the major difficulties with the MS analysis of lipids are the ionization efficiency as well as tedious sample preparation procedures when real samples are analyzed. This has motivated a continuous search for new ionization mechanisms and approaches in MS analysis. Among the new MS ionization methods is DESI, which allows the direct analysis of analytes from sample surfaces (Takáts et al. 2004; Williams, Scrivens 2005; Rodriguez-Cruz 2006; Leuthold et al. 2006; Talaty et al. 2008; Wood et al. 2009; Luosujärvi et al. 2009). In DESI a charged solvent spray is aimed at the sample surface, causing simultaneous desorption and ionization. DESI has shown a potential in wide range of applications in e.g. biological, pharmaceutical and forensic areas. Even though there have been various successful DESI applications on lipid analysis (Manicke et al. 2008; Girod et al. 2010; Basile et al. 2011), according to the author knowledge there has not been a systematic study of the effects of the DESI spray solvents on the desorption and ionization of lipids in DESI.

In this study the DESI spray solvent composition was searched for the analysis of different lipid classes (pecified below), since it is thought to be the parameter with the most drastic effect on the analyte ionization in DESI.

The tested solvents are shown in Table 1. The solvents were chosen to test the effect of solvent polarity, different organic modifiers, and different acidic, basic or salt additives.

Table 1. *The solvents used in the study.*

SOLVENT	SOLVENT DESCRIPTION
H_2O	Different polarity and water content (tested in positive ion mode)
H_2O/MeOH (75v/25v)	
H_2O/MeOH (50v/50v)	
H_2O/MeOH (25v/75v)	
MeOH	
H_2O/ACN (50v/50v)	Different polarity and organic components (tested in positive ion mode)
H_2O/IPA (50v/50v)	
MeOH/IPA (50v/50v)	
MeOH/CHCl$_3$ (50v/50v)	
ACN/CHCl$_3$ (50v/50v)	
H_2O/MeOH/NH$_3$ (50v/50v/0.1%) − negative ion mode	Different additives (tested in positive and negative ion modes)
H_2O/MeOH/HNO$_3$ (50v/50v/0.1%) − negative ion mode	
H_2O/MeOH/HCl (50v/50v/0.1%) − negative ion mode	
H_2O/MeOH/HCOOH (50/50/0.1%) − negative ion mode	
H_2O/MeOH/NaCl (50v/50v + 100 μM)	
H_2O/MeOH/KCl (50v/50v + 100 μM)	
H_2O/MeOH/NH$_4$Cl (50v/50v + 100 μM)	
H_2O/MeOH/HCOOH (50v/50v/0.1%)	Different acid content (tested in positive ion mode)
H_2O/MeOH/HCOOH (50v/50v/0.1%)	
H_2O/MeOH/HCOOH (50v/50v/1%)	
H_2O/MeOH/HCl (50v/50v/0.01%)	
H_2O/MeOH/HCl (50v/50v/0.1%)	
H_2O/MeOH/HCl (50v/50v/1%)	
H_2O/MeOH/HCl (25v/75v/0.1%)	
H_2O/MeOH/HCl (10v/90v/0.1%)	

Table 2 presents the analyte main ions observed with all the studied solvents. The main ion for verapamil, dipalmitoylphosphatidylcholine (DPPC), palmitic acid, vitamin D$_3$ and

sphingomyelin in positive ion mode was the protonated molecule [M+H]$^+$, for tricaprylin the ammonium adduct [M+NH$_4$]$^+$, and for cholesterol the fragment formed from the protonated molecule via water loss [MH-H$_2$O]$^+$. Cholesterol also formed the sodium adduct [M+Na]$^+$. When the solvent was including an adduct forming ion, then all the samples except verapamil, had the adduct with K$^+$, Na$^+$ and NH$_4$$^+$ ions in positive ion mode as the main ion. For verapamil the above mentioned adducts were all present but the main ion was still the protonated ion. In negative ion mode the signal is very low for all the samples (almost at noise level) therefore the signal in negative ion mode was marked as "not detected". This could be explained by the general feature of DESI to form alkali metal adducts in positive ion mode (Takáts et al. 2005).

Table 2. *The main ions of the analytes observed with the studied solvents. NEG = negative ion mode.*

Solvent\Sample	Quality of the main ion						
	Vera-pamil	DPPC	Palmitic acid	Cholesterol	Vitamin D$_3$	Tricaprylin	Sphingo-myelin
H$_2$O/MeOH (50v/50v)	[M+H]$^+$	[M+H]$^+$	not detected	[M+Na]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
H$_2$O/MeOH (75v/25v)	[M+H]$^+$	[M+H]$^+$	not detected	[M+Na]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
H$_2$O/MeOH (25v/75v)	[M+H]$^+$	[M+H]$^+$	not detected	[M+Na]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
MeOH	[M+H]$^+$	[M+H]$^+$	not detected	[M+Na]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
H$_2$O	[M+H]$^+$	[M+H]$^+$	not detected	[MH-H$_2$O]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
H$_2$O/ACN (50v/50v)	[M+H]$^+$	[M+H]$^+$	not detected	[M+Na]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
H$_2$O/IPA (50v/50v)	[M+H]$^+$	[M+H]$^+$	not detected	[M+Na]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
MeOH/IPA (50v/50v)	[M+H]$^+$	[M+H]$^+$	not detected	[M+Na]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
MeOH/CHCl$_3$ (50v/50v)	[M+H]$^+$	[M+H]$^+$	not detected	[M+Na]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
ACN/CHCl$_3$ (50v/50v)	[M+H]$^+$	[M+H]$^+$	not detected	[MH-H$_2$O]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
H$_2$O/MeOH/NaCl (50v/50v + 100 μM)	[M+H]$^+$	[M+Na]$^+$	not detected	[M+Na]$^+$	[M+Na]$^+$	[M+Na]$^+$	[M+Na]$^+$
H$_2$O/MeOH/KCl (50v/50v+ 100 μM)	[M+H]$^+$	[M+K]$^+$	not detected	[M+Na]$^+$	[M+K]$^+$	[M+K]$^+$	[M+K]$^+$
H$_2$O/MeOH/NH$_4$Cl (50v/50v + 100 μM)	[M+H]$^+$	[M+NH$_4$]$^+$	not detected	[MH-H$_2$O]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
H$_2$O/MeOH/HCOOH (50v/50v/0.01%)	[M+H]$^+$	[M+H]$^+$	not detected	[MH-H$_2$O]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
H$_2$O/MeOH/HCOOH (50v/50v/0.01%)	[M+H]$^+$	[M+H]$^+$	not detected	[MH-H$_2$O]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
H$_2$O/MeOH/HCOOH (50v/50v/0.1%)	[M+H]$^+$	[M+H]$^+$	not detected	[MH-H$_2$O]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
H$_2$O/MeOH/HCOOH (50v/50v/1%)	[M+H]$^+$	[M+H]$^+$	not detected	[MH-H$_2$O]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
H$_2$O/MeOH/HCl (50v/50v/0.01%)	[M+H]$^+$	[M+H]$^+$	not detected	[MH-H$_2$O]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
H$_2$O/MeOH/HCl (50v/50v/0.1%)	[M+H]$^+$	[M+H]$^+$	not detected	[MH-H$_2$O]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$

H$_2$O/MeOH/HCl (50v/50v/1%)	[M+H]$^+$	[M+H]$^+$	not detected	[MH-H$_2$O]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
H$_2$O/MeOH/HCl (25v/75v/0.1%)	[M+H]$^+$	[M+H]$^+$	not detected	[MH-H$_2$O]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$
H$_2$O/MeOH/HCl (10v/90v/0.1%)	[M+H]$^+$	[M+H]$^+$	not detected	[MH-H$_2$O]$^+$	[M+H]$^+$	[M+NH$_4$]$^+$	[M+H]$^+$

The studied analytes gave well-measurable signals with water/MeOH, water/ACN and water/IPA mixtures. However, the further increase in solvent unpolarity did not enhance the signal intensity of the analytes. In fact, exchanging the aqueous component in the solvent with chloroform had an opposite effect on signal intensity. This may indicate that the unpolar solvents, while enhancing the solubility of lipids, have quenching effect on protonation.

As the next step two different acids (HCl and HCOOH) were added to the MeOH/water mixtures in different concentrations. The acids were chosen due to their low toxicity. The results shown that HCl has somewhat stronger effect on the ionization of these analytes than HCOOH, however the effect was not dramatic. With low acid concentrations (less than 0.1%) the increase in acid concentration amplified the signal for DPPC and sphingomyelin. When the acid concentration was further increased it did not affect the signal intensities markedly. Increasing of the concentration of organic modifier had also positive effect of the signal intensities of DPPC and sphingomyelin. With other samples (palmitic acid, vitamin D$_3$ and tricaprylin) we may summarize that tricaprylin formed ammonium adducts as the main ion. Palmitic acid did not ionize well and gave a very low signal with all solvents. Vitamin D$_3$ formed protonated molecules in acidic solvents. To conclude that in general the best solvents for lipids in positive ion mode are H$_2$O/CH$_3$OH/HCOOH (50v/50v/0.1%) or H$_2$O/CH$_3$OH/HCl (50v/50v/0.1%). The experiments also show that both HCOOH and HCl could be successfully used for the analysis.

The formation of adducts in MS of lipids as a possible mean to increase sensitivity of the analysis has been known for years. The adduct formation is somewhat ionization mode dependant, yet general features have been reported for different ionization methods (Hsu, Turk 1999; Fuchs et al. 2010; Bowden et al. 2011). For DESI the studies are more scarce. It has been demonstrated that DESI has a relatively high salt tolerance, yet the extent of the tolerance is highly dependant of the experimental set-up (Jackson et al. 2007). It was aimed to test if the tolerance is valid for our set-up and if the presence of common cations (K^+, Na^+, NH_4^+) may, in case of tested analytes, even increase the signal intensity of the analytes.

It appears that for the majority of the tested analytes the intensity of adduct formation was lower than the intensity of the protonated ion. Yet for sphingomyelin and tricaprylin the adducts with both tested alkali metal cations were abundant and stable. The formation of sodiated adducts for both tricaprylin type of compounds (acylglycerols) and for sphingolipids has been supported by other studies with DESI (Manicke et al. 2008) but according to the knowledge of the authors there is no studies of adduct formation between K^+ and sphingomyelin and tricaprylin. The most intensive signal is the ammoniated adduct of tricaprylin. At the same time the ammoniation effect is practically unnoticeable for all other tested analytes. This phenomenon may offer possibilities for selective DESI-MS analysis of tricaprylin, however to draw conclusion further studies would be needed. Several additional DESI-MS parameters was also optimized for to find out the best signal quality and reproducibility. The results are listed in Table 3.

Table 3. *DESI-MS optimized experimental conditions.*

Spray solvent flow rate	5 µL/min
DESI spray angle	45°

Nitrogen gas pressure	10 bar
Capillary voltage	–4000 V
Skimmer voltage	40 V
EPO voltage	–500 V
Dry gas	2.0
Dry trap	285 ºC
CE voltage	199 V
Oct 1PC/oct 2PC	12.00/2.17
Trap draw	57.5
Octapole	RF
Voltage of lens 1/ voltage of lens 2	–5.0 V/–60.0 V

Despite the preliminary optimization more research is needed for lipid analysis by DESI as the experiment day-to-day repeatability was relatively poor due to ion contamination and destabilization of DESI system.

5.2.2. Development of CE lipid analysis method

The applications of CE cover a wide range of analytes. However, very hydrophobic analytes (like many lipids) are often difficult targets to analyse. In order to ensure both sufficient solubility of the analyte and repeatable analysis time the use of highly organic (more than 50%) BGEs is needed and sometimes the analytes are still adsorbing to the capillary wall. Therefore a coating of the capillary wall may be needed in addition. Yet many coatings designed for CE are not suitable to use in highly organic media. Our purpose was to develop a CE method for different lipids including very hydrophobic ones (like cardiolipin). The method development involved finding a suitable capillary coating which would be stable in organic media. The developed method was intended to apply on few target lipids and on a lipid extract from a cell membrane.

5.2.2.1. The stability of PolyE-323 coated capillaries in organic media

We selected a positively charged polymeric capillary coating (PolyE-323) which has claimed to withstand acetonitrile and methanol up to 50%. We intended to test the stability of the coating in 100% organic environment including various solvents: acetonitrile, methanol, isopropanol, chloroform and propylene carbonate.
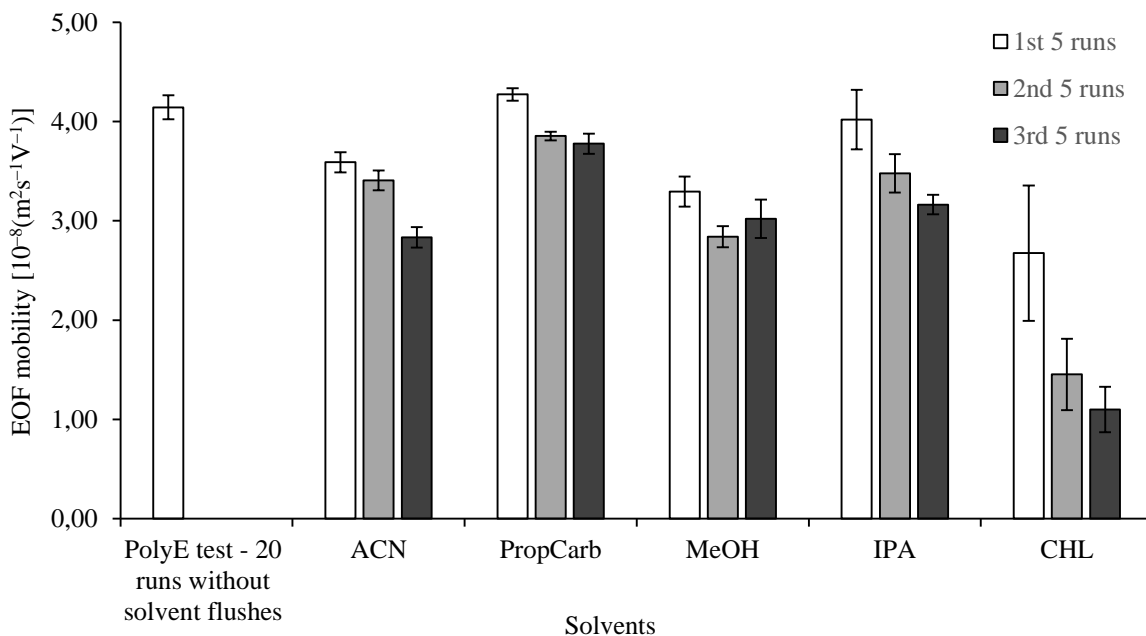
Figure 18. *The influence of exposure to different pure organic solvents on the stability of the PolyE-323 coating, assessed with EOF mobility after exposure. Exposure time was 3x 1 h, and after each hour EOF was determined 10 times. Error bars represent standard deviation which based on EOF mobility of 5 successive runs. The running BGE for EOF determination was Tris (pH 7.4 20 mM) and the running contitions: voltage −20 kV, injection 8 s at 50 mbar, detection 192.5 nm, cassette temperature 25 °C, capillary lenght 31.5/40 cm, i.d. 75 μm. (**IV**)*

Figure 18 shows how exposure to a chosen organic solvent affected the EOF mobility. It can be observed that all organic solvents affect the coating to some extent, which explains the decreasing EOF. Still the effect of acetonitrile, propylene carbonate, methanol and isopropanol on the coating was much less pronounced than that of chloroform, and strong EOF was still present after the capillary had been 3h exposed to these organic solvents (**IV**).

Based on the experiments acetonitrile, methanol, propylene carbonate and isopropanol were chosen for further studies in order to find suitable BGEs for lipids in PolyE-323 coated capillariesv (**IV**).

5.2.2.2. Cardiolipin and sphingomyelin analyses in PolyE-323 coated capillaries

Cardiolipin is a phospholipid rich in unsaturated fatty acids and localized mainly within the mitochondrial inner membrane. Changes in overall cardiolipin concentration are closely related to many pathological conditions (e.g. Barth syndrome) (Houtkooper 2009; Ho 2012). CE analysis of cardiolipin is a challenging task due to the hydrophobicity of the analyte, which causes severe interactions with the capillary wall. Moreover, cardiolipin is difficult to dissolve in aqueous media. The few works that describe cardiolipin analysis by CE have been dealing with LIF detection, using BGE additives to facilitate detection (Haddadian 1998; Duffy 2002; Fuller 2002; Danielson 2003; Zhao 2011).

Sphingolipids are structural components of the cell and participate in intra- and extracellular signalling. According literature CE analysis of sphingolipids involves analyte-dye interactions and LIF detection (Essaka 2012; Wang 2012).

As the next step a number of highly organic BGEs (Table 4) based on the results described in previous chapter were composed. The organic content in the BGEs was always 94.5%,

the rest 5.5% was an aqueous buffer (either Tris or ammonium acetate). An ionic liquid (BMI-PF$_6$) was added to selected BGEs to enhance the conductivity of the BGE. (**IV**)

Table 4. *Composition selected BGE's. BGE 1-3 were set at pH 7.4 with NaOH, BGE 8 with ammonium hydroxide. 30 µL/20 mL of the ionic liquid was added.*

BGE number	Acetonitrile (%)	Propylene carbonate (%)	Methanol (%)	Isopropanol (%)	Water (%)	Electrolyte	Ionic liquid
1	56.5			38	5.5	Tris	yes
2	56.5		38		5.5	Tris	yes
3		56.5		38	5.5	Tris	yes
4	56.5		38		5.5	Ammonium acetate	no

The performance of the BGEs was first tested with the EOF marker (DMSO) and the results (Figure 19) show that none of the composed BGEs cause destruction of the coating during 6 runs. This shows that all the BGEs listed in Table 4 could be used as BGEs in PolyE-323 coated capillaries. However, as expected, the analysis time is longer with more viscous solvents (especially with propylene carbonate) and therefore the ACN/MeOH might be preferred (**IV**).
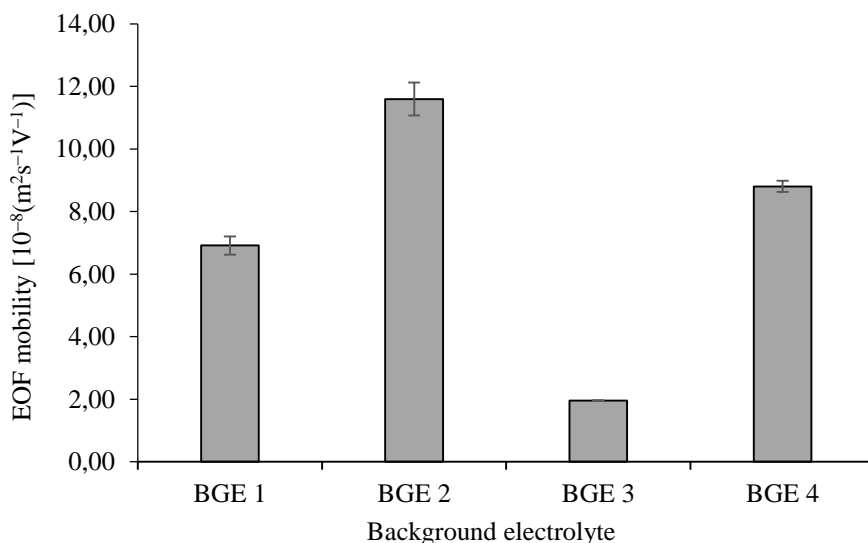
Figure 19. EOF mobility with different organic BGE mixtures (see Table 1) with the PolyE-323 coated capillary. Error bars represent standard deviation which based on EOF mobility of 6 successive runs. Running conditions as in Fig. 8.

All the BGEs presented in Table 4 were also tested for cardiolipin and sphingolipid analysis. The results presented on Figure 20 demonstrate that sphingomyelin and cardiolipin are separated from each other with all the BGEs but the separation is clearly better with Tris containing BGEs. The repeatability of the analysis was good for both lipids and the analysis time is in accordance with the solvents viscosity. Cardiolipin was anionic with the selected BGEs and migrated in front of neutral sphingomyelin. (**IV**)
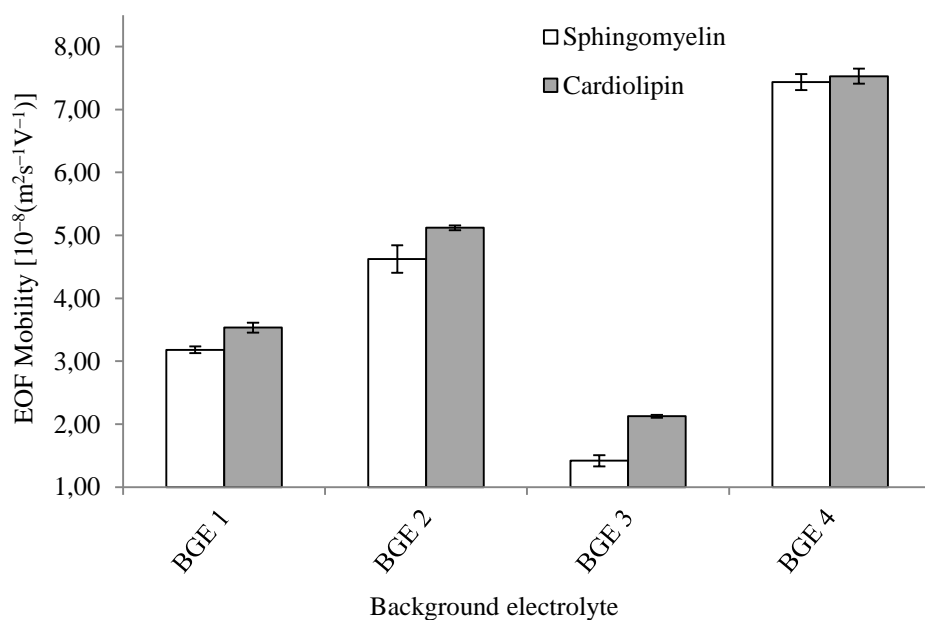

Figure 20. Cardiolipin and sphingomyelin mobilities with different organic BGE mixtures (see Table 1) with the PolyE-323 coated capillary. Error bars represent standard deviation which based on EOF mobility of 3 successive runs. Running conditions as in Fig. 19. Injected cardiolipin and sphingomyelin concentration was 1 mM. (**IV**)

The studies indicate the durability of PolyE-323 coating in organic media and its suitability for lipid analysis by CE techniques (**IV**).

5.2.2.3. Cardiolipin oxidation in PolyE-323 coated capillaries

As another test for the suitability of PolyE-323 coatings for lipid related analysis we monitored cardiolipin oxidation in PolyE-323 coated capillaries. Due to its central role in mitochondria and its composition, it is believed that oxidative stress can lead to cardiolipin oxidation and impair the mitochondrial functions (Pope et al. 2008). An ability to identify (and quantify) new cardiolipin oxidation products and their formation mechanisms could therefore help to discover more markers for various diseases and design antioxidant drugs to prevent cardiolipin oxidation.

In order to find the best environment for cardiolipin oxidation – both in terms of cardiolipin solubility and the speed of oxidation – different water-methanol mixtures (90v/10v, 50v/50v, 25v/75v) were tested (Figure 21).

As to deduce from the experiments the presence of methanol is clearly needed – in purely aqueous solutions the solubility of cardiolipin was unacceptably low. The solubility was increasing steadily with the increasing percentage of methanol. Yet if the methanol content was too high (more than 25%) then the oxidation process did not happen at acceptable speed.
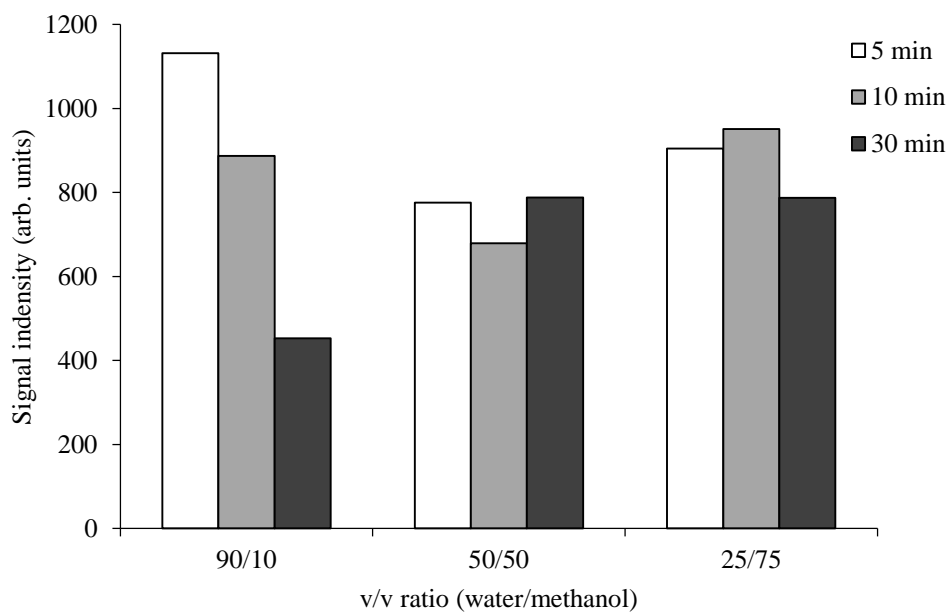
Figure 21. Cardiolipin oxidation in different water-methanol mixtures after 5, 10 and 30 minutes. Running conditions as in Fig. 9. except detection which is 192.5 nm. Time was normalized to same scale.

Based on the studies the water-methanol mixture 90v/10v was used to carry out the oxidation (Figure 22). The parallel experiments proved that the system is suitable to follow cardiolipin oxidation.
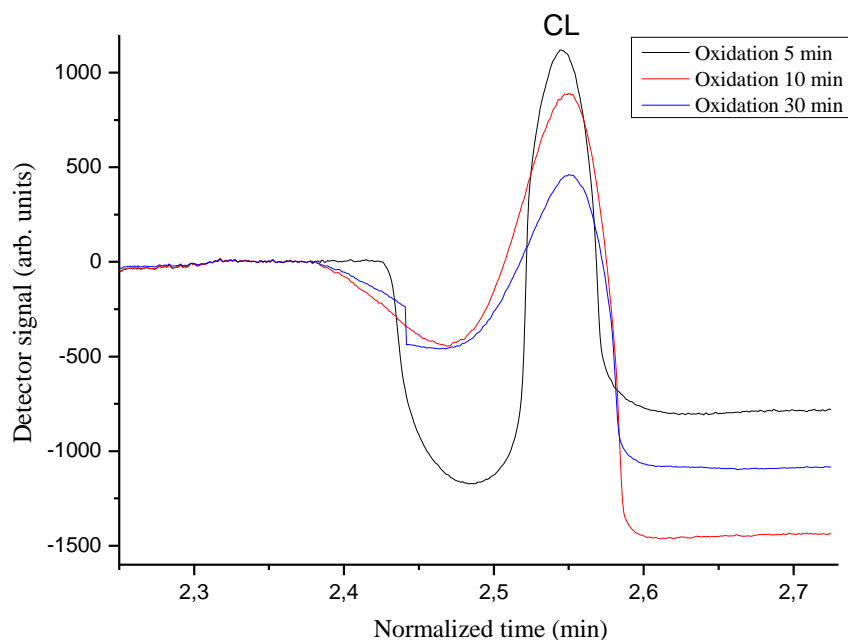
Figure 22. Cardiolipin oxidation over PolyE-323 coated capillary in water-methanol mixture 90/10 v/v. Running conditions as in Fig. 8. except detection which is 192.5 nm. Time was normalized to same scale.

5.2.3. PolyE-323 coated capillary with CE-ESI-MS

Our next goal was to show that the developed CE method (PolyE-323 capillary and highly organic BGE) is compatible with an ESI-MS system and could be used for lipid analysis. Sphingomyelin was used as the marker for the analysis performance. The first results indicate that the coating is very stable (RSD of 10 runs was 1.6%) under these conditions. (**IV**)

As a test sample a lipid extract obtained from a human primary glioblastoma cell line (U-87 MG) was analyzed, both in positive and in negative MS mode. In the positive mode, in total, 67 compounds were suggested by Agilent Masshunter "Find by Molecular Feature"

algorithm above a threshold of signal-to-noise ratio 10. Using a standard, we could putatively identify a phosphocholine (PC 18:1/16:0). (**IV**)

CONCLUSIONS

In the present work different biological materials were tested as membrane models in CEC. Different cell membrane fragments, cell membrane lipid extracts, mixtures of commercial lipids and rat liver mitochondria were immobilized to CEC capillaries either by physical adsorption, electrostatic interaction or covalent bonding. The effect of various coating parameters was tested and varied. Coating stability and durability were tested to improve coating suitability for biocompound analysis.

- A membrane model more closely resembling biological membranes than artificial lipid mixtures was created. All neuronal cell line (U-87 MG, BV-2 and SH-SY5Y) based membrane models proved to form stable and durable OT-CEC capillary coatings. In case of single coating membrane models, the best coating conditions were: BGE – TE pH 7.4, voltage 20 kV, temperature of cassete 25 °C, injection time 8 s at 20 mbar, capillary length 31.5/40 cm and i.d. 75 or 50 µm.

- Membrane model attachment via electrostatic interactions by using PolyE-323 did not improve the coating stability and were excluded from further studies. PolyD based covalent bonding showed clear improvement in membrane model stability for both lipid and membrane fragments based models. The optimization of the adhesive PolyD layer showed that the most influential variant influencing the thickness of the coating is the number of repetitions. The results also showed that the thickness needed for successful bonding of the membrane model was obtained with a single 5 h coating procedure.

- PolyE-323 coated CE studies demonstrate that the polymer is suitable for using in highly organic media and could be used for lipid analysis in nonaqueous environment. Cardiolipin oxidation was successfully monitored in PolyE-323 coated capillary. PolyE-323 coated capillaries are well-compatible with ESI-MS as no leakage of the coating was noted during the experiments.

- DESI-MS is suitable for lipid analyses but for more stable results ionization module system needs to be automated.

FUTURE PROSPECTS

The future plans involve

- Investigation of the interactions of transmembrane cell proteins (ATP-binding cassette transporters) with selected drug candidates by CEC and CEC-MS to develop new drugs.

- Application of cell membranes models on "real" samples, including tissue samples.

- Further development of CE-ESI-MS techniques for lipid analyses over coated capillaries at highly organic conditions.

KOKKUVÕTE

BIOLOOGILISTE MEMBRAANIDE JA NENDE KOMPONENTIDE UURIMINE KAPILLAARELEKTROFOREETILISTE NING MASS-SPEKTROMEETRILISTE MEETODITEGA

Bioloogilised membraanid omavad eluslooduses mitmeid väga olulisi funktsioone ning nende tähtusust on raske ülehinnata. Olles rakkude ja rakuorganellide komponendid hoiavad nad rakku ühtse tervikuna ning kontrollivad erinevate biomolekulide transporti rakku ja rakust välja. Bioloogilistes membraanides aset leidvad muutused on sageli seotud muutustega terves kehas, mistõttu uued teadmised membraanidest ja nende komponentidest avavad meile uusi võimalusi uurida erinevaid haigusi ning nende molekulaarseid mehhanisme.

Käesolevas töös kasutati raku membraanide ja nende lipiidsete komponentide uurimiseks kapillaarelektroforeesi, kapillaarelektrokromatograafiat ning mass-spektromeetriat.

Doktoriväitekirja põhieesmärgiks oli välja töötada uued kapillaarelektromigratsiooni meetodid bioloogiliste membraanide uurimiseks ning membraanis leiduvate lipiidide (kardiolipiin, sfingomüeliin, fosfaatidüülkoliinid) analüüsiks. Mass-spektromeetriat kasutati täiendavalt lipiidide analüüsiks nii eraldiseisva meetodina kui detektorina CE-ESI-MS süsteemis.

Doktoritöö põhitulemused:

- Kasutades inimese neuroblastoomi SH-SY5Y, hiire mikrogliia BV-2 ja inimese glioomi U-87 MG rakuliine loodi membraanimudelitena, funktsioneerivad kapillaarelektrokromatograafia statsionaarsed faasid. Katmisprotokolli optimiseerimise käigus leiti, et füüsikalisel adsorptsioonil põhinevate ühekordsete katete puhul andsid parimaid tulemusi järgmised katmistingimused: BGE – TE pH

7,4, pinge 20 kV, kasseti temperatuur 25 °C, proovi süstimise aeg 8 s rõhul 20 mbar, kapillaari pikkus 31,5/40 cm ja sisediameeter 50 või 75 μm.

- Membraanimudeli kõige parem katvus ja stabiilsus (hinnatud EOF liikuvuse põhjal) saavutati kahekordsel katmisel üle polüdopamiini (PolyD) kihi. Dopamiini polümerisatsioonireaktsioon vajab kiireks kulgemiseks hapniku olemasolu ning sellest tulenevalt toimus protsess kapillaaris küllaltki aeglaselt. Katmisprotsessi optimeerimise tulemustena selgus, et kõige parema aluskatte annab 5 tunnine katmisprotseduur, millele järgnevalt teostati katmisi bioloogiliste materjalidega samadel tingimustel nagu ühekordsete katete puhul. Polümeerse aluskihi kasutamine võimaldas kovalentsete sidemete loomist membraanimudeliga, mis andis tunduvalt paremaid tulemusi ning stabiilne oli nii membraani fragmentidel kui lipiididel põhinev kate.

- Polüamiin, PolyE-323, kinnitub kvartskapillaari seinale elektrostaatilise vastastikmõju tulemusena. PolyE-323 kattega teostatud CE katsed näitasid, et polümeer on stabiilne ja vastupidav ka tugevalt orgaanilises keskkonnas ning teda oleks võimalik kasutada lipiidide analüüsiks mittevesikeskkonnas. PolyE-323 kate võimaldas orgaanilises keskkonnas kapillaarelektroforeetiliselt analüüsida edukalt nii kardiolipiini kui sfingomüeliini ning täiendavalt jälgida ka kardiolipiini oksüdatsiooni. Tänu oma stabiilsusele sobib PolyE-323 hästi kasutamiseks ka CE-ESI-MS süsteemis.

- Ionisatsioonimoodul DESI on uudne täiendus mass-spektromeetrile, mis võimaldab ioniseerida proovi otse tahkest faasist viimast liigselt kahjustamata.

DESI puhul on täiendavateks võimalusteks proovile pihustatava solvendi segu varieerimine ning pihustusnurga muutmine. Lipiidide analüüsi tulemustest DESI mooduliga selgus, et süsteem on sobilik lipiidsete komponentide uurimiseks, kuid parema stabiilsuse tagamiseks vajab veel täiendavalt automatiseerimist.

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