UNIVERZA V LJUBLJANI

FAKULTETA ZA FARMACIJO

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RAZVOJ KAPILARNO ELEKTROFOREZNIH METOD ZA ANALIZO PROTEINSKIH BIOMARKERJEV NEVRODEGENERATIVNIH BOLEZNI

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DEVELOPMENT OF CAPILLARY ELECTROPHORETIC METHODS FOR THE ANALYSIS OF PROTEIN BIOMARKERS OF NEURODEGENERATIVE DISEASES

LJUBLJANA, 2013

Magistrsko nalogo sem opravljala na Fakulteti za farmacijo, Univerza v Ljubljani, pod mentorstvom prof. dr. Sama Krefta in somentorstvom Luc Denoroy, Ph.D.. Eksperimentalni del sem opravila na University Claude Bernard Lyon 1 (France), Faculty of pharmacy, Laboratory of neuropharmacology.

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Tjaša Cesar

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LIST OF ACRONYMS USED

- CE – capillary electrophoresis
- EOF – electroosmotic flow
- CZE – capillary zone electrophoresis
- CGE – capillary gel electrophoresis
- HPC – hydroxypropyl cellulose
- PEO – Poly(ethylene oxide)
- PEG – Poly(ethylene glycol)
- PVA – Polyvinyl alcohol
- MC – Methyl cellulose
- EK – electrokinetic
- HD – hydrodynamic
- AD – Alzheimer's disease
- Aβ – Amyloid-beta
- APP – amyloid precursor protein
- PD – Parkinson's disease

ABSTRACT

Neurodegenerative diseases, including Alzheimer's and Parkinson's disease (and others), are caused by misfolding and aggregation of disease specific protein. Pathological process is similar between diseases, even though different proteins are involved in each disease. Incorrectly folded proteins interact with each other, firstly a nucleus is formed and later it is extended and fibril structures are formed. In this process protein loses its biological function or it gains toxic properties. The protein involved in Alzheimer's disease is amyloid-β, which is normally present in human blood and cerebrospinal fluid. When the disease is present, amyloid aggregates are formed in brain. Our goal was to develop the method for aggregates detection as a potential diagnostic biomarker for diagnosis of Alzheimer and potentially other neurodegenerative diseases. Capillary electrophoresis was used as a technique to develop with which we would detect aggregates. In experimental part we were searching the optimal conditions for separation of aggregates and monomers using capillary electrophoresis. We worked with few model proteins with different molecular weight, and their behavior and separation was the base for evaluation of condition used. The goal was to work with native proteins and to separate them by their size. First step in development of capillary electrophoresis was the selection of buffer. After that the polymer was added to the buffer solution to provide sieving effect with which the proteins would be separated by size. Five different polymers in different concentrations were tested. To improve the separation we also performed the separation in two different diameters capillaries, 50µm and 75µm i.d.. Also, different modes of injection, coating of the capillary and addition of additives were tested. During our project we faced several problems and unexpected results but in the end we selected set of conditions provided the most prospective results: 0,5% polyethylene oxide and 2% hydroxypropyl cellulose in 50mM phosphate buffer (hydrodynamic injection, 75µm capillary) and they were further tested with insulin aggregates. In this final tests polyethylene oxide showed as the best possibility. We could conclude that capillary electrophoresis is a suitable method for separation of model proteins and insulin aggregates and it could be also suitable for amyloid aggregates separation, but further optimization of the method should be made.

Key words: Alzheimer's disease, Amyloid-beta aggregates, capillary electrophoresis, separation, proteins

POVZETEK

Vzrok za večino nevrodegenerativnih bolezni je nepravilno zvijanje in posledično združevanje le teh proteinov v oligomere in kasneje agregate, ki nato tvorijo vlakna, ki se odlagajo v možganskih tkivih. Med te bolezni spadajo Alzheimerjeva bolezen, Parkinsonova bolezen, Creutzfeldt-Jacob-sonova, Huntingtonova bolezen in druge. Pri vsaki od teh bolezni ima glavno vlogo drug protein, vendar pa je sam mehanizem tvorjenja vlaken podoben.

Sam proces se začne z nepravilno konformacijo proteina zaradi različnih vzrokov, ti proteini pa se nato združijo v jedro. Na to jedro se potem dodajajo novi nepravilno zviti proteini, veriga se veča do nastanka agregatnih struktur in vlaken. Pri tem procesu proteini izgubijo svojo fiziološko vlogo ter pridobijo toksične karakteristike.

Pri Alzheimerjevi bolezni igra glavno vlogo amiloid beta. Ta se zaradi okoliščin v možganih ali nepravilnosti v sintezi samega proteina nepravilno zvije in nato vstopi v proces agregacije. Pri tem izgubi biološko vlogo ter pridobi toksične lastnosti (na membrano, oksidativni stres, apoptoza celic), predvsem so toksični oligomeri. Amiloid beta ima glavno vlogo pri razvoju bolezni, nihanja v koncentraciji samega proteina, so lahko uporabljena kot biomarkerji pri diagnozi bolezni. Amiloidni agregati so bili temelj našega dela, saj bi z njihovo prisotnostjo v cerebospinalni tekočini ali krvi lahko dokazali prisotnost Alzheimerjeve bolezni.

Namen našega dela je bil razvoj kapilarno elektroforezne metode in s tem iskanje primernih parametrov separacije, pri katerih bi ločili modelne proteine po velikosti. Kasneje bi dobljene rezultate prenesli na analizo inzulinskih agregatov, ter nato še na bolj specifične proteine nevrodegenerativnih bolezni.

Kapilarna elektroforeza je analitska metoda z visoko učinkovitostjo, kratkim časom analize in potrebi po majhni količini vzorca in reagentov. Ločitev analiziranih spojin temelji na njihovem potovanju skozi kapilaro pod vplivom električnega polja. Hitrost potovanja analita je odvisna od samega analita, tj. njegovega naboja, velikosti in oblike in to vpliva na ločitev analiziranih spojin. Hitrost potovanja je odvisna tudi od pufra in elektro-osmotskega toka ter napetosti, ki je aplicirana med analizo.

Med iskanjem najprimernejših pogojev za ločitev proteinskih agregatov od monomerov smo za ocenitev uspešnosti določenih okoliščin uporabljali pet modelnih proteinov z znano velikostjo. Ti proteini so bili aprotinin (5,7kDa), citokrom C (11,8kDa), ribonukleaza A (16,5kDa), karbonska anhidraza (29,1kDa) in conalbumin (77,8kDa). Njihovo obnašanje in samo separacijo smo opazovali in ovrednotili pri izbiri pufra, polimera, uporabili smo tudi dve kapilari z različnim

premer (50µm in 75µm i.d.). Ločitev smo poskušali izboljšati tudi z dodajanjem aditivov, različnimi pogoji injiciranja vzorca ter *''coatingom"* kapilare.

Prvi korak razvoja metode je bila izbira primernega pufra. Že predhodno smo se odločili, da bodo ločitve izvajane v kislem pH območju, zaradi prednosti, ki jih ta izbira prinese. Ena izmed njih je ta, da so vsi proteini pozitivno nabiti (pI>pH) in bodo pod vplivom napetosti potovali proti katodi. Poleg tega je tudi elektro-osmotski tok zmanjšan in to vodi k bolj ponovljivim separacijam. Testirani pufri so bili: citratni pufer (100mM, pH=2,50) , fosfatni pufer (250mM, pH=2,00) in mravljična kislina (1M, pH=1,8). Parametri ločitve proteinov so bili primerjani med sabo in mravljična kislina se je izkazala kot najslabša možnost, saj so bili vrhovi širši in nižji v primerjavi z ostalima dvema pufroma. Kljub majhnim razlikam med drugima dvema se je kot najboljši izkazal fosfatni pufer, ki je bil izbran za nadaljnje delo. Ta del razvoja je bil zelo pomemben, saj so bili nato polimeri in aditivi raztopljeni v tem pufru, poleg tega pa so bili vsi nadaljnji rezultati ločitev proteinov primerjani z ločitvijo proteinov zgolj v fosfatnem pufru.

Po izbiri pufra smo testirali vpliv petih različnih polimerov (v različnih koncentracijah raztopljenih v fosfatnem pufru) na ločitev. Parametri, ki smo jih ocenjevali so bili čas analize, občutljivost (višina vrha), širitev vrhov ter resolucija in selektivnost med vrhovi, učinkovitost (število teoretskih podov), ter uspešnost ločitve analiziranih proteinov po velikosti. Kot najboljši se je izkazal polietilen oksid, ki nudi kratek čas analize, ozke vrhove, dobro resolucijo in uspešno ločitev. Njegova slaba lastnost je to, da smo opazili izredno velike izgube v občutljivosti. Tudi drugi polimer, hidoksipropil celuloza se je dobro izkazal, s kratkim časom analize, manjšo izgubo v občutljivosti v primerjavi z polietilen oksidom, vendar pa je bilo tu že prisotno širjenje vrhov.

Ločitev je bila izvedena tudi v dveh različnih polietilen glikolih (molekulska masa 35 000 in 2000), ki pa nista prinesla dobrih rezultatov. Pri prvem polietilen glikolu (molekulska masa 35 000) smo opazili velik porast časa analize, širjenje vrhov in upad občutljivosti. Pri drugem (molekulska masa 2000) pa smo poleg enakih težav kot pri prvem polietilen glikolu, imeli težave še z nestabilno bazno linijo in nestabilnim električnim tokom. Tudi dekstran se je izkazal kot neprimeren polimer v našem primeru, saj nismo nikoli zaznali vrhov proteinov, tudi ko je bil uporabljen v zelo malih koncentracijah. Vzrok za tako nenavadno obnašanje bi lahko bil ta, da je bil uporabljen dekstran v obliki sulfata in je tvoril s proteini kompleks, ko ga nismo mogli detektirati.

Ločitev smo želeli še izboljšati, predvsem povečati občutljivost in zožiti vrhove. Prvi poskus je bila uporaba poliakrilamidnega coatinga kapilare, ki se je pokazal z dobrimi rezultati, a je problem v

tem, da je precej nestabilen in je začel razpadati. Zaradi te lastnosti smo tudi opustili njegovo uporabo. Pri testiranju coatinga je bil prvič uporabljen tris pufer (pH=6,8). Druga možnost izboljšanja separacije je bila uporaba aditivov: metil celuloze in polivinil alkohola. Ko smo ju testirali v tris pufru, je sprva kazalo na zelo pozitiven vpliv metil celuloze na parametre ločitve, saj se je občutljivost zelo izboljšala, pa tudi vrhovi so se zožili. Vendar pa smo po premisleku in natančnem pregledu vseh rezultatov pridobljenih s tris pufrom ugotovili, da je obnašanje (višina in širina vrha) zelo odvisno od števila ločitev, pri katerih je že bila uporabljena ista raztopina pufra. Večkrat je bila uporabljena, boljši so bili rezultati. Zaradi slabe ponovljivosti (vsakič drugačni rezultati, tudi variacije v migracijskih časih) pri uporabi tris pufra smo možnost njegove uporabe opustili. Tudi dodajanje metil celuloze ali polivinil alkohola k fosfatnem pufru, ni doprinesel k boljši separaciji, saj sta širina in višina vrhov ostali enaki, ali pa so vrhovi postali celo širši in nižji, kar je bilo v nasprotju z našimi pričakovanji.

Kot najboljše možnosti sta se pokazala 0,5% polietilen oksid in 2% hidroksipropil celuloza v 50mM fosfatnem pufru (75µm kapilara), v katerih smo nato tudi testirali ločitev inzulinskih agregatov. Tu se je polietilen oksid izkazal kot boljša izbira, saj je bila ločitev med inzulinskim monomerom in agregati neprimerno boljša kot v pufru z hidroksipropil celulozo.

Na koncu lahko zaključimo, da je kapilarna elektroforezna metoda primerna za ločitev proteinskih agregatov od monomerom in potrditev njihove prisotnosti. Kljub temu mislim, da je še veliko potenciala v razvoju metode in da naše ugotovitve lahko služijo kot osnova nadaljnjemu razvoju. Možnost izboljšanja vidim v dodatku ionov k separacijskem pufru, da bi s tem zmanjšali interakcije med proteini in kapilarno steno. Zelo zanimiva možnost je tudi prenos naše metode na kapilarno elektroforezo z fluorescenčnim detektorjem, ki ima večjo občutljivost. Na koncu je potrebno še poudariti, da je bil razvoj te metode narejen na modelnih proteinih in je potreben še prenos pridobljenih ugotovitev na analizo bolj specifičnih proteinov.

Ključne besede: Alzheimerjeva bolezen, Amiloidni agregati, kapilarna elektroforeza, ločitev, proteini

1 INTRODUCTION

Neurodegenerative disease can be described with vulnerability of neurons and degradation of specific brain areas. With aging of population they now represent an important part of health problems. Neurodegenerative diseases can often be linked to pathological proteins and their aggregation process. These diseases are Alzheimer's, Parkinson's, Creutzfeld-Jacob's, Huntington's disease, amyotrophic lateral sclerosis and others. Each disease can be associated with appearance of disease specific protein which due to the different causes aggregates. Aggregation process leads to toxicity, damage in the brain regions and to the development of the disease. Formation of fibrils and their deposit in the brain is a hallmark of neurodegenerative diseases. Studying the aggregation process and presence of the protein, specific for the disease is included in many studies because of the diagnostic and therapeutic role of these processes (Pedersen et al, 2013). Nowadays, there is a tendency to include biomarkers in diagnostic, therapeutic aspects of all diseases, not just neurodegenerative. For neurodegenerative disease, diagnosis can sometimes be difficult and long-lasting; use of biomarkers would ease and hopefully shorten the process. Our work was mostly focused on Alzheimer's disease and amyloid-β, the protein responsible for pathogenesis. Formation and presence of amyloid-β aggregates is a hallmark of the disease and if with suitable technique could be shown that the aggregates are present, Alzheimer disease could be confirmed. There are different techniques suited for aggregates analysis but capillary electrophoresis was chosen to work with. During our work we will develop this technique and search for the best conditions where presence of amyloid-β aggregates can be confirmed.

2 PROTEIN AGGREGATION IN NEURODEGENERATIVE DISEASE

2.1 PROTEIN AGGREGATION

The cause for most neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Creutzfeldt-Jacob's disease and Huntington's disease, is misfolding and aggregation of the disease specific protein (Pedersen et al, 2013). These diseases are conformational diseases. Even though different proteins are involved in each pathology as we can see in table 1, they result in fibril formation and formed fibrils are similar in their morphology and structural properties. Of course, conformational diseases are also found in other body region, not just brain, one of most known is diabetes (DeToma et al, 2012).

Table 1: *Review of the amyloid diseases (Other diseases besides neurodegenerative are included)*

Disease	Involved protein	Structure of aggregates
Alzheimer's disease	Amyloid-β/ Tau protein	Amyloid/amorphous
Parkinson's disease	α-synuclein	Amorphous/amyloid
Huntington's disease	huntingtin	Nuclear inclusion bodies
Prion disease	Prion protein	Amyloid/amorphous
Type 2 Diabetes	Islet amyloid polypeptide (amylin)	amyloid
Dialysis related amyloidosis	β2-Microglobulin	Amyloid

Proteins are one of the most abundant molecules in biological system and they have an important role in different biological functions. They are composed of 20 different amino acids, which are joined together in different sequences and combinations. Under physiological conditions polypeptides are folded in a specific three-dimensional structure from random coil. The thermodynamically stable and biologically active structure is called ''native''. It is important that the proteins are folded in the correct 3D structure, because native structure has proper physiological function (Sgarbossa, 2012). The 3D structure of the protein depends on the amino acids sequence and also on physico-chemical properties of the solvent. The solvent is water or lipid bilayer and important properties that affect structure are concentration of salts, the pH, the temperature, the possible presence of the cofactors or molecular chaperones or other macromolecules. Different interactions are possible between amino acids side chains and they determine the secondary structure and later the tertiary structure of proteins. These interactions

are hydrogen bonds, ion pairs, van der Waals forces and hydrophobic interactions (Sgarbossa, 2012; Takalo et al, 2013).

Aggregation process is started by interactions between proteins or protein complexes when normally hidden regions of protein are exposed and specific, but unwanted contacts are made. The result of this contact is self-assembly and formation of insoluble structures (Invernizzi et al, 2012). Normally these structures are highly regular and known as amyloid fibrils. Amyloid fibrils have a ''cross-β'' structure (Invernizzi et al, 2012).

Incorrectly folded proteins have exposed hydrophobic amino acid residues, which are in native form buried within the protein structure. As a consequence, proteins which have exposed hydrophobic regions interact with each other by hydrophobic interactions. As a result fibril aggregates are formed. They have mostly β-sheet secondary structure in which β-strand run perpendicular to the long fibril axis, cross β-sheet structure (Sgarbossa, 2012).

The reason for exposure of normally hidden areas of proteins is most often that the protein is misfolded (Takalo et al, 2013). Inappropriately folded protein intermediates may interact with each other or they interact with other cellular components. In misfolding process proteins lose their biological function and/or gain toxic properties (DeToma et al, 2012). The reasons for misfolding can be genetic or different environmental properties such as oxidative or metabolic stress (Takalo et al, 2013). Around 30% of newly synthesized proteins are incorrectly folded and in this case, cellular chaperone machinery plays a key role in reducing the number of errors (Sgarbossa, 2012; Takalo et al, 2013).

Protein folding is a complex process, and it depends on right function of each part of the process. Because of that, there are different reasons why proteins sometimes don't fold correctly (Takalo et al, 2013). The problem can be in somatic mutation in gene sequence; in this case, the protein is unable to fold in a native form. Another potential reasons are errors in transcription or translation processes. The problem can also lie in non-functioning folding chaperone machinery. The protein can misfold also due to the changes in the environment or there may be an induction of protein misfolding by seeding or cross-seeding mechanisms (Moreno-Gozalez et al, 2011).

Since, there are many reasons why protein misfolds and it is not a rare event, protein quality control systems are important to maintain protein homeostasis. The first solution for misfolded proteins is refolding and recovering normal conformation and function. In this process heat shock proteins (molecular chaperones), play an important role. The second option is degradation of

inappropriately folded protein where ubiquintin-proteasome system (UPS) or aggresome-lysosome systems (ALP) take place. The third option is that misfolded protein is sequestered as specific protein inclusion whitin the cells if the first two options are not possible (Takalo et al, 2013). If the misfolded proteins escape the intracellular quality control system or the system doesn't work well, these proteins tend to aggregate in water-insoluble clusters and this leads to neurodegenerative disease (Sgarbossa, 2012; DeToma et al, 2012).

2.2 KINETICS OF AGGREGATION

Fibrillogenesis is a complex multistep process that normally can be described as ''nucleation-dependent polymerization'' (Picou et al, 2012; DeToma et al, 2012). It is suggested to be a three-stage process and can be described with a sigmoid curve. The three main steps in fibrilogenesis are protein misfolding, nucleation and fibril elongation (Invernizzi et al, 2012).

The first phase is called ''lag phase''. The process begins when the native monomer misfolds and forms a partially folded intermediate. Unfolded and misfolded proteins then self-assemble and the result is forming the nucleus.1 A transition from soluble, native monomers to insoluble oligomeric species with β-sheet conformation occurs (Sgarbossa, 2012). Lag phase is a thermodynamically disfavored and it influences the overall kinetics of the amyloid reaction (Invernizzi et al, 2012). Reaction rate depends on the concentration of protein. Native proteins are in equilibrium with nucleus.

The second phase is ''exponential phase'' or ''growth phase'' (Sgarbossa, 2012). A nucleus which is formed in a lag phase is in rapidly extended. Soluble species are progressively arranged at the ends of preformed structures. This process is thermodynamically favorable (Invernizzi et al, 2012). Protofibrils are formed and they are the first stable elements in fibril formation (DeToma et al, 2012). They have β-sheet rich structure and interactions between proteins are hydrophobic interactions, hydrogen bonding and stacking. Fibrils that have already been formed can be fragmented and be a source for new fibrils and with that they accelerate polymerization process end result in exponential growth (Invernizzi et al, 2012)

The last phase is ''saturation phase'', where an exhaustion of monomers stops elongation of preformed fibrils. Fibrils mature usually by their lateral association (Invernizzi et al, 2012). Still, even though mature fibrils are the end point of the process, molecular recycling within fibrils is still present (Invernizzi et al, 2012). Fibrils are approximately 100nm long and 10nm wide.

2.3 PROTEIN AGGREGATION ALZHEIMER'S DISEASE

Alzheimer's disease (AD), a neurodegenerative disease is one of the most common forms of dementia. It is estimated that 1 in 9 people over 65 years have AD and this number increases with age of observed group of people. It is a chronic, slowly progressive disease which leads to decline in cognitive and intellectual abilities, change in patient's behavior and later on physical dysfunction and death (Emeršič, 2013). At the moment the diagnosis bases on physician observation of patient, patient's family history, cognitive tests and potential magnetic resonance (Alzheimer's association, 2013). The precise conformation of AD is possible postmortem on a neuropathological basis (Pedersen et al, 2013). Because of that there is a goal is to include biomarkers of the disease in diagnosis process (Alzheimer's association, 2013).

There are two main pathological hallmarks in the brain of patients with AD, and these are extracellular amyloid plaques and intracellular neurofibrillary tangles (Finder, 2010; Picou et al, 2012). The key player is amyloid-beta peptide (Aβ) which is normally present in human plasma and cerebrospinal fluid (Sabella et al, 2004). The physiological function of Aβ is not known very well but researches showed that lower concentrations of Aβ than normal lead to neuronal death and they affect learning and memory. On the other hand, if there is an excess of Aβ, the peptides aggregate and gain toxic characteristics and lead to AD (Finder, 2010).

Aβ peptides are composed from 38 to 43 amino acids and are the product of enzymatic cleavage of larger amyloid-β precursor protein (APP) (DeToma et al, 2012). The most abundant variants are Aβ1-40 and Aβ1-42. Aβ1-42 is considered to be the main player in protein aggregation and in the initiation of AD (Finder, 2010). APP is a source of beta-amyloids and it is expressed in a variety of cells throughout our body in different isoforms. It is composed of 695 to 770 amino acid residues and the most common form in the brain is the shortest one, APP695 (DeToma et al, 2012). APP is a glycosylated transmembrane protein and its role in the brain it's not completely known. It has been suggested that it has an important role for the proper development of neurons, synaptic plasticity, cell adhesion and it could be involved in metal ion transport and homeostasis (Finder, 2010; Zhang H et al, 2012).

APP is posttranslational metabolized in two major pathways, non-amyloidogenic or amyloidogenic pathway. In the non-amyloidogenic pathway, APP is metabolized by sequential α- and γ-secretases cleavage. α-secretase cuts APP in two parts, sAPPα and C83, a C-terminal fragment, which remains in the membrane and it is later degraded by γ-secretase. In this case P3 peptide and APP intracellular domain are released, but both of them are degraded quickly (Zhang H et al,

2012). In amyloidogenic pathway, which is disease related, the APP is processed by sequential cleavage of β and γ- secretase, resulting in amyloids-β (Zhang Y et al, 2011). The products of first secretase (β) are sAPP-β and C terminal membrane fragment with 99 amino acids residues. The later fragment is then degraded by γ-secretase into Aβ and APP intracellular domain which is rapidly degraded while Aβ can either perform their physiological or pathological role (Zhang H et al, 2012; Finder, 2010).

Normally, the concentration of Aβ is steady and it is regulated by several proteases which are responsible for peptide degradation (Finder, 2010). The most important proteases are insulin degrading enzyme, neprilysin, plasmin, cathepsin B and endothelin converting enzyme (Kurz et al, 2011). Aβ concentration is also regulated with transition across blood-brain barrier which is mediated by the low-density lipoprotein receptor related protein and by the receptors for advanced glycosylation end products (Finder, 2010). After Aβ reaches the periphery it is degraded in the liver or in the kidneys (Finder, 2010).

The disease is not caused by Aβ peptide itself, but it is caused by toxic Aβ oligomers and aggregates. So, the reason for pathological effects of Aβ can lie in imbalance of Aβ production and clearance or in different circumstances that trigger misfolding of Aβ or aggregation. ***15*** Factors that induce misfolding can be separates into two groups, intrinsic or extrinsic. The first one in based on amino acid sequence, which means that charge, hydrophobicity or secondary structure of Aβ can increase the tendency of aggregation. Extrinsic factors such as temperature, pH, ionic strength, oxidative stress or excess of the protein, also triggers aggregation process (Finder, 2010).

Aβ is the main actor in AD, but also, neurofibrillary tangles in pyramidal neurons are one of the signs of the disease. They are composed of hyperphosphorylated tau protein. Tau protein is normally present in human brain. It has a role in vesicle transport, microtubule assembly and stabilization. It tau protein is hyperphosporylated it spontaneously aggregates and forms NFTs. As with Aβ, oligomers exhibit cytotoxicity and cause cognitive defects (Finder, 2010).

Nowadays, oligomers are defined as the main toxic specie in pathomechanism. Aβ plaques are a final waste deposit, but also, they can be a source of toxic species (adsorption-desorption mechanisms). One of the toxic effects of oligomers is on synapses. They are thought to be synaptotoxic which can lead to death of neurons. Number of synapses is reduced in AD and it correlates with cognitive defects. The mechanism is in particular associated with interference of normal activities (Finder, 2010). Aβ oligomers also cause membrane disruption by forming the

pores in the membrane which are permeable for ions and disturbed ion homeostasis cause apoptosis of the cell. The main ion involved is Ca^{2+}. Another mechanism of toxicity is oxidative stress in the brain which leads to change and damage in different molecules in the brain such as proteins, nucleic acids and lipids. The reason for oxidative stress can lie in dysfunction of mitochondria where energy metabolism is not normal and the result is reactive oxygen species (Finder, 2010). All this leads to dysfunction of neurons, cell death, dysfunction of signal transfer which is seen as dementia, a clinical sign (Emeršič, 2013).

2.4 PARKINSON DISEASE

Parkinson's disease (PD) is the second most common progressive neurodegenerative disease (after AD). Clinical signs of the disease are tremor, rigidity and bradykinesia. Lewy bodies are the pathological hallmark of PD. The main components of Lewy's bodies are synucleins, of which α-synuclein has the main role. Components of Lewy's bodies are also other proteins, neurofilaments, ubiquitin-proteasome system proteins (Chánez-Cárdenas et al, 2012).

α- Synuclein, the main protein involved in PD, is a presynaptic protein composed of 140 amino acids residues. Monomer protein does not have specific structure and it can be folded in a variety of tertiary structures. Also, α-synuclein aggregates vary in their structure, from amorphous to amyloid-like aggregates, and also oligomers (Chánez-Cárdenas, 2012). There are two possibilities of α-synuclein position in the cell, it can be either bound to the membrane, or it is soluble in cytoplasma (Chánez-Cárdenas et al, 2012). Its physiological role is not known very well; most likely it is involved in regulation of release of synaptic vesicles and stabilization of SNARE complex (Invernizzi et al, 2012).

There are different causes for aggregation of α-synuclein into aggregates and fibrils and probably they all contribute to disease development and progression. The possible causes are:

- An increase in concentration of α-synuclein
- Un favorable interactions with other protein
- Posttranslational modifications (phosphorylation, glycosylation, nitration, ubiquitination and partial cleavage)
- Oxidative modifications
- Insufficient degradation mechanisms

PD is divided in two forms, sporadic and familial PD. The later one depends more on genetic factor and mutation and overexpression (duplication or triplication of the gene) of the protein are the

main causes. In sporadic PD, the cause is more likely damage of the protein and failure in degradation paths (Chánez-Cárdenas et al, 2012).

When α-synuclein is 'changed' and aggregation process is started, firstly nucleuses and oligomers are formed and later on fibrils.

3 BIOMARKERS OF NEUROLOGICAL DISEASES

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of normal biological process, pathogenic process or pharmacologic response to therapeutic intervention (Strimbu et al, 2010). They can provide important information in disease diagnosis, progress of the disease, state of the disease or success of treatment. In some areas, biomarker are already well accepted and used, as for example in managing cardiovascular diseases, infections, genetic disorders and cancer (Mayeux, 2004). In neurodegenerative disorders some biomarkers are already known and used.

One of the advantages of biomarkers is a potential to identify the presence of the neurological disease at early stages. The author Mayeux classifies biomarkers of neurological diseases into two groups. The first one is group of biomarkers of exposure. The goal is to investigate presence of potential genetic mutation that promotes the disease or exposure to environmental factors and evaluate the exposure and predict the risk of the disease. The second group is the group of biomarkers of the disease. Biological fluids are used to determine either presence of the disease or subclinical manifestation of the disease (Mayeux, 2004).

Use of biomarkers in diagnosis process brings many advantages, but there are also many potential difficulties in this process that must be taken into account. Inter- and Intra-individual variability is one of the concerns. Also, the biomarker must have a certain diagnostic specificity and diagnostic sensitivity as well as proper positive and negative predictive values.

The diagnosis of Alzheimer disease, one of the neurological disorders, at the moment bases on physician observation of patient, patient's family history, cognitive tests and potential magnetic resonance (Alzheimer's association, 2013). The precise conformation of AD is possible postmortem if histological examination is performed (Pedersen et al, 2013). To confirm the presence of AD senile plaques and intracellular neurofibrillary tangles must be present in sufficient amount (Rosén et al, 2013). Since it is known that pathological processes start years or decades before there are some clinical signs of disease there is a goal is to include biomarkers in

diagnosis process in different stages of disease process (Alzheimer's association, 2013, Rosén et al 2013). AD can be divided into three stages; preclinical AD, mild cognitive impairment due to the AD and AD with dementia and there are different biomarkers that could be applied in different stages (Rosén et al, 2013).

In AD with dementia, there are different biomarkers. Firstly, there are elevated levels of T-tau and P-tau in CFS. However, the problem is that these values can be elevated also with other dementias or if head trauma or stroke occurs. Secondly, low levels of Aβ-42 are one indicator of AD because they are accumulated in senile plaques (Rosén et al, 2013). Another indicator of AD can be the ratio Aβ-42/Aβ-40 and Aβ-40 itself. Concentration of all monomeric proteins is decreased due their bonding into aggregates. The disadvantage of these biomarkers is lack of sensitivity; the concentration has to be decrease to certain level so the disease can be confirmed. There are also problems with standardization as global references are still not well defined (Rosén et al, 2013).

To continue, there are a large number of candidate biomarkers of AD, whose relevance has yet to be implied. One of them is also presence of aggregates which is included in our research. If we consider hallmarks of AD, we can see that Aβ aggregates are the most significant hallmarks (Finder, 2010). The results show CFS samples off patient with AD contain much higher concentration of Aβ aggregates as the patient without AD (Pedersen et al, 2013). Other candidate biomarkers are also activity of β-secretase involved in metabolism of APP, levels of sAPPα and sAPPβ, Aβ oligomers and other (Rosén et al, 2013).

3.1 METHODS FOR ANALYSIS OF BIOMARKERS OF NEUROLOGICAL DISEASES

Biomarkers of neurodegenerative diseases can be most commonly found and analyzed in cerebrospinal fluid or blood. There are different techniques available for analyzing presence of Aβ, Aβ aggregates or oligomers. In our interest are the techniques that can detect the presence of monomers and aggregates and that the quantity assessment can be made. Here, some techniques that can be suitable for aggregates analysis are presented.

3.1.1 ELECTROPHORESIS

There are different modes of electrophoresis, but overall, size, shape and charge are the properties of molecules that define analysis. Analytes must be soluble, stable under separation conditions and detectable. One of the modes is capillary electrophoresis, which will be used in our research and it will be more precisely described in the next chapters. Advantages of CE are that only small volume of sample is necessary, it can be coupled with UV or LIF (laser-induced

fluorescence) detectors. Also, affinity-CE is possible. Another mode is gel electrophoresis. It can be performed in different ways, one being native gel electrophoresis, which is suitable for analysis of not so stable aggregates under denaturating conditions. Also, denaturating gel electrophoresis, for example SDS-PAGE, can be used. The problem of all native forms of electrophoresis is that migration depends on mass, charge and ratio, and if relation of these parameters does not differ enough it is difficult to separate analytes (Pedersen et al, 2013).

3.1.2 ELISA (Enzyme-Linked Immunosorbent assay)

ELISA test is commonly used for measuring concentration of CFS levels of Aβ-42 (Rosén et al, 2013). Antibody can specifically detect presence of Aβ-42 monomer (it recognizes first and last amino acid), but also the can identify oligomers and aggregates (Rosén et al, 2013). To analyze oligomers sandwich ELISA may be used in which case the same antibodies can be used (there are more epitops). Possible are also different modes of AD where specific antibodies are designed to recognize oligomers, aggregates or fibrils. One big advantage of ELISA is low sample volume necessary and another is high specificity and selectivity (Pedersen et al, 2013). Also, analysis can be performed in complex samples or it can be combined with other techniques (Pryor et al, 2012).

3.1.3 SIZE-EXCLUSION CHROMATOGRAPHY

Size and shape of analyte determine the time necessary to the detection window in this technique. It is a liquid flow-driven system. Molecules with smaller hydrodynamic volumes (determined by size and shape) are detected later because they have larger distribution volume in separation column. There are pores which smaller molecules can enter but larger cant (Pedersen et al, 2013).

3.1.4 ANALYTICAL ULTRACENTRIFUGATION

Centrifugation is one of the methods that can determine the size of Aβ (Pryor et al, 2012). The separation is based on analytes velocity under influence radial acceleration. The separation depends on sedimentation coefficient, which correlates with molecular mass, and it is inversely proportional to frictional coefficient, which depends on molecular size. One possibility of performing ultracentrifugation is independent on molecular size, this is when lower speed of rotor is used (Pedersen et al, 2013). With this method, size of protein, or protein aggregates can be determined, but quite large amount of the sample are needed, and also, complex samples are not appropriate for analysis. Proteins can be used in their native form (Pedersen et al, 2013). It can be coupled to SDS-PAGE, Westrn blot or other technique (Pryor et al, 2012).

3.1.5 MASS SPECTROMETRY

In this method the sample is vaporized into gas phase and ionized by electric beam. They are separated based on their mass-to-charge ratio when they are under influence of electromagnetic field (Pryor et al, 2012). Obtained signal is then converted into mass spectrum of the sample and mass characteristic of the sample are provided. Mass spectrometry is selective method, only a small amount of the sample is needed and it can analyze complex samples (Pedersen et al, 2013). It must be added that there are different types of sources that enable ionization and also, different analyzers that transform ion signal into spectrum (Pryor et al, 2012).

3.1.6 DYES

Presence of amyloids can also be confirmed with help of two dyes, Congo Red and Thioflavin T. Both of them bind to amyloid structure and the first one produces apple-green birefringence undet polarized light while binding of Thioflavin T can be measured by fluorescence (Pedersen et al, 2013). Use of Thioflavin T can be used combined with CE coupled with laser induced fluorescence detector (Picou et al, 2012).

4 AIM OF THE STUDY

AD, PD and other neurodegenerative disease present considerable percentage of diseases nowadays, but their diagnosis is difficult. Aβ, protein that plays main role in AD, aggregates and presence of oligomers, aggregates and fibrils is and indicator of the disease. Our goal is to prepare a separation process by which we will be able to detect the presence of Aβ oligomers and aggregates. Chosen technique to work with is capillary electrophoresis. Our experimental part will be based on observing the behavior of few model proteins which are easier accessible than Aβ. The proteins will be separated in their native form. The goal is to explore and evaluate different modes of CE, especially CZE and CGE and in the end to establish conditions of separation where separation of model protein is the best. Conditions defined as the best will be also evaluated by testing insulin aggregates. Later on, but not in the scope of this thesis, these conditions will be transferred on analysis and separation of Aβ and in the end, to establish if CE is suitable for determination of Aβ aggregates.

5 MATERIALS AND METHODS

5.1 CHEMICALS AND REAGENTS

5.1.1 PROTEINS

- Aprotinin, bovine, CAS: 11070-73-8, Sigma-Aldrich, USA
- Ribonuclease A, bovine, CAS: 9001-99-4, Sigma-Aldrich, USA
- Cytochrome C, horse, CAS: 9007-43-6, Sigma-Aldrich, USA
- Carbonic Anhydrase, bovine, CAS: 9001-03-0, Sigma-Aldrich, USA
- Conalbumin, chicken egg, CAS: 1391-06-6, Sigma-Aldrich, USA
- Insulin, bovine, CAS: 11070-73-8, Sigma-Aldrich, USA
- Trypsinogen, bovine, CAS: 9002-08-8, Sigma Aldrich, USA

All proteins were diluted in water in concentration 10mg/ml. In some analysis they were diluted with water to obtain concentration 10^{-4}M, but in large part of analysis they were used undiluted.

5.1.2 POLYMERS AND ADDITIVES

- Polyvinyl alcohol 15000, Fluka
- Methyl cellulose, lot: 05219LW, Aldrich Chemical Company, Inc., USA
- Poly(ethylene oxide), average M_w=8 000 000, batch: MKBC8679, Sigma-Aldrich, USA
- Hydroxypropyl cellulose, average Mw=100 000, batch: MKBF826V, Sigma-Aldrich, USA
- Poly(ethylene glycol) #1, Mw=35 000, batch: BCBH6884V, Sigma-Aldrich, Germany
- Poly(ethylene glycol) #2, Mw=1900-2200
- Dextran (dextran sulfate sodium salt from Lenconostoc spp.), Mw>5000 000, batch: SLBD3148V, Sigma-Aldrich, Denmark

All polymers were diluted in desired concentration in 50 or 250mM phosphate buffer pH=2,00 (depends on capillary diameter). In the process of dissolving polymers the solutions were agitated with magnetic stirrer or sonicated in ultrasound bath.

5.1.3 OTHER REAGENTS

- Benzyl alcohol, lot: BCBF7667V, Sigma-Aldrich, Germany
- Ammonium Persulfate, Mw=228,20, CAS: 7727-54-0, BIO-RAD.
- TEMED: N, N, N’, N’-tetra-methyl-ethylenediamine, CAS: 110-18-9, BIO-RAD
- (3-Aminopropyl)triethoxysilane, batch: BCBG0033V, CAS: 919-30-2, Sigma-Aldrich, USA
- Ultra Pure Water (Milli-Q, Millipore, USA)

- Citrate buffer 100mM, pH=2,5
- Tris HCl buffer 50mM, pH=6,8
- Sodium Hydroxide 1M, Sodium Hydroxide 5M

5M Sodium hydroxide was prepared by weighting 30g of NaOH and dissolving it in 150mL of water. That solution was diluted 5 times to prepare 1M Sodium Hydroxide.

- Phosphate buffer 250mM, pH=2,00 and phosphate buffer 50mM, pH=2,00

To prepare 500mL of 250mM phosphate buffer we added to 450ml of water 2,812 ml of pure phosphoric acid, adjusted pH to 2,00 with 1M NaOH and then added the water to 500mL. When we used 50mM phosphate buffer we diluted 250mM buffer 5 times with water.

- Formic acid 1M, pH=1,82

4,16mL of formic acid was diluted in 100mL of water.

- Acetic acid: 20%CH3COOH, 100mM NaCl, pH=2,00

584,4 mg of NaCl was dissolved in ~50 ml of water. Then 20 ml of CH3COOH (≥99%) was added and we adjusted the pH to 2.00 with NaOH 1 M and NaOH 0.2M. Volume was completed to 100 ml.

- Phosphate buffer saline: 0,1% NaN_3, 0,14M NaCl, pH=7,40

5.2 INSTRUMENTS

Experiments were carried out on P/ACE^{Tm} MDQ Capillary Electrophoresis System (Beckman-Coulter, USA). The system is equipped with on line UV detector and electropherograms were acquired by frequency 16Hz. We used fused silica capillaries with internal diameter 50µm (50µm id x 375µm od) or 75µm (75µm id x 375µm od) and they were bought at Composite Mental Service Ltd (Great Britain). New capillaries were cut to a total length 58,5cm and the length to the detector was 48cm with detector window 0,5cm. The capillary temperature was 25,0°C and the samples were stored at 6,0°C. The wavelength of detection was 214nm.

Other instruments that we used were:

- pH meter: Beckman, USA
- balances: Mettler AC100, Mettler PE 2000
- sonicator: Bransonic 52

- Micro centrifuge: CarlRoth, Speed=6,000PRM
- Vortex Mixer: StarLab Velp Scientifica

5.3 PRINCIPLE OF CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) is an analytical method which was developed by merging two technologies; conventional slab-gel electrophoresis and high-performance liquid chromatography (Takagi, 1997). CE is widely used for analysis of different biopolymers such as proteins and DNA, but it is also suitable for analysis of smaller molecules like inorganic ions or drugs (Tagliaro et al, 1998). Benefits of CE are high efficiency, short analyses times, small volumes of sample and reagents, and it is cheaper compared to conventional electrophoresis or HPLC (Xu, 1996).

Separation is based on migration of charged analytes dissolved in a conductive solution under influence of electric field (Eu. Phr.). Electrophoretic velocity depends on the electrophoretic mobility of the analyte, on electro-osmotic mobility of the buffer and on strength of electric field. It if expressed by following equation, where ν is velocity of solute, μ is electrophoretic mobility of the solute and E is electric field strength (EU. Phr.).

$$\nu = \mu * E = \left(\frac{q}{6\pi\eta r}\right) * (\frac{V}{Lt})$$

Electrophoretic mobility of the analyte results from characteristics of the analyte (electric charge, size and molecular shape) and from the properties of buffer (pH, ionic strength, viscosity, additives) as we can see from the equation above (Eu. Phr). It is proportional to charge (q) of the solute, and inversely proportional to friction coefficient which is related to the viscosity of buffer (η) and hydrodynamic radius of the solute (r). (Xu, 1996) Electric field strength can be expressed by applied voltage (V) and total length of the capillary (L_t) (Eu. Phr).

Electroosmotic flow (EOF) has big impact on migration of solutes and buffer itself. Fused silica capillaries are most commonly used in CE and there are silanol groups exposed on the inner surface of the capillary (Petersen et al, 2001). These silanol groups lose a proton above pH 3 and they create negative charge on the capillary wall. This results in electrical double layer because cations from the buffer are attracted as counterions (Petersen et al, 2001). The layer closest to the wall is strongly bound and immobile while further from the wall, there is a compact but mobile layer with mainly cationic character. This layer is free to move (Weinberger, 1993). When the voltage is applied, free cations migrate towards cathode (negative electrode), and because they are solvated they drag the bulk solution with them (Weinberger, 1993) as it is presented in

figure 1. The velocity (v(EOF)) of EOF depends on electroosmotic mobility μ(EOF) and electric field strength. EOF can be expressed by Smoluchowski equation (Petersen et al, 2001):

$$v(EOF) = \mu(EOF) * E = \frac{\varepsilon * \zeta}{\eta} * E$$

where ε is the dielectric constant of the solution, η is the viscosity of the solution, E is the field strength, and ζ is the zeta potential. In fused silica capillaries, the EOF can be reduced by lowering the pH value of the buffer, when SiO- groups surface to SiOH and the zeta potential is decreased. With increasing ionic strength of the buffer, electric double layer collapse and EOF is reduced. Another way to influence EOF is to create a coating of the capillary wall and with that suppress ionization of silanol groups (Xu, 1996).

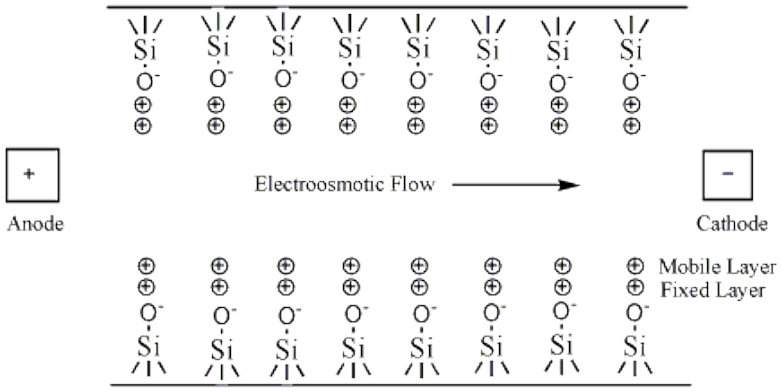


***Figure 1**: Presentation of charge of silanol groups and EOF in capillary electrophoresis. (http://en.wikipedia.org/wiki/Capillary_electrophoresis)*

Mobility of the analyte and EOF mobility may act in the same direction or in the opposite directions. Normally anions migrate towards anode, positively charged electrode, but their velocity is smaller than velocity of EOF, so they migrate towards cathode. In the case of cations the velocities of EOF and analyte add up. The velocity of neutral molecule is the same as the velocity of EOF. The apparent mobility (μApp) of the solute, which is measured by migration time, is the sum of mobility of EOF (μ(EOF)) and electrophoretic mobility (μ(E)). It can be calculated by following equation where Ld is length to the detector (cm), Lt is total length of the capillary (cm), V is voltage (V) and t is migration time (s) (Altria, 1996, Xu, 1996).

$$\mu(App) = \mu(E) + \mu(EOF) = \frac{LdLt}{Vt}$$

The basic instrumental setup of CE consists of high voltage power supply (delivering up to 30kV), a separation capillary, an injection system, two buffer reservoirs, two electrodes assemblies

(cathode and anode), a thermostatic system, an on-column detector and a recorder with computer. System of capillary electrophoresis is shown in figure 2 (Eu. Ph; Xu, 1996; Tagliaro et al, 1998).

A CE system can be carried out in different modes and so the analytical problem can be approached in variety of ways. The choice of mode is based on the analytical problem under consideration. In table 2 there are presented different modes of CE and their mechanism of separation. In our work, we worked with capillary zone electrophoresis (CZE) and capillary gel electrophoresis (CGE).

***Table 2**: Modes of CE, their acronyms and mechanisms of separation*

Mode of CE	Acronym	Mechanism of separation
Capillary zone electrophoresis	CZE	Charge-to-mass ratio
Capillary gel electrophoresis	CGE	sieving
Capillary isoelectric focusing	CIEF	Isoelectric point (pI)
Micellar electrokinetic cromatography	MEKC	Distribution of analytes between aqueous buffer and micelles
Capillary Isotachophoresis	CITP	Mobility of analyte between two different buffer systems
Chiral electrophoresis	/	Tendency of enantiomers to associate with chiral selectors
Capillary electrocromatography	CEC	Distribution between stationary and a moving phase

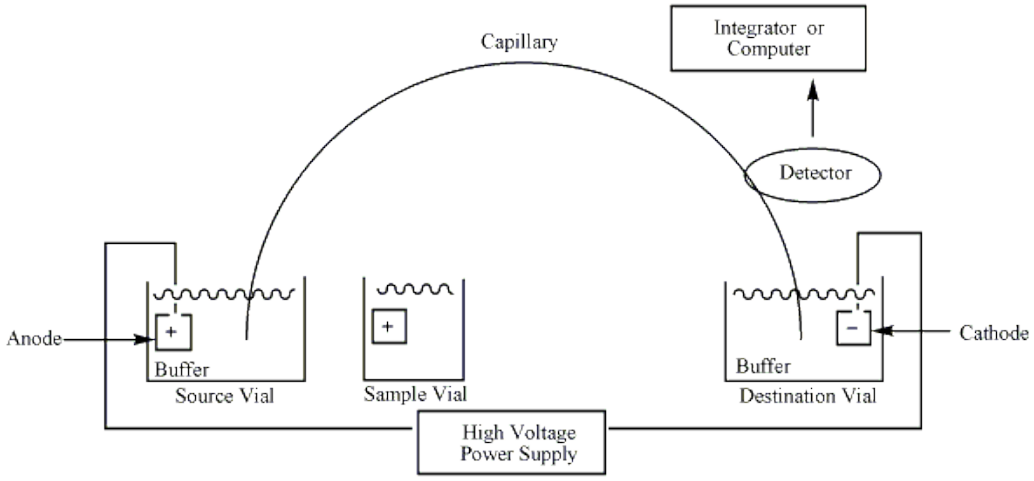


***Figure 2** : Setup of capillary electrophoresis (Wikipedia)*

5.3.1 CAPILLARY ZONE ELECTROPHORESIS

Capillary zone electrophoresis is the simplest mode of CE. It is performed in a homogenous carrier electrolyte (Weinberger, 1993). When electric field is applied, each analyte migrates according to its apparent mobility which depends on its individual mobility and EOF (Xu, 1996). CZE is a high efficient method and the components with minor differences in their charge-to-mass ratio can be separated, but neutral molecules cannot be separated because they all migrate with velocity of EOF (Xu, 1996).

In CZE optimization of separation can be achieved by modifying instrumental and buffer solution parameters. The pH has great impact on charge and consequently on mobility of the analyte, on EOF, current and so it is very important experimental variable. It needs to be properly controlled to provide reproducibility. Another variable is buffer concentration. More concentrated buffer decreases EOF, analyte-wall interactions and it increases the current. To improve the separation additives may be used. Instrumental parameters that can be modified are voltage, temperature, capillary, injection and polarity of the electrodes (Weinberger, 1993, Eu. Phr.).

5.3.2 CAPILLARY GEL ELECTROPHORESIS

In capillary gel electrophoresis (CGE) capillary is filled with gel or viscous solution. They act as a molecular sieve, minimize solute diffusion that causes zone broadening, prevent interactions between capillary and solutes, suppress EOF and limit the heat transfer by slowing down the molecules (Takagi, 1997). If the molecules have similar charge-to-mass ratio, they can be separated according to their size which is often the case in large biopolymers such as DNAs or proteins (Xu, 1996). The solutes migrate towards appropriate electrode through the gel matrix and under properly controlled conditions the solute's mobility is inversely proportional to its size (Weinberger, 1993). Small molecules migrate through the pores faster because they can move freely, while larger ones tend to be retarded (Xu, 1996; Eu. Phr.).

There are two types of gels used in CGE. The first option is use of permanently coated gels such as cross linked polyacryamide. It is prepared by polymerization in situ. The problem is if the gel fails, the entire capillary must be changed. Other option is use of dynamically coated gels. The polymers such as dextran, PEG, PEO, linear polyacrylamide, cellulose derivates, are dissolved (concentration of polymer above entanglement threshold) in separation buffer and are prepared in a vial. They are pumped into the capillary for each analysis which improves the reproducibility of the method. Another advantage of pumpable gels is that both forms of injections

(electrokinetic and hydrodynamic) are possible while in permanently coated gels only electrokinetic injection can be used (Eu. Phr.).

5.4 PROCEDURE OF CAPILLARY ELECTROPHORESIS

5.4.1 TREATMENT OF CAPILLARIES

The new capillaries were cut in desired length. The total length of the capillaries was 58,5 cm. Detector window was situated at 48 cm from the inlet of the capillary. It is necessary that it is transparent and to achieve transparency, we removed external coating by burning it and cleaning it with methanol. Approximate length of detector window was 0,5cm. Then, each new capillary was pretreated by sequential washes with Sodium Hydroxide 5M and 1M, distillated water and separation buffer. The 50µm capillaries were washed for 10min and 75µm capillaries for 5 minutes by each reagent.

Every day in the morning rinse program was used to prepare the capillary. Also, in the end of the day the capillaries were washed with water for 5 minutes. Before each run, the capillary was also rinsed with shorter program as we can see in Table 3. In all rinse procedures, the pressure of washing was 20psi. The buffer used to do washes was the buffer we were currently using for analysis. Also, with each buffer the current was checked if it follows Ohm law linearly. The voltage was chosen regarding stability of the current and current value which was desirably between 50 and 60µA. Most often, the voltage we worked with was 25 or 30kV.

***Table 3** : Washing programs for capillaries (time is expressed in minutes, always 20psi pressure was applied)*

	Morning Rinse		In between runs Rinse			End rinse
Reagent/Time	50µm capillary	75µm capillary	50µm capillary	75µm capillary	Coated capillary	All capillaries
NaOH 1M	5	2,5	3	1,5	/	/
H_2O	5	2,5	3	1,5	1,5	5
BUFFER	5	2,5	5	2,5	2,5	/
	10kV for 1 min		separation			10kV for 1 min

5.4.2 INJECTION STUDY

In CE there are very small volumes of samples injected, normally in range between picolitres and nanolitres (Xu, 1996). Most commonly two types of injection are used, hydrodynamic and electrokinetic injection. In hydrodynamic injecton the injection is accomplished by applying

pressure difference between two ends of the capillary. This can be achieved by applying pressure on injection side, by applying vacuum at the detector side or by lifting the sample vial above the electrolyte level (Kriváscy et al, 1999). The amount of sample injected can be calculated by Poiseuille equation (Xu, 1996, Petersen et al, 2001):

$$V = \frac{\Delta P d^4 \pi t}{128 \eta L}$$

In this equation, ΔP is the pressure drop between the ends of the capillary (Pascals), *d* is the capillary inner diameter (m), *t* is the time of injection (s), η is the viscosity (Pascal-seconds), and *L* is the total length of the capillary (m) (Xu, 1996). The two parameters we can change and control are applied pressure and time of injection.

In the electrokinetic injection the sample introduction is achieved by applying a low voltage for a short period of time in sample vial. The quantity of injected sample can be calculated by following equation (Tagliaro et al, 1998):

$$Q = \frac{\big(\mu(ep) + \mu(EOF)\big)\pi r^2}{L} VCt$$

In this equation Q is quantity of injected sample, μ(EP) is electrophoretic mobility of the analyte, μ(EOF) is electroosmotic flow, r is inner radius of the capillary V is applied voltage, C is concentration of the sample t is time of injection and L is length of the capillary. As we can see the volume of injected sample in this mode of injection is a function of mobility and that means that injection is discriminative. Analytes with higher mobility will be injected in larger quantity under the same conditions (Tagliaro et al, 1998, Weinberger, 1993). If the analysis is qualitative, this does not present a problem, but in quantitative analysis it does because injected concentration does not represent original concentration (Xu, 1996).

During our research work we performed two injection studies. The first has been associated with providing the most similar conditions of injection between capillaries of different inner diameter, 50μm and 75μm. The second one was performed to determine the cause of loss in sensitivity when we add polymers to separating buffers. There may be less sample injected due to the increased viscosity of the buffer solution when the polymer is added. The other possible reason is connected with the absorbance of polymer itself or potential interactions between polymers and proteins.

PART 1: Differences between 50µm and 75µm diameter capillaries

The diameter of the capillary has big influence on the amount of the sample injected as we can see in Poiseuille equation. If we double the diameter of the capillary, volume of the injected sample is enlarged 16 times (Petersen et al, 2001). In the beginning we worked with 50µm capillary and later with 75µm capillary and since we wanted to provide as similar conditions as possible between capillaries, we decided to modify injection. We targeted to inject approximately the same amount of the sample and also obtain the peak with similar properties as with 50µm i.d. capillaries.

The two parameters of injection that we could control were the pressure applied during injection and the time of injection. In the beginning we calculated the volume of the sample injected with the aid of a program (CE Expert, Beckman-Coulter). According to calculations, which showed that the volume injected in 75µm capillary under the same conditions (0,5psi, 5sec) is about 5 times bigger than in 50µm, we decided to lower one of the parameters by 5. After the analysis under new conditions (0,1psi, 5sec) we established that the results cannot be compared, because height of the peak was much lower (10 times) that is should be, since, theoretically, the same amount of sample is injected.

Then, the linearity of injection was studied by changing both parameters while always injecting the same protein - aprotinin. We applied pressure between 0,2 and 0,5 psi for 2 to 5 seconds. Regardless which parameter was increased, the height of the peak increased and the peak got wider, but the change was not always linear. As we can see in the Figure 3, there problem can lie in pressure. In the first graph, we can see, that if the variable is time of injection, the height correspond more or less linearly. On the other hand, if the variable is pressure, and time of injection is constant, the change is not linear. That means, that when are smaller pressures applied, we do not succeed to inject as much sample as expected if we extrapolate linear curve from higher pressures.

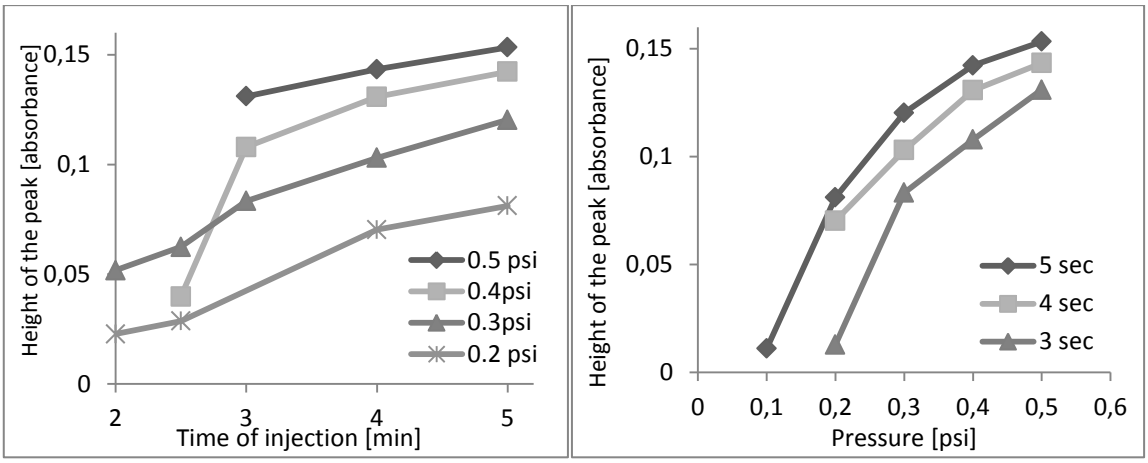


Figure 3 *: First graph represents change in the height of the peak while increasing time of injection and pressure of injection is stable and the second represents the change in the peak's height regarding pressure applied for 3, 4 or 5 seconds. Aprotinin, 30kV, 50mM phosphate buffer pH=2,00, 75µm capillary.*

After performing these experiments we chose new conditions of injection in 75µm capillary, namely 0,3 psi and 3 seconds. If we calculate volume of injected sample we can see that in these conditions there is more sample injected like in smaller capillary (volume is 1,82 bigger than in 50µm capillary). However, the response (peak's height) was comparable which was not the case during conditions when there should be the same amount of the sample injected. The reason for that could be inability of small pressure to provide as much sample as expected (Beckman-Coulter program).

Also, if the same amount of sample is injected in 50 and 75µm capillary, the response should be bigger in 75µm capillary due to the increased path length of the light of detector. Since the effective path length is about 63,5% of capillary diameter (Petersen et al, 2001), the length in a 75µm capillary *enlarged* from 37,75µm to 47,625µm as compared to 75µm i.d. capillary.

Unfortunately, relying on peak height to achieve similar conditions has led to the increased injection plug length. If we calculate injection plug length in both conditions that we used – 0,5psi 5 sec in 50µm capillary and 0,3psi 3sec in 75µm capillary we can see that the plug length is longer for 2,14mm (increase is 82%) in 75µm capillary. Since injection plug length has big impact on band broadening this is an undesired fact for separation in 75µm capillary. However, experimental data showed that in chosen conditions width of the peak (depends on band broadening and injection plug length) was increased compared to the smaller capillary but not as much as theoretical calculations predict. That is probably due to non-linear relationship between applied pressure and response which is more problematic at lower pressures.

PART 2: Electrokinetic and hydrodynamic injection

During performing our experiments, we often noticed that with increasing concentration of polymer, the height of the peak decreases significantly. The question that we have set ourselves was if the loss in sensitivity is due to the polymer absorbance, potential interaction between polymer and proteins or the amount of sample injected is reduced due to the viscosity of the polymer. To find the answer to that question we tested electrokinetic injection to see, if there is also a decrease in peak height when polymer is added.

In the beginning, we wanted to find the conditions of electrokinetic injection which provide the amount of sample injected that can be compared to hydrodynamic injection. After that, we compared the change in peak's height as a function of concentration of polymer for both modes of injection. In case of hydrodynamic injection height of the peak decreases dramatically by adding polymer to the separation buffer while on the other hand with electrokinetic injection that change is not present as we can see in figure 4.

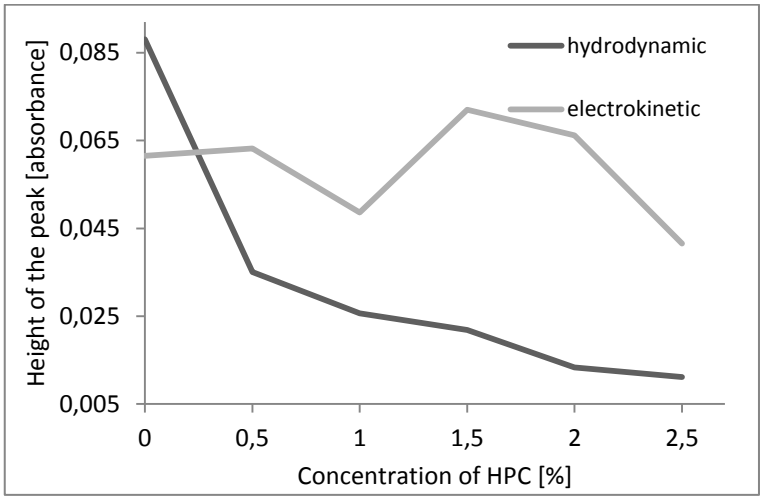


Figure 4 *: Height of the peak (aprotinin) in a relationship with concentration of HPC when injection is either hydrodynamic, either electrokinetic. 75µm capillary, 30kV, protein used is aprotinin (10mg/ml), 0.5, 1, 1.5, 2 and 2.5% HPC in 50mM phosphate buffer pH=2,00. Hydrodynamic injection: 0,3psi, 3sec; electrokinetic: 7kV, 7sec.*

On the figure 4 we can see that with hydrodynamic injection the peak's height is decreasing with increasing concentration of polymer. With electrokinetic injection, that trend is not present. We can see that height of the peaks is not constant but there is not a notable trend of progressive loss in height when concentration and with that viscosity is increased. After *observing* these results we can say that the loss in sensitivity when the polymer is added is due to the viscosity of polymer (hydrodynamic injection) not to the interaction between polymer and proteins or high

absorbance of polymer. When hydrodynamic injection is used, viscosity influences amount of sample injected, which can be also confirmed with Poiseuille equation. Volume of injected sample is inversely proportional to viscosity of the separation solution, so if the viscosity is increased, volume of sample injected is reduced. This represents the problem in analysis because there may be too little sample injected to ensure acceptable sensitivity. One way to pass it is use of electrokinetic injection, which is not affected by viscosity and other solution is to change injection conditions to inject more sample.

Although electrokinetic injection could represent the solution when polymer is too viscous for successful hydrodynamic injection (Xu, 1996), there are some other disadvantages of electrokinetic injection that we have observed.

We have often noticed that peaks obtained strange shape after electrokinetic injection when polymer was added to the separation buffer. Peaks did not have their normal, bell shape but more triangle shape (sharp). Unexpectedly peaks sometimes got even higher than without polymer and also wider. We can see strange shape of the peak in the figure 5 if we observe peak 3, this is the peak of cytochrome C, where we can see that it is very high and wide. This sort of peak shape was even more obvious when proteins were injected alone.

When a mix of proteins was injected we could observe that the peak's height correspond to the mobility of the solute. That means that the peaks of proteins that move faster (aprotinin) were higher than the height of the peaks that move slower (conalbumin). Furthermore, there was no peak of carbonic anhydrase in 2% HPC with EK injection while with HD the peak was present. That was due to the fact, that injection with EK depends on mobility of the solutes and injected sample. There is more injected more ions of high mobility and the sample is not always representative (Kriváscy et al, 1999). It is interesting why than there was a peak of conalbumin with even slower mobility. Differences between injections can be seen in figures 5 and 6.

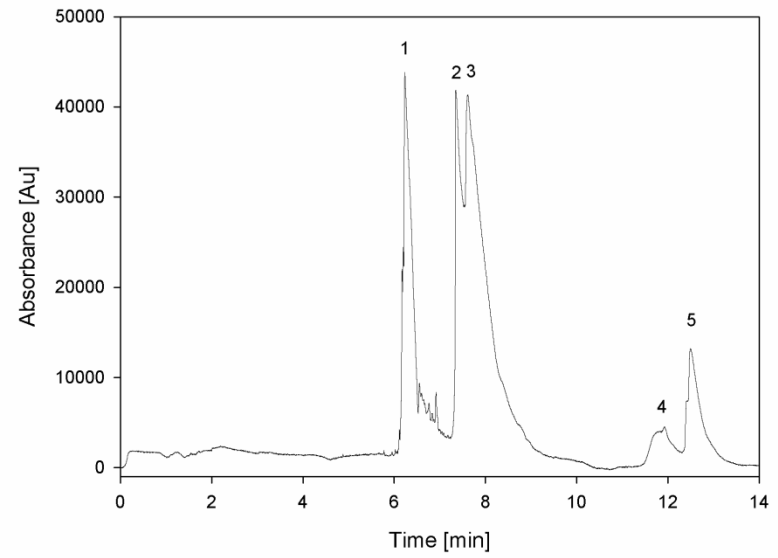


***Figure 5** : Separation of mix of proteins in 2% HPC in 50mM phosphate buffer, pH=2,00, 75µm capillary, 25kV with electrokinetic injection (7kV, 3sec). 1 – aprotinin, 2 – ribonuclease A, 3 – cytochrome C, 4 – carbonic anhydrase, 5 – conalbumin; concentration of all proteins is 10mg/ml.*

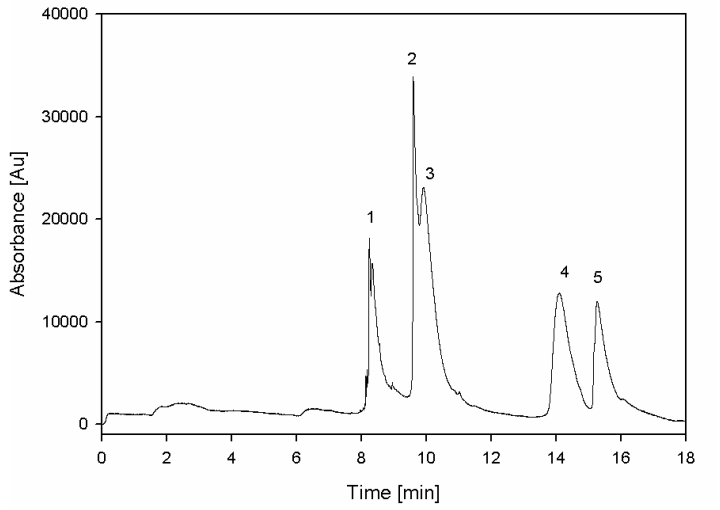


***Figure 6** : Separation of mix of proteins in 2% HPC in 50mM phosphate buffer, pH=2,00, 75µm capillary, 25kV with hydrodynamic injection (0,3psi, 3sec). 1 – aprotinin, 2 – ribonuclease A, 3 – cytochrome C, 4 – carbonic anhydrase, 5 – conalbumin; concentration of all proteins is 10mg/ml.*

Electrokinetic injection affected also time of migration of proteins. When the separations were carried out in a buffer without polymer, the differences between migration times of the same protein injected EK or HD were minor, less than 3% with exception of cytochrome C whose time was shortened for 11%. However, differences got bigger when polymer was added. For example, in 0,5% PEO, time of cytochrome C was for 0,64 minute shorter with EK injection than HD.

Furthermore, it was detected before aprotinin who was always detected the first and should migrate as the fastest. Despite that cytochrome C was the most extreme case; the differences were present also with other proteins (from 4 to 7%). Also, when a mix of proteins was injected, we could observe high diversity in migration times of all proteins between two consecutive runs.

During the analysis of insulin aggregates prepared in phosphate buffer saline, we noticed, that EK injection did not provide any peaks, while HD did. The reason for that is dependence of injection volume in EK on mobility of solutes. The pH of sample is 7,4 and if we assume that pI of insulin aggregates is the same as pI of insulin monomer, which is 5,30, that means that aggregates are negatively charged in sample vial. Since amount of solute injected is proportional to sample apparent mobility (mobility of aggregates is towards the anode and EOF is very low), it is possible that there is no sample injected. This represents a setback for EK because in this particular case, injection is not representative at all and we were unable to perform analysis with EK injection. Use of EK injection limits required pH of the sample.

To recap, in our case, EK injection did not show as the most effective. There were many undesirable properties found with EK injection compared to HD injection. Strange shape of the peaks, diverse migration times of the same protein and conditions between runs and unrepresentative sample injection present relevant parameters to consider. Even though the loss in sensitivity is present, we found HD injection more suitable for our analysis because the most importantly, it is more constant and we can rely on it.

5.4.3 DETERMINATION OF EOF

EOF contributes a lot to the migration velocity of molecules. Measurement of EOF is necessary to calculate absolute mobility of the solute. With measurement of EOF we can ensure the integrity of separation. EOF can be an indicator if the conditions of the capillary are changing and consequently it is an indicator of reproducibility.

To determine EOF we used neutral solute, benzyl alcohol, as a marker. We measured the time it takes the marker to transit to detector and then calculated EOF by following equation:

$$\mu(EOF) = \frac{v(EOF)}{E} = \frac{\frac{l}{tm}}{\frac{V}{L}}$$

In this equation μ(EOF) is electroosmotic flow, v(EOF) is velocity of EOF, E is electric field strength(V/cm), *l* and *L* are lengths to the detector and total length respectively (cm). V is applied

voltage (volts) and tm is migration time of marker expressed in seconds. The unit of μ(EOF) is cm^2/Vs.

5.4.4 COATING

In our study we decided to investigate the effect of coating on protein separation. Coating is one of the approaches to limit the effect of protein bonding to capillary surface due to the electrostatic, hydrogen or hydrophobic interactions between proteins and silanol groups of capillary. This leads to poor repeatability and separation. Besides coating, extreme pH buffers or additives can be used to limit these effects and they were also used in our research. Coating can be either covalently bound either physically adsorbed to the capillary surface (Nehme et al, 2011).

The coating was performed according to the Hjerten protocol (Hjerten, 1987). 80μL of (3-aminopropyl)triethoxysilane was mixed with 20mL of water, which had been adjusted to pH 3,5 by acetic acid. Then prepared silane solution was sucked into capillary. After one hour, the capillary was washed with water and later filler with acrylamide solution. This solution contained 4% acrylamide (w/v), 4μL TEMED reagent and 4% ammonium persulfate (w/v) and it was deaerated. After 30 minutes the capillaries were rinsed with water and in the end dried with air. The protocol can be seen in the table 4 (Hjerten, 1987).

***Table 4** : Hjerten protocol for polyacrylamide coating*

Reagent	Time [min]	Pressure [psi]
Silane solution	5	20
	55	1
Water	15	20
Acrylamide solution	5	20
	25	1
Water	15	20
Air	5	20

5.4.5 CHOICE OF PROTEINS

The aim of my study is to prepare the conditions to separate amyloid aggregates according to their size by capillary electrophoresis. During the development of the method and searching for optimal conditions, we worked with proteins which are easier accessible and their size is known. In the beginning we chose 4 proteins: insulin (MW=5,77kDa), aprotinin (MW=6,51kDa), ribonuclease A (16,46kDa) and trypsinogen (26,29kDa). After first few analyses we decided to stop working with insulin because we were not sure about its size. It behaved as it is the largest, not

the smallest of the proteins with long migration time. The reason for this behavior can result from the fact that insulin may not be in its monomeric form. Probably insulin aggregated into dimmers or small oligomers. Instead of insulin we selected conalbumin with molecular weight 77,7kDa.

For a series of experiments we worked with aprotinin, ribonuclease A, trypsinogen and conalbumin but we often observed strange behavior of trypsinogen. During the analyses we noticed that trypsinogen and ribonuclease A were not separated and they migrated together or even trypsinogen was detected earlier that ribonuclease A even though it is bigger. In a large number of experiments trypsinogen was migrating a lot faster than we expected based on its molecular weight, particularly with addition of polymers where size should have more impact on migration than charge.

We decided to review the properties of proteins, especially the properties that influence separation. In table 5 there are presented properties of proteins which have been discussed. We wanted to reselect proteins which have similar charge-to-mass ratio so their separation can be based on size in native form in CGE.

***Table 5**: Properties of proteins (AA- amino acids residuer, AR – average radius, [+] – number of positive charges)*

	MW[kDa] - Uniprot	pI	Number of AA	Number of Basic AA	Average radius [Å]	AR/MW	[+]/AR
INSULIN	5,773	5,30					
APROTININ	6,51144	10,50	58	10	14,8	2,27292273	0,74324324
CYTOCHROME C	11,832	9,59	105	21	18,1	1,52974983	1,21546961
RIBONUCLEASE	16,46	8,93	150	16	17	1,0328068	1,0000000
TRYPSINOGEN	26,294	4,61	247	17	19,6	0,74541721	0,91836735
CARBONIC ANHYDRASE	29,113	6,41	260	25	26,1	0,89650672	0,99616858
CONALBUMIN	77,776	6,85	705	88			

The mobility of proteins depends on their charge, which is in acidic pH correlated with amount of positively charged basic acid residues. Other important property for CE separation is size of the protein in combination with molecular weight, 3D structure of the protein and hydrodynamic radius. Firstly we counted the number of amino acid residues with positive charge at acidic pH which are arginine, lysine and histidine. To that number we added also the positive charge of

amino group at the end of the chain. In different literature sources we found the data about hydrodynamic radius and then evaluated the data. The two parameters we took into account the most were the ratio between hydrodynamic radios and molecular weight (AR/MW) and the ratio between number of positive charges and hydrodynamic radius ([+]/AR).

In the case of trypsinogen we observed that it is very condensed, its radius is very small in a relationship to its molecular weight. That means trypsinogen moves faster because it is smaller and there are less frictional forces to stop it. On the other hand, trypsinogen does not have a lot of basic amino acid residues compared to the number of all amino acids. But the ratio between number of basic amino acid residues and radius does not differ a lot from other proteins. With addition of polymers, where sieving effect takes place, the first parameter, the radius has more importance and with that the faster movement of trypsinogen can be explained. Another fact that may contribute to the switch of ribonuclease A and trypsinogen is also bigger ratio between radius and molecular weight of ribonuclease A compared to trypsinogen. So, ribonuclease A also contributed to undesired behavior and to conclude it was an unfortunate choice of these two proteins and separation was not successful.

After reviewing all properties of proteins we reselected five proteins to work with which were aprotinin, ribonuclease A, conalbumin, cytochrome C and carbonic anhydrase. Their sizes vary from 6,5kDa to 77,8kDa. Even though we carefully selected these proteins, we sometimes observed *similar* behavior to trypsinogen of cytochrome C. With addition of polymer (HPC) its time of migration was longer than the time of ribonuclease A which has larger molecular weight. Cytochrome has big radius compared to its size, so there are more frictional forces to slow down the protein.

Even though that proteins did not always behave as we would like that is not such a big problem, because when we will analyze aggregates and monomers of β-amyloid peptides, they base on the same amino acid chain and so their properties should be more alike.

5.5 ANALYSIS OF DATA

To analyze the results we measured different parameters in electropherograms and evaluated them and later compared with each other. The first parameter was ***migration time*** of the analyte. The migration time is the time it takes the solute to transit from the beginning of the capillary to the detector window (Weinberger, 1993). It is measured as a distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak and it is expressed as a function of time in minutes. The second important data is **sensitivity** of the analysis which

was obtained by measuring height of the peak. In the electropherogram the height is measured from the maximum of the peak to the extrapolated baseline. It is expressed in units of absorbance. Another important information is **broadening** of the peak. That parameter we evaluated by measuring width of the peak on baseline. We also measured the **noise**. Noise represents range of the background in electropherogram. It is measured after injection of a blank from maximum to minimum of baseline and it is expressed in absorbance unit.

Since we wanted to get good separation of proteins analyzed at the same time, **resolution** and **selectivity** are also parameters with big importance. Resolution tells if two adjacent peaks are well separated. It is defined as the difference between migration times of two peaks, divided by combined widths of the two peaks. Selectivity is the ratio between the two migration times. We can see both equations below, where t_2 and t_1 are migration times of proteins, and w1 and w2 are widths of the peaks of two proteins.

$$S = \frac{t2}{t1} \qquad\qquad R = \frac{t2 - t1}{1/2(w1 + w2)}$$

Efficiency of the analysis can be expressed with number of theoretical plates. The apparent number of theoretical plates can be calculated by the equation below where *N* is the number of theoretical plates, *t1* is migration time of the analyte and w_1 is width of the peak at the half-width and w is the width of the peak on baseline.

$$N = 5{,}54\left(\frac{t1}{w1}\right)^2 = 16\left(\frac{t1}{w}\right)^2$$

6 RESULTS AND DISCUSSION

The focus of my study was to prepare conditions in which amyloid aggregates can be successfully separated by their size in native form. To select the most appropriate conditions we tested the separation of 4 or 5 proteins (with different molecular weight) in various buffers, polymers, in two different diameters of capillaries and we also tried to improve the separation with coating, additives and different modes of injection.

6.1 THE BUFFER SELECTION

The first step in development of our method was choice of buffer to work with. The pH of the buffer was selected to be acidic. Even though it is not the most often choice in CE, it has significant advantages. Firstly, all proteins are positively charged (pH<pI) and they all move towards the cathode when the voltage is applied. Furthermore, EOF is reduced and this leads to more reproducible separations (McCormick, 1988).

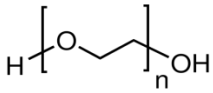
We tested three different buffer solutions with the pH between 1,8 and 2,5. Tested buffers were citrate buffer (100mM, pH=2,50), phosphate buffer (250mM, pH=2,00) and formic acid (1M, pH=1,8). We performed analysis of 4 proteins (aprotinin, ribonuclease A, trypsinogen and insulin) in all three buffers and then compared their characteristic of separation between buffers (the capillary was 50µm i.d.). The differences were not big, but nevertheless, phosphate buffer was selected. The parameters we evaluated and compared between all three buffers were EOF, noise level, and properties of the peaks (migration time, width and height of the peak). In the beginning we eliminated formic acid, since it is not really a buffer and all the parameters were the worse in comparison with other two buffers. The peaks were relatively wide and sensitivity was rather low, that can be due to the absorbance of formic acid itself. Other two buffers offered quiet similar results. Height and width of the peaks were sometimes better in one buffer and sometimes in the other, depending on the protein. Noise was slightly bigger with phosphate buffer but there was more reduction in EOF which was one of the most important parameters. One of the factors that influenced our decision (since other parameters were pretty similar) was the difference in elution times of all proteins. In citrate buffer the time of analysis for analyzed proteins was 5,9 minute and in phosphate buffer it was 7,6 minute. The difference between first and last protein eluted was less than 0,85 minute in citrate buffer while on the other hand in phosphate buffer it was 1,5 minute. This is an advantage in performing successful separation because if proteins migrate with moderate velocity, they will have more time to be sieved by the polymer network.

Choice of phosphate buffer was the first step in development of our method. Properties of this separation were the basis to evaluate other conditions and improvements.

6.2 THE POLYMER SELECTION

6.2.1 POLYETHYLENE OXIDE

Polyethylene oxide (PEO) was one of the polymers we tested. It is a synthetic polymer, a polyether and it is available in a range of molecular weights. Its structure can be seen below (source: Sigma-Aldrich web site). PEO is chemically the same as PEG but, molecules with molecular weight below 20,000g/mol are named glycols, and above are oxides.



The experiments with PEO began in **50µm capillary**. The concentrations of PEO used were 0,2% and 0,5% (diluted in 250mM phosphate buffer, pH=2,00). We tried to successfully separate 4 proteins according to their size. The proteins we worked with were aprotinin, ribonuclease A, trypsinogen and conalbumin. With addition of polymer the time of migration for each protein was prolonged as we can see in figure 7. Still, the proteins are not separated by their size, due to the trypsinogen and ribonuclease which are switched due to the different ratio of amino acid residues and radius (see chapter Materials and methods).

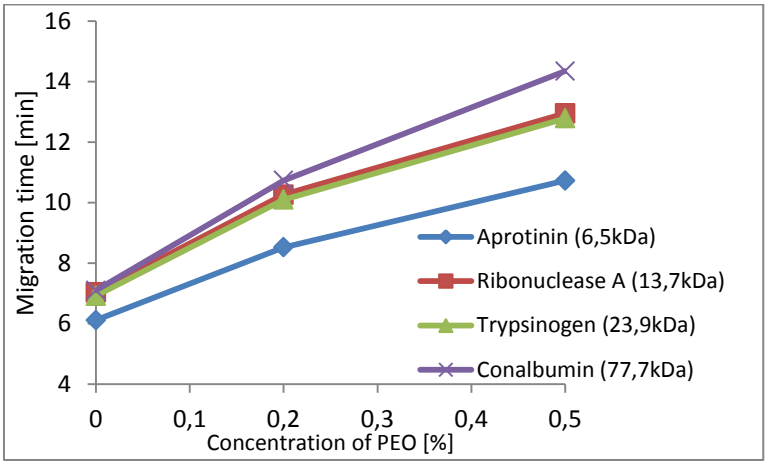


Figure 7: *Relationship between polymer concentration and migration time of protein in PEO. 50µm capillary, 0%, 0,2% or 0,5% of PEO in 250mM phosphate buffer, pH=2.00, 25kV, injection: 0,5psi, 5sec.*

Another important fact that we were observing was sensitivity, which is evaluated by height of the peaks. On the figure 8 we can see that the addition of PEO reduced the height of the peaks. In

0,2% PEO, the height is not reduced a lot, but in 0,5% PEO it is reduced on average for 69,4%. The width of the three protein peaks did not change a lot when the polymer was added, there were just minor variations. An exception is conalbumin, which width increased two 2,5 times.

Calculated values of resolution and selectivity increased with raising amount of PEO for pairs aprotinin-trypsinogen and trypsinogen-conalbumin. In these conditions we were not able to separate trypsinogen and ribonuclease A and so, the resolution did not improve. Furthermore, these two proteins did not migrate according to their size since trypsinogen, which has higher molecular weight (26,29kDa), is eluted earlier than ribonuclease A (16,46kDa).

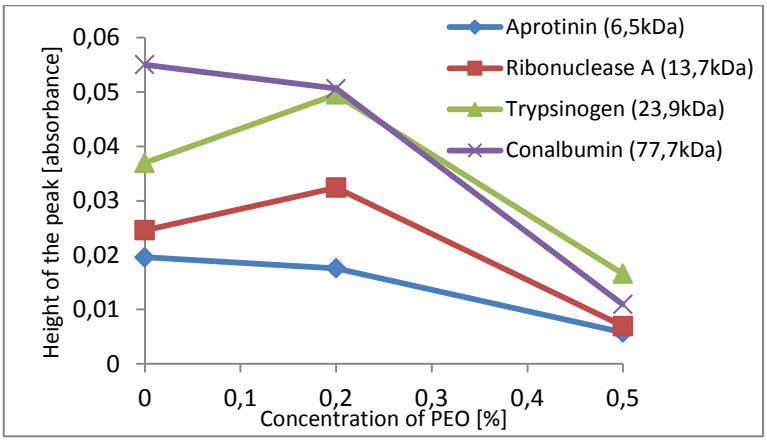


***Figure 8** : Relationship between concentration of PEO and height of the peak. 50µm capillary, 0%, 0,2% or 0,5% of PEO in 250mM phosphate buffer, pH=2.00, 25kV, injection: 0,5psi, 5sec.*

Then the experiments were done in **75µm capillary** with the same concentrations of PEO (50mM phosphate buffer, pH=2,00). If we compare the ionic strength of the buffer we can see that it is 5 times lower than in 50µm capillary. This is due to the increased conductivity in larger diameter capillary and consequently high current. To normalize values of current, ionic strength of the buffer was decreased by diluting the buffer 5 times (from 250mM to 50mM). The proteins used were aprotenin, cytochrome C, ribonuclease A, carbonic anhydrase and conalbumin. As expected, with addition of PEO the migration times of all proteins got longer. In the process of increasing concentration of PEO, there is a loss in sensitivity. With 0,2% PEO the peak's height is lower for average 70% and for 0,5% is lower for 91%. The width of the peaks was decreasing while increasing the concentration of PEO which is a huge advantage. The exception was cytochrome, whose width increased in 0,5% PEO.

PEO had positive effect on separation of proteins in relation to their size as the proteins migrated in their size order which was not the case without polymer. Resolution improved significantly for all adjacent pairs of proteins. Without polymer, resolution is for all proteins below 1,2 which

means that they are poorly separated, just the peak's tops may be resolved but not in the case of all pairs. In 0,5% PEO resolution rises above 1,5 for all pairs except carbonic anhidrase and conalbumin. For them the separation conditions do not provide sufficient separation.

We also tried to increase the concentration of PEO above 0,5% but the solution was too viscous to be successfully injected in capillary.

If we summarize the success of separation with PEO as a polymer, we can conclude that overall PEO has positive impact. Resolution improves nicely between adjacent peaks and proteins migrate accordingly to their size. One of the advantages compared to other polymers is that the peaks are getting narrower, not wider. Also, the time of analysis is short. The only disadvantage is huge loss in sensitivity, but however, this can be overcome by adjusting injection conditions. The concentration offering the best separation is 0,5% and it can be seen in figure 9.

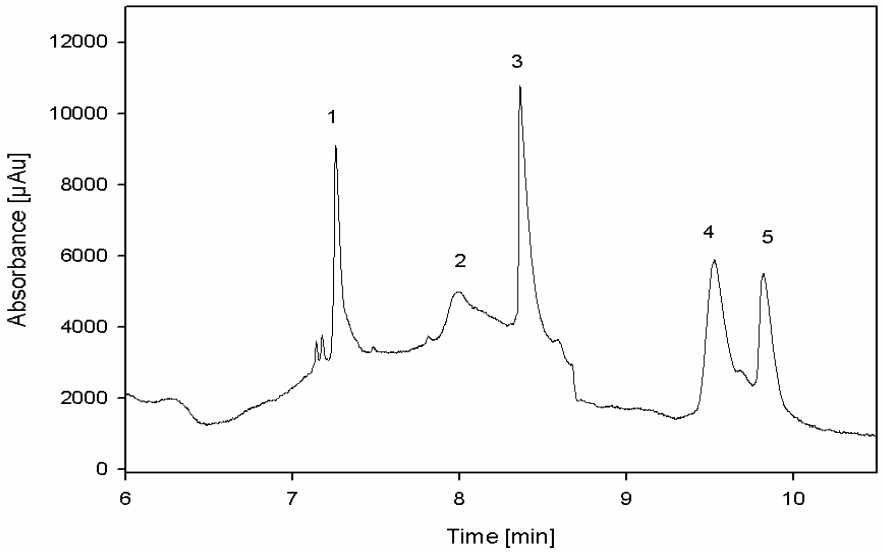
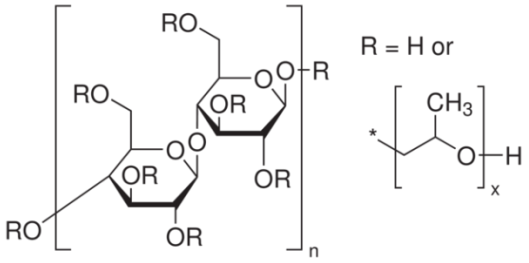


Figure 9*: Separation of proteins in 0,5% PEO, 75µm capillary, 50mM phosphate buffer, pH=2,00, injection: 0,3psi,3sec, 25kV. 1 – aprotinin, 2 – cytochrome C, 3 – ribonuclease A, 4 – carbonic anhydrase, 5 – conalbumin.*

6.2.2 HYDROXYPROPYL CELLULOSE

Hydroxypropyl cellulose (HPC) is a derivate of cellulose. It is ether of cellulose and some hydroxyl group of repeating glucose unit have been hydroxypropylated (Wikipedia). The structure is presented below (Sigma-Aldrich web site).



In the beginning, we started to test HPC in **50µm capillary**. The concentrations of HPC we worked with were 0,5%, 1% and 1,5% (in 250mM phosphate buffer, pH=2,00). Since HPC is a polymer, it brought some changes in separation characteristics compared to separation without polymer. The time of migration increased for each protein, but even with that increase, the analysis can be done in short time (in 1,5% HPC in 15min). In the process of increasing concentration of HPC the height of the peaks, which represent sensitivity, is getting smaller by 66% in 0,5% HPC, by 82% in 1% HPC and by 87% in 1,5% HPC. The peaks width is also increasing but compared to separation with certain other polymer that increase is not so big. In 1,5% HPC width of aprotinin and trypsinogen increased approximately twice, and of ribonuclease and conalbumin around 3 times.

If the effect of HPC is evaluated with resolution, we can say that the separation is improved if we do not take into account ribonuclease A and trypsinogen (discussed in chapter materials and methods). When we calculate the number of theoretical plates, we can see that the number is decreased by adding HPC. However, overall effect must be evaluated and in this case we gain a lot by adding HPC in the field of resolution and successful separation of proteins. On the other hand the loss in sensitivity and efficiency is present but they are not as important since we can try to improve these parameters during further development and most importantly, without HPC separation was not successful.

In 50µm capillary, performing analysis with HPC concentration above 1,5% was not possible due to the strange absorbance profile of baseline (blank) after 12th minute of each run, and the peaks were not detected. The reason for that can be that the buffer with 1,5% HPS is to viscous for 50µm capillary.

Since the results were good in 50µm capillary, the experiments were done also in **75µm capillary**, and the analyses were done with 5 proteins. Polymer was diluted in chosen concentrations (0%, 0,5%, 1%, 1,5%, 2% and 2,5%) in 50mM phosphate buffer (pH=2,00). As in smaller capillary the migration times of proteins increased and also, the loss in sensitivity was present. The effect of polymer on peak's width was not identical for all proteins. For 3 proteins (cytochrome C, conalbumin and carbonic anhydrase), the width was increasing by rising the concentration of HPC.

However, the peaks of aprotinin and ribonuclease were getting narrower up to the concentration of 2% HPC but they got wider with 2,5% HPC. If we compare the noise in different concentrations we can say that HPC has no influence in that area since noise value is more or less the same between all concentrations.

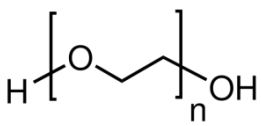
To continue, resolution was successfully improved. Without HPC the proteins are not separated, just resolved and the resolution between adjacent peaks was always below 1,2. In the case of the largest two proteins (anhydrase and conalbumin) without polymer there was only one peak they were even not resolved. The separations are presented in appendix. With adding polymer we managed to separate most proteins (resolution above 1,5). Like in other cases, there were some problems with cytochrome and ribonuclease which did not separate successfully, or even in higher concentrations of HPC they switched and did not migrate accordingly to their sizes (discussed in chapter Material and methods). Also, the resolution between anhydrase and conalbumin was not as high as we would want (only 1,12) but that was mainly due to the increased peak's widths.

It is interesting that cytochrome C and ribonuclease A switched in buffer with HPC but not in buffer with PEO. Possible explanation for this behavior is potential interaction of one protein, probably cytochrome C, which moves slower that it should regarding its size, with HPC and that interaction results in slower migration.

The concentration of HPC that offers the best results is 2%. This concentration provide good sieving effect, proteins are separated (exception of cytochrome C and ribonuclease A) and compared to 2,5% HPC broadening is not as obvious and also, sensitivity is better. Also, efficiency expressed as a number of theoretical plates is the best in 2% HPC for most proteins.

6.2.3 POLYETHYLENE GLYCOL

Polyethylene glycol (PEG) is a linear polyether substance. Two different kinds of PEG were used in our experiments and both sets of experiments were carried out in 50µm capillary. The structure of PEG is the same as PEO, but it has lower molecular weight (Sigma Aldrich web site).



The experiments with the **first PEG** (Mw=35 000) were done in three different concentrations of PEG, 15%, 20% and 25%. With increasing the concentration of PEG the migration time of each

protein increased, due to the sieving effect of polymer. Compared to the analysis with other polymers, migration times really increased a lot, from less than 10 minutes without polymer to almost 60 minutes with 25% PEG. With addition of polymer loss in sensitivity was present. Height of the peaks was smaller for 46%, 78% and 84% in 15%, 20% and 25% PEG respectively as we can see in figure X. Also, with increasing concentration of polymer band broadening occurred and peaks got wider. Especially the peaks broadened during the analysis with 25% PEG, where aprotinin gets wider 9 times but the broadening was even more severe with protein with higher molecular weight, such as conalbumin who got wider 21 times. Effects of PEG on migration time and width of the peaks can be seen in the figure 10 and on height of the peaks in figure 11.

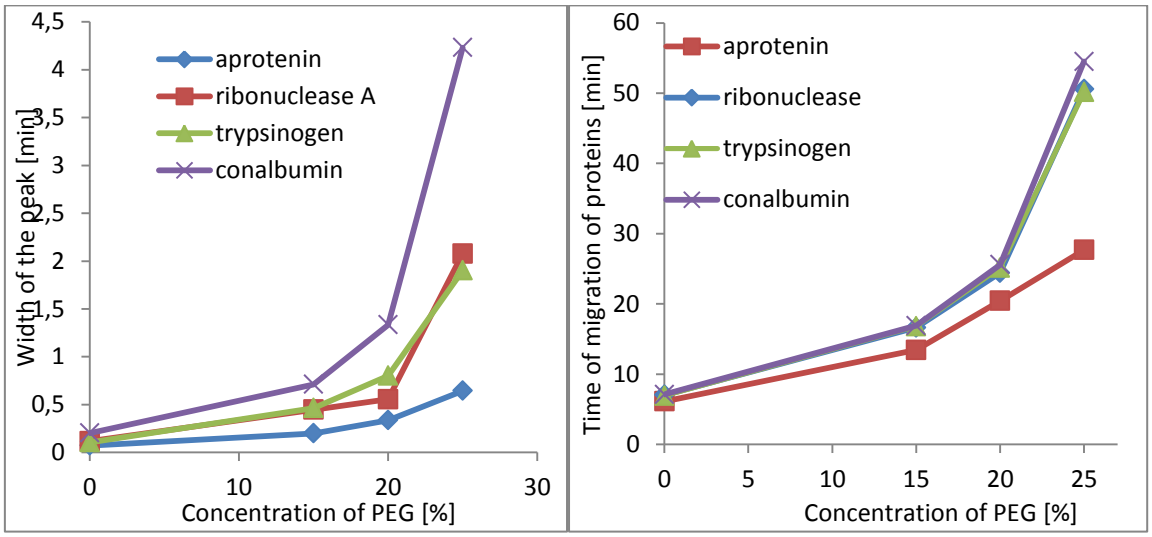


***Figure 10** : In graph on the left we can see migration time as a function of PEG concentration. In graph on the right, we can see change in width of the peaks as a function of PEG concentration. 15, 20 and 25% PEG in 250mM phosphate buffer, pH=2,00, 50µm capillary, injection 0,5psi for 5sec, separation voltage is 30kV. Proteins are diluted in water in concentration 10-4M.*

The resolution between peaks did not improve with increasing concentration of PEG. One pair of adjacent peaks had been already well separated without polymer (resolution above 1,5); this pair was aprotinin and trypsinogen. Raising resolution above that value does not make much sense, since the separation had been already good. On the other hand, other pairs had not been separated at all and with adding polymer resolution did not improve. There was an exception with pair trypsinogen and conalbumin at 15% PEG where resolution improved, but in higher concentration of PEG it was again reduced (less than 1). The main problem is that the peaks got a lot wider and since resolution is inversely proportional to peak's width, it did not improve. Other parameter influencing resolution and selectivity, the time of migration is also problematic. Bigger three proteins, and especially trypsinogen and ribonuclease A did not differ much in their

migration times like expected with addition of polymer. Efficiency of separation, evaluated with the number of theoretical plates, decreased with increasing concentration of polymer. The main factor affecting this parameter was very increased width of the peaks.

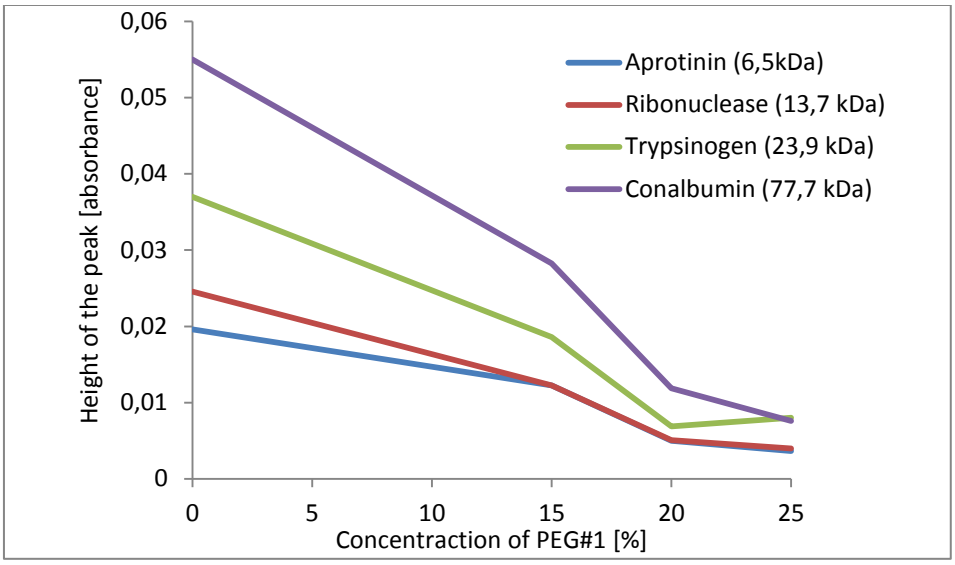


***Figure 11** : Change in height of the peak in a relationship with concentration of PEG#1. 15, 20 and 25% PEG in 250mM phosphate buffer, pH=2,00, 50µm capillary, injection 0,5psi for 5sec, separation voltage is 30kV. Proteins are diluted in water in concentration 10-4M*

In conclusion, the first PEG does not offer satisfactory results. Addition of PEG#1 does not improve separation between proteins as expected. Even though time of migration increased a lot, the difference between migration times of different proteins is still not big enough to provide successful separation. The main factor contributing to unsuccessful separation and also to inferior efficiency of the method is broadening of the peaks. Another disadvantage of PEG#1 is also loss in sensitivity. Furthermore, analysis time is rather long compared to analysis with other polymers. All these effects combined result in poor separation which leads to big setback of PEG#1 as a sieving matrix in the development of our method.

The **second PEG** (Mw=1900-2200) was tested at concentrations 5 and 10%. During analysis, when we added PEG to the buffer solution we observed very unstable current and unstable baseline, especially with 10% PEG. Another problem we had was inability to detect peaks of proteins at 10% PEG. The reason for these problems can lay in potential inhomogenity of the separation medium or in the non-constant interactions between polymer and capillary wall (Krizek, 2010). Also, formation of some kind of blockage in the capillary can be possible cause of unstable current.

During the experiments with 5% PEG#2, compared to the experiments without polymer, the migration times increased, as expected. The loss in sensitivity was on average 74,5% and the peaks got a lot wider, especially with the proteins with longer migration times. For example, in the case of carbonic anhidrase, the peak got wider for 5,75 times. If we assess the separation with resolution, we can say that it is improved with addition of 5% PEG. The efficiency expressed in the number of theoretical plates is improved with some proteins, but decreased with others and in the end there is no overall significant change in that parameter.

With analyses of 10% PEG#2 the problems with unstable current and baseline were severe and since the analysis could not be performed under so unstable conditions we decided to try to overcome these problems by applying pressure on the inlet end of the capillary during the analysis. (Krizek, 2010) With applying pressure of 1,2psi during the separation with 10% PEG#2 there was huge improvement in stability of current and baseline. With lower pressure applied, stability was not accomplished, but we managed to get a peak with 0,2psi which was not the case without pressure. While searching for optimal pressure value we observed the impact of pressure on parameters of migration (these experiments were done with aprotinin). The migration time of the aprotinin was reduced while increasing pressure applied as it is presented in figure 12. Height did not change significantly with different pressure adjustments whereas peaks got wider with increasing pressure.

During the use of 10% PEG#2, we got the results, peaks of the proteins, only when we applied pressure and so it is difficult to separate the effect of PEG on separation from the effect of pressure. As far as migration time, we would expect that increasing the concentration of polymer increases it, and this can be confirmed by longer migration times when only small amount of pressure is applied (Figure X). However, the pressure surpasses the effect of polymer and with 1,2psi pressure the times of migration are similar with 5% PEG. On one of the most important parameters, the height of the peak, applied pressure does not have much impact on, but polymer does as the height of the peak is reduced 10 times compared to the 5% PEG and 22 times compared to analysis without PEG. This is a huge loss and the sensitivity is very low. Number of theoretical plates decreased significantly with 10% PEG.

Both, the pressure as well as polymer have the same impact on broadening the peaks. Band broadening can be explained by change in flow profile when the pressure is applied. Normally in CE, the velocity of liquid is almost uniform across the capillary and this is called plug flow. In pressure driven systems such as HPLC, the velocity is not uniform across the diameter of the tube,

it is bigger in the middle of the tube than near the wall, and this is laminar or parabolic flow. The plug flow is one of the reasons for high efficiency of CE since it greatly reduces band broadening and with applying pressure during the separation we lose that property (Petersen et al, 2001).

Aprotinin peaks showed a tendency to get wider when pressure is applied (with 1,5psi it was wider almost 2 times compared to 0,2psi, when the first peak was seen), but they got wider also with 5% PEG. So, broadening of the peaks results from both, added polymer and applied pressure. To say for sure what was the main cause of excessive broadening, especially in case of the biggest two proteins, further analysis should be done.

If we evaluate the separation efficiency with the number of theoretical plates we can see that with increasing pressure the efficiency decreases. The number of theoretical plates proportional to the time of migration and inversely proportional to the width of the peaks the loss in efficiency makes sense since the time of migration is shorter and the width did not change dramatically.

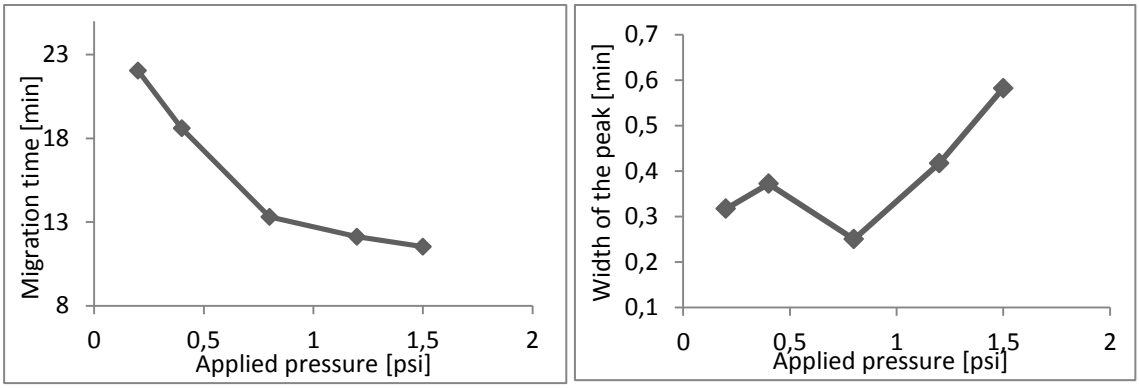
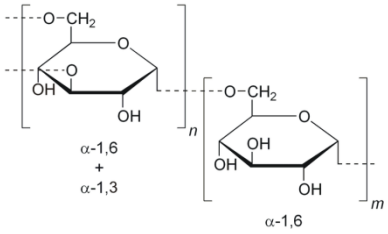


***Figure 12**: Influence of increasing pressure on the inlet vial during the separation on time of migration and on width of the peak of aprotinin.10% PEG #2 in 50mM phosphate buffer pH=2,00, 50µm capillary, injection 0,5psi, 5 seconds, separation voltage is 30kV. Applied pressure varies from 0,2psi to 1,5 psi.*

If we do an overall review of influence of pressure on separation, there are positive and negative characteristics but increased pressure in a combination with PEG did not bring prospective results. Applied pressure enabled the analysis which was not possible otherwise due to the unstable current and baseline. However, severe band broadening of the peaks and low sensitivity do not allow satisfactory separation. The main problem is polymer itself since the largest negative effect of applied pressure is contribution to band broadening and it does not affect sensitivity.

6.2.4 DEXTRAN

Dextran is a polysaharide with linear backbone of α-linked d-glucopyranosyl repeating units (Sigma-Aldrich).



One of the polymers we tried to test was also dextran but unfortunately we could not perform separation with that polymer due to its characteristics. Firstly, we worked with 5% dextran in acidic phosphate buffer (250mM), but there were several problems. One of them was high current and we had to apply low voltage – 10kV to obtain current around 60µA and that prolonged the time of analysis. Another problem was the noise present and we were unable to detect proteins. We managed to eliminate the noise by filtering and sonicating dextran buffer but even with that step there was no peak of proteins. While searching for reason that there are no peaks, we tested the solubility of proteins in 1% dextran buffer and we found out that proteins are not soluble in acidic pH dextran solution. After injection they precipitate when they encounter the separation buffer which contains dextran and consequently there is no successful migration, separation or detection.

We later decided to perform analysis with dextran in tris buffer with pH=6,8. First, we checked if the proteins are soluble in these conditions. Aprotinin, which was tested, dissolved in dextran buffer solution after mixing it by vortex for a while. Also, we had to dilute tris buffer to 12,5mM due to the high conductivity of buffer when dextran is added. Despite that, there were no peaks of proteins even when really small concentrations of dextran were used. The smallest concentration we tested was $12,5*10^{-5}$% dextran and even with that there were no peaks. We also performed analysis of neutral marker (benzyl alcohol) in dextran where we detected a peak.

It is unexpected that we were unable to detect protein peaks since the marker was detected, the proteins are soluble and really low concentration of dextran was used. Even more, there are several publications where dextran has been used as a successful polymer (Weinbereger, 1993; Takagi, 1997). The reason for our results may be that dextran we used was sulfated. Sulfate groups may interact with proteins and a complex between dextran and proteins may be formed.

Dextran was the last polymer we tested and in table 6 we can see comparison of some parameters of separation in potential usable buffer solution for further analysis.

***Table 6**: Comparison of 4 different parameters (time of migration, width, height and efficiency) of 5 proteins in different separation buffers. Buffer – 50mM phosphate buffer, pH=2,00; 0,5% PEO, 2%HPC were also dissolved in 50mM phosphate buffer, pH=2,00 (75µm capillary, HD injection (0,3psi, 3sec), 25kV). PEG#2 was tested in 50µm capillary (250mM phosphate buffer, pH=2,00, injection: 0,5psi, 5sec). Efficiency is expressed as N – number of theoretical plates.*

Protein	Polymer	time [min]	height [absorbance]	width [min]	N
Aprotenin (6,5kDa)	*buffer*	*4,907*	*0,083306*	*0,452*	*1885,7*
	0,5% PEO	*7,276*	*0,006873*	*0,196*	*22049,2*
	2% HPC	*8,265*	*0,021346*	*0,176*	*35284,2*
	5% PEG #2	*11,776*	*0,053331*	*0,407*	*2173,9*
Cytochrome C (11,8kDa)	*buffer*	*4,914*	*0,070087*	*0,496*	*1570,5*
	0,5% PEO	*7,941*	*0,004051*	*0,634*	*2510,1*
	2% HPC	*10,102*	*0,013974*	*0,584*	*4787,5*
	5% PEG #2	*18,465*	*0,016289*	*1,049*	*1059,7*
Ribonuclease A (16,5kDa)	*buffer*	*5,041*	*0,151036*	*0,63*	*1024,4*
	0,5% PEO	*8,396*	*0,005888*	*0,303*	*12285,1*
	2% HPC	*9,652*	*0,015806*	*0,195*	*39199,9*
	5% PEG #2	*16,388*	*0,032415*	*0,87*	*2277,2*
Carbonic anhydrase (29,1kDa)	*buffer*	*5,683*	*0,051696*	*0,553*	*1689,8*
	0,5% PEO	*9,532*	*0,004525*	*0,36*	*11217,2*
	2% HPC	*14,211*	*0,007325*	*1,244*	*2087,99*
	5% PEG #2	*24,223*	*0,013401*	*2,147*	*401,4*
Conalbumin (77,8kDa)	*buffer*	*5,622*	*0,082024*	*0,506*	*1975,2*
	0,5% PEO	*9,659*	*0,014407*	*0,504*	*5876,6*
	2% HPC	*15,815*	*0,008616*	*1,206*	*2751,5*
	5% PEG #2	*25,73*	*0,024213*	*1,603*	*347,8*

6.3 COATED CAPILLARY

Coating of the capillary is a possible solution to eliminate unwanted interactions between proteins and capillary wall which lead to band broadening. We used polyacrylamide coating and the goal was to provide stable surface which could reduce protein–wall interactions and decrease EOF (Weinberger, 1993). While testing effectiveness of capillary coating, Tris buffer (25mM, pH=6,8) was used and later on we wished to test behavior of coating in acidic phosphate buffer with polymers.

Our coating successfully decreased EOF. In Tris buffer and uncoated capillary, the EOF was $2{,}634*10^{-4}$ cm^2/Vs and in coated capillary it was decreased to $8{,}721*10^{-5}$ cm^2/Vs. If we take a look in protein separation we can say that it was improved. Firstly, the height of the peaks was enlarged for all proteins. Also, the peaks became narrower. Since the volume of injection was the same for coated and uncoated capillary it is from this data apparent that the retention of proteins on the capillary wall was eliminated and the peaks got higher and narrower. In table 7 we can compare migration properties between coated and un-coated capillary.

The effect on coating on migration time was unclear. It was increased for aprotinin, ribonuclease A and conalbumin, but decreased for cytochrome C and carbonic anhydrase. The resolution is not good because all proteins are detected in very short time. With that being said, we can say that coating of the capillary offers high sensitivity, narrow peaks and it has good potential for improving separations. However, there is one inconvenient and unwanted characteristic of polyacrylamide coating and this is its instability. It is very unstable in high pH, so during the use of this coating we eliminated wash with NaOH. Even with that precaution step after few days our coated capillary did not perform as it should. The current was very unstable and there were problems with peak detection. It is possible that the coating *cracks* and it starts to migrate itself and it affects detection and current. It is difficult to work with unstable method so we removed the coating.

***Table 7**: Difference in protein behavior between coated and coating-free capillary.*

	TRIS BUFFER			COATED CAPILLARY		
	time	height	width	time	height	width
Aprotinin (6,5kDa)	4,043	0,028492	0,687	8,403	0,104395	0,368
Cytochrome C (11,8kDa)	6,065	0,091805	0,62	6,575	0,123926	0,205
Ribonuclease A (16,5kDaa)	7,639	0,026741	1,557	8,351	0,194099	0,497
Carbonic anhydrae (29,1kDa)	10,149	0,018278	1,612	8,909	0,096508	0,695
Conalbumine (77,8kDa)	8,438	0,060606	0,97	8,536	0,152655	0,593

To summarize, polyacrylamide coating offers good solution for protein-wall interaction but it is not possible to work with due to its instability. It is possible that stability can be improved with other coating protocol and it could provide good separations and it would be interesting to see the separation with polymer in coated capillary.

6.4 TRIS BUFFER

The reason we used tris buffer was application of coated capillary and after that we performed a set of other experiments with this buffer.

Firstly we would like to compare separation in tris buffer to separations in acidic phosphate buffer. We can see that under the same injection conditions the sensitivity is deteriorated, on average the peaks were lower for almost 60%, an exception here was the peak of cytochrome C, which was higher for 30% than in phosphate buffer. Also, we could see that peaks were wider for 1,2 to 3 times, depending on the protein. *Broadening was not symmetrical*, in tris buffer, tailing of the peaks was very obvious. Migration times of proteins were very different from the times previously found in phosphate buffer, with carbonic anhydrase being detected the last, 2 minutes after conalbumin. When a mix of 5 proteins was analyzed, the times of migration changed a lot regarding the times when proteins were injected alone, so we cannot say with certainty to which peak certain protein belongs. Another confirmation of irreproducible separation is calculated relative standard deviation of marker (benzyl alcohol) which is 17,9%. Besides, peaks were not resolved and resolution was not good.

During performing analysis with tris buffer we noticed some unexpected changes. After using the same solution for several runs, there was a big change in behavior of proteins. As has been already mentioned, the migration times of protein changed, and when the mix of proteins was used, (11^{th} run) times were completely different as the times of individual analysis. Furthermore, the height of the peaks was increased dramatically. For instance, in 10th run height of aprotinin was 6,8 times bigger than in the first run. Also, the width of the peak was reduced for 55%. For other proteins, effect on height was the same as on aprotinin as we can see in figure 13. On the contrary, effect on peaks width was not so obvious because in some cases it was reduced significantly, but in some it stayed the same in all analysis.

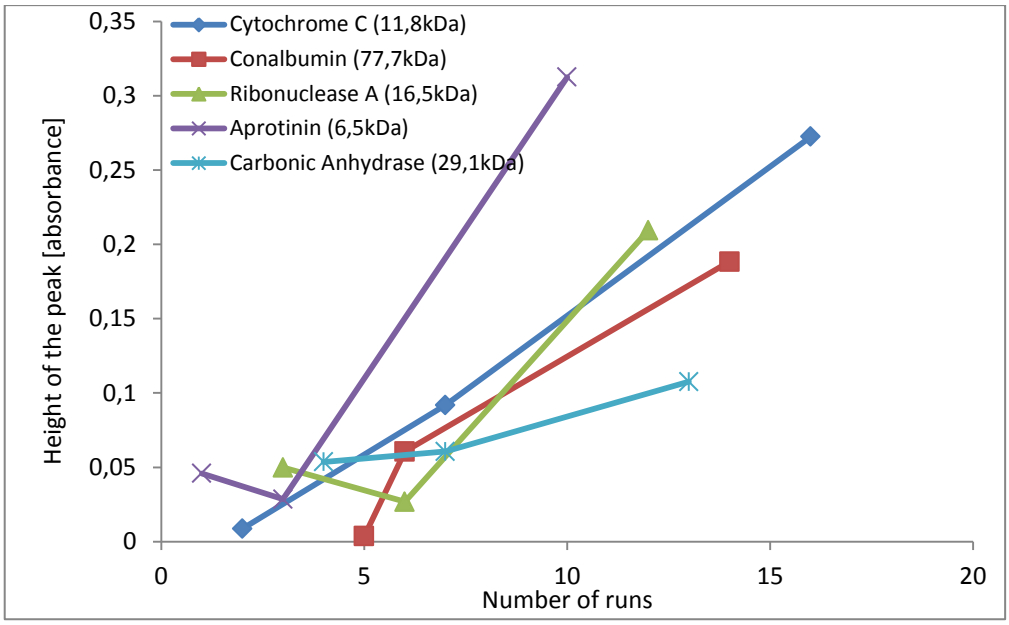


***Figure 13**: Effect of number of runs performed with the same buffer solution on height of the peak for all 5 proteins. Tris buffer, 25mM, pH=6,8, 30kV, 0,5psi, 3sec, 75µm capillary.*

There was another phenomenon we observed while implementing analysis with tris buffer. We worked with 25mM buffer who provided good conditions for application of high voltage. When voltage was 30kV, current was 56,1µA and there was a linearity of Ohm's law (R^2=0,9998). However, during each run, we could see that current was not flat, there was an upwards drift. That change was not so big during one run, around 3µA, but with each following the current got higher.

If we firstly address to the behavior of proteins in fresh buffer, where a lot peaks with excessive tailing were present, a lot of issues can be explained with effect of pH. The pH of the buffer, as had been already mentioned, has big impact on separation. One of the first things it has an effect on is charge of the proteins. To ensure that protein is positively charged, pH should be for at least two units lower than isoelectric point which does not happen in the case of carbonic anhydrase (pH> pI), insulin (analysis of insulin aggregates, pH>pI) and conalbumin (pH≈pI). That means that carbonic anhydrase migrates to the cathode because of electroosmotic flow since its net charge is positive. In the case of conalbumin, pH is close to pI, so the positive charge is not completely eliminated. This can explain migration times, especially why carbonic anhydrase is detected the last. Wider peaks can be explained with the presence of EOF which is higher than in phosphate buffer. Almost neutral pH of this buffer also influences ionization of silanol groups of the capillary wall, which offers possibility for electrostatic interactions between proteins and wall which can be cause of tailing (constant adsorption - desorption process) .

To continue, increase in current with each analysis is unusual. Ohm's law equation shows us that current is proportional to applied voltage and inversely proportional to resistance of the fluid medium. So, drift in current can be caused to increased voltage or decrease in resistance. Since voltage was stable the cause for change in current must lie in change of resistance. One possible reason is not properly working thermostating system of the CE and the capillary heats up and resistance drops. However, if there was heating inside the capillary, there would be contrary effect on shape of the peaks – they would get wider, which was not the case. Also, that behavior was noticed only with tris buffer, with others separation buffers the results were constant and there was no change observed with larger multiplicity of runs with the same buffer. So all that brings us to think that the reason for this unusual behavior must be linked to the tris buffer. Possibility is that there is change in buffer composition. Applied voltage may trigger some chemical changes of buffer components, probably some red-ox reaction. Since otherwise we were working with acidic pH buffer there is a question if that change is due to the buffer itself or to the pH of the buffer. However, since buffer with pH close to neutral are often used in CE, it makes more sense that the reason lies in tris buffer, not pH.

To summarize, if we look just into separations when buffer has been already used for several runs (7-10), we can say that this separation is quite good, with really good sensitivity. However, there are many other characteristics which present an obstacle when tris buffer is used. These obstacles are very bad reproducibility in migration times, ascending current and bad separation when buffer has not been already used for several runs. Another disadvantage is pH of the buffer itself due to changed net charge in some proteins and increased EOF and the consequences that follow.

6.5 ADDITIVES

During the development of CE we tested two additives, polyvinyl alcohol (PVA) and methyl cellulose (MC), and with that attempted to improve separation. Additives are used to influence different parameters. Our goal was to decrease EOF and reduce protein-wall interactions and with that influence width of the peaks. (Weinberger, 1993)

6.5.1 MC AND PVA IN TRIS BUFFER

The additives were firstly tested in tris buffer. At first glance, the results we got with 0,05% MC were very promising. The first data we got showed us incredible positive effect of MC on peak's height and width. Height was increased almost 8 times for aprotinin and ribonuclease, 6 times for conalbumin, 4,5 times for carbonic anhydrase and 2,3 times for cytochrome C. Width of the peaks

was reduced for more than 50% for all protein (on average for 63,5%). MC also prolonged migration time, which was expected due to its influence on EOF.

However, during performance of our experiments in tris buffer we came across that there must be some change in buffer itself which influences migration and separation of proteins. With that information in mind the firstly promising results were reevaluated. The results are nicely presented in figure X, where we can see that increase in height of the peaks depends on the number of runs already made with the same buffer. Furthermore, buffer alone brings better results like buffer with added MC. The same phenomenon we can observe on Figure 14, where width of the peak is decreased dependently on the number of runs and presence of MC does not have big impact.

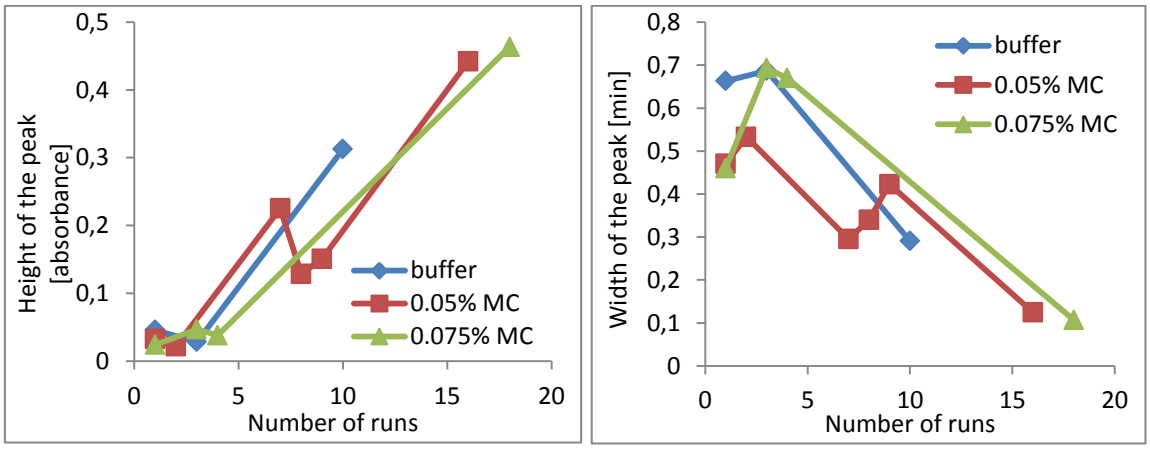


***Figure 14** : On the left we can see height of the peak in a relation with number of runs and on the right relationship between width of the peak and number of runs with the same buffer solution. The protein tested was aprotinin, in 25mM Tris buffer pH=6,8, 30kV, injection 0,5psi, 3sec, 75µm capillary.*

Regarding the literature it is expected that PVA dynamic coating doesn't work in neutral pH because adsorption of polymer is too weak (the surface is not enough negatively charged) and we decided to test it at acidic pH (Gilges et al, 1994).

6.5.2 MC AND PVA IN PHOSPHATE BUFFER

Whereas it can be explained why PVA didn't perform as expected in neutral pH buffer, we tested its effect on separation also in acidic phosphate buffer. Effect of MC was also tested in a combination with polymers.

MC affected time of migration which was a bit longer than in separation in phosphate buffer without additives. Influence on height of the peaks was not uniform, it was increased for two proteins (carbonic anhydrase and conalbumin) but decreased for other three (aprotinin,

ribonuclease A, cytochrome C). The biggest change was in the case of cytochrome C and ribonuclease A, where height decreased for 70% and 58% respectively. With other three proteins change in either direction was less than 32%. Effect of MC on width of the peaks was not very big. We can see that there was a slight increase in width of all proteins except aprotinin.

With PVA as an additive to phosphate buffer the results were pretty similar to the results with MC. Times of migration were prolonged (from 1 to 1,5minute). Sensitivity was degraded with height of the peaks getting smaller for more than 50% in the case of ribonuclease A, conalbumin and carbonic anhydrase and for 12% in the case of aprotinin. The exception was cytochrome whose peak got higher for 54%. The effect of PVA on width of the peaks was uniform, all peaks got wider.

MC was also added to 1% HPC and 0,5% PEO buffers. We evaluated influence of MC as an additive to buffer with polymer – the data was compared to buffer with the same concentration of polymer. In 1% HPC with 0,05%MC, MC did not influence much time of migration, it stayed almost the same for smaller three proteins and increased a little with larger two. With all 5 proteins we observed decrease in peak's height, on average for 52%. Width of the peaks stayed the same for 3 proteins, and increased in the case of carbonic anhydrase and ribonuclease A. If the resolution between 1% HPC with and without 0,05%MC is compared, we can see that without MC there are 5 protein peaks (even though they were not baseline resolved) but when MC was added there were only 4 peaks and so, MC did not improve separation of proteins.

Similar behavior of proteins was noticed in 0,5% PEO with 0,05% MC. There was an unexpected decrease in migration time of all proteins. Time of migration was shortened up to 1,7 minute. Change in height of the peaks is very diverse. Peaks of two proteins, aprotinin and ribonuclease A, increased in height approximately 3 times. On the other hand, height of carbonic anhydrase and cytochrome C changed minimally and height of conalbumin was lower for 50%. Width of the peaks stayed the same for 4 proteins; an exception was cytochrome C whose peak got wider for more than 2 times. The effect on resolution was negative since we detected only 3 protein peaks when a mix of proteins was injected, while without MC there was 5 peaks detected.

The results we have got with MC and PVA as an additive were not what we had expected. We expected positive effect especially on peak broadening. As a contrary of our expectations the peaks width did not change a lot. Lose in resolution and uneven effects on peak's height bring undesired properties of separation. Because there was no big positive influence on separation we decided to not include an additive to separation buffer. The possible reason for poor outcome of

separations with additives may be rinse with NaOH. After rinse with NaOH, rinse with water and buffer followed but it is possible that all effect of NaOH was not neutralized. The basic pH makes silanol groups uncharged and potentially if hydroxylic polymer cannot adsorb (Gilges et al, 1994). To confirm this hypothesis it would make sense to prolong rinse with water and buffer, or to eliminate rinse with NaOH. If we eliminate rinse with NaOH it is not sure that all remains from previous runs are eliminated. Another possible solution is to include rise with HCl (NaOH-water-HCl-water-buffer) where capillary surface is certainly regenerated and also, effect of NaOH is eliminated.

6.6 COMMON ISSUES

- **Loss in sensitivity**

One of the main undesired events during the use of polymers was loss in sensitivity. With all polymers there was a huge loss in sensitivity. One of the reasons for that is definitely smaller volume injected due to the increased viscosity of separation medium when polymer is added (discussed in chapter Materials and methods). Other possible reason is absorbance of polymer. In this case background obscures the absorbance of proteins.

What contributes to lower peaks is also migration of protein. When polymer is added, often the peak gets wider. Polymer presents a sieving network for protein and injected proteins reach the detector during longer period of time. That means that the same amount of protein is detected during longer period (broadening) of time and that the highest concentration that reaches the detector is lower. That can be also explained by longer injection plug length due to the viscosity of the buffer. If the concentration is smaller than consequently means that signal – absorbance is also smaller which can be confirmed with Beer- Lambert law: $A = c * l * \varepsilon$.

- **Band broadening**

Band broadening was one of the most common undesirable events during our experiments. It has huge effect on separation efficiency and resolution between proteins and since our goal was to have good resolution between peaks and high efficient method, we wanted to find conditions where band broadening is suppressed or at least not too evident.

In theory, the peak broadening is mainly caused by molecular diffusion of the solute along the capillary. (Eu. Phr.) But in practice there are several other factors that may affect this phenomenon. One of them is interaction between proteins and capillary wall. One of the ways that protein can bind to the capillary inner surface is hydrophobic interaction. They occur

between epoxide parts of the capillary surface and hydrophobic regions of the protein. Other potential interaction is electrostatic interaction. At acidic pH proteins are positively charged (pH is below pI) and they can interact with negatively charged silanol groups of the wall. But in our case, with lowering pH below 3 the surface was not negatively charged and these interactions are not possible. However there are still some ionized silanol groups that can react with proteins, but large part of them is eliminated. If with low pH electrostatic interactions are minimized, hydrophobic are not and they can affect band broadening. Absorption – desorption process between proteins and capillary wall is constantly present and it affects peak's width. In some cases it can also result in peak's tailing.

Another possible factors affecting band broadening is injection of too much sample. Volume of injection affects injection plug length which affects width of the peak. Another potential reason is formation of hydrostatic flow which can be caused by Joule heating. This can happen if the separation buffer is very conductive and as a result the current is very high and more heat is produced. If that heat cannot be removed the internal temperature rises. This leads to laminar flow because the temperature in the middle of the capillary is higher than on capillary wall. However, this is avoided by performing Ohm's law plot with each used buffer solution. We apply voltages from 5 to 30kV measure current and check if there is a linear curve. If the chosen voltage lies in linear range Joule heating is avoided. Since this procedure was performed with our experiments we can say that peaks did not broad due to the Joule heating.

One of the reasons for band broadening can be protein itself. It is possible that proteins are not completely pure, there may be some impurities present and also there can be different form of protein present. Different forms do not necessary move with the same velocity and these results either in presence of more peaks of the same protein (if the difference in velocity is big) either in wider peak.

At this point it can be also added that all proteins did not always behaved the same. Often we saw that there was a difference between aprotinin and ribonuclease and other three proteins. Peaks of the first two proteins did not broaden as much as with other three. One of the reasons for that may be the purity of protein, it is possible that aprotinin and ribonuclease were purer and the composition of powder was uniform – there was just one form of protein. Other possible reason may be the structure of proteins. If the protein has more hydrophobic regions, it can interact with the wall more which results in wider peaks.

The proteins we used were prepared in concentration 10mg/ml. In the beginning they were diluted to reach concentration 10^{-4}M but with use of polymers, when sensitivity was worsened, undiluted proteins were used. With that procedure stacking effect was reduced. Moreover, there was a similar effect when buffer was diluted from 250mM to 50mM. Higher concentration of buffer decrease adsorption of proteins on the capillary wall and in wider capillary this effect is reduced (Weinberger, 1993).

To conclude, there are many factors that all affect broadening of the peaks. With our conditions some should be reduced (effect of EOF, Joule heating, electrostatic adsorption), but others are still present or may be even more expressed, especially in 75µm capillary. Broadening is the sum of all potential factors and it is more obvious in some conditions than in others.

- **Effect of capillary diameter (migration time, buffer)**

Capillary diameter has big impact on several parameters of separation and changing capillary diameter for just a little can make big change in analysis conditions and results. Firstly, we had to change concentration of buffer. 250mM buffer that we were using in 50µm capillary was too conductive in 75µm capillary since it produced higher current while applying the same voltage (60µA in 50µm and 130µA in 75µm capillary at 25kV). To get in the range of acceptable voltage-current relationship (voltage 25-30kV and current up to 60µA), we diluted the buffer 5 times, to 50mM. With that action we eliminated otherwise potential negative effect of Joule heating which would be a consequence of high current (Weinberger, 1993).

The second modification we had to make was adjusting conditions of injection which has already been discussed in… Also, we modified wash conditions of the capillary, because the volume of the capillary is replaced faster in wider capillary. Another thing we noticed was shortening of migration time for each protein. Since velocity of the solutes depends on electric field strength one of the reasons for shorter time is increased voltage from 25kV to 30kV. Furthermore, with diluting the buffer we increased EOF who also influences migration of proteins (Weinberger, 1993).

Experiments with PEO and HPC were done in both capillaries. The main advantage of 75µm capillary is ability to use buffer with higher viscosity. Also, the migration of proteins is quicker and that shortens the time of analysis. On the negative side, there is a slight widening of the peaks present in 75µm capillary. In our research in 50µm capillary only a small part of experiments were performed and only 4 proteins were used. Looking back on results it would be interesting to test 5

proteins also in this capillary mainly because of smaller broadening of the peaks and consequently higher efficiency.

6.7 THE SELECTION OF BEST CONDITIONS FOR MODEL PROTEIN SEPARATION

The final part of our work was to select the best conditions for separation from all different possibilities that have been tested. The goal was to find high efficient method, with good sensitivity, efficiency and most importantly, good separation between proteins. The main purpose of the study was to transfer selected conditions to analysis of aggregates of biomarker proteins and later on to use them for diagnosis of neurodegenerative disease. Overlooking all results we acquired during our experiments as the best condition was chosen 0,5% PEO in 50mM phosphate buffer in 75µm capillary. Good results were also obtained with 2% HPC, but nevertheless, 0,5% PEO offers better results. On the side of advantages of PEO is definitely absence of band broadening which as a result provide narrow peaks. Also, sieving effect of this polymer is the best, with proteins being nicely detected according to their size. Furthermore, analysis time is short, out test proteins were detected in less than 12 minutes, so we can predict short separation of aggregates. Noise level was the same like with other proteins. On the negative side, there is enormous loss in sensitivity with addition of PEO. Despite of this shortcoming of PEO, separation in PEO is much better on all levels compared to other polymers and its influence on separation can be seen in figure 15.

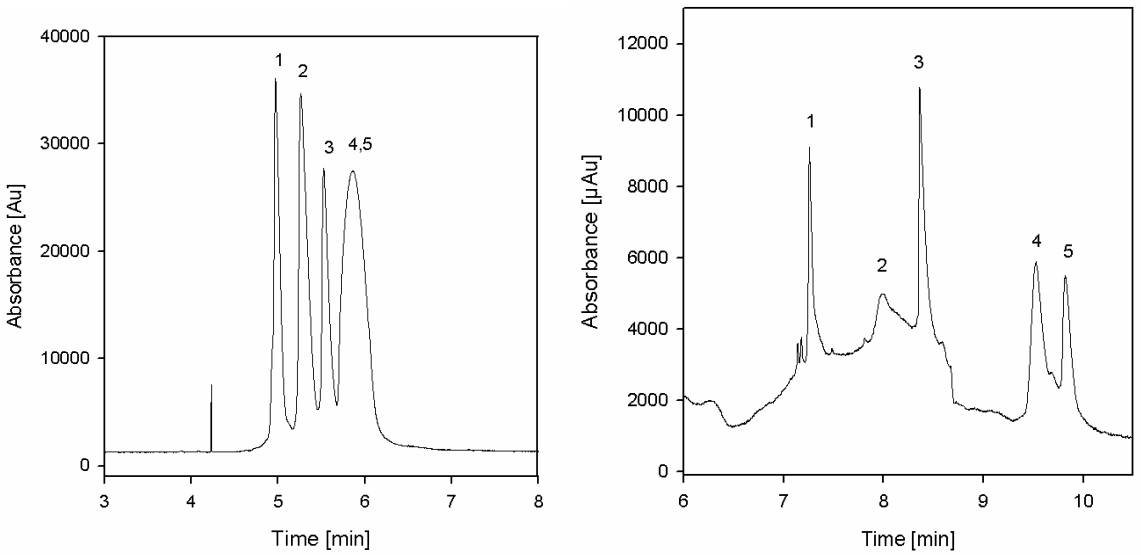


Figure 15: *Separation of proteins in phosphate buffer without polymer on the left and in 0,5% PEO on right figure. 75µm capillary, 50mM phosphate buffer, pH=2,00, injection: 0,3psi,3sec, 25kV. 1 – aprotinin, 2 – cytochrome C, 3 – ribonuclease A, 4 – carbonic anhydrase, 5 – conalbumin.*

Close to 0,5% PEO came 2% HPC (also some of the aggregates were tested with HPC) who offers compared to PEO much better sensitivity. For that reason, insulin aggregates were tested also in this buffer. Effect of HPC on model protein separation is presented in appendix.

Even though we tested impact of two additives on separation, none additive was chosen to be *part of the best conditions*. Unfortunately, additives did not behave as we expected and hoped; they did not influence much the separation or even worsened it.

6.8 AGGREGATES

After we have chosen the best conditions, we wanted to test behavior of aggregates in them. Since amyloid aggregates are harder to access, we evaluated success of separation with insulin aggregates and in the future transfer it on amyloid aggregates. Insulin aggregates was prepared either in acetic acid either in phosphate saline buffer (protocol of preparation based on articles: Amdursky et al, 2012; Brange et al, 1997 and Nielsen et al, 2001). When analyzing them with CE-UV we came across different obstacles. We started the analysis with aggregates prepared in acetic acid but we did not manage to get any peaks at 214nm (50mM phosphate buffer, 75µm). The reason for this is high absorbance of acetic acid which did not allow to detect potential peaks of aggregates. We performed ultrafiltration (30kDa cut-off) and with that action changed matrix in which aggregates were dissolved from high absorbing acetic acid to water. After the matrix was successfully changed, we started another set of analysis of insulin aggregates dissolved in water.

There was another problem with insolubility of aggregates in water. Firstly they sank in the bottom of the tube and secondly, they formed a firm cluster which could not be dissolved by vortexing or sonicating. We decided to remove the water (aggregates were on the bottom) and replace it with phosphate buffer (50mM, pH=2,00) and with that improve solubility (presence of ions, same pH as with acetic acid). However, the aggregates were still not dissolved (the solution was also vortexed and sonicated), but we managed to dissolving aggregates with sonication inside the vial. On the electropherograms of this sample we could see fairly large number of very high spikes (50mM phosphate buffer). Moreover that the shape of the peaks was quite unusual for protein peaks, with each analysis, the results were not identical. The number and position of peaks was changing both with EK and HD injection and we could not be sure that there are some aggregates present or not. When separation was performed in buffer with 0,5% PEO or 2% HPC (50mM phosphate buffer), there were no peaks detected.

Aggregates prepared in **PBS** showed different behavior. There was only one peak detected and it was wide and more protein like. That peak is wider than peaks of other analyzed protein is

expected because there may be different aggregates species present in the sample (not all aggregates consist of the same number of monomers and the result is variance in molecular weight). If we compare the peak of aggregates sample it differs a lot from the non-incubated insulin. It is wider, lower and overall shape is very unalike to non-incubated insulin and, the time of migration is different, so we can assume that aggregates are present in sample. Aggregates were injected with HD injection because EK does not work due to the higher pH that pI of proteins. Also, volume of injection was modified, to obtain significant peak (low concentration of aggregates).

Since the peak of aggregates was seen we tried to perform the separation with 0,5% PEO and 2% HPC, where 0,5% PEO has proved to be much better separation polymer. For easier comparison between insulin monomer and aggregates aprotinin was added each time as marker. In 0,5% PEO (50mM phosphate buffer), ratio of time of migration between insulin monomers and aprotinin was 1,37 and between aggregates and aprotinin it was 1,61. In 2% HPC, that ratio was 1,61 and 1,72. As we can see the difference between ratios is much smaller in 2% HPC.

To conclude, 0,5% PEO has shown as efficient for separating aggregates from monomers and it is suitable for further development. Aggregates are separated from monomers as we can see in figure 16.

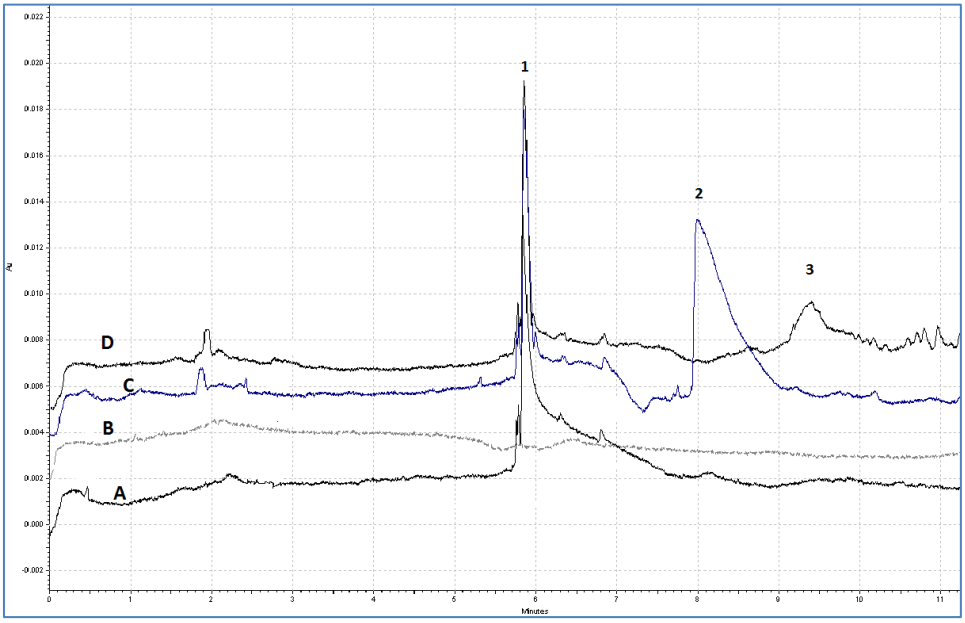


***Figure 16** : Separation of insulin aggregates (prepared in PBS) and insulin in 0,5%PEO in 50mM phosphate buffer pH=2,00, 75µm capillary, HD injection, 25kV. 1 – aprotinin (marker), 2 – insulin monomers (non-incubated), 3 – insulin aggregates; A - aprotinin, B – blank, C – aprotinin and non-incubated insulin, D – aprotinin and insulin aggregates.*

7 CONCLUSION

We can conclude that CE is a suitable technique for separation of monomers from oligomers and protein aggregates. By selecting appropriate conditions they can be separated. As prospective possibilities we can name two polymers: PEO and HPC. They provide separation with acceptable time of analysis, resolution, sensitivity and relative absence of broadening of peaks. Other three polymers, both PEGs and dextran did not bring results we would like and need for their further incorporation as a sieving medium in buffer solution.

With all proteins we observed some similarities when polymers were added to separation buffer:

- *Increase in migration time* which also depended on polymer concentration – higher polymer concentration, longer migration time. This is expected since a sieving effect was added to the separation medium.
- *Loss in sensitivity*. This can be caused either by smaller injection volume of sample (due to the viscosity of separation buffer) or polymers absorbance as well and the sensitivity decreases.

Unfortunately, as we intended to improve the separation with additives or coating, we did not reach our expectations and we did not include them in final testing. With EK injection we also did not reach our goals as it did not bring expected or prospective results. In the end, when insulin aggregates were tested, 0,5% PEO was evaluated as the best since it provided the best separation between monomers and aggregates.

With our project we found the base for future development of the method. Our selected final conditions, 0,5% PEO in 50mM phosphate buffer (75µm i.d.), provide the best conditions to separate monomers from aggregates. However, I think there is space for improvement. It would be interesting to add ions to the solutions to reduce interactions between capillary wall and proteins and with that to reduce broadening of the peaks. This could potentially also be accomplished in smaller diameter capillary - 50µm. Another prospective possibility is to transfer this method to CE with LIF detector instead of UV, where sensitivity is higher and this could bring good results. It should *be emphasized* that the research was done on model proteins and that for diagnosis of AD and other neurodegenerative diseases further researches should be done to transfer our conclusions to more specific protein types, Aβ, α-synucleins and others.

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9 APPENDIX

1. PROTOCOL FOR AGGREGATE PREPARATION

A. AGGREGATES IN PHOSPHATE BUFFER SALINE

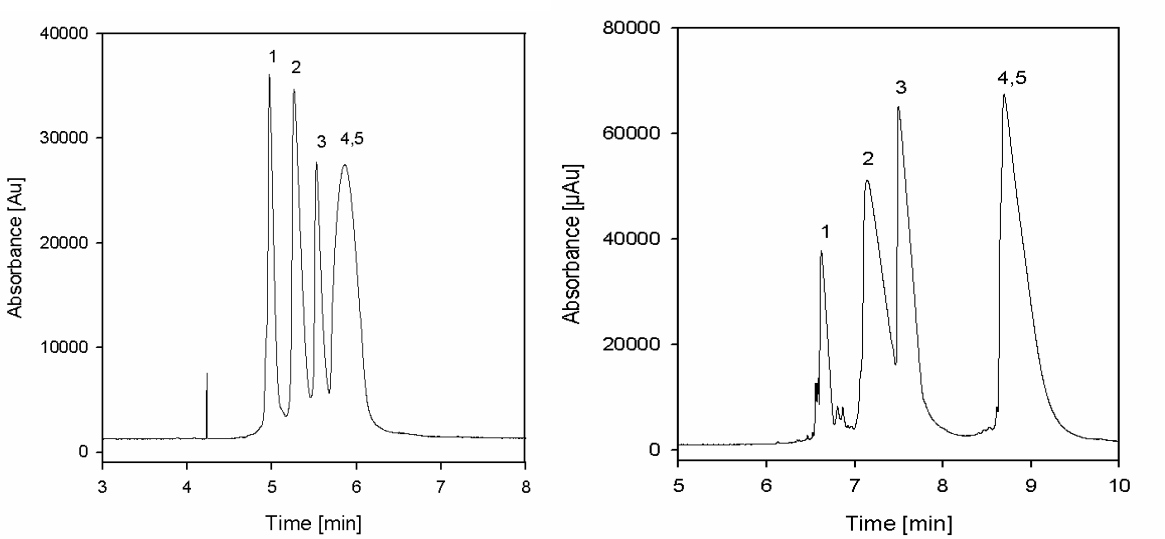
Filtrated bovine insulin incubated at 37°C in PBS for 32 days. Concentration is 2mg/ml (348µM)

B. AGGREGATES IN ACETIC ACID

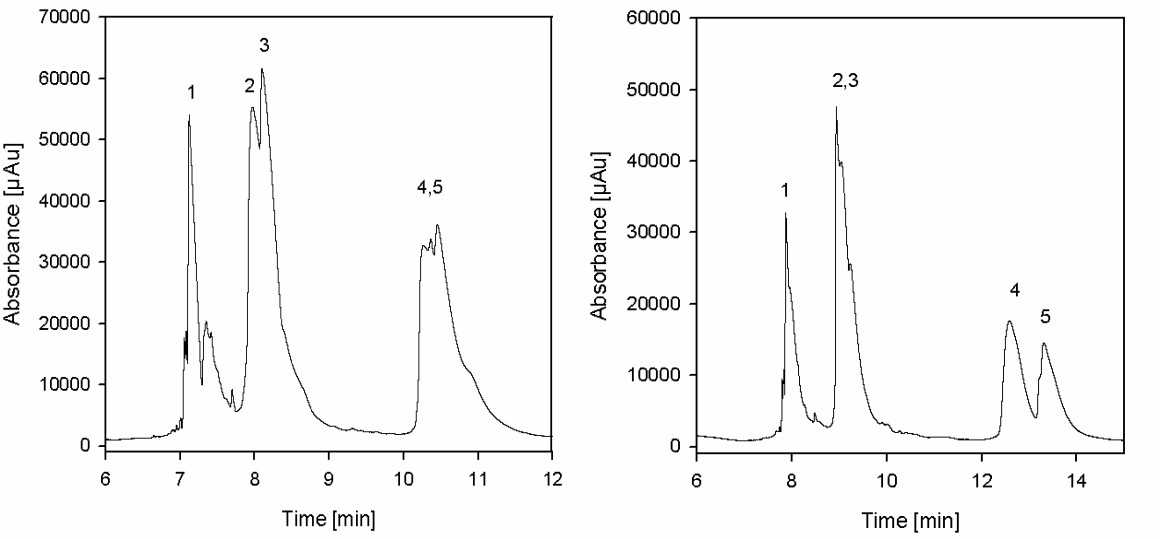
Bovine insulin incubated in 20% CH3COOH – 10mM NaCl at 37°C. Concentration is 200µM. Incubation lasted from 5 to 60 days, depending to the vial analyzed.

2. *SEPARATION IN HPC (from 0% to 2,5% HPC in 50mM phosphate buffer, pH=2,00, 75µm capillary, 25kV, injection: 0,3psi for 3sec). 1- aprotinin, 2-cytochrome C, 3- ribonuclease A, 4-carbonic anhydrase, 5-conalbumin.*

- 0% HPC on the left and 0,5% HPC on the right



- 1% HPC on the left and 1,5% HPC on the right



- 2% HPC on the left and 2,5% HPC on the right