UNIVERSITY OF NOVA GORICA
GRADUATE SCHOOL

SEPARATION OF SMALL MOLECULES ON MONOLITHIC CHROMATOGRAPHIC MATERIALS

MASTER'S THESIS

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Abstract

In this work, the isocratic separation of oligonucleotides in the ion-exchange mode on thin layered glycidyl methacrylate-ethylene glycol dimethacrylate (GMA-EDMA) polymeric material in the form of commercially available CIM® (Convective Interaction Media) disks and in the form of custom-made GMA-EDMA disks is presented. It was found that isocratic separation occurs even on monoliths with a thickness of only 0.75 mm. Peak broadening of the components retained on the monolith is proportional to the retention time, which in turn is proportional to the thickness of the monolith. Peak height is inversely proportional to the retention time. From these results it can be concluded that the mechanism of the separation on such monolithic chromatographic material is similar to that in HPLC columns filled with conventional porous particles. The HETP value of GMA-EDMA monoliths is calculated to be 18.0 μm. It was also found that the difference between peak retention volumes slightly increases with the flow rate when the experiments are performed in the range from 0.5 to 7 mL/min. From the similarities between the isocratic separations on conventional columns and on thin GMA-EDMA monoliths it is reasonable to believe that the separation based on a multiple adsorption/desorption process also occurs in thin monoliths.

Furthermore, GMA-EDMA monolith for preparative scale in a shape of hollow cylinder was prepared. To run this monolith in a radial flow mode, a special housing was also constructed. When both parts were combined, a chromatographic column capable of extremely high throughput was obtained and tested. The column exhibits constant efficiency in the range of flow rate from 5 up to 70 CV/min. Pressure drop at 50 CV/min was below 15 bar. The effect of column packing was tested, however no effect on column performance was found. The packing procedure was completed within 2 minutes. Afterwards protein sample BSA was applied on the column and the separation of BSA aggregates from monomer at the flow rate of 70 CV/min in less than 1 minute was obtained.

Keywords: GMA-EDMA monoliths; CIM® (Convective Interaction Media) disks; isocratic separation; oligonucleotides; radial flow chromatography; column packing

Statement

I am the original author of this work.

Janez Jančar

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List of Abbreviations, Acronyms and, Symbols:

BSA	bovine serum albumin
ºC	Celsius
CA	California
cm	centimeter
cm/h	centimeter/hour
CIM®	Convective Interaction Media
CV/min	column volume/minute
DEAE	diethylamine active groups
DNA	deoxyribonucleic acid
EMG	exponentially modified Gaussian
Eq	equation
GMA-EDMA	glycidyl methacrylate-ethylene glycol dimethacrylate
GMBH	gesellschaft mit beschränkter Haftung
h_{max}	maximum peak height
Hz	Hertz
HETP	height equivalent to a theoretical plate
HPLC	high performance liquid chromatography
I.D.	internal diameter
inj	injection
kDa	kilodalton
L	separation unit length
M	molar
mg/mL	milligram/milliliter
mL	milliliter
mL/min	milliliter/minute
mM	millimolar
mm	millimeter
mmol/g	millimol/gram
N	number of theoretical plate
nm	nanometer
PEEK	polyetheretherketon
pH	measure of the activity of the hydrogen ion
QA	trimethylamine active groups

R	Probability of the molecule to be in the mobile phase
R^2	R-square
RNA	ribonucleic acid
RSD	relative standard deviation
s	second
SDS–PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SS	stainless steel
STI	soybean trypsin inhibitor
TC	Tri-clamp
t_r	retention time
UK	United Kingdom
USA	United States of America
UV	ultraviolet
v	linear velocity of the mobile phase
V/V	volume/volume
μg/mL	microgram/milliliter
μL	microliter
μm	micrometer
π	pi
σ_t	peak width
%	percent

List of Chemicals:

trade name	**IUPAC name**	**Molecular formula**
Glycidyl methacrylate (**GMA**)	oxiran-2-ylmethyl 2-methylprop-2-enoate	$C_7H_{10}O_3$
Ethylene glycol dimethacrylate (EDMA)	2-(2-Methyl-acryloyloxy)ethyl 2-methyl-acrylate	$C_{10}H_{14}O_4$
Cyclohexanol	Cyclohexanol	$C_6H_{12}O$
Dodecanol	Dodecan-1-ol	$C_{12}H_{26}O$
Benzoyl peroxide	dibenzoyl peroxide	$C_{14}H_{10}O_4$
Acetone	2-Propanone	C_3H_6O
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol	$C_4H_{11}NO_3$
sodium chloride	sodium chloride	NaCl
hydrochloric acid	hydrochloric acid	HCl
myoglobin		
conalbumin		
soybean trypsin inhibitor		
bovine serum albumin		
10–200 kDa standard		
Commasie blue		

oligodeoxynucleotide 8 (oligo 8): C CAT GTC T$^{3'}$

oligodeoxynucleotide 10 (oligo 10): GTC CAT GTC T$^{3'}$

oligodeoxynucleotide 12 (oligo 12): AG GTC CAT GTC T$^{3'}$

oligodeoxynucleotide 14 (oligo 14): C GAG GTC CAT GTC T$^{3'}$

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Pregled Magistrske Naloge

(SUMMARY IN SLOVENIAN LANGUAGE)

SEPARACIJA MAJHNIH MOLEKUL NA MONOLITNIH KROMATOGRAFSKIH MATERIALIH

Tekočinska kromatografija visoke ločljivosti (HPLC) je ena izmed najučinkovitejših separacijskih tehnik za čiščenje velikih bioloških molekul. Ker so potrebe po učinkovitih postopkih čiščenja velikih bioloških molekul predvsem v farmacevtski industriji vedno večje, so bili v zadnjem času razviti tudi povsem novi kromatografski materiali, namenjeni prav tem aplikacijam. Monolitni kromatografski materiali CIM® (Convective Interaction Media) predstavljajo enega od teh novih pristopov v tekočinski kromatografiji velikih biomolekul.

V nasprotju s tradicioalnimi kromatografskimi materiali, ki imajo obliko zelo majhnih poroznih delcev, je CIM® "monolit" visoko porozen enovit kos polimernega materiala. Pri monolitnih kromatografskih nosilcih se tako aktivne skupine ne nahajajo več v slepih porah, kar je značilno za delčne nosilce, ampak na površini odprtih visokopretočnih kanalov. Izmenjava snovi med mobilno in stacionarno fazo pri monolitnih ne poteka več s pomočjo difuzije kot pri delčnih nosilcih, ampak s pomočjo konvekcije. Konvektivni prenos snovi je v primerjavi z difuzijo bistveno hitrejši proces, kar še posebej pride do izraza v primeru velikih biomolekul, katerih difuzivnost je zelo nizka. Posledica konvektivnega prenosa snovi je tako bistveno hitrejša izmenjava molekul med mobilno in stacionarno fazo, kar daje monolitnim kromatografskim materialom kar nekaj prednosti pred tradicionalnimi nosilci. Za monolitne nosilce je značilno, da tako ločljivost kakor tudi dinamična vezna kapaciteta nista več odvisni od linearne hitrosti mobilne faze, kar je značilnost delčnih nosilcev.

Kot že rečeno, so bili monolitni kromatografski nosilci razviti z namenom izboljšati kromatografski proces ločevanja velikih bioloških molekul. Kromatografski procesi ločevanja velikih biomolekul ne potekajo na izokratski način, ki je značilen za ločevanje majnih molekul. Za monolitne kromatografske nosilce tako praktično ni bilo nikakršnih podatkov, ki bi podrobneje obravnavali ločevanje majhnih molekul, zaslediti pa je bilo celo mnenja, da na monolitnih kromatografskih nosilcih ne poteka kromatografski proces.

V prvem sklopu te naloge so tako predstavljeni podatki o ločevanju štirih oligonukleotidov (oligo 8, oligo 10, oligo 12 in oligo 14) pri različnih pogojih. Ločevanje oligonukleotidov je potekalo na CIM® QA ionsko izmenjevalnih kromatografskih materialih, ki so imeli obliko diska. Pri različnih eksperimentih ločevanja štirih oligonukleotidov je bil uporabljen en ali več diskov, ki so bili vstavljeni v ustrezno kromatografsko ohišje. CIM® disk, vstavljen v kromatografsko ohišje, tvori tako imenovano kratko monolitno kromatografsko kolono. CIM® disk je kratek valj premera 16 mm, njegova dolžina pa se po potrebi lahko spreminja (**Scheme 4)**.

V prvem eksperimentu je prikazano ločevanje vseh štirih oligonukleotidov na CIM® QA disku dolžine 3 mm. Med ločevanjem je bila sestava mobilne faze ves čas enaka, kar je značilno za izokratski način ločevanja.Vsi štirje oligonukleotidi se lepo ločijo do osnovne črte in to v relativno kratkem času 2,5 minut. Iz kromatograma je razvidno, da z naraščajočim zadrževalnim časom posameznega oligonukleotida narašča tudi širina vrha (**Figure 6**).

V drugem eksperimentu so prikazani zadrževalni časi in oblika vrhov oligonukleotida 8 na CIM® QA disku dolžine 3 mm v odvisnosti od sestave mobilne faze. Iz kromatograma je razvidno, da z nižanjem koncentracije soli v mobilni fazi zadrževalni čas oligonukleotida 8 narašča. Z naraščanjem zadrževalnega časa narašča tudi širina posameznega vrha, medtem ko njegova višina pada. Obe odvisnosti sta premosorazmerni, kar predpostavlja tudi model teoretičnih prekatov, ki opisuje dogajanje pri izokratskem kromatografskem načinu ločevanj (**Figure 7, 8**).

V tretjem eksperimentu je prikazana ločljivost zgoraj omenjenih štirih oligonukleotidov v odvisosti od dolžine separacijske poti (dolžine diska). Uporabljenih je bilo 6 različnih dolžin CIM® QA diskov; 0.75, 1.5, 3, 6, 9 in 12 mm. Iz kromatogramov je razvidno, da se ločljivost z daljšanjem separacijske poti (dolžine diska) izboljšuje. Z daljšanjem separacijske poti zadrževalni časi posameznega oligonukleotida naraščajo, odvisnost pa je premosorazmerna. Tudi ta odvisnost je v skladu z modelom teoretičnih prekatov.

Zaradi že naštetih prednosti so CIM® monoliti zelo zanimivi za uporabo v industrijskih aplikacijah čiščenja velikih bioloških molekul. V industriji pa kromatografske kolon dosegajo velikosti tudi do več 10 litrov. Ker je izdelava monolitnih kromatografskih kolone velikega volumna v obliki diska zaradi težav z enakomerno distribucijo mobilne faze in mehanskih lastnosti samih diskov zelo zapletena, v industriji srečujemo samo tako imenovane radialne monolitne kromatografske kolone (**Scheme 2**). Zaradi specifičnega načina delovanja, radialna kromatografska kolona potrebuje tudi posebno kromatografsko ohišje .

V drugem sklopu te naloge je predstavljena nova oblika radialnega kromatografskega ohišja. Novo kromatrografsko ohišje je zasnovano tako, da omogoča ustrezno distribucijo mobilne faze tudi pri kolonah večjega volumna (**Scheme 7**). Izdelan in testiran je bil tudi prototip kolone, ki že vključuje novo obliko ohišja. Kolona ima volumen 8 mL, in je napolnjena s CIM® QA monolitom. Prvi ekperiment drugega sklopa naloge prikazuje teste stopenjskih motenj, opravljenih pri različnih pretokih. Namen tega testa je bil preveriti ustreznost distribucije ohišja v odvisnosti od pretoka (**Figure 13**). Stopenjske motnje so bile narejene pri šestih pretokih (20, 30, 40, 50 60, 70 CV/min), kot sledilec pa je bila uporabljena 16,6 % raztopina aceton. Iz kromatograma je razvidno, da se z večanjem pretoka število teoretičnih prekatov vrhov zmanjšuje, relativni standardni odmik znaša 20,7 %. Pregled kromatogramov je pokazal, da se z večanjem pretoka višine vrhov znižujejo, kar je tudi vzrok za nižanje števila teoretičnih prekatov.

Vzrok za zniževanje števila teoretičnih prekatov z višanjem pretoka je bil pojasnjen z četrtim eksperimentom drugega sklopa (**Figure 16**). Izkazalo se je, da za nižanje števila teoretičnih prekatov ni kriva slaba distribucija kromatografskega ohišja, ampak neustreznost kromatografskeka sistema. Največje število podatkov, ki jih lahko kromatografski sistem zabeleži, je 10 Hz, kar pa pri tako velikih pretokih ne zadostuje. Maksimalen odziv detektorja se izgubi med dvema sosednjima podatkoma in višina vrha je tako navidezno nižja, število teoretičnih prekatov pa manjše.

Kromatografske lastnosti kolone so prikazane v eksperimentih petem in šestem eksperimentu drugega skolpa. Kolona omogoča separacijo treh standardnih proteinov tudi pri pretoku 50 CV/min (**Figure 17**). Ločljivost in dinamična kapaciteta pa sta neodvisni od pretoka še pri pretokih 40 CV/min.

Pakiranje kolone je zelo enostavno in hitro, sam proces pa je zelo robusten, saj je relativni standardni odmik stopenjskih motenj, ki so bile opravljene po vsakem polnenju 4,3 % (**Figure 20**).

Na koloni je bil opravljen tudi eksperiment realnega čiščenja, v katerem je bilo prikazano ločevanje agregatov proteina BSA od osnovnega monomera. Separacija je bila opravljena pri pretoku 70 CV/min v času 2 minut (**Figure 21**).

1. INTRODUCTION

In the recent years, a demand for separation of different substances has increased dramatically. With the development of so called second-generation therapeutics particularly strong interest for fast and efficient separation and purification of proteins, polynucleotides like DNA and RNA and other large biological molecules in general has been observed. Separation or purification steps are now a bottleneck of the entire process of biopharmaceutical therapeutics production. Therefore, it is not surprising that the development of the abovementioned areas was, and still is, accompanied with the development of different separation techniques, among which High Performance Liquid Chromatography (HPLC) plays a very important role. Since one of the key components of HLPC technic is a HPLC column, many new stationary phases were introduced. The main target was to increase the stationary phase chemical stability as well as to enable fast and efficient separations. To achieve the first goal, stationary phases based on polymers, zirconia or combination of silica and polymer, just to mention some of them were introduced [1]. To accomplish the second goal, besides optimizing the chemical composition, an optimization of the matrix structure itself is a key feature [2].

One of these new stationary phases is also glycidyl methacrylate-ethylene glycol dimethacrylate (GMA-EDMA) polymeric material, so called CIM® (Convective Interaction Media) monolith. Monoliths are a type of convective chromatographic supports which exhibit flow unaffected chromatographic properties like resolution and dynamic binding capacity [3]. Since their introduction, monoliths have been successfully applied in various chromatographic separations of large biomolecules using gradient elution in extremely short analysis times [4,5]. The main difference between monoliths and conventional HPLC columns lies in the structure of the support. Traditional columns are packed with highly porous particles with a diameter in the range of 3 - 10 μm, up to 40 μm in industrial application. Most of the active groups are located within the pores which represent more than 90 % of

the total accessible surface area and provide a high specific surface area for interactions between molecules in the mobile and stationary phases.

For separations under isocratic flow conditions multiple steps of the adsorption/desorption process should take place. Conventional HPLC columns are, therefore, normally fairly narrow (2 - 4.6 mm I.D. for most analytical purposes) and rather long (10 - 25 cm), thus providing a long enough path across the separation layer necessary for the high resolution separations of different molecules.

In the case of monolithic column, the length of the separation layer is much shorter (only up to few mm). For glycidyl methacrylate - ethylene glycol dimethacrylate (GMA-EDMA) monolithic supports the maximum thickness was defined to be 15 mm [6]. Because of the short separation layer lengths and the resulting short residence times of the molecules within the separation layer, the multiple steps of the adsorption/desorption process were usually not considered as a possible mechanism for the separation. It was even suggested that GMA-EDMA monoliths do not fall into the category of real chromatography, since this basic chromatographic feature is missing [7]. In fact, the separations of large biomolecules on short columns are achieved by selective gradient elution based on the so-called "on-off" mechanism [8,9]. Only in a few cases of some difficult separations of proteins a combination of both "on-off" and multiple step (differential migration) adsorption/desorption was applied [8].

However, isocratic separation of plasmid DNA conformers under isocratic flow conditions on a 3 mm thick CIM QA (CIM - Convective Interaction Media, QA - quaternary ammonium active groups) monolithic disk was presented [10], but no clear explanation of the phenomena governing the separation mechanism was provided.

In this work, isocratic separation of oligonucleotides in the ion-exchange mode is presented. The effects of the thickness of the separation layer and the mobile phase composition, as well as the effects of the flow rate on the separation and peak spreading are discussed in terms of the theory of isocratic separations on conventional HPLC columns.

Due to the short separation layer and high porosity, disk - shaped monolithic columns also exhibit very high throughput possibilities and thus short residence times of adsorbed molecules. In case of industrial purification processes relating to production of therapeutics the residence time plays a very important role. Since the residence time of the molecules should be as low as possible because it prevents their degradation, monolithic columns with its high throughput possibility can be a very useful tool. However, if we want to use short monolithic columns in the shape of a disk in industrial scale we can easily face serious problems with a uniform distribution of the liqui.

In order to elucidate the possibility of using thin layered monolithic materials also in preparative and industrial applications, a new approach to chromatographic housing design enabling high throughputs is also presented in this work. Furthermore, a preparative column combining a thin-layered cylindrical GMA-EDMA monolith and new housing design was prepared. The behavior of the new column in terms of resolution and dynamic binding capacity as well as real sample separation at extremely high flow rates is presented.

An additional advantage of monolith technology is that it enables in-situ preparation of the chromatographic resin – monolith within the column housing. Because of that, packing can be completely omitted [11]. This is especially advantageous in the case of microchips, capillaries and, to some extent, analytical columns, where the cost of housing is significantly lower than the matrix itself. On the other hand, for larger, semi-preparative and preparative columns, the cost of column hardware is significant and chromatographic media should therefore be

refilled. In this case it would be advantageous also for monolithic matrix to be exchanged after its performance declines.

Column packing is one of the key factors to get high efficiency chromatographic column since heterogeneity of packed bed layer results in poorer resolution [12-13]. In recent years significant progress in developing procedures which are related to chromatographic column packing has been made. However, several factors such as column loading apparatus design, loading pressure and way of slurry preparation, just to mention a few of them, are still to be considered [14]. When all the parameters are optimized excellent reproducibility can be achieved even on a preparative scale [15]. Since monolith matrix is composed of a single piece of material one can speculate that packing would have no effect. However, monolith has to be properly tightened into the housing to avoid any by-pass of the mobile phase; therefore some shrinking of the monolith might occur, which can influence its performance.

With this in view the effect of refilling a new preparative column in terms of efficiency, reproducibility and speed of packing is also presented.

2. THEORETICAL BACKGROUND

2.1. High performance liquid chromatography - HPLC

Chromatography is a general term which gathers a variety of separation techniques. In chromatographic process a mixture of molecules to be separated is distributed between the mobile and the stationary phase. Due to different affinity to the stationary phase, one type of mixture molecules will be almost entirely adsorbed onto the surface of the stationary phase whilst the other type of molecules will mostly stay unadsorbed in the mobile phase. The mobile phase flows along the stationary phase and elutes the adsorbed sample molecules. Consequently molecules held preferentially in the stationary phase are retained longer in the distribution system than those that are mainly distributed in the mobile phase, and separation takes place.

One of the most recognizable chromatographic techniques is High Performance Liquid Chromatography or HPLC. In HPLC the distribution system is in a form of chromatographic column - cylinder packed with a stationary phase, where liquid mobile phase under pressure passes along the stationary phase and elutes the sample molecules. Chromatographic column design must fulfill two main functions to produce effective separation. The first function is separation itself, and is a result of different affinity forces between each molecular type and a stationary phase. Each solute is retained to a different extent, thus the one more weakly held will elute first and the more strongly bounded will elute last. Consequently, each solute will be eluted from the column in the reverse order of the magnitude of the interacting force between each solute and the stationary phase. This function is mainly dependent of appropriate distribution system (stationary phase / mobile phase) for selected molecule mixture.

The second function is minimum solute band spreading, and is achieved by selecting the optimal column physical properties like column diameter, column

length, mobile phase distribution system, stationary phase shape, linear flow velocity of the mobile phase etc [16].

2.1.1. Isocratic elution chromatography – multiple adsorption desorption process

The Theoretical plate concept is rather obsolete; however it introduces parameters (the plate height) which can be used to characterize chromatographic zone spreading and the resolution. In practice the plate height is used to describe the all zone spreading phenomenon [17].

According to theoretical plate theory, zone development in the first several plates gives high discontinuous concentration profile following the Poisson distribution. After further migration of the zone to the next plates (up to 50 plates), zone concentration profile loses its discontinuance, however concentration profile can still be followed by Poisson distribution. At the following plates (after 50) concentration profile becomes smooth and follows the Gaussian (normal) distribution [17]. The standard Gaussian deviation σ is:

$$\sigma = \sqrt{HL} \qquad 1$$

Where H ($HETP$) is the plate height and L the distance migrated by the center of the zone. Even the plate theory does not describe the basic chromatographic process; the plate height is a useful and widely accepted parameter to describe zone spreading and resolution including also nonequilibrium and longitudinal diffusion effect [17]. The plate height can be defined as:

$$H = \frac{\sigma^2}{L} \qquad 2$$

In elution chromatography the zone profile evolves at the column end with time as a variable. The concentration-time profile has a standard deviation $of\ \sigma_t$, analogous to σ except having the dimension of time. Both deviations are related:

$$\sigma_t = \frac{\sigma}{Rv} \qquad 3$$

Where R is retention ratio characterizing migration rates and v is regional velocity of the mobile phase. Combining equations 2 and 3, the equation for plate height gets form:

$$HETP = \frac{(Rv\sigma_t)^2}{L} \qquad 4$$

Plate length over zone velocity is simply the elution (retention) time t_r. Retention time is proportional to separation lyre length[17].

$$t_r = \frac{L}{Rv} \qquad 5$$

Introducing plate length over zone velocity as a retention time and combining equation 4 and 5, the standard deviation or zone width gets form:

$$\sigma_t = \sqrt{\frac{HETP}{L}} \cdot t_r \qquad 6$$

2.2. Monolithic chromatographic materials

In HPLC distribution system stationary phases are mainly found in the shape of very small porous spherical particles to achieve their large surface area and consequently incised adsorption possibilities.

Monolithic chromatographic supports represent a novel advanced type of stationary phases used in chromatography. Monolithic stationary phase appears as a homogenous continuous support and is an alternative to the conventional stationary phases having the shape of very small porous particles the size of few micrometers up to 100 micrometers. Their unique continuous bed structure provides a high rate of mass transfer, which is driven by convection at relatively low back pressure drop as well as high efficiency even at very high flow rates. As such, monoliths are especially useful for separation of large biological molecules whose mobility is limited due to their size, and convective mass transfer particularly turns out to be important. Their appearance as a single piece of highly porous structure also gives rise to the formation of the name - monolithic chromatographic stationary phases.

2.2.1. GMA-EDMA monolithic chromatographic materials

One of the most significant representatives of monolithic chromatographic stationary phases are also GMA-EDMA porous polymeric materials.
Glycidyl methacrylate-co-ethylene dimethacrylate (GMA-EDMA) monolithic supports are prepared by free radical polymerization of two monomers; a glycidyl methacrylate containing reactive epoxy group and ethylene glycol dimethacrylate as a cross-linking agent. Reaction mixture also contains benzoyl peroxide as an initiator and porogenic solvents cyclohexanol and 1-dodecanol as pore forming agents.

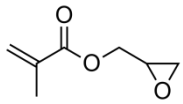
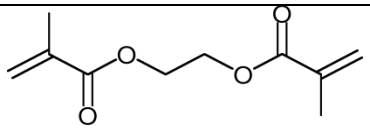
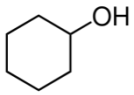
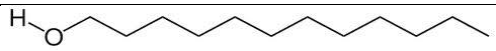
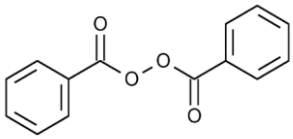
GMA	
EDMA	
Cyclohexanol	
1-Dodecanol	
benzoyl peroxide	

Table 1: **List of chemical that make up common polymerization mixture for preparation of GMA-EDMA monolithic chromatographic supports.**

2.2.2. GMA-EDMA chromatographic materials and column preparation process

Polymerization of GMA-EDMA material takes place in polymerization molds with different shapes and sizes. Polymerization molds, after they are filled with polymerization mixture, are exposed to an elevated temperature. Due to a heat, decomposition of initiator starts and results formation of polymeric structure. After polymerization is completed, the formed rigid block of polymer is moved from the mold and precisely cut to get the appropriate shape. Cut monolith is further mounted into a special housing and pore forming agents 1-dodenanol and cyclohexanol are washed out from the matrix. After washing, rigid highly cross-linked highly porous monolithic polyglycidyl methacrylate-co-ethylene glycol dimethacrylate polymeric material is obtained (**Figure 1**) [18].

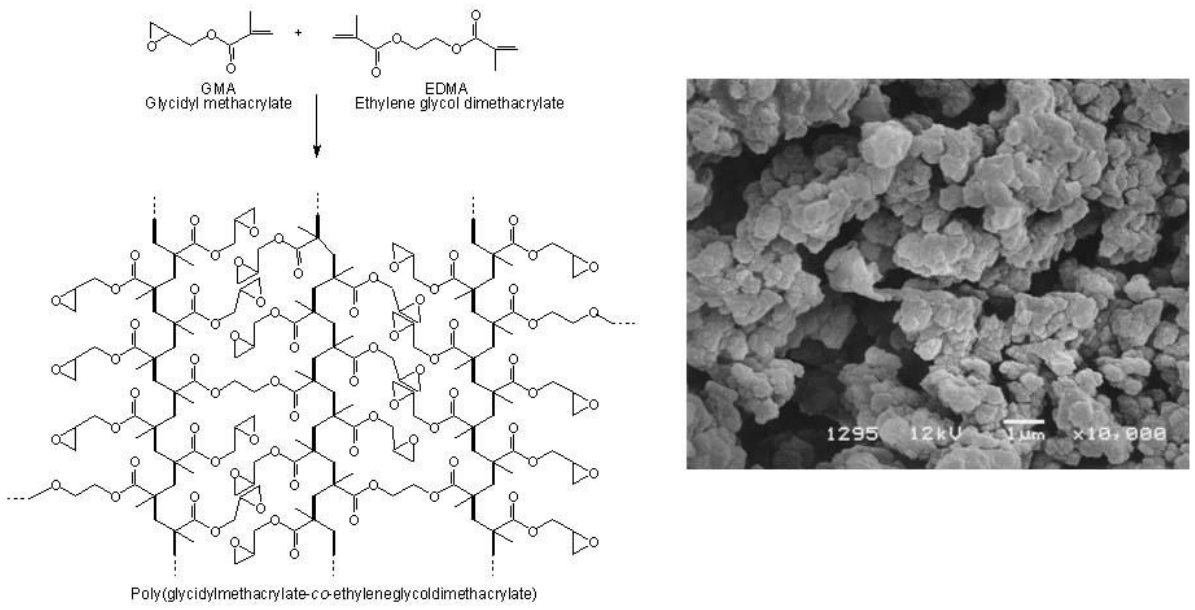


Figure 1: **Structural formula (left) and SEM picture (right) of common GMA-EDMA monolithic chromatographic support.**

Further, the monolithic polymeric material containing epoxy groups is ready for derivatization or immobilization to desired chemistry. GMA-EDMA polymeric skeleton contains active epoxide groups, which can be easily modified using various chemicals e.g. diethylamine (DEAE) and trimethylamine hydrochloride (QA) to obtain ion exchange chromatographic stationary phases. The DEAE groups (weak anion exchange groups) are introduced to the epoxy material by placing it into diethylamine for 24 hours at a room temperature. QA (strong anion exchange groups) groups are introduced in the epoxy matrix in a similar way as in the case of DEAE. Epoxy material is immersed in reaction mixture of quaternary amine solution for 4 hours. After the modification procedure is completed GMA-EDMA monolith can be packed into appropriate chromatographic housing to get the chromatographic column.

The preparation of monolithic columns is mainly considered an easy and straightforward process, especially when compared to the sensitive and time-consuming preparation of spherical particles and subsequent packing of these particles into appropriate chromatographic housing. In principle, this is true while

we are dealing with small size units where volume of monolithic polymeric block is relatively small (up to a few mL in volume). However, the preparation of large volume monolithic columns (which consist of large blocks of polymer) with a well-defined and homogeneous structure still represents a big challenge. In contrast to the scale up of particle columns, which is obtained by packing these very small particles in larger chromatographic housing, large-scale monolithic columns are obtained by producing a large block of polymer cast in a large size mold. The main problems that occur during this process are connected to the heat release (**Figure 2**) and heat dissipation during polymerization [19].

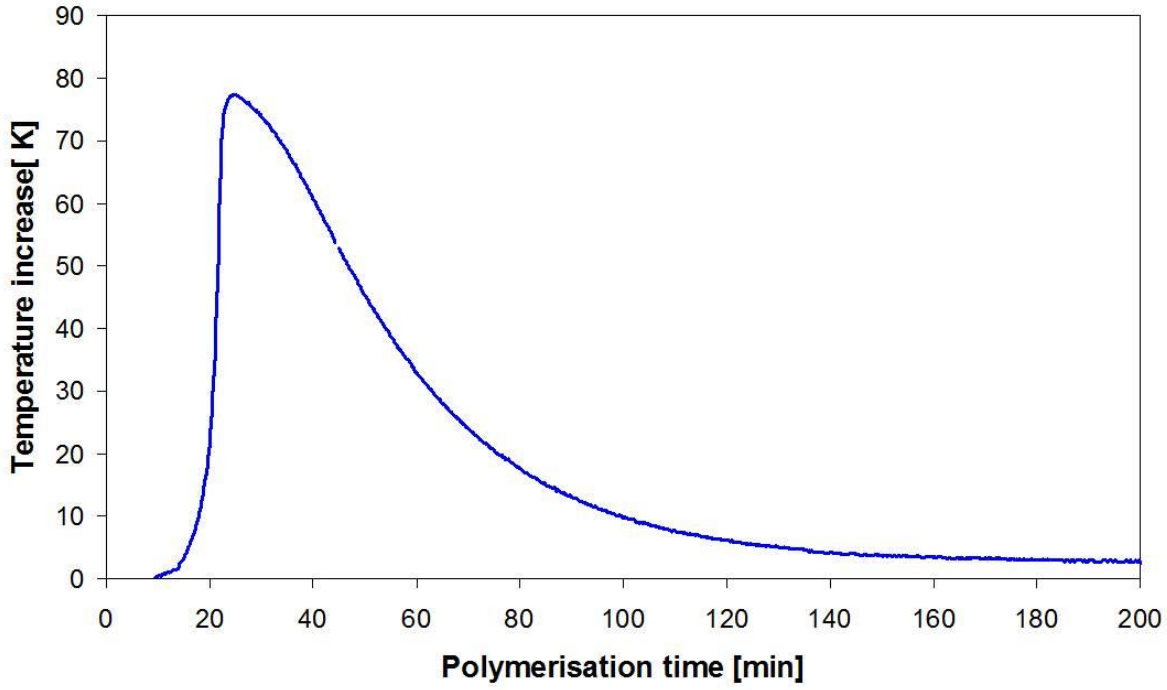


Figure 2: **Temperature increase during GMA-EDMA polymerization in cylindrical mold with diameter of 50 mm.**

Together with the progress of polymerization reaction a significant amount of heat is released (polymerization reaction is strongly exothermic) and consequently the occurrence of large temperature gradient inside the polymeric mixture can be observed. Since the pore size distribution is extremely sensitive to any temperature fluctuation during polymerization the temperature gradient results in a non-

uniform pore structure [20, 21]. As can be seen (**Figure 3**), a change in temperature of only 8 °K shifts a pore diameter from 400 to 850 nm and completely changes the flow characteristics of such a porous polymer. Therefore, the polymerization temperature is a powerful tool for controlling pore formation, while on the other hand temperature fluctuations during polymerization process present a major problem in the preparation of GMA-EDMA monoliths of large volumes.

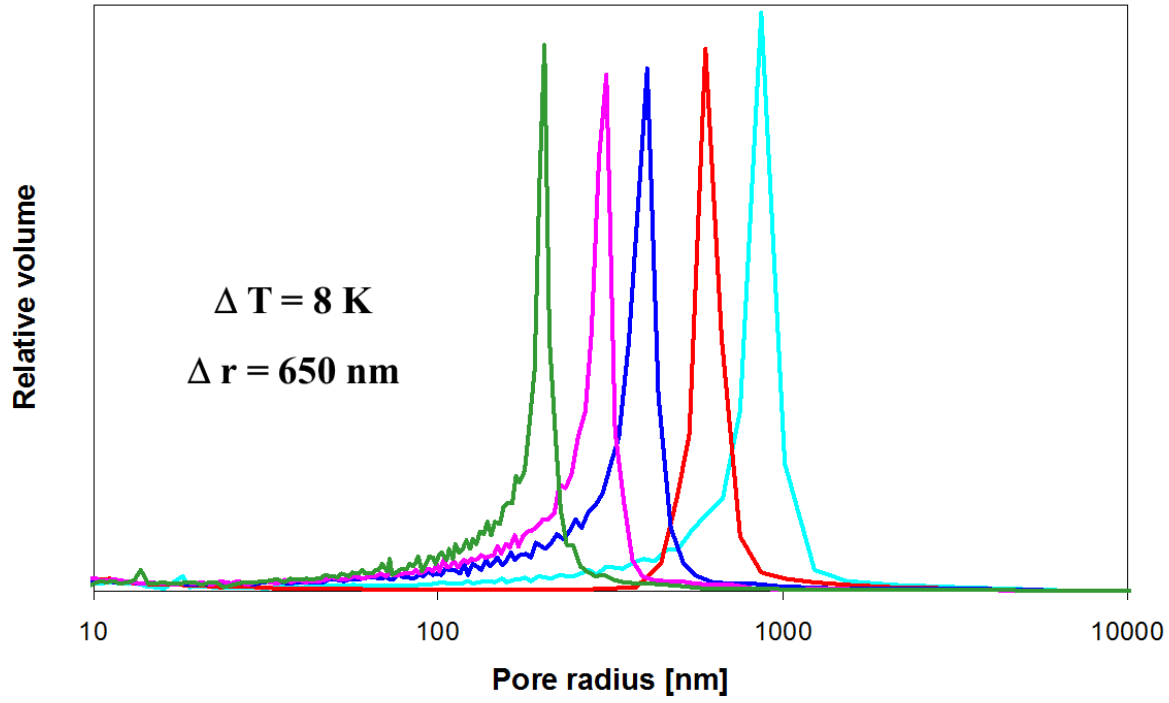
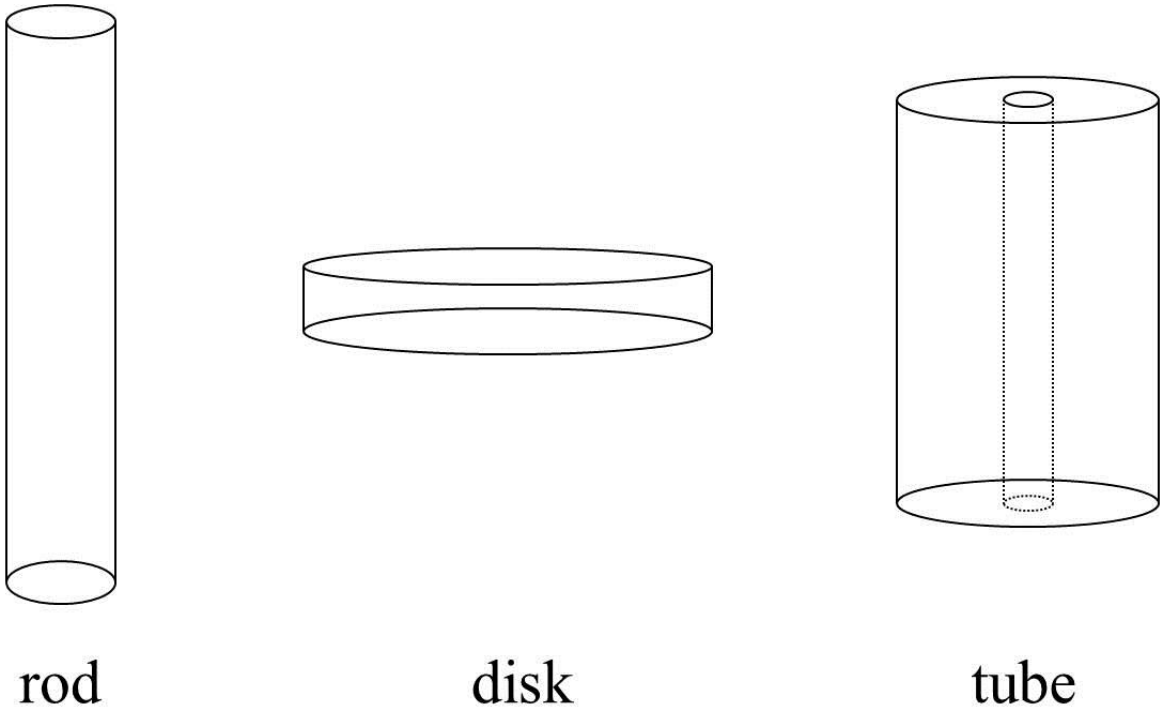


Figure 3: **Influence of polymerization temperature on a pore size.**

There are two approaches to overcoming this problem; the first one is by decreasing the polymerization reaction rate and consequently decreasing the amount of heat released per time unit by slowly adding both monomers into the reaction mixture, and the second one by adjusting the thickness of polymeric block (tin layered polymeric block) in order to decrease the temperature rise inside the mold and thus avoid the problem of heat dissipation [20].

If we adopt the second scenario, we can quickly find three appropriate geometries; a relatively long rod with a small diameter, a disk shape geometry of small thickness and a relatively large diameter and finally to thin walled hollow tube (**Scheme 1**).



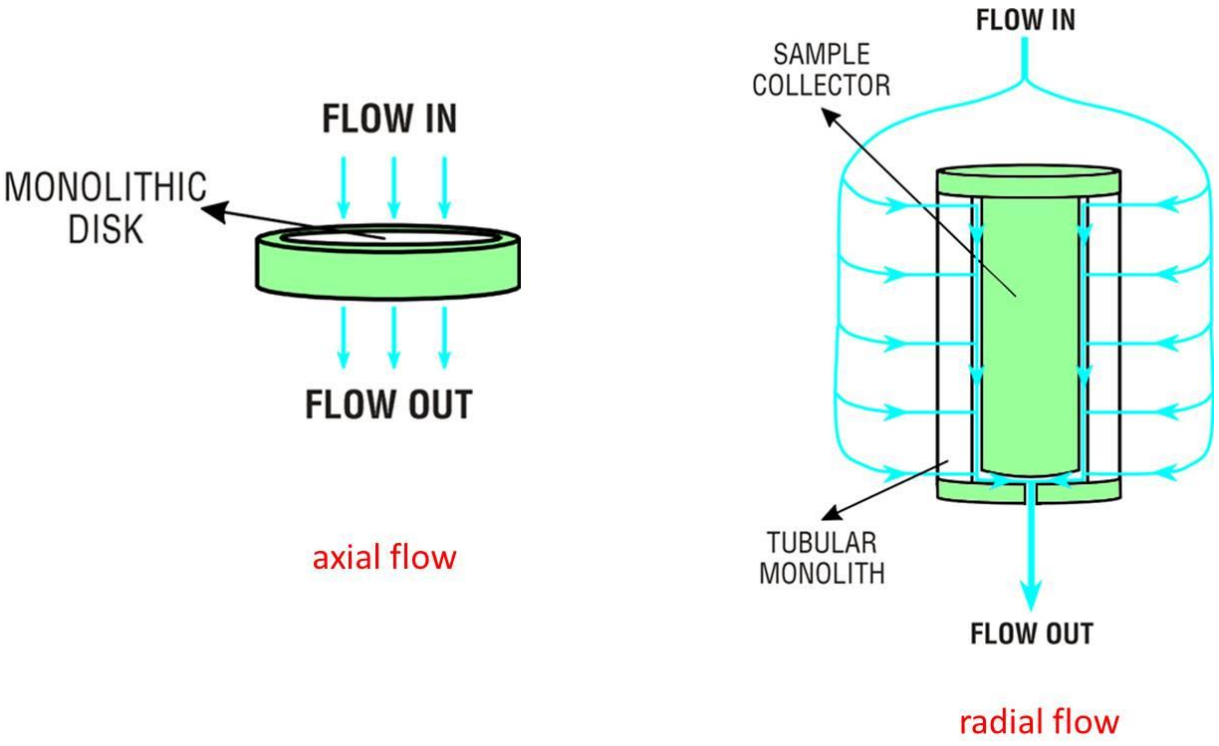
Scheme 1: **Three different alternatives for preparation of scale-up GMA-EDMA chromatographic polymers.**

2.3. Radial flow geometry

GMA-EDMA monolithic chromatographic material with its convective mass transferee feature was developed and optimized for fast and efficient separation of large biological molecules. Large biological molecules usually exhibit very steep adsorption isotherm, which means that they almost irreversibly bind to the matrix under the bounding conditions. Therefore a change of the mobile phase composition is normally required for the sufficient elution, which is carried out through linear or step gradient (gradient elution mode chromatography).

Separation of large biological molecules is thus almost exclusively based on a gradient elution and column length has no significant effect on resolution of separation. In fact longer columns might even result in additional band spreading and lowering resolution. Additionally, increased length results in increased backpressure.

Consequently, tin layered monolithic block strategy to overcome heat relishing problems during polymerization procedure perfectly fits into gradient elution mode concept. Following the idea of thin layered polymeric block, the smaller units of GMA-EDMA monolithic columns are produced in the form of disks and the larger units in the form of tubes (**Scheme 2**), whilst rods due to their long length (lower resolution and higher backpressure) do not fit into the concept [22].



Scheme 2: **Axial (monolithic disk) and radial (tube monolith) flow mode; two alternative patterns of flow through the stationary phase at the chromatographic columns.**

At the disk shape, mobile phase is entering in the direction of the cylinder (disk) axis. The liquid is led into the column through the small diameter hole and then equally spread across the entire disk surface. If we want to scale up the disk whilst considering the requirement to maintain a relatively small disk thickness, only an increase of the diameter is possible. Due to the problems with mechanical stability of large diameter disks and uniform liquid distribution across the entire disk surface, they are exclusively used in small scale.

On the other hand, tubular shape approach offers a higher degree of freedom since inner and outer diameter as well as cylinder height can be varied. Tubular shape also exhibits greater mechanical stability, regardless of the size. It was also shown that certain diameters can give the lowest pressure drop for a given flow rate. In tubular-shaped columns, the mobile phase is uniformly spread over the entire outer monolith surface, passing through the monolith, and is collected in the central hole

from where it exits the column. According to the outlined path of the mobile phase they enter into the monolith perpendicular to the tube axis. Since the development of the distribution system and further chromatographic housing design for radial flow columns is part of this work, more data will be presented in Results and Discussion chapter.

3. EXPERIMENTAL WORK

3.1. Equipment

Analytical gradient HPLC system

A gradient HPLC system built with two Pumps 64 enabling maximum flow rate of 10 mL/min at a maximum pressure of 400 bar, an injection valve with a 20 μl stainless steel sample loop, a variable wavelength monitor set to 260 nm and response time of 0.15 s, a flow-cell with a 10-mm optical path and volume of 10 μ L, connected by means of 0.25 mm I.D. PEEK (polyetheretherketon) capillary tubes and HPLC hardware/software (data acquisition and control station), all from Knauer (Berlin, Germany) were used when GMA-EDMA monolithic chromatographic material in shape of CIM® disks were tested. Knauer mixing chamber with its relatively large dead volume was replaced by the PEEK Mixing Tee with an extra low-dead volume (Jour Research, Uppsala, Sweden).

Preparative gradient HPLC system

All characterization of GMA-EDMA polymeric chromatographic material in shape of CIM® 8 mL tube monolithic column was performed with preparative HPLC system made by Knauer (Berlin, Germany). The system was built of two K-1800 preparative pumps (each pump contains SS pump head enabling maximum flow rate of up to 1000 mL/min at a maximum pressure of 50 bar), T shaped static mixing chamber, manually driven injection valve with 1 mL sample loop, UV/Visible spectrophotometer model K-2500 with time constant set to 0,1 and wavelength set to 280 nm, a flow cell with optical path of 2 mm, interface box and Eurochrom 2000 software installed on a personal computer for real time data acquisition. Data collecting was set to maximum possible value of 10 Hz.

Flow monitoring

During the experiments with CIM® monolithic columns, where the influence of the flow rate on the separation was studied, a validated digital flow meter (K-3773, Phase Separations Limited, UK) was additionally introduced to monitor possible discrepancies between set and real flow rates.

Pressure drop monitoring

Pressure drop measurements were performed with PE300 digital manometer from Hittinger Baldwin Messtechnik GMBH (Darmstadt, Germany).

Electrophoresis

SDS–PAGE electrophoresis was performed using a Mini-Protean II electrophoresis Cell made by Bio-Rad (CA, USA) on 20% separation gel with 4% stacking gel.

3.2. GMA-EDMA monolithic chromatographic columns

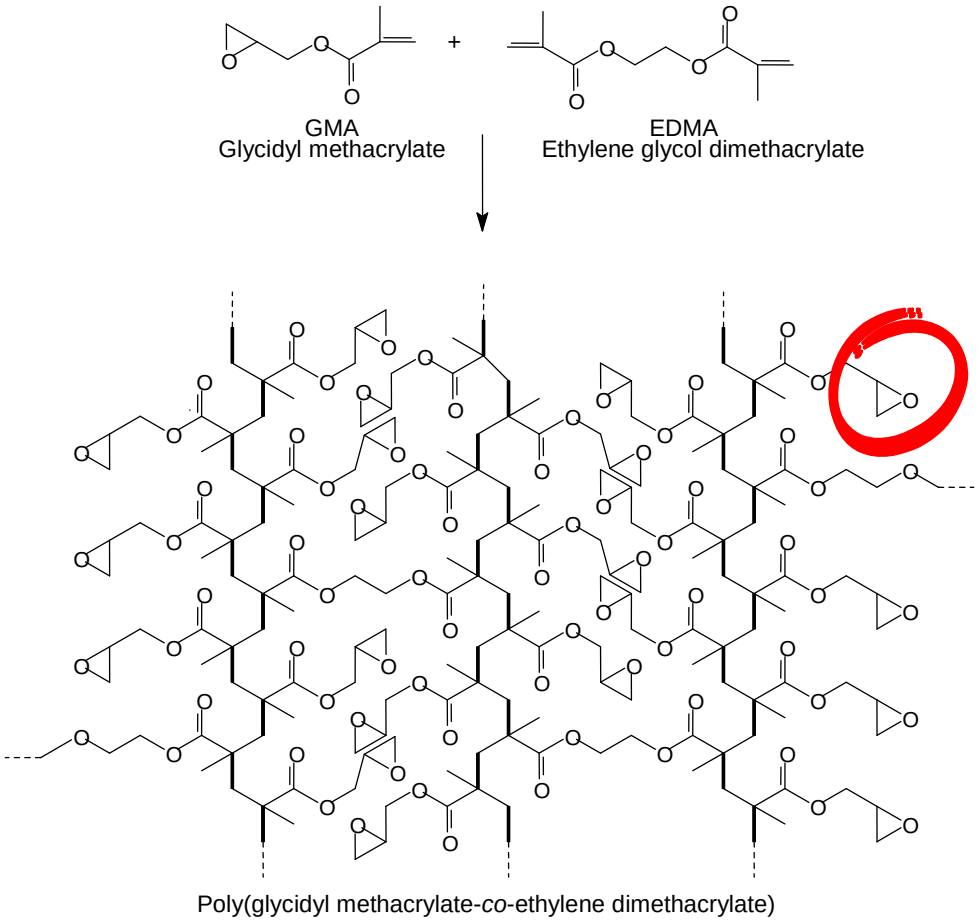
GMA-EDMA monolithic chromatographic material

All CIM® monolithic chromatographic columns used in this work were produced on platform of glycidyl methacrylate - ethylene glycol dimethacrylate polymeric material. The initial epoxy GMA-EDMA chromatographic material was prepared by the radical copolymerization of glycidyl methacrylate (Aldrich, Steinheim, Germany) and ethylene glycol dimethacrylate (Aldrich, Steinheim, Germany) in the presence of pore producing solvents. A detailed polymerization procedure is described at theoretical background in chapter 2.2.2. [18]. The resulting polymeric material contains reactive epoxy groups which can be easily modified into alternative chemical groups to obtain materials with desirable chromatographic properties. Concentration of epoxy groups of initial material was always 4,22 mmol/g of dry support. Porosity of epoxy monolithic material was 60% whilst

average pore radius was find to be 700 nm. The surface area of prepared epoxy GMA-EDMA chromatographic material was 7,48 m^2 of dry support.

Table 2: **Characteristics of initial epoxy GMA-EDMA monolithic chromatographic material.**

Epoxy GMA-EDMA material	
Porosity (%)	60
Average pore radius (nm)	700
Specific Surface area (m2/g dry support)	7,48
Epoxy group concentration (mmol/g dry support)	4,22

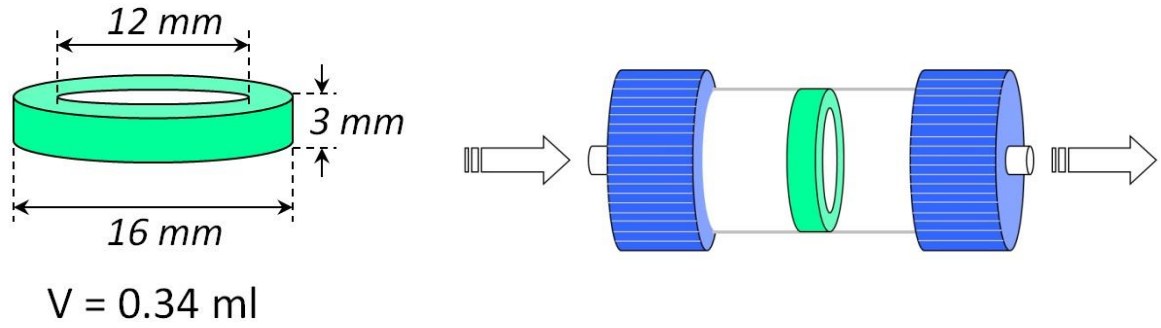


Scheme 3: Structural formula of Poly(glycidyl methacrylate-co-ethylene dimethacrylate) polymer (above in scheme 3). Modification reaction of epoxy group to obtain DEAE and QA chromatographic materials (below in scheme 3).

The DEAE groups (weak anion exchange groups) were introduced to the CIM® epoxy disk by placing the disk into pure diethylamin (Fluka, Buchs, Switzerland) for 24 hours at room temperature. The DEAE group density, obtained by a mass difference of the initial and modified disk, taking into account the reaction stoichiometry, was 2.3 mmol/g dry support which correspond to 55% conversion of epoxy group. Introduction of QA (strong anion exchange groups) groups in the matrix of 8 mL tubular shaped epoxy monolith was performed in similar way as in the case of CIM® epoxy disk modification procedure. Tubular monolith was immersed in reaction mixture of quaternary amine (Merck, Darmstadt, Germany) solution for 4 hours whilst temperature was 35 ºC (**Scheme 3**). The QA group density, obtained by a mass difference of the initial and modified tube was 2.0 mmol/g of dry support corresponding of 47% conversion of epoxy group.

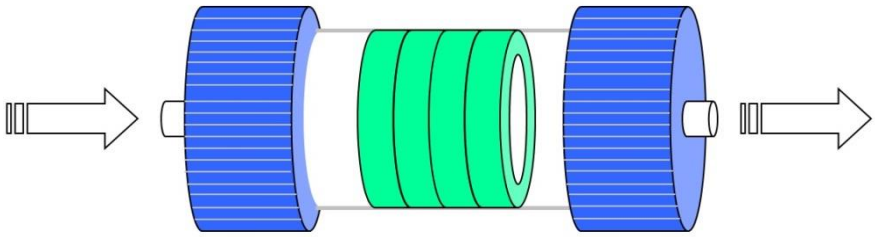
CIM® Disks – axial flow mode monolithic chromatographic column

Separation of oligonucleotides was mainly performed on commercially available CIM® DEAE (DEAE - diethylaminoethyl groups) disk columns (BIA Separations d.o.o., Slovenia). CIM® disk column consists of one or more CIM® disks packed into commercially available CIM® chromatographic housing (BIA Separations, Ljubljana, Slovenia) (**Figure 4**). Up to 4 disks can be packed into a single chromatographic housing. The bed volume of commercially available CIM® disk is 0.34 mL, outer diameter of the disk bed is 12 mm and the length of the separations layer (thickness) is 3 mm (**Scheme 4**).



Scheme 4: CIM® disk (left) and schematic presentation of CIM® disk unit packed in CIM® housing (right).

Additionally, when the effect of the length of the separation layer on a separation of oligonucleotides was studied, custom made CIM® DEAE disks with a thickness of 0,75 mm and 1,5 mm were also prepared. The length of the separation layer was also altered by placing a different number of commercially available CIM® disks units in the same housing [8]. In this way the length of the separation layer was easily prolonged (**Scheme 5**).

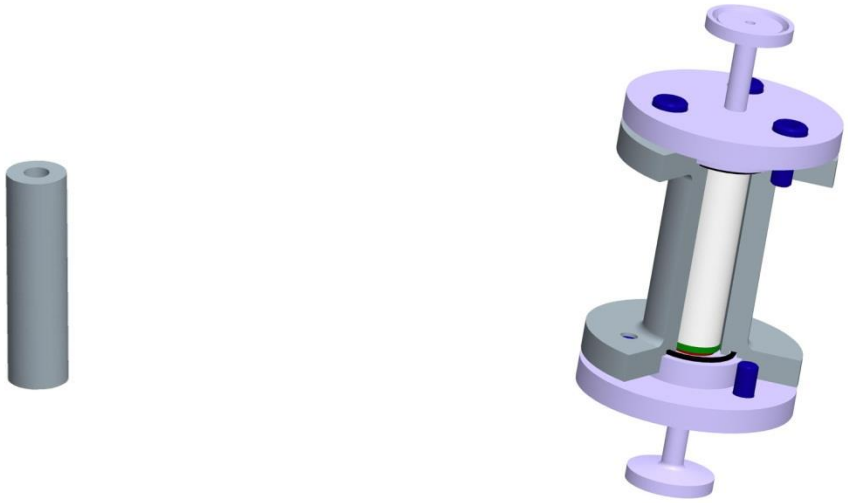


Scheme 5: Schematic presentation of 4 CIM® disks units packed in a CIM® housing.

Figure 4: Picture of complete (left) and dismounted CIM® housing.

CIM® 8 mL Tube – radial flow mode monolithic chromatographic column

All experiments concerning the possible use of thin layered GMA-EDMA monolithic chromatographic materials as high-throughput columns were performed on a novel 8 mL CIM **QA** ion-exchange radial flow monolithic column. The bed volume of CIM® 8 mL column is 8 mL. Outer diameter of the tubular monolith is 14,4 mm, inner diameter is 6,5 mm and height is 55 mm. Length of the separations layer (thickness) is 3,95 mm (**Scheme 6**). Tubular monolith was packed in a specially designed chromatographic housing with extra low dead volume. Housing was made of high grade stainless still (**Figure 5**).



Scheme 6: **CIM® 8 mL tubular monolith (left) and schematic presentation of CIM® 8 mL chromatographic column (right).**

Figure 5: **Picture of CIM® 8 mL tube monolithic chromatographic column.**

3.3. Oligonucleotide samples

The oligonucleotides, synthetized at the National Institute of Chemistry, Ljubljana, Slovenia, were of the following lengths and structure:
oligodeoxynucleotide 8 (oligo 8): C CAT GTC $T^{3'}$
oligodeoxynucleotide 10 (oligo 10): GTC CAT GTC $T^{3'}$
oligodeoxynucleotide 12 (oligo 12): AG GTC CAT GTC $T^{3'}$
oligodeoxynucleotide 14 (oligo 14): C GAG GTC CAT GTC $T^{3'}$

3.4. Mobile phases used for oligonucleotide separations

Mobile phase was a 20 mM Tris-HCl buffer, pH 8.5 with different concentrations of NaCl.

Purified water and all buffer solutions for chromatographic experiments were filtered through a 0.45 μm pore size filter composed of Sartolon polyamide (Sartorius, Goettingen, Germany).

3.5. Methods used for characterization of CIM® monolithic chromatographic columns

Oligonucleotide separations
The sample of each substance was dissolved in the appropriate mobile phase used for particular experiment and injected into the disk monolithic column under isocratic conditions. The mobile phase composition (ionic strength) was then changed in order to vary the retention time of the particular substance.

Pulse response experiments

1 mL of 16.6 % (V/V) solution of acetone in deionized water was injected as a tracer. Mobile phase was deionized water. Wavelength on detector was set to 280 nm, data acquisition frequency was 10 Hz and detector time constant was set to 0.1 to obtain the highest data density and the fastest detector response. Number of theoretical plate (N) was calculated with PeakFit software, SeaSolve Software, Inc. (Framingham, USA) Relative standard deviation (RSD) was calculated on the target set of data using Microsoft Office Excel software. (Microsoft)

Protein separations

Effect of the flow-rate on protein separation was performed by injecting 1 mL of protein mixture containing myoglobin (0.7 mg/mL), conalbumin (2.7 mg/mL) and soybean trypsin inhibitor (2,7 mg/mL) dissolved in 20 mM Tris-HCl, pH 7.4.
Separation was performed at 7 different flow rates (16, 40, 80, 160, 240, 320, 400 mL/min being equal to 2, 5, 10, 20, 30, 40 and 50 CV/min) by applying the linear gradient (from 0 M NaCl to 1 M NaCl in 20 mM Tris-HCl, pH 7.4), which was calculated for each flow-rate to be constant and equal to 8 CV.

Separation of BSA aggregates from BSA monomer was performed by injecting 1 mL of loading buffer (20 mM Tris-HCl+ 0,2 M NaCl, pH 7.4) containing BSA with concentration of 10 mg/mL on the 8 mL GMA-EDMA column. Linear gradient from 0,2 M to 0,8 M NaCl was performed in 46 sec. Flow-rate was 560 mL/min (70 CV/min). Absorbance at 280 nm was monitored.

Dynamic binding capacity

Effect of the flow-rate on protein dynamic capacity was determined by frontal analysis experiments. The column was first equilibrated with loading buffer (20 mM Tris-HCl, pH 7.4) and then loaded with protein solution (1 mg/mL BSA in 20 mM Tris-HCl buffer, pH 7.4) at flow rates from 7.5 (approximately 1 CV) to 358 (approximately 45 CV) mL/min. The absorbance at 280 nm was measured and the protein capacity at 50 % breakthrough was calculated.

Pressure drop measurement

Pressure drop was measured at different flow rates (50, 100, 150, 200, 250, 300, 350, 400 mL/min) using purified water as a mobile phase. Pressure sensor was connected at the column outlet. Experiment was repeated after disconnecting monolithic column from the HPLC system to determine pressure of the HPLC system alone. Both values were subtracted to get pressure drop on the monolithic column.

3.6. Instrumentation methods

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Efficiency of protein separation was analyzed by loading collected fractions on 20 % separation gel with 4 % stacking gel. Electrophoresis was run at 200 V for 50 min. Band detection was performed with gel staining for 2 hours in Commassie brilliant blue and washing in distillate water over the night.

3.7. Chemicals

All solutions were prepared using deionized water purified by Watek IWA-80 roi purification system (Ledeč nad Sázavon, the Czeck Republic)

Acetone was purchased from Rathburn Chemicals (Walkerburn, Scotland). Tris(hydroxymethyl)aminomethane (Tris), sodium chloride, hydrochloric acid (37 %) were obtained from Merck (Darmstadt, Germany). Proteins myoglobin, conalbumin and soybean trypsin inhibitor (STI) were purchased from Sigma-Aldrich Inc. (St. Louis, USA) while bovine serum albumin (BSA) was purchased from Fluka (Buchs, Switzerland). Buffer solutions were made by adding a known mass of buffering species to 80% of the desired final volume of purified water. Tris-HCl buffers were then titrated with HCl water solution (1:1). Molecular

weight standards LowRange Biorad (CA, USA) and 10–200 kDa MBI Fermentas (Vilnus, Lithuania) were used for electrophoreses. Commasie blue was used for protein bands detection according to the manufacturer's instructions for the PlusOne Amersham Bioscience (Uppsala, Sweden).

4. EXPERIMENTAL WORK

4.1. Isocratic separation of oligonucleotides on CIM® disks axial flow mode monolithic chromatographic column

To verify the possibility of performing isocratic separations of oligonucleotides on GMA-EDMA monolithic chromatographic material, commercially available CIM® DEAE disk was applied. The sample was a mixture of four oligonucleotides with different chain lengths: oligo 8, oligo 10, oligo 12 and oligo 14. The results are shown in **Figure 6** where all four oligonucleotides are well separated within 3 minutes.

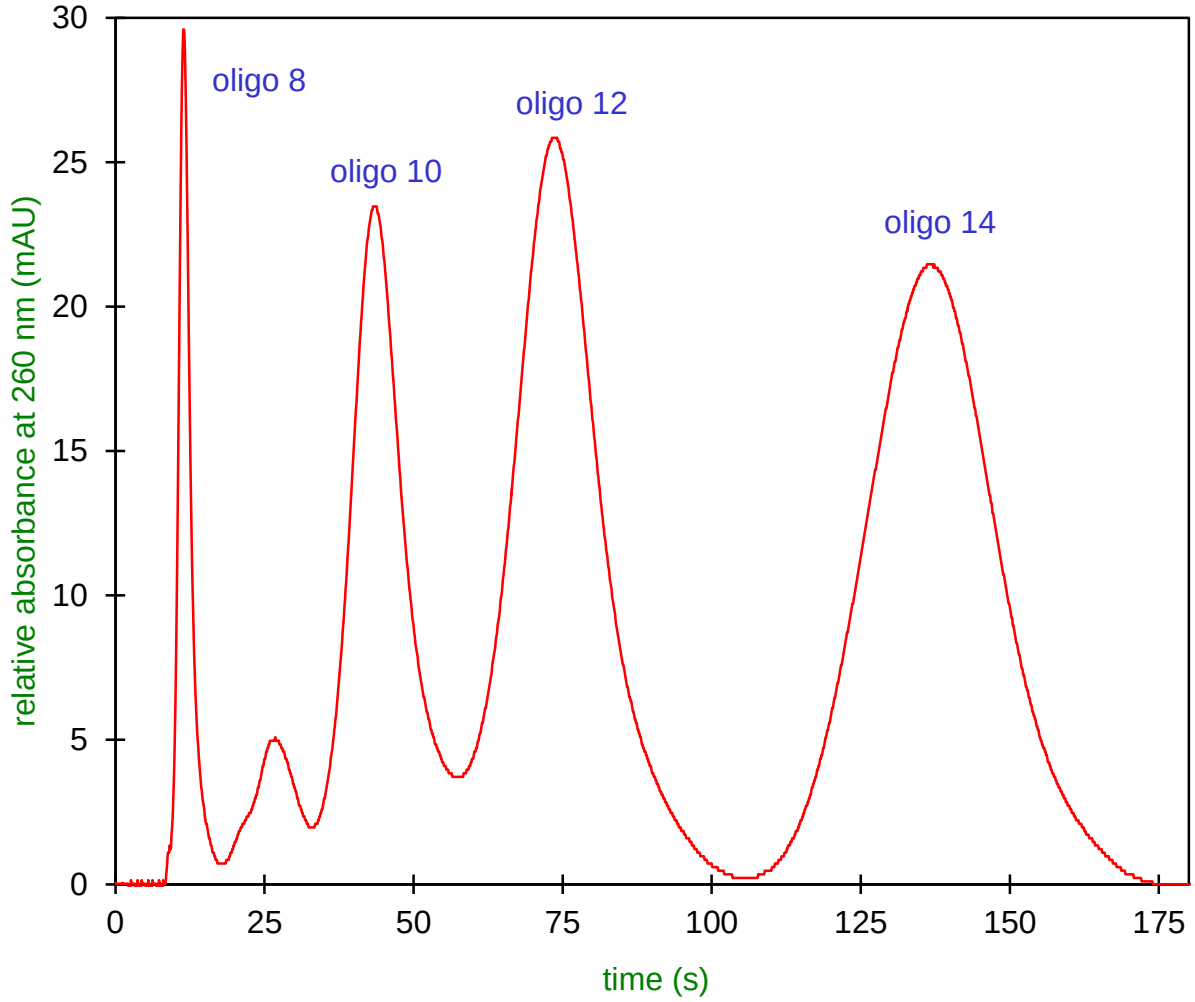


Figure 6: **Isocratic separation of oligonucleotides on a CIM® DEAE disk with the thickness of 3 mm.**

Conditions: Mobile phase: 0,46 M NaCl in 20 mM Tris-HCl buffer, pH 8.5; Flow rate: 9 mL/min; Sample: 50 μg/mL of oligo 8, 150 μg/mL of oligo 10, 450 μg/mL of oligo 12 and 750 μg/mL of oligo 14 in buffer A; Injection volume: 20 μL; Detection: UV at 260 nm.

4.1.1. Peak width of oligo 8 is proportional to its retention time

From **Figure 6** we can deduce that retention time of oligonucleotides increases with molecular weight. We could also observe a pronounced broadening of peaks with increased retention time. To investigate the correlation between peak width and the retention time isocratic runs of a single oligonucleotide oligo 8 as a sample, using mobile phase with different NaCl concentrations ranging from 1 M

to 0,38 M, were performed. The resulting chromatograms are presented in **Figure 7**.

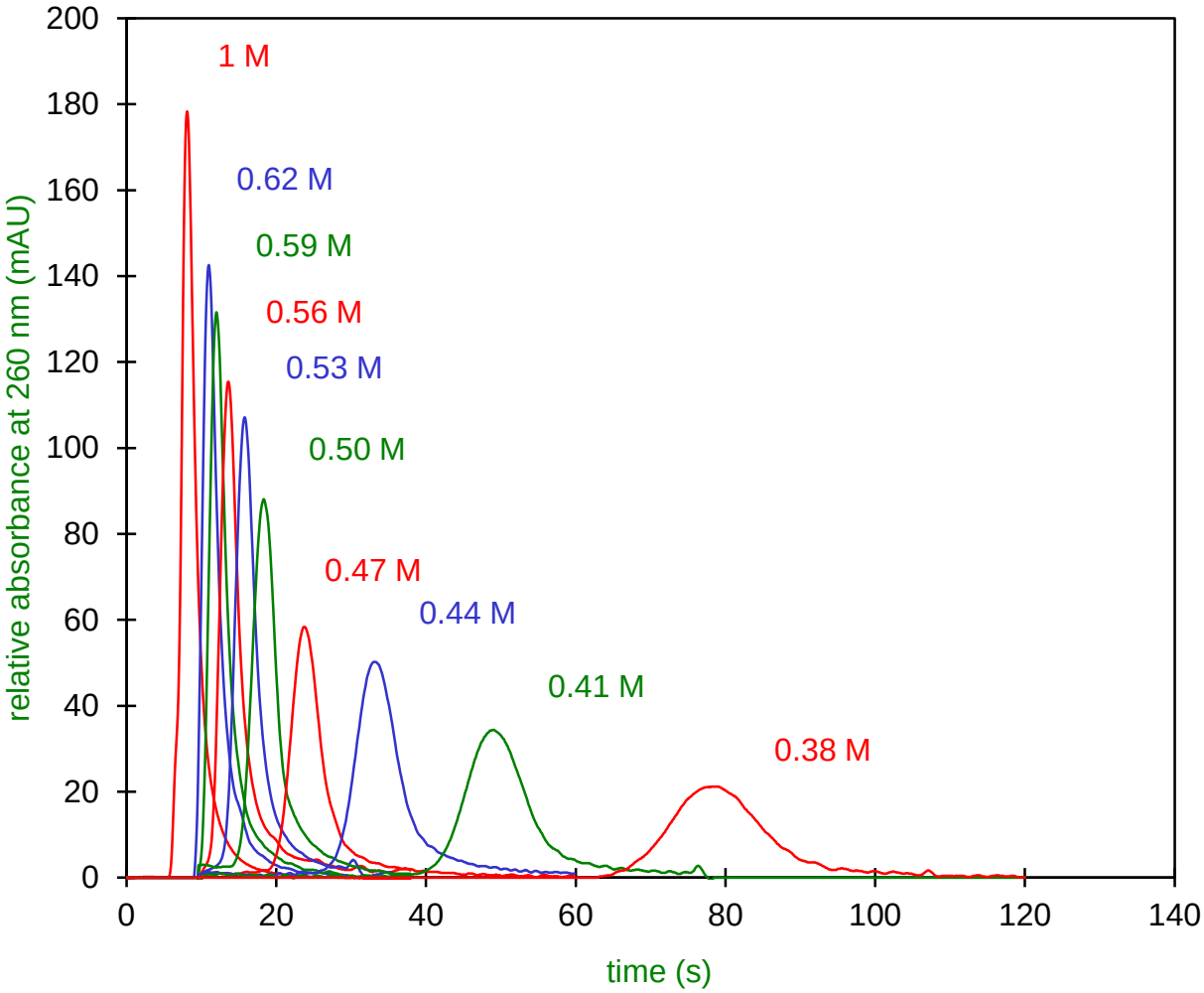


Figure 7: Effect of mobile phase composition on the retention time and peak shape of oligo 8 on a 3 mm CIM® DEAE disk.

Conditions: Mobile phase: 20 mM Tris-HCl buffer, pH 8.5 + different concentrations of NaCl (actual mobile phase composition is shown in the Figure); Flow rate: 3 mL/min; Sample: 50 μg/mL of oligo 8 in buffer A; Injection volume: 20 μl; Detection: UV at 260 nm.

The increase of salt concentration in the mobile phase decreases the retention time. When 1 M NaCl in buffer is used oligo 8 does not bind to the matrix and is eluted with the front. At very low salt concentration (only buffer A) oligo 8 binds irreversibly to the support and is not eluted at all.

According to the theory of isocratic multiple adsorption/desorption separation process, the peak width (σ_t) is proportional to the retention time (t_r) via equation

$$\sigma_t = \sqrt{\frac{HETP}{L}} \cdot t_r \qquad (6)$$

[17]:

The experimentally determined peak width, σ_t, was measured from the oligo 8 peaks at different molar concentrations of sodium chloride. The resulting values of σ_t are plotted against the retention time, t_r, in the **Figure 8**.

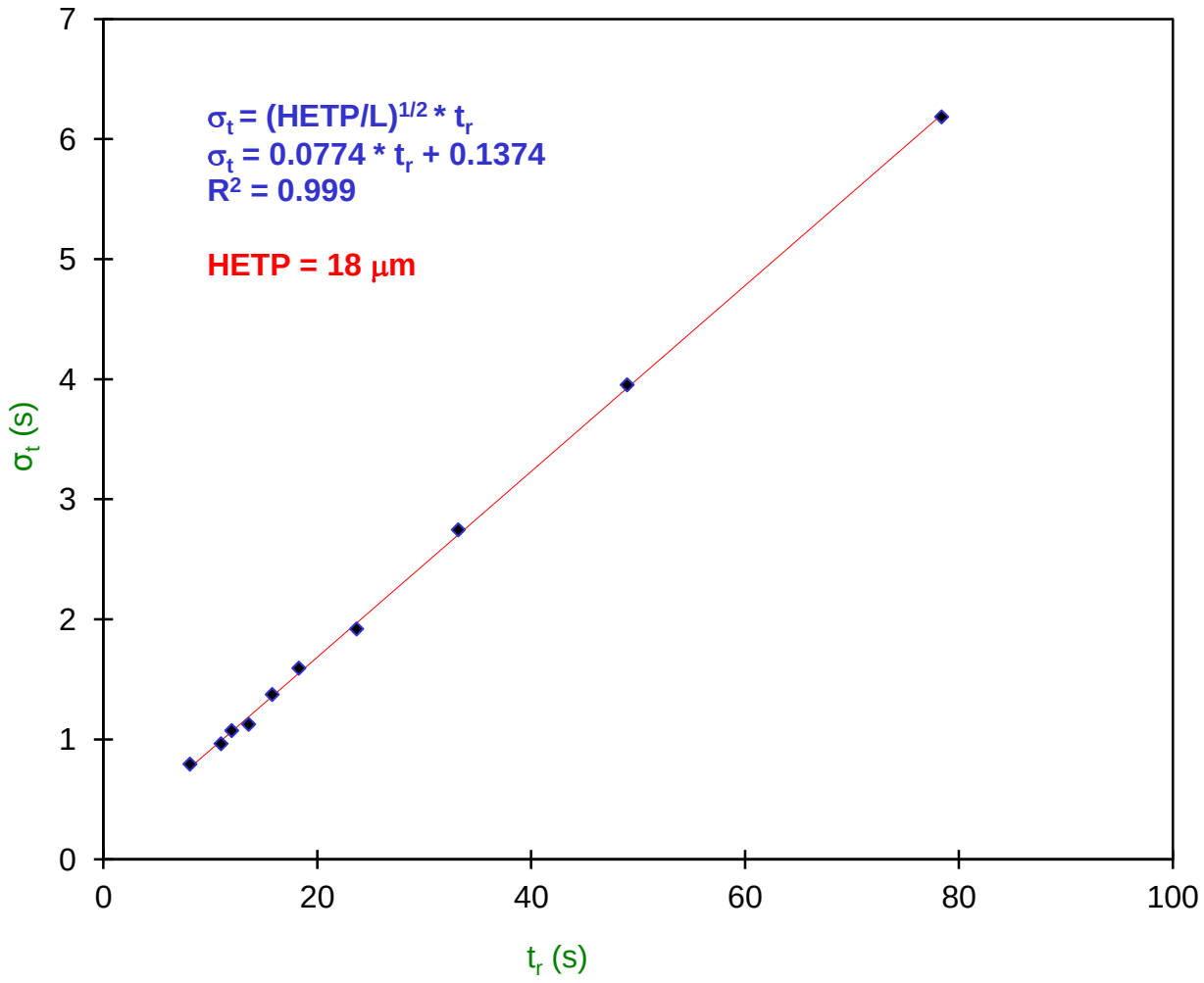


Figure 8: **Standar deviation (σ_t) of peak as a function of the retention time (t_t). Data was derived from chromatograms presented in figure 7.**

The linear relationship between σ_t and t_r as predicted by Eq. (6) is clearly demonstrated (R^2 = 0.999). The slope of the line is equal to 0.0774, which translates to the HETP (height equivalent to the theoretical plate) value corresponding to 18.0 μm. This value approaches the values of HETP for conventional HPLC columns filled with 5-7 μm porous particles.

4.1.2. Peak height of oligo 8 is inversely proportional to its retention time

Furthermore, for the peaks that can be described by a Gaussian function, characteristic for chromatographic processes based on multiple adsorption/desorption steps, the maximum peak height (h_{max}) is inversely proportional to the retention time [17]:

$$h_{max} = \frac{1}{R \cdot v \cdot \sqrt{2\pi \cdot \frac{HETP}{L}}} \cdot \frac{1}{t_r} \qquad (7)$$

Experimental values of peak heights of oligo 8 correlated to reciprocal values of the retention times can be successfully fitted with equation (7) giving the correlation index R^2 = 0.988.

From these results a close similarity between the isocratic separation mechanism on conventional HPLC columns based on multiple adsorption/desorption steps and isocratic separation on the thin GMA-EDMA monolithic chromatographic material is indicated.

4.1.3. Effect of the monolith thickness (column length) on a resolution

If multiple adsorption/desorption steps occur in thin GMA-EDMA monoliths, the retention time should be proportional to the monolith thickness [17]. To investigate this possibility, monoliths of different lengths were prepared. Firstly, one disk was placed in the appropriate housing and then the separation of oligonucleotides was carried out. In the next experiment, an additional disk was placed in the same housing. This progression was continued by placing 3 and 4 disks in the same housing. In this way monoliths with different lengths were prepared, with a thickness ranging from 3 mm (one disk in the housing) to 12 mm (4 disks in the housing), which should be equivalent to a single monolith of the same thickness assuming that the interface effects can be neglected. This assumption is justified by the similarity existing between isocratic separation on a single 3 mm disk and a combination of two disks with the thickness of 1.5 mm in the same housing. In addition, specially prepared thin monolithic disks with a thickness of only 1.5 and 0.75 mm were also applied.

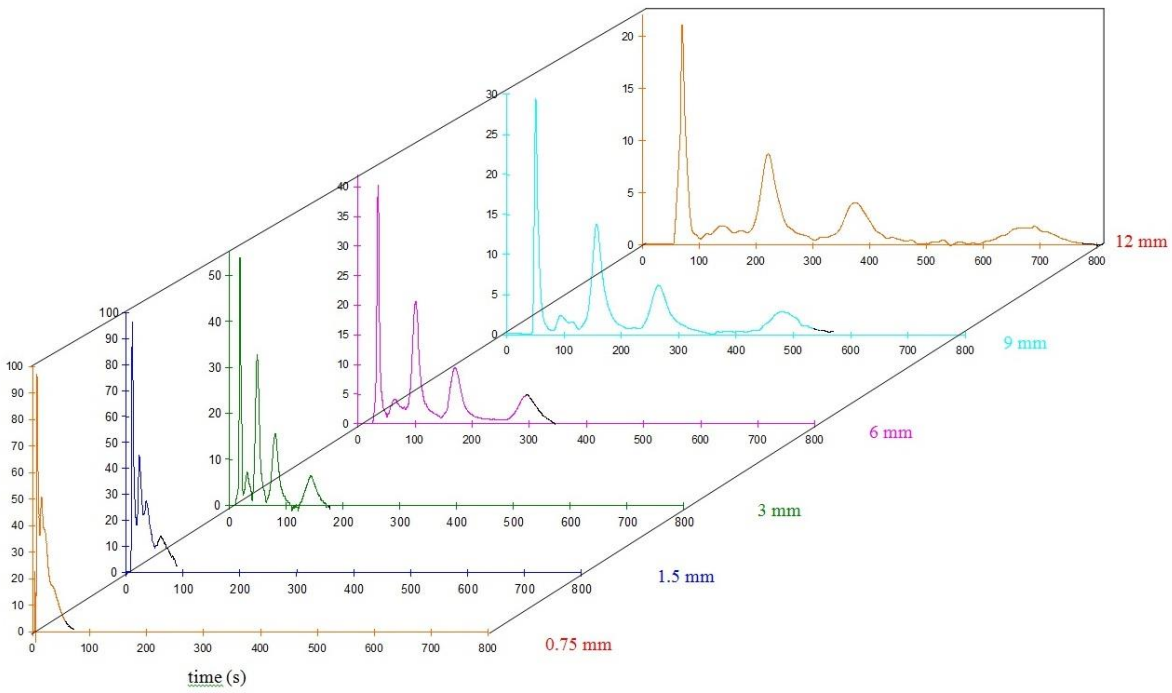


Figure 9: **The effect of the monolith thickness (column length) on the resolution of the oligonucleotide isocratic separation.**

Conditions: Mobile phase: 0,5 M NaCl in 20 mM Tris-HCl buffer, pH 8.5; Separation unit: one or more CIM DEAE disks in the same housing (one 0,75 mm disk or one 1,5mm disk or one 3 mm disk or two 3 mm disks to form 6 mm column length or three 3 mm disks to form 9 mm column length or four 3 mm disks to form 12 mm column length); Flow rate: 3mL/min; Sample: 50 μg/mL of oligo 8 (1st peak; in the insert), oligo 10 (2nd peak; in the insert), oligo 12 (3rd peak; in the insert) and oligo 14 (4th peak; in the insert) in buffer A; Injection volume: 20 μL; Detection: UV at 260 nm.

Figure 9 shows that the separation improves significantly with the increase of the column length. In fact, fitting the relationship between the retention times of certain oligonucleotide and a particular column length, the straight line obtained provides a correlation index higher than 0.99 for all four oligonucleotides. The dependency of the retention times and the length of the column for all four oligonucleotides is presented in **Figure 10.**

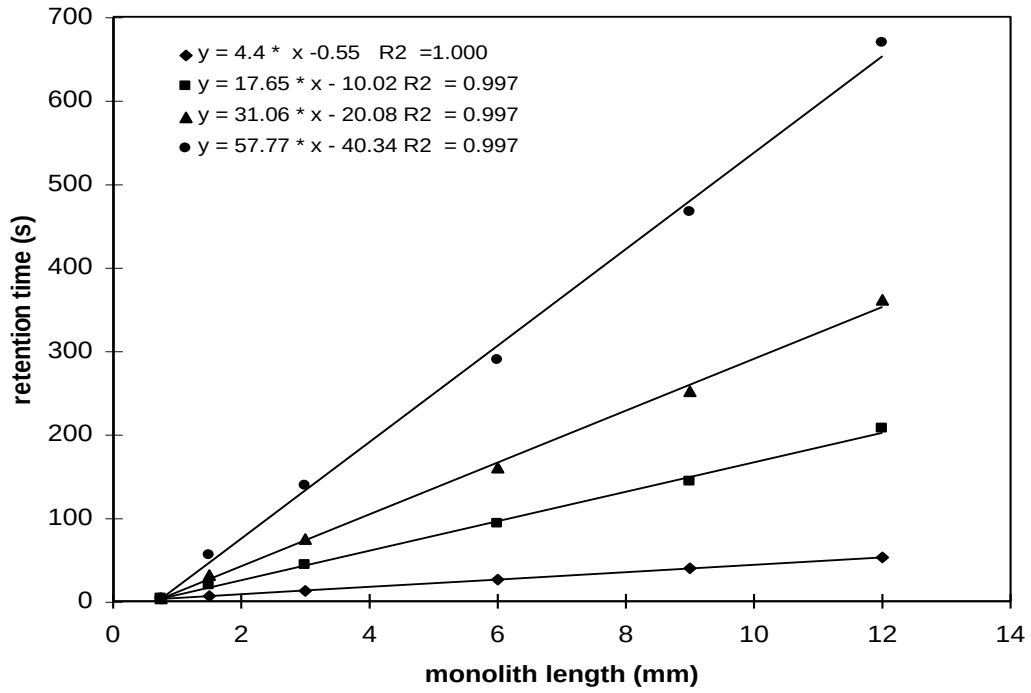


Figure 10: **The retention time dependency on the monolith thickness.**

Under this salt concentration, separation on the 0.75 mm GMA-EDMA monolith was not achieved. However, after the optimization of the mobile phase composition, taking into account the special requirements of the disk related to its thickness, the separation of all oligonucleotides was successfully carried out also on the ultra-thin 0.75 mm GMA-EDMA monolith (**Figure 11)**.

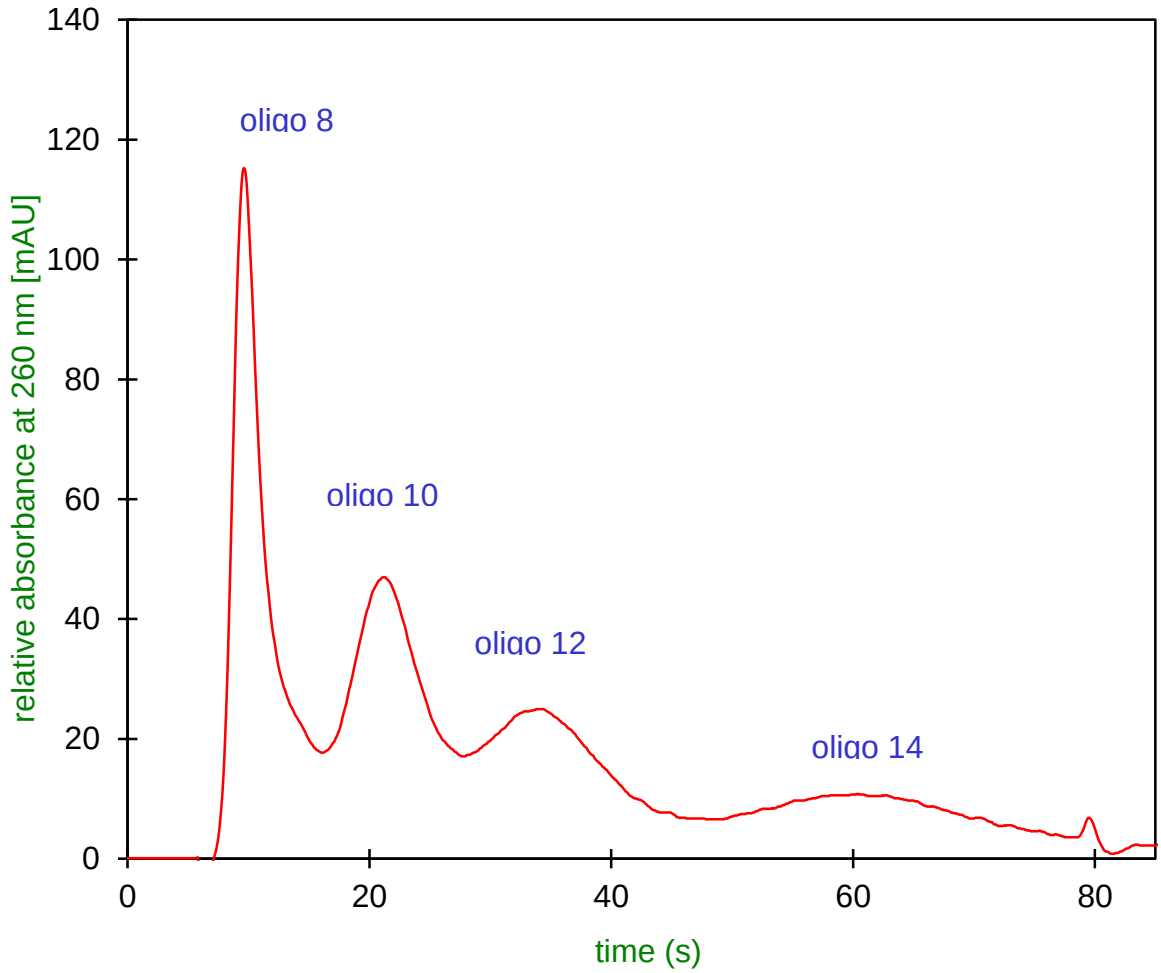


Figure 11: **Isocratic separation of oligonucleotides on a CIM DEAE disk with the thickness of 0.75 mm.**

Conditions: Mobile phase: 0,37 M NaCl in 20 mM Tris-HCl buffer, pH 8.5; Flow rate: 3 mL/min; Sample: 50 μg/mL of oligo 8, oligo 10, oligo 12 and oligo 14 in buffer A; Injection volume: 20 μL; Detection: UV at 260 nm.

These data demonstrate that also very thin GMA-EDMA monoliths enable isocratic separation. This rather surprising result could be ascribed to the particular matrix structure.

4.1.4. Effect of the flow rate on a resolution

Since monoliths have low diffusion resistance due to convective transport inside the flow-through pores it was shown that the flow rate has no pronounced effect on

the resolution [3]. In contrast to this fact, in the case of isocratic separation of plasmid DNA conformers it was found that there is an optimum flow rate around 1 mL/min [10]. Therefore, the effect of the flow rate on the isocratic separation of 4 oligonucleotides was also investigated. The flow rate was measured by a validated flow meter to provide accurate data for further analysis, where the results are normalized to the volume of mobile phase run through the GMA-EDMA monolith. The effect of the flow rate was investigated on CIM DEAE disk of 3 mm thickness.

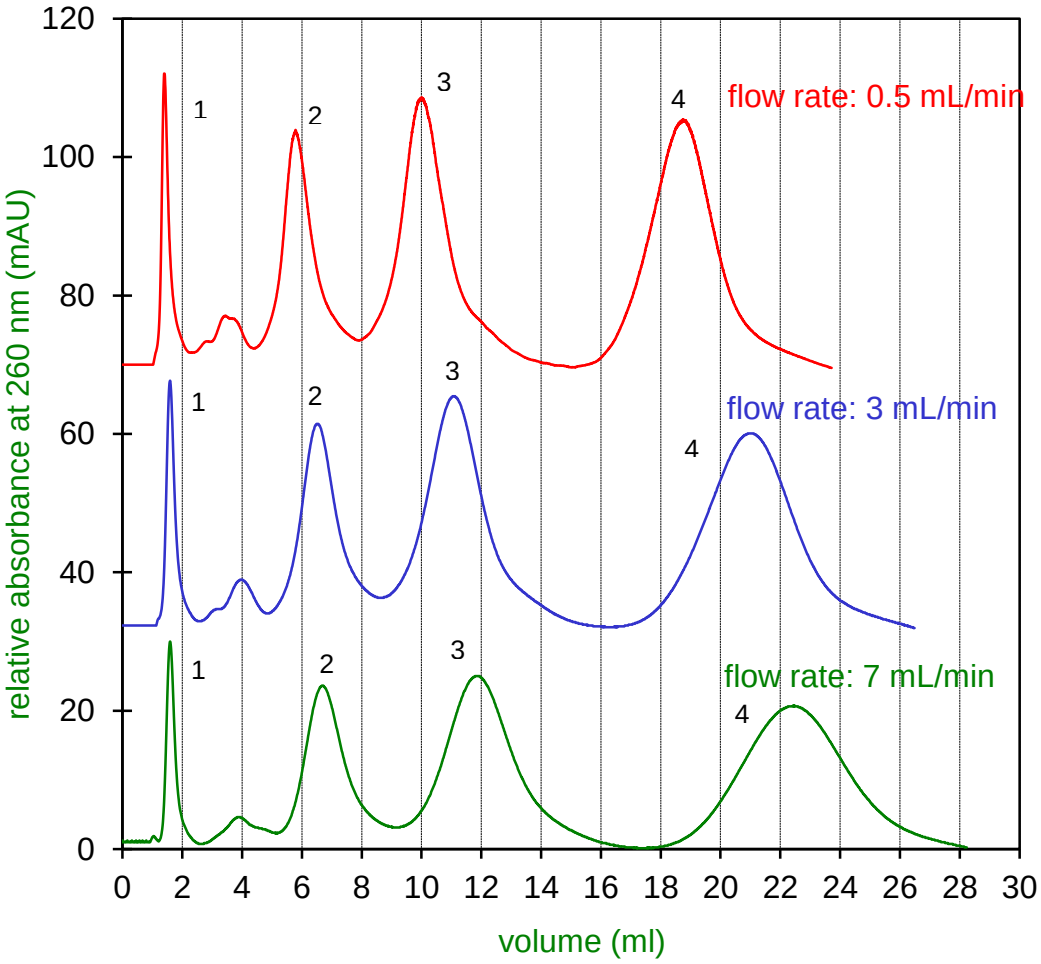


Figure 12: **Effect of the flow rate on the isocratic separation of oligonucleotides on a CIM DEAE disk with the thickness of 3 mm.**

Conditions: Mobile phase: 0,46 M NaCl in 20 mM Tris-HCl buffer, pH 8.5; Sample: 1: 50 μg/mL of oligo 8, 2: 150 μg/mL of oligo10, 3: 450 μg/mL of oligo12 and 4: 750 μg/mL of oligo 14 in buffer A; Injection volume: 20 μL; Detection: UV at 260 nm.

Surprisingly, it was found that an increase in flow rate has a beneficial effect in terms of the difference between peak retention volumes. This phenomenon is presented in **Figure 12** and can possibly be explained from the hydrodynamic point of view. Higher flow rate causes higher back pressure drop on the monolith. As a consequence, mobile phase containing the molecules to be separated can penetrate into pores of smaller diameter [9,10]. In this way, the active surface available for the adsorption/desorption process is larger, possibly resulting in longer retention times.

From these results, another confirmation of the separation process based on multiple adsorption/desorption mechanisms can be derived. Some speculated the possibility that the separation under isocratic condition occurs due to shear forces.

In contrast to separations carried out in conventional HPLC columns, where fluid in the pores is stagnant, in monoliths there is a laminar convective flow throughout the pores. This flow could cause shear forces relative to the surface where the molecules are adsorbed. It is possible that these forces could cause some kind of erosion of adsorbed molecules from the surface. Since the molecules are bound to the surface to a different degree because of a varying number of binding sites, they can be selectively desorbed. This mechanism would lead to two effects: since the shear forces increase with increased flow rate, it is reasonable to speculate that the resolution would decrease. On the other hand, at very low flow rates shear forces would be probably too low to desorb some strongly bound molecules. However, these two effects were not observed either at a very low flow rate, where also all molecules desorb from the matrix, or at higher flow rates, where the separation even improves and no reduction in retention volume is observed (**Figure 12**). Since both effects are different from the expected one in the case of the shear rate elution, the multiple adsorption/desorption process is the most probable phenomenon occurring.

All data show that equations derived for isocratic separations based on multiple adsorption/desorption steps can also be efficiently applied for isocratic separations

on thin monoliths. Therefore, it is reasonable to assume that similar mechanisms also occur in monoliths. It is surprising, however, that the 0.75 mm thick monolith can provide sufficient travel length for this phenomenon to occur. There are two characteristics of the monoliths that might help to explain these results. The flow-through pores in the GMA-EDMA monoliths have a broad size distribution [18]. In addition, they are highly interconnected. Therefore, the molecules travelling through the monolith have to pass many constrictions and tortuosities. As a consequence, the real path through the monolith is longer than its physical thickness. Thus, the molecules have more time to interact with the surface. The second characteristic of the monoliths is the reduced diffusion resistance. In the case of particle supports the diffusion through the stagnant liquid inside the pores is a major bottleneck of the mass transfer exchange between the mobile phase and the surface of the support where the active groups are located. In the case of monoliths, this stagnant layer is extremely reduced and the mass exchange is much faster. The main result is that on the same length of the separation layer, many more adsorption/desorption steps can take place in monoliths than in the conventional porous supports. Thus, a much thinner monolith is sufficient for achieving the same number of adsorption/desorption steps.

In addition, by comparing the separation of plasmid DNA conformers [10] and oligonucleotides, better separation in the case of the latter was achieved. This might be due to the smaller molecular size of the oligonucleotides and, consequently, faster diffusion. It can be concluded that, in contrast to the selective gradient elution where the separation resolution commonly improves with increased molecular weight [9], in the case of isocratic separations smaller molecules can be separated more easily.

4.2. Design and characterization of high throughput CIM® 8 mL tube radial flow mode monolithic chromatographic column

In the first part of the wok it was shown that GMA-EDMA monoliths material can be prepared in extremely thin forms and then used as a stationary phase for preparation of extremely short HPLC columns. In spite of very short separation path due to their unique structure GMA-EDMA monolithic columns still enable very good separation of oligonucleotides in isocratic mode.

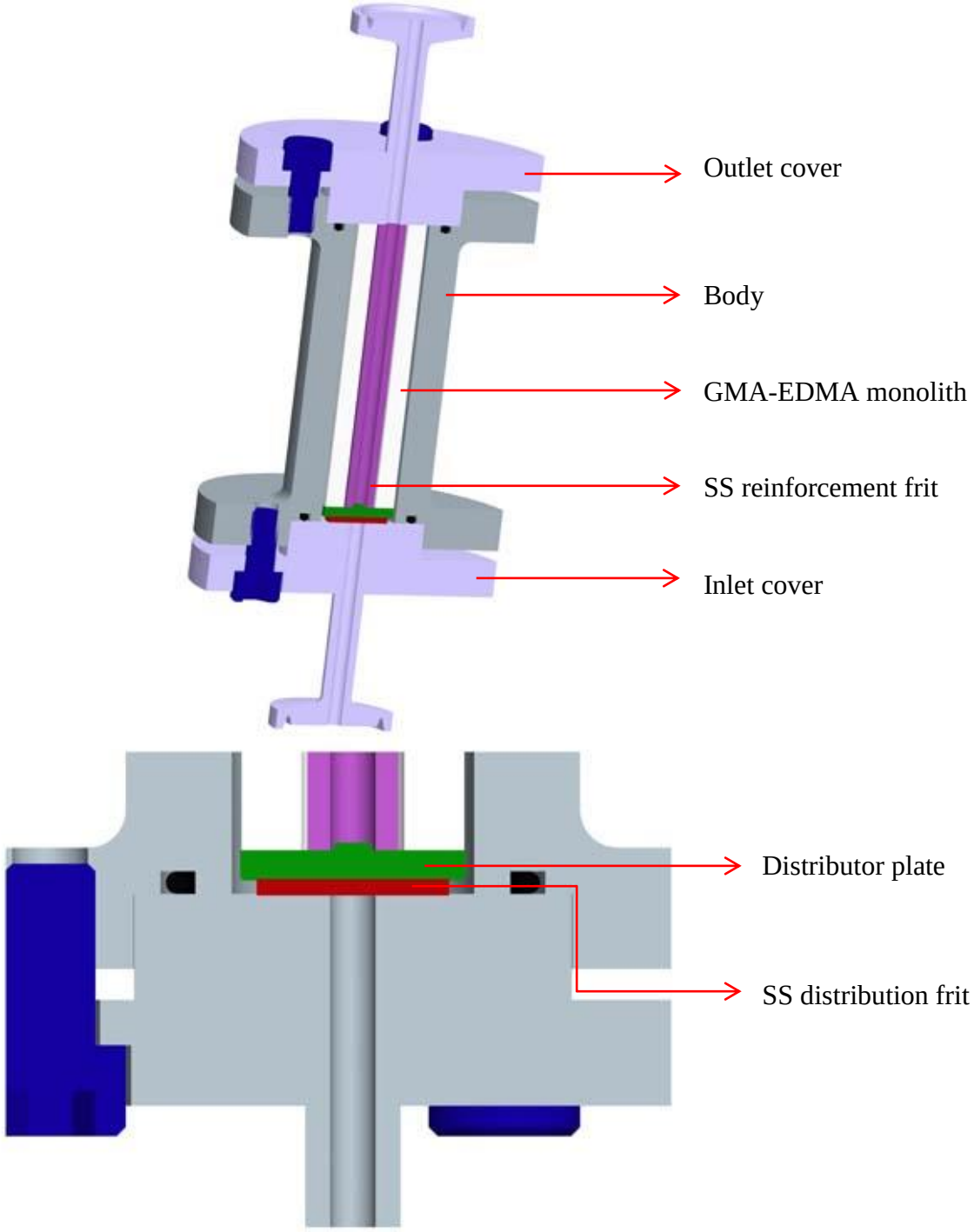
Additionally, monolithic chromatographic columns, when used in a gradient separation mode, also provide an excellent tool for fast and efficient separations of very large biological molecules. If it were possible to design very thin layered monoliths also on preparative or even industrial scale, significant benefits can be expected. However, to develop such chromatographic columns appropriate chromatographic housing must be designed.

On the market there are different designs of chromatographic columns, which can be divided into two main groups. Most of today's HPLC column belongs to the group of so-called axial mode operating columns, while the radial one with a radial flow pattern are more rear. Which type performs better depends on the particular case [23] but it seems that radial operating columns attracting interest since they exhibit some beneficial features [24].

One of the main problems of radial operating chromatographic columns is the changing of a mobile phase linear velocity over the chromatographic bed. Because of that, matrix efficiency for porous particulate supports varies by its position within the bed, and overall performance is more difficult to predict. This problem is not present when the monolithic supports are used, since it was demonstrated that their chromatographic properties are flow unaffected even at the extreme linear velocity of 50000 cm/h [25] which was confirmed also for radial operating mode [26]. It was demonstrated that even in the range of applicable flow-rates, bed

utilization for monolithic column operating in a radial mode is still close to 100 % [27]. Therefore, one can hardly distinguish between axial and radial operating monolithic chromatographic column of the same bed length. This, together with lower pressure drop of a radial design [28], was the motivation for designing a new monolithic column in a radial operating mode.

The monolith and housing were designed for extremely high flow rates, up to 70 CV/min which is the range of the flow-rates applied on membrane columns. This was achieved by proper monolith dimensions with the height of 55 mm, inner diameter of 6.0 mm and thickness of just 4.5 mm. The construction of radial flow monolithic column is shown in **Scheme 7**.



Scheme 7: **Sliced design of 8 mL radial monolithic column with indicated parts.**

The monolith is fixed between two stainless steel (SS) covers with TC connectors that prevent bypassing. Two covers close the column on both sides and are fixed to the SS column body with SS bolts. Inside the monolith, there is a SS reinforcement frit. The monolith is surrounded by the SS body cylinder and has a flow distributor

that uniformly spreads the mobile phase over the entire outer surface of the monolith.

4.3. Properties of high throughput CIM® 8 mL tube radial flow mode monolithic chromatographic column

4.3.1. Mobile phase distribution as a function of flow rate

To investigate the properties of the described design several pulse response tests at different flow rates, from 20 up to 70 CV/min, were performed. Such a broad range of flow rates could affect distribution of the mobile phase over the outer surface of the monolith and consequently influence column efficiency. To investigate all these data, pulse response experiments were normalized to the elution volume and plotted in the same chromatogram (**Figure 13**).

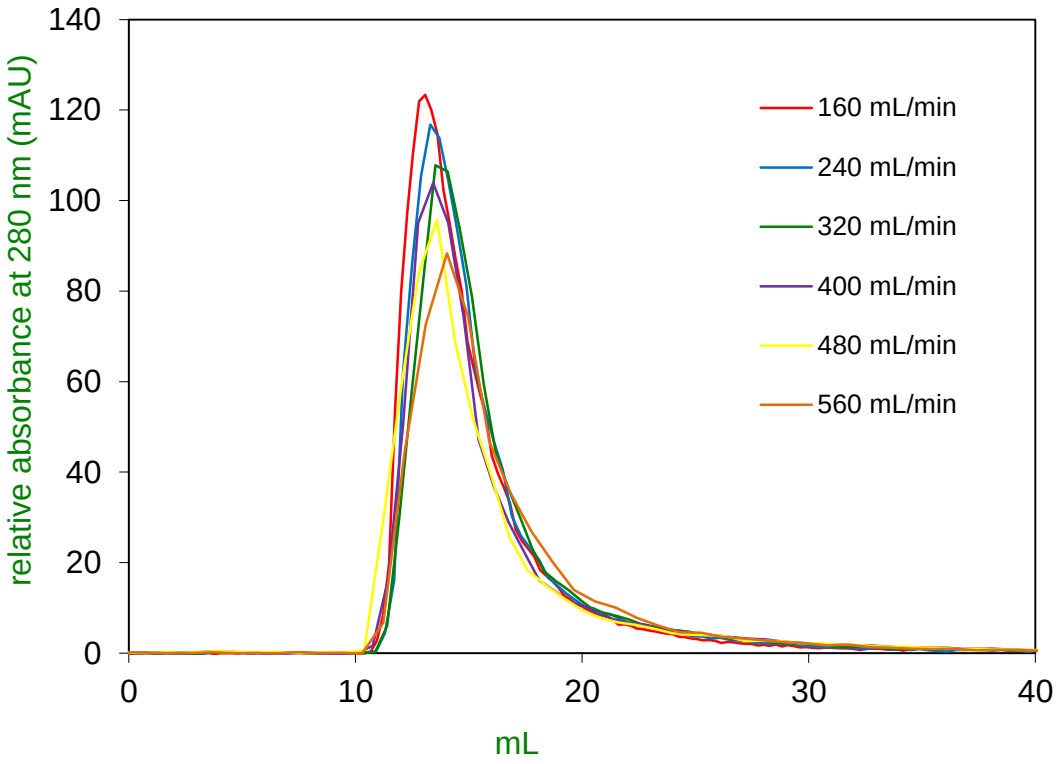


Figure 13: **Pulse response experiment as a function of flow rate.**

Conditions: Mobile phase: deionized water; Flow rate: 160, 240, 320, 400, 480, 560 mL/min; Sample: 1 mL of 16,6 % acetone solution; Injection volume: 1 mL; Detection: UV at 280 nm; Data acquisition frequency was 10 Hz and detector time constant was set to 0,1

At **Figure 13** we can see from the peak overlapping that there was a slight trend of efficiency change over the entire range, also confirmed by calculation on a number of theoretical plates (N-plate). Values were scattered within 20,7 % RSD (**Table 2**).

Table 3: **Scattering of N-plate values in pulse response experiment as a function of flow rate.**

Flow rate (mL/min)	N-plate (EMG)
160	93,7
240	81,4
320	77,5
400	73,0
480	68,8
560	47,9

RSD	20,7

To investigate whether this was due to changes in column performance or due to small variations in HPLC system performance consecutive pulse response injections were performed at fixed flow-rate of 10 and 30 CV/min (**Figures 14 and 15**).

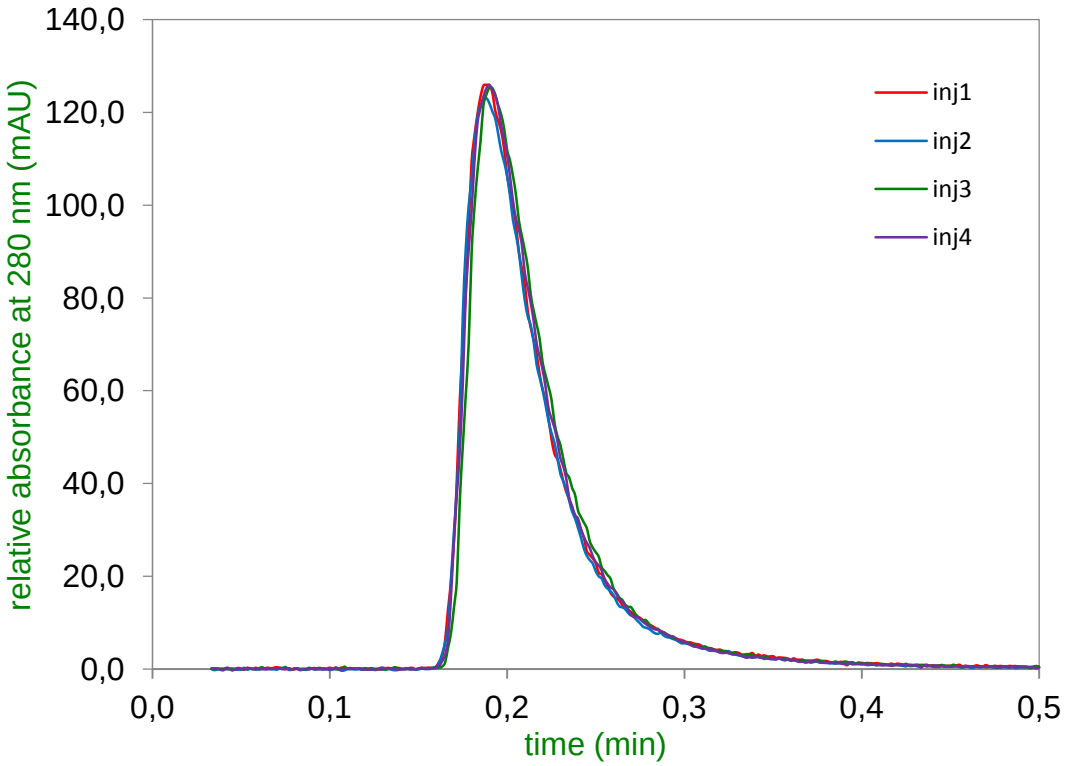


Figure 14: **Pulse response experiment at 80 mL/min as a function of injections.**

Conditions: Mobile phase: deionized water; Flow rate: 80 mL/min; Sample: 1 mL of 16,6 % acetone solution; Injection volume: 1 mL; Detection: UV at 280 nm; Data acquisition frequency was 10 Hz and detector time constant was set to 0,1

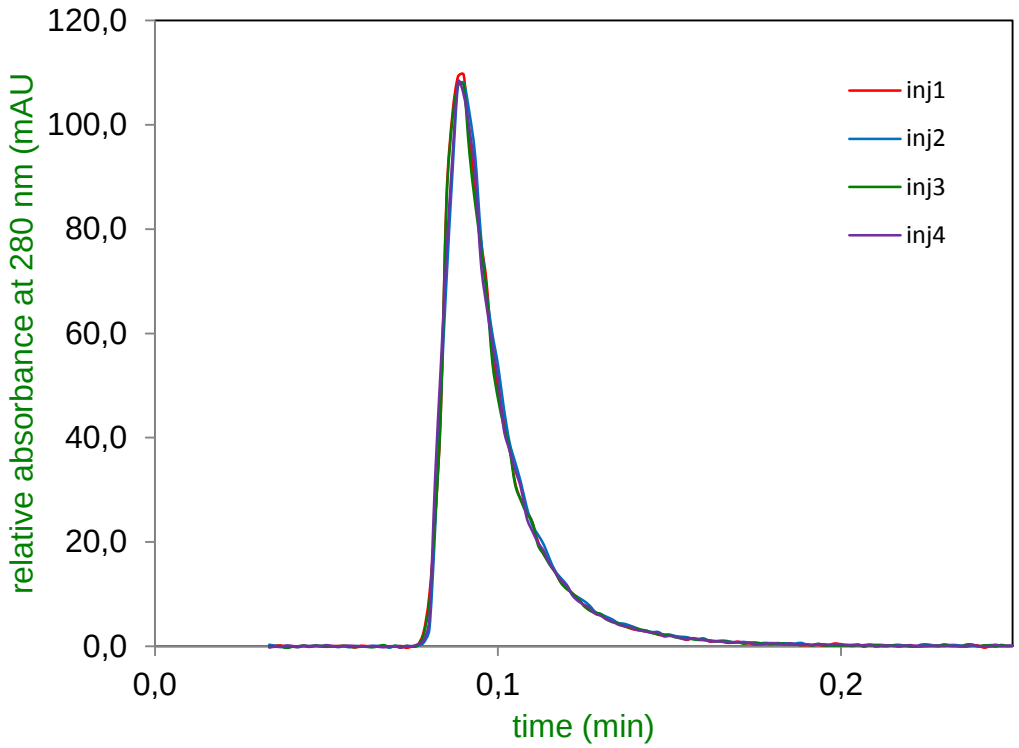


Figure 15: **Pulse response experiment at 240 mL/min as a function of injections.**

Conditions: Mobile phase: deionized water; Flow rate: 240 mL/min; Sample: 1 mL of 16,6 % acetone solution; Injection volume: 1 mL; Detection: UV at 280 nm; Data acquisition frequency was 10 Hz and detector time constant was set to 0,1

Table 4: **Scattering of N-plate values in pulse response experiment as a function of injection at 80 and 240 mL/min.**

80 mL/min

Serial injection	N-plate (EMG)
Inj 1	114,8
Inj 2	108,2
Inj 3	115,4
Inj 4	110,8
RSD	3,8

240 mL/min

Serial injection	N-plate (EMG)
Inj 1	176,8
Inj 2	188,4
Inj 3	178,0
Inj 4	188,2
RSD	3,5

The number of theoretical plates together with RSD was found to be **3,8 %** at 10 CV/min and **3,5 %** at 30 CV/min. This indicates that injection to injection reproducibility indeed causes constant variation of performance in a range of about 4 % regardless of applied flow-rate, on the other hand it is still not so high as we

can observe in **Figure 13,** where pulse response experiment as a function of flow rate was performed.

A close look back to **Figure 13** reveals a constant decrease of the peak height and an increase of the flow rate. To investigate the cause of such behavior the column was replaced with a union fitting and pulse response tests at different flow rates were repeated.

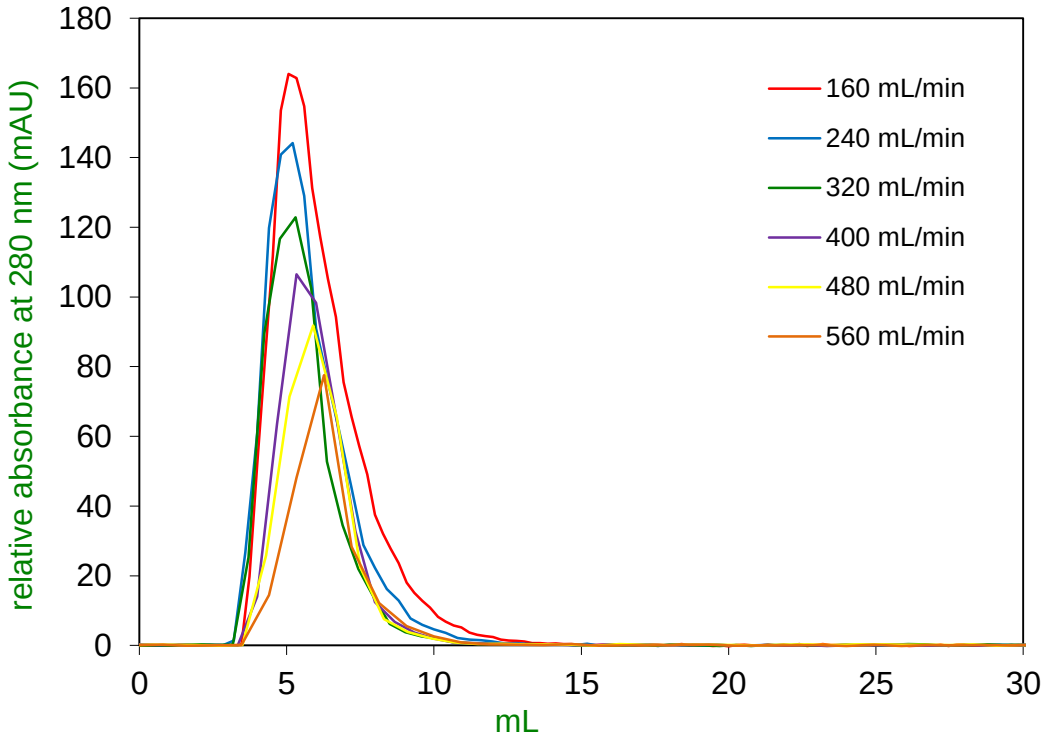


Figure 16: **Pulse response experiment without column as a function of flow rate.**

Conditions: Column: union was used instead of column; Mobile phase: deionized water; Flow rate: 160, 240, 320, 400, 480, 560 mL/min; Sample: 1.mL of 16,6 % acetone solution; Injection volume: 1 mL; Detection: UV at 280 nm; Data acquisition frequency was 10 Hz and detector time constant was set to 0,1

Table 5: **Scattering of N-plate values in pulse response experiment as a function of flow rate.**

Flow rate (mL/min)	N-plate (EMG)
160	95,4
240	73,0
320	69,4
400	53,0
480	55,6
560	53,2
RSD	23,9

A very similar trend of peak height decrease in case of higher flow rates was observed in experiment when the chromatographic column was replaced with the union fitting (**Figure 16** and **Table 4**). This deviation probably needs to be ascribed to the limitation of data collecting capability of detector since peak width at higher flow rates was in range of second. The peaks are actually so sharp that the maximum of the peak can be lost between two neighboring data points, as already described in literature [29]. These data demonstrate that variations are in fact caused by HPLC system itself while column performance is preserved over the entire tested flow rate.

4.3.2. Chromatographic properties

The performance of developed column was further tested by investigating its chromatographic properties. In **Figure 17** a separation of standard protein mixture at various flow rates (up to 50 CV/min) normalized to elution volume is presented.

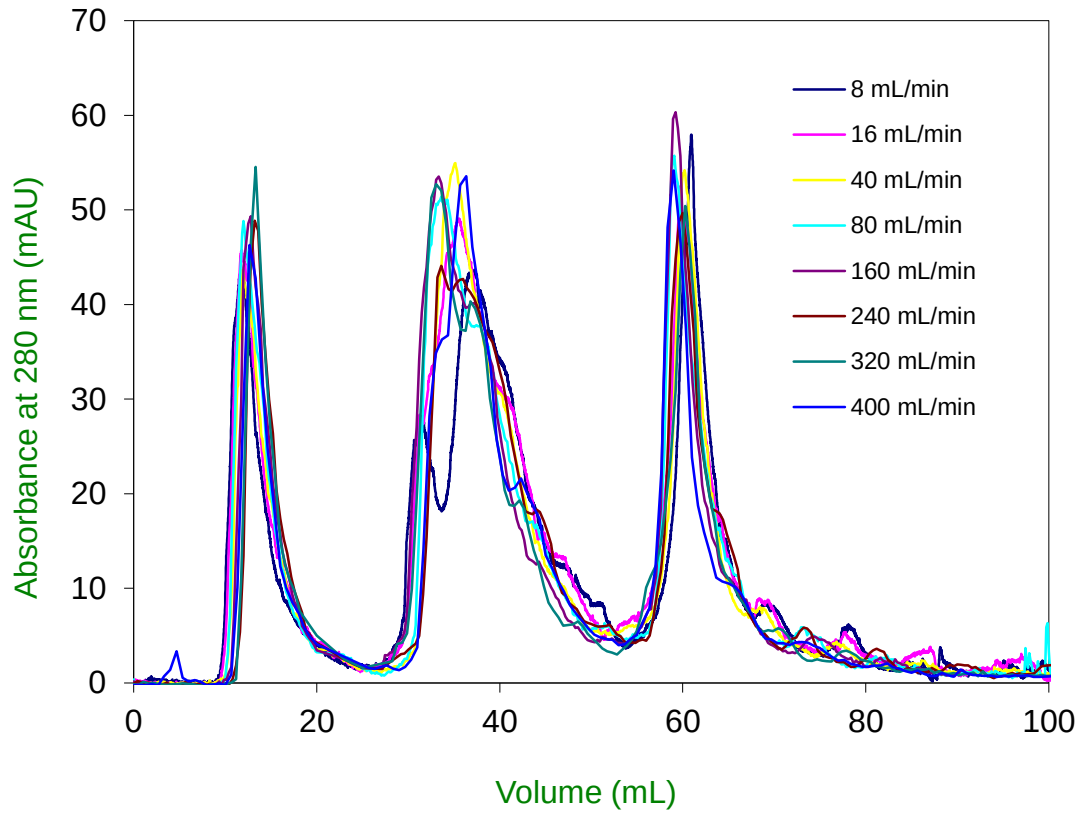


Figure 17: **Effect of flow rate on protein separations.**

Conditions: Mobile phase: mobile phase A, 20mM Tris-HCl buffer pH 7.4; mobile phase B, 1M NaCl in buffer A, pH 7.4; Flow rate: 16, 40, 80, 160, 240, 320, 400 mL/min; Sample: myoglobin (0,7 mg/mL), conalbumin (2,7 mg/mL) and STI (2,7 mg/mL) dissolved in mobile phase A; Injection volume: 1 mL; Linear gradient from 0-100% buffer B in 8 CV; Detection: UV at 280 nm; Data acquisition frequency was 10 Hz and detector time constant was set to 0,1

Again it can be noted that no significant change in resolution is brought about by changing the flow rate. A similar conclusion can be arrived at based on the dynamic binding capacity measurements performed by loading BSA on the monolithic column at different flow rates (**Figure 18**).

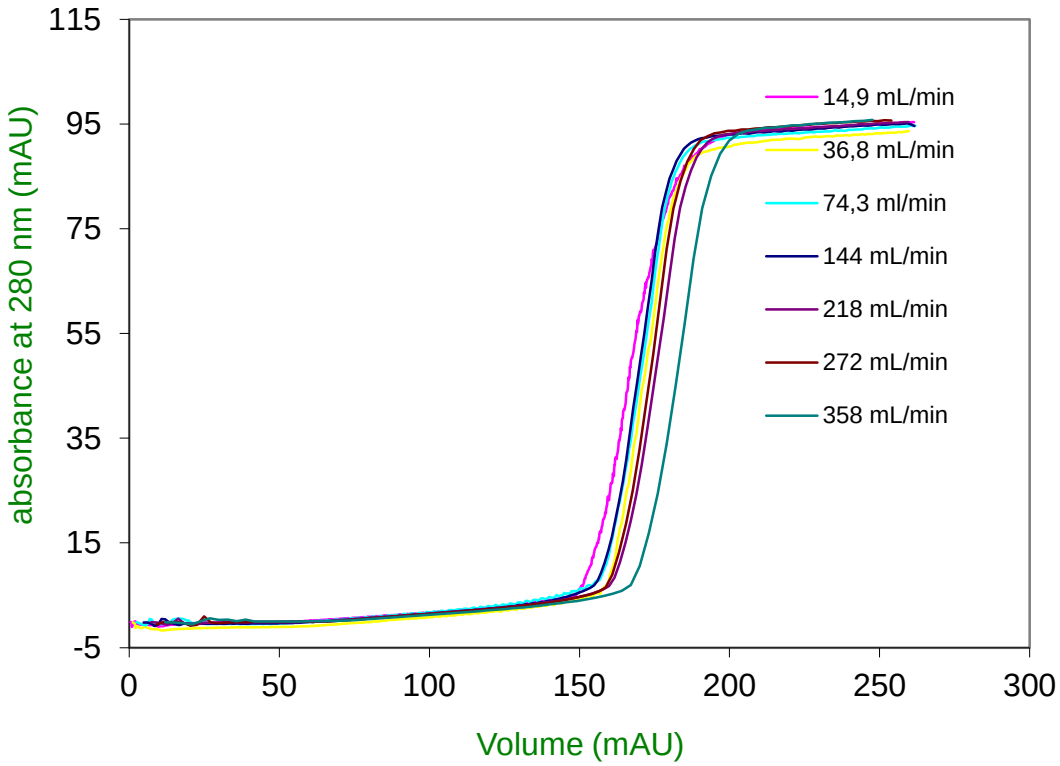


Figure 18: **Effect of flow rate in dynamic binding capacity.**

Conditions: Mobile phase: 1mg/mL BSA dissolved in 20mM Tris-HCl buffer pH 7.4; Flow rate: 15, 37, 74, 144, 218, 272, 358 mL/min; Detection: UV at 280 nm

Despite a very high flow rate, pressure drop vs. linear velocity curve is straight (**Figure 19**), demonstrating that no compression of monolith is present. Furthermore, pressure drop at the highest flow rate was only 15 bars.

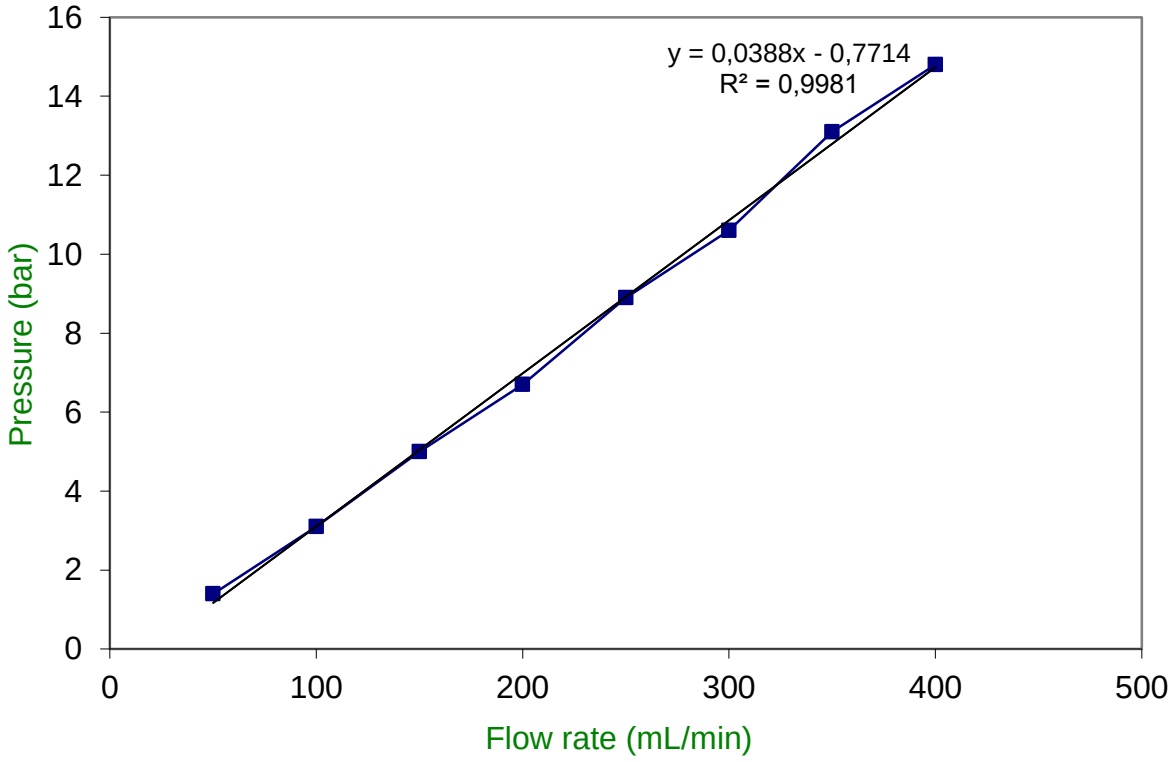


Figure 19: **Pressure drop as a functions of flow rate.**

Conditions: Deionized water was used as a mobile phase

4.3.3. Packing and refill

Such column design also allows a refill of the monolith by easily taking out an old monolith and exchanging it with a new one. To investigate how reproducible such a procedure can be the column was disconnected from the HPLC system, the outlet cover was unscrewed from the column (**Scheme 7**), the monolith, was taken out than placed back into the same housing, the outlet cover was again tightened and the column was reconnected to the HPLC system. The entire procedure was completed within 2 minutes. After each refill, pulse response test procedure at the flow rate of 30 CV/min was performed and the number of theoretical plates (N) was calculated (**Figure 20** and **Table 5**).

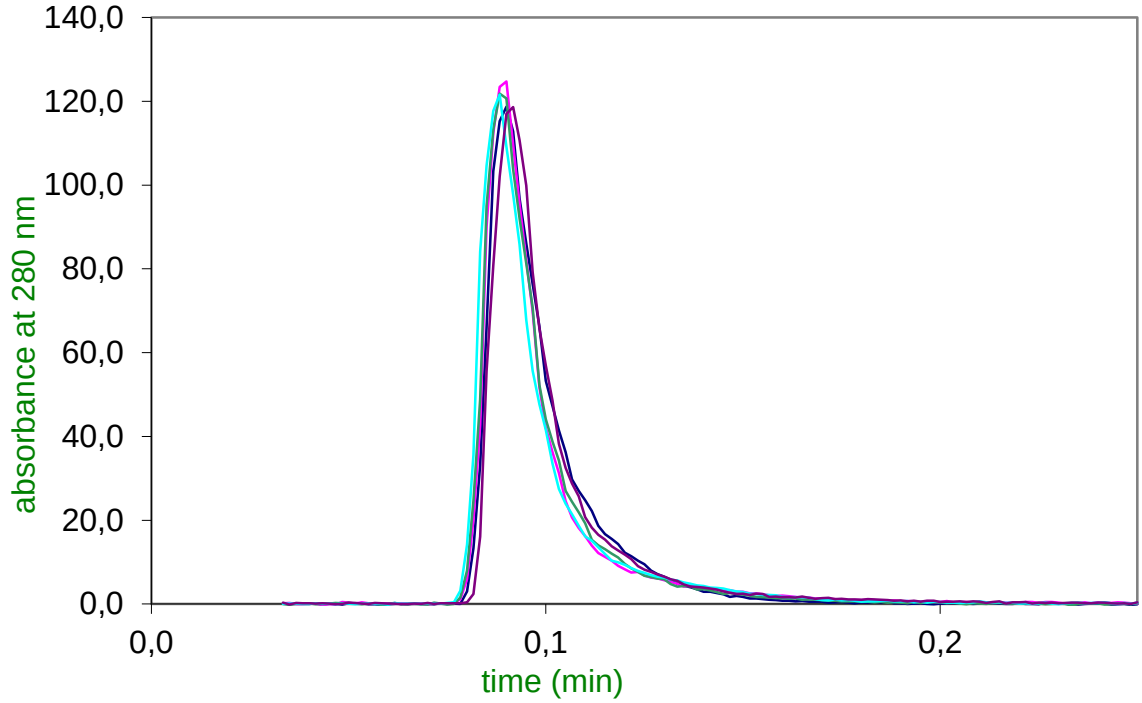


Figure 20: **Effect of column refill on column efficiency.**

Conditions: Mobile phase: deionized water; Flow rate: 240 mL/min; Sample: 1 mL of 16,6 % acetone solution; Injection volume: 1 mL; Detection: UV at 280 nm; Data acquisition frequency was 10 Hz and detector time constant was set to 0,1

Table 6: **Scattering of N-plate values in pulse response experiment as a function of flow rate.**

Flow rate (mL/min)	N-plate (EMG)
packing 1	195,9
packing 2	222,7
packing 3	212,6
packing 4	216,6
packing 5	217,0

RSD	4,3

RSD of N was calculated to be 4.3 %, which is a value comparable to the one obtained by the consecutive injections performed at constant flow rate described above. Obviously, column filling procedure has no detectable influence on column performance, therefore refilling of monolithic columns can be considered as extremely reproducible, easy and fast.

4.3.4. Separation of real sample

Finally on developed monolithic column separation of protein aggregates from a monomer protein as example of macromolecule removal using high throughput monolithic radial column was tested as well. For this purpose lyophilized protein BSA was used, which is known to contain aggregates [30], and dissolved it in a loading buffer. BSA dimers and aggregates interact slightly stronger with the anion exchange matrix therefore they can be separated by implementation of salt gradient. A typical chromatogram is shown in **Figure 21**.

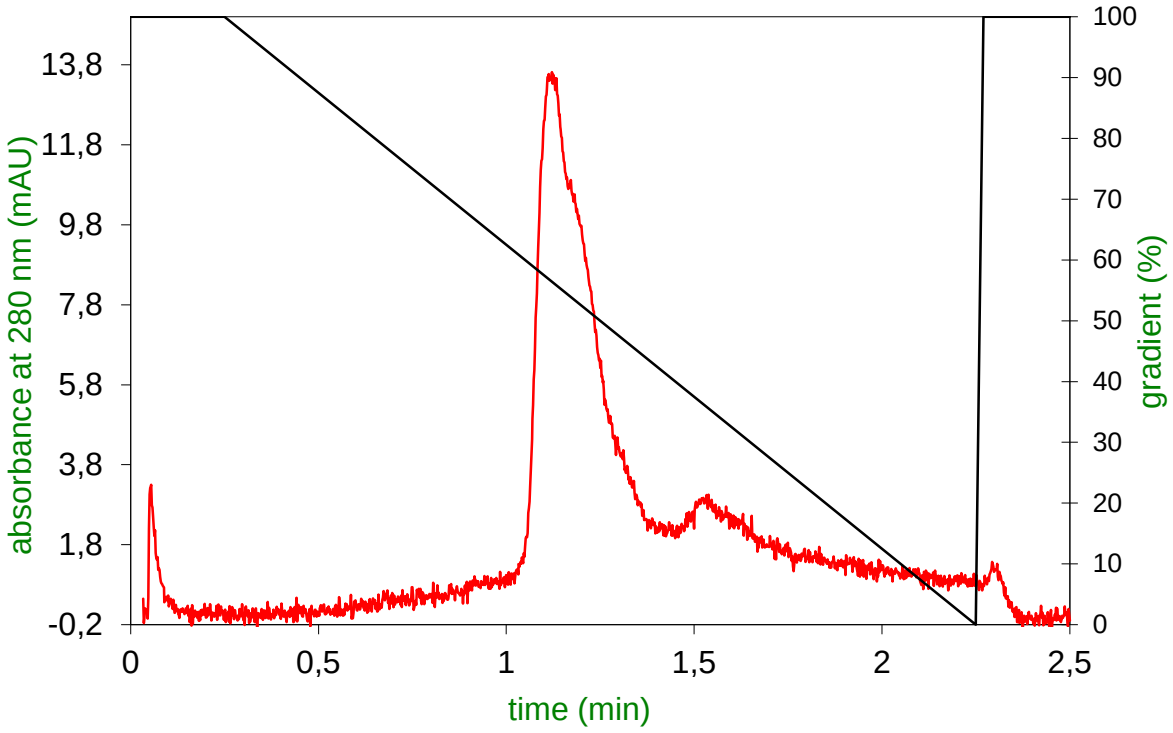


Figure 21: **Separation of BSA monomer from BSA aggrgates.**

Conditions: Mobile phase: mobile phase A, 20 mM Tris-HCl buffer + 0,2 M NaCl pH 7.4; mobile phase B, 1M NaCl in buffer A, pH 7.4; Flow rate: 560 mL/min; Sample BSA (10 mg/mL) dissolved in buffer A Injection volume: 1 mL; Linear gradient from 0-100 % buffer B in 46 s; Detection: UV at 280 nm; Data acquisition frequency was 10 Hz and detector time constant was set to 0,1

Separation was performed at extremely high flow rate of 70 CV/min and completed within 46 seconds. Such short time implies the possibility of implementing this type of column for in-process control during preparative

aggregate removal but it can also be used for removal itself since the separation was efficient as indicated in **Figure 22**.

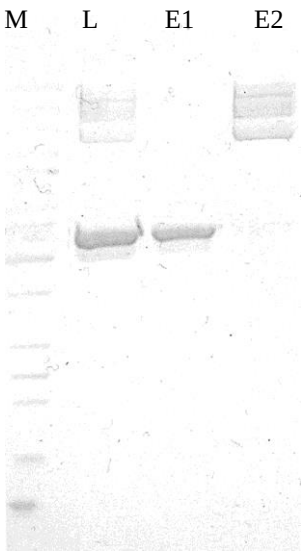


Figure 22: **SDS–PAGE analysis: M: molecular weight standard, L: load, E1: peak 1 , E2: peak 2.**

5. CONCLUSIONS

It was shown that it is possible to isocratically separate oligonucleotides on anion-exchange CIM® DEAE disks and that the performance of the monolith stationary phase is similar to those of 5 - 7 μm porous particles. From the behaviour of the isocratic separations it was concluded that the mechanism of the separation on thin glycidyl methacrylate-ethylene glycol dimethacrylate (GMA-EDMA) monoliths is very similar to the one on conventional columns.

All these observations indicate that isocratic separation on thin GMA-EDMA monoliths is probably the result of the multistep adsorption/desorption process. The fact that isocratic separation on the monolith with a thickness of less than 1 mm occurs, may be ascribed to convective mass transport through the flow-through pores, which causes an increase in the number of adsorption/desorption steps on shorter monolith lengths. This phenomenon opens many new areas of fast separations or purification on this type of supports. For example, when comparing the separation of plasmid DNA conformers with that of oligonucleotides much better separation was achieved in the latter case. This is probably due to the lower molecular weight of oligonucleotides and, consequently, higher diffusion coefficients. According to this analogy one can speculate also the possibility of efficient isocratic separation of peptides. Furthermore, it is reasonable to assume, that also other smaller molecules having even higher diffusivities,might also be separated isocratically.

It was also shown that thin layered GMA-EDMA monoliths can also be applied in preparative scale format. Combining radial flow pattern together with appropriate housing design, columns capable of very high throughput can be prepared, making them comparable to the membrane one. From the experiments it can be concluded that resolution of columns is unaffected in a broad range of flow rates, making them the material of choice for various applications, especially for purification of macromolecules and nanoparticles on an industrial scale. The column packing and

refilling process is very fast and relatively unsophisticated, making thin layered GMA-EDMA monoliths even more attractive for industrial application.

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