Masaryk University
Faculty of Science
Department of Chemistry

Polymer-based monolithic stationary phases in the separation of small molecules

Habilitation thesis

Brno 2017 Jiří Urban

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Bibliografický záznam

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Název práce:	Separace malých molekul na monolitických stacionárních fázích
Obor:	Analytická chemie
Rok:	2017
Počet stran:	45
Klíčová slova:	kapilární kapalinová chromatografie, monolitické stacionární fáze, malé molekuly, vysoké zesítění

Bibliographic Entry

Author:	RNDr. Jiří Urban, Ph.D. Faculty of Science, Masaryk University Department of Chemistry

Title:	Polymer-based monolithic stationary phases in the separation of small molecules
Field of Study:	Analytical Chemistry
Year:	2017
Number of Pages:	45
Keywords:	capillary liquid chromatography, monolithic stationary phases, small molecules, hypercrosslinking

Ivě, Kateřině a Barboře

„Challenges are what make life interesting;
overcoming them is what makes life meaningful.“

Joshua J. Marine

First and foremost, I would like to express my gratitude to my wife Iva. Without your understanding and helpfulness this work would have never become a reality. I really appreciate your supportive attitude, I thank you so very much!

I would also like to thank Prof. Jan Preisler and Prof. Viktor Kanický for the chance they gave me and the confidence they showed in the realization of this work.

I was lucky to have a chance to work with many excellent scientists. Above all I would like to name Prof. Pavel Jandera, Prof. Peter Schoenmakers, and Prof. Frantisek Svec. Each of them taught me something, and all together, they taught me a lot.

I am grateful to my closest associates for their contribution to the papers discussed in this work. I would like to thank Veronika Škeříková, Simona Janků, Martina Komendová, and Radovan Metelka.

I would also like to thank the Dean of the Faculty of Science Assoc. Prof. Jaromír Leichmann and the Czech Science Foundation for their financial support.

Jiří Urban

Brno, September 11, 2017

Abstract

This habilitation thesis deals with a preparation, characterization, and application of polymer-based monolithic stationary phases with a special focus on the separation of small molecules in capillary liquid chromatography. Work is presented as a commented collection of selected peer-reviewed scientific papers. First, pore formation and characterization is described. Then, protocols allowing preparation of monolithic capillary columns suitable for a separation of small molecules are mentioned, including effect of functional monomer and post-polymerization hypercrosslinking modification. Final part of the thesis focuses on the development of monolithic capillary column integrating sample preparation, separation, and electrochemical detection of polar neurotransmitters in one single unit. Conclusions and future aspects are mentioned in the last part of the thesis.

Abstrakt

Tato habilitační práce je prezentována jako komentovaný soubor vědeckých publikací a zabývá se přípravou a charakterizací polymerních monolitických stacionárních fází vhodných pro separace malých molekul. První část komentáře se věnuje kontrole tvorby pórů v monolitickém materiálu a možnostmi jejich charakterizace. Dále se práce věnuje popisu retenčních vlastností připravených kolon, a to zejména pomocí vhodného výběru funkčního monomeru a také využitím vysokého zesítění povrchu stacionární fáze. Poslední část komentáře se zabývá přípravou monolitické kapilární kolony, která by integrovala přípravu vzorku nervových přenašečů, jejich vlastní separaci a následnou elektrochemickou detekci do jednoho celku. Možnosti budoucího vývoje monolitických stacionárních fází jsou shrnuty na konci komentáře.

Abbreviations

2D LC – Two-Dimensional Liquid Chromatography

HILIC – Hydrophilic Interaction Liquid Chromatography

HPLC – High Performance Liquid Chromatography

NMR – Nuclear Magnetic Resonance

RP – Reversed-Phase Retention

RSD – Relative Standard Deviation

SEC – Size-Exclusion Chromatography

Content

1 Introduction

When Mikhail Semenovich Tswett described a new analytical method for the first time at the beginning of 20th century, he used grinded calcium carbonate as a sorbent to separate plant pigments[1]. The method has been named chromatography and irregularly shaped stationary phases evolved rapidly in fine spherical particles within a well-defined diameter, porosity, specific area, and surface chemistry. Nowadays, high performance liquid chromatography (HPLC) utilizes cylindrical columns packed with these particles and belongs to one of the most powerful analytical techniques allowing fast and efficient separations of complex mixtures.

For several decades, spherical particles served as stationary phases with steady optimization of all possible physico-chemical properties but their shape. In 1990s, a new type of stationary phases emerged that consisted of one piece of porous material that fills the whole space defined by a confinement of column. These materials can be prepared from both inorganic and organic precursors and are called *monolithic stationary phases*. Introduction of monolithic stationary phases in column liquid chromatography is the major change in column technology since Tswett invention[2].

1.1 Organization of thesis

The thesis is presented as a commented collection of selected peer-reviewed scientific papers published between years 2006 and 2017 that are attached in the appendix. To facilitate orientation within the other cited works these papers are listed as the first seventeen references and are marked **bold** thorough the text. Since all discussed manuscripts are attached in the appendix, in following text I am discussing only main achievements and general conclusions.

1.2 Aim of the work

The aim of this thesis is to present the results of my research from a little bit over the last decade concerning preparation, characterization, and application of polymer-based monolithic

[1] M. S. Tsvett, Berichte der deutschen botanischen gesellschaft 24 (1906) 316 – 323.
[2] G. Guiochon in *J. Chromatogr. A* 975 (2002) 275 – 284.

stationary phases with a special focus on their ability to separate small molecules in a capillary liquid chromatography.

At first, a general introduction is presented to compare the main structural differences in between conventional chromatographic columns packed with spherical particles and new types of monolithic stationary phases. This part is concluded with a description of monolithic stationary phase's preparation with a special attention to polymer-based organic monoliths.

Pore size distribution of prepared monolithic stationary phases controls the hydrodynamic properties of prepared capillary columns such as porosity and permeability. Therefore, part of this chapter is also devoted to a detailed studies focused on the formation of both large flow-through [**1**] and small mesopores [**2**] and equally important characterization of porous properties [**3**] and accessibility of the mesopores for small and large molecules [**4**].

Separation of small molecules on monolithic capillary columns is a hallmark of a presented thesis. At first, various protocols utilized for the preparation of polymer-based monolithic stationary phases for an isocratic separation of small molecules are reviewed [**5**]. Then, composition of the polymerization mixture used for the preparation of stationary phases is discussed. Change of the functional monomer is the easiest way how to control surface chemistry of prepared stationary phases. I have studied an effect of functional monomer polarity on the retention properties of monolithic columns in both reversed-phase [**6**] and hydrophilic interaction [**7**] capillary liquid chromatography. I have also described a retention of small molecules on the columns prepared with an addition of thermally responsive monomer in the polymerization mixture at various composition of the mobile phase and working temperatures [**8**].

During my post-doctoral research stay at The University of California in Berkeley I have introduced hypercrosslinking modification to the polymer-based monoliths and used them for the first time for the separation of small molecules [**9**]. In the following years, I have focused on the optimization of the hypercrosslinking modification reaction to further improve separation properties of hypercrosslinked stationary phases [**10**,**11**].

Since hypercrosslinked polymers are usually prepared from reactive 4-vinylbenzyl chloride monomer, their surface polarity can be further alternate by a post-polymerization modification. I have used thermally-initiated grafting [**12**] and nucleophilic substitution [**13**] to prepare hypercrosslinked monolithic capillary columns applicable in the separation of small polar compounds in hydrophilic interaction liquid chromatography (HILIC).

To further demonstrate applicability of hypercrosslinked stationary phases in fast separations of small molecules I have hyphenated them for the first time with a remote nuclear magnetic resonance (NMR) detection [**14**]. Last, but not least, hypercrosslinking modification was also used to prepare monolithic capillary columns with a longitudinal gradient of porosity that were successfully used in a two-dimensional liquid chromatography of polymers [**15**].

During the last couple of years, I moved my research attention towards the preparation of monolithic capillary column applicable in the analysis of neurotransmitters, in particularly dopamine metabolites. Dopamine belongs to one of the most important neurotransmitters and undesired changes in its metabolism result in serious illnesses such as depression, schizophrenia, Parkinson disease, and tumors.

I have developed an online solid-phase extraction with liquid chromatography method based solely on polymer monoliths and used it to both determination of dopamine in urine and continuous monitoring of dopamine in a flowing system [**16**]. Very recently, I have introduced concept of an integrated monolithic capillary column that combines chromatographic separation and electrochemical detection in one capillary device [**17**]. When combined with (also integrated) sample preparation, such column can be used for unsupervised determination of neurotransmitters in various biological samples.

1.3 Author contribution

So far, I have published 33 papers in impacted peer-reviewed journals. Seventeen of them are selected to be part of this thesis, here I am the first author of twelve of them – including one equal contribution of the first authors [**1 – 7**, **9 – 11**, **14**, **15**], and the corresponding author of nine of them [**4**, **5**, **8**, **11 – 13**, **15 – 17**].

The following tables summarizes my contribution to the selected works with special attention to amount of performed experiments, supervision of students, definition of research direction, and my contribution to manuscript preparation.

1. J. Urban, D. Moravcová, P. Jandera, A model of flow-through pore formation in methacrylate ester-based monolithic columns, *J. Sep. Sci.* 29 (2006) 1064-1073.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
80	20	20	40	**40**

2. J. Urban, P. Jandera, P. Schoenmakers, Preparation of monolithic columns with target mesopore-size distribution for potential use in size-exclusion chromatography, *J. Chromatogr. A* 1150 (2007) 279-289.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
100	-	40	40	**60**

3. J. Urban, S. Eeltink, P. Jandera, P. Schoenmakers, Characterization of polymer-based monolithic capillary columns by inverse size-exclusion chromatography and mercury-intrusion porosimetry, *J. Chromatogr. A* 1182 (2008) 161-168.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
100	-	25	40	**55**

4. J. Urban, Pore volume accessibility of particulate and monolithic stationary phases, *J. Chromatogr. A* 1396 (2015) 54-61.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
100	-	100	100	**100**

5. J. Urban, Current trends in the development of porous polymer monoliths for the separation of small molecules, *J. Sep. Sci.* 39 (2016) 51-68.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
100	-	100	100	**100**

6. J. Urban, P. Jandera, P. Langmaier, Effects of functional monomers on retention behavior of small and large molecules in monolithic capillary columns at isocratic and gradient conditions, *J. Sep. Sci.* 34 (2011) 2054-2062.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
10	80	10	40	**35**

7. J. Urban, V. Škeříková, P. Jandera, R. Kubíčková, M. Pospíšilová, Preparation and characterization of polymethacrylate monolithic capillary columns with dual hydrophilic interaction reversed-phase retention mechanism for polar compounds, *J. Sep. Sci.* 32 (2009) 2530-2543.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
30	75	20	25	**37.5**

8. M. Chocholoušková, M. Komendová, J. Urban, Retention of small molecules on polymethacrylate monolithic capillary columns*, J. Chromatogr. A* 1488 (2017) 85-92.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
10	90	100	100	**75**

9. J. Urban, F. Svec, J.M.J. Fréchet, Efficient separation of small molecules using a large surface area hypercrosslinked monolithic polymer capillary column, *Anal. Chem.* 82 (2010) 1621-1623.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
100	-	25	40	**55**

10. J. Urban, F. Svec, J.M.J. Fréchet, Hypercrosslinking: New approach to porous polymer monolithic capillary columns with large surface area for the highly efficient separation of small molecules, *J. Chromatogr. A* 1217 (2010) 8212-8221.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
100	-	25	40	**55**

11. J. Urban, V. Škeříková, Effect of hypercrosslinking conditions on pore size distribution and efficiency of monolithic stationary phases, *J. Sep. Sci.* 37 (2014) 3082-3089.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
90	10	100	100	**75**

12. V. Škeříková, J. Urban, Highly stable surface modification of hypercrosslinked monolithic capillary columns and their application in hydrophilic interaction chromatography, *J. Sep. Sci.* 36 (2013) 2806-2812.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
10	90	100	100	**75**

13. S. Janků, V. Škeříková, J. Urban, Nucleophilic substitution in preparation and surface modification of hypercrosslinked stationary phases, *J. Chromatogr. A* 1388 (2015) 151 - 157.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
10	100	100	100	**77.5**

14. T.Z. Teisseyre, J. Urban, N.W. Halpern-Manners, S.D. Chambers, V.S. Bajaj, F. Svec, A. Pines, Remotely detected NMR for the characterization of flow and fast chromatographic separations using organic polymer monoliths, *Anal. Chem.* 83 (2011) 6004-6010.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
25	-	20	30	**25**

15. J. Urban, T. Hájek, F. Svec, Monolithic stationary phases with a longitudinal gradient of porosity, *J. Sep. Sci.* 40 (2017) 1703-1709.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
90	10	90	90	**70**

16. S. Janků, M. Komendová, J. Urban, Development of an online solid-phase extraction with liquid chromatography method based on polymer monoliths for the determination of dopamine, *J. Sep. Sci.* 39 (2016) 4107-4115.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
20	80	100	100	**75**

17. M. Komendová, R. Metelka, J. Urban, Monolithic capillary column with an integrated electrochemical detector, *J. Chromatogr. A* 1509 (2017) 171 – 175.

Experiments, %	Supervision, %	Research direction, %	Manuscript, %	Total, %
5	85	90	100	**70**

1.4 Funded projects

Since 2012 I have worked as Principal Investigator of following projects, with the final evaluation of the first two projects being fulfilled.

- Improving the performance of hypercrosslinked monolithic stationary phases and their application in separations of polar compounds, Czech Science Foundation, postdoctoral project P206/12/P049 (2012 – 2014).

- Development of a multifunctional monolithic capillary column with integrated sample focusing, separation, and on-column electrochemical detection, Czech Science Foundation, standard project 14-22426S (2014 – 2016).
- Tailoring selectivity of polymer-based monolithic stationary phases, Czech Science Foundation, standard project 17-11252S (2017 – 2019).

2 Monolithic stationary phases

2.1 Internal structure

The most important advances in chromatography have been always related to the development of novel separation media featuring higher efficiency and better selectivity. The development of high performance liquid chromatography was triggered by the introduction of a broad range of small-particle separation media prepared from silica gel, polymers, and inorganic oxides. To minimize the pore diffusion and mass transfer resistance, non-porous and superficially porous particles with a solid central core and a very thin porous outer layer of the stationary phase were also introduced [18].

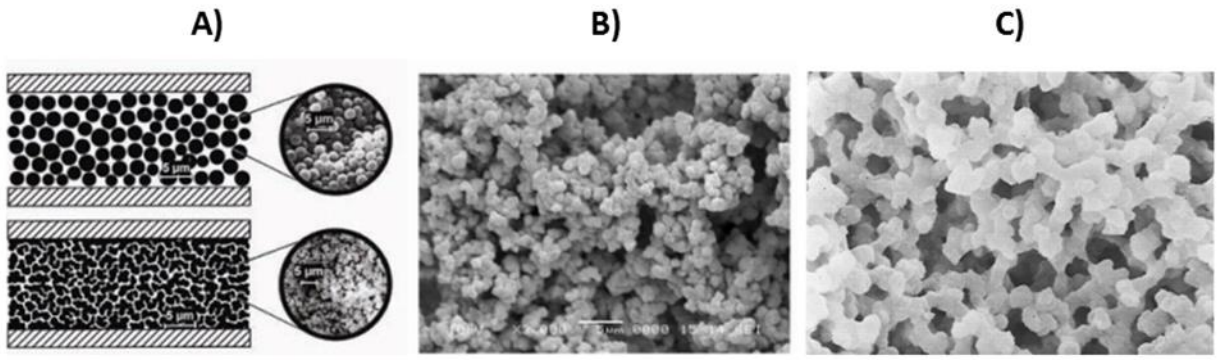


Figure 1. Comparison of an internal structure for packed (A, top) and monolithic (A, bottom) stationary phase [19], together with representation of organic polymer-based (B) and inorganic silica-based (C) monolithic stationary phase [20,21].

In recent years, continuous separation media have attracted considerable attention because of the advantages they offer over packed columns [**5**,22,23]. This research resulted in two useful monolithic material types, the first based on modified silica gel and the second on organic

polymers. The monoliths consist of a single piece of highly porous material and can be compared to a single large “particle” that does not contain interparticular voids typical of packed beds. **Figure 1** highlights difference in between packed and monolithic stationary phases and compares an internal structure of organic and inorganic monolithic stationary phase.

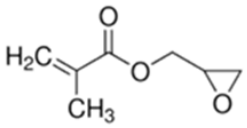
2.2 Preparation of polymer monoliths

Monolithic stationary phases based on organic polymers are generally prepared by a free radical polymerization although several other approaches were also introduced [24]. The polymerization mixture contains radical initiator to start the reaction, functional and/or crosslinking monomer, and pore forming solvents (e.g. short alcohols) that do not participate in the polymerization reaction but are responsible for phase separation during the reaction and formation of the resulting polymer [25]. Generally, a homogenous polymerization mixture is filled in the capillary where monolithic material is formed by a free radical reaction initiated by an elevated temperature or a UV light [18,26]. **Figure 2** shows several examples of both methacrylate and styrene-based functional and crosslinking monomers and radical initiators used for the preparation of polymer monoliths.

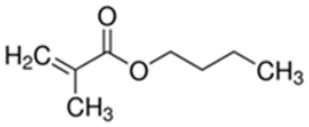
The formation and growth of polymeric monolithic stationary phase was already described in 1995 [27] and recently further studied by thermal analysis and scanning electron microscopy [28]. During the early stages of the polymerization reaction, polymer growth and branching is homogeneously initiated throughout reaction medium leading to a formation of compact microgel particles that crosslink and form polymer microglobule.

At this stage, phase separation occurs, which might happen as little as 10 minutes when the polymerization reaction is running at 70 °C. Then, pre-gel network is formed by interconnected globules and at around 45 – 60 minutes of polymerization reaction macroscopic network of interconnected globules is already formed throughout the capillary.

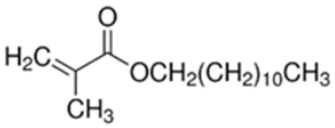
Functional monomers



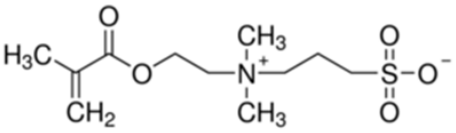
Glycidyl methacrylate



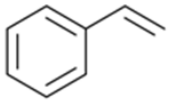
Butyl methacrylate



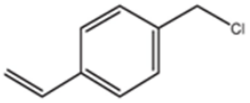
Lauryl methacrylate



[2-(Methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide

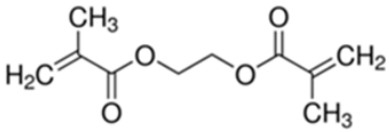


Styrene

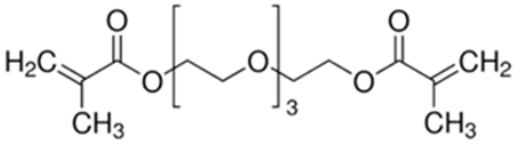


4-Vinylbenzyl chloride

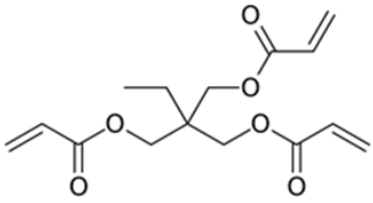
Crosslinking monomers



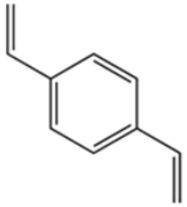
Ethylene dimethacrylate



Tetraethylene dimethacrylate

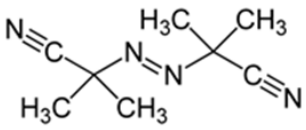


Trimethylolpropane trimethacrylate

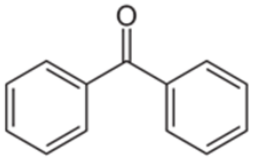


Divinylbenzene

Initiators



Azobisisobutyronitrile



Benzophenone

Figure 2. Examples of functional and crosslinking monomers, together with radical initiators, used for the preparation of polymer-based monolithic stationary phases.

After a couple of hours, previously formed gel links together and continuously fills the capillary confinement with a macroscopic network structure. At this point, polymer microglobules further grow by a coalescence with newly formed microglobules and reduce the size of flow-through pores. The total conversion at this stage of the polymerization reaction is generally over 60% and reaches 95% after 24 hours [28].

Compared to that, inorganic silica monoliths are prepared by the classical sol–gel process of sequential hydrolysis and polycondensation of organo-silicium compounds, as the same protocol is used for the preparation of fine silica particles packed in currently available conventional HPLC columns [23].

2.4 Pore formation

Pores inside the monolith are open, forming a highly interconnected network of channels. There are two main types of pores in the structure of monolithic columns, i.e. (i) flow-through pores enabling an easy flow of the mobile phase and (ii) micropores and mesopores filled with a "stagnant" mobile phase in which the solute molecules migrate to access the adsorption sites. According to the IUPAC classification, micropores are pores with a diameter smaller than 2 nm, mesopores have diameters between 2 and 50 nm, and flow-through pores are larger than 50 nm [29].

Pore size distribution of monolithic stationary phases can be controlled in experimental conditions including polymerization reaction and time, and by the composition of the polymerization mixture. We have thoroughly investigated the effect of polymerization mixture composition on the formation of both flow-through pores [**1**] and mesopores [**2**] in polymethacrylate polymer monoliths.

We systematically varied the concentrations of butyl methacrylate as functional and ethylene dimethacrylate as crosslinking monomer in the presence of 1,4-butanediol and 1-propanol as the porogen solvents to find significant factors affecting pore formation in monolithic capillary columns. To distinguish the role of the four components of the polymerization mixture in the pore formation process, multivariate analysis of polynomial models allows investigation not only of the

separate effects of the individual components, but also of the combined synergistic or antagonistic effects [**1**,**2**]. After a mathematical optimization of polynomial models, the mean error of prediction was lower than 8% for flow-through pore formation [**1**] while it was 32% for model describing formation of mesopores [**2**]. It should be pointed out, however, that absolute values of mesopore volume are significantly lower than those of flow-through pores.

According to the models derived based on multivariate data analysis, individual components of the polymerization mixture provide complementary contribution of the formation of the pores. Increasing the sum of the concentrations of the porogen solvents (1,4-butanediol and 1-propanol) in the polymerization mixture increases the content of flow-through pores in the monolithic separation medium. On the other hand, increased concentrations of butyl methacrylate monomer and of 1-propanol show a combined negative effect on the flow-through pore formation. Hence, the concentration of 1,4-butanediol in the porogen solvent mixture is the main factor enhancing the flow-through pore formation [**1**]. Oppositely, the main role in the mesopore formation plays the combination of the hydrophobic butyl methacrylate monomer and less polar 1-propanol as the porogen solvent. Increasing concentration of 1-propanol and decreasing concentration ratios of the cross-linker to monomer and of 1,4-butanediol to 1-propanol solvents leads to higher incorporation of the mesopores in the monolithic capillary columns [**2**].

The pore formation is controlled by a phase separation governed by a polarity of the polymerization mixture. With higher polarity of the mixture the phase separation starts at early stages of the reaction allowing diffusion of monomers over a longer distance to the first regions of a polymeric phase, so that the polymerization can proceed preferentially at the phase interface. Consequently, a network with relatively large flow-through pores is formed. On the other hand, later phase separation provides denser network of crosslinked polymers and therefore denser polymer with a lower concentration of flow-through pores arises.

The presented models can be used to predict the porosity of flow-through pores and mesopores within a limited, well defined space of polymerization mixture composition. Alternatively, they can be also applied in a tailored preparation of monolithic capillary columns with desired pore size distribution. For example, monolithic stationary phase with a dominant flow-through pores and absent mesopores can be successfully used in fast gradient separations

of both small and large molecules [**1**]. On the other hand, monolith with a substantial volume of mesopores might provide selectivity necessary for a size-exclusion type of the separation. Hence, we have prepared monolithic columns with large volume of mesopores, however further optimization is necessary to prepare columns with mesopore distribution suitable for efficient and selective practical SEC separation of polymers according to their size in specific molar mass range [**2**].

2.5 Characterization of porous properties

Various methods have been used to determine the porous properties of chromatographic stationary phases. The most common methods include nitrogen adsorption using the Brunauer, Emmett, Teller (BET) equation and mercury-intrusion porosimetry [**3**]. Alternatively, inverse size-exclusion chromatography that utilizes a set of well-defined molecular probes with widely varying sizes can be used to determine pore dimensions [30].

A major question in characterizing the porous properties of monolithic beds is, however, the extent to which the porous properties of the "dry" monoliths are indicative of the chromatographic performance under "wet" (swollen) conditions. Therefore, we have prepared polymethacrylate monolithic stationary phases and determined their porous properties by inverse size-exclusion chromatography and mercury-intrusion porosimetry method [**3**].

Both techniques are complementary; while mercury-porosimetry measures the entire range of pore sizes and provides more physical information on the monoliths, inverse size-exclusion chromatography is suitable for determining the size of mesopores in the swollen monoliths. Additionally, inverse size-exclusion chromatography has some advantages in comparison with mercury intrusion porosimetry such as limited accuracy of the mercury porosimetry at elevated pressures (for characterizing small pores) and the high toxicity of mercury can be avoided by using inverse size-exclusion chromatography.

As expected, the porosities, permeabilities, flow-through pore diameters, pore volumes, surface areas, and polymer densities were found to depend on the composition of the polymerization mixture. The concentration of the porogen solvents in the mixture affected

the total and flow-through porosities most significantly. With higher concentrations of the porogen solvents in the mixture, very porous monolithic materials with high permeabilities and large flow-through pore diameter were obtained.

The total porosity of polymer monolith measured with inverse size-exclusion chromatography decreased in increasing concentration of the monomers in the polymerization mixture. However, the decrease in the total porosity determined using mercury intrusion porosimetry was much less significant. The differences between the results obtained using the two techniques may be possibly attributed to swelling of the monolithic beds when tetrahydrofuran is used as the mobile phase in inverse-size exclusion chromatography and confirms once again the importance of determination of pore characteristics directly from the size-exclusion data in the wet (swollen) state, like the conditions of practical use of the columns for chromatographic separations [**3**].

In size-exclusion chromatography, compounds are eluted with respect to their size that controls accessible pore volume. While small molecules penetrate to all pores and elute last, large molecules are excluded from the pores smaller than their hydrodynamic diameter and elute first. I have used size-exclusion chromatography to determine the pore volume fractions of various stationary phases that are accessible to small and large molecules [**4**], i.e. accessible porosity, and correlated it with the size of the pores from which individual compounds are excluded as schematically shows **Figure 3**.

Such evaluation allows simple and straightforward comparison of various types of stationary phases with different structural, porous, and hydrodynamic properties. To prove this concept, I have first determined pore volume accessibility of commercially available columns packed with fully and superficially porous particles, as well as with silica-based monolithic stationary phase. Pore volume accessibility correlated well with the internal structure of both particulate and monolithic stationary phases highlighting morphological differences of individual stationary phases.

Then, I have used this approach to study pore formation in polymer-based monoliths. In polymethacrylate monolithic stationary phases, the mesopores were formed at the very beginning of the polymerization reaction followed by the development of micro-pores during later stages of the polymerization reaction. The same applies also for hypercrosslinking modification, where mainly mesopores were formed during the first 60 min of hypercrosslinking and micropores were formed when the hypercrosslinking modification was carried out for more than 60 min. When hypercrosslinking modification followed early termination of the polymerization reaction, the pore volume accessibility decreased with longer time of the polymerization reaction for styrene-based polymerization mixture [**4**].

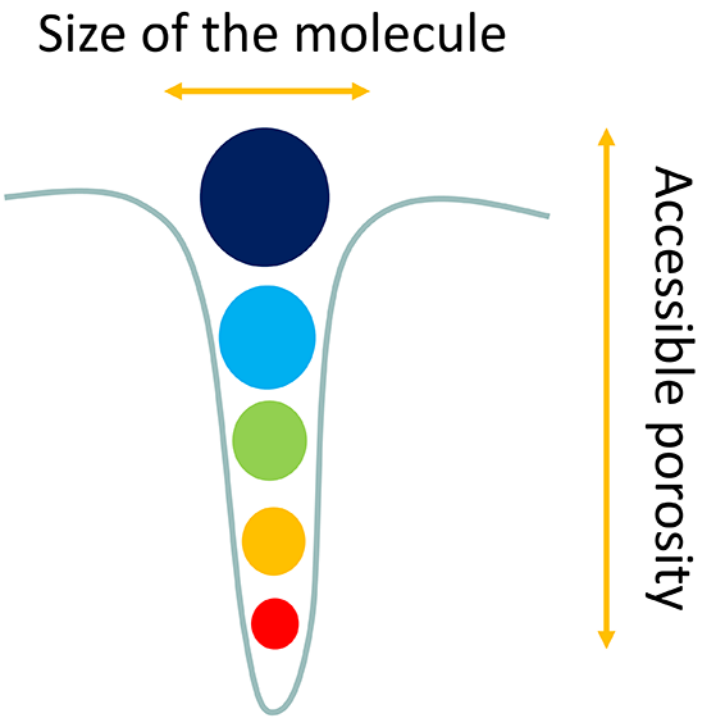


Figure 3. Schematic representation of pore volume accessibility determination. Size of the molecule is expressed as a hydrodynamic diameter of the molecule (nm), while accessible porosity is determined as a fraction of internal pores that are accessible for individual compounds (%) [**4**].

I believe, that this simple protocol can be applied not only to studies concerned with pore volume of stationary phases for liquid chromatography but also for non-destructive characterization of any porous material such as heterogeneous catalysts and adsorbents. Additionally, in case of polymer-based monoliths, this method provides fast, simple, and straightforward information about pore formation and efficiency during the fabrication process.

2.3 Repeatability of columns preparation

Repeatability and mechanical stability of polymer monoliths have always been considered less robust when compared to both silica-based monoliths and particulate stationary phases. However, several studies already demonstrated that the repeatability of the preparation and mechanical stability of polymer-based monolithic stationary phases is (at least) comparable to other types of the stationary phases conveniently used in liquid chromatography [31-33].

We have showed, that hypercrosslinked polymer monoliths with grafted polar functionality do not change its chromatographic properties even after 10 000 injections of test compounds [**12**]. Additionally, we also demonstrated that a combination of an early termination of polymerization reaction and post-polymerization surface modification provides repeatable and robust column properties with plate height RSD lower than 6.5% [**13**].

Most articles about polymer monoliths published nowadays provide a part devoted to columns preparation repeatability showing that polymer-based monoliths are very robust and stable stationary phases.

3 Separation of small molecules

Since their introduction, the main application area of porous polymer monoliths has been the fast gradient separations of synthetic and natural polymers. On the other hand, preparation of polymer monoliths providing column efficiency comparable with particulate and monolithic silica-based stationary phases has been proven to be difficult.

During the last decade, several experimental approaches were performed that aimed to improve this property of polymer monoliths which I have summarized in recent review [**5**]. These protocols include fine tuning of the polymerization mixture composition, preparation of monolithic stationary phases at limited conversion of the polymerization reaction, application of novel, highly ordered, nanomaterials, and/or hypercrosslinking surface modification controlling the crosslink density of the prepared monoliths. By using some of these approaches,

monolithic stationary phases with column efficiency reaching 200 000 plates/m for low-molecular-weight compounds have been prepared [**5**].

This part of the thesis focuses on two approaches leading to the preparation of polymer monoliths suitable for an isocratic separation of small molecules: tuning a composition of the polymerization mixture and hypercrosslinking modification of polymer surface.

3.1 Effect of functional monomer on the retention of small molecules

The composition of the polymerization mixture is the easiest way to change and control the properties of polymer-based monoliths. While the concentration and polarity of pore forming solvents affects phase separation hence porous and hydrodynamic properties of the prepared monoliths, type of the functional and crosslinking monomer controls the surface chemistry of the stationary phase that is responsible for chromatographic selectivity.

The disadvantage is the time-consuming optimization of experimental conditions. Even small changes in the concentration of individual parts of the mixture provide the monolithic material with different properties. Changes in the concentration, or even a replacement of monomer or pore forming solvent must be followed by a new optimization of preparation and experimental conditions to provide stationary phase with the desired properties.

By a proper selection of functional monomers monolithic capillary columns applicable in reversed-phase [**6**] and hydrophilic interaction [**7**] liquid chromatography can be prepared. It is also possible to utilize a thermally-responsive monomer to prepare monolithic stationary phases suitable for the analysis of small molecules [**8**].

To compare polarity and efficiency of polymethacrylate monolithic capillary columns, we have prepared stationary phases with butyl, cyclohexyl, 2-ethylhexyl, lauryl, and stearyl methacrylate functional monomers and tested them in reversed-phase chromatography of low-molecular alkylbenzenes and in gradient elution of proteins [**6**].

The non-polar chromatographic selectivity can be tuned by choosing the alkylmethacrylate monomer used. The lauryl methacrylate column showed the lowest methylene and phenyl

selectivity for small molecules, whereas the butyl methacrylate column showed high phenyl selectivity and the column with stearyl methacrylate had a high methylene selectivity. On the other hand, there were no direct correlations between the size of the functional monomer and the methylene and phenyl selectivity. This may be possibly caused by the differences in the skeleton conformation (folding) affecting the shape and accessibility of pores of the monolithic media for alkylbenzenes.

Even though the efficiencies of most polymethacrylate columns prepared with various functional monomers for alkylbenzenes were low, the monolithic columns prepared with lauryl methacrylate monomer showed markedly better efficiency than the other columns, which might be possibly attributed to different degree of lauryl methacrylate stationary phase swelling and hence its accessibility for low molecular alkylbenzenes.

Generally, the organic polymer monolithic columns show much better performance (efficiency) for biopolymers than for low-molecular samples. The linear solvent strength gradient model showed good fit to the retention data of proteins in gradient elution. We have found that the retention and the concentration of acetonitrile at the time of elution of proteins increased for functional monomers with longer alkyl chains, especially for large proteins. The model allowed simple prediction of the effects of the gradient program on the elution volumes and bandwidth in gradient liquid chromatography.

All the tested columns could be successfully used for fast gradient separations of proteins. However, the lauryl methacrylate column showed the lowest hydrophobicity and best efficiency for alkylbenzenes and proteins [**6**].

Previously discussed monolithic capillary columns were applied in the separations of moderately polar compounds following reversed-phase liquid chromatography retention mechanism. However, very polar compounds do not provide any retention in reversed-phase chromatography and therefore it is very difficult to separate them properly. One of the possibilities of how to overcome this drawback is to utilize polar stationary phase together with highly organic mobile phase and separate polar compounds in HILIC [34].

Thus, we aimed to prepare monolithic capillary column based on zwitterionic [2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide monomer suitable for the separation of polar phenolic compounds [**7**]. The columns prepared with methanol as the only pore forming solvent [35] did not provide satisfactory permeability. Therefore, we have tested various polar porogen solvents including methanol, ethanol, tetrahydrofuran, 1-propanol, 1,4-butanediol, and their mixtures with water to prepare monolithic HILIC columns. We found that addition of water to the porogen solvent mixture enhances the solubility of the zwitterionic sulfobetaine monomer and therefore permits the use of organic solvents less polar than methanol to adjust the porosity [**7**].

Polymethacrylate monolithic capillary columns with zwitterionic sulfobetaine groups incorporated into the polymer structure were prepared using binary or ternary porogen solvent mixtures containing water. Porosity, permeability, selectivity, and retention characteristics of prepared columns depended on the composition of the polymerization mixture. The main factors affecting the selectivity of monolithic stationary phase were the concentration of zwitterion monomer and the composition of the porogen solvents in the polymerization mixture.

The retention of phenol and phenolic acids on the monolithic columns showed a dual RP-HILIC separation mechanism, depending on the concentration of acetonitrile in the mobile phase (reversed-phase in water-rich mobile phases and HILIC at high concentrations of acetonitrile in aqueous–organic mobile phases) that could be successfully described by the model assuming the additivity of the HILIC and RP contributions to the retention.

The monolithic sulfobetaine columns show a separation selectivity for phenolic acids comparable to that of the commercial column with sulfobetaine stationary phase chemically bonded to silica gel particles in the HILIC mode, but provided significantly higher retention and separation selectivity than the silica-based column in the reversed-phase mode in highly aqueous mobile phases.

Monolithic capillary columns with a dual retention mechanism offer the possibility of selecting between the two retention modes with different separation selectivity on a single column,

just by changing the composition of the mobile phase, which is advantageous for separations of samples containing compounds with significant differences in polarities.

N-isopropylacrylamide belongs to the family of monomers for the preparation of thermally responsive materials that change their solvation and associated polarity based on external temperature: when heated in an aqueous solution over 32°C (lower critical solution temperature, LCST) it reversibly loses over 90% of volume and changes from a swollen hydrated (hydrophilic) monomer to a shrunken dehydrated (hydrophobic) one [36]. Unfortunately, this behavior is observed mainly in pure water and presence of high concentration of organic solvent eliminates the effect.

We have explored an effect of composition of the polymerization mixture, concentration of acetonitrile in the mobile phase, and working temperature on the retention of small molecules on polymethacrylate monolithic capillary columns prepared with N-isopropylacrylamide in the polymerization mixture [**8**]. Optimization of a second-order polynomial retention model revealed that the most significant parameter controlling the retention of small molecules is the concentration of acetonitrile in the mobile phase, and this effect is more dominant for less polar compounds.

Working temperature and combined parameter of acetonitrile concentration and elevated temperature contribute only a little to the retention. The possible explanation is that quite low concentrations of added N-isopropylacrylamide were unable to form sufficiently long polymer sequences inside the monolithic phase. This drawback can be easily overcome by an application of a grafting reaction and attach poly(N-isopropylacrylamide) chains at the surface of generic monolith.

This chapter confirms that by a proper optimization of polymerization mixture composition, particularly by a selection of type and chemistry of functional monomer, monolithic capillary columns applicable in reversed-phase and hydrophilic interaction liquid chromatography of small molecules can be successfully prepared [**6-8**].

It should be pointed out again however, that when functional monomer in the polymerization mixture is replaced by another one, new optimization must be performed to prepare monolithic

stationary phase with desired efficiency and selectivity. To avoid this, generic monolithic capillary column allowing efficient separation of small molecules should be prepared and its chemistry (eventually) controlled by a post-polymerization surface modification either via direct chemical modification or by surface initiated grafting of selected functional groups. Preparation of such generic columns allows for example post-polymerization hypercrosslinking modification that is thoroughly discussed in next chapter of this thesis.

3.2 Hypercrosslinking surface modification

3.2.1 Hypercrosslinked monolithic stationary phases

Several decades ago, Davankov prepared large surface area materials from preformed polymer precursors in a technique termed "hypercrosslinking" [37]. The original implementation used linear polystyrene, which was crosslinked via Friedel-Crafts alkylation to afford materials containing mostly small pores. This approach was later extended to crosslinked porous poly(styrene-*co*-divinylbenzene) particles and led to products containing both the original pores and an extensive network of additional micro- and mesopores generated during hypercrosslinking [38].

Hypercrosslinking modification allows tailored preparation of functional materials suitable for various applications, including HPLC, biomedical applications in hemodialysis, solid-phase extraction, and large-scale liquid adsorption chromatography [39-41].

In 2010, we demonstrated for the first time the preparation of hypercrosslinked porous polymer monoliths exhibiting a large surface area and their use in capillary columns for the separation of small molecules as well as for rapid size-exclusion chromatography [**9**]. Since then, we have further optimized the experimental conditions to improve column efficiency [**10**,**11**], tuned surface polarity of hypercrosslinked monoliths [**12**,**13**], coupled hypercrosslinked monolithic columns with an online NMR detection [**14**], and utilized hypercrosslinking modification to prepare monolithic capillary columns with a longitudinal gradient of small pores [**15**].

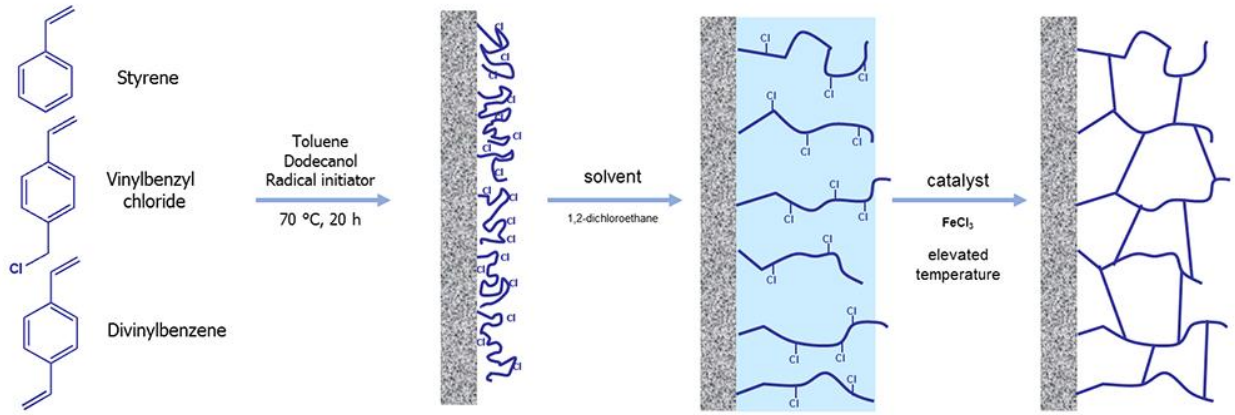


Figure 4. Individual steps in the preparation of hypercrosslinked monolithic stationary phases. In the first step, generic monolith is formed by a free-radical polymerization. Then, stationary phase is swollen in thermodynamically good solvents and loose polymer chains are fixed by Friedel-Crafts alkylation [**9**,**10**].

Hypercrosslinking modification utilizes the fact, that bifunctional divinyl monomer polymerizes faster and hence the surface of the monolith at the end of the polymerization reaction is formed mainly by slightly cross-linked chains attached to the surface of highly crosslinked microglobular scaffolds. After the swelling of a monolith in a thermodynamically good solvent, the loose polymeric chains at the surface of monolithic stationary phases are fixed by a nucleophilic substitution reaction catalyzed by a Lewis acid. A thin layer of small pores is then formed at the surface of the monolithic material, thus providing a higher column efficiency and selectivity in the separation of small molecules. **Figure 4** provides description of individual steps in the preparation of hypercrosslinked monolithic stationary phases.

Despite its recent introduction to the field of polymer monoliths [**9**], hypercrosslinking modification has already attracted considerable attention [42]. The hypercrosslinked stationary phases have been used for sample clean up [43], to separate compounds in capillary LC [44] and capillary electrochromatography [45], modified with gold nanoparticles [46], used as stationary phases in TLC of peptides and proteins [47] or as a media for a gas storage [48].

We have illustrated the significant effects of the polymerization conditions and the post-polymerization hypercrosslinking process on the chromatographic performance of the resulting monolithic poly(styrene-*co*-vinylbenzyl chloride-*co*-divinylbenzene) stationary phase [**10**].

The comprehensive optimization based on mathematical design of the composition of the polymerization mixture used to prepare the precursor monoliths, and temperature, at which the hypercrosslinking is carried out, led to an equation which facilitated the calculation of conditions that should afford columns with predetermined efficiencies. Specifically, this approach enabled the reproducible preparation of a column for (i) isocratic reversed phase separation of alkylbenzenes characterized by its high plate count, (ii) gradient elution of peptides, and (iii) very efficient size-exclusion chromatography of polystyrene standards in organic solvent. The use of the high temperature and of a ternary mobile phase permits a significant acceleration of the separations.

The proper combination of the swelling solvent and Friedel-Crafts catalyst can be used for the preparation of hypercrosslinked monolithic stationary phases with controlled hydrodynamic and porous properties for a separation of small molecules [**11**]. Three dihalogenic solvents differing in the length of alkyl chain (1,2-dichloroethane, 1,4-dichlorobutane, and 1,6-dichlorohexane) with three Friedel–Crafts alkylation catalysts varying in reactivity ($AlCl_3$ > $FeCl_3$ > $SnCl_4$) have been used to prepare hypercrosslinked poly(styrene-*co*-vinylbenzyl chloride-*co*-divinylbenzene) columns.

The column permeability is significantly affected by the combination of swelling solvent and reaction catalyst applied. With increasing reactivity of the alkylation catalysts and increasing length of alkyl chain in the swelling solvent, the column permeability decreases. The column efficiency is higher for columns modified with highly reactive catalyst in the presence of the solvent with the longest alkyl chain. The column efficiency however decreases with the higher catalyst reactivity for 1,4-dichlorobutane. The column efficiency also increases for columns with longer time of hypercrosslinking modification, lower concentration of reaction catalyst, and at elevated reaction temperature.

The pore volume of micropores smaller than 2 nm can be successfully linked to the efficiency of hypercrosslinked stationary phases. The higher their pore volume in the monolithic material, the better was the column efficiency. This pore volume segment is probably associated to so-called gel porosity, introduced earlier [49] as a general property responsible for an efficiency of organic polymer-based monoliths in the separation of small molecules [50].

3.2.2 Surface modification of hypercrosslinked monoliths

Due to the low polarity of scaffold styrenic monolith, hypercrosslinked monolithic stationary phases have been mainly used in reversed-phase liquid chromatography retention mechanism [**9-11**,44,45]. Since not all chloromethyl groups of generic hypercrosslinked monolith are involved in Friedel-Crafts alkylation, residual reactive groups can be utilized for further surface modification.

A two-step surface modification of poly(styrene-*co*-vinylbenzyl chloride-*co*-divinylbenzene) monolithic stationary phases, including hypercrosslinking and grafting of [2-(methacryloyloxy) ethyl]dimethyl(3-sulfopropyl)ammonium hydroxide, provided hypercrosslinked monolithic capillary columns for the isocratic separation of small polar compounds in HILIC [**12**].

According to response surface methodology, the main factor in the surface modification of hypercrosslinked monoliths was the synergistic effect of grafting time. Modified columns provided a dual retention mechanism, including RP and HILIC, which can be controlled by the composition of the mobile phase. Hypercrosslinked stationary phases with zwitterion functionality have been used for isocratic separation of polar phenolic acids in HILIC, and applied in one- and two-dimensional liquid chromatography.

We also demonstrated an application of nucleophilic substitution reaction in the preparation of hypercrosslinked monolithic stationary phases [**13**]. We have used linear diaminoalkanes differing in the number of methylene units in between terminal amino groups to crosslink swollen polymer material. The column efficiency of the prepared columns increased with the length of diaminoalkane. We have optimized the modification reaction conditions, such as time, temperature, and concentration of the crosslinking agent. To improve the permeability of prepared columns, we have hypercrosslinked monolithic material after only 2 h of polymerization reaction.

Furthermore, we have modified the polymer hypercrosslinked by nucleophilic substitution reaction with 2-aminoethanesulfonicacid (taurine) to prepare capillary columns suitable for analysis of low-molecular polar compounds. We have optimized reaction conditions and used prepared capillary columns in the isocratic separation of polar phenolic compounds [**13**].

Although presented protocol is more complex than a single-step polymerization reaction, our results clearly demonstrate the versatility of hypercrosslinked monolithic stationary phases for separation of small molecules with superior stability and robustness.

3.2.3 Fast separations on hypercrosslinked stationary phases

Hypercrosslinked modification allows preparation of stationary phases providing fast and efficient separation. Hence, they find applications in an online hyphenation of LC with a remote NMR detection [**14**] or industrial applications where fast isocratic separation of polymers is needed, such as the second dimension in 2D LC [**15**].

Although UV-visible spectroscopy is the most common method of monitoring chromatographic separations in real-time, it requires well resolved analyte peaks and the use of chemical standards to correlate species as they elute from the column. Mass spectrometry can also be used for real-time detection, providing an additional method to confirm peak assignments. However, the chemical information provided through mass spectrometry is limited to the mass-to-charge ratio of the individual compounds, unless secondary processes such as fragmentation are utilized. Neither detection method can perform multidimensional imaging of flow or separations directly on the column. One powerful alternative to these detection methods is nuclear magnetic resonance.

As the sensitivity of NMR detection is directly proportional to the filling factor of the coil (the fraction of the coil volume occupied by the sample), a microsolenoid with diameter comparable to the inaccessible microporous features of the column will afford the highest sensitivity. As an alternative, remotely detected NMR provides an improved filling factor and a tremendous sensitivity enhancement when detecting small volumes of flowing liquid. Remote detection separates and optimizes the components of a magnetic resonance experiment (polarization, encoding, and detection). This is possible because the steps are correlated in both space and time by the flowing fluid, whose spin degrees of freedom act like a magnetic recording tape in which information can be stored, at one time and place, and read out later (**Figure 5A**).

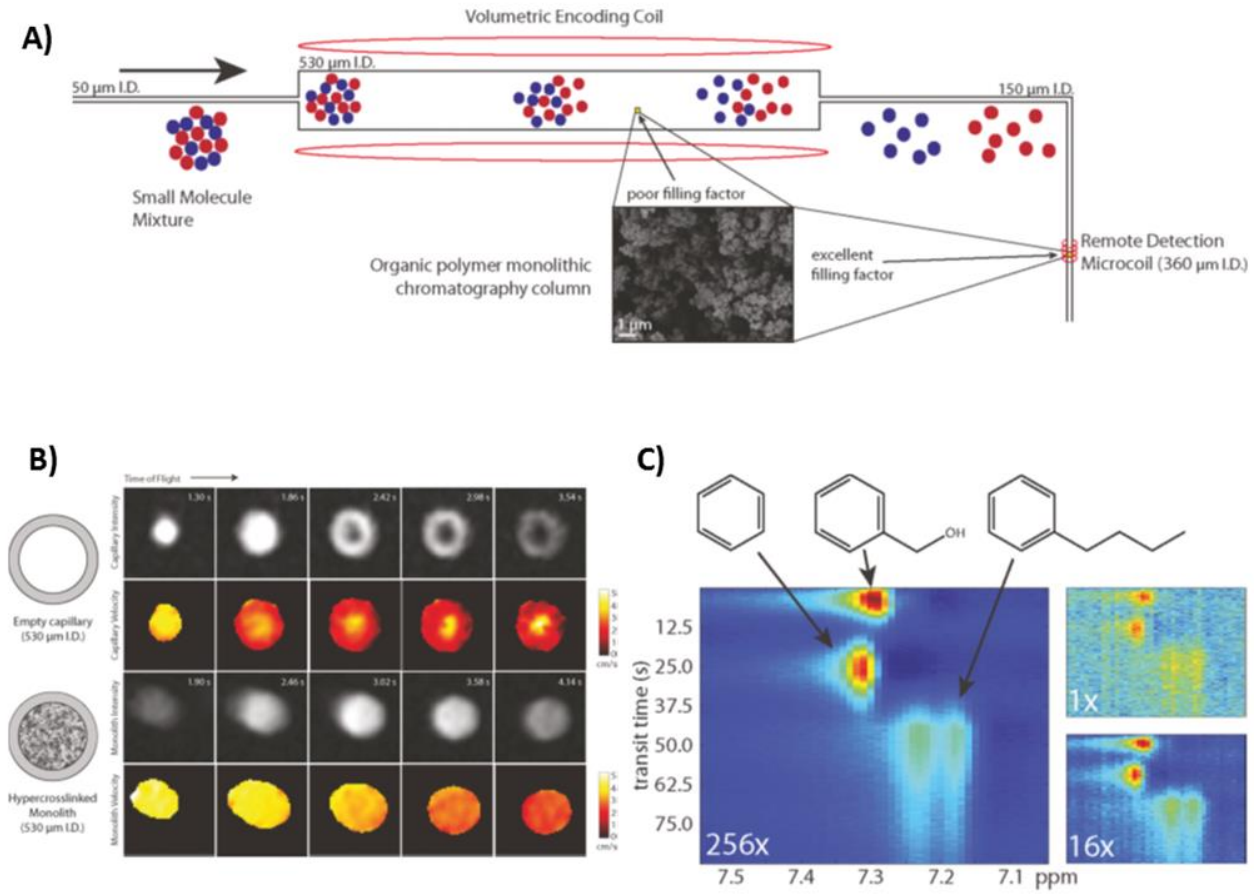


Figure 5. (A) Illustration of the remote detection experiment, as applied to an organic polymer monolithic column, (B) Axial images illustrating intensity and velocity for acetonitrile flowing through the open capillary (top) and monolith (bottom), (C) Two-dimensional plot illustrating the separation of small molecules using a hypercrosslinked monolithic chromatography column. The horizontal axis corresponds to the NMR chemical shift, while the vertical axis represents the transit time of compounds [**14**].

We have demonstrated an application of remotely detected magnetic resonance imaging for the characterization of flow and the detection of fast, small molecule separations within hypercrosslinked polymer monoliths [**14**]. Magnetic resonance imaging enabled high resolution intensity and velocity-encoded images of mobile phase flow through the monolith. The images confirm that the presence of a polymer monolith within the capillary disrupts the parabolic laminar flow profile that is characteristic of mobile phase flow within an open tube. As a result, the mobile phase and analytes are equally distributed in the radial direction throughout the monolith (**Figure 5B**). We have also shown in-line monitoring of chromatographic separations

of small molecules at high flow rates (**Figure 5C**). These experiments demonstrate the unique power of the magnetic resonance, both direct and remote, in studying chromatographic processes. Hypercrosslinked monoliths shown to be robust media for the rapid separation of small molecules with a transit time relative standard deviation of less than 2.1%, even after more than 300 column volumes were pumped through at high pressure and flow.

Furthermore, in separating the polarization, encoding, and detection steps of an NMR experiment, remote detection enables development of truly portable LC-NMR instrumentation when advantageously coupled with nonmetallic monolithic capillary columns.

Generally, we use isotropic columns in LC and control their separation performance by tuning external conditions. However, because of the simple and straightforward preparation, monolithic stationary phases offer elegant way how to prepare columns with longitudinal gradients of column properties.

We demonstrated the preparation of monolithic stationary phases with longitudinal gradient of porosity. For this, we used hypercrosslinking modification and controlled the extent of formation of small pores by the change in reaction time [**15**]. Segments of five columns hypercrosslinked for 30–360 min were coupled via zero-volume unions to prepare columns with segmented porosity gradients. The quality of isocratic separation for both low-molecular weight alkylbenzenes and high molar mass polystyrene standards improved significantly with the steepness of porosity gradient.

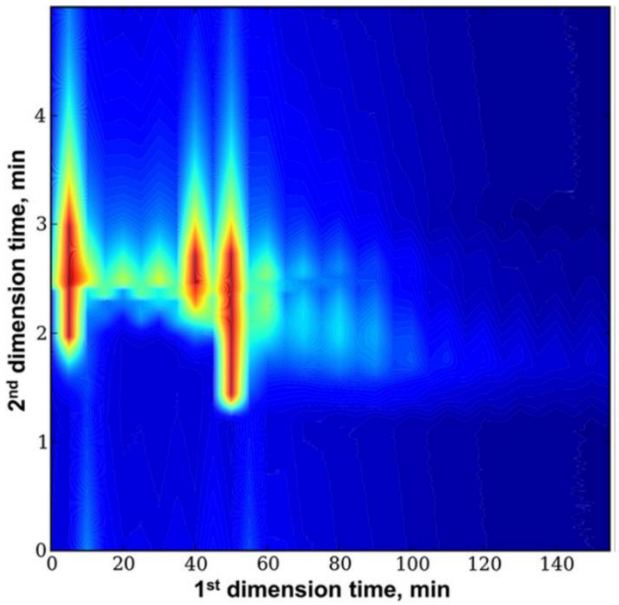


Figure 6. Online 2D LC of polystyrene standards using completely hypercrosslinked monolithic column combined with monolithic column with a gradient of porosity [**15**].

We have also prepared two individual columns with continuous gradient of porosity differing in the steepness. Column with a shallower porosity gradient enabled separation of polystyrene

standards comparable to that of hypercrosslinked column with no porosity gradient. However, compared to completely hypercrosslinked column, due to higher pore volume of permeable macropores, column with porosity gradient exhibited 1.5 times better column permeability and allowed fast separation of high molar mass polystyrenes.

Finally, we coupled completely hypercrosslinked column with porosity gradient column in 2D LC and demonstrated suitability of these columns for application in 2D separation of polymers combining on-column precipitation–redissolution and SEC separation modes as demonstrates **Figure 6**.

4 Towards multifunctional monolithic capillary column

Neurotransmission is a chemical communication which is controlled by small molecules called neurotransmitters that travel across the synaptic gap to transmit the desired information to other neurons. Catecholamine dopamine belongs to one of the most important neurotransmitters since it plays a major role in reward-motivated behavior. Undesired changes in dopamine metabolism result in serious illnesses such as depression, schizophrenia, Parkinson's disease, and tumors [51,52]. Therefore, it is necessary to develop fast, simple, and reliable analytical methods for its precise determination in various biological samples.

To reach this goal, numerous protocols are developed daily that include several subsequent steps and apply various experimental methods. One of the current trends in analytical chemistry is to integrate individual steps of sample analysis into one online and comprehensive workflow, which eliminates tedious and expensive sample manipulation. Yet, another trend is to miniaturize these methods and systems, and to develop online miniaturized capillary and microfluidic analytical systems.

4.1 Sample extraction and focusing

We have used polymer monoliths to develop an online solid-phase extraction with liquid chromatography method for determination of dopamine in a urine sample [**16**]. A polymerization

mixture containing 4-vinylphenylboronic acid monomer has been used to prepare a trapping column. Boronic acid functionality covalently bonds to cis-diol functionality at neutral pH, while acidic pH allows reversible de-attachment of selectively trapped compounds. Additionally, a monolithic stationary phase with zwitterion functionality has been used to prepare capillary column for the separation of dopamine.

One-variable-at-a-time approach has been used to optimize experimental conditions including molarity, pH, and flow rate of the loading buffer together with a time switching valve to provide the highest dopamine recovery. The best results were achieved for dopamine loaded at 0.5 μL/min in 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer with pH 7. By using these conditions, the concentration of dopamine in urine sample was determined to be 1.19 and 1.28 mg/L with a calibration curve method and a standard addition method, respectively.

In the next step, an experimental design has been used to optimize an online coupling of two extraction loops with a separation monolithic capillary column to allow comprehensive monitoring of dopamine in a flowing system. Sample loading flow rate and a flow rate of buffer back-flush were determined as the most significant variables. Finally, the degradation of unstabilized dopamine in a buffer was continuously studied by an optimized experimental setup that allowed the determination of both rate and half-time of dopamine degradation.

Although presented for dopamine only, other cis-diol containing compounds can be also determined. Optimized results will be used in the development of monolithic capillary column integrating both sample focusing and separation of cis-diol neurotransmitters in a single capillary unit. For that, we plan to attach 4-vinylphenylboronic acid monomer at the surface of a generic monolithic stationary phase by a spatially controlled UV-initiated grafting. Our yet unpublished results show that modification of one third of the column length with 4-vinylphenylboronic acid monomer allows fabrication of dual-function monolithic capillary column providing both sample focusing (extraction) of the desired compounds and their subsequent separation in one single analysis.

4.2 Integrated electrochemical detector

Very recently, we have introduced a concept of an integration of an electrochemical detector inside the monolithic capillary column [**17**]. Both carbon fiber and silver microwire were used as a working and a pseudoreference electrode, respectively, and inserted into the ending of capillary to prepare a monolithic capillary column with an integrated electrochemical detector.

Carbon fiber as a working electrode and silver microwire as a pseudoreference electrode with a diameter of 7 μm and 25 μm, respectively, were attached to contact silver-plated wires using conductive silver paint and allowed to dry at room temperature. Afterwards, the microelectrodes were cut to desired length with a razor blade and fixed in parallel on ceramic slides using cyanoacrylate adhesive (**Figure 7A**). The capillary monolithic column was carefully slid to the required length onto the working and reference electrode with the aid of micromamipulator under microscopic observation using an optical stereomicroscope (**Figure 7B**). The position of the capillary with embedded microelectrodes on a ceramic support was fixed afterwards with cyanoacrylate adhesive (**Figure 7C**).

Prepared capillary devices offered stable and robust results with relative standard deviations of retention, resolution, and detection signal lower than 1.5, 5.5, and 5.0%, respectively. To further increase the sensitivity of developed electrochemical microdetector, a multiple pulse amperometry detection mode has been used. An optimized integrated device provided a reliable chromatographic separation of mixture of neurotransmitters (**Figure 7D**) with a calibration curve for dopamine linear from 0.5 to 20.0 mg/L and an instrumental limit of detection as low as 24 pg of injected dopamine.

Finally, a developed capillary column was applied to successfully determine the dopamine in a human urine. By using both a calibration curve and a standard addition method, the dopamine level was determined to be 0.74 ± 0.03 mg/L and 0.71 ± 0.02 mg/L, respectively. Triplicates of dopamine analysis provided relative standard deviations lower than 2.7% for intraday analyses, while interday relative standard deviations were lower than 3.6% for five consecutive days.

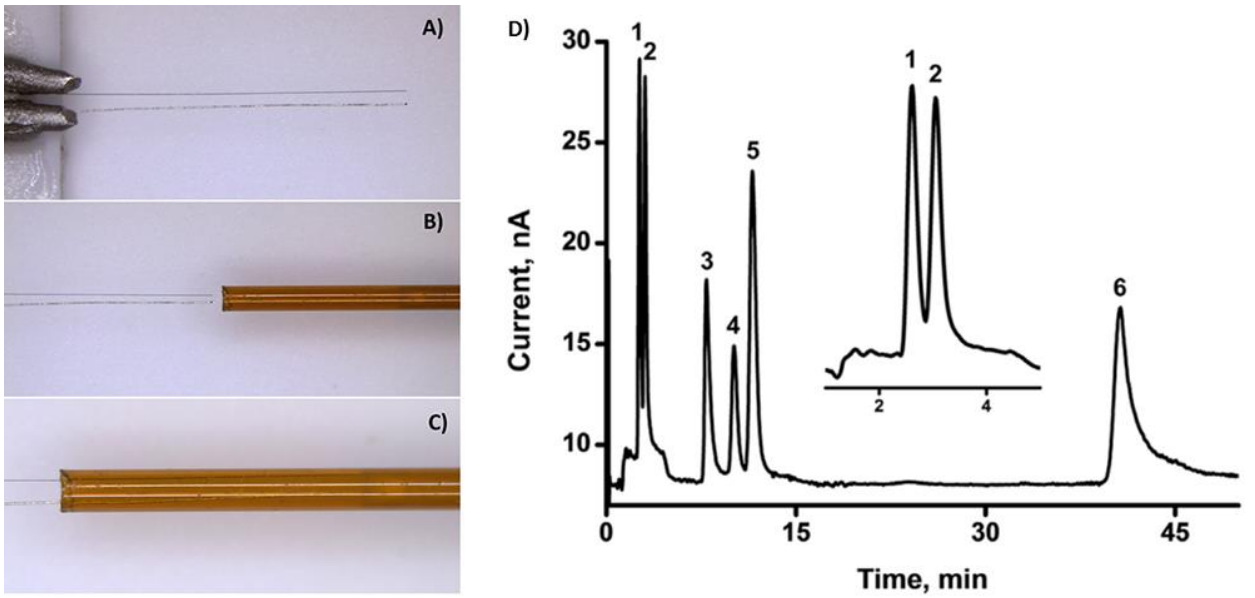


Figure 7. Preparation of integrated electrochemical detector: (A) working (carbon fiber, up) and pseudoreference (silver microwire, down) electrodes glued to the silver-plated wires, (B) insertion of microelectrodes into the end of fused-silica capillary with monolithic stationary phase, and (C) microelectrodes inside the end of the capillary. (D) Separation of mixture of neurotransmitters on monolithic capillary column with an integrated electrochemical detector in 5% acetonitrile with 0.1% TFA. Analytes: (1) epinephrine, (2) dopamine, (3) serotonin, (4) homovanillic acid, (5) 5-hydroxyindol-3-acetic acid, (6) 3,4-dihydroxyphenyl acetic acid [**17**].

The presented instrumental limit of detection calculated from a regression line is slightly higher when compared to other HPLC systems with electrochemical detection where limits of detection are determined from the noise of the baseline [53]. However, in this proof-of-concept study we aimed mainly in the utilization of straightforward preparation of monolithic stationary phases and simple miniaturization of an electrochemical detection.

Advantageous combination of these techniques allows further development of tailored analytical systems. For example, the coupling of an integrated electrochemical detector to a dual-function monolithic capillary column mentioned in Chapter 4.1 provides multifunctional three-in-one monolithic capillary column offering sample focusing, separation, and separation in one single analysis.

5 Conclusions & Future aspects

Since their introduction in the 1990s, monolithic stationary phases caught considerable attention as an alternative to widespread particulate stationary phases. The internal porous

structure of an interconnected network of channels allows convective flow of the mobile phase through the column and provides fast isocratic and gradient separations of both small and large molecules. While inorganic-based silica monoliths excel in the isocratic separations of small molecules, the power of polymer-based monoliths is in the fast gradient separations of large molecules. Up to now, various preparation protocols have been introduced to increase the efficiency of polymer monoliths in the separation of small molecules, as also demonstrated in this thesis.

Nowadays, the efficiency of polymer-based monolithic stationary phases cannot compete with commercially available particulate stationary phases, especially those with superficially porous particles. One of the possibilities of how to improve efficiency of monolithic stationary phases is a utilization of highly ordered materials, such as polyhedral oligomeric silsesquioxanes or metal-organic frameworks and prepare homogeneous composite stationary phases [**5**]. A very promising technique for future fabrication of monolithic stationary phases is additive manufacturing, also known as 3D printing, especially when combined with post-processing surface modification with a polymer layer providing desired selectivity.

On the other hand, applicability of current polymer monoliths is in their very simple preparation and straightforward surface modification. Without exaggerating, almost any functional moiety can by attached at the surface of monolith. Additionally, spatial control of UV-initiated grafting allows segmented modification leading to multifunctional devices. Possible control of monolithic stationary phase selectivity via tailored preparation might provide – after thorough optimization of experimental conditions – solution for almost any separation problem.

Another advantage of polymer monoliths is that they can be very easily prepared in any size and shape, including capillary, chip, and planar format. This makes them ideal materials for development of lab-on-chip systems integrating all necessary steps of sample analysis (sampling, sample transport, filtration, dilution, chemical reactions, separation, and detection) in one integrated total analytical system. Such miniaturized and portable systems offer analysis results at the point of interest and eliminate time-consuming, long-distance, and expensive sample transport and preparation.

I believe that my involvement in polymer-based monolithic stationary phases including a description of pore formation and its characterization, preparation of polymer monoliths for analysis of polar compounds, introduction of hypercrosslinked monolithic stationary phases, and the development of an integrated monolithic capillary column significantly contributed to the current state of the art of polymer-based monolithic stationary phases research.

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Appendix

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