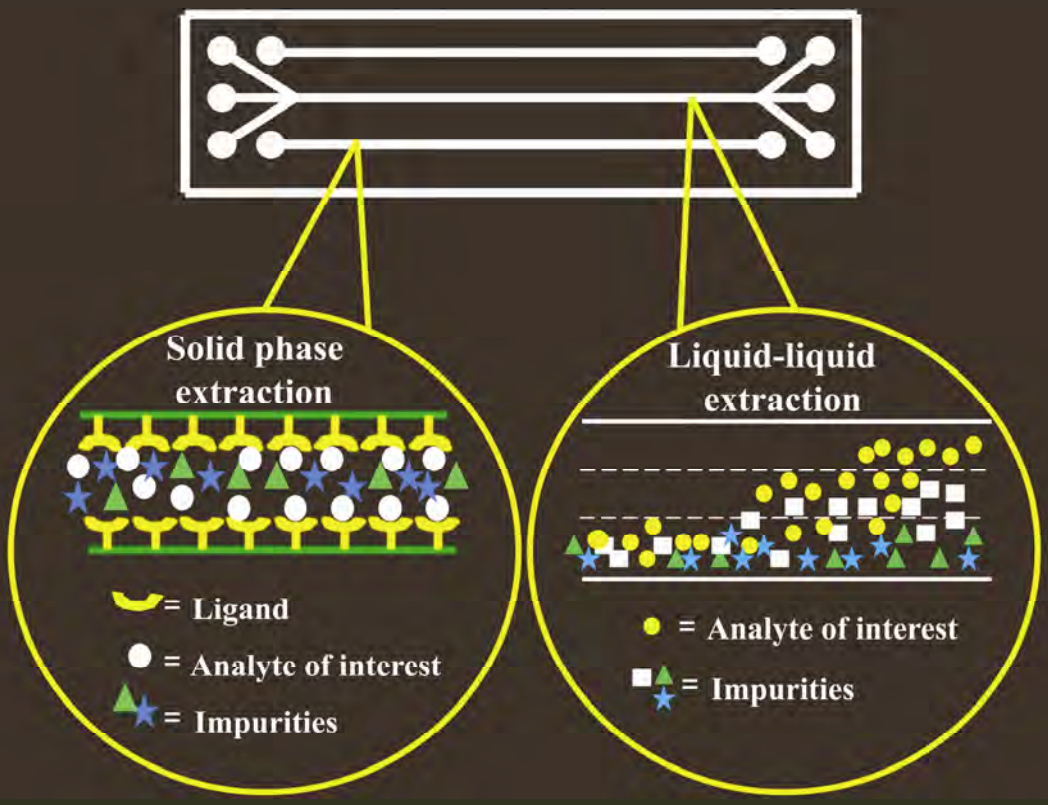
Microfluidic devices for sample clean-up and screening of biological samples



Kali Kishore Reddy Tetala

Propositions

1. Carbohydrate monolithic columns appear to have considerable merit for applications in diagnostics and possibly even treatment of Guillain-Barré syndrome (*this thesis*)

2. Modeling can help to quickly optimize the chip design and experimental conditions to improve extraction of analytes in two- and three-phase microchips (*this thesis*)

3. In current research scenarios, micro and nanotechnologies offer an inspiring interface between various branches of life sciences

4. Microfluidics have, like everything else, their advantages and disadvantages

5. Microalgae are a better choice than food plants for biofuel production as societal and ecological costs are reduced

   Williams, P. J. L. *Nature*, *450*, **2007**, 478

6. One's scientific findings become much more valuable, if they relate with the essence of mankind's problems

7. Complexity of human relationships is like chemical bonds

Propositions belong to the Ph. D. thesis

"Microfluidic devices for sample clean-up and screening of biological samples"

Wageningen, 27th February 2009

Kali Kishore Reddy Tetala

Microfluidic devices for sample clean-up and screening of biological samples

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Microfluidic devices for sample clean-up and screening of biological samples

Kali Kishore Reddy Tetala

Proefschrift
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To my beloved parents

ప్రియమైన అమ్మ, నాన్నకి

To be good and to do good, that is the whole of religion

- Swami Vivekananda

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1

General Introduction

Abstract

This chapter gives an insight into the origin of the μTAS (miniaturized total analysis systems) concept and a wide range of available materials. The application of microfluidic devices in the field of chemistry is exemplified. Further, the scope for potential research in the field of analytical chemistry is described. The thesis outline is presented at the end of this chapter.

There's Plenty of Room at the Bottom
Richard Feynman

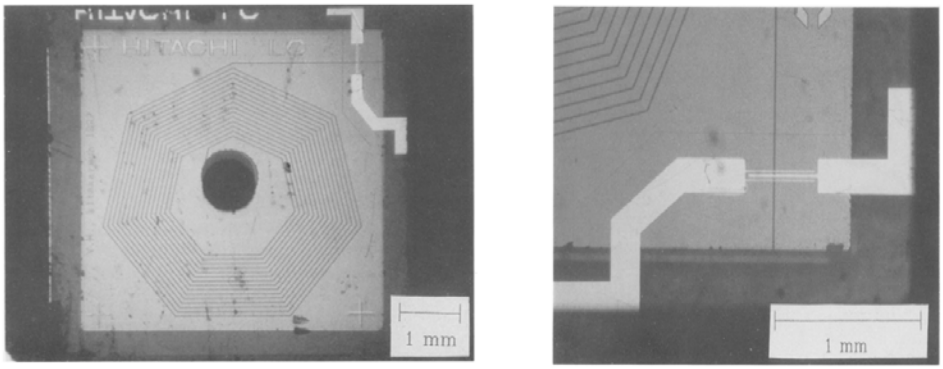
1.1 Introduction

Microfluidics caught the attention of researchers with the concept to fabricate entire lab-on-a-chip or miniaturized total analysis systems (μTAS). It is a rapidly growing technology in the 21st century due to its tremendous applications in diverse research areas of life sciences (medicine, biotechnology, pharmaceutical industry, and chemistry). By taking a closer look at the rapid growth of this technology, it is predicted that microfluidics will result into a separate branch of life sciences in the near future (Santini et al., 2000; Stone and Kim, 2001; Reyes et al., 2002; Khandurina and Guttman., 2002; Schulte et al., 2002; Verpoorte, 2002; Huikko et al., 2003; Erickson and Li, 2004; Marle and Greenway, 2005; Walt, 2005; Yager et al., 2006; Whitesides, 2006; Haeberle and Zengerle, 2007). At the moment chemists, biologists, physicists and engineers around the globe are working together on diverse challenging projects to develop various microfluidic systems.

Microfluidics is a technology tool box comprising a network of pumps, filters and valves connected to chips fabricated with single or multiple channels of several millimeters long and diameters in the range of ten to hundreds of micrometers. These chips can handle small amounts of fluids ranging from 10^{-6} to 10^{-18} litres (Whitesides, 2006).

The first seed for miniaturization was sown on 16th Dec 1947 with the invention of the first point-contact transistor at Bell laboratories (U.S) in the field of electronics. This intriguing invention along with other fruitful miniaturizations in the field of electronics fueled the concept of miniaturization in other fields (Geschke et al., 2004). The foundation for microfluidic devices in chemistry was laid by analytical chemists in 1979 with miniaturization of a gas chromatograph (GC) on a silicon wafer (Terry et al., 1979). The success of this device was limited due to the lack of technical experience to work with such miniaturized devices. In the mean time, engineers devoted more time to develop different fabrication techniques for microfluidic components (micro-pumps, microvalves, and chemical sensors) on silicon to pump fluid through the channels, mixing, to control and measure fluid flow in these devices (van Lintel et al., 1988; van de Pol et al., 1989; Smits, 1990; Shoji et al., 1990; van de Pol et al., 1990; Esashi, 1990). In 1990, Manz et al. successfully miniaturized HPLC using silicon-Pyrex technology (Manz et al., 1990). The schematic design of this chip is depicted in fig 1.1. The success of this device ensued in the renaissance of microfluidic devices in the field of chemistry with silicon technology. In the same year, Manz et al. proposed the μTAS concept (miniaturized total analysis systems), a modified version of the existing TAS concept (total analysis systems), to integrate sample clean-up, separation and analysis on a single chip (Manz et al., 1990a). The reason for the concept of miniaturization is to improve the analytical performance of the devices. The envisioned concept of Manz et al. has the advantages mentioned below.

1. Consumption of small volumes of reagents and solvents
2. Shorter reaction and analysis times
3. Perform multiple reactions using a single chip
4. Efficient separation and detection of samples on-chip



a) b)

Figure 1.1 *Miniaturized HPLC design: a) Top view of the chip (5 mm x 5 mm) with channel dimensions 6 µm x 12 µm x 15 cm. The column inlet is located at centre of the chip (the big black round spot). Conventional HPLC pump is employed for the fluid flow b) Detection area includes "column outlet and two electrodes (platinum was the electrode material)" (reproduced with permission from Manz et al., 1990).*

Table 1.1 *Various materials employed in preparing microfluidic devices and their advantages*

Materials	Advantages
Silicon	1) Mechanical (hard, strong) and electrical properties
	2) Purity of the material (99.99999 %)
	3) Clean room compatibility (no pollution)
Glass	1) Chemical resistance, thermal stability and relative biocompatibility
	2) Optical transparency (optical detection and visual inspection)
	3) Dielectric (can withstand high voltages)
Polymers	1) Flexible material properties and surface chemistry
	2) Low electrical conductivity and good biocompatibility
	3) Low material and microfabrication cost makes manufacturing easy
Hydrogels	1) Flexible material with varying degree of porosity
	2) Biocompatible, degradable and easy to handle
	3) Mechanical property (soft)

The following years witnessed the development of diverse microfluidic devices and in most cases miniaturization of various analytical devices as first realized on silicon (prototype of a

micro Polymerase Chain Reaction device, flow injection analysis on a chip, and microfilters with controlled pore-size) (van der Schoot et al., 1991; van Rijn et al., 1997; Northrup et al., 1998). Because microfluidic devices took advantage of silicon at the proof of principle stage, the commercialization of these devices was limited due to the enormous production costs (material and fabrication). Commercialization of these devices was successfully realized using other materials like glass, polymers, and hydrogels. In general, polymer materials are classified into three types based on the interaction between polymer chains: thermoplastics (PMMA (polymethylmethacrylate), PC (polycarbonate), and PS (polystyrene)), thermosets (some polyimides and polyesters), and elastomers (PDMS (polydimethylsiloxane)). Besides controlling production costs of microfluidic systems, another reason to replace silicon with other materials is the diverse advantages these materials offer (see table 1.1) (Hoffman, 2001; Oosterbroek and van den Berg, 2003; Geschke et al., 2004).

1.2 Applications and scope for potential research

In this section, some important contributions of microfluidic devices to the field of chemistry are discussed. Subsequently, the reason that prompted me to pursue the presented research in this thesis using microfluidics is mentioned. The scope for further research using microfluidic devices is highlighted.

Chemical industry is constantly seeking new technologies which can facilitate a safe environment for working with highly reactive and toxic intermediates (e.g. cyanides, peroxides, azides), achieve high yields and high selectivity of products, suppress undesired side reactions, and accomplish high-throughput (Jensen, 1999; Fletcher et al., 2002; Jähnisch et al., 2004; Watts and Haswell, 2005; Pennathur et al., 2008). Microreactor technology appears the way to go due to the following appealing features:

1. High surface area to volume ratio (SAV) - high heat-exchange efficiency allows for fast heating and cooling in reaction mixtures
2. High mass transport - small channel dimensions ensue in short diffusion times
3. Easily controllable parameters (pressure, temperature, residence time, and flow rate)
4. Undesired side reactions are impeded by suppressing hot spot developments

Organic chemists repeated some of the well known chemical syntheses (Wittig reaction, Aldol condensation, Diels-Alder reaction, and Suzuki coupling) successfully using microreactors and in most cases with improved yields. The application of microreactors was further extended to other known chemical reaction types such as elimination, nucleophilic substitutions on aliphatics, electrophilic substitutions on aromatics, cycloadditions, etc. (Jähnisch et al., 2000; Fletcher et al., 2002; Jähnisch et al., 2004). Fluorination of organic compounds using elemental fluorine was successfully achieved by employing a specially

designed microreactor (Chambers and Spink, 1999). Synthesis of methyl isocyanate (MIC), a highly toxic chemical, was successfully demonstrated by Ryley's team (DuPont) in a silicon microreactor indicating microreactors are safe to work with (Service, 1998). Powerful explosive materials (used by terrorists during attacks) can now be analyzed more efficiently and faster utilizing small amounts of samples with microchip electrophoresis devices (Pumera, 2006).

Analytical chemistry is a broad field due to the fact that analytes of interest from complex matrixes (e.g. plant extracts, blood) can be identified with different techniques. The analytical process can mainly be subdivided as follows (see fig 1.2) (Lichtenberg et al., 2002; De Mello and Beard, 2003):

1. Analytical procedure (starting from sample preparation till obtaining final information)
2. Analytical method (pretreatment and interpretation) and
3. Analytical principle (measurements with device)

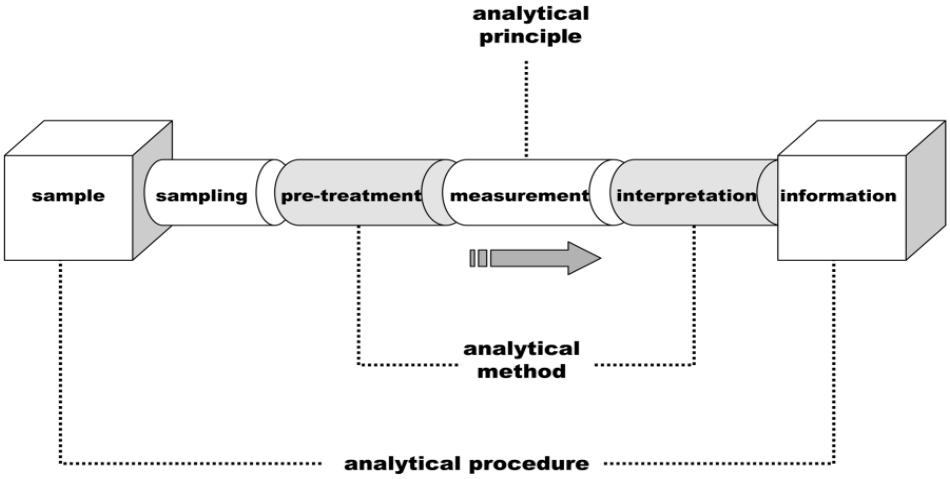


Figure 1.2 *Schematic representation of the subdivision of an analytical process (reproduced with permission from De Mello, 2003).*

After the introduction of the μTAS concept, miniaturization of analytical principles was demonstrated successfully and in particular integrated microchip capillary electrophoresis (CE) was reported from time to time (Jacobson et al., 1994; He et al., 1998; Jacobson et al., 1998; Khandurina et al., 2000; Liu et al., 2000; Ericson et al., 2000; Gottschlich et al., 2001; Lagally et al., 2001; Yu et al., 2001; Slentz et al., 2002; Jemere et al., 2002; Throckmorton et al., 2002; Ramsey et al., 2003; Lee et al., 2005; Liu et al., 2006).

Today, the big challenge in front of analytical chemists is to develop an integrated system which can extract/isolate analytes of interest from raw samples efficiently and perform on-

chip analytical processes. Extracting analytes of interest from complex fluids is performed by sample clean-up (pretreatment) process, which itself involves various techniques such as filtration, liquid-liquid extraction, solid phase extraction, and cell lysis. From various literature sources on chip-based pretreatment, it is clear that the extraction technique to be employed depends on the sample and the analytical method chosen, as there is no one universal technique for all samples (Xu et al., 1998; Kamholz et al., 1999; He et al., 1999; Tokeshi et al., 2000; Oleschuk et al., 2000; Minagawa et al., 2001; Jiang et al., 2001; Burns and Ramshaw, 2001; Lichtenberg et al., 2002; Auroux et al., 2002; Jemere et al., 2002; Xiao et al., 2006; Znidarsic-Plazl and Plazl, 2007). For analytical chemists, the goal of an adequate μTAS system is still a far-away one. This renders the opportunity for scientists to "improve and develop an integrated microfluidic system that is suitable for sample clean-up of raw samples". This motivated me to pursue the research presented in this thesis.

I aimed to contribute to the development of a microfluidic system, which can be used for selective sample clean-up prior to a detection step (e.g. for diagnostics or chemical analysis) and enrichment of valuable constituents through affinity chromatography.
For the development of such a microfluidic system, a systematic step-wise research plan was proposed, which is presented below.

a) Focus to develop a device that can selectively adsorb products from a process stream (plant extracts and serum samples). Lectins (proteins) found in plants, animals and microorganisms were chosen as model compounds. The reason to choose lectins is their specific interaction with carbohydrates, the information that can be extracted to interpret "how carbohydrates bind to proteins" and their commercial availability. To selectively capture lectins from a process stream using a microfluidic system, the interior glass wall of microchannel should be coated (immobilized) with carbohydrates. As carbohydrate immobilization protocols on glass are not widely available, a detailed study to optimize a feasible route for immobilization of carbohydrates on microscopic glass slides is required. After standardization of the protocol, carbohydrate coated microchannels can be developed to study carbohydrate-lectin interactions using the affinity chromatography technique.

b) Develop a three phase microchip (aqueous-organic-aqueous) for the sample clean-up of acidic or basic molecules in aqueous solution by extraction. The idea is to employ this reactor for the investigation of compounds of interest when present in small quantities e.g. to investigate the contents of a single plant cell for the presence of alkaloids.

For the initial experiments, a simple two phase system (100 μm width, 40 μm depth and 32 mm long) and commercially available alkaloids (strychnine nitrate, strychnine) were chosen. After acquiring sufficient experience and optimization of extraction conditions from a simple

two phase system, plant extracts containing alkaloids can be tested using a more complicated two x two phase microchip (shown in fig 1.3) and finally proceed to a three phase microchip. Fig 1.3 gives an overview of the basic idea for alkaloid purification from plant extracts using the two x two phase microchip design. On-line detection can be performed with UV or MS or the purified analytes can be transferred to a micro HPLC column.

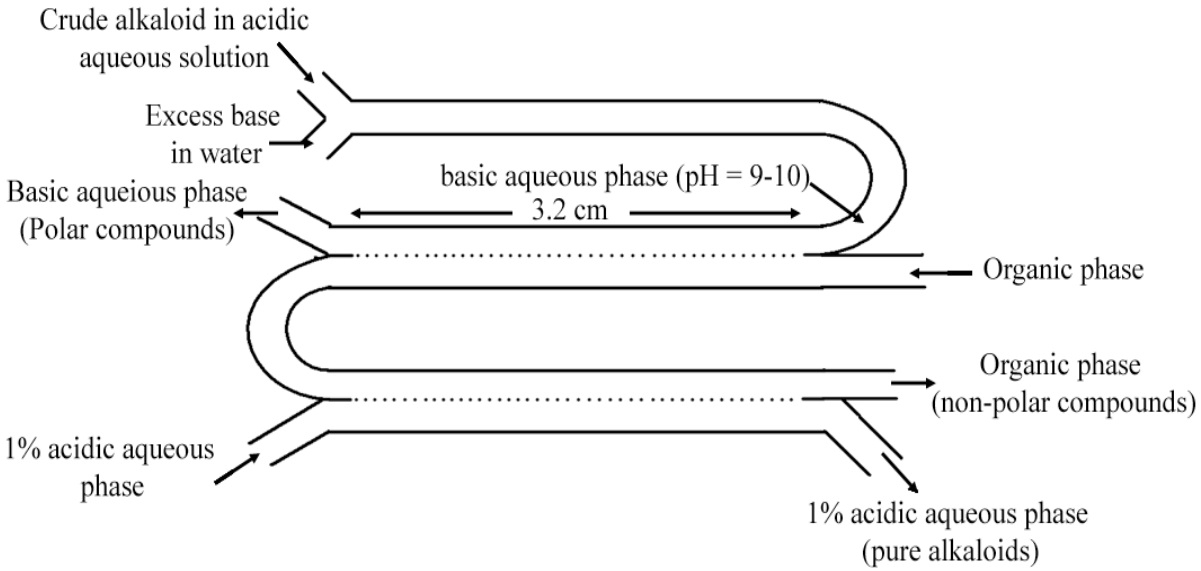


Figure 1.3 *Principle of alkaloid extraction from plant extracts using a two x two phase microchip (chip dimension of a single channel: 100 µm width, 40 µm depth and 3.2 cm long).*

1.5 Thesis outlook

This section elaborates on the plans to pursue the aim of this thesis. The task for **chapter 2-5** is to "develop a carbohydrate microfluidic chip for the sample clean-up of analytes from plant extracts and human serum".

The focus in **chapter 2** is to formulate a suitable carbohydrate immobilization protocol and validate the affinity of lectins towards carbohydrates. The step-wise plan is:

1. Prepare carbohydrate microarray on a glass surface
2. Characterize the surface modification with different techniques
3. Screening of lectins using a carbohydrate microarray with fluorescence microscopy and DESI-MS (Desorption electro spray ionization-mass spectrometry)
4. Transfer the developed protocol to prepare a carbohydrate microfluidic chip and study carbohydrate-lectin interactions using frontal affinity chromatography (FAC)

In **chapter 3** and **chapter 4**, to develop an affinity stationary phase via different synthetic routes in fused silica capillary columns. The detailed study involves:

1. Physical characterization of the stationary phase
2. Acquire affinity data of carbohydrates towards lectins using FAC and fluorescence microscopy
3. Elution of bound lectins from the column using various eluents
4. Study carbohydrate-lectin dissociation constants
5. Prepare carbohydrate microfluidic chips

The focus in **chapter 5** is:

1. Choose one of the feasible protocols developed for carbohydrate immobilizations as described in chapter 2, 3, and 4 for oligosaccharide column preparation
2. Utilize those columns to capture antibodies (IgM and IgG) through affinity interactions from serum samples of patients suffering with Guillain Barré syndrome (GBS) disease
3. The affinity of carbohydrates towards antibodies can be measured using ELISA (Enzyme linked immunosorbent assay) and fluorescence microscopy
4. Further, tests should be conducted to validate the use of these affinity columns as a diagnostic tool

At a later stage use the acquired experience in preparing affinity columns to develop an affinity array chip (see fig 1.4) in order to screen lectins (plant extracts) and antibodies (serum) using nano ESI-MS.

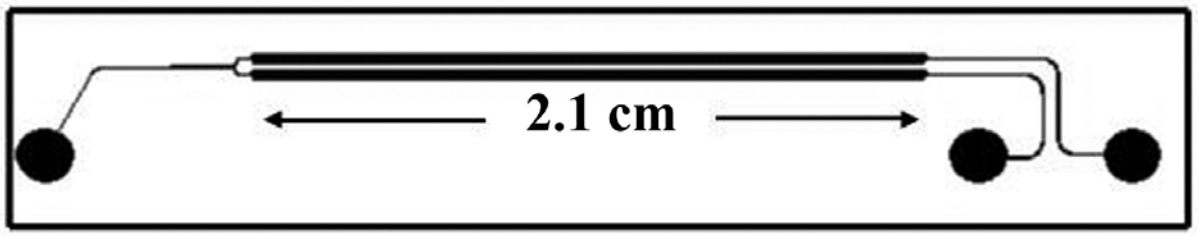


Figure 1.4 *Affinity array chip to screen lectins from plant extract. The channel dimensions of the affinity stationary phase are 200 µm x 80 µm x 2.1 cm. The channel dimensions connecting the stationary phase are 100 µm x 40 µm.*

The task in **Chapter 6** is to explore the possibility to develop a three phase microchip for the sample clean-up of acidic or basic molecules in aqueous solutions.

1. Optimize the extraction conditions of alkaloids using a two phase chip
2. Study the influence of the channel length for extraction efficiency
3. Compare experimental results with modeling studies using partition coefficients

4. Employ the optimized extraction condition for two x two phase microchip (see fig 1.3) and a three phase microchip to extract alkaloids from plant extracts
5. Monitor the extraction of plant extracts using on-line analysis (UV, LC or MS)
6. Perform on-chip separation of alkaloids using Capillary electrophoresis (CE)

Chapter 7 draws conclusions on the results obtained in various chapters of this thesis and compares the targets achieved with the desired goal. Subsequently, some future perspectives to further continue this diverse research are outlined.

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2

Carbohydrate modified capillary columns for interaction studies with lectins

Abstract

In this chapter, a brief description of lectins and their interaction with carbohydrates is given followed by the concept of frontal affinity chromatography (FAC). The experimental research in this chapter was carried out in two parts:

In the first part, the development of a carbohydrate immobilization protocol on a glass surface is described in combination with the study of specific carbohydrate-lectin interactions. The glass surface was modified with an aldehyde terminated linker group with varying chain lengths. A carbohydrate with an amino-terminated alkyl spacer was coupled to the aldehyde groups on the glass surface via reductive amination resulting in carbohydrate microarrays. Fluorescently labeled (FITC) lectins (concanavalin A and *Arachis hypogaea*) were used to study specific carbohydrate-lectin interactions. Contact angle and Atomic force microscopy (AFM) measurements were used to monitor the modification of the glass. Confocal laser fluorescence microscopy (CLFM) was employed to study the selective binding of lectins to the carbohydrate microarray.

In the second step, the developed carbohydrate immobilization protocol was applied to prepare α-mannose coated capillary columns. Frontal affinity chromatography (FAC) was employed to study the interaction of α-mannose with concanavalin A (Con A).

A part of this chapter is based on: Tetala, K. K. R., Giesbers, M., Visser, G. M., van Beek, T. A., Sudhölter, E. J. R. *Nat. Product Commun. 2*, ***2007***, 391-394.

2.1 Introduction

Lectins are non-enzymatic proteins found in plants, animals and microorganisms and are widely recognized as carbohydrate binding proteins. Carbohydrate-lectin interactions play a pivotal role in understanding fundamental aspects of "how carbohydrates bind to proteins" and a wide variety of biological information can be obtained in various fields such as oncology, plant pathology and medicine. Because of their biological functions dealing with cell-cell recognition, lymphocyte homing, cell agglutination and cancer metastasis, they are considered valuable biochemical tools (Weis and Drickamer, 1996; Lis and Sharon, 1998; Lee and Lee, 2000; Sharon and Lis, 2002; Goldstein, 2002; Ambrosi et al., 2005; Komath et al., 2006; Sharon, 2007). Lectins are classified into three main groups based on some common structural features (Lis and Sharon, 1998):

1. Simple lectins (legume, cereal, Amaryllidaceae, Moraceae, Euphorbiaceae, galectins and pentraxins)
2. Mosaic lectins (viral hemagglutinins, C-type, I-type and P-type)
3. Macromolecular assemblies (found in bacteria in the form of fimbriae or pili)

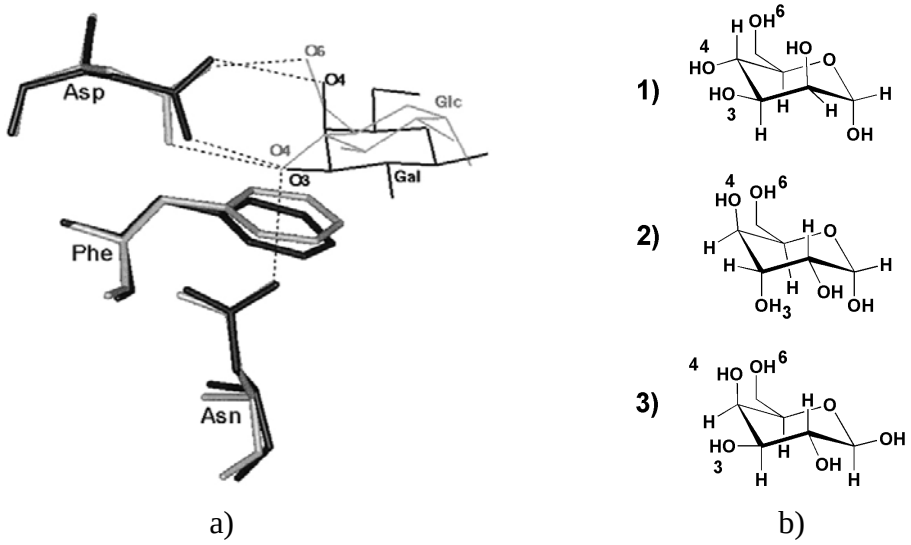
The family of legume lectins (available from plants) was considered as model lectins for studies due to their ease of availability in pure form. Concanavalin A (Con A) was the first legume lectin to be isolated in pure form from *Canavalia ensiformis* (Jack bean) in 1919 and was recognized for its affinity towards α-mannose/α-glucose. So far nearly 100 lectins in this family have been identified and characterized.

Simple lectins contain two or four subunits either identical or slightly different, each with a molecular weight below 40 kDa. Each subunit is comprised of a single polypeptide chain of about 250 amino acids and consists of a carbohydrate binding site with metal ions, calcium (Ca^{2+}) and manganese (Mn^{2+}). Carbohydrate-lectin interaction occurs via hydrogen bonds, Van der Waals and hydrophobic interactions, and metal coordination bonds. Four invariant amino acids (aspartic acid (Asp), asparagine (Asn), glycine (Gly), and an aromatic amino acid or leucine (Leu)) take part in the carbohydrate binding event. The metal ions are located at a distance of 4.25 Å and are in close vicinity (9-13 Å) to the carbohydrate binding site and help amino acids to position themselves in such a way that they can interact with carbohydrates (Lis and Sharon, 1998; Sharon and Lis, 2002; Ambrosi et al., 2005; Kommath et al., 2006).

An intriguing question for researchers was "how lectins discriminate between various epimers of carbohydrates to choose the carbohydrate of their interest". 3D-structural studies carried out by varying ligand (different carbohydrates) positions in different orientations revealed interesting facts about lectin specificity. In fig 2.1a the image acquired by superimposing β-galactose bound to EcorL on α-glucose bound to LoL-I (Banerjee et al., 1996; Sharon and

Lis, 2002) and in fig 2.1b the structure of three monosaccharides are depicted. A few interesting points observed in this study are:

1. The 4-OH & 6-OH groups of α-glucose and the 3-OH & 4-OH groups of β-galactose interact with oxygen of the aspartic acid (Asp) side chain via formation of hydrogen bonds
2. The 4-OH group of α-glucose and the 3-OH group of β-galactose interact with the amide of asparagine (Asn)
3. The 3-OH of α-glucose interact with the amide of glycine (Gly) and the 3-OH group of β-galactose interact with the NH of glycine (Gly)



a) b)

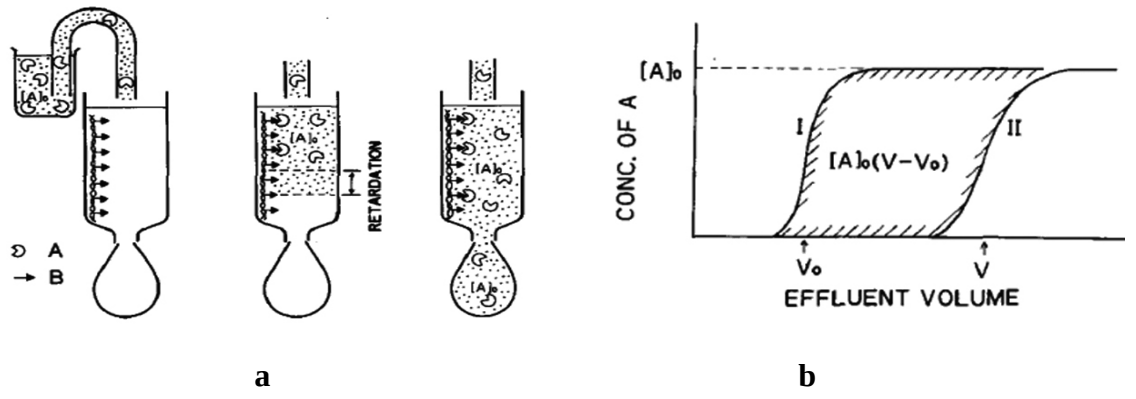
Figure 2.1 a) *Comparison of β-galactose and α-glucose interaction towards the same amino acid groups involved for carbohydrate binding to lectins (Sharon, 2002). b) Structures of three sugars are presented above (α-mannose (1), α-glucose (2), and β-galactose (3)).*

Screening and purification of proteins is carried out by various techniques: microarray plates, bioaffinity mass spectrometry, and frontal affinity chromatography (FAC) (Nilson, 2003). New lectins with interesting properties are constantly being isolated and characterized using crystallography and NMR. The limiting factor for these techniques is the requirement of a large amount of sample. Mass Spectrometry (MS) requires small amounts of sample and offers a wide range of information. These three techniques are complementary to one another. The task to researchers is "develop a method to screen and purify new lectins from a process stream". FAC with MS as an interface would be an ideal technique to screen lectins adequately.

Frontal affinity chromatography (FAC)

Kasai et al. introduced the concept of frontal affinity chromatography (FAC) in the year 1986. The principle of FAC is based on the study of molecular interaction between different biomolecules. With FAC, not only quantitative information but also insight into dissociation constants (K_d) of various biomolecules can be obtained (Kasai et al., 1986; Hirabayashi et al., 2000; Arata et al., 2001). In this method, an excess volume of analytes is continuously infused through a column with an immobilized ligand (fig 2.2a). Each analyte elutes at a different time depending on its affinity towards the ligand (fig 2.2b). The elution profile consists of an elution front and a plateau. These elution curves are called breakthrough volume curves. The column capacity can be determined by using the 50% point of breakthrough volume curve (fig 2.2b, V & V_0) and the initial concentration of the analyte ($[A]_0$) in the equation:

$$Capacity = [A]_0(V - V_0) \qquad (2.1)$$



a **b**

Figure 2.2 S*chematic representations of the FAC technique: a) Continuous infusion of analyte solution through the column, b) Comparison of two different analytes elution profiles and calculating column capacity (reproduced with permission from Kasai, 1986).*

As explained in chapter 1 (section 1.2), to capture lectins from a process stream, carbohydrates should be immobilized covalently on the column. However, a protocol to covalently immobilize carbohydrates onto a glass surface is not widely available. So far in literature covalent immobilization of carbohydrates was carried out on gold (Houseman and Mrksich, 2002; Park and Shin, 2002; Houseman et al., 2003; Galanina et al., 2003) and polystyrene surfaces (Love and seeberger, 2002), but only non-covalently on glass (Wang et al., 2002; Ratner et al., 2004). It is surprising that not much work has been carried out to covalently link carbohydrates on glass surfaces (Biskup et al., 2005) considering their advantages over other surfaces (Cheung et al., 1999; Perruchot et al., 2000; Li et al., 2001):

1. Low background fluorescence
2. Chemical inertness
3. Resistance to high temperatures and

4. Easy availability and low costs compared to other surfaces

Selecting a route to immobilize carbohydrates

Glass surfaces with active terminal functional groups are widely developed and employed for immobilization of various other biomolecules than carbohydrates (Ramsay, 1998; MacBeath and schreiber, 2000; Schaeferling et al., 2002; Lesaicherre et al., 2002; Glökler and Angenendt, 2003; Angenendt, 2005). To choose a feasible protocol several points were considered:

1. Easy synthetic route to prepare carbohydrates with useful terminal functional group
2. The terminal functional group (amine, carbonyl or hydroxyl, etc.) to be chosen, depends on the active group present on the carbohydrate and on the glass surface
3. Avoid non-specific protein adsorption on the surface during interaction studies

Synthesis of carbohydrates with various functional groups has been studied extensively (Lindhorst, 2001). Carbohydrates with amino termination are easy to synthesize and can be obtained in high yields (Ziegler et al., 1994; Lindhorst, 2001; Houseman and Mrksich, 2001). Li and coworkers reported a protocol for covalent immobilization of DNA on glass surfaces via aldehyde groups present on the glass (Li et al., 2001). This approach seems easy to reproduce, flexible and the linker chain length may prevent non-specific protein adsorption on the surface. Most importantly, the active terminal aldehyde on the glass surface can covalently bind to the carbohydrate with an amine termination via a Schiff base reaction. This protocol was selected for covalent immobilization of carbohydrates on glass surfaces, on the inside of capillary columns (intermediate step to study carbohydrate-lectin interactions using FAC) and finally within the channels of microfluidic chips.

2.2 General information

Materials and methods

Fused-silica capillaries with 75 μm i.d. and 375 μm o.d. were from Polymicro Technologies (Phoenix, AZ, USA). All chemicals were purchased from Sigma (The Netherlands).

^{1}H NMR and ***^{13}C NMR spectra*** were recorded either on Bruker DPX 300 (300 MHz) or DPX 400 (400 MHz). $CDCl_3$ (dried over mol. sieves) and D_2O were the solvents.

Contact angle measurements were performed using a Krüss instrument. MilliQ water was deposited on the surface using a micropipette (volume range: 0.5-50 μL). The average value of 3 droplets in discrete positions on the surface is reported.

Atomic force microscope (AFM) measurements were performed using a Nanoscope Digital instrument with a Si_3N_4 tip cantilever. The images were recorded in Contact mode using 256 pixels per line with a scan rate of 1.96 Hz. The surface roughness was determined with a scale of 5 μm (X-axis) and 0-20 or 0-30 nm (Y-axis).

Confocal laser fluorescence scanning microscope (CLFSM) measurements were performed with a confocal laser fluorescence microscope (LSM 510). An Argon ion laser was the source with an excitation wavelength of 488 nm. Images were recorded with an objective of Plan-Neofluar (10 X/0.3) and 512 pixels with 2.56 μs pixel time.

Frontal affinity chromatography (FAC) was performed using a syringe pump (Harvard 11 PicoPlus, dual syringe, VWR international, The Netherlands) connected to a micro injector (2 μL loop size) (7725, Rheodyne, USA). The capillary column was connected to the micro injector at one end and interfaced with a UV detector at the other end.

The working conditions for FAC were: Con A (0.1 mg/mL) in both water and binding buffer was used. The flow rate of the syringe pump was 0.2 μL/min. On-capillary measurements were performed with a K-2501 UV detector (Knauer, Germany) at 218 nm. The detection window was created on-column by local heating to remove the polyimide coating on the outside of the capillary.

Synthesis of amino-terminated carbohydrates

Figure 2.3 depicts the structure of β- & α-carbohydrates with an amino-terminated spacer (β-gal, β-glc, α-man, α-gal & α-glc), which were synthesized according to a literature procedure (Ziegler, 1994; Vermeer et al., 2000; Lindhorst, 2001; Houseman and Mrksich, 2001).

Synthesis of β-carbohydrates with amino terminated alkyl spacer

5-Amino-1-pentanol (67.9 mmol) was added to sodium bicarbonate (23.8 mmol) in water (400 mL) in a 500 mL round bottom flask and stirred until a clear solution was obtained. To this solution, benzyl chloroformate (102.9 mmol) was added dropwise and the mixture was stirred for 6 h. The reaction mixture was then extracted with ethyl acetate (3 x 70 mL). The collected organic layers were combined and extracted with water (15 mL), brine (15 mL) and then dried over sodium sulphate. The collected organic layer was concentrated to a crude product. The crude product was purified with column chromatography (petroleum ether: ethyl acetate (4:1)) to obtain compound **1** (81.3% yield) in pure form.

5-(Benzyloxycarbonylamino)pentanol 1

^{1}H-NMR (300 MHz, $CDCl_3$) δ= 7.30-7.58 (5H, m), 5.07 (2H, s), 4.93 (1H, bs), 3.56-3.63 (2H, m), 3.12-3.22 (2H, m), 1.24-1.73 (6H, m) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$) δ= 156.57, 136.59, 128.52, 128.10, 66.63, 62.52, 40.92, 32.17, 29.71, 22.87 ppm.

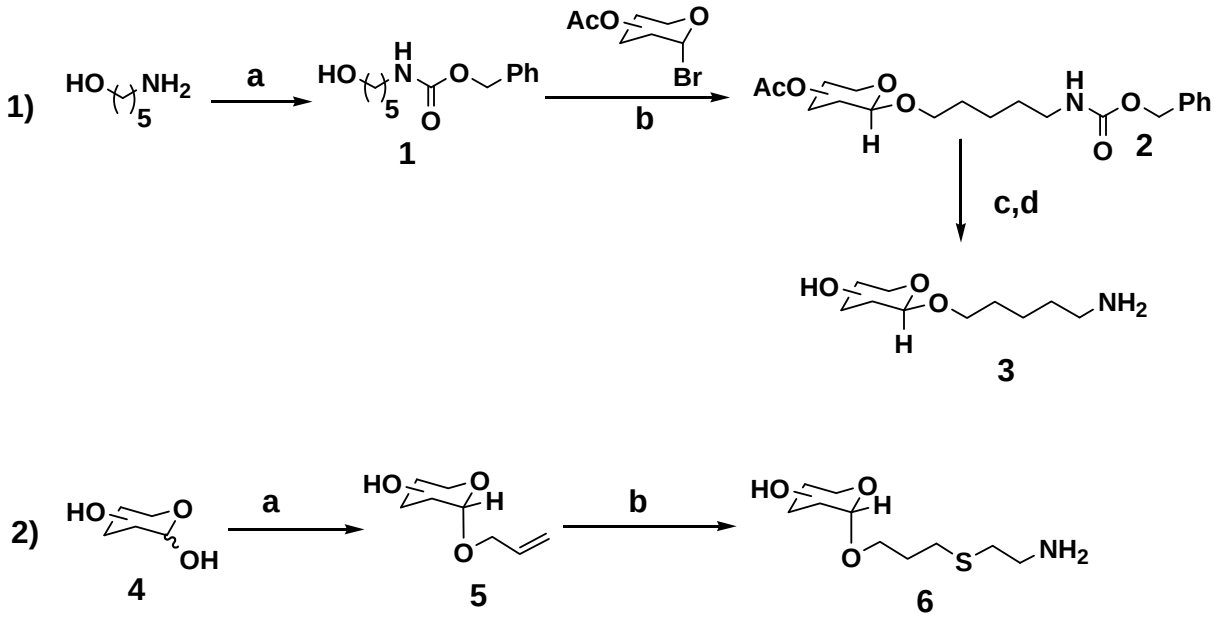


Figure 2.3 *1) Synthesis of β-amino carbohydrates: a) benzyl chloroformate, $NaHCO_3$, H_2O, RT, 6 h; b) $Hg(CN)_2$, C_6H_6-CH_3NO_2 (1:1), 24 h, RT; c) NaOMe/MeOH; d) H_2/Pd (10% activated charcoal, DeGussa type), 3-4 h. 2) Synthesis of α-amino carbohydrates: a) allyl alcohol, acetyl chloride, 60 °C, 4 h; b) cysteamine hydrochloride, H_2O, UV-254 nm, 4 h.*

5-(Benzyloxycarbonylamino)pentyl glycosides 2

A mixture of **1** (6 mmol) and $Hg(CN)_2$ (6 mmol) in benzene-nitromethane (1:1, 37.5 mL) was taken in a 100 mL round bottom flask and stirred under argon for 30 min. To this, a solution of per-O-acetylglycosyl bromide (5 mmol) in benzene-nitromethane (1:1, 12 mL) was added with a syringe. The reaction mixture was stirred for 24 h. After completion of the reaction, the reaction mixture was filtered to remove $Hg(CN)_2$. The organic layer was evaporated to an oil, which was dissolved in dichloromethane (70 mL) and filtered again. The organic layer was then treated with brine (3 x 25 mL) and dried over sodium sulfate, which was then concentrated to a crude product. The crude product was purified with column chromatography (petroleum ether: ethyl acetate (4:6)) to obtain compound **2** (a-64.5% & b-67.3% yield) in pure form.

5-(Benzyloxycarbonylamino)pentyl-2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside 2a

^{1}H-NMR (400 MHz, $CDCl_3$) δ= 7.3-7.58 (5H,m), 5.04 (2H, s), 4.41 (1H, d, J = 7.8 Hz), 3.83-4.13 (6H, m), 3.42-3.48 (2H, m), 3.16-3.12 (2H, m), 2.09 (3H, s), 2.03 (3H, s), 1.99 (3H, s), 1.93 (3H, s), 1.45-1.56 (4H, m), 1.30-1.32 (2H, m) ppm.

^{13}C-NMR (100 MHz, $CDCl_3$) δ= 171.51, 170.57, 170.51, 170.41, 156.84, 137.05, 128.84, 128.40, 101.59, 71.7, 70.62, 69.7, 69.14, 67.86, 66.87, 62.45, 61.10, 41.22, 29.91, 29.32, 21.36, 20.98, 20.92, 14.53 ppm.

5-(Benzyloxycarbonylamino)pentyl-2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside 2b

^{1}H-NMR (400 MHz, $CDCl_3$) δ= 7.2-7.34 (5H, m), 5.19 (1H, d, J = 8 Hz), 5.09 (2H, s), 4.91-4.97 (2H, m), 3.68-4.26 (6H, m), 3.44-3.5 (2H, m), 2.18 (3H, s), 2.07 (3H, s), 2.03 (3H, s), 2.02 (3H, s), 1.63-1.73 (4H, m), 1.25-1.37 (2H, m) ppm.

^{13}C-NMR(100 MHz, $CDCl_3$) δ= 171.63, 171.12, 169.83, 169.76, 156.86, 137.01, 128.90, 128.49, 101.13, 73.22, 72.14, 71.72, 70.20, 69.3, 68.95, 64.11, 61.84, 60.8, 41.92, 29.92, 29.33, 23.51, 21.37, 14.58 ppm.

5-Aminopentyl-β-D-glycosides 3

To compound **2 (a** or **b)** (1 mmol), sodium methoxide (1 mmol) in methanol (10 mL) was added and stirred for 10 min. To this mixture Dowex-50 WX (H^+) was added and stirred for 10 min. The reaction mixture was filtered and washed with methanol (5 mL). The filtrates were combined and treated with palladium (0.2 g, 10% on activated charcoal) and hydrogenated for 2 h. This resulted in the desired compound **3 (a** or **b)**. The compounds were used for further tests without purification.

5-Aminopentyl-β-D-galactose 3a

^{1}H-NMR (400 MHz, $CDCl_3$) δ= 4.09 (1H, d, J = 8 Hz), 3.64-3.73 (2H, m), 3.21-3.54 (6H, m), 2.67-2.71 (2H, m), 1.45-1.56 (4H, m), 1.25-1.30 (2H, m) ppm.

^{13}C-NMR(100 MHz, $CDCl_3$) δ= 103.65, 77.49, 75.58, 73.84, 71.70, 71.34, 69.09, 39.86, 39.65, 39.44, 39.23 ppm.

5-Aminopentyl-β-D-glucose 3b

^{1}H-NMR (400 MHz, $CDCl_3$) δ= 4.2 (1H, d, J = 6 Hz), 3.5-3.74 (2H, m), 3.16-3.48 (6H, m), 2.6-3.68 (2H, m), 1.47-1.62 (4H, m), 1.26-1.51 (2H, m) ppm.

^{13}C-NMR(100 MHz, $CDCl_3$) δ= 103.34, 77.30, 77.24, 73.9, 70.54, 61.52, 61.09, 40.80, 40.52, 40.24, 39.97 ppm.

Synthesis of 3-(2-Aminoethylthio)propyl-α-L-glycosides 6

Allyl-α-L-glycosides 5

To a solution of allyl alcohol (1.03 mol), acetyl chloride (60 mmol) was added dropwise at 0 °C. To the above solution, compound **4 (a, b** or **c)** (20 mmol) was added. The reaction mixture was stirred at 70 °C for 4 h. Excess allyl alcohol was removed under reduced pressure resulting in crude product. The crude product was purified with column chromatography (ethyl acetate (100%) and ethyl acetate: methanol (17:3)) to obtain compound **5** (a-55.2%; b-54.5%; c-57.6% yield) in pure form.

Allyl-α-L-glucose 5a

^{1}H-NMR (300 MHz, D_2O) δ= 5.92-5.97 (1H, m), 5.32 (2H, m), 4.90 (1H, d, J = 3Hz), 4.68-4.87 (2H, m), 3.28-4.16 (6H, m) ppm.

^{13}C-NMR (75 MHz, D_2O) δ= 133.96, 118.55, 97.72, 76.29, 73.50, 72.23, 71.62, 70.61, 60.92 ppm.

Allyl-α-L-galactose 5b

^{1}H-NMR (300 MHz, D_2O) δ= 5.76-5.91 (1H, m), 5.15-5.24 (2H, m), 4.85 (1H, d, J = 3.4 Hz), 4.62-4.75 (2H, m), 3.41-4.28 (6H, m) ppm.

^{13}C-NMR (75 MHz, D_2O) δ= 133.84, 118.50, 97.78, 71.25, 70.88, 69.77, 69.52, 69.15, 68.78 ppm.

Allyl-α-L-mannose 5c

^{1}H-NMR (300 MHz, D_2O) δ= 5.85-5.9 (1H, m), 5.26 (1H, dd, J = 6.2 Hz, 5.84 Hz), 5.2 (1H, dd, J = 5.46 Hz, 6.12 Hz), 4.82 (1H, d, J = 2 Hz), 4.01-4.14 (2H, m), 3.26-3.81 (6H, m) ppm.

^{13}C-NMR (75 MHz, D_2O) δ= 133.58, 118.81, 99.33, 72.91, 70.90, 70.12, 68.19, 66.24, 61.24 ppm.

3-(2-Aminoethylthio)propyl-α-L-glycosides 6

Compound **5 (a, b** or **c)** (8.6 mmol) was added to cysteamine hydrochloride (25.8 mmol) in water (120 mL) and irradiated at 254 nm for 5 h under stirring. Excess cysteamine hydrochloride was removed under reduced pressure resulting in crude product. The crude product was purified using column chromatography (ethyl acetate: isopropyl alcohol: water (9:4:2)) to obtain compound **6** (a-48.4%; b-47.5%; c-49.6% yield) in pure form.

3-(2-Aminoethylthio)propyl-α-L-glucose 6a

^{1}H-NMR (300 MHz, D_2O) δ= 4.84 (1H, d, J = 3.6 Hz), 3.31-3.5 (6H, m), 3.11-3.18 (2H, m), 2.93-2.97 (2H, m), 2.77-2.82 (2H, m), 2.61-2.76 (2H, m), 1.17-1.24 (2H, m) ppm.

^{13}C-NMR (75 MHz, D_2O) δ= 98.92, 76.67, 73.94, 72.66, 72.10, 70.48, 61.48, 42.76, 38.75, 34.42, 29.28 ppm.

3-(2-Aminoethylthio)propyl-α-L-galactose 6b

^{1}H-NMR (300 MHz, D_2O) δ= 4.88 (1H, d, J = 3 Hz), 3.66-3.91 (6H, m), 3.32-3.36 (2H, m), 3.14-3.19 (2H, m), 2.94-2.98 (2H, m), 2.64-2.82 (2H, m), 1.88-1.90 (2H, m) ppm.

^{13}C-NMR (75 MHz, D_2O) δ= 98.7, 71.34, 69.9, 69.64, 68.66, 66.83, 64.65, 49.27, 38.73, 33.68, 28.80 ppm.

3-(2-Aminoethylthio)propyl-α-L-mannose 6c

^{1}H-NMR (400 MHz, D_2O) δ= 5.03 (1H, d, J = 3 Hz), 3.56-3.87 (6H, m), 3.28-3.34 (2H, m), 3.14-3.16 (2H, m), 2.94-2.98 (2H, m), 2.77-2.82 (2H, m), 1.85-1.87 (2H, m) ppm.
^{13}C-NMR (100 MHz, D_2O) δ= 100.08, 73.16, 70.95, 70.37, 67.09, 66.41, 61.30, 38.67, 38.05, 33.63, 28.65 ppm.

Surface modification of glass

All solutions were freshly prepared. Glass slides were immersed in solution in an inclined position. Afterwards slides were rinsed and dried under a stream of "dust free" nitrogen. Carbohydrates were patterned using a Finntip 250 universal pipette (volume range 0.5-250 µL). Figure 2.4 depicts the scheme to prepare carbohydrate microarrays.

Linker group L_1

Glass slides were immersed in piranha solution [conc. H_2SO_4: 30% H_2O_2 (7:3 (v/v))] for 1 h, rinsed with water (2 x 5 min), dried and placed in an oven (T = 120 °C) for 10 min. Subsequently, these slides were immersed in 1% (w/v) N-(2-aminoethyl)-3-aminopropylmethyldimethoxy silane in 95% aq. acetone solution for 15 min, rinsed with acetone (2 x 5 min), dried and placed in oven (T = 120 °C) for 40 min. The silanized slides were immersed in 5% (v/v) glutaraldehyde in PBS (phosphate buffered saline, pH=7.2) solution for 2 h, rinsed with water (2 x 5 min) and dried.

Linker group L_2

The aldehyde terminated slides (L_1) were immersed in 5% aq. 2,2'-(ethylenedioxy) diethyl amine solution for 2 h, rinsed with water (2 x 5 min) and dried. The slides were immersed in 5% (v/v) glutaraldehyde in PBS solution for 2 h, rinsed with water (2 x 5 min) and dried.

Carbohydrate immobilization procedure

Carbohydrates (0.5-2 µL of a 5 mM solution in sodium carbonate buffer, pH=9) were spotted on specific locations of the aldehyde-terminated slides L_1 & L_2. The slides were placed in a wet box with 100% humidity for 1 h at room temperature. The box was placed in a water bath (T = 37-38 °C) for 2 h. The slides were rinsed with 1% SDS (sodium dodecyl sulfate) solution and distilled water (2 x 5 min). The carbohydrate microarrays were immersed in $NaBH_4$ (0.28 g, 75 mL PBS, 25 mL ethanol) solution for 15 min and rinsed with water (2 x 5 min) and dried. This resulted in the carbohydrate microarray.

Carbohydrate-Lectin interaction

The carbohydrate microarray was incubated for 1 h in 0.3% Tween 20 in PBS (1 mM Ca^{2+}, 1 mM Mn^{2+} & 1 mM Mg^{2+}) containing FITC labeled lectin (1 µg/mL) solution. Subsequently, the slides were rinsed gently with 0.3% Tween 20 in PBS (1 mM Ca^{2+}, 1 mM Mn^{2+} & 1 mM

Mg^{2+}) solution for 3 x 5 min, dried and scanned with a Confocal laser fluorescence microscope.

Carbohydrate capillary coating

Surface modification of the fused-silica capillary was performed as described in literature (Ericson, 1997) with slight modifications: acetone (20 min), distilled water (20 min), 0.1 M HCl (20 min), 0.1 M NaOH (1 h), distilled water (20 min) and acetone (20 min). The slides were then dried with nitrogen. Next the capillary was treated with 5% (w/v) N-(2-aminoethyl)-3-aminopropylmethyldimethoxysilane in 95% aq. acetone solution for 12 h followed by washing with acetone for 30 min. The silanized capillary was then treated with 10% (v/v) glutaraldehyde in PBS solution for 4 h, followed by washing with water (30 min). The aldehyde active capillary was then treated with 5 mM α-mannose in sodium carbonate buffer (pH=9) for 15 h, followed by treatment with $NaBH_4$ (0.28 g, 75 mL PBS, 25 mL ethanol) solution for 1 h and finally with water (30 min).

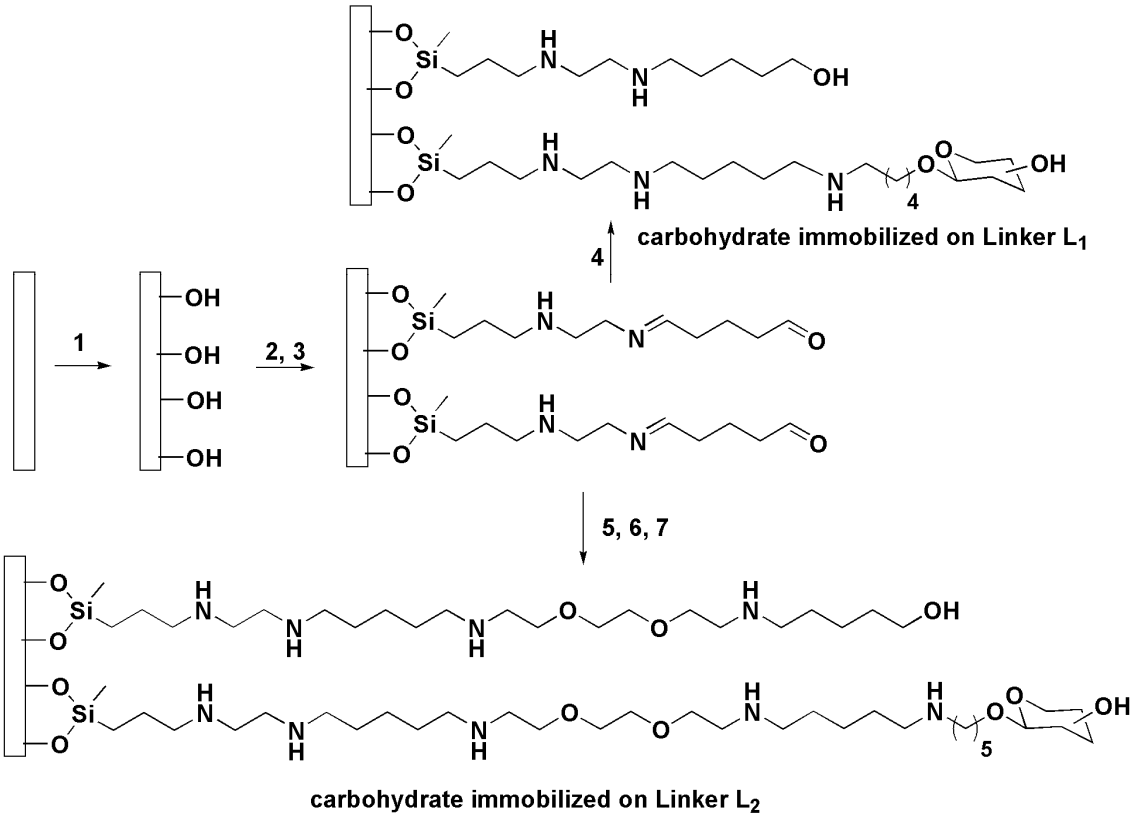


Figure 2.4 *Synthetic procedure to prepare carbohydrate arrays on glass surfaces 1) Piranha solution; 2) N-(2-aminoethyl)-3-aminopropylmethyldimethoxysilane; 3) glutaraldehyde; 4) Coupling and reduction; 5) 2,2`-(ethylenedioxy)diethylamine; 6) glutaraldehyde; 7) Coupling and reduction.*

2.3 Results and Discussion

After the successful synthesis of amino-terminated carbohydrates, a glass surface was modified with various linker groups and carbohydrates as depicted in fig 2.4. Contact angle and AFM techniques were used to study the change in surface morphology of glass with varying functionality on the surface.

Contact angle and AFM

Table 2.1 shows water contact angle values (θ) of glass and various functionalized glass surfaces. Commercial untreated glass is rather hydrophobic due to Si-O-Si bonds on the surface. Cleaning with piranha solution removes impurities and creates free silanol groups and increases the hydrophilic character (decrease of the contact angle). Treatment of the glass surface after cleaning with N-(2-aminoethyl)-3-aminopropylmethyldimethoxysilane, followed by reaction with glutaric dialdehyde led to linker L_1 with some hydrophilic character. Further treatment of L_1 with 2,2`-(ethylenedioxy)diethylamine followed by glutaric dialdehyde attachment via imine linkage resulted in linker L_2 (fig 2.4).

Table 2.1: *Contact angle values of various functionalized glass surfaces*

Functionalized surface	**Contact angle (θ°)**
Commercial glass before cleaning	33.5 ± 0.5 °
Glass surface after cleaning	9.0 ± 1.0 °
AEAPS surface	49.5 ± 0.5 °
Glutaric dialdehyde (L_1)	45.5 ± 0.5 °
2,2'-(Ethylenedioxy)diethylamine	50.5 ± 0.5 °
Glutaric dialdehyde (L_2)	49.5 ± 0.5 °
α-Mannose	58.5 ± 0.5 °
β-Galactose	61.0 ± 1.0 °
α-Galactose	57.5 ± 2.5 °
β-Glucose	56.5 ± 0.5 °
α-Glucose	56.5 ± 1.5 °

The morphology of the surface changed as a result of the attachment of the different linker groups (Perruchot et al., 2000). AFM was employed to obtain more detailed information on the surface modification (fig 2.5). The roughness of clean glass (≈ 5 nm) was reduced after

treatment with N-(2-aminoethyl)-3-aminopropyl methyl dimethoxy silane resulting in a soft silane layer on the surface (fig 2.5b). A slight increase of surface roughness was observed after the glutaric dialdehyde treatment (L_1) (fig 2.5c). After further elongation of the chain, it was difficult to observe additional changes of the surface.

From the contact angle and AFM measurements, it is clear that the glass surface was successfully modified. Initially, to study the carbohydrate immobilization step, each of the carbohydrate was individually coated on the slides with linker L_1 via imine linkage followed by reductive amination resulting in a carbohydrate coated glass surface. The contact angle of the surface increased after carbohydrate immobilization on linker L_1. Each carbohydrate slide exhibited different contact angles (Table 2.1). The AFM image of the α-mannose coated slide shows a slight increase of surface roughness (fig 2.5d).

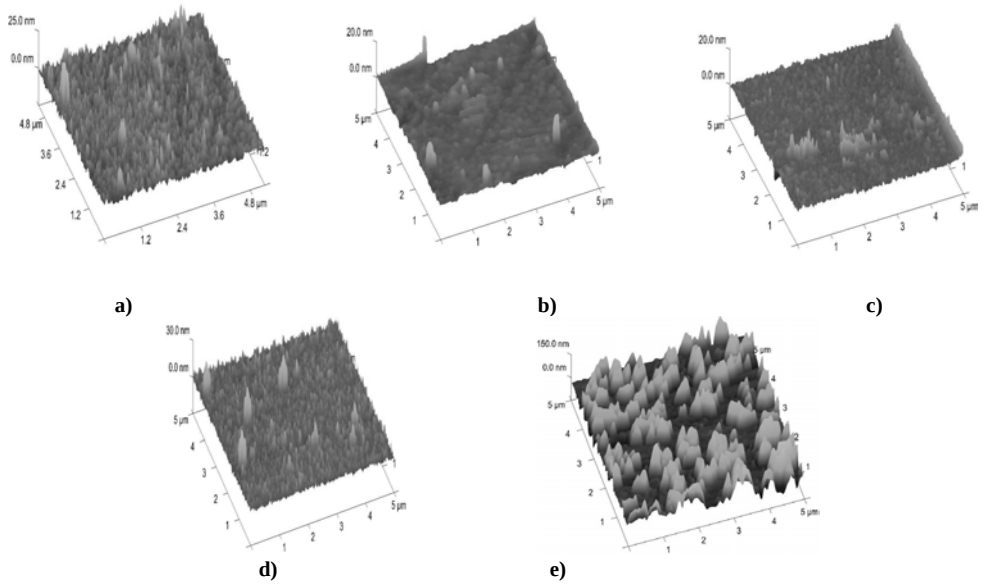


Figure 2.5 *3D-AFM images of glass surface morphology: a) clean glass slide with hydroxyl groups b) silane coated slide c) aldehyde coated slide L_1 d) α-mannose attached to linker L_1 coated slide and e) Con A bound to α-mannose present on the surface (see Appendix for colour pictures).*

Carbohydrate-lectin interaction

A carbohydrate microarray was prepared by spotting 0.5-2 μL of a solution of various carbohydrates on glass slides with a Finntip pipette. The remaining free aldehyde groups on the slides were reduced to hydroxyl groups along with reductive amination of the linker chain using $NaBH_4$ solution. The hydroxyl groups formed on the surface act as protective groups to prevent non-specific adsorption of lectins to the surface (see scheme 1). After this process, the microarray slides were incubated for 1 h in PBS buffer (0.3 % Tween 20) containing

fluorescently labelled lectins (FITC Concanavalin A and FITC *Arachis hypogaea* (PNA)). Tween 20 was used to reduce the background fluorescence of the slide (Park, 2002). The slides were washed with PBS buffer, dried and imaged using AFM and confocal laser fluorescence microscopy.

Fig 2.5e shows a drastic increase of surface roughness to 100 nm due to a Con A - α-mannose interaction on the glass surface. It is evident from the fluorescence images that lectins were bound selectively to the carbohydrates (fig 2.6a-j). When the carbohydrate microarray is treated with FITC labeled lectins, it is observed that Con A binds to α-mannose and α-glucose and does not show any interaction with other carbohydrates (β-gal, α-gal, β-glc) present on the surface (fig 2.6a-e). The other lectin, *Arachis hypogaea* binds to β- and α-galactose only (fig 2.6i-j). No fluorescence was observed in any region other than that of the carbohydrate spots.

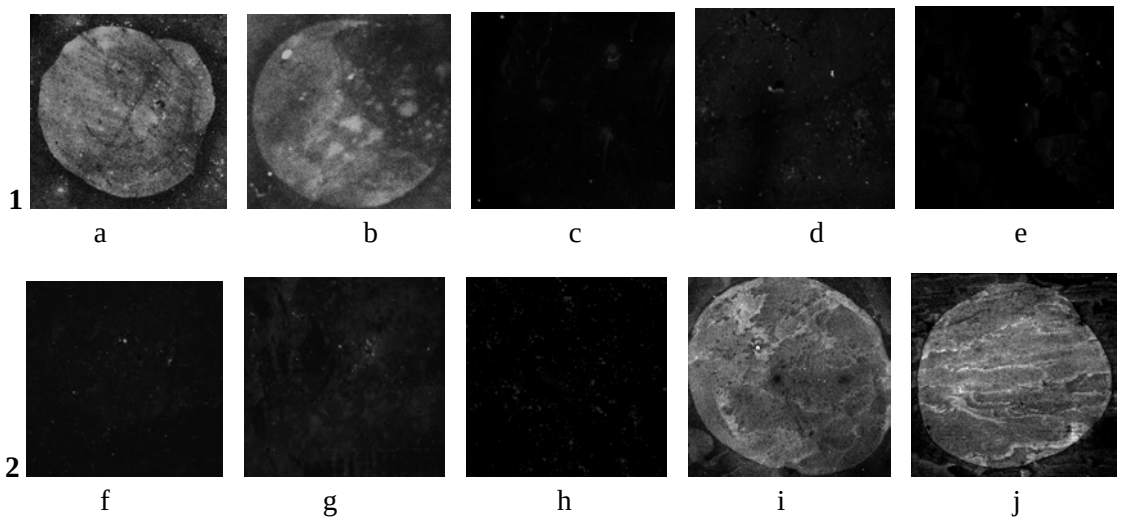


Figure 2.6 *CLFM images of carbohydrate-lectin interactions on the glass surface: 1) Con A interaction with a) α-mannose, b) α-glucose, c) β-glucose, d) β-galactose and e) α-galactose. 2) PNA interaction with f) α-mannose, g) α-glucose, h) β-glucose, i) β-galactose and j) α-galactose (see Appendix for colour pictures).*

The influence of the linker chain lengths on the non-specific adsorption of lectins was examined. The fluorescence results obtained from carbohydrate linker L_2 microarray showed similar results to that of carbohydrate linker L_1 microarray, which indicates that linker L_1 is long enough to prevent non-specific adsorption of lectins to the surface.

α-Mannose interaction with Con A in a capillary column

For proof of principle, the above developed carbohydrate immobilization protocol (linker L_1) was chosen to prepare carbohydrate modified capillary columns to capture lectins. α-Mannose was chosen as ligand in this study. The α-mannose modified capillary column prepared was

employed to study the interaction of Con A using frontal affinity chromatography (FAC) (fig 2.7). Dimethyl sulfoxide (DMSO) was used to determine the total system dead volume. Con A in water and binding buffer were used to verify the specific interaction of Con A with α-mannose in presence and absence of the proper metal ions.

As described in section 2.1, with frontal affinity chromatography, the interaction of Con A (analyte) and α-mannose (ligand) immobilized in the column can be determined using the 50% breakthrough point of the frontal affinity chromatogram. As seen in fig 2.7, Con A in water (**B**) and Con A in binding buffer (**C**) eluted at the same retention time as DMSO (**A**). This result indicates that although α-mannose was successfully bound to the capillary column, the affinity column is not able to capture Con A due to a too low column capacity.

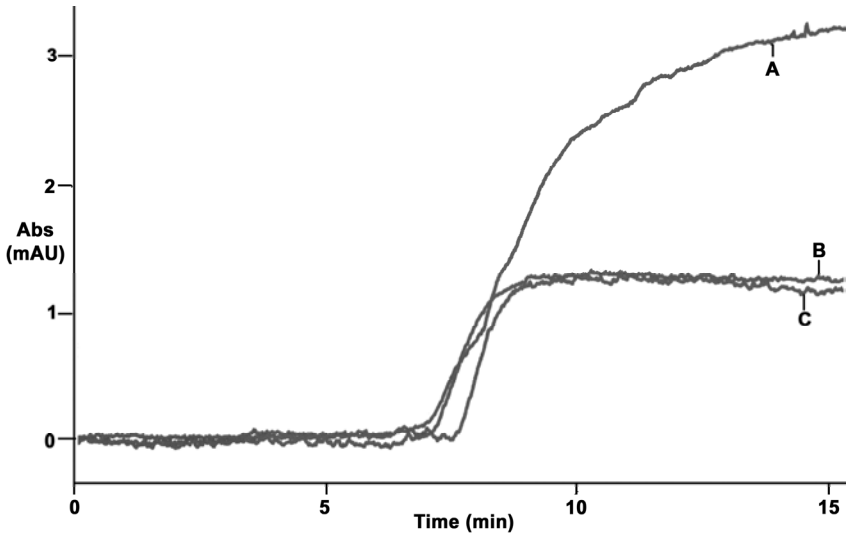


Figure 2.7 *Frontal affinity chromatogram of α-mannose coated capillary column (75 µm i.d, 36 cm effective length, 45 cm total length) in both aqueous and binding buffer (B.B) solution. A) DMSO in water; B) Con A in water and C) Con A in B.B.*

2.4 Conclusions

A simple method to covalently immobilize carbohydrates on a glass surface was developed. Contact angle and AFM measurements indicate a successful surface modification of the glass surface. The carbohydrate microarray showed successful selective binding of FITC Con A and PNA with carbohydrates of their interest. Linker group (L_1) is long enough to prevent non-specific adsorption of lectins on the glass surface. The AFM image showed a drastic increase of surface roughness due to Con A interaction with α-mannose on the glass surface.

The advantages of this method are:

1. The linker groups and carbohydrates are easy to synthesize and

2. No special protective groups are required to end-cap the free aldehyde groups on the surface after the carbohydrate immobilization step

The α-mannose capillary column prepared is not effective in capturing Con A due to a low capacity of the column. To enhance the capacity of such affinity columns, further research should be carried out using various strategies.

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3

Carbohydrate monolithic capillary columns for affinity chromatography of lectins

Abstract

A brief overview about monolithic materials, their preparation and characterization is given in this chapter. Subsequently, a three step approach to prepare carbohydrate monolithic capillary columns to study affinity chromatography of lectins is investigated. α-Mannose with an amino terminated alkyl spacer was chosen as a ligand in this study. The monolithic material was comprised of HEMA (2-hydroxyethyl methacrylate) as a monomer in combination with DATD ((+)-N,N-diallyltartardiamide) and PDA (piperazine diacrylamide) as crosslinkers. After oxidation of DATD with periodate, α-mannose with spacer was bound to the aldehyde groups of the polymeric skeleton via reductive amination to form an affinity column. The columns showed good permeability. Scanning electron microscopy (SEM) and inverse size exclusion chromatography (ISEC) were employed to investigate the porosity of the monolithic materials. The affinity of the monolith was evaluated by frontal affinity chromatography (FAC). FAC showed specific interaction of two different lectins with the α-mannose modified monolith. Adsorption and desorption of concanavalin A (fluorescently labeled) from the column was investigated using fluorescence microscopy.

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3.1 Introduction

The carbohydrate coating on the capillary wall was found not to be effective to capture lectins due to a low capacity of the column (chapter 2). Various approaches for carbohydrate immobilization to increase capacity were considered.

1. Packing of the column with a particulate stationary phase. However this has the disadvantage of difficulty with filling of the column and also other tedious practical problems (Maruŝka and Kornyŝova, 2004).
2. Immobilization of carbohydrates on various soft gels. The limiting factor is the poor mechanical strength of these soft matrixes (Charan et al., 2000; Ooi et al., 2004).
3. The use of monolithic columns. The last decade witnessed the growth of these new chromatographic media. These materials generated lots of interest in the field of separation science with their distinct advantages and applications (Gagnon, 2006). Considering their success in diverse fields, these materials were chosen for carbohydrate immobilization.

Monoliths - A new generation of chromatographic materials

The quest to find alternative materials for chromatographic media (packed columns) began with an increase in demand to improve the separation of biomolecules (in the field of separation science) and to overcome the limitations of particulate stationary phases (Maruŝka Maruŝka and Kornyŝova, 2004; Podgornik and Strancar, 2005; Tennikova and Reusch, 2005; Ŝvec and Huber, 2006; Gagnon, 2006). In 1967, Kubín and co-workers developed a polymer matrix from 2-hydroxyethyl methacrylate hydrogel. However, the material was soft and showed very poor permeability (Kubín et al., 1967). Later the focus turned towards the use of open-pore polyurethane-based materials. The success of these materials was however moderate. The first breakthrough came in the year 1989 with the work of Hjertén and co-workers. They prepared a continuous polymer bed from N,N'-methylene bisacrylamide and acrylic acid in the presence of a salt (ammonium sulfate) (Hjertén et al., 1989). This material showed a very good permeability. The success was extended further by Ŝvec and Fréchet with the development of continuous methacrylate rods from glycidyl methacrylate and ethylene glycol dimethacrylate as monomer and crosslinker (Ŝvec and Fréchet, 1992). These two pioneering works played a crucial role in providing the platform for non-particulate stationary phases and generated the name "monoliths". Fig 3.1 shows the difference between a packed column and a monolithic column.

Monoliths are defined as "continuous stationary phases that form as a homogenous column in a single piece and prepared in various dimensions with agglomeration-type or fibrous microstructures" (Podgornik and Strancar, 2005). These are categorized into organic and inorganic, dependent on the materials the columns are made from. Organic monoliths are

prepared by *in situ* polymerization of monomers, crosslinkers, porogens and an initiator. Inorganic monoliths (silica) are prepared by the sol-gel method. Organic polymer beds demonstrated their efficiency in the fast separation of large biomolecules, while inorganic polymer beds were proven favourable for the separation of small molecules (Legido-Quigley et al., 2003; Ŝvec and Huber, 2006; Zhu et al., 2007).

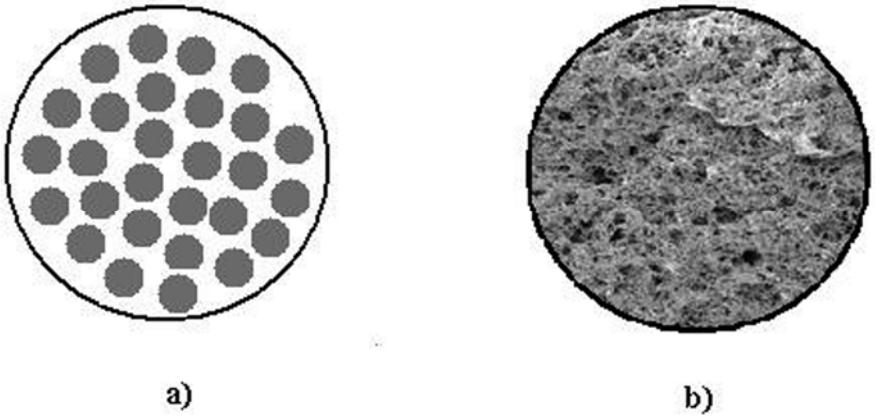


Figure 3.1 *Packed column vs monolithic column a) particulate stationary phase with large void volume and less-efficient packing b) highly interconnected network of pores for monolithic material results in high permeability (adapted from Ŝvec, 2006).*

Organic monolithic materials

The last decade witnessed a tremendous growth of various organic monolithic columns from materials like acrylamide, polystyrene and methacrylate derivatives. The reason for this is the ease with which these columns can be prepared (Legido-Quigley et al., 2003). The organic monolithic bed preparation can be mainly classified into two groups (Maruŝka and Kornyŝova, 2004):

I. Continuous beds prepared by incorporating polar and amphiphilic water soluble acrylic comonomers in the presence of a salt or a polymer
II. Polymeric beds synthesized from organic soluble comonomers in the presence of a co-solvent

The advantages of carrying out the polymerization in aqueous solutions are biocompatibility and the easy preparation of the monolithic materials on a large scale. The majority of monomers employed in group I can be used in group II as well, but after a solubility check. The distinct advantages of these materials are listed below:

1. The starting materials can be easily introduced into the microchannels (capillary and chip)

2. The monoliths show a high mass transport and permeability (low back pressures at high flow rates)
3. They show a high mechanical, chemical and thermal stability
4. A high capacity column is obtained in comparison to open tubular columns
5. They have a tunable surface chemistry by the use of a combination of various monomers, crosslinkers and porogens

Monolithic columns with diverse functional groups can be prepared due to the availability of a wide range of monomers and crosslinkers. This feature makes monolithic materials more flexible to work with. The important point to be considered while preparing these columns is that the polymerization conditions developed for one system can be transferred to another system only after further experimentation. In fig 3.2 some of the mostly commonly used monomers and crosslinkers described in the literature are depicted (Ŝvec and Fréchet, 1999; Merhar et al., 2003; Ro et al., 2006, Nordborg et al., 2005).

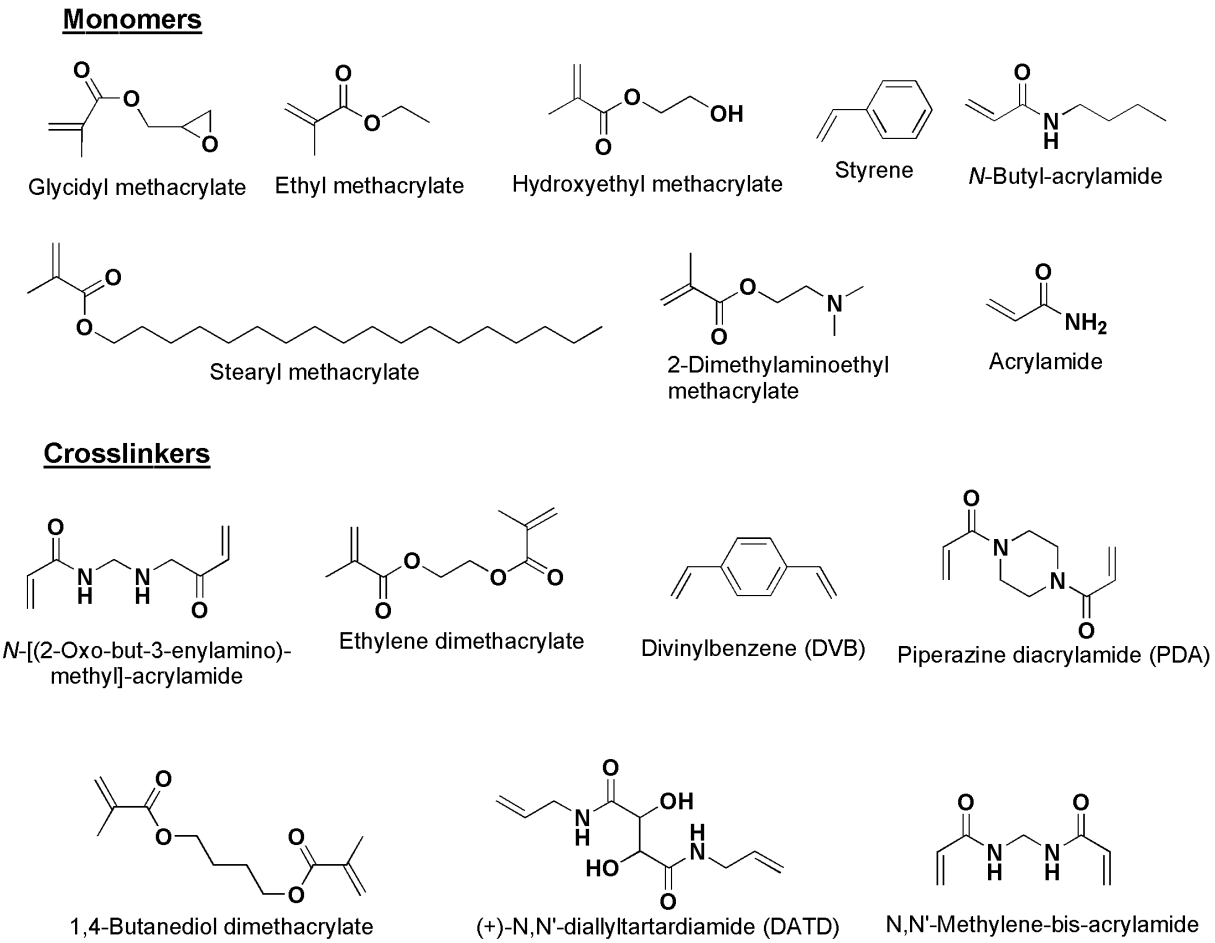


Figure 3.2 *Monomers and crosslinkers available to develop different organic monolithic columns.*

***In situ* preparation of continuous beds**

a) Continuous bed preparation of group I

The acrylate activated capillary column is filled with a liquid precursor of the stationary phase comprised of monomers, crosslinkers and salt in a buffer solution. The polymerization is performed with ammonium peroxydisulfate (APS) as initiator and N,N,N',N'-tetramethyl ethylene diamine (TEMED) as accelerator. The radical co-polymerization was carried out overnight at room temperature (Maruŝka and Kornyŝova, 2004; Ledigo-Quigley et al., 2003).

b) Continuous bed preparation of group II

The liquid precursor of the stationary phase is comprised of monomers, crosslinkers and an initiator in the presence of a porogen or a combination of different porogens. This reaction mixture is poured into a sealed mold (stainless steel, PEEK or glass) where polymerization takes place. Upon completion of polymerization, the seals are removed and the column is sealed with fittings. The radical co-polymerization can be initiated in four ways: by heat, UV light, chemical agents or gamma rays (Ŝvec and Fréchet, 1999; Ŝvec, 2003; Buchmeiser, 2007).

The use of liquid precursors for the stationary phase avoids various tedious procedures (uniform packing, fixing frits, and avoid bubble formation) that are employed in preparing particulate stationary phases. Monolithic materials do not possess interparticular voids, which are typical for particulate stationary phases. The absence of these interparticular voids accelerates the mass transfer by convection during the liquid flow through the open and highly interconnected network of pore channels (creates high surface accessibility) resulting in fast and efficient separation of various biomolecules like proteins, polynucleotides and viruses (Maruŝka and Kornyŝova, 2004; Gagnon, 2006; Vlakh and Tennikova, 2007).

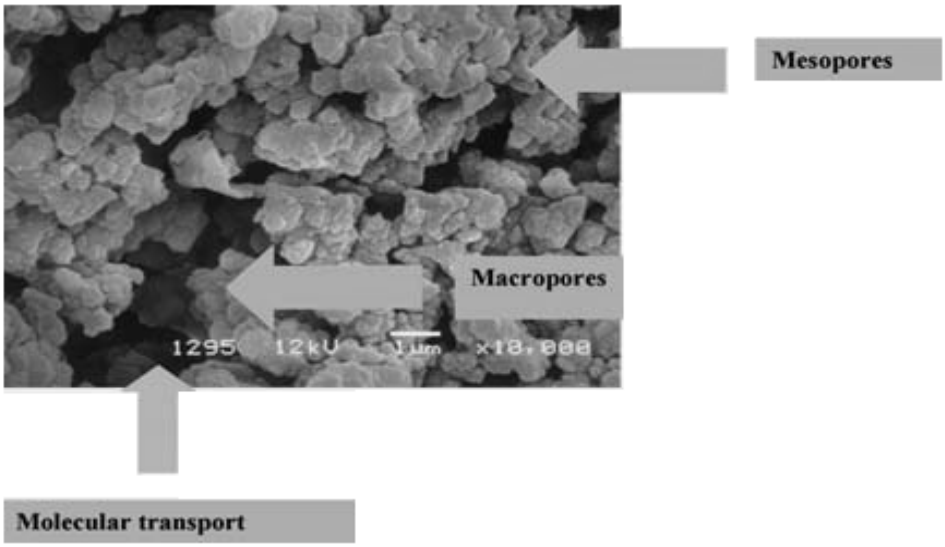


Figure 3.3 *Highly interconnected network of monolithic material with macropores and mesopores (Tennikova, 2002).*

The mechanism for monolith pore formation is as follows (Ŝvec and Fréchet, 1999; Legido-Quigley et al., 2003; Buchmeiser, 2007; Vlakh and Tennikova, 2007): when a certain temperature is reached, the initiator starts decomposing and triggers the polymerization. This leads to polymer growth via crosslinking and the formation of insoluble nuclei, which are poorly soluble in the porogen and precipitate. At this juncture, the nuclei are surrounded by remaining monomer and crosslinker, which now act as solvating agents. The polymerization is faster within the nuclei than in the solution due to an excess amount of monomer in the nuclei. At the end of the polymerization, the increasing size of the nuclei ensues in the formation of the final monolithic structure with an interconnected large diameter microglobules network with large void volumes. This void volume fraction corresponds to the volume fraction of the porogen. Fig 3.3 depicts the final structure of the monolithic phase with macropores and micropores, followed by a schematic diagram indicating the mechanism of monolith formation (fig 3.4).

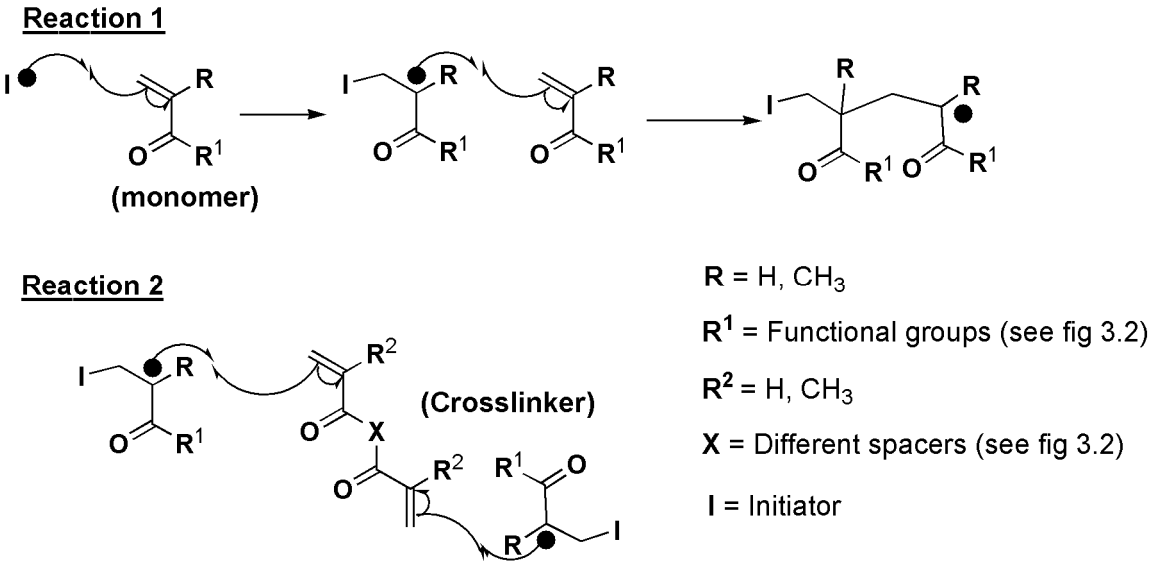


Figure 3.4 *Schematic representation of monolith formation mechanism: Reaction 1: Radical polymerization leading to formation of polymer chains. Reaction 2: crosslinking of monomers by means of crosslinkers (adapted from Ledigo-Quigley, 2003).*

Morphological study of monolithic materials

The pores in the monolithic materials are classified into two types: a) macropores (the flow through pores) and b) micropores and mesopores. The macropores consists of pores larger than 50 nm and enable the flow of mobile phase through the column directing the sample to access the network of mesopores, which have pore diameter in the range of 2-50 nm. Micropores have pores smaller than 2 nm. The characterization of these monolithic pore structures can be carried out using various techniques: Scanning electron microscopy (SEM), Mercury intrusion porosimetry (MIP), Nitrogen sorption, and Inverse size exclusion chromatography (ISEC). MIP and nitrogen sorption techniques require special

instrumentation, high skills to handle the instrument and the interpretation of the results obtained. ISEC chromatography is a simple technique that does not require special instruments to carry out the measurements and is now widely employed (Guan-sajonz et al., 1997; Urban et al., 2008).

Inverse size exclusion chromatography (ISEC) - Theory

ISEC is opposite of the widely known Size Exclusion Chromatography (SEC) technique. In SEC, the molecular weight distribution of a polymer mixture is determined by infusing it through a solid stationary phase, whose pore structure is known. In ISEC, the pore size distribution of solid stationary phase is not known and is determined by infusing a series of "monodisperse" polymers (e.g. standard polystyrene samples) with a wide range of known molecular weights. Some important points that need to be considered for these measurements are listed below (Halász and Martin, 1978; Guan and Guiochon, 1996; Guan-sajonz et al., 1997; Al-Bokari et al., 2002):

1. The polymers chosen should not be adsorbed on the matrix surface.
2. The solvent chosen should not be adsorbed on the matrix surface as well. Tetrahydrofuran (THF) or dichloromethane (DCM) are usually preferred.
3. The elution peak profile should relate closely to a Gaussian curve.
4. The matrix under investigation should not swell or shrink. The experimental conditions (temperature and mobile phase) should remain unchanged.

Polystyrenes with varying molecular weights (M_r) are widely employed in such studies. Toluene or benzene is chosen as standard sample to record the volume of most retained sample. An ISEC calibration graph is drawn by plotting elution volumes of the sample peaks against the logarithm of the molecular weights (log M_r) of the sample. From these measurements external (ε_e), internal (ε_i) and total porosity (ε_T) of the stationary phase are calculated by using the following formulas:

$$\varepsilon_T = \frac{V_T}{V_g}; \varepsilon_e = \frac{V_e}{V_g}; \varepsilon_i = \varepsilon_T - \varepsilon_e \qquad (3.1)$$

Where V_T is volume of most retained sample (toluene or benzene); V_g is total volume of the column derived by using the formulae ($V_g = \pi r^2 L$) and V_e is retention volume of the excluded molecular mass. From the calibration graph, the volume of the pores (V_p), volume of stationary phase (V_s) and volume of column channels (V_{ch}) can also be determined.

The molecular weight (M_r) of polystyrene samples employed correlates with the pore size of the matrix they have been excluded from using the equation (Halász and Martin, 1978; Guan and Guiochon, 1996):

$$\phi_n \simeq 0.627 \quad (M_r)^{0.588} \tag{3.2}$$

The pore size distribution of the matrix can be determined by assuming that "the fractional volume (V_n) corresponds to the pores of the matrix with size equal to or larger than ϕ_n (ϕ is the pore size diameter (Å) and calculated as in eq. 3.2). Similarly, all pores that have a size equal to or larger than ϕ_{n+1} have fractional volume V_{n+1} ($\phi_{n+1} > \phi_n$)". The volume of the pores that have a size larger than ϕ_n and smaller than ϕ_{n+1} is derived by:

$$\Delta V_{n+1,\,n} = V_{n+1} - V_n \tag{3.3}$$

$\Delta V_{n+1,\,n}$ can be obtained from the ISEC calibration graph.

Choosing an appropriate synthetic route to immobilize carbohydrates

In general, to prepare a suitable affinity sorbent the ligand of interest should be immobilized on the surface of the chromatographic material. Active functional groups present on the sorbent are utilized for the covalent linkage of the ligand. Glycidyl methacrylate is widely employed due to the (active) terminal epoxide group (Ŝvec and Fréchet, 1999; Ro et al., 2006; Zhu et al., 2007; Buchmeiser, 2007). Bedair and El Rassi developed monolithic capillary columns for affinity-based separations of lectins in both nano-LC and CEC modes. In their approach glycidyl ether and ethylene dimethacrylate were incorporated as monomer and crosslinker in their monolith. Subsequently the mannose was immobilized directly or after attachment of a spacer by a nucleophilic attack on the epoxide ring under basic conditions (Bedair and El Rassi, 2004). At the same time, Kornyšova et al. reported on a simpler method for attaching ligands to monoliths. Their approach was to include (+)-N,N'-diallyltartardiamide (DATD) as a crosslinker. DATD can be oxidized with periodate to generate *in situ* aldehyde groups. These groups can be used to attach the ligand covalently on the monoliths (Kornyŝova et al., 2004). The advantages of this approach are the increase in porosity of the polymeric skeleton, and absence of basic conditions.

Two different routes were designed to prepare carbohydrate monolithic columns:

1. A three step approach: the DATD approach mentioned above was chosen to immobilize carbohydrates on the monolithic bed.
2. Single step synthesis of affinity columns: Use carbohydrates as a functional monomer in the monolithic solution (will be discussed in chapter 4).

The focus in this chapter is to prepare affinity columns via a three step process to study affinity of α-mannose with different lectins (concanavalin A (Con A); *Arachis hypogaea* (PNA) and *Lens culinaris* (LCA)).

3.2 General information

Materials and methods

Polystyrene standards (M_r 707 to 1,530,000) were from Polymer Standards Service (Mainz, Germany).

Scanning electron (SE) microscopy (JEOL 6300f, Tokyo, Japan) measurements were carried out by sputter coating the capillary with 10 nm platinum in a preparation chamber (Oxford instruments, CT 1500 HF, England). Dry samples of the monolithic phases were measured at room temperature at a working distance of 8-16 mm with SE detection at 3.5-5 kV. Images were recorded at a scan rate of 100 seconds (full frame) using Orion software. The images were optimized and resized with Adobe Photoshop CS.

Flow rate vs back pressure measuring system consisted of an HPLC pump [(Applied Biosystems, Netherlands) water is the solvent] to deliver mobile phase and read backpressure, a T-shaped connector for splitting, an HPLC column to provide backpressure on the split exit, and a 10 µL micro-syringe connected to the end of the monolith for accurate monitoring of the flow rate.

Inverse size exclusion chromatography (ISEC) measurements were carried out with polystyrenes (M_r range from 707-1,530,000) out at a wavelength (λ) of 262 nm at a flow rate of 0.2 µL/min by using a syringe pump (Harvard pump) connected to a nano-injector (10 nL internal loop) [MX7984, Rheodyne, USA] and a Spectra 200 UV-VIS detector (Spectra-Physics, San Jose, CA, USA) modified for on-capillary detection. Tetrahydrofuran (THF) was the mobile phase. The elution volume obtained is an average of three sets of values for each polystyrene standard. Exactly the same column was used (original monolith, periodate treated, α-mannose modified) for the ISEC measurements.

Fluorescence measurements (FM) were performed using a stereo fluorescence microscope using a GFP filter [470 nm (excitation wavelength) and 525 nm (emission wavelength)].

Frontal affinity chromatography (FAC) was performed using a nano-HPLC system made in-house consisting of an HPLC pump (Separations analytical instruments, Netherlands) connected to a T-shape splitter with an HPLC column (Spherisorb S5-RP-8, 250 x 3.0 mm) at one end to provide back pressure on the split exit and a micro-injector (2 µL loop size) (7725, Rheodyne, USA) at the other end. The monolithic capillary column was connected to the

micro-injector. On-capillary measurements were performed with a K-2501 UV detector (Knauer, Germany) at 218 nm. The detection window was created on-column by local heating to remove the polymer bed and the polyimide coating outside the capillary. The column was washed with water to locally hydrolyze the polymer and to avoid polymer ash formation during the heating process.

The working conditions were as follows: detection on-column, UV-218 nm; HPLC pump flow rate: ~1 µL/min. Test solutions of lectins were 1 mg/mL in aqueous solution for the investigation of non-specific adsorption of lectins to monoliths and 1 mg/mL lectins in binding buffer solution for the investigation of specific affinity adsorption of lectins to affinity monoliths. Recording the breakthrough curve, defining the 50% breakthrough point as described in (Arata et al., 2001) and calculating the affinity capacity of the monolith were done with a chromatographic workstation (Eurochrom 2000, Knauer, Germany).

Affinity monolithic capillary column preparation

Capillary pretreatment

Surface modification of fused-silica capillary and chip inner surface was performed as described in (Ericson et al., 1997) with slight modification: acetone (20 min), distilled water (20 min), 0.1 M HCl (20 min), 0.1 M NaOH (1 h), distilled water (20 min) and acetone (20 min), followed by drying with nitrogen. Treatment of hydroxyl terminated surface with a 30% (v/v) solution of 3-(trimethoxysilyl)propyl methacrylate in acetone by closing both ends for 12 h resulted in acrylate groups on the surface, which can co-polymerize with the monomers (Kornyšova et al., 2001).

***In situ* polymerization**

In situ polymerization was accomplished as described in (Kornyšova et al., 2004) with slight modification. *Reaction mixture*: 30 µL of HEMA, 20 mg of DATD, 12.5 mg of $(NH_4)_2SO_4$ and 250 µL of PDA buffer solution (160 mg of PDA was dissolved in 2 mL of 50 mM sodium phosphate buffer, pH 7.0) were taken in an Eppendorf tube and mixed well. The reaction mixture was then degassed by vacuum for 5 min. 10 µL of 10% (w/v) aqueous AMPA solution (initiator) was added to the above reaction mixture and mixed well. No extra porogen was added. The resulting polymerization mixture was taken in a disposable syringe and injected during 5 min into the capillary by using a syringe pump at a flow rate of 1 µL/min. The capillary ends were closed with a GC septum and placed in an oven (T = 65 °C) for 12 h. The resulting monolithic column was washed with distilled water and acetonitrile successively. A schematic picture of monolith formation is depicted in fig 3.5.

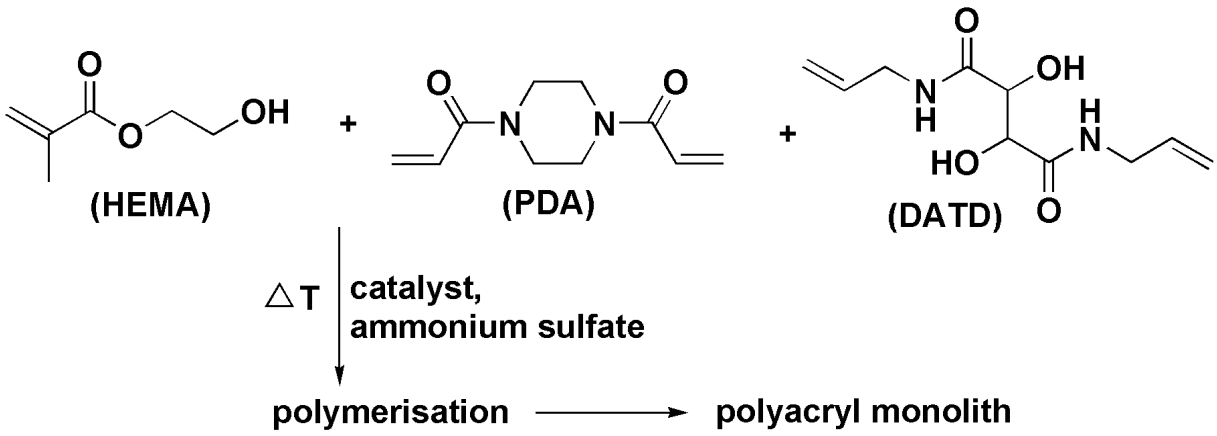


Figure 3.5 *Schematic representation of monolithic bed formation.*

Attachment of α-mannose to monolithic bed

Immobilization of the affinity ligands via reductive amination was carried out as described in (Wikstrom et al., 2000). Monolithic columns were treated with 70 mM $NaIO_4$ solution in H_2O-MeOH (4:1) for 2 h with a flow of 2 μL/min, followed by washing with distilled water for 2 h (2 μL/min) resulting in an aldehyde terminated monolithic column. Subsequently, the column was treated with a solution of 3.5 mM α-mannose with a spacer containing a terminal amino group in 6 mM $NaBH_3CN$ solution (50 mM sodium phosphate, pH 7) for 24 h [0.2 μL/min], followed by washing with 10 mM $NaBH_3CN$ solution (50 mM sodium phosphate, pH 3) for 2 h [2 μL/min] and distilled water for 2 h (2 μL/min) respectively.

Fluorescence microscopy (FM) of fluorescein-isothiocyanate (FITC) labeled Con A: Affinity adsorption and desorption conditions

The procedure for binding Con A on the affinity monolithic column was as follows: first the column was washed with distilled water for 20 min (1 μL/min) followed by binding buffer (10 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 6-7, adjusted with HCl) including 1 mM Mg^{2+}, 1 mM Mn^{2+} and 1 mM Ca^{2+}) for 60 min (1 μL/min) and treated with FITC Con A (9 μg/mL) in binding buffer for 60 min (0.5 μL/min). The column was washed with binding buffer for 60 min (1 μL/min) and finally with distilled water for 30 min (1 μL/min). After binding, the fluorescence caused by FITC Con A on the monolith was measured.

Methyl-α-D-mannopyranoside and α-glucose were utilized to elute Con A from the monoliths. The procedure was as follows: column A & B were washed with distilled water for 30 min (1 μL/min) followed by 100 mM methyl-α-D-mannopyranoside (column A) and 100 mM α-glucose (column B) in binding buffer, for 60 min (1 μL/min) and finally washed with water for 30 min (1 μL/min). After elution the fluorescence intensity caused by FITC Con A on the monoliths (column A & B) was evaluated.

3.3 Results and discussion

Permeability of columns

By measuring the relationship between flow rate and backpressure the permeability of the different monolithic beds was evaluated (fig 3.6). The results indicated a high permeability of the columns. A particulate column of similar dimensions filled with 5 µm particles is expected to generate a 10 times higher back pressure with the same solvent at the same flow rate (Waters Column Backpressure Calculator Software 2001, Waters Cooperation, USA). The differences between the three columns were marginal with the mannose-modified column being slightly less permeable. The permeability is predominantly determined by the large flow-through (macro) pores and the % mobile phase volume (V_{mob}) is similar for the three columns.

Porosimetric calculations of various monolithic beds

Scanning electron microscopy (SEM) (Luo et al., 2002) and Inverse size exclusion chromatography (ISEC) [Kornyšova et al., 2004; Al-Bokari et al., 2002; Guan and Guiochon, 1996) were used to study the porosity differences of the three monolithic beds synthesized.

Scanning electron microscopy (SEM)

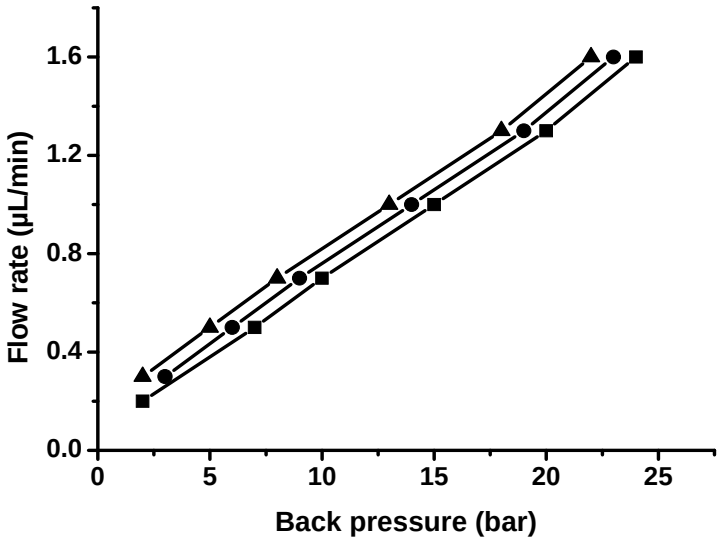
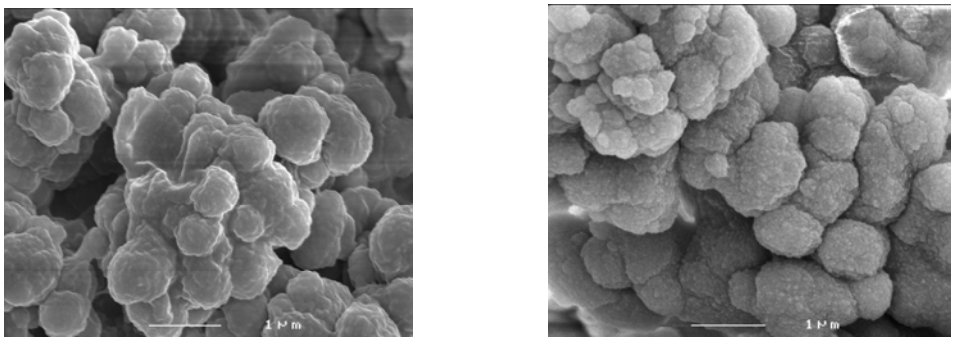


Figure 3.6 *Relationship between flow rate and back pressure of polymeric bed, periodate treated bed and mannose immobilized bed (75 µm i.d. × 12 cm) [distilled water was the solvent] -▲-: original bed; -■-: periodate treated bed; -●-: mannose immobilized bed.*

SEM images of original bed and mannose immobilized bed are shown in fig 3.7a and 3.7b. Visually there are differences between the two monolithic beds, with the original monolith showing larger structures and macropores of 2 µm and even larger and the mannose-modified

monolith show many smaller globular structures and more flow-through pores (macro) in the order of 1 µm. However, these visual differences are not reflected in significant differences in permeability or % mobile phase volume.



(a) (b)

Figure 3.7 *Scanning electron microcopy photos of monolithic bed (AMPA initiator) (a) Original polymeric bed & (b) Affinity monolithic bed.*

Inverse size exclusion chromatography (ISEC)

Using ISEC, the difference in porosity between the original polymeric bed, periodate treated bed and mannose immobilized beds were investigated.

Figure 3.8 summarizes the ISEC results of the original bed, the periodate treated bed and the mannose immobilized bed. Based on the ISEC data, average pore size diameter (Ds), pore size polydispersity (U), and the surface/pore volume ratio of the three beds were obtained (see table 3.1). The relative volumes of mobile and stationary phases were obtained as well. The original bed has a very broad pore size distribution (polydispersity factor U = 2.5) with a dominating contribution of the macropores (D_s > 50 nm) in the polymeric skeleton. The mean pore size for this bed is quite high with D_s = 89 nm. It is worth mentioning that the pore size polydispersity for particulate HPLC stationary phases rarely exceeds 1.1 - 1.2. For packings with a monodisperse pore size U = 1. After the periodate treatment, the pore size polydispersity increased even further to U = 6.0. Cleavage of the DATD crosslinker caused partial dissolution of the skeleton. The volume of the stationary phase dropped from 15.5 % to 10.1 %. As shown in fig 3.9, the pore size distribution changed dramatically resulting in a larger contribution by micropores. However, also a considerable fraction of mesopores was found to be present. Overall this greatly increased the available surface area for attachment of the mannose with the amine-terminated spacer. This is also reflected in an increase of the stationary phase fraction from to 10.1 to 14.2 %. Immobilization of mannose reduced the skeleton porosity and prevented access to the micropores (no Ds = 3 nm fraction in the pore size distribution curve of the mannose immobilized bed, see fig 3.10). This is also clear from the increase of the average pore size from 9 nm to 21 nm and the reduced pore size

polydispersity. This was expected as the total length of the mannose unit with spacer is estimated at 1.7 nm.

Table 3.1: ISEC data and porosity characteristics

Parameter	**Original bed**	**Periodate treated bed**	**Mannose immobilized bed**
Mean pore size D_s, nm	89	9	21
Pore size polydispersity, U	2.5	6.0	2.0
Surface/pore volume, m^2/ml	22	225	94
V_{mob}, %[a)]	84.5	89.9	85.8
V_s, %[b)]	15.5	10.1	14.2
Skeleton porosity, %[c)]	37.9	36.7	33.4

a) $V_{mob}\%=[(V_p+V_{ch}) / V_c] \times 100$; b) $V_s\%=(V_s / V_c) \times 100$; c) Skeleton porosity $P\%= [V_p / (V_s+V_p)] \times 100$

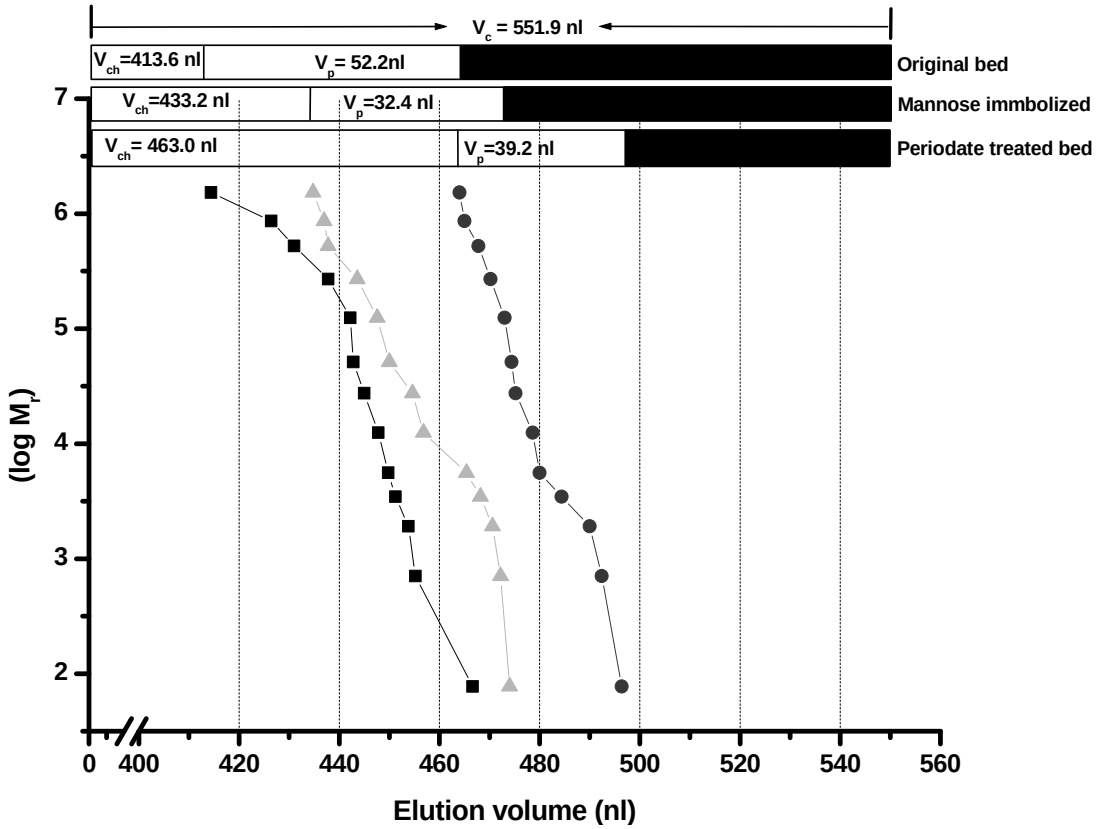


Figure 3.8 *ISEC calibration graphs of -■-: Original bed; -●- Periodate treated bed; -▲- Mannose immobilized bed (L_{eff} : 12.5 cm, 75 µm ID) with total internal volume of the column (V_c), and volumes of column channels (V_{ch}), of pores (V_p) and stationary phase (V_s) respectively. Total mobile phase volume (V_p + V_{ch}) was determined with benzene. V_{ch} was determined with the largest polystyrene.*

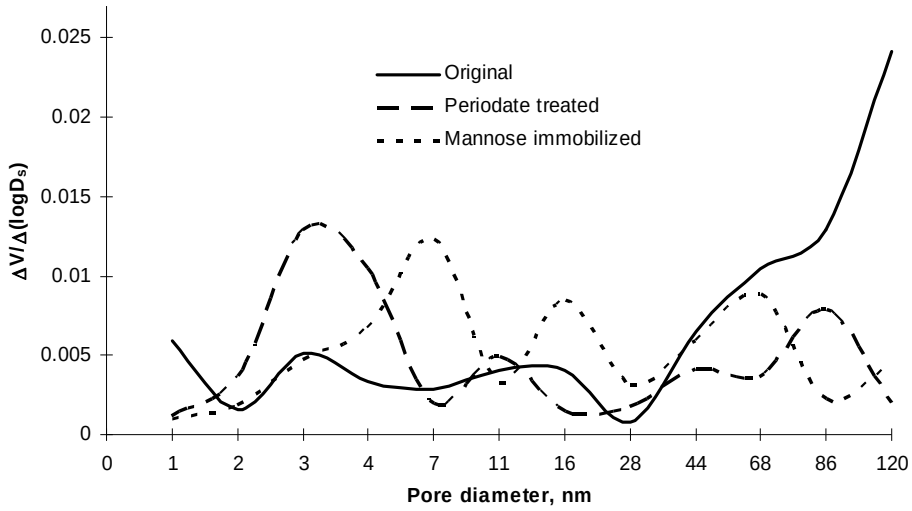


Figure 3.9 *Pore size distribution curves of original bed; periodate treated bed and mannose immobilized bed are depicted as derivatives of cumulative curves of the calibration graphs.*

Evaluation of the affinity

The affinity adsorption and desorption FM images of the monoliths are shown in fig 3.10 and 3.11. The results show an even distribution of the affinity sites over the monolithic bed and a strong affinity for Con A (fig 3.10). For affinity chromatography, Con A attached on the affinity monolith could be eluted completely by a 100 mM methyl-α-D-mannopyranoside solution containing 1 mM Mg^{2+}, 1 mM Mn^{2+} and 1 mM Ca^{2+} (fig 3.11). Methyl-α-D-mannopyranoside solution is an effective competitive displacer in affinity chromatography of mannose-specific lectins [Hilder et al., 2004]. As shown in fig 3.11, α-glucose was not successful in eluting Con A from the affinity monolithic bed, due to the weak interaction of α-glucose with Con A [Schwarz et al., 1993].

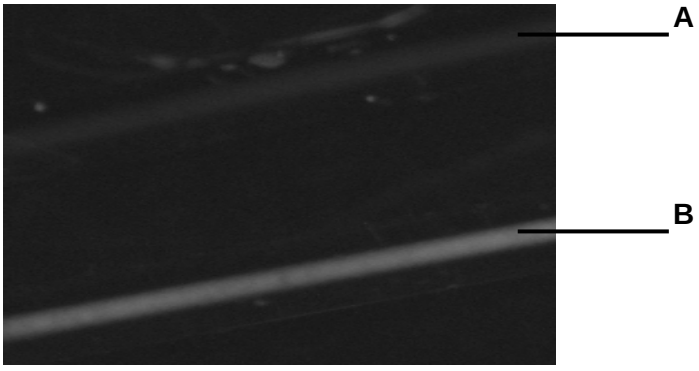


Figure 3.10 *Fluorescence microscopy images of the monolithic bed. A) Affinity monolith prior to Con A (labeled FITC) binding; B) Affinity monolith with bound Con A labeled FITC (see Appendix for colour pictures).*

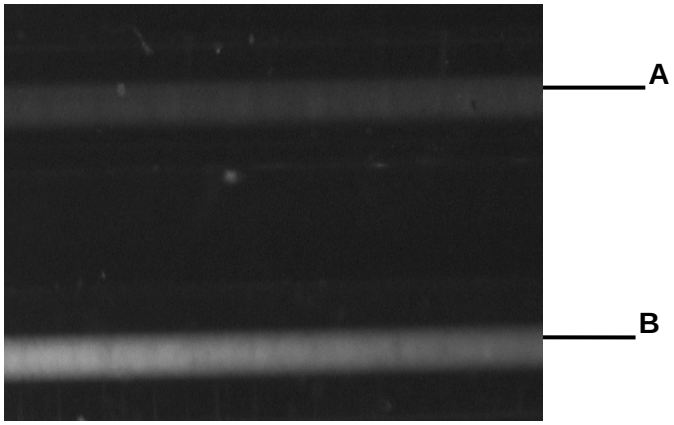


Figure 3.11 *Fluorescence microscopy images of the effect of different carbohydrates as eluents. A)Affinity monolith with bound Con A after washing with methyl-α-D-mannopyranoside solution as eluent; B) Affinity monolith with bound Con A after washing with α-glucose solution as eluent (see Appendix for colour pictures).*

Frontal analysis of α-Mannose-lectin interactions

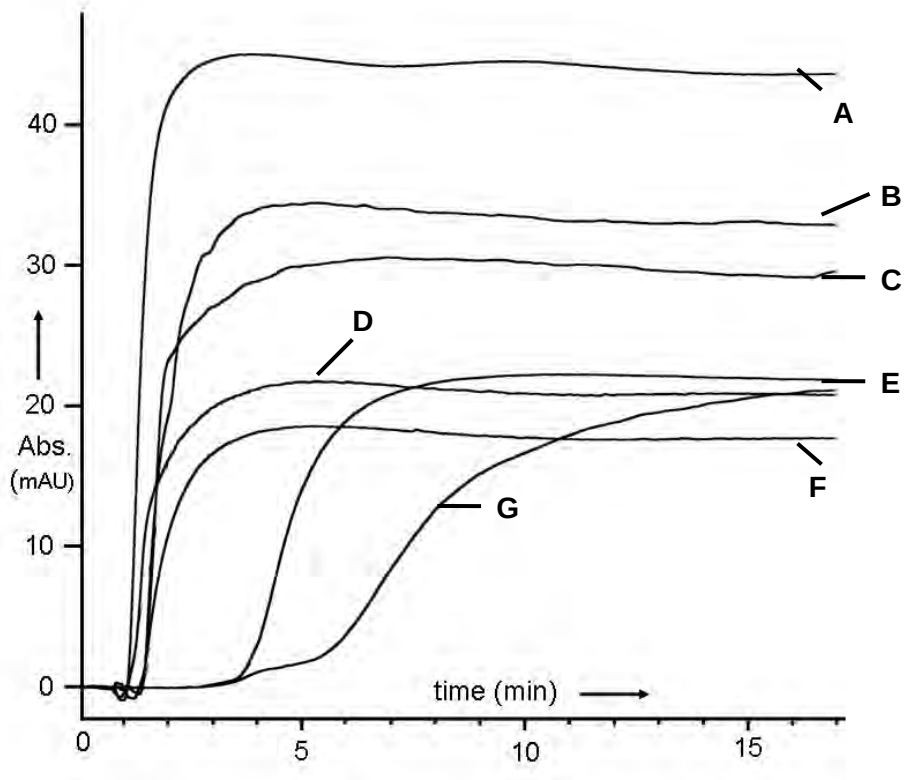


Figure 3.12 *Chromatograms of frontal analyses with UV detection at 218 nm [length - 13.5 cm; detection window 7.5 - 8 cm] A: DMSO in H_2O; B: Con A in H_2O; C: PNA in H_2O; D: LCA in H_2O; E: LCA in binding buffer (b.b); F: PNA in b.b; G: Con A in b.b.*

Frontal analysis was used to study the specific interaction of different lectins with α-mannose immobilized in the column. Fig 3.12 shows typical frontal analysis chromatograms of the column. Dimethyl sulfoxide (DMSO) was used to determine the total system dead volume. After subtracting the dead volume, the non-specific and specific affinity interactions of lectins were investigated. In binding buffer as mobile phase, the order of affinity of lectins towards α-mannose is as follows: Con A > LCA > PNA (see fig 3.12). In the presence of only aqueous mobile phase, there is no specific interaction of lectins with α-mannose. The results obtained are in full agreement with those reported elsewhere (Schwarz et al., 1993; Osawa, 1989).

Depending on the 50% breakthrough point of the frontal affinity chromatogram, the affinity capacity of the monolith was calculated (Arata et al., 2001). The capacity of an affinity column of 13.5 cm length and 75 µm i.d. prepared according to the conditions described in the experimental section was 7.4 ± 0.6 µg Con A.

3.4 Conclusion

A three step method to prepare carbohydrate monolithic beds in capillary columns was demonstrated successfully. The monolithic columns prepared by this method showed good permeability and affinity capacity for α-mannose-specific lectins. Con A showed high affinity towards α-mannose followed by *Lens culinaris* (LCA). Elution of Con A from the affinity monolithic bed was successful using 100 mM methyl-α-D-mannopyranoside. The easy preparation of these efficient columns opened up an alternative route for the preparation of affinity columns in three steps.

3.5 References

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4

Single step preparation of carbohydrate monolithic columns in a capillary and on a microchip

Abstract

A single step preparation of carbohydrate monolithic beds in both capillaries and microchips to study affinity chromatography of lectins is described. In this method, carbohydrates (β-galactose and α-mannose) with an easy to synthesize alkene terminated tetraethylene glycol spacer were used as functional monomers along with the monomer 2-hydroxyethyl methacrylate (HEMA). Besides, (+)-N,N-diallyltartardiamide (DATD) and 1,4-bisacryloyl-piperazine (PDA) were used as crosslinkers. Scanning electron microscopy (SEM) showed the successful formation of monolithic beds in the capillary columns. The columns showed good permeability. The specific interaction of lectins (concanavalin A (Con A), Lens *culinaris* (LCA) and *Arachis hypogaea* (PNA)) with the carbohydrate stationary phase was studied by frontal affinity chromatography (FAC). Con A and LCA were successfully eluted from the column using 100 mM methyl-α-mannopyranoside and PNA was eluted using 100 mM β-galactose. Carbohydrate-lectin dissociation constants (K_d) were determined and compared with literature. A β-galactose monolith was successfully introduced in the channel of a microchip. FAC studies showed the specific interaction of PNA with β-galactose in the microchip.

A part of this chapter is based on: Tetala, K. K. R., Chen, B., Visser, G. M., van Beek, T. A. *J. Sep. Sci. 30*, ***2007***, 2828-2835.

4.1 Introduction

In chapter 3, two approaches were mentioned to prepare carbohydrate monolithic columns. The first approach to prepare an affinity column via a three step synthesis was successful (Tetala et al., 2007). The overall time to prepare such an affinity monolithic column was roughly 2-3 days. To shorten this period, the possibility to prepare affinity monolithic columns in a single step (approach 2) utilizing carbohydrates with an alkene terminated tetra ethylene glycol spacer as a functional monomer along with HEMA (monomer), PDA and DATD as crosslinkers was studied in this chapter. The practical added values of this approach are:

1. One synthetic step less to prepare carbohydrate monomers compared to the previous protocol (chapter 2) and
2. Preparation of monolithic beds with various biomolecules in less time than with other protocols

These carbohydrate columns prepared through approach 2 were applied in various studies

1. Specific interactions of carbohydrates with various lectins (Con A, PNA and LCA)
2. Elution of bound lectins from the columns and
3. Determination of dissociation constants (K_d) for interaction of lectins and immobilized carbohydrates using capillary columns

4.2 General information

Materials and Methods

Fused-silica capillaries with 75 μm i.d and 375 μm o.d. were from Polymicro Technologies (Phoenix, AZ, USA). All chemicals were purchased from Sigma (The Netherlands).

^{1}H and ^{13}C NMR spectra were recorded either on Bruker DPX 300 (300 MHz) or DPX 400 (400 MHz) NMR spectrometers. $CDCl_3$ (dried over mol. sieves) and D_2O were the solvents.

Scanning electron microscopy (SEM), ***Frontal affinity chromatography (FAC)*** and ***Permeability*** measurements were carried out as described in chapter 3.

Synthesis of carbohydrates with an alkene terminated tetraethylene glycol

Carbohydrates (β-gal and α-man) with an alkene terminated tetraethylene glycol spacer were synthesized according to literature procedures reported earlier (Prime and Whitesides, 1993; Lin et al., 2002; Houseman and Mrksich, 2002). The schematic representation of oligosaccharide syntheses is depicted in fig 4.1.

Pent-4-enyl-tetraethylene glycol 3

To compound **1** (110 mmol) in a 50 mL round bottom flask, aqueous sodium hydroxide (12.7 M, 13.2 mmol) was added and stirred for 30 min at 100 °C. To this solution, compound **2** (13.2 mmol) was added and the mixture was stirred for 21 h at 100 °C. The reaction mixture was treated with water (20 mL) and ethyl acetate (15 mL). The aqueous layer was further treated with ethyl acetate (3 x 15 mL). All collected organic layers were combined and concentrated to a crude product. The crude product was purified with column chromatography (100% ethyl acetate) to obtain pure compound **3** (65.4% yield) in pure form.

^{1}H-NMR (300 MHz, $CDCl_3$) δ = 5.75-5.91 (1H, m), 4.9-5.1 (2H, m), 3.59-3.69 (16H, m), 3.45-3.5 (2H, m), 2.29 (1H, s), 2.10-2.13 (2H, m), 1.67-1.72 (2H, m) ppm.
^{13}C-NMR (75 MHz, $CDCl_3$) δ = 138.25, 114.68, 72.57, 72.50, 70.69, 70.60, 70.55, 70.30, 70.20, 70.07, 61.69, 30.19, 28.72 ppm.

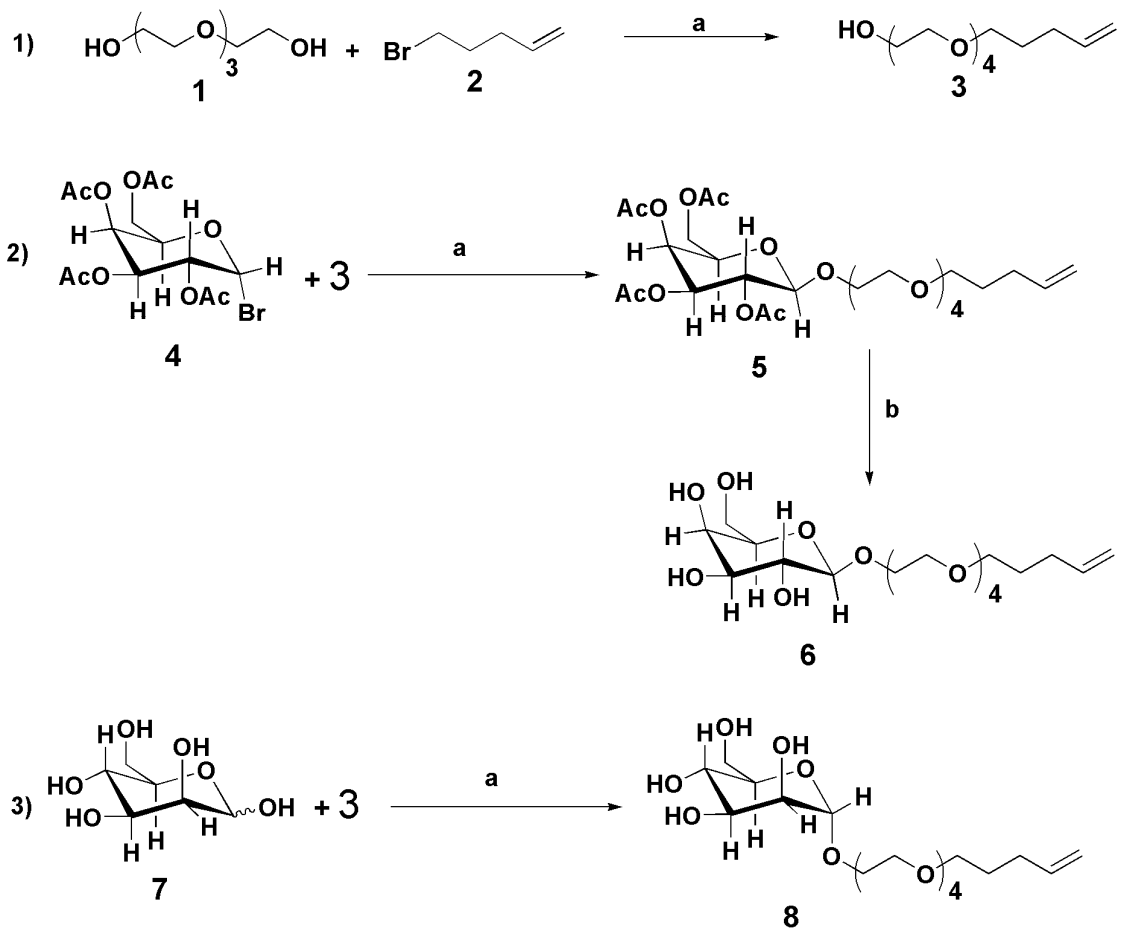


Figure 4.1 *1) Synthesis of pent-4-enyl-tetraethylene glycol: a) aq. NaOH, 100 °C, 21 h. 2) Synthesis of β-galactose with an alkene terminated tetraethylene glycol spacer: a) Ag_2CO_3, Drierite, DCM. b) Dowex-8X (H^+); NaOMe/MeOH, 40 min. 3) Synthesis of α-mannose with an alkene terminated tetraethylene glycol spacer: a) acetyl chloride, 70 °C, 4 h.*

Pent-4-enyl-tetraethylene glycol 2,3,4,6-tetra-O-acetyl-β-galactose 5

Compound **4** (2.7 mmol) was taken in an 100 mL round bottom flask containing **3** (3.8 mmol), drierite (0.3 g) and Ag_2CO_3 (3.02 mmol) in DCM (20 mL). The reaction mixture was stirred for 20 h. Subsequently, the reaction mixture was diluted with ethyl acetate (20 mL) and filtered to remove Ag_2CO_3 and concentrated to a crude product. The crude product was purified with column chromatography (100% ethyl acetate) to obtain compound **5** (yield 71.5%) in pure form.

^{1}H-NMR (400 MHz, $CDCl_3$) δ = 5.7-5.9 (1H, m), 5.12-5.23 (2H, m), 4.92-4.98 (2H, m), 4.50 (1H, d, J=9 Hz), 4.04-4.10 (3H, m), 3.84-3.87 (1H, m), 3.51-3.59 (16H, m), 3.38-3.42 (2H, m), 1.98-2.1 (2H, m), 2.08 (3H, s), 1.98 (3H, s), 1.97 (3H, s), 1.91 (3H, s), 1.59-1.63 (2H, m) ppm.
^{13}C-NMR (100 MHz, $CDCl_3$) δ = 170.38, 170.27, 170.15, 169.48, 138.29, 114.70, 101.38, 70.94, 70.71, 70.66, 70.61, 70.28, 70.10, 69.07, 68.83, 67.09, 61.31, 30.22, 28.79, 20.66 ppm.

Pent-4-enyl-tetraethylene glycol-β-galactose 6

To compound **5**, sodium methoxide (0.5 mmol) in methanol (10 mL) was added and the reaction mixture was stirred for 40 min. After confirmation with TLC, Dowex-8X (H^+) [prewashed with methanol] was introduced to the reaction mixture and stirred for a further 15 min. Dowex-8X (H^+) was removed by filtration. The concentrated product was analyzed with NMR, which confirmed the successful formation of compound **6** (yield 58.7%) which was used without further purification.

^{1}H-NMR (300 MHz, D_2O) δ = 5.8-5.89 (1H, m), 4.92-5.01 (2H, m), 4.35 (1H, d, J = 9 Hz), 3.84-4.1 (6H, m), 3.57-3.78 (16H, m), 3.42-3.56 (2H, m), 2.01-2.08 (2H, m), 1.57-1.66 (2H, m) ppm.
^{13}C-NMR (75 MHz, $CDCl_3$) δ = 135.74, 112.17, 98.31, 70.31, 69.24, 68.74, 68.17, 68.15, 68.04, 67.74, 67.55, 66.53, 59.42, 27.68, 26.23 ppm.

Pent-4-enyl-tetraethylene glycol-*α*-mannose 8

To compound **3** (0.11 mol), acetyl chloride (6 mmol) was added dropwise at 0 °C. To this, **7** (2 mmol) was added and the reaction mixture was stirred for 4 h at 70 °C. After confirmation with TLC, the crude product was purified with column chromatography (100% ethyl acetate) to obtain compound **8** (yield 63.2%) in pure form.

^{1}H-NMR (400 MHz, D_2O) δ = 5.78-5.88 (1H, m), 5.12 (1 H, d, J = 1.2 Hz), 4.97-5.05 (2H,m), 4.6-4.89 (6H, m), 3.93-4.26 (16H, m), 3.46-3.73 (2H, m), 2.01-2.13 (2H, m), 1.64-1.79 (2H, m) ppm.

^{13}C-NMR (100 MHz, D_2O) δ =121.84, 114.69, 97.39, 76.32, 71.74, 70.71, 70.61, 70.11, 69.57, 65.57, 62.42, 61.93, 60.51, 30.23, 24.49 ppm.

***In situ* preparation of affinity monolithic beds in capillaries and microchips**

The fused silica capillary (75 µm i.d) was activated with 3-(trimethoxysilyl)propyl methacrylate as described in chapter 3. To the monomer HEMA (30 µL) in an Eppendorf tube, carbohydrate (**6** or **8**) (5 mg), ammonium sulfate (12.5 mg), DATD (20 mg) and PDA (16 mg in 250 µL in phosphate buffer, pH 7.0) were added and mixed well followed by de-aeration for a period of 5 min. Subsequently, the initiator AMPA (10 µL, 10% v/v in water) was added and the solution was infused into the acrylate terminated capillary using a syringe pump for a period of 5 min. Afterwards the capillary ends were sealed with a GC septum and the capillary was placed in an oven (T = 65 °C) for 12 h resulting in an affinity monolithic bed. The resulting affinity monolithic column was washed with water and acetonitrile:water (50:50) each for 2 h at 2 µL/min.

The microchip with channel dimensions of 3.5 cm x 100 µm x 40 µm was placed in a chip holder and connected to a syringe pump using capillary columns (75 µm i.d, 20 cm long). After activation of the channel wall with acrylate groups, the monolith solution (prepared as described above) was infused through the channel using a syringe pump for a period of 5 min. The capillaries connected to the chip were sealed with a GC septum and the chip was covered with aluminum foil exposing roughly 2.3 cm of the chip to UV light (λ = 366 nm) for 1 h 30 min (fig 4.2). This resulted in the polymerization of the monolith solution (~2.3 cm long). The monolith formed in the column was washed with water and acetonitrile:water (50:50, v/v) each for 2 h at 0.5 µL/min flow to remove the non-polymerized monolith solution.

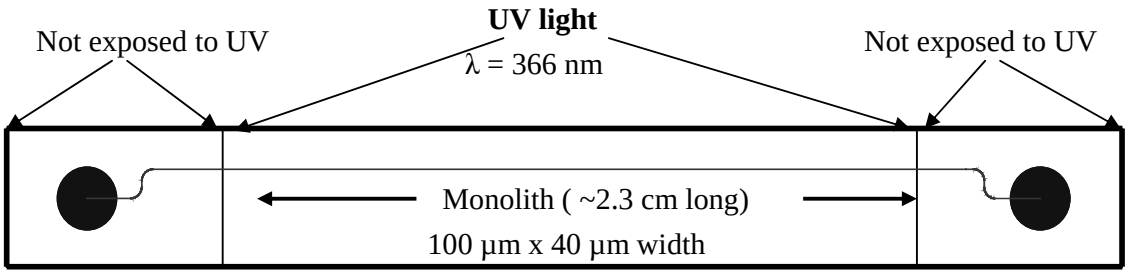


Figure 4.2 *Preparation of a monolithic column in a microchip using UV light.*

Adsorption and desorption of lectins using FAC

Frontal affinity chromatography (FAC) was used to investigate specific interactions of various carbohydrate (β-gal and α-man) monolithic beds (in capillaries and microchips) with lectins (Con A, LCA and PNA) and to determine the capacity of the monolithic beds.

Working conditions for affinity monolith in capillary:
Lectin solutions (1 mg/mL) in both water and binding buffer (10 mM tris(hydroxymethyl) aminomethane (Tris) buffer (pH 6-7, adjusted with HCl) including 1 mM Mg^{2+}, 1 mM Mn^{2+} and 1 mM Ca^{2+}) were prepared for this study. The flow rate for these measurements was roughly 1 µL/min. The 50% breakthrough point was used to determine the capacity of the column (Arata et al., 2001).

Working conditions for affinity monolith in microchip:
Lectins (0.5 mg/mL) in both water and binding buffer (Tris buffer) were used. The flow rate of the syringe pump was 0.5 µL/min. On-line UV detection (Capillary cell, 458-200.625, Sunchrom, Germany) was performed to record the chromatograms.

Elution of the lectins from monolithic columns was attempted using two eluting agents: 100 mM methyl-α-mannopyranoside (for Con A and LCA) and 100 mM β-galactose (for PNA) in binding buffer. The procedure was as follows: after adsorption of the lectins onto the column, 2 µL of the eluting agent was injected by means of a sample loop and the eluate was monitored with a UV detector at 218 nm. The flow rate of the system was roughly 1 µL/min.

Determination of carbohydrate-lectins dissociation constants (K_d)
Carbohydrate-lectins dissociation constants (K_d) were determined using carbohydrate monolithic capillary columns according to literature procedures reported earlier (Kasai et al., 1986; Arata et al., 2001; Chan et al., 2003). The FAC system with a slight modification (syringe pump was used instead of HPLC pump) was utilized for these measurements. The concentration of lectins varied from 0.3 to 1.0 mg/mL in binding buffer. The flow rate of the system was 1 µL/min with binding buffer as the mobile phase.

4.3 Results and Discussion

Physical characteristics of the monolithic beds
The permeability and the attachment of the monolithic bed to the column inner wall was evaluated using flow rate *vs* back pressure experiments and scanning electron microscopy (SEM).

Fig 4.3 depicts the flow rate *vs* back pressure results obtained for the two carbohydrate monolithic beds. At flow rates of 2.3-2.6 µL/min, these columns generated back pressures in the range of 30-38 bar. The back pressures generated by these columns were comparable with those of other polymethacrylate stationary phase beds reported earlier (Bisjak et al., 2005; Bedair and Oleschuk, 2006) and with the columns prepared in chapter 3. A column of the same inner diameter packed with a 5 µm sized particulate stationary phase with the same solvent and flow rate would generate a 10 times higher back pressure (Waters Column

Backpressure Calculator Software 2001, Waters Cooperation, USA). In conclusion, these carbohydrate monolithic columns showed good permeability.

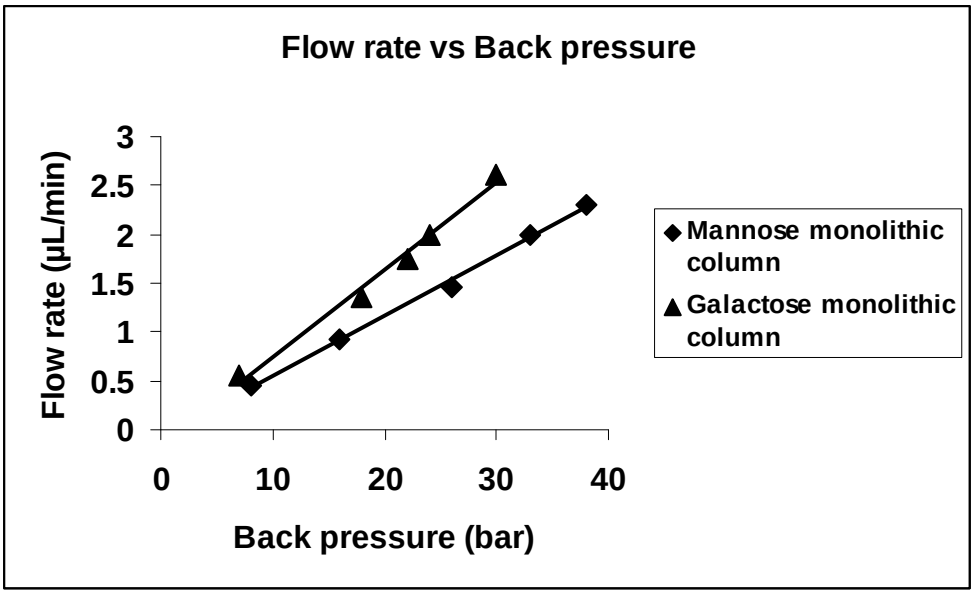
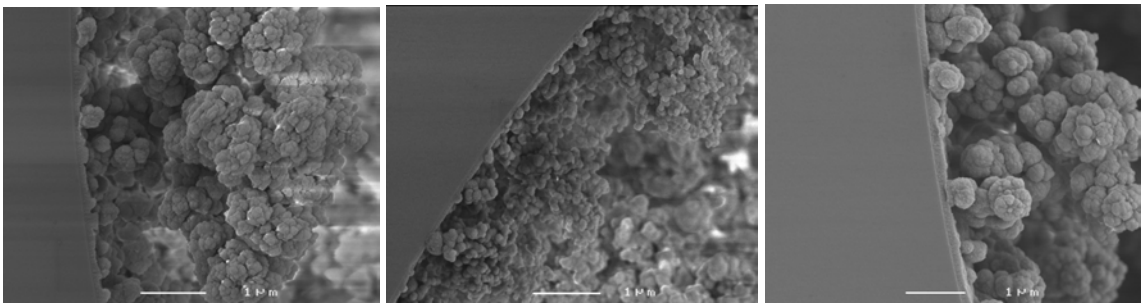


Figure 4.3 *Flow rate vs back pressure graph of various carbohydrate (β-gal and α-man) monolithic columns (75 µm i.d, 12 cm).*

The SEM images of the two carbohydrate monolithic columns and the original monolithic bed (i.e. without carbohydrates) were recorded. From these images (fig 4.4), it is clear that the monoliths were successfully attached to the capillary wall.



a) b) c)

Figure 4.4 *SEM images a) Monolithic bed in the absence of functional monomer (carbohydrate); b) β-galactose monolith, c) α-mannose monolith.*

Frontal affinity chromatography of lectins

Carbohydrate-lectin interactions were investigated using three different lectins (Con A, LCA and PNA) both in aqueous and binding buffer solution with two carbohydrate coated capillary columns. The total system dead volume was determined using 2.5% DMSO in water.

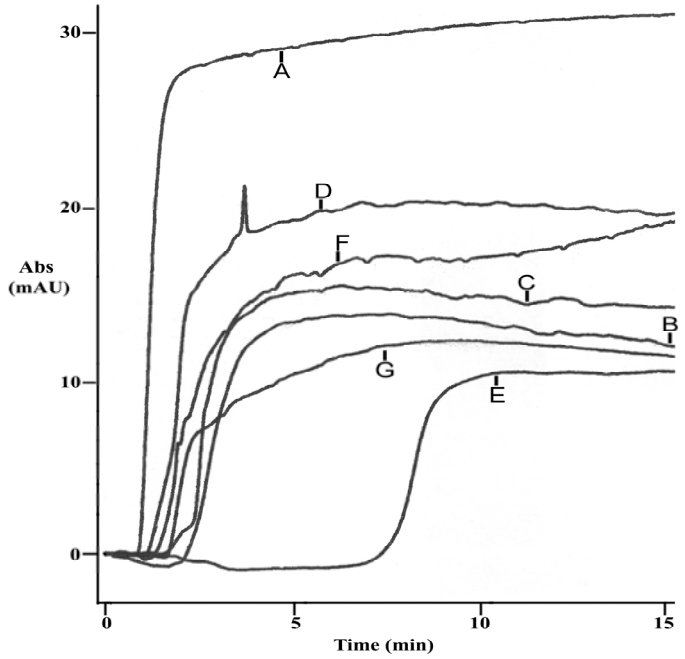
Figure 4.5 shows the frontal affinity chromatograms obtained for β-galactose and α-mannose monolithic columns respectively. The elution front of Con A, LCA and PNA in aqueous solution showed that there is no significant interaction of lectins with carbohydrates in absence of proper metal ions. PNA in binding buffer showed interaction with β-galactose but no interaction was observed with either Con A or LCA (fig 4.5a). The α-mannose monolithic column successfully retained Con A and LCA but not PNA (fig 4.5b). The capacity of the columns was determined according to the literature (Arata et al., 2001). 4.25 ± 0.25 µg of Con A and 2.25 ± 0.25 µg LCA were bound to the α-mannose monolithic column. 6.7 ± 0.2 µg of PNA was bound to the β-galactose monolithic column.

From these results, it can be concluded that carbohydrates with an alkene terminated tetraethylene glycol spacer were successfully incorporated in the monolithic bed. PNA showed interaction with β-galactose only. α-Mannose bound strongly to Con A and weakly to LCA. This is in accordance with the literature data available on carbohydrate-lectin specific interactions (Houseman and Mrksich, 2002; Ambrosi et al., 2005) and with our findings in chapter 3 (fig 3.12).

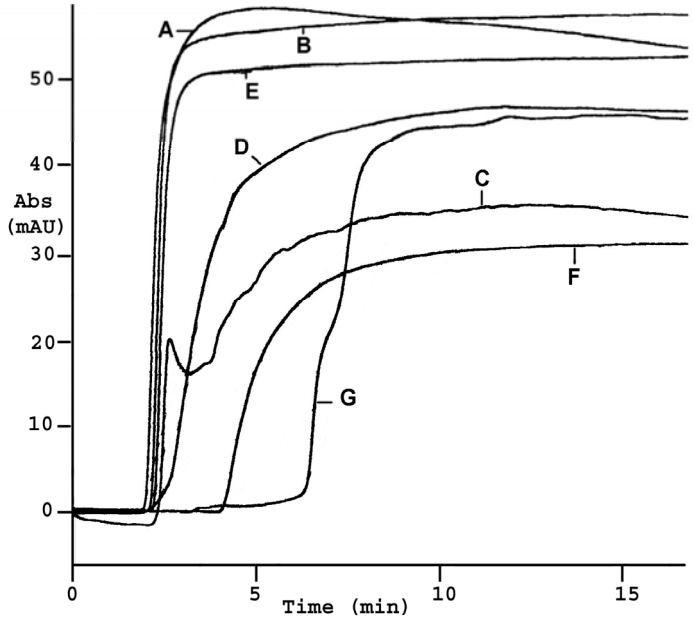
Elution of bound lectins

To test if these affinity columns can be used for the enrichment of lectins, lectins in binding buffer were loaded onto the monolithic columns, and subsequently eluted from the columns using various eluting agents such as carbohydrates in the millimolar range (100 mM methyl-α-mannopyranoside and 100 mM β-galactose).

Figure 4.6a, b and c show the chromatograms of eluted Con A, LCA and PNA. A blank experiment was performed using only binding buffer as eluent to confirm that the sugars in the eluting solution are responsible for the displacement of lectins from the column (fig 4.6d). 100 mM methyl-α-mannopyranoside eluted Con A (fig 4.6a) and LCA (fig 4.6b), while 100 mM β-galactose eluted PNA (fig 4.6c).



a)



b)

Figure 4.5 *Frontal affinity chromatograms of a) β-galactose monolith (75 µm i.d, 11.5 cm effective length and 18 cm total length) and b) α-mannose monolith (75 µm .id, 9.5 cm effective length and 16 cm total length) with PNA, LCA and Con A in water and binding buffer (B.B) medium. A) DMSO in water; B) PNA in water; C) LCA in water; D) Con A in water; E) PNA in B.B; F) LCA in B.B G) Con A in B.B.*

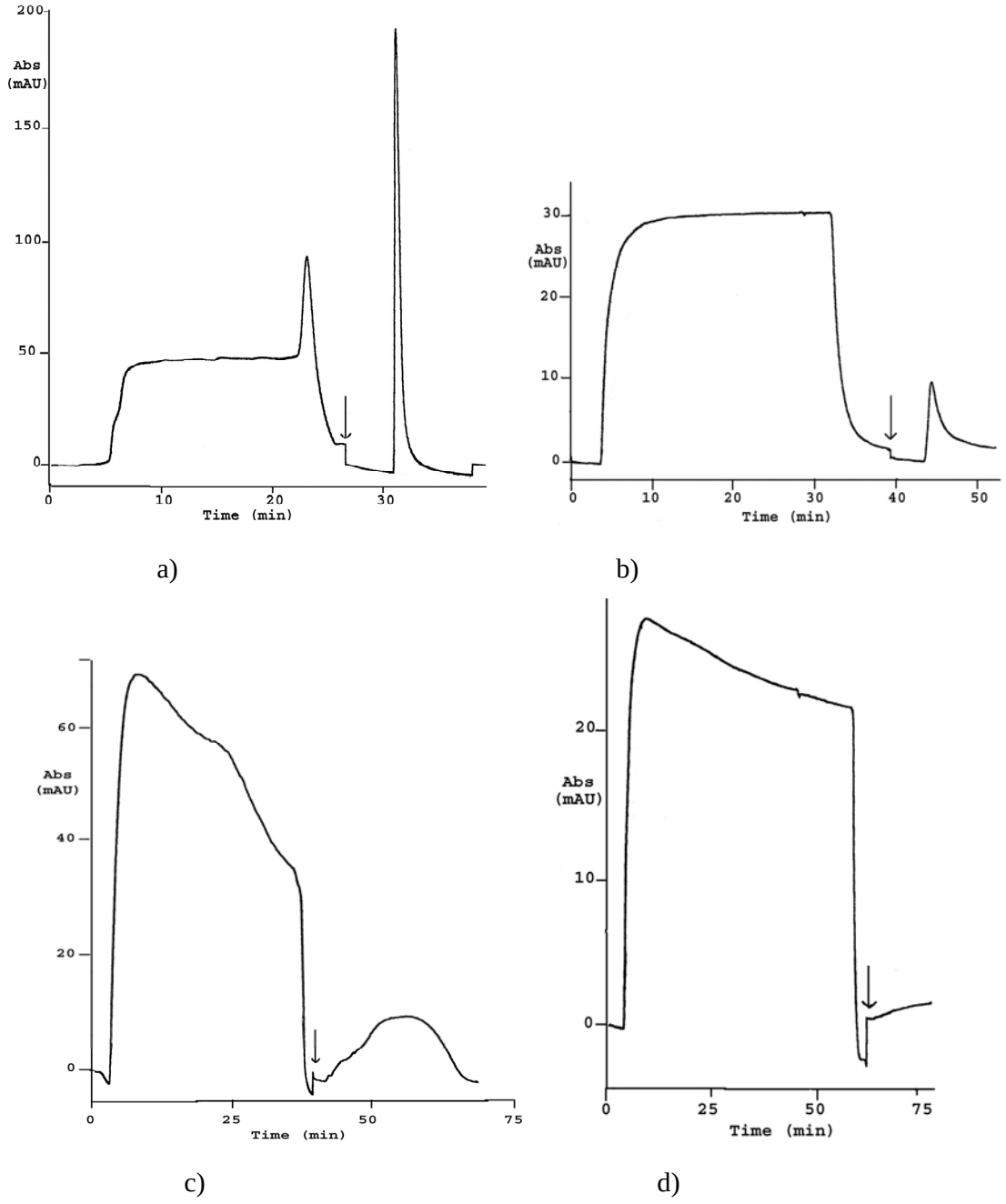


Figure 4.6 *Chromatograms of lectins eluted from β-galactose and α-mannose monolithic columns. Binding buffer was the mobile phase (flow rate is roughly 1 µL/min). eluting agents: 100 m M methyl-α-mannopyranoside for α-mannose monolithic column and 100 mM β-galactose for β-galactose monolithic column. Arrows in the chromatogram indicate eluting agent injection time: a) Con A bound (elution front: 0 to 25 min) to the column was eluted at 31 min; b) LCA bound (elution front: 0 to 38 min) to the column was eluted at 43 min; c) PNA bound (elution front: 0 to 39 min) to the column started eluting at 43 min and d) PNA bound to the column (elution front) was not eluted with binding buffer as eluting agent.*

Determination of carbohydrate-lectin dissociation constant (K_d)

Monosaccharide - lectin interactions in solution are considered to be weak with dissociation constants in the millimolar range (Mandal et al., 1994; Ambrosi et al., 2005). However it has been reported that binding of lectins by immobilized sugars on surfaces is different from the interaction in solution and also different from the interaction between immobilized lectins and sugar in solution (Tang et al., 1998). This is caused by a multidentate interaction of one lectin molecule with different immobilized sugars, i.e. the number of binding sites affects the binding constant (the avidity effect). A similar increase of binding strength is known to take place with dendrimers (Pukin et al., 2007).

Monolithic carbohydrate columns can be used for the screening of lectins, and it is expected that sugars immobilized on a surface resemble the natural situation, where saccharides are on the outside of cell membranes, more closely than sugars in solution. Smith et al. studied the mannose - Con A interaction with carbohydrate arrays on gold films using SPR (Smith et al., 2005). They observed a K_d of 1.78×10^{-7} M. Zhang et al. reported recently on the interaction between lectins and immobilized saccharides on a gold surface (Zhang et al., 2006). By means of QCM and SPR they found K_d values of 1.14×10^{-6} M and 2.56×10^{-7} M respectively for the interaction of mannose and Con A. The SPR data of these reports are comparable.

The dissociation constant (K_d) for the interaction of lectins with the monolithic columns was determined using FAC. The binding constant measurements were carried out as described by Chan and co-workers with lectins being infused starting from low concentration to high concentration however in our case a washing with buffer in between each infusion was applied (Chan et al., 2003). The values obtained were plotted in a graph with $1/[A]_0$ $(V\text{-}V_0)$ $(pmol^{-1})$ *vs* $1/[A]_0$ (μM^{-1}) (fig 4.7) (Kasai et al., 1986), where V is the elution front volume of the lectin retained on the column, V_0 is the elution front volume of the void marker (in this case DMSO) and $[A]_0$ is the initial infusion concentration. The slope of the graph provides the value of K_d from the intercept on the abscissa and B_t, the amount of ligand immobilized in the column, can be obtained from the intercept of the ordinate. The K_d and B_t for PNA and Con A are 3.7×10^{-6} M; 28.5 pmol (fig 4.7a) and 8.3×10^{-6} M; 125 pmol (fig 4.7b). If we assume that mannose (MW = 180 Da) is the active ligand, this corresponds with 22.5 ng of mannose being available for binding Con A. Earlier the capacity of the column was determined at 4.25 µg Con A (*vide supra*). Taking as MW for Con A 102,000 Da and assuming a tetradentate interaction between mannose and Con A this translates to 29 ng of mannose which fits the amount of mannose calculated on the basis of B_t rather nicely. Differences could be due to measuring errors, or deviations from the ideal 4:1 ratio between mannose and Con A. Both figures deviate substantially from the amount of Con A expected to be bound based on the original concentration of mannose in the monomer solution (5 mg/290 µL) and the column volume (420 nL). Based on those figures one would expect 630 µg of bound Con A, i.e. 150

times more. The discrepancy must be due to mannose in the monolithic bed being unavailable for binding due to small pore sizes. The Con A tetramer has dimensions of 12.4 x 12.9 x 6.7 nm (Naismith et al., 1994). In chapter 3, it has been shown that a significant percentage of the pore sizes of these types of columns are below 15 nm. Another reason could be due to losses during the polymerization process.

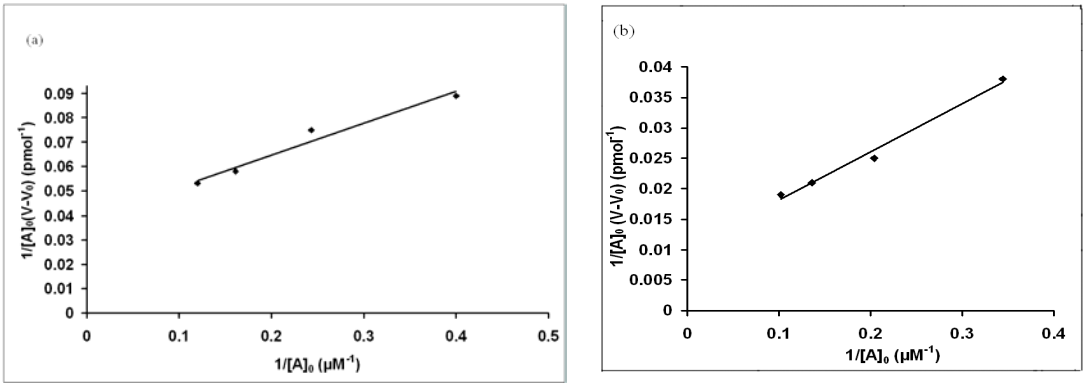


Figure 4.7 *The graph of $1/[A]_0$ $(V-V_0)$ $(pmol^{-1})$ vs $1/[A]_0$ (μM^{-1}) provides the dissociation constant (K_d) and amount of ligand immobilized (B_t) for a) PNA using a β-galactose column and b) Con A using an α-mannose column.*

Once the B_t value is determined for a particular column, dissociation constants of other ligands can be obtained easily using the equation (Sueyoshi et al., 1988)

$$K_d = 1/((B_t/V\text{-}V_0)\text{-}[A]_0) \qquad (4.1)$$

From this equation, the K_d of LCA was calculated to be $2.8 \pm 0.95 \times 10^{-6}$ M. The observed K_d values are much lower than reported using a lectin immobilized column or in solution (Dulaney, 1979; Schwarz et al., 1993; Ambrosi et al., 2005) but comparable to the values reported by Zhang and Smith using the SPR technique especially when one takes into account the very different surface and immobilization technique. Thus the monolithic columns appear suitable for the study of lectin-sugar interactions.

Carbohydrate monolithic array chip

A β-galactose monolithic microchip (~2.3 cm long) was employed to study the interaction of PNA and Con A in both aqueous and binding buffer solutions using FAC (fig 4.8). The results obtained with a β-galactose monolithic microchip were identical to those of a β-galactose monolithic capillary column, in their capacity to distinguish between the two lectins (Con A and PNA). It is clear that a β-galactose monolith was successfully incorporated in the microchip (fig 4.9a).

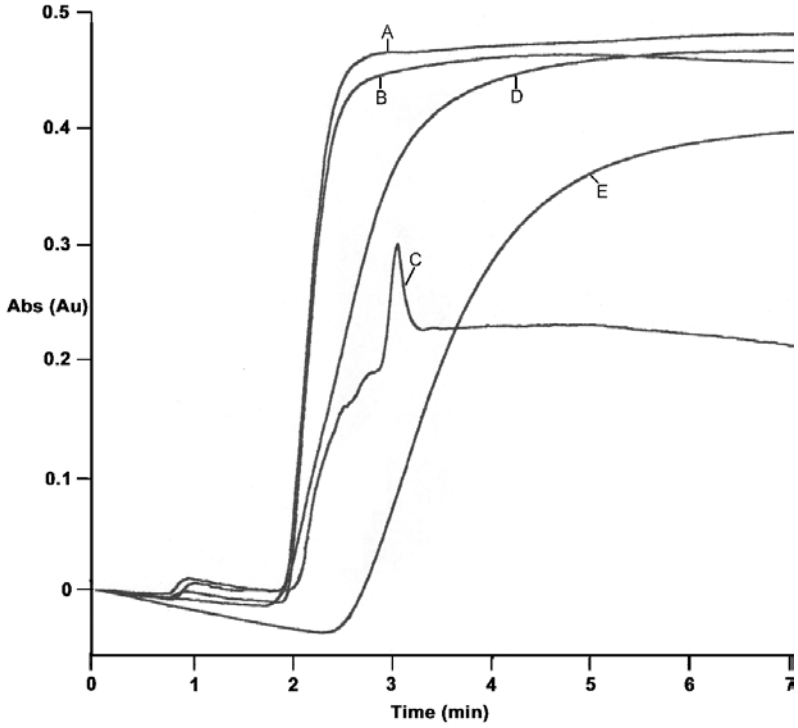
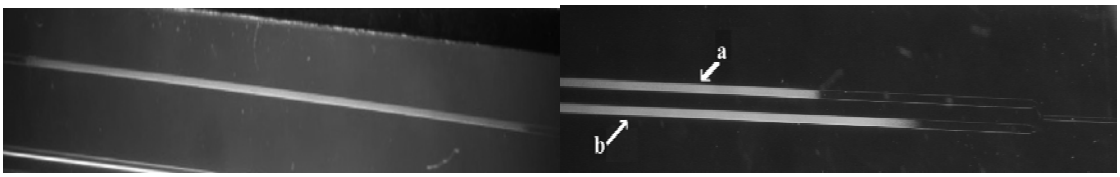


Figure 4.8 *FAC of the β-galactose monolith in a microchip (100 µm x 40 µm x 2.3 cm effective length and 3.2 cm total length) with PNA, LCA and Con A in water and binding buffer (B.B) medium. A) DMSO in water; B) Con A in water; C) PNA in water; D) Con A in B.B; E) PNA in B.B.*

The protocol developed for the monolithic chip was used to prepare an affinity monolithic array chip (β-gal and α-man). As seen in fig 4.9b, the monolithic array chip was successfully prepared, but differences in back pressure (fig 4.3) between the β-galactose and α-mannose monolithic columns resulted in 65% of the infused flow going through the β-galactose column (**b**). To employ this monolithic array chip to screen lectins from plant extracts, further research should be carried out to obtain equal permeability in the two columns for equal distribution of liquid flow.



a) b)

Figure 4.9 *Microscopic image of a monolith prepared in a microchip a) β-galactose monolithic column (2.3 cm long) and b) β-galactose (**a**) and α-mannose (**b**) monolith array chip (see Appendix for colour pictures).*

4.4 Conclusion

Carbohydrate monolithic beds were synthesized in a single step by *in situ* polymerization in capillary columns. These monolithic beds with high permeability showed specific lectin interactions and low dissociation constant values i.e. strong binding. The developed monolith protocol was reproduced successfully in a microchip. The monolith array chip requires further fine tuning of pressure between two columns to obtain equal distribution of flow.

Looking into future research prospects of these affinity columns, they could be applied for the enrichment of lectins and sugar-binding toxins from crude mixtures e.g. plant extracts and serum samples. Additionally, they can be used to determine carbohydrate-protein dissociation constants in a simple manner requiring minimal amounts of proteins. The sensitivity and throughput could be further enhanced by interfacing with nanospray ESI-MS.

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5

Synthesis of affinity monolithic columns for depleting antibodies against gangliosides in human serum

Abstract

The focus in this chapter is on capturing anti-ganglioside antibodies from serum samples of patients suffering from Guillain-Barré syndrome (GBS) using ganglioside monolithic columns. Gangliosides (GM1, GM2 and GM3) with an alkene terminated alkyl spacer were used as functional monomers along with another monomer (HEMA), and crosslinkers (PDA and DATD) to prepare ganglioside monolithic capillary columns. Serum samples obtained from patients, whose sera had high titers of antibodies against GM1, GM2 and GQ1b gangliosides were utilized in this study. ELISA (Enzyme linked immunosorbent assay) results showed that human anti-ganglioside IgM antibodies (against GM1 and GM2) are specifically adsorbed by GM1 and GM2 monolithic columns only and not by blank monolithic or GM3 monolithic columns. “Anti-human antibodies with FITC label” showed that the gangliosides are evenly distributed in the columns. The high capacity (>90 to 94% of high titer antibodies are depleted after an incubation of < 2 min) of these columns shows the potential of these columns as a diagnostic tool in future.

Tetala, K. K. R et. al., *Manuscript in preparation*

5.1 Introduction

Antibodies to human peripheral nerve gangliosides are frequently found in various forms of immune-mediated neuropathy and may be directly involved in nerve damaging. Examples of such neuropathies are the Guillain-Barré syndrome (GBS) and multifocal motor neuropathy in which at least half of the patients have antibodies to various types of gangliosides. GBS is an acute form of neuropathy which is frequently preceded by infections with *Campylobacter jejuni* (Ang et al., 2004). In this syndrome, the antibodies released to fight the infection attack the lipooligosaccharides (gangliosides) present on the nerve cell membrane as well as the gangliosides present on the outer cell wall of the *Campylobacter jejuni*. The typical symptoms of GBS are weakness, areflexia, and loss of control over limbs (Hughes and Cornblath, 2005; Ang et al., 2004). The gangliosides are complex carbohydrates with a sialic acid group and are found abundantly in the nervous system. Fig 5.1 depicts the most widely known gangliosides: GM1, GM1b, GM2, GQ1b, GalnAc-GD1a, GQ1b, GalC, GT1a and GD1a (Willison and Yuki, 2002; Ang et al., 2004; Hughes and Cornblath, 2005). Some of these gangliosides also act as receptors for *cholera* toxin (CT) (binds to GM1 and other gangliosides) and *Escherichia coli* heat-labile enterotoxin (LT) (binds to GM1, GM2) (Cuatreca, 1973; Holmgren et al., 1985).

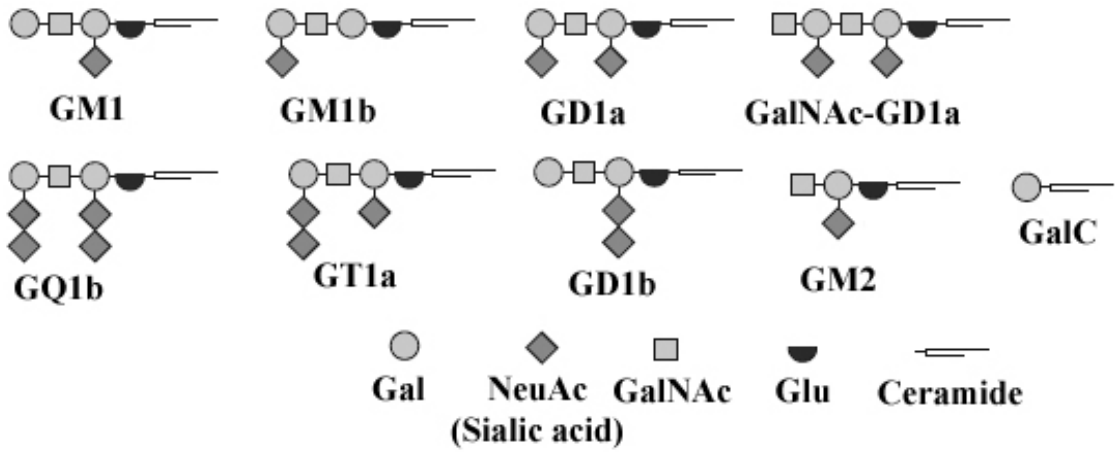

Figure 5.1 *Structures of various gangliosides (adapted from Hughes, 2005).*

Synthetic carbohydrate mimics possess various advantages over the natural structures (high affinity, more stable, and improved bioavailability) and could be used to develop therapeutic agents and diagnostic tools (Sears and Wong, 1999). In the case of GBS, bovine brain derived gangliosides were used in ELISA studies for the identification of the antibodies. These gangliosides are difficult to purify due to the presence of other gangliosides, are expensive and may be potentially infected. The molecular mimicry of such natural gangliosides can help researchers to solve these problems (Kuijf et al., 2005). Recently, Pukin et al. synthesized analogs of various gangliosides (GM1, GM2 and GM3) with various terminal functional groups (alkene, alkyne and azide) in pure form. These ganglioside mimics showed high

affinity towards CT and LT and similar affinity towards anti-GM1 GBS-related IgM and IgG antibodies in comparison with bovine GM1 (Pukin et al., 2008). Some literature exists on the use of carbohydrates as ligands carrying various functional groups, for e.g. microarrays on gold, silicon, glass surfaces (Park and Shin, 2002; Faber et al., 2005, Tetala et al., 2007), and affinity monolithic columns (Bedair and Oleschuk, 2004; Tetala et al., 2007a; Tetala et al., 2007b). This opens the route to use these ganglioside mimics in monolithic columns as a diagnostic tool for GBS.

In this chapter, the research focused on obtaining proof of principle that "ganglioside mimic" monolithic materials can efficiently capture antibodies from sera samples. GM1, GM2 and GM3 with an alkene termination were chosen as ligands and the affinity monolithic column protocol as described in chapter 4 was chosen for ligand immobilization. To determine the efficiency of these affinity columns, sera obtained from patients suffering from GBS and a healthy patient were used.

5.2 Materials and methods

Fused-silica capillaries with 250 µm i.d. and 375 µm o.d. were obtained from Polymicro Technologies (Phoenix, AZ, USA). Bovine serum albumin (BSA) and O-phenylenediamine tablets were purchased from Sigma Aldrich, The Netherlands. Hydrogen peroxide, citric acid, and ganglioside were purchased from Merck, The Netherlands. Serum samples (see Table 5.1) were provided by Rotterdam Erasmus Medical Centre, The Netherlands. Gangliosides (GM1, GM2 & GM3) with an alkene-terminated alkyl spacer were synthesized at the Laboratory of Organic chemistry of Wageningen University (Pukin et al., 2008). Gas tight syringes (500 µL) were purchased from Alltech, The Netherlands. Syringe pumps (Harvard 11 PicoPlus, dual syringe) were purchased from VWR International, The Netherlands.

Table 5.1 *Diagnosis and anti-ganglioside antibody reactivity of tested serum samples*

Serum code	**Antibody subclass**	**Antibody reactivity to Gangliosides**	**Diagnosis**
F325	IgM	GM2	GBS
PP019	IgM	GM2	Polyneuropathy and monoclonal gammopathy
PFKEY	IgM, IgG	GM1	GBS
F226	IgG	GM1	GBS
G119	IgM	GQ1b	GBS
G156	IgG	GM2	GBS
NC030	IgM	None	Healthy control

Fluorescence measurements (FM) were carried out as described in chapter 3.

Enzyme linked immunosorbent assay (ELISA) was used to measure the residual antibody activity against gangliosides (GM1, GM2 and GM3). The walls of a 96-well plate (Nunc, Maxisorp) were coated either with ethanol or 300 pmoL of ganglioside dissolved in ethanol and evaporated to dryness. All the wells were then blocked with a solution of PBS (phosphate-buffered saline, pH = 7.8) containing 1% (w/v) of BSA for 2 h at room temperature and a further 2 h at 4 ºC. The plates were then emptied by flicking and incubated with the samples, which were diluted, initially 1:100, in PBS - 1% BSA, overnight at 4 ºC. Samples were added to 4 wells, 2 coated with GM1, GM2 or GM3 and 2 with ethanol only. In this way duplicate values were obtained and any activity attributed to an assay component other than GM1, GM2 or GM3 could be subtracted from the total activity.

The following day, the plates were washed with an automated ELISA-washer (Elx50, Bio-Tek) with PBS solution and filled with a solution of peroxidase-conjugated secondary step antibodies (Jackson Immuno Research Labs) diluted to 1:2500 in PBS - 1% BSA for 1.5 h at room temperature. After washing once again with PBS solution, the plates were developed by adding a substrate solution comprising one O-phenylenediamine tablet (5 mg dissolved in citrate buffer with hydrogen peroxide). The reaction was stopped by the addition of hydrochloric acid and the optical densities (OD) were read in an automated reader at 490 nm. The mean OD's of the blank (ethanol-coated) wells were subtracted from the mean OD's of the GM2-coated wells to obtain a specific OD.

***In situ* preparation of ganglioside monolithic columns**

A fused silica capillary (250 µm i.d) was activated with 3-(trimethoxysilyl)propyl methacrylate as described in chapter 3. A solution of ganglioside ((GM1, GM2 or GM3), 5 mg) in methanol (30 µL) was added to a mixture of HEMA (30 µL), ammonium sulfate (8 mg), DATD (20 mg) and PDA (16 mg in 250 µL of phosphate buffer, pH = 7.0) in an Eppendorf tube and mixed well followed by de-aeration for a period of 5 min. Subsequently, the initiator AMPA (10 µL, 10% v/v in water) was added. The monolith solution was then sucked into the acrylate terminated capillary using vacuum and both ends of the capillary were sealed with a GC septum. The column was placed in an oven (T = 65 °C) for 12 h, which resulted in a ganglioside-containing monolithic column. The column was washed with water for 2 h (2 µL/min).

Depletion of antibodies from serum samples

A three step procedure to capture antibodies (IgM & IgG) from serum samples was developed. Various serum dilutions (F325 (1:50 & 1:5), PP019 (1:100), G119 (1:10), NCO30 (1:10), G156 (1:10), PFKEY (1:200); F226 (1:100) in PBS buffer (pH-7.8)) were prepared. A

500 µL syringe was used to infuse the solutions into the columns. The protocol was as follows:

Step 1: The columns were washed with PBS buffer (pH = 7.8) for 1 h (1.4 µL/min)
Step 2: The serum samples in PBS buffer were infused for 3 h (1 µL/min)
Step 3: After 3 h, the columns were washed with PBS buffer for 1 h (1.4 µL/min)

For convenient analysis of the samples using ELISA, the samples collected during the experiments were given codes as:

"**A**" for initial serum sample concentration before serum infusion in the column
"**B**" for sample collected during serum infusion; and
"**C**" for sample collected during the column wash with buffer solution

Fluorescence microscope measurements
The homogeneous distribution of the ganglioside monolithic columns was tested using either "goat anti-human IgM with FITC label" or "goat anti-human IgG with FITC label". The procedure for this experiment was as follows:

Step 1: The column was washed with PBS buffer (pH = 7.8) for 30 min (1 µL/min)
Step 2: Treating the column with "anti-human antibodies with FITC labeled" solutions for 1h (1 µL/min)
Step 3: Wash the column with PBS buffer (pH = 7.8) for 45 min at (1µL/min)
Step 4: Analyze the samples with fluorescence microscopy

5.3 Results and Discussion

Depletion of IgM and IgG antibodies using GM2 monolithic columns
The prepared GM2 monolithic column was employed to deplete IgM and IgG antibodies from human serum samples (F325 (IgM), PP019 (IgM) and G156 (IgG)) of patients, whose serum had high titer of antibodies against GM2. Figure 5.2 depicts the data obtained from ELISA measurements. From the data, it can be seen that IgM antibodies in sera F325 and PP019 were successfully depleted (the decrease of the IgM concentration was ~94 % in F325 (1:50) and ~86 % in PP019 (1:100) in sample B). Still ~75% of IgM antibodies were depleted when the sera dilution went from 1:50 to 1:5. This indicates that the GM2 column has a high capacity. However, the GM2 column was not successful in depleting IgG antibodies from G156 sera.

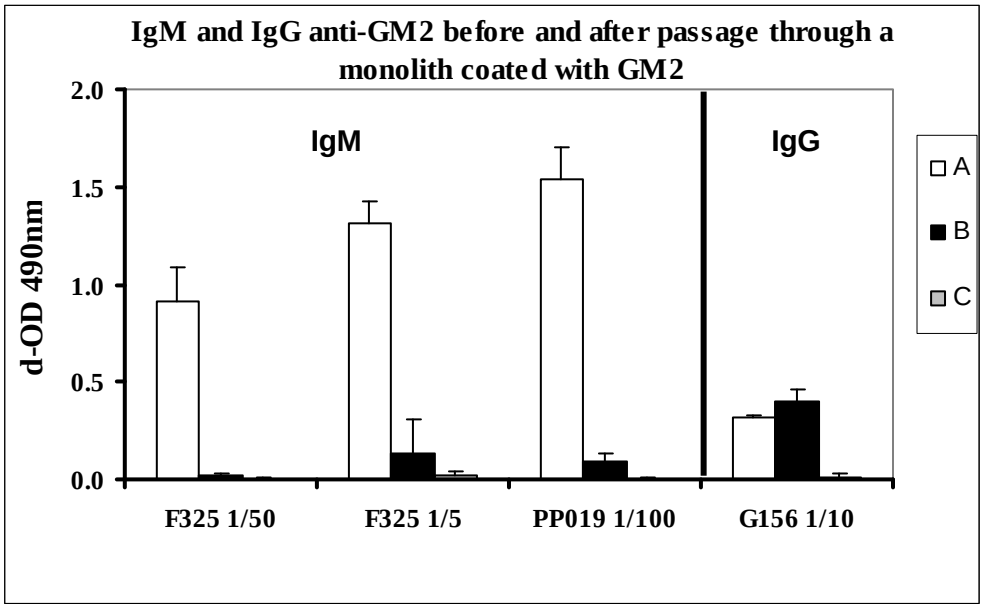

Figure 5.2 *ELISA results of GM2 monolithic capillary column tested against various serum samples (F325, PP019 and G156) to capture IgM and IgG antibodies. "**A** - intial serum concentration before serum infusion in the column; **B** - sample collected during serum infusion and **C** - sample collected during the column wash with buffer solution"*

To check the homogeneity of GM2 distribution on the capillary and to obtain proof of IgM/IgG antibody depletion by GM2 monolithic columns, "anti-human IgM/IgG with FITC label" were infused into the column after the binding of antibodies. These anti-human antibodies bind only if there are any antibodies present in the column. The GM2 column treated with sera F325 (1:50 and 1:5 dilution), G156 (1:10 dilution) and a blank GM2 column (not treated with any sera) were used in this study. The columns were viewed under a fluorescence microscopy and the recorded images are depicted in fig 5.3.

The "GM2 column-F325 (1:50 dilution)" (fig 5.3I) and "GM2 column-F325 (1:5 dilution)" (fig 5.3II) treated with "goat anti-human IgM with FITC" showed an increase of fluorescence intensity along the channel when the serum concentration increased. This result indicates the homogeneity and high capacity of the GM2 column. However, the "GM2 column-G156 (1:10 dilution)" (see fig 5.3IV) treated with "goat anti-human IgG with FITC", did not show any fluorescence i.e. GM2 did not capture any IgG from G156 sera. These results (fig 5.3I, II, and IV) are in full agreement with the results obtained from ELISA measurements (see fig 5.2). Blank GM2 monolith (not treated with any sera) did not show any binding of "anti-human IgM with FITC label" to the column (fig 5.3III). This is due to the absence of any IgM in the blank GM2 column.

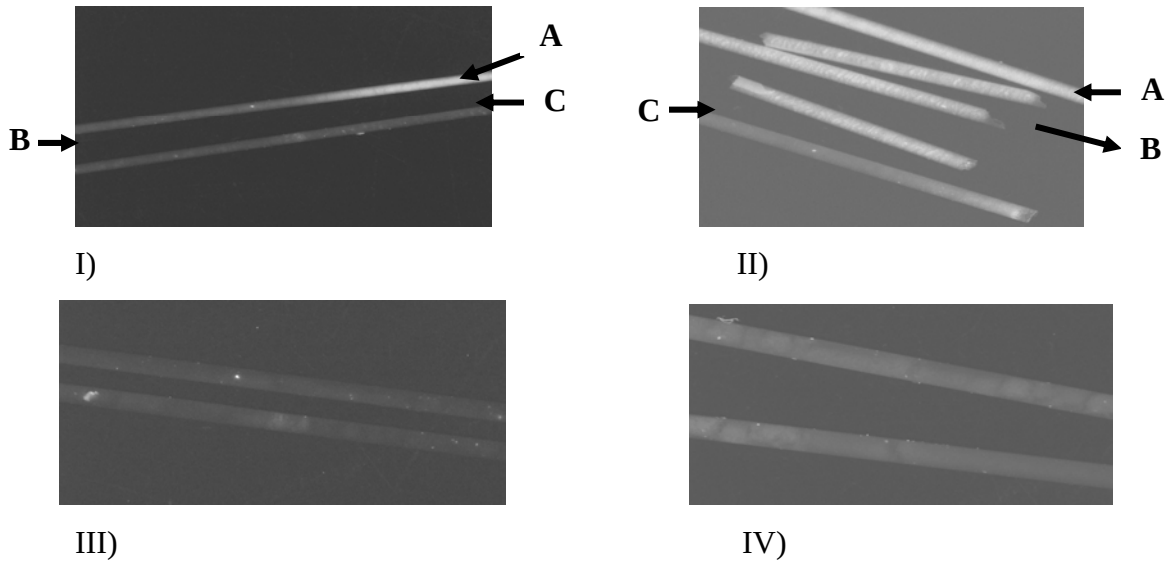

Figure 5.3 *Fluorescence microscopy images of I) GM2 column-F325 (1:50 dilution), II) GM2 column-F325 (1:5 dilution) and III) GM2 column (not treated with any sera) treated with "anti-human IgM with FITC label". IV) GM2 column-G156 (1:10 dilution) treated with anti-human IgG with FITC label. "**A** - start of the column; **B** - middle of the column and **C** - end of the column" (see Appendix for colour pictures).*

Depletion of IgM & IgG antibodies using GM1 monolithic columns

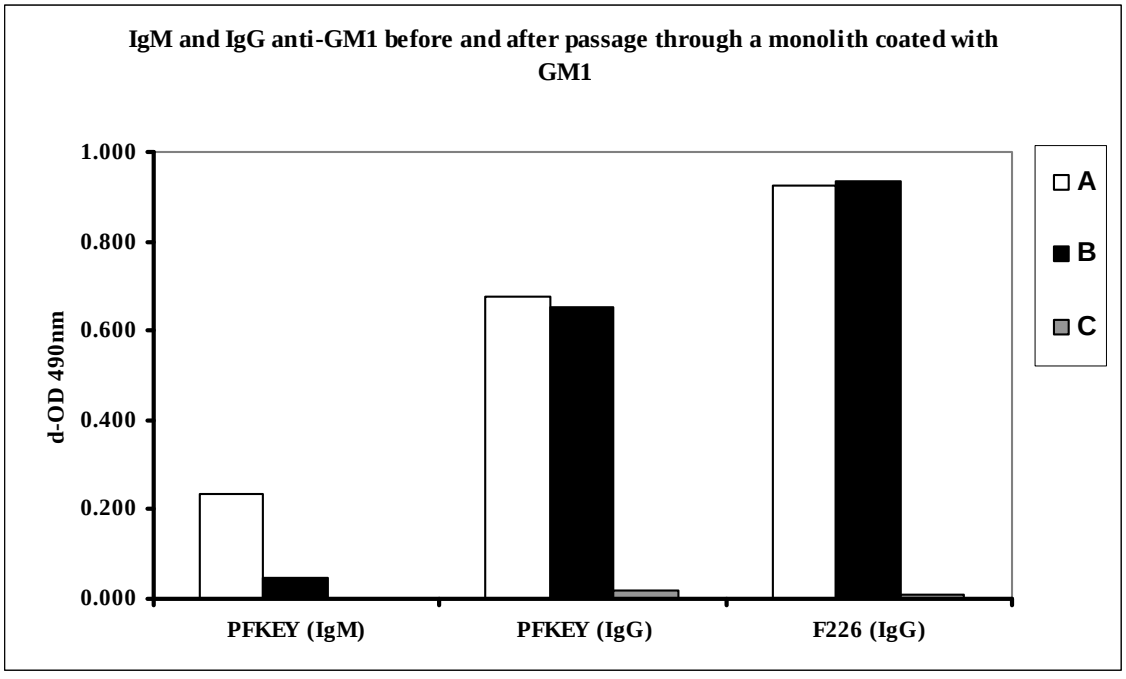

Figure 5.4 *ELISA results GM1 monolithic column used to deplete IgM and IgG antibodies of sera PFKEY (1:200) and F226 (1:100). "**A** - intial serum concentration before serum infusion in the column; **B** - sample collected during serum infusion and **C** - sample collected during the column wash with buffer solution"*

After the successful depletion of IgM from serum samples using a GM2 monolithic column the next step was to study whether a GM1 monolithic column could also capture IgM and IgG antibodies from sera of patients suffering from GBS. The GM1 monolithic column was treated with two serum samples (PFKEY (contain IgM and IgG) and F226 (contain IgG)), whose sera had high titers of antibodies against GM1. The ELISA results obtained are depicted in fig 5.4. The GM1 column successfully captured IgM from PFKEY sera (decrease of IgM concentration in sample B) from the sera, but was unsuccessful in capturing IgG (no decrease of IgG concentration in sample B) from both serum samples (PFKEY and F226).

It is surprising that both GM1 and GM2 columns were not able to deplete IgG antibodies but were effective in depleting IgM antibodies present in the same serum sample. The fact that IgG did not bind to GM1 and GM2 may relate to two factors:

1. Size difference - IgG is a monomer (150 kDa, ~3.5 nm) with two active sites, whereas IgM is a pentamer (900 kDa, ~ 10 nm) with ten active sites (Edberg et al., 1972; Allansmith et al., 1979). There can be a competition between IgG and IgM to bind with the ligand, but IgM with more active sites is more likely to bind with GM1/GM2 than IgG
2. Effect of temperature - In general, hemagglutination of antibodies occurs strongly at room temperature or in the cold (4 °C) (Van Regenmortel, 1992). Taking this property of antibody into consideration, IgG may bind more effectively to GM1/GM2 in the cold (4 °C) than at room temperature

Approaches to deplete IgG antibodies from the serum sample are mentioned below:

1. The serum incubation time should be increased by reducing the flow rate from 1 µL/min to 0.2 or 0.1µL/min (flow rates lower than 0.1 µL/min are not feasible with the current syringe pumps)
2. Multi-ligand (dendritic) approach - Use of dendrimers with functional GM1 and GM2 (a dimer or a tetramer) as ligands in the monolithic column may enhance the column capacity there by increasing the chance of IgG binding to GM1 and GM2
3. Monolithic powder - a finely powdered GM1/GM2 monolithic material in an Eppendorf tube could deplete IgG at room temperature or at 4 °C (longer incubation). This increase in contact time between IgG in solution and GM1/GM2 in the monolith will be effective to deplete IgG
4. Molecular imprinting - Formation of an IgG template within the monolithic column obtained during an *in situ* polymerization process in the presence of IgG (control: IgM) may be effective to deplete IgG antibodies

Control experiments

Two sets of control experiments were performed to demonstrate that

1. IgM antibodies against GM2 in sera F325 and PP019 can only be depleted by GM2 monolithic columns and not by either a blank monolithic column or another ganglioside (GM3) monolithic column
2. GM2 monolithic columns can deplete only IgM antibodies against GM2 and not IgM antibodies against other gangliosides (e.g. $GQ1b^{+}$) nor various antibodies from a healthy person

The ELISA data obtained from these measurements is depicted in fig 5.5. There is no decrease of the IgM concentration in F325 and PP019 sera in sample B. This indicates that IgM in sera F325 and PP019 cannot be depleted by either an uncoated monolithic material or the GM3 monolithic column. It can only be depleted with a GM2 column (see fig 5.2).

In case of control experiments for the GM2 monolithic column, IgM against GQ1b ganglioside of sera G119 was not captured by the GM2 column indicating that the GM2 column can capture IgM against GM2 only. As the healthy patient does not have IgM against GM2, the GM2 column treated with "healthy" serum did not deplete any antibodies, as there are no infected antibodies present in the serum.

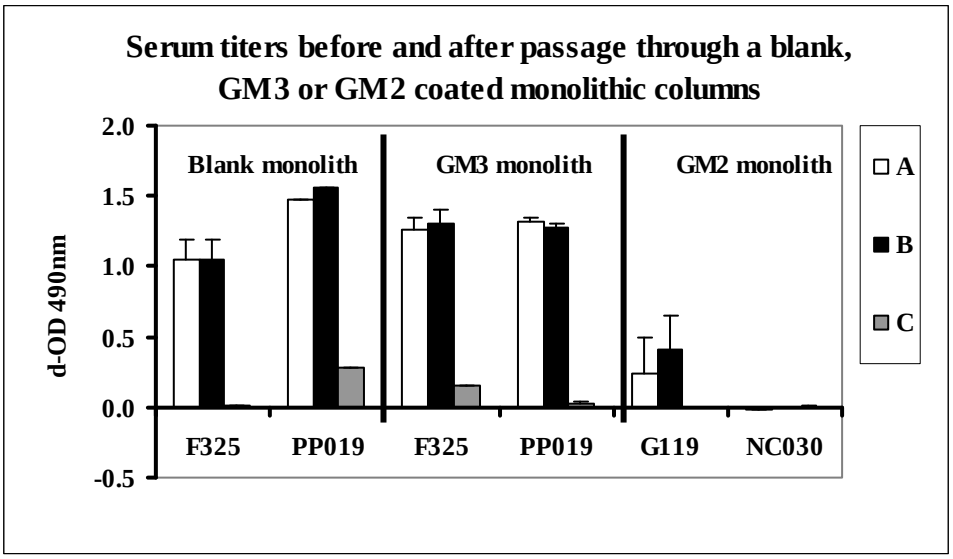

Figure 5.5 *Control experiments to study: a) IgM binding towards blank monolithic and GM3 monolithic columns towards F325 and PP019 sera and b) GM2 monolithic column against IgM of ganglioside GQ1b and healthy patient (NC030).* ***A*** *- intial serum concentration before serum infusion in the column;* ***B*** *- sample collected during serum infusion and* ***C*** *- sample collected during the column wash with buffer solution"*

Fig 5.6 depicts the fluorescence microscopy results obtained for GM3 monolithic column-F325 (fig 5.6a), blank monolithic column-F325 (fig 5.6b), GM2 monolithic column-G119 (fig 5.6c) and GM2 monolithic column-NC030 (fig 5.6d) treated with "goat anti-human IgM with FITC label". From the images, it is clear that anti-human IgM did not bind to any of the columns. These results prove that there is no IgM bound to any of the columns (blank monolith, GM3 monolith and GM2 monolith) and these results are in full agreement with the ELISA data shown in fig 5.5.

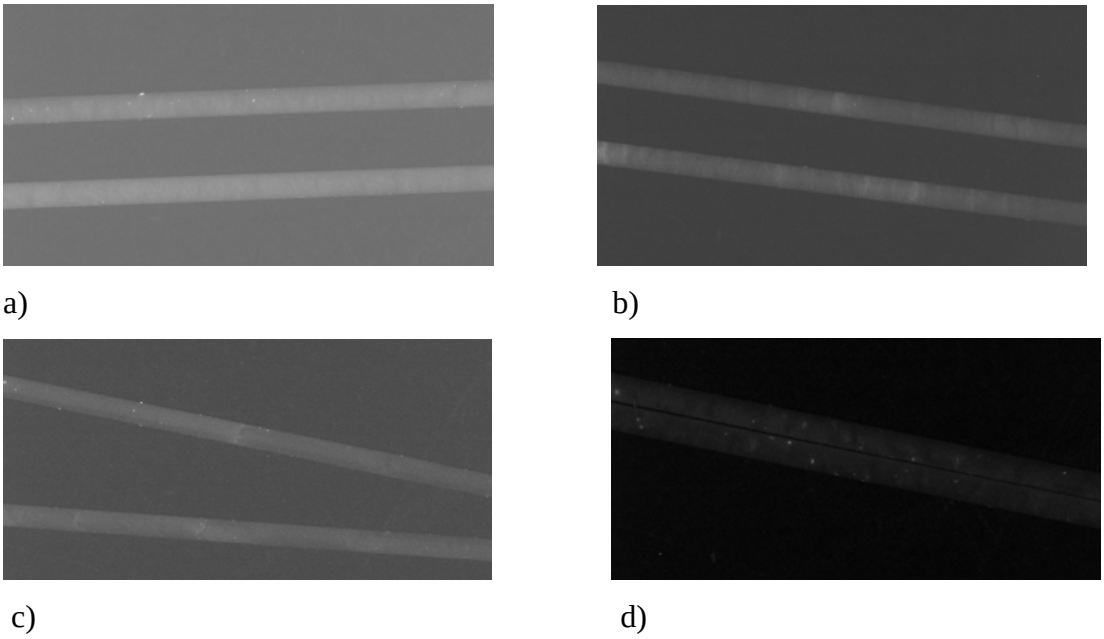

Figure 5.6 *Fluorescence microscope images of a) GM3 monolithic column-F325, b) Blank monolithic column-F325, c) GM2 monolithic column-G119 and d) GM2 monolithic column-NC030 treated with anti-human IgM with FITC label (see Appendix for colour pictures).*

The overall results obtained indicate the efficiency and selectivity of GM1 and GM2 monolithic columns to capture IgM antibodies from serum samples containing high titers of antibodies against gangliosides.

5.4 Conclusions

Ganglioside monolithic capillary columns can be prepared using a single step *in situ* polymerization protocol described in chapter 4. GM1 and GM2 columns effectively depleted IgM antibodies, but not IgG antibodies. GM2 molecules are evenly distributed across the column and cannot deplete antibodies against other gangliosides nor any from a healthy person. GM2 columns showed a high capacity to deplete IgM antibodies (>90 to 94% of high titers of antibodies are depleted after an incubation time of < 2 min). IgM antibodies did not show any aspecific binding towards the monolithic matrix (blank monolith) and GM3

monolithic columns. Overall, these columns can be used to deplete IgM antibodies from serum samples of patients suffering from Guillain-Barré syndrome (GBS).
Future research should focus on:

1. Focus on the approaches mentioned to deplete IgG antibodies from serum samples
2. Develop an elution protocol that can elute the bound antibodies, so that the re-generated monolithic column can be used again.
3. Validate the use of these ganglioside monolithic columns as a diagnostic tool

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6

A three-phase microfluidic chip for rapid sample clean-up of alkaloids from plant extracts

Abstract

A three-phase microchip was developed for the rapid and efficient small-scale purification of alkaloids from plant extracts. As part of the development of such a three-phase microchip, first a two-phase microchip with two channels (3.2 cm and 9.3 cm) was used to study the extraction efficiency of strychnine at various flow rates. Strychnine was extracted from a basic aqueous phase to a chloroform phase (extraction) or strychnine was extracted from a chloroform phase into an acidic aqueous phase (back extraction). Subsequently, the "simultaneous extraction and back extraction" of strychnine was carried out in a three-phase microchip. The experimental extraction rate and yield were compared with model data. In general, a good correlation was found between experimental results and model data for both two- and three-phase extractions. Finally, the three-phase microchip was employed in the purification of alkaloids (strychnine and brucine) from Strychnos seed extracts.

Tetala, K. K. R., Swarts, J., Chen, B., Janssen, A. M., van Beek, T. A. *Manuscript submitted to "Lab on a Chip"*

6.1 Introduction

Sample clean-up (pre-treatment) is employed to purify analytes of interest from complex fluids (blood, urine and plant extracts), before they are analyzed by high-resolution chromatographic techniques like HPLC, GC or CE. Various techniques such as filtration, centrifugation, liquid-liquid extraction (LLE), solid phase extraction (SPE), dialysis, precipitation and cell lysis fall in this category. Classical LLE is one of the most used sample pretreatment methods to purify a wide range of compounds. However, the disadvantage of classical LLE is the formation of emulsions during purification, automation difficulties, use of large amounts of organic solvents (costs, safety risks, health and environmental considerations) and sample, introduction of impurities, sometimes dilution and amount of time for performing LLE. The challenge to analytical chemists is thus to develop an integrated miniaturized system, which can be hyphenated on-chip or off-chip with extraction and/or separation of analytes of interest (capillary electrophoresis (CE) or HPLC) and/or detection steps (UV or mass spectrometry (MS)) (Lichtenberg et al., 2002; De Mello and Beard, 2003; Hyotylainen, 2007).

In recent years, several groups reported various LLE microfluidic chips that can be subdivided into two types (Sato et al., 2000; Tokeshi et al., 2000; Tokeshi et al., 2000a; Hisamoto et al., 2001; Hisamoto et al., 2001a; Minagawa et al., 2001; Surmeian et al., 2001; Hibara et al., 2001; Maruyama et al., 2004; Takahashi et al., 2006; Smirnova et al., 2006; Smirnova et al., 2007; Aota et al., 2007; Znidarsic-Plazl and Plazl, 2007):

1. Miscible-miscible liquids
2. Miscible-immiscible liquids (two- or three-phase)

In these studies, researchers took advantage of low Reynolds numbers to achieve multi-phase laminar flows in microchannels. For immiscible liquids, formation of a stable liquid-liquid interface in microchip is difficult.

One of the first attempts to obtain a stable interface between two immiscible liquids in a solvent extraction microchip was reported by TeGrotenhuis et al. In their system, a porous polymeric membrane or a micro machined contractor plate was clamped between the two phases (TeGrotenhuis et al., 1999). Later the focus turned towards creating selective hydrophobic pathways using self-assembled monolayers (SAM) in a microchannel (Zhao et al., 2001; Zhao et al., 2002; Hibara et al., 2002) and solid guide structures (provide stable interface between immiscible liquids) formed during the chip fabrication process (Tokeshi et al., 2002). When there is a large difference in linear velocity or pressure between two liquids in a countercurrent flow, the guide structures are not effective enough and the fluid interface is disrupted (Kikutani et al., 2005). For chips coated with selective hydrophobic and

hydrophilic pathways, a difference in linear velocity or pressure of fluids does not seem to be a problem and still leads to a stable interface (Kikutani, 2005; Aota, 2007).

The research reported so far on two-phase microchip extraction systems focused on the extraction of a variety of samples. The first two-phase microchip was employed in the study of molecular transport of a nickel-complex from an aqueous phase to a chloroform phase using an on-chip thermal lens microscope (TLM) detection method (Sato et al., 2000). In the same year, Tokeshi et al. reported rapid extraction of an iron-complex from an aqueous phase to a chloroform phase and monitored the extraction using TLM detection method. The fluid flow was controlled by syringe pumps. (Tokeshi et al., 2000). The above intriguing results led to the use of these two-phase microchips for extraction of other metal ion complexes (Tokeshi et al., 2000; Minagawa et al., 2001; Hisamoto et al., 2001; Hisamoto et al., 2001a), several pesticides from environmental samples (Smirnova et al., 2006; Smirnova et al., 2007) and organic compounds after reaction in a chip (Takahashi et al., 2006). This indicates that the two-phase extraction microchips can extract not only charged species but also neutral species.

Research on three-phase microchips is limited compared to two-phase microchips. Hibara et al. developed the first three-phase microchip, in which an interface between water/organic/water (organic phase = acetone, xylene or ethyl acetate) demonstrated the possibility of a stable multilayer flow for both miscible and immiscible liquids in microchips (Hibara et al., 2001). Tokeshi et al. developed a "Continuous-flow chemical processing (CFCP)" methodology in which the microunit operations (MUOs) (mixing, reaction and extraction) are integrated with a multilayer flow network on a single chip. In the MUOs for Co^{2+} wet analysis, 2-nitroso-1-naphthol (NN) was extracted from a NaOH aqueous phase to an organic phase (xylene) as a metal-chelate (Co^{2+}, Cu^{2+}, Ni^{2+} and Fe^{2+}). During the decomposition and removal step (HCl/xylene/NaOH), NN was back extracted to an NaOH aqueous phase and the coexisting (Cu^{2+}, Ni^{2+} and Fe^{2+}) metal chelates dissociated. This was the first time that a stable, multilayer flow of acidic aqueous/organic/basic aqueous interface and a back extraction step was reported in microchannels (Tokeshi et al., 2002).

The unique feature of a three-phase microchip is the "simultaneous extraction and back-extraction" - the molecules fed from an aqueous phase (feed phase) are extracted rapidly across the organic phase (transport phase) to the other aqueous phase (acceptor phase). Maruyama et al. reported an efficient and selective "simultaneous extraction and backward extraction" of yttrium ions (Y^{3+}) using a liquid membrane (water/n-heptane/water) in a three phase-microchip (Maruyama et al., 2004). Y^{3+} ions were selectively extracted in the presence of Zn^{2+} ions from the feed phase to n-heptane due to presence of extractant (PC-88A) in n-heptane phase and the pH (pH = 2; 0.1 M nitric acid and 0.1 M 4-aminobutyric acid) of the feed phase. The back extraction of Y^{3+} ions from n-heptane to acceptor phase (1.0 M nitric

acid) was influenced by varying the concentration of the acid, as a high concentration of acid is required to dissociate the metal-extractant complex.

Considering the potential of two- and three-phase extraction microchips mentioned above, it is surprising to note that not much work has been carried out to utilize these two technologies for various analytical applications (e.g. purify analytes from plant extracts and biomedical samples). The only report so far was by Znidarsic-Plazl et al. In their two-phase continuous extraction microchip system (33.2 cm long), steroids (progesterone and 11α-hydroxyprogesterone) were extracted efficiently from a water phase to an ethyl acetate phase at various high flows (10 to 100 μL/min of water flow and 21 to 210 μL/min ethyl acetate flow). The main drawback in this system was the instability and slug flow formation leading to disturbance of phase separation at the channel exit, when the flow rate of water was less than 4 μL/min (Znidarsic-Plazl and Plazl, 2007).

The aim of this research is to develop a three-phase (aqueous/organic/aqueous) microchip to purify analytes of interest from plant extracts at low flow rates. Here we report the design and testing of such a microchip and present a proof-of-principle using a three-phase microchip for the sample clean-up of alkaloids (weakly basic compounds) from plant extracts (Strychnos seeds).

6.2 Experimental

Materials and methods

Strychnine nitrate, strychnine and triethylamine (TEA) were purchased from Sigma, The Netherlands. Acetonitrile and methanol (HPLC grade) were purchased from Biosolve, The Netherlands. Gas tight syringes (500 μL), ferrules, nuts, Teflon tubing, Leur lock adapters and filters were purchased from Alltech, The Netherlands. Syringe pumps (Harvard 11 PicoPlus, dual syringe) were purchased from VWR international, The Netherlands. Chips were designed using Clewin290 software and ordered from Micronit, The Netherlands. Afterwards the channel for the organic phase in each chip was provided with a stable non-polar coating by the company Future Chemistry (Nijmegen, The Netherlands) using a proprietary process.

Off-line HPLC analysis was performed to analyze strychnine in collected samples. The system was comprised of an HPLC pump (Separation analytical instruments, The Netherlands) interfaced with an injector (10 μL loop, 7725, Rheodyne, USA) to which a C18 column (Alltima, 4.6 mm i.d, 250 mm length, Alltech, The Netherlands) was connected. The column outlet was interfaced with a UV detector (λ = 254 nm, Gilson 115, The Netherlands). The chromatograms were recorded on an integrator (C-R3A Chromatopac, Shimadzu, The Netherlands).

Eluent A: 600 mL of methanol was added to 400 mL of water containing 20 mM K_2HPO_4 and mixed well. To this solution 10 mL triethylamine (TEA) was added. The final pH of the solution was adjusted to 3.5 using phosphoric acid (H_3PO_4). This eluent was used only for the analysis of pure strychnine.

Eluent B: 100 mL of acetonitrile was added to 900 mL of water containing 20 mM K_2HPO_4 and mixed well. To this solution 10 mL triethylamine (TEA) was added. The final pH of the solution was adjusted to 3.5 using H_3PO_4. This eluent was used for the analysis of Strychnos seed extracts only.

Mass spectrometry measurements were carried out to screen extracts containing alkaloids using "Nano ESI-MS". The screening system (see fig 6.1) was comprised of three syringe pumps for the flow of basic aqueous phase, chloroform phase and acidic aqueous phase. The syringe pump with basic aqueous phase was connected to a micro injector (300 nL loop, 7725, Rheodyne, USA) followed by connection to the three-phase microchip. The capillary (100 µm i.d, 375 µm o.d, 35 cm) outlet of basic aqueous phase and chloroform phase were directed to waste and the capillary (100 µm i.d, 190 µm o.d, 35 cm) outlet of acidic aqueous phase was interfaced with the MS. The flow rate of the system was set at 1:2:1 µL/min for the basic aqueous / chloroform / acidic aqueous flow respectively.

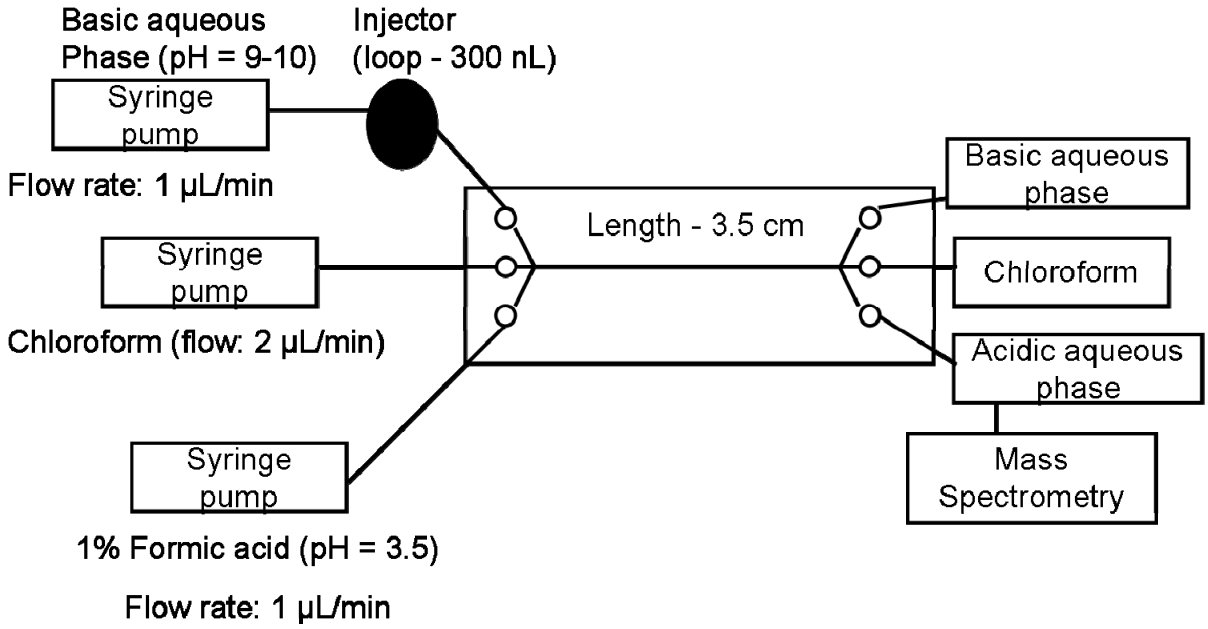


Figure 6.1 *Schematic design of on-line screening of plant extracts using three-phase chip interfaced with nano spray ESI-MS.*

Chip fabrication and design

Fig 6.2 depicts the schematic design of a two-phase, a two x two-phase and a three-phase extraction chip employed in this study. These chips were produced from borosilicate glass by photolithographic fabrication methods (Micronit, The Netherlands). The two-phase chip (fig 6.2a) had two inlets and two outlets. Chips with two effective lengths (3.2 cm and 9.3 cm, where the two liquids will be in contact with each other) were used. The two x two phase chip (fig 6.2b) had three inlets and three outlets with an effective contact length of 3.2 cm for each channel. The three-phase chip (fig 6.2c) was comprised of three inlets and three outlets with an effective contact length of 3.56 cm. Each channel diameter for all three chips was approximately 100 µm (width) x 40 µm (depth).

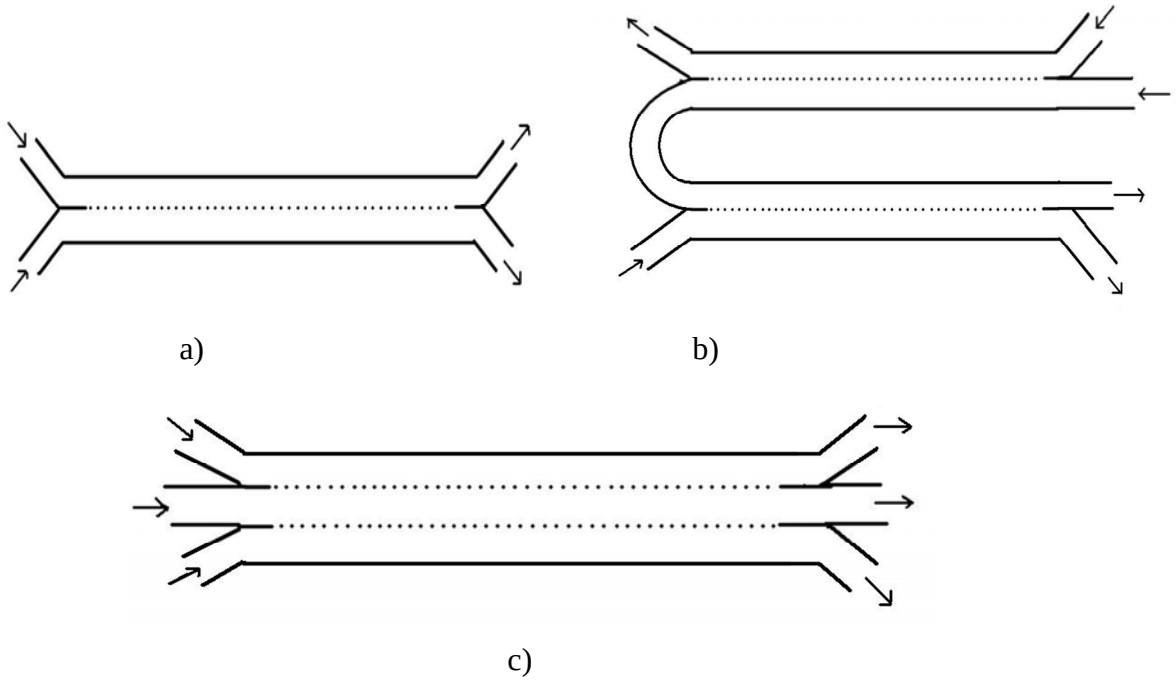


Figure 6.2 *Schematic representations of the two and three phase extraction chips with pillars (represented by dots above) located at equal distance: a) two phase chip with 3.2 cm effective length b) two x two phase chip with 2 x 3.2 cm effective length and c) three phase chip with 3.56 cm effective length. The arrow (→) indicates the direction of flow.*

Partition coefficient determination of strychnine

The partition coefficients (P) of strychnine for basic aqueous/organic and organic/acidic aqueous were determined using various concentrations of strychnine in base form ranging from 0.05 mM to 0.25 mM. A calibration graph was obtained by analyzing all samples with HPLC (eluent A).

Step 1: 5.0 mL of an acidic aqueous solution (1% formic acid) was added to a 5.0 mL of chloroform in a 20 mL vial and stirred overnight. After stirring, the two phases were

separated. 4.0 mL of chloroform phase was collected, dried over sodium sulfate, filtered and evaporated. To this vial, 4.0 mL of 1% formic acid aqueous solution was added. A 4.0 mL of acidic aqueous phase was also collected.

Step 2: 5.0 mL of basic aqueous solution was added to a 5.0 mL of chloroform in a 20 mL vial and stirred overnight. After stirring, the two phases were separated. 4.0 mL of chloroform phase was collected, dried over sodium sulfate, filtered and evaporated. To this vial, 4.0 mL of 1% formic acid aqueous solution was added. A 4.0 mL of basic aqueous phase was also collected.

After extraction, all samples were analyzed using HPLC (eluent A). The partition coefficient (P) of strychnine in chloroform/acidic aqueous and a chloroform/basic aqueous system were calculated using:

$$\mathbf{P_{aqueous\ /\ chloroform}} = \mathbf{A_{aqueous}} / \mathbf{A_{chloroform}} \qquad (6.1)$$

Where $\mathbf{A_{aqueous}}$ is the peak area of strychnine in either acidic or basic aqueous phase and $\mathbf{A_{chloroform}}$ is the peak area of strychnine in chloroform phase.

Evaluation of alkaloid extraction efficiency in two-phase and three-phase chip

Initial extraction experiments were performed using commercially available strychnine nitrate and strychnine as model compounds in two-phase chips (3.2 cm and 9.3 cm long) in two steps. The flows of basic aqueous phase/ acidic aqueous phase were half the flow of chloroform phase. Extraction efficiency was tested at various flow rates.

Step 1: A stream of pure chloroform was introduced in parallel to a stream of basic aqueous phase [1% formic acid:1% ammonia (1:2, v/v), pH = 9-10] containing 0.2 mM strychnine nitrate at various flow rates (aqueous flow : chloroform flow - 4:8 µL/min; 2:4 µL/min; 1:2 µL/min; 0.5:1 µL/min, and 0.25:0.5 µL/min respectively).

Step 2: A stream of 0.2 mM strychnine in chloroform was introduced in parallel to a stream of acidic aqueous phase (1% formic acid, pH = 3.5) in the chip at various flow rates (aqueous flow : chloroform flow - 4:8 µL/min; 2:4 µL/min; 1:2 µL/min; 0.5:1 µL/min, and 0.25:0.5 µL/min respectively).

The three-phase chip was tested using 0.2 mM strychnine nitrate in basic aqueous phase at various flow rates (aqueous flow : chloroform flow : aqueous flow - 4:8:4 µL/min; 2:4:2 µL/min; 1:2:1 µL/min; 0.5:1:0.5 µL/min, and 0.25:0.5:0.25 µL/min).

For this study, a volume of 80 µL of chloroform phase and 40 µL of basic/acidic aqueous phases was collected in Eppendorf tubes from the channel exit. The collected chloroform solution was evaporated and the extracted strychnine was redissolved in 80 µL of 1% formic acid solution. The samples were analyzed with HPLC (eluent A).

Collected aqueous and chloroform phases were weighed and divided by the collection time. Due to post-chip evaporation of chloroform, the exact chloroform flow rates were determined by deducting the water flow rate value from the total set flow rate (water + chloroform or water + chloroform + water) value.

Modeling studies

The two- and three-phase extraction was modeled using commercial CFD software COMSOL Multiphysics 3.3 Comsol, Inc., Burlington, MA, USA). The complex three dimensional structure was simplified to a two dimensional structure. Taking all curvatures in the channel bottom (due to isotropic etching) and pillars into account, an average diffusion distance with a constant depth of 40 µm was calculated. The total two-or three-phase channel diameter is fixed, but the position of the fluid interfaces was determined by visual inspection of photographs and averaging the position. These estimations resulted in a two dimensional model with channel widths of 96.5-96.5 µm (for two-phase extraction) and 95.5-112-95.5 µm (for three-phase extraction).

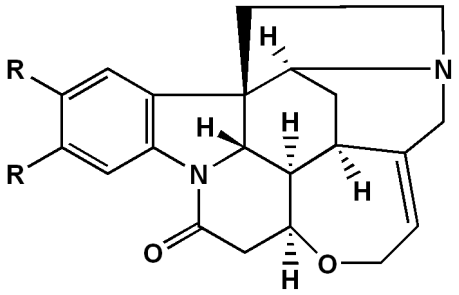
In the models, the pillars and the moving fluid interface in between were replaced by a zero velocity interface and a diffusive component flux reduced by the portion of the total interface blocked by the pillars area (approximately 21%). These model simplifications did not influence the results significantly (results not shown). The flow rates and partition coefficients used in the model were determined experimentally (*vide supra*). Diffusion coefficients were calculated using the Wilke-Chang equation (Wilke & Chang, 1955).

Purification of alkaloids from Strychnos seeds using a three-phase microchip

Purification of the alkaloids strychnine and brucine (found abundantly in Strychnos seed extracts) was performed in a three-phase microchip (effective channel length of 3.56 cm) at a flow rate of 1:2:1 µL/min.

Preparation of Strychnos seed extract in basic aqueous phase: 0.5 g of Strychnos seeds was stirred in 16.6 mL of 1% formic acid solution for 20 min. This solution was then filtered to remove the undissolved seed particles. To this solution, 33.4 mL of 1% ammonia solution was added. The final pH of the solution was 9-10 and this solution was used for extraction studies. The structure of the two main alkaloids (strychnine and brucine) present in Strychnos seeds is depicted in fig 6.3.

The purification was carried out by continuously introducing Strychnos seed extract in basic aqueous phase (feed phase) in parallel to streams of pure chloroform (transport phase) and acid aqueous phase (acceptor phase). All three phases were analyzed with HPLC (eluent B).

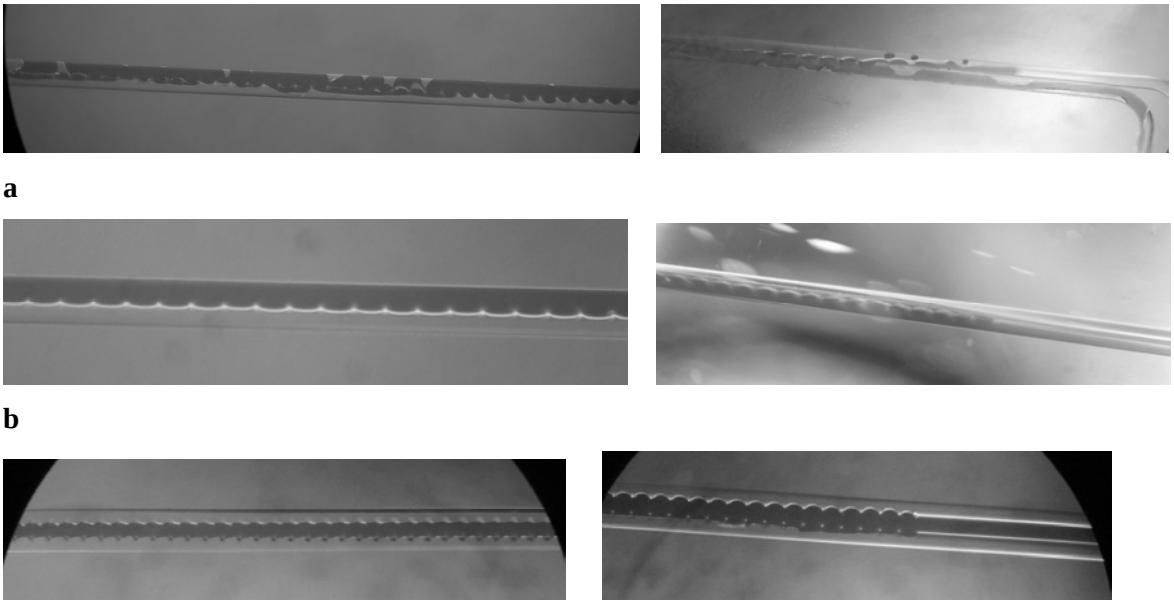


R = H (strychnine) or OCH_3 (brucine)

Figure 6.3 *Structures of alkaloids of interest in Strychnos seeds (strychnine and brucine).*

6.3 Results and Discussion

Phase separation of fluid in uncoated and coated two phase chips



a

b

c

Figure 6.4 *Streams of water and chloroform (Sudan dye) for a) an uncoated two-phase chip, b) a selectively non-polar coated two-phase chip, and c) a selectively non-polar coated three-phase chip. In picture "a" and "b", the flow rate of the system are 4:8 µL/min and in picture "c" the flow rate of the system are1:2:1 µL/min (see Appendix for colour pictures).*

The fluid flow in microchannels is laminar due to the low Reynolds number, indicating that multiple streams of liquid in contact can be obtained without turbulent mixing. A stream of pure chloroform was continuously infused in parallel to a stream of water in the two-phase

chip at a flow rate of 8:8 µL/min and monitored under a microscope (fig 6.4a). To distinguish between the two phases, chloroform phase was coloured with Sudan dye. The images obtained indicate that a good phase separation for liquids with different viscosities is difficult due to constant interference of both phases along the channel and at the channel exit (see fig 6.4a). Even the decrease of water phase flow rate to half that of the chloroform phase (4:8 µL/min) or lowering the flow rates further (0.25:0.5 µL/min) did not result in a good phase separation.

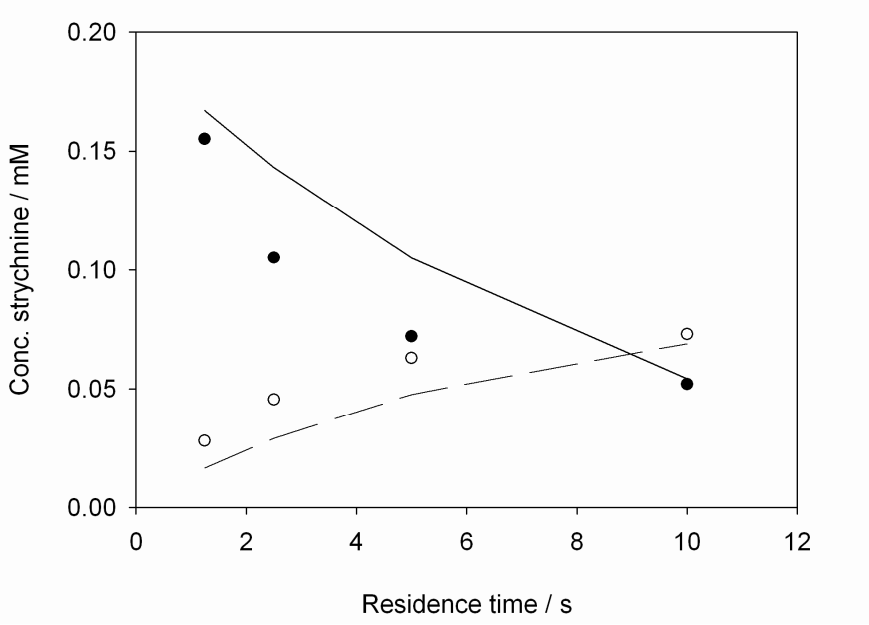
The company "Future Chemistry" (Nijmegen, The Netherlands) provided a selective, stable non-polar coating for a single channel of the microchip. The organic phase was introduced in the coated channel and water in the uncoated channel of the chip at a flow rate of 4:8 µL/min. A good phase separation with stable flows for liquids with different dynamic viscosities (water: 1×10^{-3} Pa·s, chloroform: 0.54×10^{-3} Pa·s) was obtained in two-and three-phase microchips (see fig 6.4b & 6.4c). However, a good phase separation was not achieved with the two x two-phase microchip (see fig 6.2b for design), due to constant disturbance of the two phases at channel outlets and sometimes across the channel as well. This is most likely due to pressure differences at the channel outlets or poor coating. Therefore, the two x two-phase microchip was not used in further experiments.

Partition coefficient

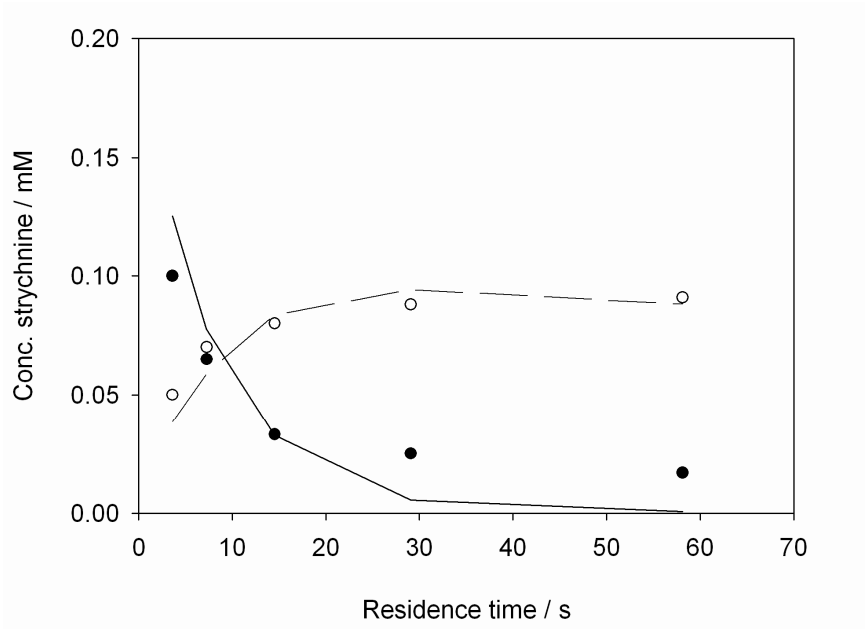
The transport of a molecule from one phase to another phase is not only by passive diffusion but also depends on the partition coefficient (P) of the molecule in transport between the two phases i.e. depends on the affinity of the molecule for the two solvents. The partition coefficient (P) of strychnine was experimentally determined as $P_{\text{basic aqueous/chloroform}}$ is 1 : 133 (±11.5 for 95% certainty) and $P_{\text{chloroform/acidic aqueous}}$ is 1 : 20.6 (±0.72 for 95% certainty) and used in the modeling studies to compare experimental results with modeling data.

Extraction studies of strychnine in two-phase microchip

Before studying the extraction efficiency of strychnine in a three-phase microchip, a two-phase microchip was used to study the extraction efficiency of strychnine in two separate steps. The first step involved the extraction of strychnine from basic aqueous phase to chloroform phase and the second step involved the back extraction of strychnine from chloroform phase to acidic aqueous phase. Two-phase microchips with 3.2 cm and 9.3 cm effective channel lengths were used to study the influence of the channel length (i.e. longer contact time of the two liquids) at various flow rates. The experimental results obtained were then compared with the model data and are depicted in fig 6.5a, b and 6.6a, b.



a)



b)

Figure 6.5 *Extraction efficiency of strychnine from basic aqueous phase to chloroform (3.2 cm (a) and 9.3 cm (b) effective channel length) in two-phase microchip. Experimental data (● - basic aqueous phase experimental; ○ - chloroform experimental) and model data (-- is chloroform model data; – is basic aqueous phase model data).*

Extraction of strychnine: In the experimental extraction results of strychnine from basic aqueous phase to chloroform phase, it is observed that extraction of strychnine to the chloroform phase increased with the increase of contact time between the two liquids. These experimental results were plotted against the predicted model data (see fig 6.5a & 6.5 b). The

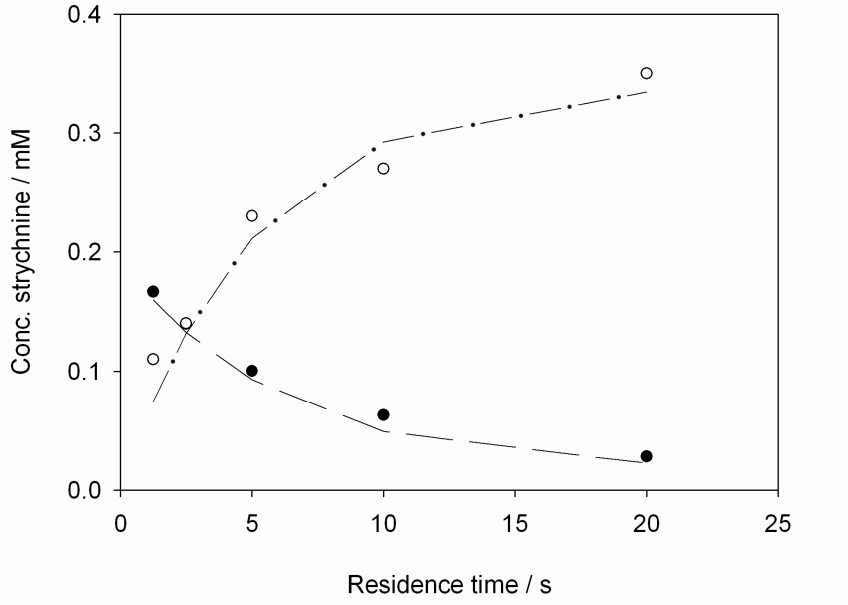
model data predicts the extraction trend very well. The experimental results and model data in fig 6.5a are not in full agreement, except for the data point at a residence time of 10 sec. In fig 6.5b, the model predicts near to complete extraction of strychnine with a residence time of 60 sec, whereas the experimental data indicate that extraction is slow. These overall deviations in the results maybe due to some minor fluctuation between the two phases at the channel exit (although a stable phase separation was present across the channel) leading to formation of tiny droplets of chloroform in the aqueous phase (not visible to the naked eye) in the collecting Eppendorf tubes. To check, whether this assumption was correct, a Sudan dye test was carried out. These tiny droplets will have big impact because of the high strychnine concentration in the chloroform phase. A stream of chloroform phase coloured with Sudan dye was introduced in parallel to a stream of basic aqueous phase. The collecting solutions were monitored during 30 min. After 30 min, small tiny droplets of chloroform coloured with Sudan dye were observed in the basic aqueous phase. This result could explain the deviation of experimental data with model data for forward extraction studies.

Back extraction study: As seen from both fig 6.6a & fig 6.6b, the experimental extraction of strychnine to the acidic aqueous phase increased with longer residence times. The extraction of strychnine from the chloroform phase to the acidic aqueous phase reached its maximum at a contact time of 60 sec. The experimental extraction results of strychnine from chloroform phase to the acidic aqueous phase correlate quite nicely with the model data, except for some minor deviation for the acidic aqueous phase in fig 6.6a.

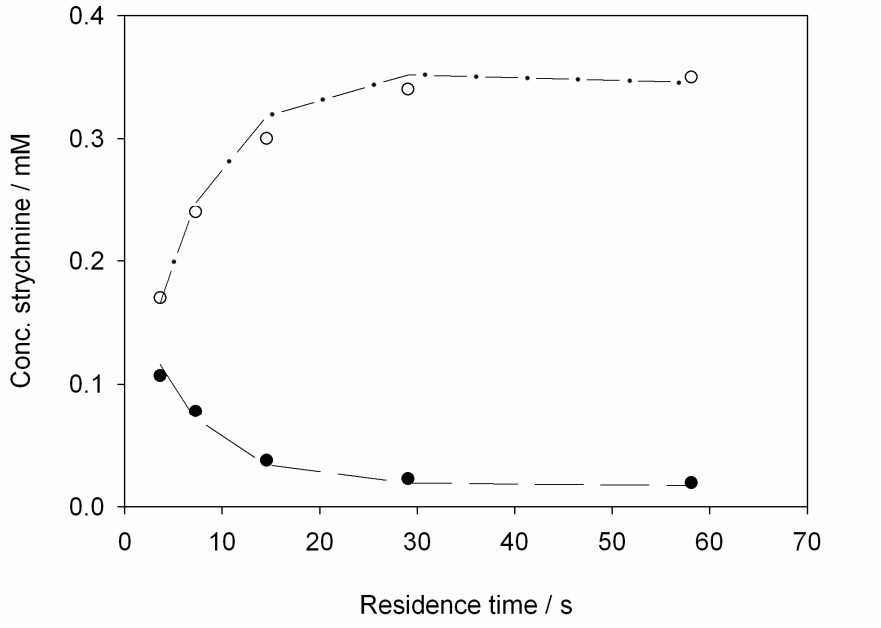
Extraction studies of strychnine in three-phase microchip

The extraction efficiency of strychnine from the feed phase (basic aqueous phase) across the transport phase (chloroform phase) to the acceptor phase (acidic aqueous phase) was studied in the three-phase microchip. The experimental results obtained were compared to the model data, which is depicted in fig 6.7a. A representation of numerical model results predicting the extraction of strychnine in the three-phase microchip at a flow rate of 0.5:1:0.5 µL/min is shown in fig 6.7b.

From fig 6.7a, it is clear that the overall correlation between experimental results and model data is fairly good, except for the experimental data at a residence time of 12.4 seconds (0.5:1:0.5 µL/min flow rate) which seem to be a bit out of range from the model prediction. By taking a closer look at the feed phase data, it appears that experimental variation could be a reason for this deviation, as the extraction of strychnine should follow a smooth line in time. The overall outcome of this study indicates that other experimental set-ups (e.g. a change of solvent or flow rates) can be tested with this model to predict the extraction efficiency of the three-phase microchip, as long as the flow-profile of the microchip remains the same.



a)



b)

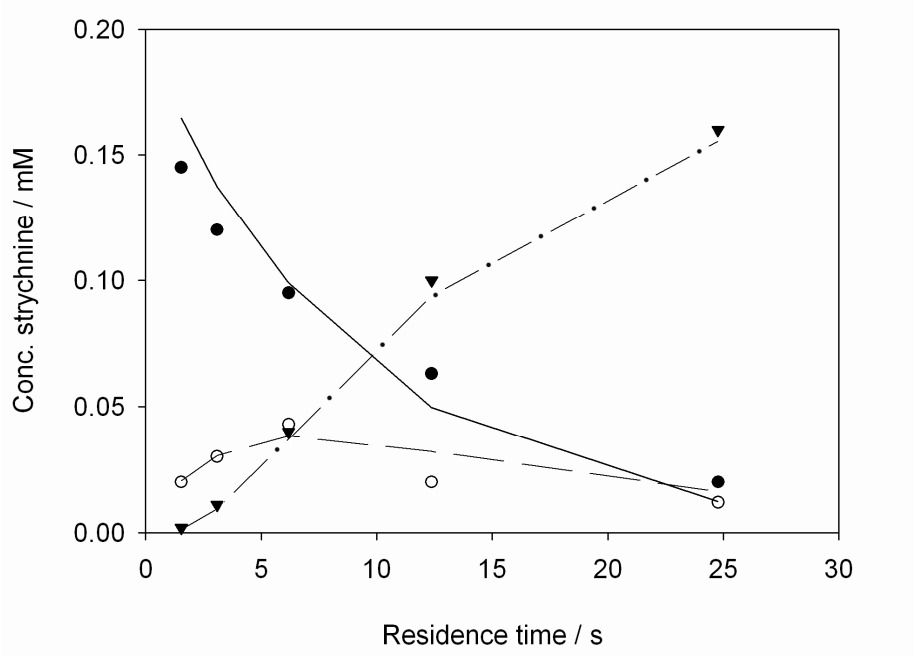
Figure 6.6 *Extraction efficiency of strychnine from chloroform phase to acidic aqueous phase (3.2 cm (a) and 9.3 cm (b) effective channel length) in two-phase microchip. Experimental data (○ - acidic aqueous phase experimental; ● - chloroform experimental) and model data (- - is chloroform model; -•- is acidic aqueous phase model).*

Purification of strychnine and brucine from Strychnos seed extracts using the three-phase microchip

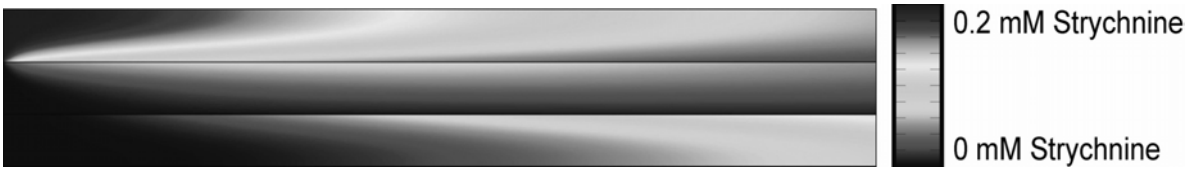
To demonstrate the efficiency of the developed three-phase microchip for purification of plant extracts, the Strychnos seed extract (containing the alkaloids of interest strychnine and brucine) was chosen. The chromatogram of Strychnos seed extract in basic aqueous phase (pH

9-10; feed phase) is depicted in fig 6.8a. The chromatogram recorded showed some polar compounds at 10.6 min. A good separation of strychnine (28.8 min) and brucine (48.0 min) was obtained using eluent B.

The extraction is carried out with flow rates set at 1:2:1 µL/min (feed phase: transport phase: acceptor phase). The out coming solutions were collected and analyzed with HPLC. The results obtained are depicted in fig 6.8b to fig 6.8d indicate that the purification of Strychnos seed extracts was successful, as pure strychnine and brucine were extracted to the acceptor phase (fig 6.8d) and transport phase (fig 6.8c) leaving polar compounds in the feed phase (fig 6.8b). Considering the results obtained in fig 6.7a, maximum extraction of strychnine and brucine can be achieved at low flow rates i.e. longer residence times.



a)



b)

Figure 6.7a) *Extraction of strychnine using three phase microchip (3.56 cm length) from basic phase through chloroform to acidic phase at different residence times.* b) *A three-phase extraction model (0.5:1:0.5 µL/min flow rate, 12.5 sec residence time), transition of red to blue colour or vice versa indicates strychnine concentration in feed, transport or acceptor phases. Experimental data (● - basic aq. phase exp.; ○ - chloroform exp.; ▼- acidic aq. exp.) and model data (– is basic aq. phase model data; -- is chloroform model data; -•- is acidic aq. phase model data) (see Appendix for colour pictures).*

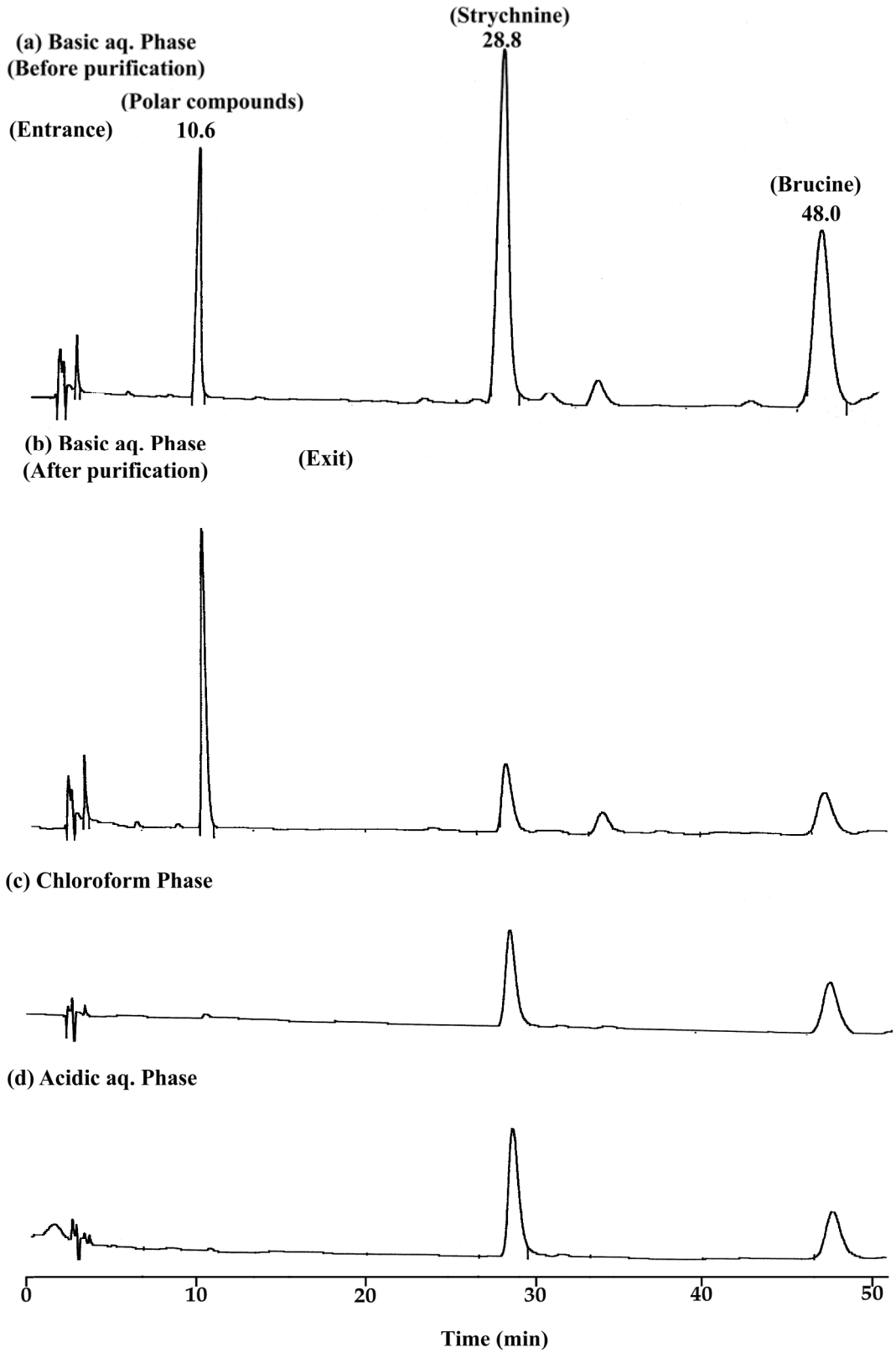


Figure 6.8 *Chromatogram of a Strychnos seed extract before and after purification using a three-phase microchip (3.56 cm length) at a flow rate of 1:2:1 µL/min: a) basic aqueous phase (feed phase) before purification, b) basic aqueous phase (feed phase) after purification, c) chloroform phase (transport phase) and d) acid aqueous phase (acceptor phase).*

Modeling to predict extraction efficiency of three-phase microchip

The modeling was successful in predicting strychnine extraction in two- and three-phase microchips. For the optimisation of the chip design and experimental parameters, it is interesting to calculate the extraction efficiency of strychnine in three-phase microchips using the developed numerical model by changing a few parameters like e.g. channel thickness (narrow organic phase compartment), viscosity of organic solvent equal to aqueous phase, organic solvents with different polarity and chemical characteristics (change of partition coefficient), and channel length. The results obtained will provide more insight into the organic solvents to be chosen and future chip design, when one considers using this three-phase microchip for sample clean-up of various biological samples. The channel width (95.5-112-95.5 µm) of the three-phase microchip was kept the same as in previous measurements except for the narrow organic channel model. Fig 6.9 depicts the predicted model data of strychnine extraction to the acceptor phase with varying different parameters in comparison with the standard case. More detailed model data for each individual parameter change is provided in appendix (see page 111-113).

Channel thickness: The width of the chloroform phase (transport phase) compartment was reduced from 112 µm to 56 µm keeping the depth of the channel as 40 µm. The channel dimensions of the three-phase microchip were set at 95.5-50-95.5 µm (total width of 241 µm). The flow rate of the system was fixed as 1:2:1. At a residence time of 25 sec, 0.162 mM of strychnine was extracted to the acidic aqueous phase, which is 0.003 mM higher than that of larger film thickness chip (0.159 mM; fig 6.7a). This result indicates that an increase of 1.5% strychnine extraction can be obtained if the transport phase compartment is reduced.

Organic solvent: The organic solvent plays a major role in multilayer flow chips, which influences the extraction efficiency of strychnine. We studied the effect of changing the viscosity and using another organic solvent.

a) In this case, we assumed the use of an organic solvent with the viscosity of water and the partition coefficient of chloroform. The flow rates in the 3 channels were then assumed to be equal (1:1:1) in all the three channels. A close observation of Fig. 8, shows that at a residence time of 25 sec (i.e. lower flow rate) 79% of strychnine was extracted to the acidic aqueous phase, which is 0.5% less compared to the value obtained when pure chloroform is used as organic phase (79.5% of strychnine at residence time of 25 sec).
b) Other organic solvents: Chloroform is a very special solvent for alkaloids, but considering its toxicity it is good to search for alternative organic solvents which are more environmentally friendly like e.g. ethyl acetate. With the change of organic solvent, both partition coefficients (P) will change (Znidarsic-Plazl and Plazl, 2007).

We assume that the "P" of water/organic solvent is half that of the "P" of water/chloroform i.e. $P_{\text{base aqueous/org.solvent}}$ is 1 : 66.7; $P_{\text{org. solvent/acid aqueous}}$ is 1 : 41.2. The flow rate of the system is then assumed to be 1:2.1:1. At a residence time of 25 sec, 0.170 mM (85%) strychnine was extracted to the acidic aqueous phase, which is 0.011 mM higher (5.5% extra) compared with that of using chloroform as organic phase (79.5% of strychnine, at residence time of 25 sec).

Figure 6.9 compares the transport of strychnine into the acceptor phase (acidic aqueous phase) at various residence times by changing the parameters (film thickness and viscosity) relative to the standard case. At longer residence times (25 sec) the extraction of strychnine decreases in the following order:

change of partition coefficient > narrow chloroform phase compartment > standard case ≈ change of viscosity.

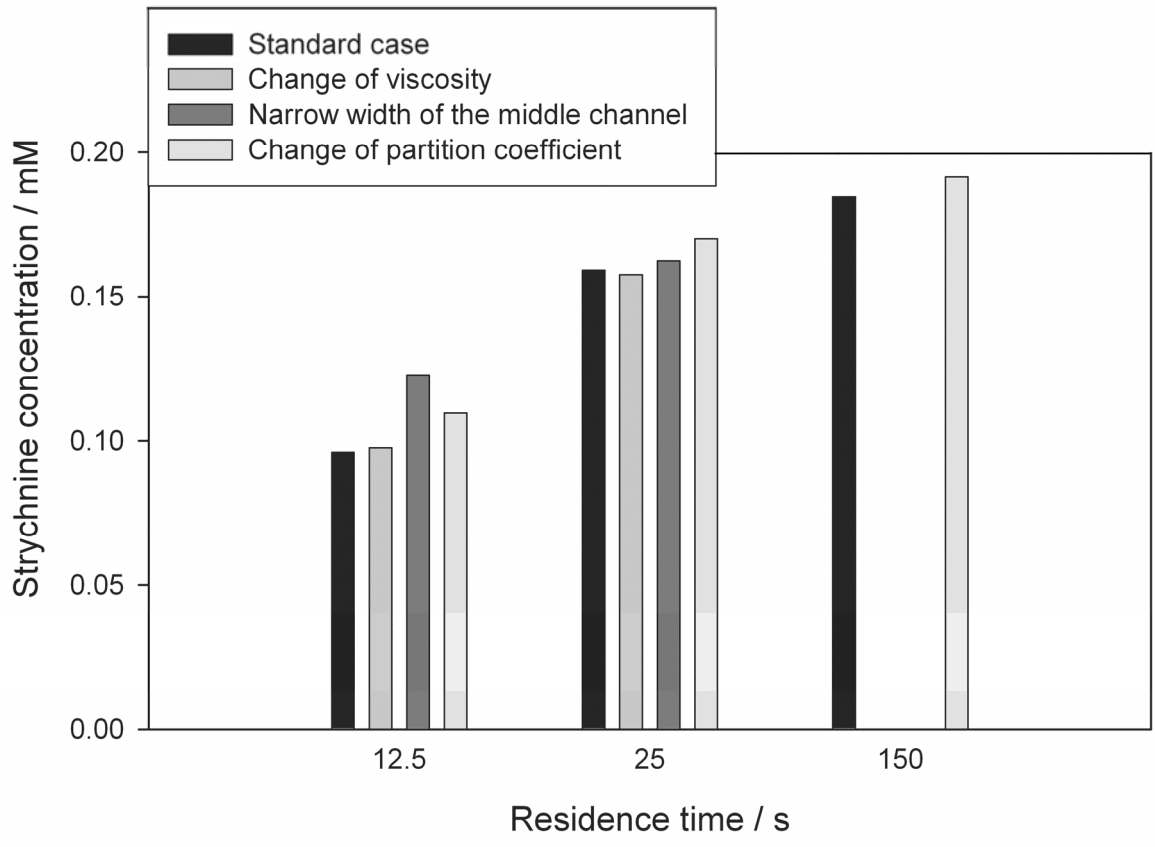


Fig. 6.9 *Comparison of model data obtained by changing various parameters (channel thickness, viscosity, partition coefficient, and channel length) with standard case.*

Channel length: The model was also used to study the extraction efficiency of strychnine by increasing channel length from 3.56 cm to 9.3 cm. Two cases were studied by fixing the standard channel width of 95.5-112-95.5 µm (total channel width was 303 µm).

a) Chloroform as organic solvent
b) Change of organic solvent e.g. ethyl acetate

In case of chloroform as organic solvent, 0.185 mM strychnine was extracted at a residence time of 150 sec (flow of 0.1:0.21:0.1 µL/min). When the organic solvent was changed from chloroform to e.g. ethyl acetate, 0.19 mM strychnine was extracted at a residence time of 150 sec (flow of 0.1:0.21:0.1 µL/min). It can be noted that a maximum of 95 % strychnine can be extracted to the acidic aqueous phase at longer residence times if e.g. ethyl acetate is used as organic solvent. The same 95 % of strychnine extraction can be achieved with a channel length of 3.56 cm, but require flow rates lower than 0.1:0.21:0.1µL/min, which is not feasible with the current syringe pumps. From this it is clear, it is not the channel length that influences the extraction but a high $P_{org.\ solvent/acidic\ aqueous}$ 1 : 41.2 and the flow rates of the three phases, which allow the liquids to be in contact for longer time.

On-line screening of plant extracts with nano ESI-MS

Now that the three-phase microchip is capable of purifying alkaloids from plant extracts, our future research is to focus on the development of an integrated miniaturized system. Approaches towards the integration of this sample clean-up chip are:

1. Screening the extraction of plant extracts using on-line detection (UV or MS)
2. Separation of analytes either on-chip (CE) or off-chip (LC)

The initial step towards the integration of this chip was carried out by on-line screening of plant extracts using nano ESI-MS. The system set-up for on-line screening is as shown in fig 6.1.

Prior to the screening study of plant extracts using the three-phase microchip, a screening of pure strychnine was carried out in a two-phase microchip. ESI-MS of pure strychnine in 1% formic acid was performed. The fused silica capillary of the ESI-MS spray was directly connected to the outlet of the acidic aqueous phase of the microchip avoiding connections to prevent possible back pressure in the system that may disturb the phase separation across the channel. The length of the capillaries for the other phases was exactly the same as the capillary interfaced with MS. Although some minor fluctuation of the two phases (acidic aqueous phase interfering with chloroform phase) was observed at the channel exit due to a pressure difference between the two channels, the on-line screening of pure strychnine was

successful in a two-phase microchip. During these measurements, the flow of acidic aqueous phase was 1 µL/min and the chloroform phase was 2 µL/min. The mass spectra recorded during these measurements are depicted in fig 6.10.

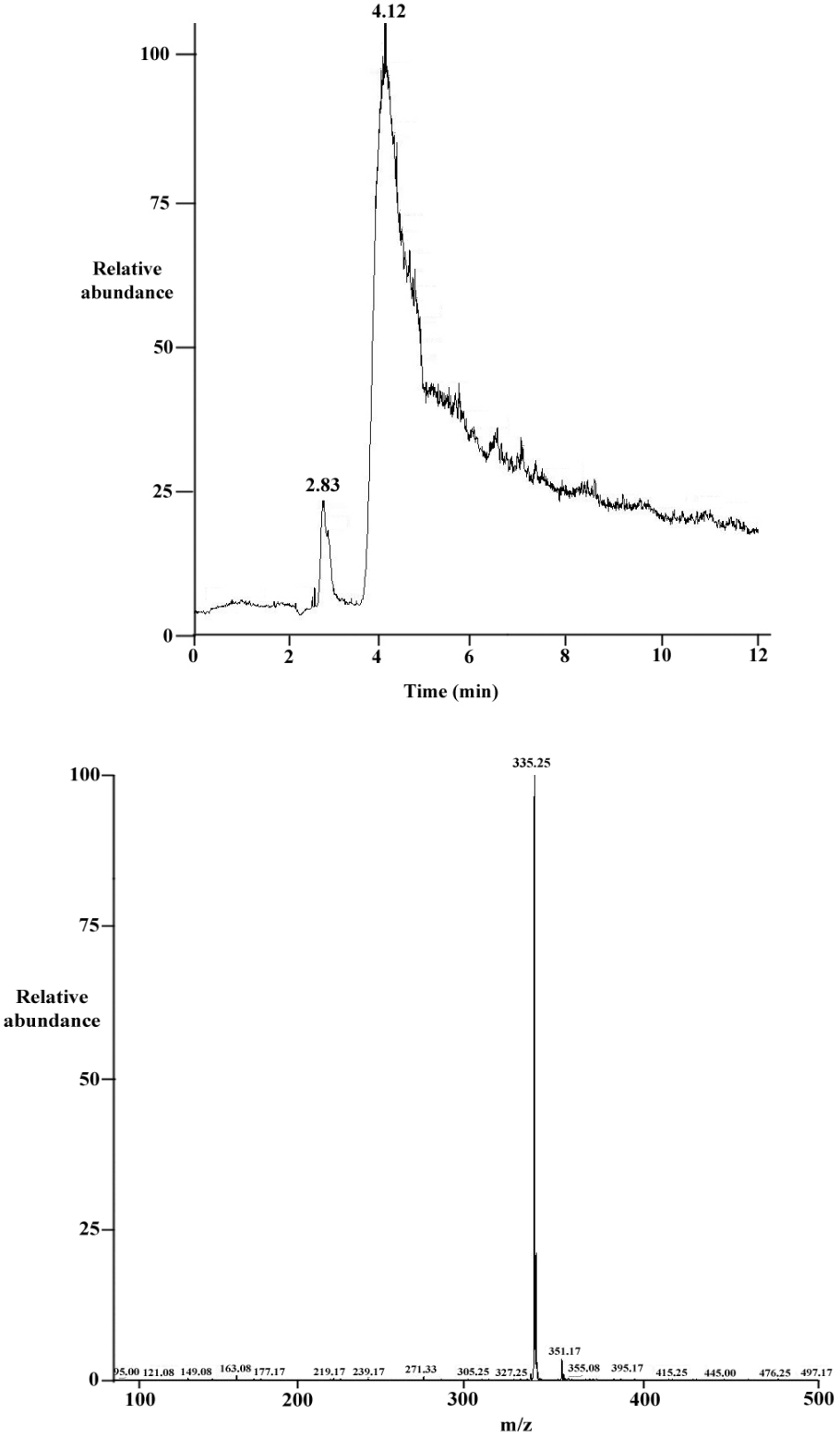


Figure 6.10 *Mass spectra recorded during on-line screening of pure strychnine extraction from chloroform phase to acid aqueous phase in a two-phase microchip.*

An attempt to perform on-line screening of strychnine using a three-phase microchip was carried out as follows: to a stream of feed phase and acceptor phase (flow of 1 µL/min each), a stream of chloroform was introduced in parallel at a flow of 2 µL/min. In the first 5 min, a stable phase separation was achieved, but with passage of time the acceptor phase started coming out with the transport phase and finally led to the disturbance of the phase separation across the channel in the microchip due to the pressure difference in the three channels.

Although, the initial measurements to use the three-phase microchip for on-line screening were not successful, the results obtained with two-phase microchip (see fig 6.11) suggest that further fine tuning of the three-phase microchip connections with MS is required to obtain an equal back pressure for all the three channels.

6.4 Conclusions

In this chapter, an efficient three-phase microchip for sample clean-up with the unique feature of "simultaneous extraction and back extraction" of alkaloids from plant extracts was developed. Selective, non-polar coated two-phase and three-phase microchips with a good phase separation of immiscible liquids (water and chloroform) showed maximum extraction of strychnine at longer residence times i.e. lower flow rates. The obtained experimental results nicely fitted with the CFD model data. Sample clean-up of a Strychnos seed extract was successful with the developed three-phase microchip. The developed model can be used to optimize various parameters (e.g. chip design, viscosity of organic solvent, partition coefficient values, flow rates and channel length) to determine the optimal extraction experimental conditions. On-line screening of strychnine with nanospray ESI-MS using two-phase microchip was successful. Further research should be carried out on:

1. Investigation of organic solvents (e.g. ethyl acetate) to validate the obtained model results
2. On-chip neutralization of crude alkaloid extracts in acidic aqueous phase with excess basic aqueous phase before extraction step (see chapter 1, fig 1.3)
3. Integrate the three-phase microchip with either MS or HPLC for rapid on-line screening of plant extracts.

Considering the overall performance of this microchip in terms of obtaining a stable phase separation between immiscible liquids with varying viscosities, ruggedness, its extraction efficiency and the purification of alkaloids from plant extracts, this three-phase microchip in future can be employed in the sample clean-up of various acidic or basic molecules in aqueous solutions, other alkaloids, basic or acidic drugs in biological fluids such as plasma, urine, and milk.

6.5 References

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7

Conclusions and future research

The research presented in this thesis (see chapter 1) aims towards the development of a microfluidic device that can be used for selective sample clean-up prior to a detection step (e.g. for diagnostics or chemical analysis) and enrichment of valuable constituents through affinity chromatography. Both solid phase extraction and liquid-liquid extraction have been investigated.

1. Solid phase extraction (SPE) microfluidic chip

The aim was to develop an affinity microfluidic chip that can selectively adsorb and screen analytes of interest from a process stream (plant extract and serum samples).

Achieved

First, a carbohydrate immobilization protocol for glass surfaces was developed to prepare carbohydrate microarrays to study specific carbohydrate-lectin interactions. It was found that due to a low column capacity the carbohydrate coated capillary was not effective in capturing lectins. To improve the column capacity, monoliths, a new generation of stationary phases, were chosen for carbohydrate immobilization. Two protocols were developed (a three-step protocol and a single step protocol). The advantage of the single step protocol is the lower amount of time needed to prepare an affinity monolithic column. The carbohydrate monolithic columns efficiently captured lectins (α-mannose column captured Concanavalin A and *Lens culinaris,* β-galactose column captured *Arachis hypogaea*), and antibodies (GM1 and GM2 columns specifically captured IgM antibodies) from serum samples of patients suffering from Guillain-Barré syndrome (GBS). These columns can also be used to study the dissociation constants (K_d) of carbohydrate-lectin interactions. The carbohydrate monolith prepared in a microchip gave identical results as the carbohydrate monolith in a capillary. The initial attempt to prepare a carbohydrate monolithic array microfluidic chip was successful. However, the fluid flow in the two channels varied due to the difference in permeability of the two affinity columns. Further fine tuning of the permeability of the two columns is necessary to use this array microfluidic chip for screening of analytes.

2. Liquid-liquid extraction (LLE) microfluidic chip

The aim was to develop a three-phase microfluidic chip for efficient purification and screening of acidic or basic molecules in aqueous solution for e.g. alkaloids, weakly basic compounds found abundantly in plants.

Achieved

The extraction of strychnine was studied using a two-phase microchip, followed by "simultaneous extraction and back extraction" of strychnine using a three-phase microchip. Maximum extraction of strychnine was achieved at longer residence times i.e. lower flow rates. A good correlation between experimental results and model data was found for both two-phase and three-phase microchips. Sample clean-up of a Strychnos seed extract was successful with the three-phase microchip. The developed model can be used to predict the extraction outcome by changing various parameters e.g. chip design, partition coefficient, and viscosity. On-line screening of strychnine was demonstrated by interfacing the two-phase microchip with nanospray ESI-MS. The initial interfacing of a three-phase microchip with nanospray ESI-MS led to the disturbance of the phase separation in the chip due to pressure

differences in the three channels. Fine tuning of the interface connections between microchip and MS could enhance the chance of using this three-phase microchip for on-line screening of plant extracts.

The next steps to carry out this research are:

1. To develop an affinity monolithic array microfluidic chip for screening of analytes of interest from plant extracts and serum samples using nanospray ESI-MS
2. Either on-chip separation (CE) or interface with MS or HPLC to screen analytes of interest from plant extracts

Outlook

The aim to develop an "integrated microfluidic chip" is not accomplished in this thesis. However looking into the results obtained from chapter 2 to 6, a few important points can be noted from these findings for future research:

1. Microarray - the developed protocol has various advantages and in future could be used as a biosensor chip to screen various ligand-analyte interactions from biological samples using DESI-MS (Desorption Electro-Spray Ionization Mass Spectrometer)
2. Wall coated columns - not efficient to capture analytes from a process stream
3. Affinity monolithic columns - the two protocols developed (three-step and single-step) to prepare affinity columns showed their efficiency to capture analytes selectively from a process stream. These monolithic materials may further be used for immobilization of various ligands (e.g. proteins, enzymes, antibodies) to screen ligand-analyte interactions, separation of enantiomers etc.
4. Three-phase microchip - an efficient and rapid sample clean-up of alkaloids, weakly basic compounds, from plant extracts indicates that in future this microchip can be used for efficient sample clean-up of basic or acidic molecules from a stream of aqueous solution
5. Two- and three-phase microchips - could also be used for sample clean-up of various dirty samples, milk, blood, urine, etc.
6. Model - the developed numerical CFD model has the potential to overview and optimize the chip design and experimental conditions to improve the extraction efficiency of analytes

Though there are quite a few hurdles to realize the dream of accomplishing a true µTAS, by taking inspiration from the famous quote of great scientist Dr. Albert Einstein, "*I think that only daring speculation can lead us further and not accumulation of facts*" I strongly believe the day for scientific community to realize µTAS is not too far.

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Summary

In recent years, the concept of “lab on a chip” (microfluidic devices) gained momentum due to its diverse advantages and interlinking of various branches of life sciences. The main component of a microfluidic device is the chip, which can be made of various materials like glass, silicon, or polymers. Its numerous channels of varying diameters are made in µm sizes and can handle amounts of liquids as low as 10^{-18} litres. In the field of chemistry, such kind of devices were introduced by analytical chemists in 1979, but it was only in 1990 when chemists realized the importance of these miniaturized systems and introduced the µTAS concept (miniaturized total analysis systems), a modified version of the existing TAS concept (total analysis systems).

Analytical chemistry plays a pivotal role in the separation and identification of analytes from complex matrixes (e.g. plant extracts, blood). The initial research focused on the development of miniaturized analytical instruments (e.g. microchip capillary electrophoresis) and sample pre-treatment on-chip. From literature, it is known that the method to be chosen solely depends on the sample, as there is not one universal method for all samples. The big challenge for analytical chemists is “to develop an integrated system which can extract/isolate analytes of interest from crude samples efficiently and perform on-chip analytical processes”.

The research performed in this thesis, aims towards the development of

1. A microfluidic chip that can selectively adsorb products from a process stream (plant extracts and serum samples) through molecular (ligand-analyte) interactions
2. A three-phase (liquid-liquid extraction) microchip for efficient sample clean-up and screening of acidic or basic molecules in aqueous solution

Chapter 1 highlights the concept of µTAS (miniaturized total analysis systems) and various materials used to prepare microfluidic devices and their advantages. Subsequently, the application of microfluidic devices in organic and analytical chemistry is illustrated. Further, the scope for potential research in the field of analytical chemistry is described. The research plan of this thesis is mentioned at the end of chapter 1.

In ***Chapter 2***, the focus is to develop a protocol for carbohydrate immobilization on glass surfaces and use the developed protocol to prepare carbohydrate modified microchips to study specific carbohydrate-lectin interactions. Various techniques (contact angle measurements and atomic force microscopy) were employed to study glass surface modification with different functional groups (amine and aldehyde). Lectins (Concanavalin A (Con A) and *Arachis hypogaea* (PNA)) with FITC tags were used in this study. Confocal laser fluorescence

microscopy (CLFM) was employed to study specific carbohydrate-lectin interactions. The developed protocol was used to prepare α-mannose modified capillary columns to study the α-mannose-Con A interaction, but due to a low column capacity, the α-mannose modified column was not effective in capturing Con A. To increase the column capacity, different carbohydrate immobilization supports (matrix) were reviewed. Out of various stationary phases, monolithic materials appeared to be the most promising.

The aim in ***Chapter 3 & 4*** is to develop various protocols to prepare affinity monolithic columns to study carbohydrate-lectin interactions. In *chapter 3*, a three step protocol to prepare affinity monolithic columns and in *chapter 4*, a single step protocol to prepare affinity monolithic columns are described. These affinity columns displayed good permeability (low back pressures at high flow rates). ISEC (Inverse size exclusion chromatography) is used to study the pore size distribution in the columns prepared in *chapter 3*. Carbohydrate-lectin interactions are studied successfully with frontal affinity chromatography (FAC) technique (Con A and LCA bound to α-mannose and PNA to β-galactose). Elution of bound lectins is successful with carbohydrate solutions as eluents (Con A and LCA are eluted using 100 mM methyl-α-mannopyranoside and PNA is eluted using 100 mM β-galactose). The carbohydrate-lectin dissociation constant (K_d) is studied using monolithic columns prepared in *chapter 4*. The advantage of the protocol in *chapter 4* over *chapter 3* is the time spent to prepare an affinity monolithic column. The protocol developed in *chapter 4* was repeated successfully in a microchip. However, the prepared affinity monolithic array microchip needs further tuning of the permeability of the two monolithic columns to be able to use this array microchip for screening of plant extracts with nano ESI-MS.

In ***Chapter 5***, ganglioside (GM1, GM2 and GM3) monolithic columns are prepared according to the protocol developed in *chapter 4*. These columns are employed to capture IgM & IgG antibodies from sera of patients suffering from Guillain-Barré syndrome (GBS), whose sera have a high concentration of antibodies against GM1 and GM2. ELISA (Enzyme linked immunosorbent assay) is used to study the depletion of IgM and IgG using ganglioside columns. Both GM1 and GM2 monolithic columns captured IgM efficiently from different sera, but were not successful in capturing IgG. Control experiments carried out to test the depletion of IgM antibodies using blank monolithic and GM3 monolithic columns did not capture IgM from sera specific for GM2. It is also shown that GM2 monolithic columns can capture only IgM antibody specific for GM2 and not IgM antibodies specific for other gangliosides (GQ1b) and those of a healthy patient. Fluorescence images obtained using anti-human IgM/IgG with FITC tags demonstrated the homogeneity (GM2 molecules are evenly distributed along the column) of the GM2 monolithic columns. The results obtained demonstrate the efficiency of ganglioside monolithic columns in capturing IgM antibodies.

Further studies should be carried out to capture IgG antibodies and develop a ganglioside monolithic array microchip to screen serum samples using nano ESI-MS.

The focus in ***Chapter 6*** is to develop a three-phase microchip for sample clean-up of acidic or basic molecules in aqueous solution by extraction. For proof of principle, alkaloids (weakly basic molecules) from plant extracts are chosen as model compounds in this study. A good phase separation of organic and aqueous phases is achieved using a selective, stable non-polar coated chips. First a two-phase microchip is used to study the extraction of strychnine from a basic aqueous phase to a chloroform phase and secondly, from a chloroform phase to an acidic aqueous phase. Subsequently, the extraction of strychnine from feed phase (basic aqueous phase) through transport phase (chloroform phase) to the acceptor phase (acidic aqueous phase) is performed in a three-phase microchip. The experimental data is compared with modeling data (a numerical model is developed using partition coefficients of strychnine obtained in macro-scale experiments). Overall, a good correlation is observed between model and experimental results. Purification of Strychnos seed extract is carried out using the developed three-phase microchip. The developed numerical model is used to predict the outcome of strychnine extraction by changing a few parameters (channel thickness, viscosity of organic solvent, partition coefficient values, flow rate and channel lengths). The first attempt to screen a Strychnos extract on-line using nano ESI-MS is not fully successful due to pressure problems in the system resulting in disturbance of the parallel flows in the three-phase chip.

Chapter 7 highlights the achieved targets from the main aim of this thesis work “to develop a miniaturized system that can be used for sample clean-up and screening of biological samples” and puts the results in a broader context.

Samenvatting

Recentelijk is er meer en meer aandacht voor het concept van “Lab on a Chip” als gevolg van de vele voordelen van de toepassing van microfluïde apparatuur in diverse gebieden van de levenswetenschappen. Het hart van microfluïde apparatuur is de chip die van uiteenlopende materialen gemaakt kan zijn, zoals glas, silicium of kunststof. Elke chip bevat kanalen in de grootte orde van micrometers en kan volumina zo klein als 10^{-18} liter verwerken. Analytisch chemici introduceerden zulke apparatuur in 1979 maar het duurde tot 1990 voordat zij zich het belang van zulke systemen realiseerden. Het μTAS (micro totaal analyse systemen) concept werd toen geïntroduceerd.

Analytische chemie speelt een doorslaggevende rol in de scheiding en identificatie van componenten in complexe mengsels (bijv. planten-extracten of bloed). In het begin richtte het onderzoek zich op de ontwikkeling van geminiaturiseerde analytische systemen (bijv. capillaire electroforese op een chip) en monster-voorbewerking op een chip. Uit de literatuur was bekend dat de te kiezen methode grotendeels bepaald wordt door het monster aangezien er geen universele methode is voor alle bestaande soorten monsters. De grote uitdaging voor analytisch chemici is om een geïntegreerd en efficiënt systeem te ontwikkelen dat ruwe monsters kan extraheren en op een chip kan analyseren.

Het onderzoek dat is uitgevoerd in het kader van dit project had als doelstelling de ontwikkeling van:

1. een microfluïde chip die selectief producten kan adsorberen uit complexe monsters zoals plantenextracten en serum door selectieve chemische interacties

2. een 3-fasen (vloeistof – vloeistof – vloeistof extractie) chip voor de monstervoorbewerking van zure of basische stoffen voorkomend in waterige oplossingen.

Hoofdstuk 1 behandelt het μTAS concept, de voordelen van deze techniek en de componenten die nodig zijn voor het fabriceren van microfluïde apparatuur. Er worden voorbeelden gegeven van de toepassing van microfluïde apparatuur in de organische en analytische chemie. De onderzoeksplanning wordt gepresenteerd.

In ***Hoofdstuk 2*** ligt het accent op het ontwikkelen van een protocol voor het chemisch binden van suikers op glasoppervlakken. Met de ontwikkelde methodologie worden diverse microchips met suikers gefunctionaliseerd om specifieke interacties tussen lectine eiwitten en suikers te onderzoeken. Verschillende technieken, o.a. contacthoekmetingen en atomaire kracht microscopie, werden gebruikt om de verschillend gemodificeerde glasoppervlakken

(amine en aldehyde) te onderzoeken. De lectines concanavaline A (Con A) en *Arachis hypogaea* (PNA) voorzien van een fluorescerend FITC label werden gebruikt om met confocale laser fluorescentie microscopie de suiker – lectine interacties en dus de kwaliteit van de modificatie te testen. Het protocol werd gebruikt om de binnenwand van een fused silica capillair met α-mannose te modificeren en vervolgens de interactie met ConA te meten. Helaas was het capillair vanwege een te gering wandoppervlak niet in staat een meetbare hoeveelheid ConA te binden. Om de capaciteit te vergroten werd literatuuronderzoek verricht naar de verschillende soorten stationaire fasen waaraan suikers kunnen worden gebonden. Uit dit onderzoek kwamen monolithische fasen als meest geschikt naar voren.

Het doel in ***Hoofdstuk 3 en 4*** is om verschillende methodes te ontwikkelen voor het fabriceren van suiker-gemodificeerde monolitische affiniteits kolommen om daarmee lectine – suiker interacties te bestuderen. In *hoofdstuk 3 en 4* worden respectievelijk een 3-staps methode en een 1-staps methode beschreven voor het maken van dergelijke kolommen. Deze affiniteits kolommen waren zeer doorlatend (weinig tegendruk bij een hoge vloeistofsnelheid). ISEC (omgekeerde gelpermeatie chromatografie) is gebruikt om de groottespreiding van de monoliet kanaaltjes in de kolommen te meten. Interacties tussen suikers en lectines konden goed gemeten worden met FAC (frontale affiniteits chromatografie). ConA en LCA hadden interactie met α-mannose en PNA met β-galactose. Gebonden lectines konden vervolgens verdrongen worden door andere suikers aan het elutiemiddel toe te voegen: ConA en LCA met 100 mM methyl-α-mannopyranoside en PNA met 100 mM β-galactose. De suiker – lectine dissociatieconstante (K_d) werd bepaald met de monolitische kolommen vervaardigd in *hoofdstuk 4*. Het voordeel van de methode in *hoofdstuk 4* boven die in *hoofdstuk 3* is de tijd nodig om een affiniteitschromatografie-kolom te produceren. Met de methode van *hoofdstuk 4* is het ook gelukt om kanalen in een microchip te vullen met monolitische fase. Echter aanvullend onderzoek is nodig om de doorlaatbaarheid van de verschillende kanalen beter onder controle te krijgen. Dan kan dit microchip type gebruikt worden om plantenextracten te testen op de aanwezigheid van lectines in combinatie met nano-electrospray ionisatie massaspectrometrie (nanoESI-MS) als detectiemethode.

In ***Hoofdstuk 5*** werden capillaire monolithische kolommen voorzien van drie verschillend gangliosides (GM1, GM2, GM3) met behulp van het protocol uit *hoofdstuk 4*. Deze kolommen werden vervolgens gebruikt om IgM en IgG antilichamen afkomstig van sera van Guillain-Barré syndroom patiënten te adsorberen. Hun sera bevatten hoge concentraties aan antilichamen tegen GM1 en GM2. ELISA (enzym gebonden immunoassays) testen zijn gebruikt om het verwijderen van IgM en IgG antilichamen uit sera met behulp van de kolommen te volgen. Zowel GM1 als GM2 monolithische kolommen waren in staat IgM antilichamen effectief te adsorberen maar niet de IgM antilichamen. Controle experimenten met ongemodificeerde kolommen en GM3 kolommen lieten zien dat deze kolommen geen

IgM adsorbeerden van sera met hoge concentraties antilichamen tegen GM2. Verder adsorbeerden GM2 kolommen uitsluitend IgM antilichamen tegen GM2 en geen IgM antilichamen tegen bijvoorbeeld andere gangliosides zoals GQ1b of antilichamen van gezonde mensen. Fluorescentie afbeeldingen gemaakt met fluorescerende anti-antilichamen tegen humane IgM/IgG lieten zien dat de GM2 kolommen homogeen waren: alle GM2 moleculen waren gelijkmatig verspreid over de kolom. Alle resultaten samengevat laten zien dat de monolithische ganglioside kolommen zeer effectief IgM antilichamen kunnen verwijderen uit menselijk serum. Meer onderzoek is nodig om kolommen te maken die IgG antilichamen kunnen adsorberen en een microchip te maken waarmee sera van GB patiënten onderzocht kunnen worden op de aanwezigheid van antilichamen, mogelijk in combinatie met nanoESI-MS als detectiemethode.

In ***Hoofdstuk 6*** wordt geprobeerd een 3-fasen microchip te ontwikkelen voor de zuivering van basische of zure moleculen middels extractie. In essentie vervangt een dergelijke chip twee gescheiden vloeistof – vloeistof extracties. Om de haalbaarheid van dit concept te testen is strychnine (een alkaloid) als modelstof uitgekozen. Strychnine komt in noten van de Strychnos-boom voor. Een goede fasenscheiding tussen de twee waterige fasen en de organische fase werd bereikt door selectief een stabiele apolaire coating aan te brengen op de wand van het middelste microkanaal van de chip. In eerste instantie werd een 2-fasen chip gebruikt om de extractie van strychnine van een basische waterige fase naar een organische fase (chloroform) en van de chloroform fase naar een zure waterige fase te volgen. Daarna werd de extractie van strychnine van een basische waterige fase (aanvoerfase) via de organische tussenfase (transportfase) naar een zure waterige fase (afvangfase) bestudeerd in drie parallelle vloeistoffasen. Dit ging snel en efficiënt met minimaal gebruik van oplosmiddelen. De experimentele gegevens werden vervolgens vergeleken met wat theoretisch te verwachten is. Hiervoor werd speciale computerprogrammatuur gebruikt in combinatie met experimenteel verkregen verdelingsconstanten. Er was een prima correlatie tussen de theoretische en experimentele waardes. De zuivering van strychnine uit een Strychnos extract kon succesvol worden uitgevoerd met de ontwikkelde 3-fasen chip. Het ontwikkelde theoretische model kon gebruikt worden om de extractie-efficiëntie te berekenen van nog niet bestaande chips waarin bepaalde parameters veranderd zijn ten opzichte van de bestaande 3-fasen chip zoals kanaalbreedte, viscositeit van het organisch oplosmiddel, verdelingsconstantes, vloeistof stroomsnelheid en kanaallengte. Een eerste poging een 3-fasen chip aan te sluiten op een massaspectrometer faalde als gevolg van drukproblemen. Deze veroorzaakten een verstoring van de vloeistofstromen.

Hoofdstuk 7 kijkt terug op alle behaalde resultaten en vergelijkt deze met de hoofddoelstelling van dit onderzoek: de ontwikkeling van chip-gebaseerde analytische systemen die gebruikt kunnen worden voor het testen op aanwezigheid van bepaalde

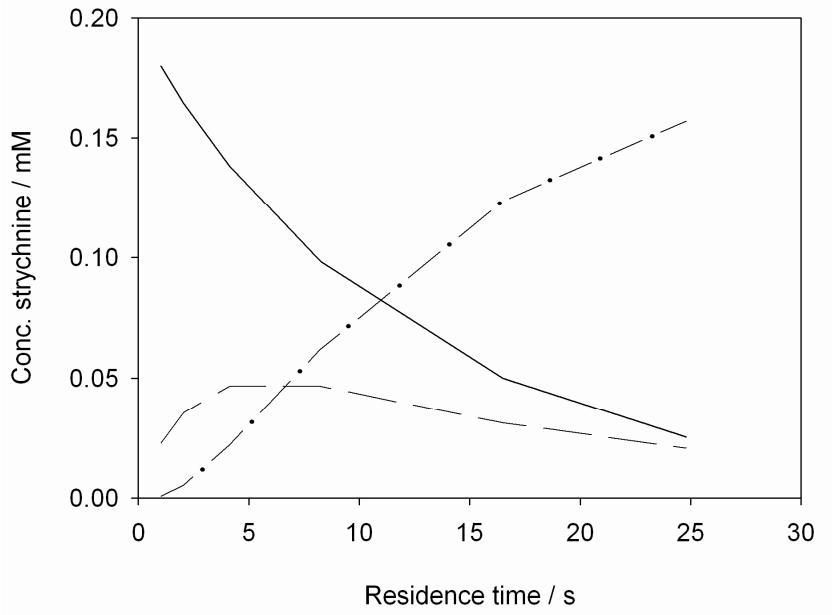
componenten en monstervoorbewerking van biologische monsters. Er worden conclusies getrokken en suggesties gedaan voor mogelijk vervolgonderzoek.

Appendix

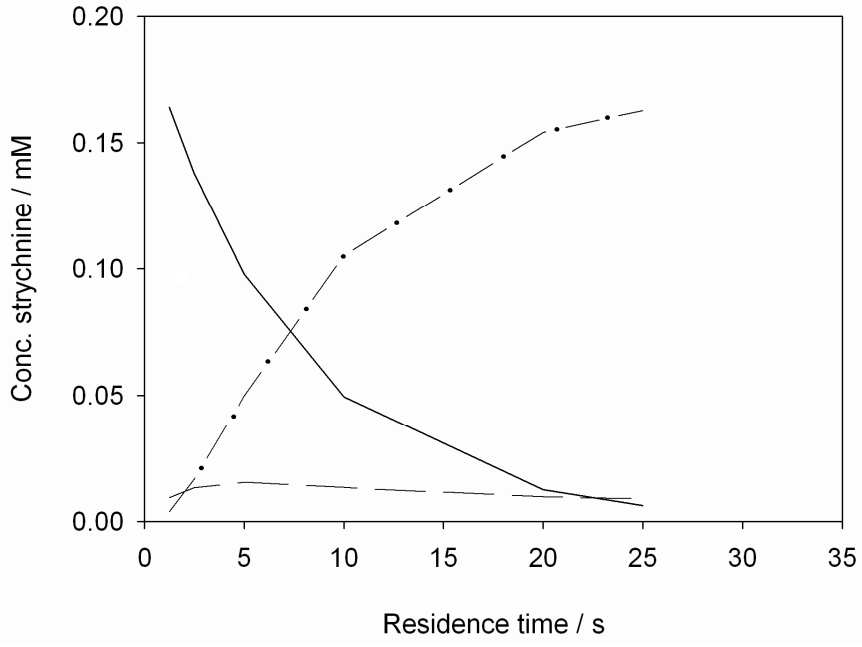
Model data obtained with change of various parameters

Modeling results obtained by changing two parameters (viscosity of organic solvent and flow rate of the system) (– is basic aq. phase model data; -- is chloroform model data; -•- is acidic aq. phase model data).

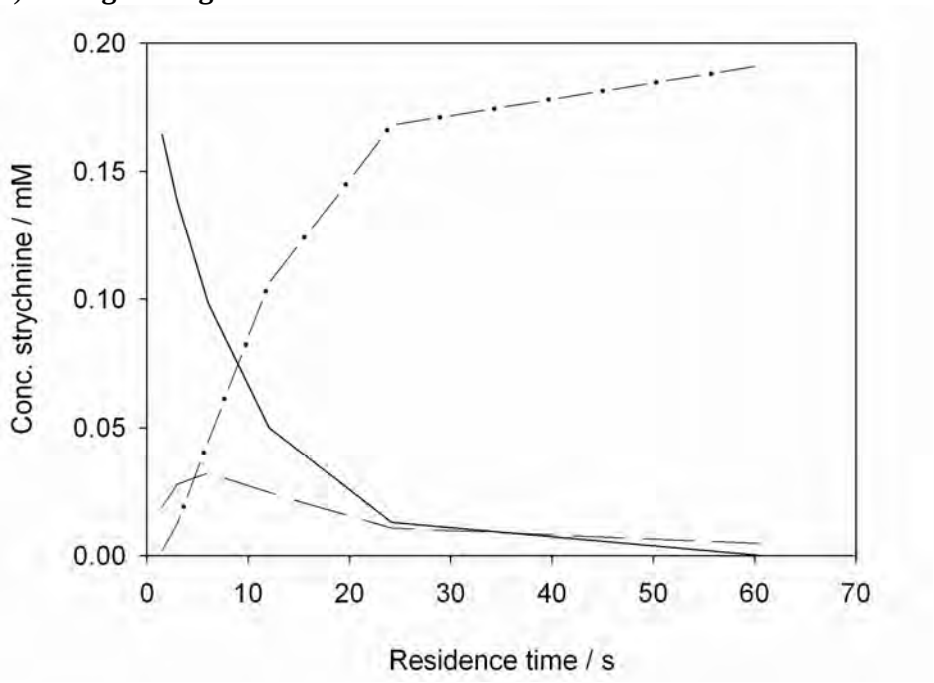
a) **Flow rate of the system 1:1:1 µL/min**



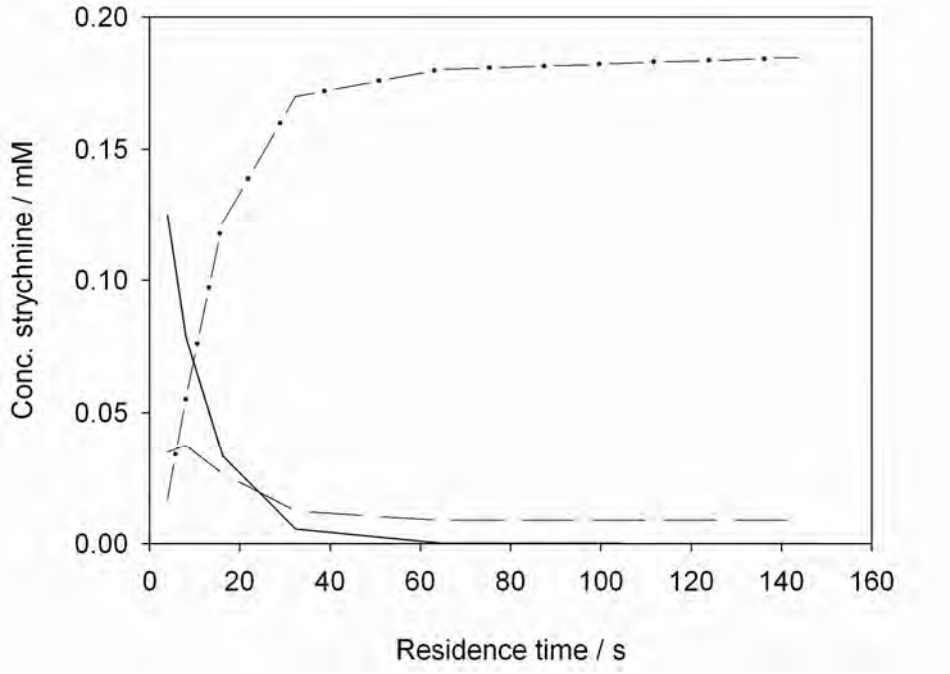
b) **Narrow width of the middle(organic) channel**



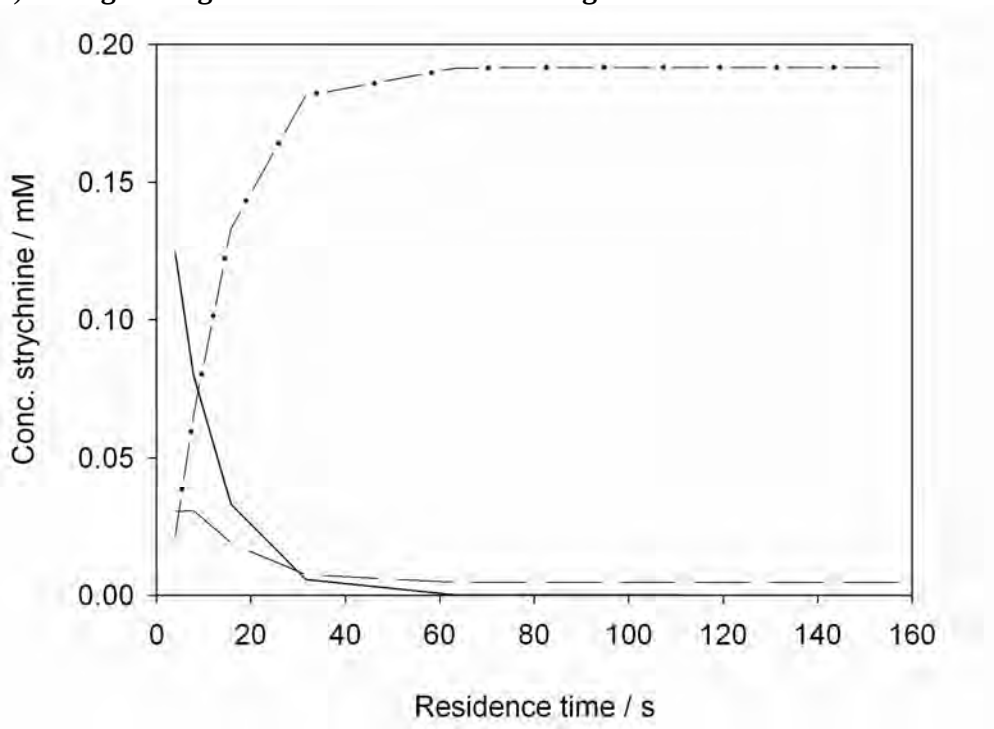
c) **Change of organic solvent**



d) **Standard case with channel length - 9.3 cm**



e) **change of organic solvent with channel length - 9.3 cm**



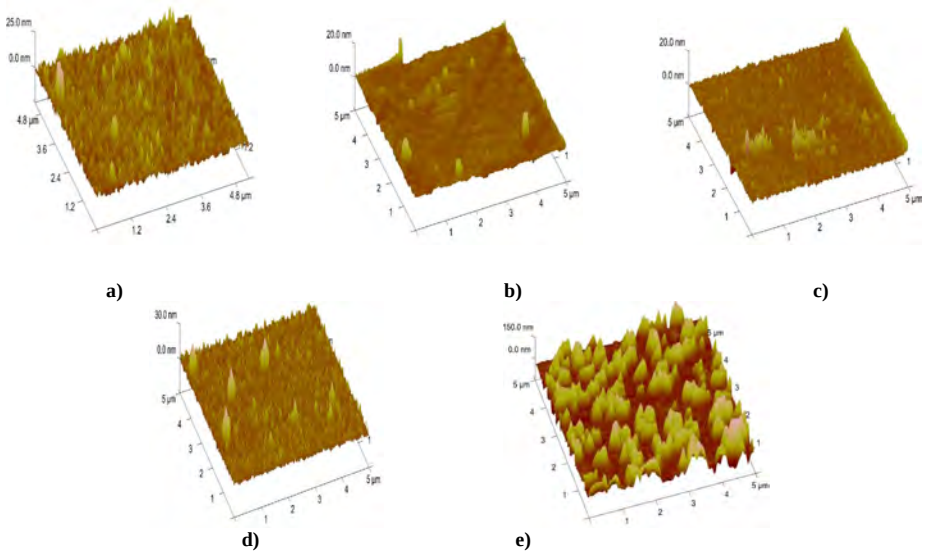


Figure 2.5 *(chapter 2, page no. 25).*

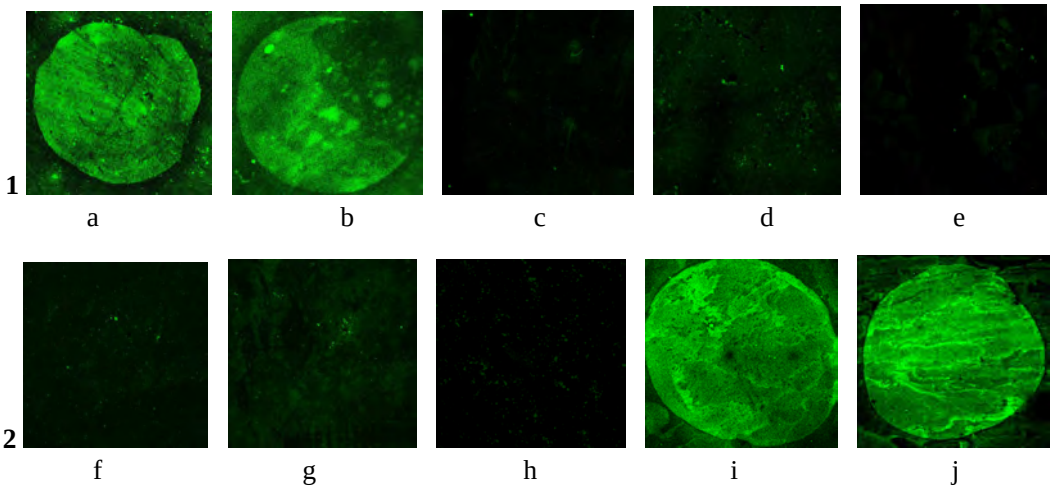


Figure 2.6 *(Chapter 2, page no. 26)*

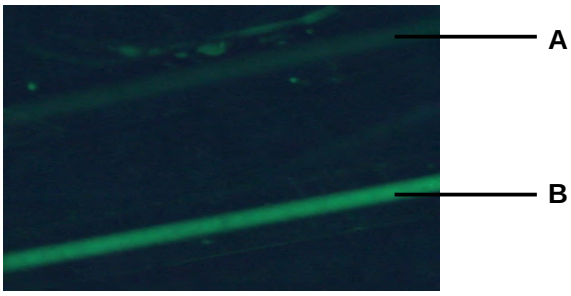


Figure 3.10 *(Chapter 3, page no. 45).*

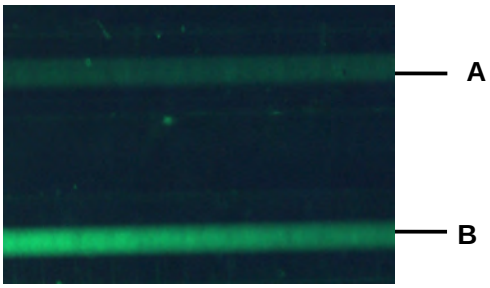


Figure 3.11 *(chapter 3, page no.46).*

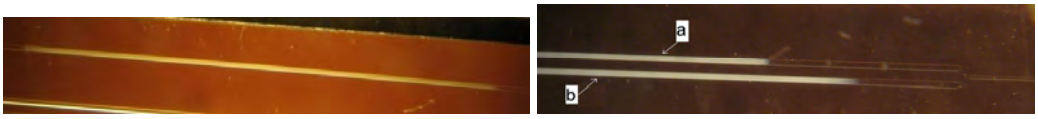


Figure 4.9 *(chapter 4, page no.61)*

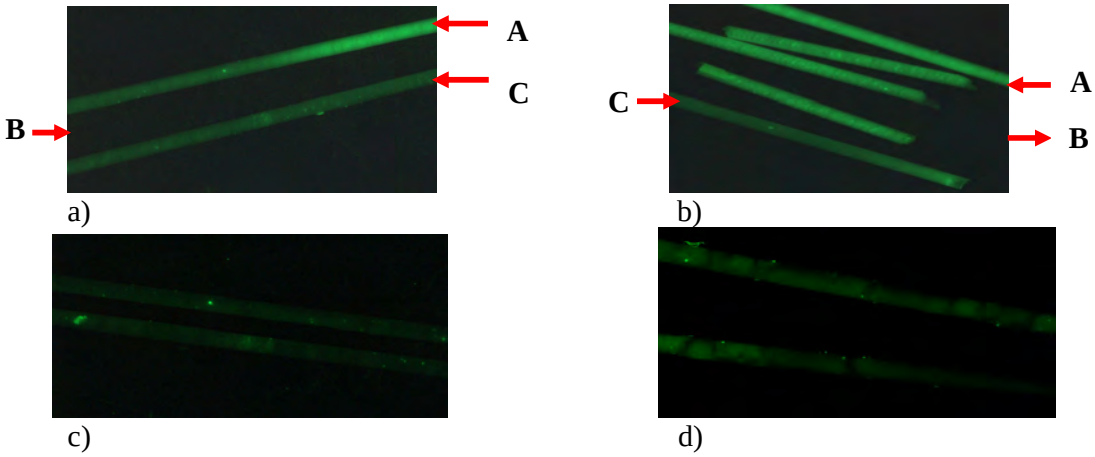


Figure 5.3 *(chapter 5, page no.71)*

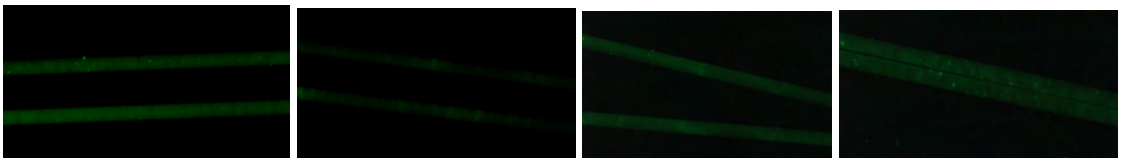


Figure 5.6 *(chapter 5, page no. 74).*

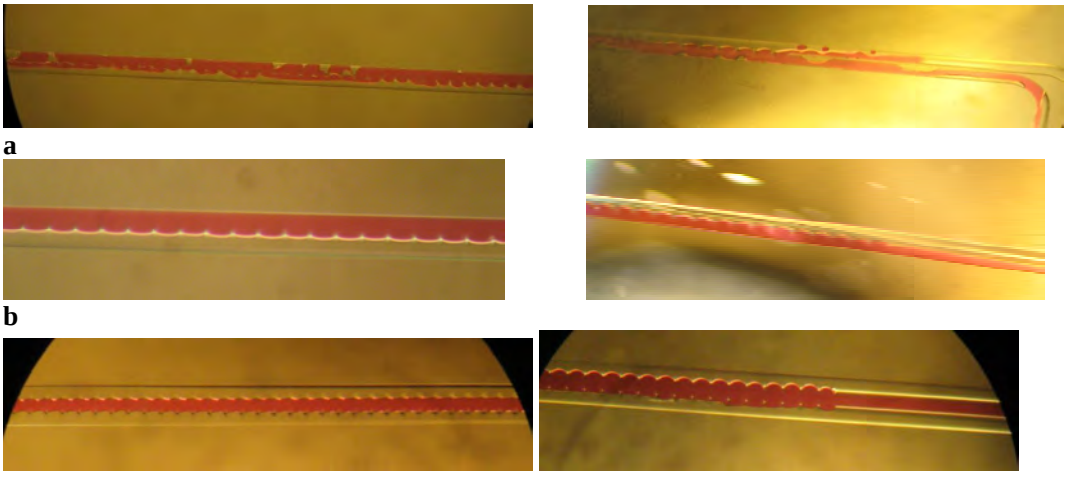


Figure 6.4 *(chapter 6, page no. 85).*

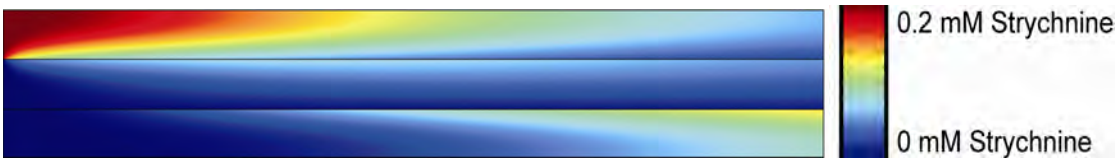


Figure 6.7b *(chapter 6, page no. 90)*

Curriculum Vitae

Kali Kishore Reddy Tetala, was born in Penugonda, Andhra Pradesh, India on 5th August 1979. He graduated with a Bachelor of Science (B.Sc) degree in 1999 from A.V. College, Osmania University, India. He obtained his Master degree (M.Sc) in Organic Chemistry from K.G.R.L College, Andhra University, India. He was awarded the Gold medal for outstanding performance during his M.Sc. work from K.G.R.L College. In Oct 2002, he started working for his M.Phil. (Master of Philosophy) degree with thesis entitled "Palladium catalyzed synthesis of amidines and imidates" in the group of Prof. Dr. Richard J. Whitby, Organic Chemistry department, Southampton University, U.K. In June 2004, he started his research at the Laboratory of Organic Chemistry, Wageningen University for Ph.D. degree under the supervision of Dr. Gerben M. Visser, Dr. Teris A. van Beek and Prof. Dr. Ernst J. R. Sudhölter. From 1st July 2008 onwards, he is working as a post doctoral fellow with the Dutch Separation Technology Institute (DSTI), The Netherlands.

Publication list

Tetala, K. K. R., Chen, B., Visser, G. M., Maruška, A., Kornyšova, O., van Beek, T. A., Sudhölter, E. J. R., Preparation of a monolithic capillary column with immobilized α-mannose for affinity chromatography of lectins, *J. Biochem. Biophys. Methods 70*, 2007, 63-69.

Tetala, K. K. R., Giesbers. M., Visser, G. M., Sudhölter, E. J. R., van Beek, T. A., Carbohydrate microarray on glass: a tool for carbohydrate-lectin interactions, *Nat. Product Commun*. *2*, 2007, 391-394.

Tetala, K. K. R., Chen, B., Visser, G. M., van Beek, T. A., Single step synthesis of carbohydrate monolithic capillary columns for affinity chromatography of lectins, *J. Sep. Sci. 30*, 2007, 2828-2835.

Tetala, K. K. R., Swarts. J. M., Chen, B., Janssen, A. E. M., van Beek, T. A., A three-phase microchip for sample clean-up of alkaloids from plant extracts, *submitted to "Lab on a Chip"*

Tetala, K. K. R. et al., Ganglioside (GM2) monolithic capillary columns to capture antibodies from serum samples, *manuscript in preparation to submit in J. Am. Chem. Soc.*

A chapter in the book "Monolithic Chromatography and Its Modern Applications"
Tetala, K. K. R., van Beek, T. A., Bio- and lectin affinity monolithic chromatography, *manuscript in preparation*

van Beek T. A., Tetala, K. K. R., Koleva, I. I., Dapkevicius, A., Exarchou, V., Jeurissen, S. M. F., Claassen, F. W., van der klift, E. J. C. Recent developments in the rapid analysis of plants and tracking their bioactive constituents, *Phytochem. Rev., accepted*, 2009.

<u>Publication from M. Phil</u>
Tetala, K. K. R., Whitby, R. J., Light, M. E., Hurtshouse, M. B. Palladium-catalysed three component synthesis of α, β-unsaturated amidines and imidates. *Tetrahedron Lett. 45*, 2004, 6991-6994.

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Looking back into 4 years of my life in Wageningen, in general, I have had a very enjoyable and satisfying journey, better than I had dreamt of. This short journey has fetched me good friends and my interactions with them and others I worked in close collaboration with, have taught me important lessons in life, including organizing skills, thinking simple, and ability to deal with problems maturely and with patience. I take this opportunity to thank all those who helped me or made me feel better whenever I faced challenging situations.

First of all, I would like to thank my M.Phil supervisor, Prof. Dr. Richard Whitby (Southampton University). The great learning experience I had in his lab helped improve my knowledge in chemistry and gave me enough confidence to think in the direction of continuing research for Ph.D.

I thank my supervisors, Prof. Dr. Ernst Sudhölter, Dr. Gerben Visser and Dr. Teris van Beek for giving me the opportunity to work with them and for the faith they placed on me, that I would succeed in my Ph.D. project. Ernst, thank you for the scientific interaction all these years and valuable comments during thesis writing. Special thanks for keeping an update about my work even after moving to Delft and helping to organize Ph.D. study trip abroad.

Throughout our lives, we make new contacts but only a few make a strong impact on us with their attitude. Two such persons I met in Wageningen were my daily supervisors, Dr. Teris van Beek and Dr. Gerben Visser. Teris, you gave me enough freedom to work on my own and at the same time, you were always there to help me with your ideas and practical help whenever I knock on your door for suggestions. You have been very critical in correcting my manuscripts, to find out even very minute mistakes in them, making me realize that I have to be very attentive, self-critical and proud about my work while documenting it. Apart from science, I learned important lessons in life from you during our interaction in travelling for project meetings: “when one’s plate is full, better be satisfied rather than trying to overfill it” and “to solve a problem, we should be simple in thinking and to the point”. I had a memorable time during the touristic trips you organized every year for foreign people in your group and the Phytochemistry group (*sjoel*) events. Special thanks to your family too. I thank Geb and you for being caring and friendly supervisors.

Geb, thank you for taking care and making me feel comfortable at work in the beginning of my Ph.D. with your daily questions … how is life? … how is chemistry going? You have been very supportive and motivating with your enthusiasm. Apart from carbohydrate chemistry, from you I learned that I should never lose hope and enthusiasm and should take

things slow when I run into problems. I feel sad that you couldn't be in my defense committee. Nevertheless, you will always be in my thoughts when thinking about these years.

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"Finding good friends to share our sorrows and happiness is difficult". I feel myself lucky to find some good friends with whom I had splendid and most memorable moments in Wageningen. Special thanks to the cooking group members, whom I felt as my family in Wageningen: Arun, Shibu, Byjesh, Srinu, Nagesh, and Feana for the fun and time we spent together cooking every day and especially endless discussions during Friday and Saturday evenings on various topics ranging from science to politics to entertainment. From each one

of you I learned some positive things towards life. Thanks to Milena and Michel for their special friendship, the fun we had during the touristic trip in USA and especially "SALSA" is just unforgettable. I wish you both a happy life in the years to come. Thanks to Ganesan and Senthilkumar for their friendship and encouragement with nice suggestions.

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Besides research, playing "Badminton" on Friday evenings in sports centre became a part of my life in Wageningen. I would like to thank all the people I met during the game.

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I thank God for a wonderful life and for giving me the courage and wisdom to choose the right path whenever I faced difficult situations.

Kishore

Overview of completed training activities

Discipline specific activities

Courses

Ischia Advanced School in Organic Chemistry (IASOC), Italy, 18-23rd Sept. 2004
Lab on a chip course, MESA+, University of Twente, May-June 2006
Future trends in Phytochemistry, Italy, 6-9th June 2007
Bio-Nanotechnology course, VLAG, Wageningen University, 8-11th May 2006

Meetings

Advances in microarray technology conference, U.K., 11-13th Oct. 2005
Bionanotechnology symposium, Wageningen University, 25th Nov. 2004
Wageningen Bionanotechnology symposium, 88th Dies Natalis, WUR, 9th March 2006
Annual NWO conference (Organic Chemistry), Lunteren, 2004, 2005, 2006
Annual NWO conference (Analytical Chemistry), Lunteren, 2005, 2007
Nanoned/Microned symposium, Groningen, 8-9th Dec. 2005

General courses

VLAG Ph.D. week, March 2006
NWO Talent Day, 5th Dec. 2007
Techniques for scientific writing and presenting paper, Wageningen University, Feb. 2008
Organizing and supervising B.Sc. thesis project, Wageningen University, 2006-07

Optionals

Preparation Ph.D. research proposal
Group meetings laboratory of Organic chemistry, 2004-2008,
Ph.D. study trip, organized by Laboratory of Organic chemistry, USA, 9th-22nd April 2005
Ph.D. study trip, organized by Laboratory of Organic chemistry, Sweden, 14-26th April 2007
Literature study, Laboratory of Organic Chemistry, 2004
Euregio project meetings, 2004-2008
Supervising students for the course “Research Methods in Organic Chemistry”, 2004-07

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