Miniaturization of the Bioanalytical Process

by

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Doctor of Philosophy

UNIVERSITY OF TASMANIA

School of Chemistry
University of Tasmania
Hobart
Submitted November 2011
DECLARATION

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_“But He said to me, ‘My grace is sufficient for you, for My power is made perfect in weakness.’” (2 Corinthians 12:9)._
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<thead>
<tr>
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<th>Definition</th>
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<tr>
<td>ADME</td>
<td>Absorption, distribution, metabolism, and excretion</td>
</tr>
<tr>
<td>AIBN</td>
<td>$\alpha,\alpha'$-azoisobutyronitrile</td>
</tr>
<tr>
<td>BMA</td>
<td>Butylmethacrylate</td>
</tr>
<tr>
<td>BVBDMS</td>
<td>Bis(p-vinylbenzyl)dimethylsilane</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CEC</td>
<td>Capillary electrochromatography</td>
</tr>
<tr>
<td>DMN-H6</td>
<td>1,4,4a,5,8,8a-hexahydro-1, 4, 5, 8-exo,endo-Dimethanonaphthalene</td>
</tr>
<tr>
<td>EDMA</td>
<td>Ethylene dimethacrylate</td>
</tr>
<tr>
<td>ERPA</td>
<td>Extended range proteomic analysis</td>
</tr>
<tr>
<td>GDMA</td>
<td>1,3-glycerol dimethacrylate</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>i.d.</td>
<td>Internal diameter</td>
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<tr>
<td>IE</td>
<td>Ion-exchange</td>
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<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
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<tr>
<td>LMA</td>
<td>Lauryl methacrylate</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>NBE</td>
<td>Norborn-2-ene</td>
</tr>
<tr>
<td>PAA</td>
<td>Polyacrylamide</td>
</tr>
<tr>
<td>RPLC</td>
<td>Reversed-phase liquid chromatography</td>
</tr>
<tr>
<td>RAFT</td>
<td>Reversible addition fragmentation chain transfer</td>
</tr>
<tr>
<td>ROMP</td>
<td>Ring-opening metathesis polymerization</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviations</td>
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<tr>
<td>RAM</td>
<td>Restricted access media</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid-phase extraction</td>
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<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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LIST OF PUBLICATIONS

Papers in refereed journals and in preparation


Posters and conferences


- Poster, *School of Chemistry Research Evening*, 2010, University of Tasmania, Australia.


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ABSTRACT

This work presents a systematic study of macroporous polymer monoliths and their use as stationary phases for microscale separation and as supports for immobilized trypsin digestion in pipette tip format for bioanalysis.

Reversed-phase/cation-exchange mixed-mode polymer monolithic columns were prepared in situ within fused-silica capillaries via UV-initiated free-radical polymerization reaction. Control of the porous properties was achieved by varying the ratio of porogenic solvents (e.g. 1,4-butanediol and 1-propanol), the type of porogenic solvents used and the relative amounts of functional monomers and cross-linker. These columns were successfully used for the separation of a mixture of selected acidic drugs and β-blockers, namely ketoprofen, ibuprofen, diclofenac, arterenol and propranolol. Monolithic columns with higher amounts of cross-linker were shown to give better repeatability. Furthermore, the separation mechanism of the investigated compounds was shown to be dual mode hydrophobic/ion-exchange interaction of the analytes with the hydrophobic and cation-exchange monolith. All these properties demonstrated the potential of these devices for solid-phase extraction and sample enrichment purposes in miniaturized formats.

Based on the success of the previous work a trypsin-immobilized monolithic polymer with pipette-tip format was also investigated to explore the utility of using a tip-based protein digestion methodology in a bioanalytical setting. The excellent performance of immobilized enzymatic polypropylene pipette (IMEPP) tips was characterized using MicrOTOF-Q quadrupole time-of-flight MS and triple quadrupole LC-MS/MS systems. Very high sequence coverages of over 90% were achieved for the digestion of proteins ranging from
low molecular weight to high molecular weight proteins with short contact times prior to MS analysis, which is comparable to 24 h digestion in solution. In addition, quantitative analysis of target proteins spiked in rat plasma was demonstrated for the first time with relatively good linearity over a wide range from 40-1000 ng/mL. The developed IMEPP tips exhibit high plasma loading capacity up to 40 µL of plasma that can be used to yield the highest efficiency for digestion. The IMEPP tip approach is thus rapid, reliable, and robust suggesting the potential of this approach to improve sample throughput for pharmaceutical and pharmacokinetic studies, leading to faster and safer discovery of new drugs to treat diseases.

The trypsin immobilized polymer monolith was prepared *in situ* in syringe-compatible glass tube and evaluated for the digestion of protein. Preliminary results showed that the enzyme on the immobilized bed exhibited high proteolytic performance in this special format. The present glass tube bioreactor provides a promising platform for the full automation, on-line coupling to detection systems, short sample preparation times and high-throughput protein digestion.
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Chapter 1

Introduction & Literature Review

1.1 Introduction

Reversed-phase high performance liquid chromatography (RPLC) has become one of the most popular analytical techniques over the past 30 years for quantitative analysis of a wide range of pharmaceutical compounds [1,2]. It has been used in a variety of fields, including quality control of drugs, pharmacokinetic studies, and the determination of environmental pollutants or food additives [1]. The latest paradigms in the process of drug discovery require smaller quantities of analytes to be separated in an increasingly large number of samples [3] and in a drug discovery setting, the demand for high quality bioanalytical assays has grown over recent years. The increase in the number and potency of new chemical entities and the associated decrease in sample size, have in turn driven down the limits of quantification required for many bioanalytical methods. A bioanalytical method now has to be rapid, automated, sensitive, selective, robust and applicable to large numbers of samples in order to be considered useful.

Quantitative bioanalysis is an important tool in the process of drug discovery, especially pertaining to important in vitro and in vivo studies. The data generated from bioanalytical methods are pivotal in making decisions around compound progression through the drug discovery process. The need to develop new bioanalytical methods to support the drug discovery process presents a continual challenge to the bioanalyst. For example, thousands of samples from in vitro screens need to be analyzed on a daily basis. Alternatively, quantifying pg/mL
levels of drug-related components in plasma from clinical studies, using a validated complex bioanalytical method, may be required. More considered approaches to both the extraction technique and chromatographic separation have also been successfully adopted for clinical applications.

To further improve the efficiency of the drug discovery process, bioanalysts have been expected to generate analytical data in a far more efficient manner. Because of this, the development of more selective extraction techniques and the optimization of fast chromatographic conditions have come to prominence. Other advances in analytical chemistry, such as new column chemistries, high-pressure separations and hyphenated techniques such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) are common in the development of new bioanalytical methods. LC-MS/MS is the analytical platform used most commonly for bioanalysis, as triple quadrupole mass spectrometers offer the required specificity and sensitivity of detection. LC–MS/MS is ideally suited for pharmaceutical compounds, owing to the typical compound physicochemical properties and molecular weight. In addition, LC–MS/MS is specific enough to allow very rapid chromatographic separations to be performed; it can accept small volume samples in a micro-titre plate format, and it can also be used to monitor multiple analytes in the same complex sample (such as a plasma extract). LC-MS/MS has become the most widely used analytical platform for quantifying drugs and their metabolites at low concentration levels in biological matrices [4]. For rapid LC–MS/MS bioanalysis, most laboratories use short analytical columns (3–10 cm), with relatively high flow-rates (e.g. 1 mL/min), small particles (5 μm or less) and fast gradients (10% -90% organic modifier) to achieve full analysis in less than 5 min per sample [5]. Single ion monitoring MS and multiple reaction monitoring MS/MS techniques provide great detection power. Since its
establishment in the early 1990s, LC-MS/MS has revolutionized the development of assays for new therapeutic agents in biomatrices [4,6,7], replacing more traditional high performance liquid chromatography (HPLC) detection techniques, such as ultra-violet or fluorescence.

The development of new analytical methodologies for high-throughput bioanalysis has focused on the better utilization of the significant capital investment made in LC-MS/MS instrumentation and automation. Advances in on-line and off-line sample preparation (e.g. sorbent chemistry, micro-plate formats, turbulent flow chromatography and column switching) and further advances in HPLC technology, which lead to enhancements in speed, sensitivity, and resolution (e.g. ultra-high pressure, capillary- and nano- HPLC ) [8-17], have also been implemented.

Sample preparation is a key step in quantitative bioanalysis and can often be a bottleneck in the process of developing a robust and efficient bioanalytical methodology [4,18]. The sample preparation stage is often the most labor-intensive, time-consuming and error-prone process in a bioanalytical method [4]. This can be attributed to the complex nature of macromolecular compounds, such as proteins and non-volatile endogenous substances, that have to be removed from a biological sample and separated from the analytes to eliminate matrix interferences prior to the LC-MS/MS analyses [19]. Sample preparation can also serve to pre-concentrate the sample while removing the endogenous matrix interferences at the same time. This leads to an increase in sensitivity and an ability to subsequently use faster, less well resolved chromatographic separations. Sample preparation is typically achieved by liquid-liquid extraction [20], solid-phase extraction (SPE) [13,21], protein precipitation [22], and on-line methods with HPLC [23]. In addition, as the number of samples increases, there has been a trend to
develop different formats of sample preparation devices, more selective sorbents for sample clean-up and enrichment, as well as fully automated analytical techniques [24,25]. The introduction of automated sample pre-treatment approaches has greatly increased the efficiency of the bioanalysis process [26]. Automated SPE in a 96-well plate format is now an established technique for sample clean up for biological samples [10].

One way to overcome some of the challenges associated with using fast LC/MS/MS is the use of monolithic phases. These have a number of potential advantages over more conventional silica-based particulate materials, as outlined in Table 1.1. Polymer monoliths can be adapted to most bioanalytical situations, such as larger scale sample preparation and small-scale capillary- or nano- HPLC separations, and therefore their use for bioanalysis has increased over recent years. These materials have some interesting properties when compared with the more traditional particulate materials, such as their high permeability for liquids and biological samples, thus making them ideal for SPE. The relative ease of synthesis, high control of shape, porosity and selectivity give the bioanalyst many options in the choice of bioanalytical applications and formats for monolithic phases. Additionally, the option of operating at high or low flow-rates, stability at high pH as compared to similar silica-based materials and low column pressure drop, can yield flexibility and speed for HPLC separations. These phases can be prepared in situ within narrow capillaries and applied to micro-scale separations and even further miniaturized for micro-fabricated applications. In summary, monolithic phases can be used in a variety of formats and can be prepared in a range of formats including HPLC columns, SPE plates, pipette tips or even synthesized in narrow capillaries for nano-flow chromatography.

Organic polymeric monoliths can take two structurally different forms: (i)
Table 1.1: Advantages, disadvantages of using monoliths as compared to alternative phases for bioanalytical applications.

<table>
<thead>
<tr>
<th><strong>Advantages</strong></th>
<th><strong>Monolithic phases</strong></th>
<th><strong>Other phases</strong></th>
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<tbody>
<tr>
<td></td>
<td>Low cost, relative ease of <em>in situ</em> preparation (e.g. thermal initiation, redox reagents, UV and gamma radiation).</td>
<td>High surface area.</td>
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<td>Mechanically robust, no void volumes formed with conventional LC flow-rates.</td>
<td>Higher column efficiencies.</td>
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<td></td>
<td>Can control the porous properties by varying the compositions of monomers and porogens for the separations of large and small-molecules.</td>
<td>Small particle sizes and high operating pressures.</td>
</tr>
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<td></td>
<td>High hydraulic permeability and the dominance of the convection over the diffusion mechanism of mass-exchange under dynamic conditions that allow the separation to be carried out at extremely high flow-rates.</td>
<td>Diverse column chemistries and HPLC column dimensions.</td>
</tr>
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<td></td>
<td>Flexible synthesis means that monoliths can be modified with almost any functionality, including ion-exchange, affinity, chiral, mixed-mode, restricted access, hydrophobic and hydrophilic to tailor the stationary phases for different analytes.</td>
<td>Validated applications/assays.</td>
</tr>
<tr>
<td></td>
<td>Can be molded into any shape (capillary, column, micropipette tips, microfluidic channel on a chip).</td>
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<tr>
<td></td>
<td>Relatively biocompatible, due to open porous structure. Could be used for SPE column for over 200 times for the analysis of human plasma without loss of efficiency.</td>
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<tr>
<td></td>
<td>Can withstand more extreme conditions (i.e. pH working range from 2 to10).</td>
<td></td>
</tr>
<tr>
<td>Disadvantages</td>
<td></td>
<td></td>
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<tr>
<td>------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Lower surface area, lower binding capacity.</td>
<td>Higher backpressure (slow diffusional mass transfer).</td>
<td></td>
</tr>
<tr>
<td>Lower column efficiencies and HPLC column-to-column repeatability.</td>
<td>For extraction, sorption materials, such as beads requires extra instruments to generate high-pressure for the sample solution to pass through the media. The intrinsic problem of all particulate media is their inability to completely fill the available space. The channeling between particles reduces extraction efficiencies and can adversely affect flow characteristics.</td>
<td></td>
</tr>
<tr>
<td>Narrow column chemistries and column dimensions commercially available.</td>
<td>Fabrication of packed capillary columns requires a tremendous amount of skill because of small i.d. particles used.</td>
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<tr>
<td>Limited use in routine analysis due to the limitation of commercial suppliers.</td>
<td>Need for frits, undesired interactions of the frits with some analytes can make the utilization of packed capillary columns very problematic.</td>
<td></td>
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<td>Narrow pH stability range.</td>
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homogeneous gels and (ii) rigid porous polymers. Homogeneous gels can be applied as visually transparent or opalescent separation media and in principle they represent an almost ideal chromatographic support because of their high porosity and the fact that the eddy diffusion is negligible. An example of a homogeneous gel is the low cross-linked polyacrylamide (PAA), which is a soft and highly swollen material. On the other hand, rigid porous polymeric monoliths consist of a single piece of highly cross-linked macroporous polymer with interconnected pores and greater structural rigidity separations with low column backpressure and fast mass transfer kinetics [1-3].

Recently, Guiochon [27], Smith and Jiang [28] and Potter and Hilder [29] have reviewed some general aspects of monolithic materials and their applications. In this chapter the focus is on recent developments in the use of monoliths (with particular emphasis on polymer monoliths) in the applied area of bioanalysis. The synthesis of polymer monoliths is discussed, followed by the use of monoliths for bioanalysis by HPLC and for SPE. Finally, some future directions are indicated.

1.2 Synthesis of organic polymer monoliths for bioanalysis

Despite the widespread progress in monolith synthesis, the preparation of monoliths may often remain a trial-and-error procedure. Furthermore, scale-up of the synthesis to larger diameter formats above typical analytical dimensions remains a challenge due to the high exothermic level of the polymerization reaction which becomes paramount in the case of large-volume monoliths. This can result in significant radial temperature gradients, leading to a non-uniform pore structure. Danquah and Forde [30] employed a novel synthetic technique using a heat expulsion mechanism to prepare a large methacrylate monolith (40 mL) with a homogeneous radial pore structure along its thickness. This large monolith was
used for the rapid purification of pDNA from clarified bacteria lysate using elevated flow-rates under moderate pressure drops. The same authors reported the preparation of macroporous methacrylate monolithic material with controlled pore structure using an un stirred mould through precise control of the polymerization kinetics and parameters. The results revealed that the control of the kinetics of the overall process through changes in reaction time, temperature and overall composition, such as cross-linker and initiator contents, allowed the fine tuning of the macroporous structure [31]. In contrast to this work, Chen et al. [32] directly prepared a supermacroporous monolithic cryogel by in situ cryo-copolymerization in a stainless steel cartridge using methacrylic acid as functional monomer and polyethylene glycol diacrylate as cross-linker. The resulting highly cross-linked (90%, molar ratio) cryogel had more uniform super macropores as compared to the poly(acrylamide)-based cryogels. The viability of this cryogel as a medium was demonstrated through the separations of lysozyme from chicken egg white and water-soluble nanoparticles from crude reaction solution.

A weak ion-exchange grafted methacrylate monolith was prepared by Frankovic et al. [3] by grafting the methacrylate monolith with glycidyl methacrylate and subsequently modifying the epoxy groups with diethylamine. A comparison of the binding capacity for the non-grafted and grafted monolith was performed using β-lactoglobulin, bovine serum albumin (BSA), thyroglobulin, and plasmid DNA (pDNA). The results revealed that the grafted monolith exhibited 2 to 3.5-fold higher capacities (as compared to non-grafted monoliths). Furthermore, the maximum pDNA binding capacity was reached using 0.1 M NaCl in the loading buffer and no degradation of the supercoiled pDNA form was detected. The grafted monolith exhibited lower efficiency than the non-grafted version,
however baseline separation of pDNA from RNA and other impurities was achieved from a real sample.

Monolithic materials have been prepared for small drug molecule bioanalysis ranging from high-throughput separations in combinatorial chemistry through to validated clinical assays. For example, Aoki et al. have described a new approach to prepare polymer monoliths with morphology tailored for HPLC application to small solutes such as drug candidates [33]. Polymer monoliths based on 1,3-glycerol dimethacrylate (GDMA) were prepared by in situ photo-initiated free radical polymerization (UV-irradiation at 365 nm). The photo-polymerization was carried out with a mono-disperse ultra-high molecular weight polystyrene solution in chlorobenzene uniquely formulated as a porogen. The poly-GDMA monoliths were prepared in bulk, rod and capillary formats and these showed a bicontinuous network-like structure featured by their fine skeletal thickness approaching sub-μm size. This monolithic structure was considered to be a time-evolved morphology frozen by UV-irradiation via visco-elastic phase separation induced by the porogenic polystyrene solution. The UV-initiated poly-GDMA capillary monolith demonstrated a sharp elution profile affording higher column efficiency and permeability as compared to the thermally prepared capillary of the same pore size. This investigation showed that poly-GDMA monoliths with a well-defined bicontinuous structure could be prepared reproducibly by photo-initiated radical polymerization via visco-elastic phase separation using the unique porogen.

Roohi et al. [34] reported an interesting alternative to reversed-phase (RP) monolithic columns using a convenient coupling route of a thermo-responsive polymer to hydrophilic silica monoliths. Poly(N-isopropylacrylamide) was polymerized in solution via a reversible addition fragmentation chain transfer (RAFT) polymerization technique and coupled then in situ onto an amino-modified
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silica monolithic column. These columns were compared with reversed-phase C18 monolithic columns in the separation of steroids under isocratic conditions using an aqueous mobile phase. The separation was optimized by changing the temperature rather than by changing the mobile phase composition. Another rapid method for profiling steroids with a wide range of polarity has been developed by Colosi et al. [35]. Utilizing HPLC equipped with MS detection and a monolithic LC column, steroids were detected and quantified using testosterone-d3 as the internal standard. The method was compared to two similar methods using a traditional particulate column in terms of number of steroids eluted, peak area repeatability, limits of detection and overall analysis time. The monolithic method eluted the steroids in a 20 min analysis time, whereas the particulate methods required up to 45 min [35].

Levkin et al. [36] prepared poly(lauryl methacrylate-co-ethylene glycol dimethacrylate) and poly(styrene-co-divinylbenzene) stationary phases in monolithic format using thermally initiated free radical polymerization within polyimide chips featuring channels having a cross-section of 200 µm x 200 µm and a length of 6.8 cm. These chips were then used for the separation of a mixture of proteins including RNase A, myoglobin, cytochrome, and ovalbumin, as well as peptides. Both the monolithic phases based on methacrylate and on styrene chemistries enabled the rapid baseline separation of most of the test mixtures. The best performance was achieved with the styrenic monolith leading to fast baseline separation of all four proteins in less than 2.5 min. The in situ monolith preparation process afforded microfluidic devices exhibiting good batch-to-batch and injection-to-injection repeatability [36]. Furthermore, Zhao et al. [37] developed a capillary chromatographic technique for the separation and detection of proteins, taking advantage of the specific affinity of aptamers and the porous property of the
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monolith. A biotinylated DNA aptamer targeting cytochrome c was successfully immobilized on a streptavidin-modified polymer monolithic capillary column. The aptamer, having a G-quartet structure, could bind to both cytochrome c and thrombin, enabling the separation of these proteins from each other and from the unretained proteins. Elution of strongly bound proteins was achieved by increasing the ionic strength of the mobile phase. The following proteins were tested using the aptamer affinity monolithic columns: human IgG, Hb, transferrin, human serum albumin, cytochrome c, and thrombin. The benefit of porous properties of the affinity monolithic column was demonstrated by the selective capture and pre-concentration of thrombin at low ionic strength and the subsequent rapid elution at high ionic strength. The combination of the polymer monolithic column and the aptamer affinities makes the aptamer-modified monolithic columns useful for protein detection and separation.

Sinner et al. [38] prepared monolithic capillary columns via ring-opening metathesis polymerization (ROMP) using norborn-2-ene (NBE) and 1,4,4a,5,8,8a-hexahydro-1, 4, 5, 8-exo,endo-dimethanonaphthalene (DMN-H₆) as monomers. The monolithic polymer was copolymerized with Grubbs-type initiator RuCl₂(PCy₃)₂(CHPh) and a suitable porogenic system within the confines of fused silica capillaries of different inner diameters (i.d.). Capillary monoliths of 200 μm i.d. showed good performance in terms of retention times, with relative standard deviations (RSD) of 1.9% for proteins and 2.2% for peptides. However, the separately synthesized capillary monoliths revealed pronounced variation in back pressure with RSD values of up to 31%. These variations were considerably reduced by the cooling of the capillaries during polymerization. Using this optimized preparation procedure capillary monoliths of 100 and 50 μm i.d. were synthesized and the effects of scaling down the column i.d. on the morphology and
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on the repeatability of the polymerization process were investigated. The applicability of ROMP-derived capillary monoliths to a separation problem common in medical research was assessed. A 200 µm i.d. monolithic column demonstrated excellent separation behavior for insulin and various insulin analogs, showing equivalent separation performance to Vydac C4 and Zorbax C3-based stationary phases. Moreover, the high permeability of monoliths enabled chromatographic separations at higher flow-rates, leading to shortened analysis times. For the analysis of insulin in human biofluid samples, enhanced sensitivity was achieved using a 50 µm i.d. ROMP-derived monolith [38].

Wieder et al. [39] prepared hydrophobic organosilane-based monolithic capillary columns by thermally initiated free radical polymerization within the confines of 200 µm i.d. fused silica capillaries. A novel cross-linker, bis(p-vinylbenzyl)dimethylsilane (BVBDMS), was copolymerized with p-methylstyrene (MS) in the presence of 2-propanol and toluene, using α,α′-azoisobutyronitrile (AIBN) as initiator. Monolithic capillary columns, differing in the total monomer, microporogen content and nature (2-propanol versus toluene, THF or CH₂Cl₂) were fabricated and the chromatographic efficiency of each monolith for the separation of proteins, peptides and oligonucleotides, was evaluated. The pressure drop versus flow-rate measurements showed the prepared poly(p-methylstyrene-co-bis(p-vinylbenzyl)dimethylsilane) (MS/BVBDMS) monoliths to be mechanically stable and swelling propensity factors of 0.78–1.10 indicated high cross-linking homogeneity [39].

For the separation of peptides with gradient-elution liquid chromatography, Pruim et al. [1] prepared a poly(butyl methacrylate-co-ethylene glycol dimethacrylate) (BMA-co-EDMA) monolithic capillary column. The conditional peak capacity was used as a metric for the performance of this column, which was
compared with a capillary column packed with C18-modified silica particles. The retention of the peptides was found to be less on the BMA-co-EDMA column than on the particulate C18 column. To obtain the same retention in isocratic elution an acetonitrile concentration approximately 15% (v/v) lower was used in the mobile phase. The retention window in gradient elution was correspondingly smaller with the BMA-co-EDMA column. The relationship between peak width and retention under gradient conditions was studied in detail. It was found that in shallow gradients, with gradient times of 30 min and more, the peak widths of the least retained compounds strongly increased with the BMA-co-EDMA column. This was attributed to the fact that these compounds were eluted with an unfavorably high retention factor. With shallow gradients the peak capacity of the BMA-co-EDMA column (=90) was clearly lower than that of a conventional packed column (=150). On the other hand, with steep gradients, when the components were eluted with a low effective retention factor, the performance of the BMA-co-EDMA column was relatively good. With a gradient time of 15 min similar peak widths and thus similar peak capacities (=75) were found for the packed and the monolithic columns. Two strategies were investigated to obtain higher peak capacities with methacrylate monolithic columns. The use of lauryl methacrylate (LMA) instead of BMA gave an increase in retention and narrower peaks for early eluted peptides. The peak capacity of the LMA column was =125 in a 60 min gradient. Another approach used a longer BMA-co-EDMA column which resulted in a peak capacity of =135 in 60 min.

### 1.3 Monolithic phases for bioanalysis using HPLC

The wide variety of applications of silica monolithic phases for HPLC has been summarized by Unger et al. [10], including high-throughput analysis of drugs and
metabolites [11-15,40], separation of complex biological samples (e.g. biofluid samples, proteins and peptides) [16,17,19-21,24,41] and separation of biological samples in more complex multi-dimensional HPLC [22,23,25,42]. High-throughput bioanalysis in a drug discovery setting has promoted the change from simple isocratic elution towards relatively higher flow-rates and fast gradient elution. Fast generic methodology with ballistic gradients, high flow-rates and shorter columns (typically of length 10-50 mm and internal diameters 2.1-4.6 mm) packed with 3-5 μm silica-based phases were used in early applications of high-throughput LC-MS/MS assays to dramatically reduce the chromatographic run times in bioanalysis and ADME (absorption, distribution, metabolism, and excretion) screening [43,44]. Ultra-fast, high-pressure chromatographic LC-MS/MS approaches with short columns and sub-2 μm particle sizes have been reported for quantitative bioanalysis [45-47]. The corresponding increase in column back pressure and smaller sample injection volumes using this approach with biological samples can be avoided using flow-porous monolithic columns. Separation using an eluent flow-rate gradient can be performed on monolithic columns due to their structure and unique hydrodynamic characteristics [48]. The use of monolithic silica columns for screening applications and discovery bioanalysis by fast gradient LC-MS/MS, is now well established [49-52].

Monolithic silica and polymer columns have been commercialized by Merck (Darmstadt, Germany) and by Dionex (Sunnyvale, USA) under the brand names of Chromolith™ (silica) and ProSwift™ respectively. Several reviews have also described the use of a gradient approach for ultra-fast bioanalysis, with run times ranging from less than 10 min for certain separations down to 30 s or less for more high-throughput applications [53-55]. Tzanavaras and Themelis [56] performed high-throughput assay of acyclovir and its major impurity guanine
using the Chromolith™ (100 mm x 4.6mm i.d., Merck) column and a flow-gradient approach in HPLC system [56]. Papp et al. [52] presented a rapid and sensitive method for quantitation of montelukast in sheep plasma using LC-MS/MS. The method used a Chromolith RP column (25 mm x 4.6 mm i.d., Merck) with the gradient concentration of acetonitrile (30-95% acetonitrile in 1 min, 95% for the next 0.2 min and re-equilibration to 30% over 0.3 min) and a simple one-step protein precipitation sample preparation step, which has a limit of quantification of 0.36 ng/mL. A total run time of 1.5 min was achieved with the precision below 5% and an overall relative standard deviation of 8%. In summary, the use of short monolithic columns with fast gradient approaches parallels the increased prominence of bioanalysis and sample numbers to be analyzed in a drug discovery setting.

Monolithic HPLC has been successfully implemented to improve the efficiency and resolution for bioanalytical HPLC assays. In particular, monolithic phase HPLC separations have been established for fast reversed-phase gradient HPLC, with an initial divert to waste period allowing for more selective separation and reduction of ion suppression phenomena with MS detection. A simplified work-flow for this type of assay is indicated in Figure 1-1. This very fast gradient HPLC system allows direct analysis of biological samples that have undergone minimal sample pre-treatment (e.g. protein precipitation using acetonitrile). The initial divert to waste period reduces the introduction of polar components, non-volatile salts, soluble proteins and other endogenous material to the MS. Employment of a fast gradient achieves some separation of the analytes from the remaining endogenous material and gives sharper eluting peaks. Therefore, these fast gradient systems generally exhibit reduced ionization suppression effects as compared to those previously employed using isocratic HPLC conditions (with a
Automated sample prep, dilution, QCs, calibration curve

Addition of analogue IS

Target drug molecule In plasma

Automated sample prep, dilution, QCs, calibration curve

LC-MS/MS quantification

Figure 1-1: Typical assay work-flow for a small molecule drug in plasma in an on-line configuration using a monolithic HPLC column.
high percentage organic mobile phase composition) [52]. The fast gradient systems are simple to set up, requiring only a binary HPLC pump and a single switching valve. A schematic diagram for this type of set-up is indicated in Figure 1-2. Miniaturization of these HPLC column separations has recently been developed, giving MS sensitivity as well as solvent and sample consumption advantages, and narrow-bore and capillary columns are being employed routinely in bioanalytical laboratories.

Miniaturization based on decreasing the i.d. of the chromatographic column has driven the development of HPLC column formats from narrow-bore (2.1 mm < i.d. < 4 mm) to nano-bore (25 μm < i.d. < 100 μm) or even smaller columns (open tubular liquid chromatography < 25 μm) for nano analysis of biological molecules. Four factors have promoted the trend to develop smaller LC columns. The recent developments in capillary-LC have been aimed to enhance the separation efficiency characteristics of capillary techniques, particularly where many analytes need to be separated. The second factor is the overwhelming growth of LC-MS/MS in drug discovery and development; this has in turn spurred the need to interface the MS source more optimally with the column flow-rates to yield more efficient ionization and sensitivity. The third factor is the requirement of capillary-LC columns to analyze smaller-sized bioanalytical samples with less mobile phase consumption. Finally, the development of the chromatographic hardware, in particular, pumps capable of reproducibly delivering flow-rates in the nL/min, has greatly enhanced the practical implementation of miniaturized systems.

Monolithic capillary columns were initially developed for capillary electrophromatography (CEC) and stimulated the introduction of this format for HPLC separations [57]. Capillary-HPLC monolithic columns provide an alternative approach to conventional phases in the analyses of protein molecules, because of
Figure 1-2: Diagram showing the divert valve set-up for a monolithic gradient HPLC system, for direct injection of biofluids (e.g. protein precipitated plasma or diluted urine). Position A is held in high aqueous conditions with the HPLC eluent being directed to waste, thereby removing polar interferences and non-volatile salts. After this hold period the valve is switched to position B to direct the eluent into the mass spectrometer and a fast gradient is performed to elute the analytes. This process is fully automated and is able to eliminate the need for a separate SPE procedure.
one-step fabrication process and faster analysis times [58]. Most of the publications to
date have focused on the synthesis and characterization of monolithic materials for
capillary- or nano-LC with internal diameters of approximately 25-320 µm [58-64]. Urban and Jandera [26] reviewed the synthetic polymethacrylate-based
monoliths in capillary-LC and their applications. Numerous highly important
applications of micro-LC are in the field of analyses of low- and high-molecular
weight biologically active solutes, typically for protein and peptides [64-66]. Zhang et al. [67] recently developed a LC-MS platform, known as the extended
range proteomic analysis (ERPA) for comprehensive protein characterization at
ultra-trace level. Large peptides with or without post-translational modifications
were finely separated with high resolution using nano-bore (20 and 50 µm i.d.)
polystyrene divinylbenzene (PS-DVB) monolithic columns. High sequence
coverage (>95%) analysis for β-casein and epidermal growth factor receptor at
low-molecular levels were achieved using the nano-bore polymer monolithic
column. This methodology has immense potential for the characterization of
biologically important proteins at trace level and the facilitation of biomarker
discovery [61]. The living functionalities of monoliths that allow grafting of
surfaces with functional polymer chains open up a new approach to capillary
column technologies and chemistries [57]. However, there are relatively few
publications that focus on the synthesis and fabrication of polymer monoliths for
the bioanalysis of small molecules using nano-bore HPLC columns.

Most monolithic columns are shorter in lengths, so as to achieve faster
separation and to ensure the consistency of the monolithic morphology throughout
the column. Hosoya et al. [68] prepared novel wired chip devices for µ-HPLC
analyses where the monolithic capillary column is 95 cm length. The latter was
prepared using a tri-functional epoxy monomer, tris(2,3-epoxypropyl)isocyanurate
with a diamine, 4-[(4-aminocyclohexyl) methyl] cyclohexylamine. The prepared column was evaluated by observing the sectional structure of the column with a scanning electron microscope and also by μ-HPLC. The repeatability in the preparation of the long capillary columns was extensively examined for applications of novel wired chip devices. Furthermore, the wired chip device column showed that its high performance was maintained even after chip preparation [68].

1.4 Monoliths for sample preparation using solid phase extraction

Solid phase extraction (SPE) has become one of the most popular sample preparation strategies for the extraction and pre-concentration of small-molecule drugs in biological samples [69,70]. SPE can offer the benefits of a selective clean-up, lower detection limits, high accuracy and precision, amenability to automation and also extended column lifetimes [71]. SPE also offers a large choice of sorbent phases and formats, allowing flexibility with respect to assay development, miniaturization and sample throughput.

SPE sorbents are commonly based on chemically bonded phases of silica, cross-linked polymers or graphitized carbon, with bonded silica-based materials especially dominating the field [72,73]. Particles of the SPE phase can be packed in a cartridge, micro-plate or column [73]. However, there are some inherent limitations of silica-based materials, which include the presence of polar silanol groups, low surface area and narrow pH stability range. Furthermore, an intrinsic problem of all particulate media is their inability to completely fill the available space, with flow-channeling between particles leading to reduced extraction efficiencies and adverse flow characteristics [71,74]. This has led to the development of alternative SPE formats, such as disk type materials with
embedded small sorbent particles [74] or micro-sized disks with bonded phases [75]. By using the disk approach, better extraction recovery can be obtained due to the superior flow control between samples [71,74,76]. The extraction disks used for SPE are normally prepared by punching from larger sheets of material, but the limitation of this approach is that it often results in low sample loading capacity [77].

*In situ* preparation of SPE materials with larger surface area and capacity can be easily achieved with the use of monolithic materials [15,74]. The unique properties of monoliths, in particular their high permeability for liquid biological samples, operation at high flow-rates without loss of efficiency [78,79], low cost, pH stability and ease of preparation, as well as adjustable shape, porosity and selectivity, allow them to meet the requirements of modern miniaturized SPE materials designed specifically for automated operation [7,71].

Many different options exist for the off-line and on-line coupling of SPE to HPLC. The most common approach is the off-line configuration, although it can be a time-consuming process to set up this methodology in an automated fashion, unless specific robotic interfaces are used. In addition, technical difficulties may arise when dealing with smaller sample volumes, and pre-concentration methods and complex elution protocols are also often required. In on-line approaches [80-82], the sample preparation step is embedded into the chromatographic system and manual intervention is minimized, thereby increasing efficiency and sample throughput [83]. Both silica- and polymer-based monolithic sorbents have significant advantages over packed particles for SPE and hence monolithic phases are becoming more popular as sorbent materials for SPE. Several reviews have addressed the applications of silica- and polymer-based monoliths for bioanalytical sample preparation [27,45,74,83].
1.4.1 Off-line sample preparation by SPE

Off-line SPE can be optimized independently from the ensuing HPLC separation and can be easily automated [84]. For this reason, off-line SPE has been frequently performed using a range of commercial disposable cartridges or disks [69,85]. SPE techniques in 96-well format are widely adopted in automated higher throughput quantitative bioanalysis and hence are also well-suited for LC-MS/MS applications [85,86]. The associated liquid transfer steps, including preparation of calibration standards and quality control samples as well as the addition of the internal standard are usually performed using robotic liquid handling systems [83,87]. The principles and application details of automated 96-well SPE have been documented in a number of publications [88,89], and a simplified work-flow for this type of assay is shown in Figure 1-3. In addition, several related small volume 96- and 384-well SPE devices have been described and these provide the benefits of lower solvent requirements, minimal desorption volumes and decreased void volume [84,90-92]. Combinations of a number of techniques (e.g. protein precipitation followed by SPE) and more intricate extraction protocols have also been developed to achieve high purity of the sample extract as well as high sample throughput [93].

Monolithic materials have recently been introduced in the field of SPE and have potentially great impact in some bioanalytical applications [82]. SPE using monolithic silica gel cast into a pipette tip was reported by Miyazaki et al. [94] for the purification of biological samples. The surface of monolithic silica can be chemically modified readily to provide selective extraction of analytes with specific characteristics (e.g. hydrophilic, hydrophobic, phosphorylated form, etc.) Polymer monoliths in SPE can be prepared in situ in a wide range of formats, including capillary, micropipette tips, and microfluidic channels and can offer
Figure 1-3: Typical assay work-flow for a small molecule drug in plasma in an off-line configuration with automated SPE.
exciting possibilities for micro-SPE [18,29,84,94]. Using this approach a wide range of surface functionalities can be accessed either through co-polymerization of suitable functional monomers, simple direct chemical modification of the base polymer or by photografting [95] or coating a suitable monolithic scaffold with functionalized nanoparticles [96,97]. The approach of coating with nanoparticles is particularly attractive as suitable nanoparticles can be synthesized with almost any functionality, including ion-exchange, affinity, chiral, mixed-mode, restricted access, hydrophobic and hydrophilic, and the same scaffold can be used for a wide range of nanoparticles with different chemistries.

There are numerous reports describing off-line SPE using polymer monoliths, with subsequent coupling to HPLC for bioanalysis [98-101]. Altun et al. and Abdel-Rehim et al. [18,102-104] introduced a sample preparation technique using a set of polypropylene tips containing a plug of an in situ polymerized methacrylate-based monolithic sorbent for use with 96-well plates. These sorbents have been used successfully for the extraction and quantification of β-blockers [104] and anaesthetics [103] from human plasma by LC-MS/MS. This novel design permitted 96 samples be extracted in approximately 2 min, with only microlitre volumes of solvent being required for elution. In addition, the unique properties of monolithic materials facilitate a low pressure drop, thus, reducing the risk of blockages due to plasma samples even at higher flow rates. Moreover, they also provide high analyte binding efficiency and yield good recoveries as a result.

This approach can be further extended to include immobilized enzymes for sample digestion. Ota et al. [105] described a novel silica monolith within a pipette tip, where trypsin was successfully immobilized via an aminopropyl group. The trypsin-immobilized monolith was used for rapid digestion of
reduced and alkylated proteins with only minimal manual operations prior to chromatographic analysis, and was evaluated for high-throughput trypsin proteolysis of bio-substances in proteomics [105].

1.4.2 On-line sample preparation SPE

In an HPLC system for bioanalytical applications, monolithic columns can serve either as an on-line extraction cartridge or analytical column, or combinations of this in a column switching or multi-dimensional set-up. Performing the SPE process as part of the HPLC system has recently gained popularity as an automated bioanalytical technique. The major advantage of this approach is that it can incorporate the sample preparation, analyte enrichment, HPLC separation and MS detection steps into a single system. In-line SPE is based on direct injection of crude biological samples, with little pre-treatment (e.g. dilution with internal standard), onto an LC-MS/MS system that incorporates switching valves and multi-column configurations [85]. In its simplest form, bioanalysis using column switching methods involves two columns, the first being used for sample clean up (SPE) and the second for chromatographic separation [45]. Various column dimensions can be configured for the fast analysis of drugs and their metabolites in biological matrices at the ng/mL level or lower. There are minimal sample preparation steps required; except for sample aliquoting, internal standard addition and centrifugation [106,107]. These on-line SPE systems can also be used in 96-well plate format in conjunction with a robotic liquid handling system to provide complete automation. The pre-treated samples are then injected directly onto the LC-MS/MS system auto-samplers with temperature control capability to prevent evaporation and sample instability issues [107]. This can provide a more environmental friendly analytical method which reduces the
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operator’s exposure to hazardous solvents, aerosols and toxins from the samples and sample preparation process [108].

Several generic approaches have recently been developed for on-line sample extraction coupled to LC-MS/MS [109]. These extraction phases include restricted access media (RAM) [110,111], large-size particle disposable cartridges and monolithic phases [88]. Zang et al. [82] described a novel on-line SPE approach integrated with a monolithic HPLC column and triple quadrupole tandem mass spectrometry for direct plasma analysis to simultaneously monitor an eight-analyte test mixture in plasma. A simple column-switching configuration that required only one six-port switching valve and one HPLC pumping system allowed both isocratic and gradient separations for on-line SPE LC-MS/MS. By using the monolithic column, the total analysis time was significantly shortened while high chromatographic separation efficiency was still maintained. This method provided the benefits of minimum sample preparation, simplified hardware configuration, increased sample throughput, efficient chromatographic capability, and a robust bioanalytical LC-MS/MS system.

An alternative on-line sample preparation based on turbulent flow chromatography (TFC) has been well-documented in recent years. A unique characteristic of this on-line sample preparation approach is the use of narrow-bore LC column (typically 1 mm x 50 mm) packed with large irregular shaped particles (typically 30-50 μm) as the stationary phase and relatively high flow-rates. The large particles permit the use of extremely high linear velocities in the ranges of 3-5 mL/min. The combination of the high flow-rate and large particle sizes promotes the rapid passage of the large biomolecules of the biological sample matrix with the simultaneous retention of the small-molecule analytes of interest. This setup
facilitates the sample extraction procedure without significant increases in column pressure or blockages. Monolithic phases have been successfully employed in TFC using high-flow on-line extraction. For example, Xu et al. described the use of a short monolithic silica column for the bioanalysis of plasma and urine [112,113]. The performance of this extraction method was compared with that of an automated liquid-liquid procedure; with both of the methods being found to give similar performance.

Commercially available automated SPE systems, such as Symbiosis™, offer the advantages of both off-line and on-line SPE. These SPE systems have disposable cartridges contained in a 96-cartridge cassette and the use of multiple cartridge trays permits more than 1000 samples to be run [81]. The Symbiosis™ system was described by Alnouti et al. [81] using both conventional C18 and monolithic columns (Chromolith™) for high-throughput direct analysis of pharmaceutical compounds in plasma. The combination of on-line SPE with monolithic columns enabled the development of high-throughput methods with 2 min total analysis time without compromising the data quality. Alternatively, these systems can also be operated in an off-line mode either for method development or routine SPE where the final elution sample is not compatible with the LC set-up (i.e. the requirement for evaporation, buffer reconstitution followed by chromatographic analysis). A multi-dimensional SPE mode allows the use of two different extraction mechanisms on two separate cartridges in one sample run, resulting in a more selective sample clean-up.

1.5 Conclusions and future possibilities

Monolithic materials provide both well-documented advantages and limitations. Future bioanalytical interest in these materials could lie in further adaptations of
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the surface chemistry to select the right phases for isolation of specific analytes for SPE, or to improve separations in HPLC. Once the phases are optimized for a particular assay, other formats and scalability could be tested, with a view to achieving miniaturize and high-throughput analyses. Pipette tip based formats offer some exciting possibilities, especially when multi-purpose devices are used. For example, enzyme digestion and selective SPE can be combined into a single pipette tip using sequential layers of specific-purpose monolithic materials. Furthermore, mixed mode adsorbents that combine ion-exchange and reversed-phase characteristics for instance, can also provide some attractive options.

The use of electrospray nano-flow interfaces with LC-MS/MS platforms has become far more prevalent, particularly in the peptide and biomarker quantification arena. Difficulties in constructing suitable small-scale analytical columns and preparative on-line clean-up columns have been encountered. The latter can comprise specific immunoaffinity extraction columns, which can be difficult to setup and integrate into a HPLC system. Most importantly, it can take a relatively long time to identify and isolate suitable antibody reagents to prepare the column. Small-scale monolithic columns could provide an alternative approach, due to their flexibility and ease of preparation.

Capillary- and nano- LC formats are attractive both from the mass sensitivity and environmental perspective. The trend in bioanalysis is leaning strongly in this direction as samples are decreasing in size with the increased usage of micro-sampling techniques, such as dried blood spot analysis. In this respect, monoliths are likely to find increasing usage in areas such as isolating small volumes for micro-sampling, tailored stationary phases and microchip-based/micro-fabricated formats.
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1.6 Project aims

As presented in the literature review, monolithic materials, especially organic polymer monoliths have been widely used for the separation and sample pretreatment process in bioanalysis. Organic polymer monoliths also possess significant potential to be developed in different formats and functionalities, which suggests their versatile applicability to all types of bioanalytical problems.

Taking into consideration the possibilities for future developments in this field as identified in the literature review above, this project aims to explore ways in which monoliths can be designed for use in high throughput absorption, distribution, metabolism and elimination (ADME) assays. The focus in all of this work will be on methods to miniaturize this analysis. The specific aims of the project are to:

- Investigate and synthesize of a range of functional polymer monoliths both in bulk and in situ within capillaries and characterize the stationary phase properties, such as porosity, morphology and ion-exchange functionality.
- Test the suitability of the monolithic phases and optimize the selective separation process of a set of target analytes of importance to ADME assays using a capillary LC system.
- Synthesize the optimized macroporous polymer monoliths in pipette tip formats and explore the possibility of using trypsin immobilized enzyme polypropylene pipette (IMEPP) tips for high throughput analyses.
- Characterize and implement the developed IMEPP tips in a real bioanalytical setting, i.e. for qualitative and quantitative analysis of target proteins in biological matrix.
- Synthesize these enzyme reactors in a range of different formats for different bioanalytical settings.
1.7 References


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Chapter 2

Experimental

This section describes the instrumentation, chemicals and procedures used throughout this research, unless otherwise specified in a particular chapter.

2.1 Instrumentation

For the preparation of the monolith phases *in situ* within capillary and pipette tips, an OAI deep UV illumination system (Model LS30/5, San Jose, CA, USA) fitted with a 500W HgXe-lamp was used for UV exposure. For calibration, the irradiation power was adjusted to 20.0 mW/cm², using an OAI model 206 intensity meter with a 260 nm probe head. The Teflon®-coated UV transparent fused-silica capillary (75 μm I.D. x 375 μm O.D.) was obtained from Polymicro Technologies Inc. (Phoenix, AZ, USA). Finntip-200 pipette tips consisting of homopolymeric isotactic polypropylene (PP) were purchased from Pathtech Pty Ltd (Victoria, Australia).

Scanning electron microscopy (SEM) was performed on a DSM 962 scanning electron microscope (Zeiss, LEO) equipped with a DX-4 X-ray detection system by EDAX. The SEM provides secondary and backscattered electron imaging of biological, geological and other specimens yielding surface information. SEM operated in low and high vacuum mode using accelerating voltages of 15, 20 and 25 kV.

The HPLC system used throughout this study was a Dionex Corporation UltiMate® 3000 capillary LC system featuring an integrated flow-splitter connected to a continuously monitored flow meter and control valve, which maintained constant flow in the range of 0.1 to 10.0 μL/min. It comprised a binary pump, a
vacuum degasser, a column thermostat, an autosampler and a photodiode-array UV detector. Chromeleon® Chromatography Management System version 6.80 was used for the system control, data acquisition and processing. OriginLab 8.0 software (Northampton, MA, USA) was also used for data analysis and graphing.

Direct infusion ESI-MS experiments were performed using a quadrupole time-of-flight MS, Bruker MicrOTOF-Q MS controlled by a MicrOTOF-Q Control software from Bruker Daltonics (Bremen, Germany). The liquid sample was delivered via a KD Scientific (Holliston, MA, USA) syringe pump. The pump was set at 180 µL/hr and an appropriate syringe from Hamilton (Reno, Nevada, USA) was employed. Acquisition time was at least 2 min for each sample using the tune wide LC method of the software.

For LC-MS separations, an Agilent 1200 Series LC system (Santa Clara, CA, USA) consisting of a binary pump, a vacuum degasser, an autosampler, a thermostatted column (30 °C) (Agilent Technologies, Palo Alto, CA) was coupled to a Bruker micrOTOF-Q operating at a resolution of 10000 in the positive ion mode. Argon was used as a nebulizing gas (1.5 bar) and nitrogen as a drying gas (8.0 L/min, 200 °C). The capillary voltage was set to +4.0 kV. The reversed-phase (RP) analytical column was a Dionex Acclaim® PolarAdvantge C16 (2.1 x 100 mm, 3 µm) coupled with binary RP solvents comprising (A) water and (B) acetonitrile, both containing 0.1% formic acid. The data were collected and handled with the Bruker Compass DataAnalysis 4.0 software.

Multiple reaction monitoring (MRM) transition development and optimization were performed on an AB SCIEX Triple Quadrupole™ 5500 mass spectrometer (Foster City, CA, USA) coupled to an Agilent 1100 binary HPLC pump. An external Valco valve (10 or 6 port valves can be used) in “back flush mode” was employed to control the column switching for both the loading and
eluting gradient. A CTC PAL autosampler (Zwingen, Switzerland) fitted with a 250 µL syringe and 250 µL sample loop (for larger volume injection) was employed for sample introduction. The system was controlled using Analyst version 1.5.1 which was also used for data acquisition and processing. An Onyx monolithic RP C18 guard column (Torrance, CA, USA) and a Waters Xbidge BEH130 C18 column (2.1 x 100 mm, 3.5 µm) (Milford, MA, USA) were used as pre-column and analytical column in addition to the RP solvents were (A) 5% acetonitrile/ 95% water and (B) 95% acetonitrile/ 5% water, both containing 0.1% formic acid. Chromatography was carried out using a fast gradient; following a 2.5 min sample loading, peptides were eluted over a 22 min gradient from 0-45% “B”. The column was washed with 95% “B” for 1 min and re-equilibrated at initial conditions for 3 min prior to the next injection.

2.2 Reagents

Unless specified otherwise, the chemicals used were of analytical grade and are listed in Table 2.1 to 2.6.
## Table 2.1: Chemicals used as buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Formula</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>CH₃COOH</td>
<td>BDH Chemicals</td>
</tr>
<tr>
<td>Formic acid</td>
<td>HCOOH</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>CF₃COOH</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>Na₂HPO₄</td>
<td>Merck</td>
</tr>
<tr>
<td>Ammonium acetate</td>
<td>CH₃CO₂NH₄</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ammonium bicarbonate</td>
<td>CH₃COONa</td>
<td>BDH Chemicals</td>
</tr>
<tr>
<td>Tris(hydroxymethyl)aminomethane hydrochloride</td>
<td>NH₂C(CH₂OH)₃·HCl</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate</td>
<td>NaH₂PO₄</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>HCl</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium hydroxide (99.99% and 99.0%)</td>
<td>NaOH</td>
<td>Sigma-Aldrich</td>
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</table>
Table 2.2: Chemicals used as analytes

<table>
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<tr>
<th>Analyte</th>
<th>Formula</th>
<th>pKₐ</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterenol, hydrochloride</td>
<td>(HO)₂C₆H₃CH(OH)C H₂NH₂ · HCl</td>
<td>8.55</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Propranolol hydrochloride</td>
<td>C₁₆H₂₁NO₂ · HCl</td>
<td>9.5</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ketoprofen,</td>
<td>C₁₈H₁₄O₃</td>
<td>5.94</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>C₁₄H₁₀Cl₂NO₂</td>
<td>4</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>C₁₃H₁₈O₂</td>
<td>4.4</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Pindolol</td>
<td>C₁₄H₂₀N₂O₂</td>
<td>9.04</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Acebutolol</td>
<td>C₁₈H₂₈N₂O₄</td>
<td>9.2</td>
<td>Sigma-Aldrich</td>
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</table>
### Table 2.3: Chemicals used for methacrylate polymerization

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Formula</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-methacryloxypropyltrimethoxysilane (γ-MAPS)</td>
<td>C₁₀H₂₀O₅Si</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>1,4-butandiol</td>
<td>HO(CH₂)₂OH</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>1-propanol</td>
<td>CH₃(CH₂)₂OH</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>2,2-dimethoxy-2-phenylacetophenone (DMAP)</td>
<td>C₆H₅COC(OCH₃)₂C₆H₅</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Methanol</td>
<td>CH₃OH</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Cyclohexanol</td>
<td>C₆H₁₂O</td>
<td>Merck</td>
</tr>
<tr>
<td>Decanol</td>
<td>C₁₀H₂₂O</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Dodecanol</td>
<td>C₁₂H₂₆O</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Basic alumina activity grade I, Type WB-2</td>
<td>Al₂O₃</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Butyl methacrylate (BMA)</td>
<td>H₂C=CH(CH₃)CO₂-(CH₂)₃CH₃</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>3-Sulfopropyl methacrylate (SPMA)</td>
<td>H₂C=CH(CH₃)CO₂(CH₂)₃SO₃K</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ethylene glycol dimethacrylate (EDMA)</td>
<td>[H₂C=CH(CH₃)CO₂CH₂]₂</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Methyl methacrylate</td>
<td>CH₂=CH(CH₃)COOCH₃</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>2-Hydroxyethyl methacrylate (HEMA)</td>
<td>H₂C=CH(CH₃)COOCH₂CH₂OH</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Azobisisobutyronitrile (AIBN)</td>
<td>(CH₃)₂C(CN)N=NC(CH₃)₂CN</td>
<td>Sigma-Aldrich</td>
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### Table 2.4: Protein samples used for studies

<table>
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<th>Protein</th>
<th>Molecular Weight (Da)</th>
<th>Source</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>23800</td>
<td>Bovine pancreas</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Melittin</td>
<td>2846.5</td>
<td>Bee venom</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Calcitonin gene related peptide</td>
<td>3806.3</td>
<td>Rat</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>11701.5</td>
<td>Equine heart</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>16951.4</td>
<td>Equine heart</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Transferrin</td>
<td>77050</td>
<td>Human</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>66000</td>
<td>Bovine</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Polyclonal IgG</td>
<td>150000</td>
<td>Human</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

### Table 2.5: Organic solvents used in this work

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Formula</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>(CH₃)₂CO</td>
<td>BDH Chemicals</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>CH₃CN</td>
<td>Merck</td>
</tr>
</tbody>
</table>
## Chapter 2

### Table 2.6: Other chemicals used in this work

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Formula</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>NH₂CONH₂</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Guanidine hydrochloride (GdnHCl)</td>
<td>CH₅N₃ · HCl</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Iodoacetamide (IAA)</td>
<td>ICH₂CONH₂</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>DL-Dithiothreitol (DTT)</td>
<td>HSCH₂CH(OH)CH(OH)C</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Methoxy polyethylene glycol 550 methacrylate (PEGMA)</td>
<td>H₂C=C(CH₃)CO₂(CH₂CH₂O)nCH₃</td>
<td>Sartomer</td>
</tr>
<tr>
<td>Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)</td>
<td>C₉H₁₅O₆P · HCl</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>4,4-Dimethyl-2-vinyl-2-oxazolin-5-one (VAL)</td>
<td>C₇H₁₉NO₂</td>
<td>Tokyo Chemical Industry Co.</td>
</tr>
<tr>
<td>Benzophenone</td>
<td>(C₆H₅)₂CO</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Copper(II) sulfate pentahydrate</td>
<td>CuSO₄·5H₂O</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>KCl</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>NH₂CH₂CH₂OH</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>
2.3 Procedures

2.3.1 Buffer and standard preparation

Buffers, analyte and protein standards were prepared with water purified by a Milli-Q water purification apparatus (Millipore, Bedford, MA, U.S.A.). All buffers were degassed using vacuum sonication and filtered with a Millex-HA 0.45 μm disc filter (Millipore). Monomers were purified prior to use by passage through a bed of basic alumina (Brockman activity I, 60-325 mesh), followed by distillation under reduced pressure. Distilled monomers were stored at -20 °C in the freezer.

2.3.2 Sample injection

For the HPLC separations, samples were injected on to the monolithic column via the HPLC autosampler. The capillary HPLC column temperature was maintained at 40 °C. The mobile phases A and B consisted of 0.1% TFA in water (v/v) and 0.1% TFA in acetonitrile (v/v), respectively. For all samples, the injected volume was 1 μL. Preliminary UV analyses were performed at several different wavelengths for each drug sample, so as to select the optimum wavelength for all the drug analytes and utilize a single wavelength UV detector optimally.

For the LC-MS separations, samples were injected on to the column via the Agilent 1200 Series autosampler.

For the Agilent 1100/ AB SCIEX Triple Quadrupole™ 5500 mass spectrometer, samples were introduced by CTC PAL auto sampler fitted with a 250 μL syringe and 250 μL sample loop.

2.3.3 Surface modification in fused-silica capillaries

In order to anchor the monolithic structure to the capillary wall by covalent bonding, the fused-silica capillaries were surface-modified using the procedure of Schaller et al. [1]. The Teflon®-coated UV transparent fused-silica capillaries were
rinsed using a Harvard syringe pump (Harvard Apparatus, Holliston, MA, USA) and a 250 μL gas-tight syringe (Hamilton Company, Reno, NE, USA) with acetone and water, activated with 0.2 mol/L NaOH for 30 min, washed with water, then with 0.2 mol/L HCl for 20 min, rinsed with water and ethanol. A 20% (w/w) solution of γ-methacryloxypropyl-trimethoxysilane (γ-MAPS) in 95% ethanol adjusted to pH 5 using acetic acid was pumped through the capillaries at a flow-rate of 15 μL/hr for 1 h. The capillary was then washed with acetone and dried with a stream of air and left at room temperature for 24 h.

2.3.4 Synthesis of polymer monoliths in capillaries

The monomers (butyl methacrylate and sulfopropyl methacrylate), cross-linker (ethylene glycol dimethacrylate), porogens (1,4-butanediol and 1-propanol) were mixed with the UV initiator 2,2-dimethoxy-2-phenylacetophenone (DMAP) to give a clear, organic solvent mixture. This mixture was sonicated for 10 min and subsequently degassed with nitrogen for 5-10 min prior to UV-initiated polymerization. The overall ratio of monomer to porogen was held constant. Only the relative composition of the porogenic solvents was varied to give a controlled porous structure. A small portion of polymerization mixture was pumped into a fused-silica capillary which had previously been derivatized with γ-MAPS. The ends of the capillary were plugged with rubber septa. The capillary containing the polymerization mixture was irradiated under constant UV irradiation for 10 min using the OAI deep UV illumination system described in Section 2.1. The resultant monolithic column was flushed with methanol for 5 h at 1200 kPa, followed by water using a syringe pump, prior to the HPLC separations.

2.3.5 Measurement of ion-exchange capacity

The ion-exchange capacity of the monolithic stationary phases synthesized in situ in the capillary was determined by copper adsorption/desorption using the liquid
chromatography system (Dionex). The capillary columns were first loaded with potassium using 0.2 mM potassium chloride solution. Excess chloride was flushed from the column using MilliQ water at a rate of 0.2 μL/min for a period of 30 min. The capillary was disconnected from the pump and the system was flushed with 0.1 mM copper(II) sulfate pentahydrate (CuSO₄·5H₂O) solution for a period of 30 min using a flow-rate of 2 μL/min. The capillary column was reconnected to the system and 0.1 mM copper solution was pumped through the capillary at a rate of 0.2 μL/min. The absorbance of the eluent was monitored at 235 nm until breakthrough was observed and the absorbance reached a stable value. The process was further repeated twice.

2.3.6 Porosimetry

Porosimetry measurements were performed by mercury intrusion (Micrometrics Pore Sizer 9310, Norcross, Georgia, USA), using a 3 cc glass penetrometer designed for powder analysis. Characterization measurements were conducted on 0.1 g of polymer material placed into the penetrometer head, before securely fastening the lid with a nut and screw cap. Pressure measurements were made from levels between 0.5 psi and 30,000 psi (2,000 bars), yielding pore size distributions in the range of 0.003 to 360 μm.

2.3.7 Surface modification in polypropylene pipette tips

Finntip pipette tips (Finntip-200) consisting of homopolymeric isotactic polypropylene (PP) [3] were used in this study. The surface modification in pipette tip was done by using both single- and two-step photografting according to the procedure described previously [3] (Figure 2-1). The photoinitiator benzophenone was used to generate radicals at the surface of PP by hydrogen abstraction (Figure 2-1). For single-step photografting, a stock solution containing methyl methacrylate (MMA) (0.485 g) and ethylene glycol dimethacrylate (EDMA) (0.485 g) with a ratio
Figure 2-1: Schematic diagram of the single- and two-step photografting procedures.
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(1:1) (% w/w) was prepared. From this stock solution, a modification mixture containing MMA:EDMA:BP (benzophenone) (3% w/w) (0.03 g) was prepared. Before use, the stock solution was vortexed and purged with nitrogen for 10 min to remove oxygen. The Finntip pipette tips were then filled with 60 µL modification mixture and sealed with parafilm [Figure 2-2 (a)]. The filled pipette tips (with tapered end sealed with parafilm) were irradiated on both sides of the tips under UV light for 12 min (or 10.5 min if EDA was used). Once the reaction was complete, the pipette tip was washed with methanol and acetone, before drying in a vacuum oven at room temperature for 10 min. For the two-step surface modification procedure, BP was first grafted to the surface of PP tips by filling the tips with 3% (w/w) BP solution in methacrylate and sealed with parafilm. The filled tip was irradiated under UV for 12 min. After photgrafting BP, the tip was rinsed with methanol and dried. Next, the tip was filled with the monomer mixture (MMA and EDMA) and irradiated under UV. Upon completion of the reaction, the pipette tip was washed with methanol and acetone, and was then dried in a vacuum oven at room temperature for 10 min.

**Figure 2-2**: Schematic diagram of the photoinduced surface modification and preparation of a monolith in an empty pipette tip. (a) Tip is filled with a BP solution in methacrylate and irradiated with UV source; (b) a grafted compatibilizing polymer layer containing free double bond is created at the surface.
2.3.8 Preparation of porous polymer monoliths in PP tips

A polymerization mixture consisting of 16% (w/w) BMA, 24% (w/w) EDMA, 12% (w/w) 1,4-butanediol, 42% (w/w) 1-propanol and 1% (w/w) DMAP (with respect to monomers) was prepared and purged with nitrogen for 10 min. The surface-modified pipette tip was filled with 20 µL of the homogenous polymerization mixture, and irradiated using an OAI deep UV illumination system. The polymerization was allowed to proceed first for 40 min with the sharp end of the tip down, and then for 25 min with the sharp end up (Figure 2-3), resulting in a 1 cm monolith support in situ polypropylene pipette tip. After photopolymerization, the pipette tip with polymer monolith was then washed with methanol for 1 h using the vacuum filtration apparatus (Figure 2-4), to allow the solvents to flow through the porous monolith. The pore size of the monolith was ~ 2.8 µm, as measured by mercury intrusion porosimetry [4].

2.3.9 Digestion protocol for immobilized enzyme polypropylene pipette (IMEPP) tip and MonoTip® Trypsin

The pipetting procedure of the IMEPP tip was done by attaching silicone tube to a curved tip syringe (Monojet 412, Figure 2-5). The IMEPP tip was equilibrated by aspirating and dispensing the digestion buffer for 10 cycles. The protein sample solution was heated in a dry bath incubator at 37 ºC prior to the digestion by IMEPP tip. Then, protein sample was aspirated and dispensed (20 cycles). The pipette tip was removed from the syringe and soaked with the sample solution for 30 min. The tip was removed and reconnected to the syringe to dispense the remaining digestion solution at the tip end. After the digestion procedure was complete, the solution with peptides was protonated by adding formic acid in order to achieve a final concentration of 0.1%. The same digestion procedure was used for the MonoTip® Trypsin except that an autopipette was used instead of the
Figure 2-3: Schematic diagram showing *in situ* preparation of polymer monoliths in polypropylene pipette tip.
Figure 2-4: A vacuum filtration apparatus was assembled using a filter flask with a side arm which permits making a connection to vacuum pump. The top opening of the suction flask accommodates a two-hole rubber stopper which in turn supports a glass tube specifically used for suction operation for pipette tips.
Figure 2-5: Silicone tube attached to curve syringe (Monojet 412) for pipetting IMEPP tip.
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curved tip syringe.

2.3.10 Scanning electron microscopy

The monolithic capillary was cut into 1 cm fragments that were mounted perpendicularly to a 12 mm pin-type aluminium stub using epoxy resin. High-resolution images were obtained by coating the capillary with gold or platinum (nominally 40 nm thick). In order to take cross-sectional SEM images of the pipette tips, samples approximately 3 mm in length were cut from the tip with a razor blade and mounted on an aluminum stub. This was coated with a thin layer of platinum using an EIKO IB5 high-resolution platinum coater.

2.4 Calculations

2.4.1 Permeability based on Darcy’s Law

The flow of solution through a porous medium is directly proportional to the pressure drop over a given distance. Using this relationship, the permeability $B_0$, which represents the resistance to mobile phase flow through the monolithic column can be calculated by pumping five different solvents through the column at different linear flow-rates according to [4] where $B_0$ is the column permeability, $v$ is the linear velocity (m/min), L is the column length (m), $\eta$ is the dynamic solvent viscosity (Pa·s) and $\Delta P$ is the column back pressure (Pa).

$$B_0 = \frac{vL\eta}{\Delta P}$$

2.4.2 Retention factors

Retention factors were calculated using the following expression:

$$k = \frac{t_R - t_0}{t_0}$$
where \( t_R \) is the elution time of the analyte and \( t_0 \) the elution time of the flow marker.

### 2.4.3 Sequence coverage

Sequence coverages were calculated using the following expression:

\[
S.C. = \frac{P_1}{P_2} \times 100\%
\]

where \( P_1 \) is the number of peptides identified, \( P_2 \) is the expected number of peptides after protein digestion. Sequence coverages of over 80% are very useful and important for proteomic studies.

### 2.5 References


Chapter 3

Chapter 3

Mixed-Mode Porous Polymer Monoliths for Separation of Small Molecule Pharmaceuticals

3.1 Introduction

The increasing cost of drug discovery and development places a significant burden on the drug candidate evaluation process. It has been estimated that discovering and bringing one new drug to the market takes an average of 14 years of research and clinical development efforts, and costs around 2 billion US dollars. Of ten thousand or more drugs tested in early drug discovery, only one may eventually lead to a drug available to the market. Therefore, high-throughput adsorption, distribution, metabolism and excretion (ADME) assays for compound drug development properties are extremely important in this process [1].

In recent years, monolithic supports as stationary phases in HPLC have gained significant interest in pharmaceutical analysis, in particular for small molecules as well as protein and peptide separations in gradient and isocratic modes [2-4]. The most prevalent hyphenated techniques, liquid chromatography coupled to tandem mass spectrometry, have become critical tools in pharmacokinetics and metabolism studies at various drug development stages [5]. Tandem mass spectrometry based methods provide unique analytical sensitivity, selectivity and speed for monitoring one specific target compound in biological samples. This has reduced the need for high resolution power in LC analysis. Despite only acceptable resolution being required in the sample analysis, certain sample preparation steps are needed to remove endogenous interference species.
from the matrix prior to the HPLC-MS/MS assay for small molecules. This prevents the HPLC column from clogging in reversed-phase (RP) chromatography and to avoid ion source contamination in the mass spectrometer [5]. In addition, the use of capillary columns with a nanospray interface gives better compatibility and sensitivity with mass spectrometry. Other advantages of the capillary column format include the small consumption of both sample and solvents and providing fast assays and short method development timelines [6]. Therefore, an increasing number of researchers are focusing on improving the capacity, selectivity and repeatability of columns in miniaturized formats [7-9].

Monolithic materials can be divided into two main categories, namely silica-based (inorganic) and rigid polymer (organic) based monoliths. The silica-based monoliths can be prepared by a sol-gel method [10] and the rigid polymer-based monoliths are generally made by in situ polymerization of monomers, cross-linkers and porogens [11]. Polymer-based monoliths have been widely used as matrices for sample preparation and separation since being first introduced by Hjerten et al. [12] and following further innovations by Svec and Fréchet [13]. Although there are some concerns of using polymer-based monoliths for sample preparation, such as low surface area and the relatively poor chromatographic performance for small molecules [6,14,15], the advantages of these materials clearly outweigh the silica-based monoliths in terms of the pH stability and a wealth of surface chemistries available [14]. Monolithic materials also offer particular advantages for high-throughput ADME assays due to high permeability (i.e. high flow-rates can be used) and excellent column stability [9,16].

Mixed-mode strong cation-exchange (SCX) and reversed-phase (RP) materials are the most often used modes of chromatography for the extraction and separation of different types of compounds of pharmaceutical interest [17]. These
approaches have been used for over 80% of the separation of target analytes in pharmaceutical analysis [18]. Several approaches have been reported to synthesize strong SCX polymer monoliths containing sulfonic acid groups, including adsorption [19], post-modification [20], and copolymerization [21]. Recently, monolithic columns with mixed-mode of strong anion-exchange (SAX) and hydrophilic (HI) or RP interactions have also been reported by several groups [20,22,23]. The mixed-mode monolithic columns with SAX interaction were used to counter undesirable electrostatic adsorption between positively charged basic compounds and the stationary phases [24,25]. These columns can be prepared using a monolithic matrix containing active groups followed by their functionalization with versatile surface chemistries [20,26]. However, the post-functionalization strategy with multiple steps can be time-consuming and precise control over the number of ionizable functionalities can be difficult to achieve. An alternative approach to obtain ion-exchange functionality monolithic columns is the direct incorporation of ion-exchange functional monomers into continuous polymer matrices by the co-polymerization of suitable monomers and cross-linkers. For example, Lin et al. [22] reported a novel a mixed-mode SAX-HI stationary phase for the separation of polar-charged nucleotides and neutral compounds. Jiang et al. [17] fabricated a SCX-RP monolithic columns for the separation of basic compounds using micro-HPLC. Both works have demonstrated the fast preparation procedures of mixed-mode functionality monolithic columns and it is easy to control the average concentration of functional groups in the monolith, thus, more repeatable and manufacturable.

Despite the enormous significance for users, only a few studies relating to the repeatability and stability of polymer-based monolithic columns are found in the literature [9,27]. Geiser et al. [9] used three standard proteins – ribonuclease A,
cytochrome c and myoglobin to investigate the stability and repeatability of poly(BMA-co-EDMA) capillary columns. They found excellent repeatability of retention times for the reparation of three proteins as evidenced by percent relative standard deviation (%RSD) values of less than 1.5%. The stability of retention times was also monitored and no significant shifts in either retention times or backpressure were observed after more than 2200 protein separations. Recently, Li et al. [28] reported a highly cross-linked network resulting from single crosslinking monomers that improved column-to-column repeatability, better mechanical stability and higher surface area of polymeric monoliths [14]. However, this polymer monolith prepared by a single monomer does not make it easily possible to provide mixed-mode functionality. To date, there are also only a few studies on the stability and repeatability of mixed-mode SCX-RP functionalities polymer-based monolithic columns for capillary LC [17,22]. Further effort is therefore needed to develop novel stationary phases with negatively charged and RP functionalities for highly efficient separation of polar analytes. In this regard, we report on the characterization, repeatability and stability of a mixed-mode SCX-RP polymeric monolithic stationary phase prepared by the copolymerization of a functional monomer, namely 3-Sulfopropyl methacrylate (SPMA) with BMA and EDMA within the confines of 75 µm i.d. fused-silica capillaries. The impact of different polymerization parameters (monomer content, porogenic solvent and cross-linker) on the porous properties, and hence on the separation selectivity for some acidic drugs and β-blockers, was studied.

3.2 Experimental

The general experimental details are described in Chapter 2. Detailed conditions are elaborated in each of the figure captions.
3.2.1 Preparation of porous polymer monoliths in fused-silica capillaries

The surface modification in fused-silica capillaries was done using the procedure of Schaller et al. [29]. The short (~10-15 cm in length) surface-modified capillary was filled by capillary action with the degassed polymerization mixture consisting of monomers, porogens together with 1% (w/w) DMAP (with respect to monomers). Several SPMA monoliths that consist of similar composition and percentages of monomers but differ in the composition of porogens were prepared (S1-S7), as described in Table 3.1. In addition, monoliths (S1M-S3M) consisting of a greater amount of cross-linker (EDMA) than the functional monomer (BMA) were prepared, as shown in Table 3.2. The short columns filled with the polymerization mixture were placed under the light source and irradiated with UV light for 10 min at a distance of 30 cm. The unreacted porogens were removed from the short monolithic columns by pumping the columns with methanol using a syringe pump at a flow-rate of 100 µL/hr for 4 h before being conditioned with water and mobile phase, both for 1 h at 30 µL/hr.

3.2.2 Bulk monolith preparation

Bulk polymers were prepared using a sandwich container (Figure 3-1) for characterization studies. The sandwich container is made of stainless steel and has a diameter of 6.35 cm. It consists of two halves; a base with a thickness of 1 cm and an upper rim which is 0.45 cm thick. The central part of the base is made of polypropylene and there is a shallow cavity which has a diameter of 3.5 cm and a depth of 2 mm. For the monolith formation, the polymerization mixture was injected into this shallow cavity, and a piece of quartz plate of 4.6 cm in diameter and 2 mm in thickness was placed in between the two halves of the container. The two halves were fastened together with three screws that are at 120 degrees from
Table 3.1: Effect of variation of the percentage of porogenic solvents on the pore size of the studied monoliths (S1-S7) prepared from a polymerization mixture consisting of 0.4% (w/w) SPMA, 23.6% (w/w) BMA and 16% (w/w) EDMA that constitute 40% (w/w) of the polymerization mixture with respect to 60% (w/w) porogenic solvents.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>1,4-butanediol [% (w/w)]</th>
<th>1-propanol [% (w/w)]</th>
<th>Water [% (w/w)]</th>
<th>Pore size [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>30</td>
<td>60</td>
<td>10</td>
<td>2.70</td>
</tr>
<tr>
<td>S2</td>
<td>20</td>
<td>70</td>
<td>10</td>
<td>2.20</td>
</tr>
<tr>
<td>S3</td>
<td>10</td>
<td>80</td>
<td>10</td>
<td>0.14</td>
</tr>
<tr>
<td>S0</td>
<td>0</td>
<td>90</td>
<td>10</td>
<td>NA</td>
</tr>
<tr>
<td>S4</td>
<td>90</td>
<td>0</td>
<td>10</td>
<td>NA</td>
</tr>
<tr>
<td>S5</td>
<td>80</td>
<td>10</td>
<td>10</td>
<td>NA</td>
</tr>
<tr>
<td>S6</td>
<td>70</td>
<td>20</td>
<td>10</td>
<td>1.06</td>
</tr>
<tr>
<td>S7</td>
<td>60</td>
<td>30</td>
<td>10</td>
<td>1.36</td>
</tr>
</tbody>
</table>

NA – Polymer does not form for measurement.
Table 3.2: Effect of variation of the percentage of porogenic solvents and alcohol chain length on the pore size of the studied monoliths (SMeth-SL) prepared from a polymerization mixture consisting of 0.4% (w/w) SPMA, 23.6% (w/w) BMA and 16% (w/w) EDMA that constitute 40% (w/w) of the polymerization mixture with respect to 60% (w/w) porogenic solvents (percentage shown in table).

<table>
<thead>
<tr>
<th>Polymer*</th>
<th>1,4-butanediol [% (w/w)]</th>
<th>Alcohol [% (w/w)]</th>
<th>Water [% (w/w)]</th>
<th>Pore size [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1Meth</td>
<td>30</td>
<td>60</td>
<td>10</td>
<td>1.23</td>
</tr>
<tr>
<td>S2Meth</td>
<td>20</td>
<td>70</td>
<td>10</td>
<td>1.68</td>
</tr>
<tr>
<td>S3Meth</td>
<td>10</td>
<td>80</td>
<td>10</td>
<td>2.24</td>
</tr>
<tr>
<td>S1C</td>
<td>30</td>
<td>60</td>
<td>10</td>
<td>1.71</td>
</tr>
<tr>
<td>S2C</td>
<td>20</td>
<td>70</td>
<td>10</td>
<td>0.97</td>
</tr>
<tr>
<td>S3C</td>
<td>10</td>
<td>80</td>
<td>10</td>
<td>0.07</td>
</tr>
<tr>
<td>S1D</td>
<td>30</td>
<td>60</td>
<td>10</td>
<td>1.90</td>
</tr>
<tr>
<td>S2D</td>
<td>20</td>
<td>70</td>
<td>10</td>
<td>NA</td>
</tr>
<tr>
<td>S3D</td>
<td>10</td>
<td>80</td>
<td>10</td>
<td>NA</td>
</tr>
<tr>
<td>S1L</td>
<td>30</td>
<td>60</td>
<td>10</td>
<td>2.07</td>
</tr>
<tr>
<td>S2L</td>
<td>20</td>
<td>70</td>
<td>10</td>
<td>NA</td>
</tr>
<tr>
<td>S3L</td>
<td>10</td>
<td>80</td>
<td>10</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA – Polymer does not form for measurement.

*Meth: methanol (C1)
C: cyclohexanol (C6)
D: decanol (C10)
L: dodecanol (C12)
one another. The polymerization mixture was inserted via a syringe fitted with a 25 gauge syringe needle in the sandwich container. With the solution in place and the two halves of the sandwich container secured, the container was then irradiated under UV for 20 min. For characterization of the porous properties, the polymer material was removed from the sandwich container and the material was extracted with methanol using Soxhlet apparatus for 12 h, before being vacuum-dried at 60 °C for a further 12 h. These materials were later used for the determination of pore size distribution and pore volume.

3.2.3 Standard solutions and sample preparation

Drug samples arterenol, propranolol and ketoprofen, diclofenac and ibuprofen were prepared in water and methanol at 1 mg/mL. All were further diluted or multicomponent standard mixtures of samples were made using ultra-pure water from the Milli-Q Element system (Millipore, Billerica, MA, USA).

Figure 3-1: Sandwich container used for bulk monolith preparation.
3.3 Results and discussion

3.3.1 Preparation of polymer monoliths

Several prerequisites should be met when designing a macroporous monolithic polymer for use in separation media and solid-phase extraction (SPE). Among these, material surface chemistry, porosity, pore size distribution and rigidity constitute the core of the monolith formation strategy [14]. In the monolithic systems studied here, the first two variables depended on the type of monomers used. SPMA was used as a functional monomer for its strong cation-exchange properties, EDMA was used as a crosslinking monomer and BMA monomer was used as a functional monomer with RP properties. Since the other variables are closely related to the porous structure of the monolith, these depend on the pore-forming solvent (porogens). Consequently, the number of variables that were used for the control of porous properties in this system was limited to: a) the ratio of monomers to pore-forming solvents in the polymerization mixture, b) the percentage of different solvents in the pore-forming mixture and c) the ratio of cross-linker to functional monomers in the polymerization mixture.

SPMA-based monolithic fused-silica capillary columns with 75 μm internal diameter and varying pore properties were prepared in situ by a UV-initiated free-radical copolymerization reaction. The mixed-phase monomers consisted of sulfopropyl methacrylate SPMA [0.4% (w/w)], butyl methacrylate BMA [23.6% (w/w)] and ethylene dimethacrylate EDMA [16% (w/w)], with 1,4-butanediol, 1-propanol and water as porogenic solvents, and 2,2-dimethoxy-2-phenylacetophenone (DMAP) as the photo initiator. As the porosity of monolithic phases can be varied by making minor alterations to the composition of the polymerization mixture, the total monomer concentration in the prepared phases was kept constant at 40% (w/w) with respect to the total porogen solvents.
consisting of 1,4-butandiol, 1-propanol and water at 60% (w/w). Thus, only the percentage of solvents in the total porogenic solvent composition, the percentage of cross-linker to functional monomers, and their effects on the pore size and separation of different compounds were studied. For comparison purposes, the alkyl chain of the alcohol in the porogenic solvent was altered in a separate experiment. Thus, either methanol (C1), cyclohexanol (C6), decanol (C10) or dodecanol (C12) was used to replace the 1-propanol in the aforementioned polymerization mixture.

Three types of polar porogen mixtures that favor early phase separation of the polymer were used for the creation of the porous structures. Thus, 1-propanol, 1,4-butandiol and water that constituted 60% (w/w) of the polymerization mixture were tested. Out of the 60% (w/w) of total porogenic solvents, the amount of water [10% (w/w)] was fixed while the ratio of 1,4-butanediol to 1-propanol was systematically varied in the total porogen solvents in an attempt to prepare the first set of capillaries, namely S1 to S7 with different properties (Table 3.1). Furthermore, the effect of alkyl chain length in the alcohol porogenic solvent mixture on the pore size and separation of the investigated drugs was studied. Thus, 1-propanol was replaced with methanol (S1meth-S3Meth), cyclohexanol (S1C-S3C), decanol (S1D-S3D) or dodecanol (S1L-S3L) using similar percentages of porogenic solvents as reported above (Table 3.2).

3.3.2 Characterization of SPMA-based polymer monoliths

3.3.2.1 Mercury intrusion porosimetry

Monoliths prepared in situ in capillaries do not have sufficient bulk for porosimetry measurements. Therefore, polymerization was carried out in a sandwich device of larger volume to obtain sufficient amount of monolithic polymer so that the mercury intrusion porosimetry measurements on bulk
material can be representative of the porous properties [30]. This technique revealed the median pore diameters from all the prepared monoliths, with the value of the median pore diameter being 2.70 µm and 2.20 µm for S1 and S2, respectively. The porogenic solvent composition of S1 and S2 consisted of 1,4-butanediol, 1-propanol and water, in the ratios of 30:60:10 and 20:70:10 (% w/w/w), respectively. On the other hand, the pore diameters of S3, S6 and S7 were 0.14 µm, 1.06 µm and 1.36 µm, respectively, and their corresponding porogenic solvent composition ratios were 10:80:10; 70:20:10; and 60:30:10 (% w/w/w), respectively (Table 3.1).

For S1 and S2, early phase separation was achieved and larger pore size was obtained. However, the relatively smaller pore sizes of S3, S6 and S7 were probably due to the simultaneous decrease in solubility of both the monomers and the polymers in the system which contained a certain percentage of porogenic solvents. When the amount of 10% (w/w) 1,4-butanediol in combination with 80% (w/w) 1-propanol was used as in S3, 70:20 [1,4-butanediol:1-propanol, % (w/w)] as in S6, or 60:30 [1,4-butanediol:1-propanol, % (w/w)] as in S7, the solubility of both monomers and the polymers decreased (Table 3.1). The polymer chains remained soluble in the mixture for a longer period prior to phase separation due to the absence of contrasting polarity within the separated nuclei and also in the surrounding solution. Monomers were not compelled to adsorb preferentially into the nuclei and the polymerization proceeded with the formation of nuclei that remained individualized. Consequently, a larger number of individual nuclei competed for the remaining monomers [31], and this led to a large number of small microglobules that aggregated to form macroporous structure with fine individual microglobules and small pore sizes (e.g. S3, S6 and S7) (Table 3.1). On the contrary, a decrease in
the solubility of the polymer formed in a system containing 30% (w/w) 1,4-butandiol relative to that of 60% (w/w) 1-propanol, as in S1, led to an early phase separation. The separated nuclei, swollen with 1,4-butandiol (a more polar solvent than 1-propanol) preferentially extract the monomers from the surrounding liquid polymerization containing the less polar 1-propanol since the monomers favor the more polar environment. The enlarged nuclei could attract and coalesce with the newly precipitated chains and further increase their size, thus leading to the formation of large microglobules and larger pores. This explanation is in agreement with the previously reported pore formation mechanism [31]. The homogenous pore size distribution curve of S1, S2 and S3 measured by mercury intrusion porosimeter is shown in Figure 3-2. Additionally, S0, S4 and S5 with 0:90, 90:0 and 80:10 [1,4-butandiol:1-propanol, % (w/w)] and a fixed 10% (w/w) water formed an emulsion in the polymerization mixture due to poor solubility of monomers (Table 3.1).

On the other hand, by varying the alkyl chain in the alcohol porogenic solvent from 1-propanol (C3) to methanol (C1), cyclohexanol (C6), decanol (C10) or dodecanol (C12), an increase in pore size was also observed from 1.23 µm for methanol (S1Meth) to 2.07 µm for dodecanol (S1L) (Table 3.2). In addition, the increase of methanol percentage relative to that of 1,4-butandiol in the porogenic solvents led to an increase in the pore sizes of the formed monoliths (Figure 3-3 and Table 3.2). This is in contrast with S1-S3 where the pore sizes decreased when increasing the amount of 1-propanol compared to methanol (Tables 3.1 and 3.2, Figure 3-3). No monolithic polymer formed for S2decanol, S3decanol, S2dodecanol and S3dodecanol due to solubility problems. Based on these results, monoliths prepared with different alcohol alkyl chains were not further considered; only monoliths prepared with 1-propanol (S1-S3, S6 and S7) were investigated further.
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Figure 3-2: Pore size distribution curve of S1, S2 and S3 measured by mercury intrusion porosimeter.

Figure 3-3: Relationship between median pore diameter and amount of alcohol porogen.
3.3.2.2 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was initially used to study the effect of variation of the weight percentage of the porogenic solvent on the morphology of the prepared monoliths. This was achieved by comparing polymers prepared in bulk and in capillary. Both showed similar SEM images regardless of the method of preparation (Figure 3-4).

The morphology of the prepared monoliths S1, S2, S6 and S7 showed that the copolymerized monolith was composed of a heterogeneous surface of spherical units agglomerated into larger clusters interdispersed by large-pore channels, a characteristic sign of monolithic structures (Figure 3-4). S3 with 10:80 1,4-butandiol to 1-propanol [% (w/w)] and a fixed 10% (w/w) of water was not permeable when a mobile phase of 50:50 acetonitrile:water [% (w/w)] was used. It was observed to be a more gel-like monolith rather than having macropores such as exhibited by the S1, S2, S6 and S7 monoliths (Figure 3-5).

![SEM images of S1 prepared (a) in situ in capillary and (b) in bulk process.](image-url)
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Figure 3-5: SEM images of S1, S2, S3, S6 and S7 prepared by in situ polymerization process showing an obvious feature of the effect of 1-propanol on the morphology of SPMA-based monolith prepared in capillary column.
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3.3.3 Chromatographic characterization of SPMA monolithic columns

3.3.3.1 Separation mechanism

The main interest in this part of the study was the development of new SCX-RP functionality polymer-based monolithic columns that could tolerate relatively large injection volumes and exhibit adequate separation performance of small molecules. The desired material would also have the capability for upscaling for preparative chromatography or downscaling for low resolution chromatography (e.g. rapid screening) or sample preparation applications. Therefore, 1 µL of sample volume was injected for all the chromatographic evaluations. By mixing SPMA (cation-exchange functional monomer) and BMA (RP functional monomer) the formation of a mixed-mode SCX-RP material resulted.

3.3.3.1.1 Reversed-phase interaction

Monolithic columns with RP retention mechanisms have been widely used for the analysis of various complex samples [32]. The hydrophobicity of the stationary phase determines the selectivity of the separation, and RP retention can be easily controlled by adjusting either the percentage of the organic modifier in the mobile phase or the hydrophobicity of the surface [33]. In order to confirm a RP separation mechanism of the prepared mixed-mode monolith, three acidic drugs (i.e. diclofenac, ibuprofen and ketoprofen) were chosen as test compounds in the capillary LC mode. At low pH, these drugs existed as neutral analytes and were separated based on RP interaction. The logarithm of the retention factor of the target analytes was plotted against volume percentage of the organic solvent (acetonitrile) in the mobile phase. As shown in Figure 3-6, as the volume of organic modifier in the mobile phases was increased, the retention for the investigated
Figure 3-6: Plot of logarithm of the retention factor of a set of acidic drugs versus logarithm of the organic solvent in the mobile phase acetonitrile:water with 0.1% trifluoroacetic acid (TFA) separated on S1 monolithic column. Capillary 12 cm x 75 µm i.d. Conditions: flow-rate, 2 µL/min; 1 µL injection volume; column temp, 40 °C; detection wavelength, 219 nm.
analytes decreased. The linearity of these plots confirms the RP nature of the separation mechanism.

3.3.3.1.2 Cation-exchange interaction and capacity

The inclusion of SPMA provides cation-exchange sites in the monolith. The ion-exchange contribution to the separation mechanism was also investigated. Ion-exchange interactions between the charged analytes and the stationary phase can be influenced by the concentration of the competing ions in the mobile phase used. A competing ion (NH$_4^+$) was chosen and its concentration was altered, with log [ammonium] plotted versus log $k$ (retention factor) (Figure 3-7) for the separation of a set of β-blockers, namely acebutolol, pindolol and propranolol using ammonium acetate buffer (pH 7.5) and acetonitrile (65/35, % v/v) with a total NH$_4^+$ concentration ranging from 5 mM to 30 mM. The results revealed that when the concentration of the competing ion increased, the retention of the investigated analytes (positively charged under the above mentioned conditions) decreased accordingly. This was due to the competition of ammonium ions (+ve) with the analytes (+ve) towards the stationary phase (-ve) which resulted in the reduction of the ion-exchange interactions of the analytes (β-blockers) with the negatively charged stationary phase.

The ion-exchange capacity of the S1 monolithic column was determined by an adsorption/elution method, as described in Chapter 2. The capacity determined for a 75 µm i.d. monolithic column was 1.3 pequiv/cm of column, corresponding to a total capacity of 15.6 pequiv for a 12 cm column. The stationary phases exhibited relatively low cation-exchange capacity due to the prepared monoliths having relatively large pores, decreasing the surface-to-volume ratio, and the degree of negative charge on the polymer was low due to the low concentration of SPMA [0.4% (w/w)] used in the polymerization mixture [7].
Figure 3-7: Plot of the logarithm of the retention factor for a set of β-blockers versus the logarithm of the competing ion concentration in the mobile phase (5 to 30 mM ammonium acetate-acetonitrile 65:35, v/v), pH adjusted to 7.5 with ammonium hydroxide, separated on S1 monolithic column [Capillary 12 cm x 75 um i.d. (capacity ≈1.3 pequiv/cm)]. Conditions: flow-rate, 0.7 µL/min. Other conditions are the same as in Figure 3-6.
3.3.3.2 Permeability of SPMA monolithic columns

An important characteristic of a column in LC is its permeability $B_0$, which represents the resistance to mobile phase flow through the column. The permeabilities $B_0$ for S1 and S2 monoliths were determined by pumping 30:70 acetonitrile:water (\% v/v) through the column at different flow-rates and calculated using Darcy’s Law as illustrated in Section 2.4.1. As can be seen in Figures 3-3 and 3-8, an increase in the size of both flow-through channels and their permeability were evidenced along the 1,4-butandiol and 1-propanol variation range. Good linearity between backpressure and the flow-rate for S1 and S2 clearly demonstrated that the monoliths had sufficient mechanical stability to withstand the pressure of the liquid passing through the column up to 3200 psi (Figure 3-8). This also indicates that the prepared monolith did not appear to swell or shrink in different flow-rates.

3.3.3.3 Method development and separation performance of SPMA monolithic columns

Compared to the number of reports of the successful separation of large-molecular-weight compounds using organic polymer monoliths, only a handful have been successful for small molecules due to the lower surface areas and generally larger pores in polymer monoliths compared to silica-based monoliths. The present SCX-RP polymer-based monoliths are very versatile stationary phases. With these phases, analytes with hydrophobic moieties could interact with the hydrophobic domain of the stationary phase (RP mechanism), while positively charged analytes could be retained by electrostatic interactions with the SCX site in the acidic to neutral pH range.

To further characterize the selectivity of the mixed-mode poly(SPMA-co-BMA-co-EDMA) monolithic columns under investigation, a set of acidic drugs
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Figure 3-8: Permeability measurement of different prepared monoliths in 12 cm x 75 µm i.d. capillary using acetonitrile: water 30:70 [% (v/v)].
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and β-blockers with different pKa values, namely diclofenac (pKa = 4), ibuprofen (pKa = 4.4), ketoprofen (pKa = 5.94), arterenol (pKa = 8.55) and propranolol (pKa = 9.5), were selected as model compounds for capillary HPLC. In terms of finding acceptable separation conditions, two parameters were selected to access the separation performance and repeatability: (i) the target analytes were separated at acceptable resolution (i.e. all target analytes were baseline separated in the chromatograms) and (ii) target analytes were separated under isocratic conditions. Isocratic elution was preferred for simple samples (i.e. less than 10 components) where the retention factor of last peak eluted at less than 5 [34].

A mobile phase composition of 30:70 acetonitrile:water [% (v/v)] containing 0.1% (v/v) trifluoroacetic acid (TFA) (pH 2) was selected based on the separation mechanism (SCX-RP) of the prepared monoliths and properties of the target analytes. Traditionally, TFA is used in the mobile phases for RP-HPLC separations to serve one or more of the following functions: (i) pH control, (ii) complexation with oppositely charged ionic groups to enhance RP retention (ion pairing), (iii) or the suppression of adverse ionic interactions between basic analytes and silanol groups on the silica to minimize peak broadening [35]. The presence of TFA as a very strong eluting additive might have affected the elution of the analytes. However, in a separate experiment, weaker acids, namely formic acid and acetic acids, were used as additives [0.1% (w/w)] in the mobile phase composition consisting of acetonitrile: water 30:70 (% v/v) for the separation of the investigated compounds. The best analytical performance was obtained when TFA was used as an additive in the mobile phase composition.

S1 monolithic column exhibited acceptable separation performance for the baseline separation of five pharmaceutical drugs (Figure 3-9). At pH 2 (pH of the used mobile phase), these β-blockers were positively charged while the acidic
Figure 3-9: Separation of some acidic drugs and β-blockers using SPMA monolithic columns (12 cm x 75 µm i.d.) Conditions: flow-rate, 0.5 µL/min; Peaks: 1, Artenerol; 2, ketoprofen; 3, ibuprofen; 4, diclofenac; 5, propranolol. Other conditions are the same as in Figure 3-6.
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drugs were neutral. In terms of properties of the investigated acidic drugs, they appeared to be hydrophobic in nature and their hydrophobicity increased from ketoprofen, ibuprofen, to the strongly retained diclofenac. In addition, an electrostatic attraction between the functional sulfopropyl anion of the monolith and the positively charged β-blockers was expected. Therefore, a mixed-mode (i.e. SCX-RP) mechanism was involved in the separation of β-blockers. Figure 3-10 shows a fast separation on the S1 monolithic column using high flow-rate (2 µL/min) and column temperature (80 °C). The target analytes were eluted within 10 min without significantly increased backpressure. On the contrary, S2, S6 and S7 showed poor separation of the studied compounds using the above mentioned mobile phase composition (Figure 3-9). The surface chemistry, concentration of sulfonic acid groups on the polymer surface as well as the size and distribution of pores played a key role in affecting the separation performance.

3.3.3.4 Repeatability of SPMA monolithic columns

Repeatability is extremely important for a developed monolithic material designed for the separation and sample preparation purposes. The repeatability of the monolithic columns S1 and S2 was assessed through the %RSD of the retention times of the five analytes mentioned above. The repeatability of the column preparation process was tested on three levels: (i) intra-batch (run-to-run and day-to-day) and (ii) inter-batch (batch-to-batch). The run-to-run and day to day reproducibilities of a given monolith were evaluated from ten and six injections, respectively, of the test analytes using the same column. Meanwhile, the column-to-column repeatability was calculated from results obtained with six different columns prepared from different polymerization mixtures with the same composition. Acceptable RSD values for intra-batch and inter-batch are 2.5% and 15%, respectively, as reported in the literature [9,36,37].
Figure 3-10: Separation of some acidic drugs and β-blockers using S1 monolithic column (12 cm x 75 µm i.d.). Conditions: mobile phase, acetonitrile/water (20:80, v/v) with 0.1% TFA (v/v/v); flow-rate, 2 µL/min; column temperature, 80 °C. Other conditions same as in Figure 3-6.
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The observed run-to-run (n=10) and day-to-day (n=6) RSD values of arterenol, ketoprofen, ibuprofen, diclofenac and propranolol on S1 and S2 columns were very low, ranging from 0.5 to 2.14%, except for arterenol which was not repeatable on the S2 column (Table 3.3). Additionally, the column-to-column (n=6) repeatability for the aforementioned analytes on S1 and S2 columns exhibited high RSD values, ranging from 28 to 43% (Table 3.3). Although columns S1 and S2 exhibited good permeability (i.e. they can withstand up to 3200 psi), these results clearly confirmed the non-robustness of the prepared monolithic columns S1 and S2 as their performance appeared to vary greatly based on the RSD values of column-to-column repeatability of the target analytes (Table 3.3 and Figure 3-11).

3.3.4 Effect of cross-linker on the modified SPMA monoliths(SM)

3.3.4.1 Combined effects of percentage variations of the cross-linker and porogenic solvents on modified SPMA monoliths

The lack of column-to-column repeatability of the S1 and S2 columns was the motivation to alter the amount of cross-linker, which was the second variable in the studied monolithic system. A higher amount of cross-linker (EDMA) over functional monomers (SPMA and BMA) in the fixed polymerization mixture [40% (w/w)] monomers with respect to porogenic solvents [60% (w/w)] may result in more robust and repeatable monolithic polymers while maintaining the SCX-RP interaction properties. It has been reported that a higher cross-linker concentration produced monoliths with improved column-to-column repeatability and better mechanical stability [28]. Similar effects were observed in the investigated system, when further experimentation was done with the same overall monomer percentage but the monomer composition percentages being switched between the functional monomer BMA and cross-linker EDMA.
Table 3.3: Repeatability of prepared SPMA-based monolithic columns S1 and S2 expressed as relative standard deviations (%RSD) of retention time (R.T.) for the separation of acidic drugs and β-blockers using mobile phase 30:70 acetonitrile:water with 0.1% TFA. Other conditions are the same as in Figure 3-9.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Analyte Conc. (mg/L)</th>
<th>Run-to-run (n=10)</th>
<th>Day-to-Day (n=6)</th>
<th>Column-to-column (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Renal</td>
<td>Retention time (R.T.)</td>
<td>Retention time (R.T.)</td>
<td>Retention time (R.T.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>%RSD</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S1</td>
<td>S2</td>
<td>S1</td>
</tr>
<tr>
<td>Arterenol</td>
<td>3.50</td>
<td>6.76</td>
<td>NA</td>
<td>1.02</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>0.70</td>
<td>7.32</td>
<td>7.10</td>
<td>1.05</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>1.75</td>
<td>8.51</td>
<td>9.83</td>
<td>1.05</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>2.45</td>
<td>9.50</td>
<td>11.51</td>
<td>0.98</td>
</tr>
<tr>
<td>Propranolol</td>
<td>3.50</td>
<td>16.55</td>
<td>13.50</td>
<td>0.83</td>
</tr>
</tbody>
</table>

NA – Analyte was not observed
Figure 3-11: Column-to-column repeatability of S1 (12 cm x 75 µm i.d.): separation of acidic drugs and β-blockers on different batches of S1 stationary phase. Other conditions are the same as in Figure 3-8.
Thus, newly modified monoliths i.e. S1M-S3M were prepared using 16% (w/w) BUMA, 0.4% (w/w) SPMA, 23.6% (w/w) EDMA [overall 40% (w/w)] compared with 23.6% (w/w) BUMA, 0.4% (w/w) SPMA, 16% (w/w) EDMA [overall 40% (w/w), as for the previously prepared S1-S7] and 60% (w/w) porogenic solvents as used previously for S1-S7 (Table 3.4).

Considering the effect of 1-propanol on the pore size shown previously in the examples S1-S7 (where higher percentage of 1-propanol led to the formation of monoliths with smaller pore size), the effect of increasing the amount of cross-linker over functional monomer might be reduced if the percentage of 1-propanol relative to that of 1,4-butandiol was varied in the preparation of the modified monoliths S1M-S3M. Thus, fixing the amount of water [10% (w/w)], the effect of the percentage variation of 1-propanol relative to that of 1,4-butandiol in the 60% (w/w) porogenic solvent and 40% (w/w) monomers compositions (16% (w/w) BMA, 0.4% (w/w) SPMA, 23.6% (w/w) EDMA) was studied in an attempt to prepare a second set of columns, namely S1M-S3M, that were intended to exhibit both rigidity and larger pore size.

3.3.4.2 Mercury intrusion porosimetry of the modified SPMA monoliths

Mercury intrusion porosimetry was used to measure the pore size of the modified SM monoliths that were prepared. The results (Table 3.4) show that the median pore diameters from all the above prepared columns (S1M-S3M) were 2.85 μm for S2M, 1.35 μm for S1M and 1.12 μm for S3M with porogenic solvents consisting of 1,4-butandiol, 1-propanol and water in the ratios of 20:70:10, 30:60:10 and 10:80:10 [% (w/w/w)], respectively. The pore diameters of the modified monoliths were not what were expected. In contrast with S1-S7, there was a higher amount of cross-linker than functional monomer in the
polymerization mixture. As the amount of 1-propanol increased relative to that of 1,4-butandiol, the pore size increased from S1M to S2M, although it decreased again in S3M (Table 3.4 and Figure 3-12). This indicated that phase separation occurred early for systems with 60 and 70% (w/w) of 1-propanol over 1,4-butandiol and with higher amount of cross-linker over functional monomer.

3.3.4.3 Scanning electron microscopy of the modified SPMA monoliths

In terms of the morphology of the prepared monoliths, SEM analysis of S1M, S2M and S3M showed the copolymerized monolith was composed of a heterogeneous surface of spherical units agglomerated into larger clusters interdispersed by large pore channels. Therefore, these monoliths (S1M-S3M) showed good permeability to solvents and can withstand backpressure of up to 3200 psi (Figure 3-8). It was interesting to see that previously the S3 was not permeable, but, it became permeable in the S3M form and macropores were observed after the composition of the cross-linker and functional monomer were switched (Figures 3-5 and 3-13).

3.3.4.4 Separation performance, repeatability and stability of modified SPMA monolithic columns

A similar set of acidic drugs and β-blockers were tested for their chromatographic separation on the studied monoliths (S1M-S3M). All acidic drugs and β-blockers studied in this investigation were baseline separated within 25 min using a mobile phase consisting of acetonitrile:water:TFA (34:66:0.1%, v/v/v) for S2M and S3M columns [Figures 3-14 (a) and 3-15 (a)]. Fast separations using S2M and S3M monolithic columns were performed using high flow-rate (2 µL/min) and column temperature (80 °C). The target analytes were eluted within 5 min [Figures 3-14 (b) and 3-15 (b)].
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Table 3.4: Effect of variation of the percentage of porogen solvents on the pore size of the studied modified SPMA monoliths (S1M-S3M) prepared from a polymerization mixture consisting of 0.4\% (w/w) SPMA, 16\% (w/w) BMA and 23.6\% (w/w) EDMA that constituted 40\% (w/w) of the polymerization mixture with respect to 60 \% (w/w) porogen solvents.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>1,4-butanediol [% (w/w)]</th>
<th>1-propanol [% (w/w)]</th>
<th>Water [% (w/w)]</th>
<th>Pore size [\mu m]</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1M</td>
<td>30</td>
<td>60</td>
<td>10</td>
<td>1.35</td>
</tr>
<tr>
<td>S2M</td>
<td>20</td>
<td>70</td>
<td>10</td>
<td>2.85</td>
</tr>
<tr>
<td>S3M</td>
<td>10</td>
<td>80</td>
<td>10</td>
<td>1.12</td>
</tr>
</tbody>
</table>

Figure 3-12: Pore size distribution curve of S1M, S2M and S3M measured by mercury intrusion porosimeter.
Figure 3-13: SEM images of modified SPMA monoliths (a) S1M, (b) S2M and (c) S3M.
Figure 3-14: Separation of some acidic drugs and β-blockers on S2M monolithic column (12 mm x 75 µm i.d.) using either mobile phase composition acetonitrile:water:TFA (a) 34:66:0.1% v/v/v; flow-rate, 0.5 µL/min; column temperature, 40 °C or (b) 30:70:0.1% v/v/v; 2µL/min; 80 °C. Other conditions are the same as in Figure 3-9.
Figure 3-15: Separation of some acidic drugs and β-blockers on S3M monolithic column (12 mm x 75 µm i.d.) using either mobile phase composition acetonitrile:water:TFA (a) 34:66:0.1% v/v/v; flow-rate, 0.5 µL/min; column temperature, 40 °C or (b) 30:70:0.1% v/v/v; 2 µL/min; 80 °C. Other conditions are the same as in Figure 3-9.
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Stability of a column and repeatability of retention times during separations are critical in the evaluation of chromatographic properties. Several batches (A-D) of S2M and S3M columns were prepared from separate polymerization mixtures under similar conditions and the separations of the test compounds were evaluated (Figures 3-16 and 3-17). The observed run-to-run (n=10) RSD values based on retention times of arterenol, ketoprofen, ibuprofen, diclofenac and propranolol on S2M and S3M columns were all within 1.2% (Table 3.5). The RSD values for day-to-day (n=6) were higher, ranging from 0.7 to 2.7% (Table 3.5). Additionally, the column-to-column (n=6) repeatability for the aforementioned analytes on S2M exhibited satisfactory RSD values, ranging from 3 to 11% (Table 3.5). The column-to-column (n=6) RSD values of S3M were also at acceptable level with all values < 15% except for propranolol (16%) (Table 3.5). The stability of the prepared S2M was impressive and acceptable separation performance of all the analytes was obtained for up to 450 injection cycles at 80 °C (Figure 3-17). Stable retention times of tested compounds featuring a RSD less than 3.5% were observed, even after 450 separations for the S2M monolithic column prepared via photopolymerization. These results clearly confirmed the robustness of the prepared monolithic column S2M as the separation performance did not appear to deteriorate with time, temperature, number of injections or column-to-column conditions (Figure 3-18).

3.4 Conclusions

Mixed-mode SPMA-based monolithic columns for HPLC separation of some acidic drugs and β-blockers were prepared. The influence of porogenic solvent composition and cross-linker on morphological, porogenic and separation properties of monoliths has been investigated. The possibility of fine-tuning of
Figure 3-16: Column-to-column repeatability of S2M: separation of acidic drugs and β-blockers on different batches of S2M stationary phase using mobile phase composition acetonitrile:water:TFA (34:66:0.1%, v/v/v). Other conditions are the same as in Figure 3-8.
Figure 3-17: Column-to-column repeatability of S3M: separation of acidic drugs and β-blockers on different batches of S3M stationary phase using mobile phase composition acetonitrile:water:TFA (34:66:0.1%, v/v/v). Other conditions are the same as in Figure 3-9.
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Table 3.5: Repeatability of prepared SPMA-based modified monolithic columns S2M and S3M expressed as relative standard deviations (%RSD) of retention time (R.T.) for the separation of acidic drugs and β-blockers. Other conditions are the same as in Figure 3-16.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Analyte Conc. (mg/L)</th>
<th>Run-to-run (n=10)</th>
<th>Day-to-Day (n=6)</th>
<th>Column-to-column (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Retention time (R.T.)</td>
<td>Retention time (R.T.)</td>
<td>Retention time (R.T.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>%RSD</td>
<td>Mean</td>
</tr>
<tr>
<td>Arterenol</td>
<td></td>
<td>S2M</td>
<td>5.92</td>
<td>6.56</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td></td>
<td>0.70</td>
<td>7.39</td>
<td>8.42</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td></td>
<td>1.75</td>
<td>9.17</td>
<td>10.71</td>
</tr>
<tr>
<td>Diclofenac</td>
<td></td>
<td>2.45</td>
<td>11.30</td>
<td>13.31</td>
</tr>
<tr>
<td>Propranolol</td>
<td></td>
<td>3.50</td>
<td>13.61</td>
<td>16.92</td>
</tr>
</tbody>
</table>
Figure 3-18: Study of the stability of S2M column after 450 injection cycles of a mixture of acidic drugs and β-blockers. Conditions: mobile phase, acetonitrile:water (30:70, v/v) with 0.1% TFA; flow-rate, 2 µL/min; 1 µL injection volume; column temp, 80 °C.
porosity and separation properties of monoliths was explored by changing the 1-propanol to 1,4-butane diole ratio to allow for the optimization of the HPLC performance of the columns. In general, at a given porogenic solvent composition, a SPMA-based monolith with a greater amount of cross-linker gave better repeatability values in separation behavior, even at high temperature (up to 80 °C). Furthermore, the separation mechanism of the investigated compounds appeared to be based on a mixed-mode hydrophobic/ion-exchange interaction of the analytes with the SCX-RP monolith. The influence of ion-exchange interaction on the retention between the β-blockers and the stationary phase could be manipulated by changing the concentration of the competing ion (5-30 mM NH₄⁺) of the mobile phase used. All these properties are promising for a broader application of mixed-mode porous polymer monoliths in low resolution chromatography and sample preparation for pharmaceutical analysis. The simplicity of the in situ preparation process also makes the monolithic columns excellent candidates for use in miniaturized devices, such as microfluidic chips and pipette tips.

3.5 References


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Development of a Trypsin-Immobilized Monolithic Polymer with Pipette-Tip Format for Protein Digestion

4.1 Introduction

There have been significant improvements in the analytical methodologies and techniques pertaining to proteomic studies in the past decades. Several strategies have emerged as efficient and indispensable techniques for the bottom-up approach of protein analysis, which involves separation, identification, and characterization of proteins in the field of functional proteomics [1,2]. Two-dimensional electrophoresis (2-DE) is one of the most powerful methods for the separation of proteins, followed by off-line digestion and further identification by MS/MS [3]. Alternatively, the hyphenation of multidimensional HPLC with MS/MS is used for the separation and identification of all the digested proteins extracted from a biological sample [4]. However, sample preparation often poses a constraint to rapid bioanalysis. The traditional protocol for protein digestion is accomplished by enzymatic hydrolysis in free solution for at least several hours. This method presents a number of drawbacks, such as proteolytic enzyme autodigestion, low efficiency, extended incubation time, insufficient sample loading capacity for direct analysis of plasma, and manual sample manipulation steps. These limit the advancement of high throughput protein identification technology [5]. To obtain rapid, sensitive and high-throughput protein digestion, research efforts have focused on the immobilization of enzymes.
Research dealing with immobilization of trypsin can be dated back to the late 1970s [6]. Immobilized enzymes have found applications in a wide variety of areas. In addition, the application of bioreactors containing immobilized enzymes is also growing as an integral part of quality control (QC) and quality assurance (QA) for products in biotechnology, chemical synthesis, and the pharmaceutical industry [7]. These enzyme reactors enable rapid screening of enzyme inhibitor candidates as well as detailed characterization of binding interactions and reaction mechanism [8,9]. Over the past few decades, various strategies enabling enzyme immobilization have been designed and developed. Enzymes can be covalently bound, trapped, or physically adsorbed onto various supports, such as particles [10], membranes [11], the inner walls of fused-silica capillaries,[12] magnetic particles[13] and monolithic materials [14]. It is also now well-known that the properties of the support such as pore size, porosity, chemistry, and mechanical strength will affect the characteristics of the immobilized enzymes [15]. That is, the activity and the applicability of the resulting bioreactor are greatly affected by the selection of the support matrix.

Currently, monoliths have been widely used as matrices for sample preparation and separation. The micrometer-sized pores of the monoliths provide a low-pressure drop for the proteins to flow convectively through the interstices and interact with the enzyme-immobilized surfaces via short diffusion distances, which may enhance the digestion efficiency. A review of the advantages of using monoliths over using alternative phases for bioanalytical applications has been reported elsewhere [16]. In addition, the wide variety of applications of silica and organic polymer monolithic phases in the preparation of immobilized enzyme reactors of different formats has been reported. A number of bioreactors prepared by inorganic silica monoliths in 4.6 mm i.d. column have been studied by
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Temporini et al. for the on-line digestion and characterization of proteins [17,18]. A solid-phase digestion tool of immobilized-trypsin on monolithic silica gel in a pipette tip has been developed [19]. Organic polymer monoliths used as supports for enzyme immobilization have been prepared in different formats, such as in situ in the channel of microfluidic devices [14], monolithic disks [20] and columns of different i.d. [7,21]. Specifically, organic polymer monoliths have gained much attention over silica-based monoliths as supports for enzyme immobilization due to properties such as high chemical stability over a wide pH range, good biocompatibility, ease of preparation in different formats and ease of modification with various functional groups [5].

A further advantage of polymer monoliths is that they can be readily modified, for example using UV light. Irradiation with UV light through a photomask allows precise patterning of the area subjected to surface modification and enables the precise placement of specific functionalities within selected areas of a single monolith for use in a variety of applications [22,23]. This process permits the introduction of multiple sites with various functionalities located next to each other or at predetermined locations in a single monolith [24]. Recently, 2-vinyl-4,4,4-dimethyazlactone (VAL) has been used for copolymerization with ethylene glycol dimethacrylate (EDMA) to obtain a reactive monolithic support for the immobilization of enzymes [25,26]. Krenkova et al. [7] described the preparation of enzymatic microreactors containing trypsin and endoproteinase LysC immobilized on a porous polymer monolith for the characterization and identification of proteins, such as cytochrome c, bovine plasma albumin, and high-molecular weight human immunoglobulin G. Subsequently, a reactor with immobilized peptide-N-glycosidase F on a porous polymer monolith in a capillary was also developed for the fast and efficient release of N-linked glycans from
immunoglobulin G molecules [7]. The monolith was first hydrophilized via photografting of poly(ethylene glycol) methacrylate (PEGMA) followed by photografting of VAL. This multistep photografting process was used to reduce non-specific adsorption of proteins and to obtain a support containing reactive azlactone functionalities so as to enable the preparation of highly active immobilized enzymes [7,26]. With the use of this system, a much shorter reaction time and a lower reaction temperature were achieved for the protein digestion.

In this chapter, a novel trypsin-immobilized organic polymer monolith based on a pipette tip format is described for the analysis of proteins. A robust and reproducible monolith formulation reported previously in Chapter 3 was used as the support for the enzyme immobilization. The tips were prepared using a porous polymer monolith, followed by photografting the pore surface of the monolith with VAL inside the pipette tip. An unstable enzyme, trypsin, was successfully immobilized covalently in the azlactone-functionalized area. The functionalized monolith was characterized, followed by evaluation of its efficiency in the rapid digestion of proteins.

4.2 Experimental

The general experimental details are described in Chapter 2. Detailed conditions are elaborated in each of the figure captions.

4.2.1 Fluorescent assay of protein absorption

4.2.1.1 Photografting of poly(ethylene glycol) methacrylate (PEGMA)

A two-step modification reaction was used for the hydrophilization of the monolithic support to prevent the non-specific adsorption of proteins to the monolith surface [26]. Briefly, the monolith in the pipette tip was flushed with a
deaerated 5% (w/w) benzophenone (BP) solution in methanol for 30 min using a vacuum filtration apparatus. It was then exposed to UV irradiation for 4 min with rotation. The tip was fitted on a rotating stirrer and was rotated at the slowest speed possible (~50 rpm). After photografting BP, the monolith was flushed with methanol for 30 min to remove the unbound initiator. Next, a deaerated 0.1 M solution of PEGMA monomer in water was pumped through the monolith for 30 min and the monolith was exposed to UV irradiation for 4 min with rotation. The monolith was then rinsed with water for 60 min to remove the unreacted PEGMA monomer.

4.2.1.2 Preparing stock solution of protein and fluorescein isothiocyanate (FITC)

A stock solution of protein was prepared by directly dissolving commercially available bovine serum albumin (BSA, Sigma Aldrich) in MilliQ water. In order to prepare 1x10^-4 M of BSA (MW 66430), 132.9 mg of BSA was dissolved in 20 mL of water using a magnetic stirrer at approximately 25 °C. Then, it was diluted to 1x10^-5 M with water. To prepare 10^-3 M of FITC (Sigma Aldrich) solution, 19.5 mg of FITC was dissolved in 50 mL of high purity (>99.8%) acetone and diluted to 5x10^-5 M with acetone.

4.2.1.3 Labeling reaction of protein with fluorescein isothiocyanate (FITC-BSA)

In order to prepare a fluorescently-labeled BSA solution with the concentration of 5x10^-6 M, 20 mL of BSA solution (1x10^-5 M) and 20 mL FITC (5x10^-5 M) were mixed (five moles of FITC were coupled to one mole of albumin) in dry 50 mL plastic tubes. Then the mixtures were wrapped with aluminum foil and kept in the drawer overnight to perform the labeling reaction (at ~20-25 °C temperature controlled environment).
4.2.1.4 Fluorescent assay of protein adsorption on the pipette tips

The extent of protein adsorption on unmodified and PEGMA-photographed monolith in situ pipette tips was evaluated using a fluorescence assay developed previously [27]. Briefly, the pipette tips were flushed with a 5x10^-6 M (~0.33 mg/mL) solution of fluorescein-labeled BSA for 1 h. The pipette tips were then rinsed with water for 30 min to remove excess BSA. After BSA exposure, the cross-section of the tip was then observed using a microscope with a blue LED array illuminating the microscope for fluorescent imaging as shown in Figure 4-1. Monolith tips with and without PEGMA-photographing were used for screening non-specific protein adsorption. Successful prevention of protein adsorption was indicated by the low fluorescence intensity observed. By contrast, high fluorescence intensity indicated substantial absorption of the fluorescent protein and poor performance of the monolith surface.

![Image of microscope with blue LED array](image-url)

Figure 4-1: The set-up of a microscope with blue LED array illuminating the microscope for fluorescent imaging.
4.2.2 Photografting of 2-vinyl-4,4-dimethylazlactone (VAL)

A mixture consisting of 25% (w/w) VAL and 0.22% (w/w) BP dissolved in 75:25 (% w/w) tert-butyl alcohol:water was pumped through the monolith for 30 min and exposed to UV irradiation for 30 min on both sides of the tips (Figure 4-2). After grafting vinyl azlactone, the monolith was washed with acetone for 1 h to remove excess reagents.

4.2.3 Immobilization of trypsin on grafted support

Trypsin (1, 2, 5, 10 and 15 mg/mL) was dissolved separately in 50 mM phosphate buffer, pH 7.2, containing 0.25 mg/mL benzamidine. Benzamidine was added to avoid undesirable autodigestion and the enzyme solution was pipetted for 15 to 20 cycles. After the immobilization reaction, the monoliths were rinsed with 1 M ethanolamine in 50 mM phosphate buffer, pH 7.2 to quench all unreacted azlactone functionalities. Finally, the monolithic reactor was washed and stored with 50 mM ammonium acetate solution pH 6.7 at 4 °C.

The amount of immobilized trypsin on the azlactone monolith in the pipette tips was determined by the modified method described previously [28]. After the immobilization process, the tip was rinsed with 20 μL of the same buffer to remove the unreacted trypsin on the surface of monolith. This eluent was collected and combined with the eluent from the immobilization and diluted to 2 mL and the absorbance was measured at 280 nm. The concentration was calculated by the difference of the amount of trypsin before and after immobilization using the calibration curve established (slope = 0.0014 abs/μg mL⁻¹).

4.2.4 Sample preparation for protein digestion

The sample preparation for protein digestion was performed according to the procedure described previously [7]. The digestion of proteins using immobilized
Figure 4-2: Schematic diagram of the photopatterning process. (a) vinylazlactone (VAL) is photopatterned onto the monolith surface and activates the surface for protein immobilization. (b) azlactone functionality reacts with amines of proteins to form a covalent amide bond between the protein and the polymer monolith surface.
enzyme polypropylene pipette (IMEPP) tips is illustrated in Figure 4-3. Briefly, cytochrome c was dissolved in 50 mM ammonium acetate, pH 8.75 containing 20% acetonitrile to a concentration of 0.5 mg/mL. 100 µL of the protein sample was incubated in a dry-bath incubator for 5 min at 37 °C before being pipetted with IMEPP tips using the protocol described in Section 2.3.9. For protein digestion with soluble enzyme, trypsin was added at a substrate-to-enzyme ratio of 50:1 (w/w) for protein digestion and the solution was incubated at 37 °C for 24 h. The proteolysis was terminated by decreasing the pH with the addition of formic acid to a final concentration of 0.1% (1 µL of 10% formic acid in water). The resultant peptide fragments from the digestion of IMEPP tips or liquid phase digestion were collected in microvials and analyzed using direct infusion MS. The mass spectra of all samples were then compared against a protein digestion database (protein prospector, http://prospector.ucsf.edu/) to identify the digest and determine the sequence coverage (Figure 4-3).

4.3 Results and discussion

4.3.1 Surface modification of the polypropylene (PP) tips

The modification of the surface of PP was first demonstrated by Stachowiak et al., who covalently attached the porous monolithic polymer to the tip wall in order to transfer the technique to the microfluidic chips [22,29]. Following this, Altun et al. have used the grafting modification to create reactive groups and they improved the wetting ability of the inner surfaces of PP tips in order to prepare monolithic pipette tips for SPE purposes in high throughput bioanalysis [29,30]. In the present work, the inner surface of PP tips was modified via grafting of monomer, followed by the preparation of a porous poly (butyl methacrylate-co-ethylene glycol dimethacrylate) monolith. The porous polymer monolith in situ PP tips can act as a
Figure 4-3: Schematic diagram for the protein digestions using trypsin-immobilized monolith tip.
support for enzyme immobilization in protein digestion. In this study, we also investigated a two-step photografting approach that involved covalent attachment of monomer to the PP surface (method described in section 2.3.7). Figure 4-4 shows the SEM images of non-modified PP tips and surface modification of tips by single- and two-step photografting using BP as initiator. The SEM images were not markedly different except that the surface of the modified tip looked smoother, as shown in Figures 4-4 (b) and 4-4 (c).

Based on the results above, the effects of single- and two-step surface modification were further investigated by synthesizing the poly (BMA-co-EDMA) in situ PP tip using UV-induced polymerization. Figure 4-5 (a) shows SEM images of the monolith inside the PP tips in the absence of surface treatment. A magnified image shown in Figure 4-5 (a) bottom indicates a large void between the PP wall of the tip and the monolith. After washing the monolith with methanol and drying it in the vacuum oven, the monolith was loosened and could slip out of the tip. By contrast, a surface grafted with monomer enabled a better attachment of the monolith either by single-step or two-step photografting methods, as shown in Figures 4-5 (b) and 4-5 (c). This can be confirmed by the presence of a thin layer of polymer gel observed from the magnified images taken between the PP wall and monolith [Figures 4-5 (b)] which can also correspond to the smoother surfaces of tip walls for single or two-step photografting. Both of the photografting methods showed excellent covalent binding of the monolith to the PP surface and good mechanical stability. When a high pressure was applied, the monolith attached well to the surface without slipping out.

In addition, the differences between using EDA (ethylene diacrylate) and EDMA for the surface modification of PP tips were compared. Previous works suggested that EDA offers the benefits of shorter exposure time which
Figure 4-4: SEM images of PP tips (above) and the magnified part (bottom): (a) no surface modification (b) single-step photografting and (c) two-step photografting.
Figure 4-5: SEM images of porous polymer monoliths prepared in PP tips (above) and the magnified part (bottom): (a) no surface modification (b) single-step photografting with MMA/EDMA 1:1 with BP [3% (w/w)] and (c) two-step photografting.
achieve a stronger surface treatment of the PP surface. However in this work, surface modification with EDA achieved only partial attachment of the monolith to the PP wall. These results are in agreement with findings reported by Stachowiak et al. who stated that using EDMA for surface modification achieved the best result, with no visible void between the PP wall and monolith [22]. Therefore, a single-step photografting with EDMA was used to simplify the preparation of monoliths in tips.

4.3.2 Fluorescence assay of protein adsorption

The hydrophilic polymer PEGMA has been widely used for modifying the surfaces of monoliths to reduce non-specific protein interactions and to permit repeated use of the reactor [7,26,31]. BSA is well-known to be a “sticky” protein and is commonly used to estimate protein adsorption onto enzyme-immobilized monolithic supports [32]. The fluorescence assay of BSA can be used to mimic the conditions for enzyme immobilization onto the monolith to obtain an enzyme reactor in pipette tip format [27]. Figure 4-6 shows the fluorescence images of a pipette tip with and without PEGMA-grafted after being flushed with fluorescein-labeled BSA and rinsed with water. The non-PEGMA-grafted tip was highly susceptible to absorption of the fluorescently-labeled protein. The monolith photografted with PEGMA [Figure 4-6 (b)] exhibited lower fluorescent intensity than the monolith which had not been photografted [Figure 4-6 (a)]. Despite the fact that this method was developed to impart hydrophilic functionalization onto monoliths in small i.d. capillaries (75 µm), these images clearly demonstrated the successful upscaling of the reaction to larger i.d. PP tips. When monolith containing FITC-BSA was washed with 20% acetonitrile in water, most of the BSA was removed, with much lower fluorescence intensity monolith observed [Figure 4-6 (c)]. The longer photografting time (from 4 min to 8 min) for PEGMA resulted in a thicker layer of PEGMA being formed on the monolith, which caused high
Figure 4-6: Fluorescence cross-section images of pipette tips. Fluorescein-labeled BSA was pumped through the polymer monolith tips (a) without and (b) PEGMA-grafted region after washed with water and (c) 20:80 (% v/v) acetonitrile:water.
backpressure. Therefore, 4 min photografting was deemed to be the optimum irradiation time.

4.3.3 Enzyme immobilization using azlactone functionalities

The application of azlactone monoliths in enzyme immobilization has been widely used in the past 10 years for the fabrication of enzymatic microreactors. The aim of this chapter was to develop an inexpensive single-use IMEEP tip, so the issue of non-specific adsorption is less of a problem in this work. Thus, we used the non-PEGMA-grafted monolithic PP tips for subsequent studies.

As a starting point, the protocols described previously [26] were used to prepare the azlactone monolith [i.e. 15% (w/w) VAL, 2 min photografting and 5 mg/mL of trypsin for immobilization]. The first demonstration of proteolytic activity for the IMEPP tip was carried out by pipetting 100 µL 0.5 mg/mL of cytochrome c for 10 cycles. However, no noteworthy enzyme activity was observed for the cytochrome c digestion using the IMEPP tip [Figure 4-7 (b)], as compared to the cytochrome c solution digested using the non-VAL-grafted monolithic PP tip [Figure 4-7 (a)]. This might be due to the fact that the UV source used for functionalization could not penetrate the large i.d. of the monolith fabricated in situ inside the PP tips, since the PP materials have a much lower UV transmission compared to the UV transparent fused-silica capillaries with smaller i.d.. Another possibility for the lack of enzyme activity might be an insufficient amount of VAL grafted onto the surface of monolith, resulting in a low capacity of enzyme immobilized on the polymer surface.

As demonstrated by Krenkova et al. [7], the concentration of VAL at 25% (w/w) or above in the monolith support clogged the column. Here, 25% (w/w) VAL was used in the photografting mixture, with 10 min of UV irradiation. The result shows that reasonable backpressure could be maintained to allow solution to pass
through the PP tips after photografting with the relatively high concentration of VAL. Cytochrome c was again used to demonstrate the proteolytic activity of the IMEPP tip grafted with higher concentration of VAL. Figure 4-7 (c) indicates that the IMEPP exhibited very high activity for the complete digestion of cytochrome c with 100% of sequence coverage achieved within few minutes.

The maximum concentration of VAL and photografting time that could be used to maximize the enzyme loading capacity of the IMEPP tip were also determined. Firstly, the photografting time was increased from 10 min to 30 min while maintaining the permeability of the monolith. However, when the concentration of VAL was increased from 25% (w/w) to 30% (w/w), permeability through the IMEPP tip was no longer observed. Therefore, 25% (w/w) VAL in the photografting mixture and 30 min photografting time were used for the following studies. Table 4.1 shows a comparison of the performance of the IMEPP tips with different numbers of pipetting cycles for the digestion of cytochrome c at 0.1 mg/mL. This table shows that the highly efficient IMEPP tip developed here could give sequence coverage of 89% even with 1 pipetting cycle (or 10 s) of digestion of low-molecular weight proteins, such as cytochrome c.

**4.3.4 Effects of trypsin concentration for immobilization on monolithic support**

A batch of three IMEPP tips immobilized with different concentrations of trypsin was prepared (2530a, b, c, d and e). Using the UV spectrometric method, the amount of immobilized trypsin on the azlactone monolith was found to be different when different concentrations of trypsin solution were employed (Table 4.2). The maximum bound amount of trypsin was limited to about 140 µg which was
Figure 4-7: ESI-TOF mass spectra obtained from cytochrome c digestion using pipette tips (a) without VAL photografting; (b) photografting under UV light at an exposure time of 2 min with 15% (w/w) VAL in photografting mixture; (c) exposure time of 30 min with 25% (w/w) VAL in photografting mixture.
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Table 4.1: Comparison of digestion performances of the IMEPP tip with different numbers of pipetting cycles for the digestion of cytochrome c.

<table>
<thead>
<tr>
<th>Label</th>
<th>2530</th>
<th>2530</th>
<th>2530</th>
<th>2530</th>
</tr>
</thead>
<tbody>
<tr>
<td>pipette cycles</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Identified peptide number</td>
<td>87/105</td>
<td>99/105</td>
<td>100/105</td>
<td>105/105</td>
</tr>
<tr>
<td>Sequence coverage (%)</td>
<td>89</td>
<td>94</td>
<td>95</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 4.2: Amount of bound trypsin and performance of the IMEPP tips immobilized with different concentrations of trypsin.

<table>
<thead>
<tr>
<th>Label</th>
<th>2530a</th>
<th>2530b</th>
<th>2530c</th>
<th>2530d</th>
<th>2530e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin conc. immobilization (mg/mL)</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Reacted amount (Trypsin µg)</td>
<td>214</td>
<td>406</td>
<td>1031</td>
<td>1809</td>
<td>2821</td>
</tr>
<tr>
<td>Bound amount (Trypsin µg)</td>
<td>18</td>
<td>71</td>
<td>138</td>
<td>142</td>
<td>135</td>
</tr>
<tr>
<td>Identified peptide number</td>
<td>54/105</td>
<td>95/105</td>
<td>100/105</td>
<td>102/105</td>
<td>99/105</td>
</tr>
<tr>
<td>Sequence coverage (%)</td>
<td>51</td>
<td>90</td>
<td>95</td>
<td>97</td>
<td>94</td>
</tr>
</tbody>
</table>
obtained using a concentration of 10 mg/mL trypsin in solution. Any trypsin concentration greater than 10 mg/mL makes it difficult to pass through the monolith because of the high backpressure stemming from the high viscosity solution. However, the amount of immobilized enzyme on the monolith in the present work was four times higher than that previously used in a capillary format [28]. Thus, it might result in much higher digestion capacity.

With regard to enzyme immobilization, it is always desirable to increase the amount of enzyme on the support in order to enhance the enzyme:substrate ratio and thus increase the protein digestion reaction rate. Two critical factors have been suggested for the optimization of trypsin bioreactor performance: accessibility to the active site related to enzyme:substrate ratio, and response intensity (i.e. sensitivity) related to the total amount of active sites [33]. As shown in Table 4.2, when the amount of trypsin used for immobilization increased, the bound amount of trypsin as well as the sequence coverage likewise increased. However, when the immobilization maximum had been reached, using 10 mg/mL of trypsin, all the available binding sites had been occupied. When 15 mg/mL of trypsin solution was used for immobilization, the sequence coverage slightly decreased. This might either be due to the high enzymatic density on the solid support which hindered the access of substrate to the active site, or perhaps the higher enzyme loading induced an aggregation and/or denaturation of the enzyme prior to or after immobilization, which led to a slight decrease in the enzyme activity [33]. Based on these results, a trypsin concentration of 10 mg/mL for immobilization was selected for studies of protein digestion.

In summary, a single-step surface modification method with EDMA, post modification of monolith support with 25% (w/w) VAL concentration and exposed under UV for 30 min, enzyme immobilization using 10 mg/mL of trypsin and 50
mM ammonium acetate solution in 20:80 acetonitrile:water, pH 8.75, were used in all our experiments.

4.4 Conclusions

In this work, a novel trypsin-immobilized polypropylene pipette tip based on an organic polymer monolith was developed. UV-initiated photografting provided a simple and versatile approach for PP surface modification that produced better attachment of the polymer monolith. Further post-modification on the pore surface of the monolith created reactive azlactone functionalities which afforded an excellent support for the immobilization of trypsin. In addition, the high surface area to volume ratio, as well as a high degree of interconnected channels produced in photopolymerized monoliths, provided rapid mass transfer for proteolytic digestor applications. The preliminary results obtained are very promising in view of the very high sequence coverage coupled with very short digestion time achieved for cytochrome c. It is likely that this developed IMEPP tip can be used for the digestion of protein samples of different sizes. This makes the developed immobilized enzyme reactor based on pipette tip format very suitable for high-throughput sample preparation required to accelerate the process of protein mapping, drug discovery and drug development. Further studies were to be focused on detailed characterization of this enzyme reactor in comparison with commercially available tryptic digest tips and traditional liquid phase digestion. In addition, the developed IMEPP tips were to be used for fast sample preparation of biological samples of pharmaceutical interest for both qualitative and quantitative bioanalysis.
Chapter 4

4.5 References

Chapter 4


Chapter 5

Characterization and Application of a Trypsin-Immobilized Monolithic Polymer with Pipette-Tip Format for Bioanalysis using LC-MS/MS

5.1 Introduction

In recent years, there has been a significant change in direction in the pharmaceutical industry and many new drugs are based on biological molecules, such as proteins and peptides. In testing the efficacy and safety of these new types of drugs, it is necessary to measure the medication levels in blood or plasma and the first step in this process is an enzymatic digestion step. Traditional enzyme digestion protocols present a number of drawbacks, such as long incubation time and also enzyme autodigestion which results in undesirable formation of additional peptides, leading to possible ionization suppression in the MS analysis and interference in the interpretation of the data [1]. Recent major research efforts have focused on the use of reactive monoliths directly polymerized in different formats (e.g. capillaries, microchips, columns, pipette tips, syringe needle and disks), acting as supports for trypsin immobilization. Such immobilized enzyme techniques have been used in the digestion of proteins, which are followed by MS determination of the peptides [2-6] and were introduced in Chapter 4 of this thesis.

These applications clearly highlight the potential of enzyme immobilization technology for fast and efficient sample preparation. However, previous work was all based on qualitative studies of proteins in standard buffer as a proof of concept of these enzyme reactors. A limited number of proteomic studies have been
reported for rapid proteolytic digestion and protein identification in serum samples [3,7]. Conversely, quantitative analyses of proteins are more applicable and important to the bioanalytical setting of the pharmaceutical industry. One of the most difficult analytical challenges in the drug discovery process is preparing samples for reliable quantification of proteins in a fast and efficient manner. These proteins are potential biomarkers of disease in biological matrices.

Tip-based methods reap numerous advantages in terms of speed, efficiency, sample clean-up and enrichment with subsequent coupling to LC-MS/MS for high-throughput bioanalysis. Altun et al. [8] and Abdel-Rehim et al. [9] introduced a sample preparation technique using a set of polypropylene tips containing a plug of an in situ polymerized methacrylate-based monolithic sorbent for use with a 96-tip robotic device. These sorbents have been used successfully for the extraction and quantification of β-blockers and anaesthetics from human plasma by LC-MS/MS. A novel trypsin-immobilized organic polymer monolith based on a pipette tip format has been developed and described in Chapter 4 for the digestion of proteins. The developed IMEPP tips exhibited high hydrolytic activity for the digestion of cytochrome c, which resulted in very high sequence coverage of over 90% with a short contact time prior to MS analysis.

The aim of this chapter is to explore the utility of tip-based protein digestion methodologies in a bioanalytical setting. Additionally, this chapter will compare and contrast the potential advantages of this technology with traditional liquid phase digestion and commercially available trypsic digest tips, such as MonoTip® Trypsin. The developed IMEPP tips were characterized using a MicrOTOF-Q quadrupole time-of-flight MS and LC-MS/MS system including an AB SCIEX Triple Quadrupole™ 5500 mass spectrometer, followed by an evaluation of its efficiency in rapid digestion of proteins by running a number of pipetting cycles. In
addition, digestion of proteins spiked in rat plasma was performed for the first time using enzyme immobilization technology for both qualitative and quantitative analysis. This method has great potential for high-throughput sample preparation, enabling a fast quantitative profiling of biological molecules for drug discovery and development in pharmaceutical industries.

5.2 Experimental

The general experimental details are described in Chapter 2. Detailed conditions are elaborated in each of the figure captions.

5.2.1 Sample preparation for qualitative analysis

5.2.1.1 Protein digestion in standard buffer

The sample preparation for protein digestion was performed according to the procedure of Krenkova et al. [10]. Briefly, protein [BSA (bovine serum albumin) or hIgG (polyclonal human immunoglobulin G)] was dissolved in 100 mM ammonium bicarbonate, pH 8.2, containing 8 M urea to obtain a solution with a concentration of 5 mg/mL. The protein was then reduced and alkylated. 1 mL of 5 mg/mL protein was added to 100 µL of 45 mM dl-dithiothreitol (DTT) at 60 °C for 40 min. After the temperature of the sample solution was reduced to room temperature, the proteins were alkylated with 100 µL of 100 mM iodoacetamide (IAA) for 30 min at room temperature. Before digestion, the reduced and alkylated protein solution was diluted to 0.5 mg/mL using 50 mM ammonium acetate, pH 8.75 containing 20% acetonitrile. Melittin (0.005 mg/mL, rat calcitonin gene-related peptide (CGRP) (0.005 mg/mL), cytochrome c (0.1 mg/mL) and myoglobin (0.1 mg/mL) without any previous treatment were dissolved in 50 mM ammonium acetate, pH 8.75 containing 20% acetonitrile. For protein digestion with soluble enzyme, denatured protein was diluted with water to achieve a final urea
concentration of 2 M and protein concentration of 1.25 mg/mL (for BSA). Trypsin was added to 200 µL of protein solution at a substrate-to-enzyme ratio of 50:1 (w/w) for protein digestion and the solution was incubated at 37 °C for 24 h. The proteolysis was terminated by a pH decrease after the addition of formic acid to a final concentration of 0.1% (1 µL of 10% formic acid in water). For the digestion of proteins using IMEPP tips, the protein was dissolved in a solution consisting of 50 mM ammonium acetate, pH 8.75, containing 20% acetonitrile, and 20 pipetting cycles were used. The peptide fragments from the digestion were collected in microvials and analyzed using the direct infusion and LC-MS methods. The mass spectra of all samples were then compared against the protein digestion database to determine the sequence coverage.

5.2.1.2 Protein digestion in rat plasma

Rat plasma samples were spiked with target proteins (melittin, rat CGRP, cytochrome c, myoglobin, BSA and IgG) individually to achieve the same concentration as described above. Then, 1 µL of plasma spiked with target protein was made up to a total volume of 100 µL using 50 mM ammonium acetate buffer with 20% acetonitrile for IMEPP tip digestion. The peptides from the digestion were collected in microvials and analyzed using the direct infusion and LC-MS methods. The peptide fragments were identified using a database search of Protein Prospector tool.

5.2.2 Protein identification and multiple reaction monitoring (MRM) design

5.2.2.1 Sample preparation for MRM development

Different proteins, including melittin, rat CGRP, cytochrome c, myoglobin, transferrin, BSA and hIgG per 20-µL aliquot of 10 mg/mL were prepared using digestion buffer (100 mM Tris-HCl/ 10 mM CaCl₂/ 20% acetonitrile). Each of the
proteins was digested individually for the development of MRM methods. For smaller-sized proteins such as melittin, rat CGRP, cytochrome c and myoglobin, each of the 20 µL aliquot protein samples was diluted with 450 µL of digestion buffer, 10 µg of trypsin added and the sample were incubated at 37 °C for 24 h on an Eppendorf heating block. For larger-sized proteins that needed to be chemically treated, such as transferrin, BSA and hIgG, the 20 µL aliquot protein samples were diluted with 25 µL of 6 M guanidine hydrochloride (GdnHCl). Samples were reduced by adding 15 µL of 7 mM TCEP, followed by 45-min incubation at 56 °C on an Eppendorf heating block. Alkylation of cysteine residues was carried out by adding 15 µL of 13 mM iodoacetamide and incubating the sample for 1 h at 37 °C. Alkylated protein samples were diluted with 450 µL of digestion buffer, 10 µg of trypsin added and the solutions were incubated at 37 °C overnight. Each of the digested proteins was further diluted 1:80 with the digestion buffer. 75 µL of protein samples (100 µL for myoglobin) were injected and analyzed by the LC-MS/MS system.

5.2.2.2 Peptide MRM development

A scheme describing the work flow for the method development of test proteins and peptides in plasma is shown in Figure 5-1. Peptide fragment masses were selected from in silico digest of each target protein using the ExPASy tool available from the website http://au.expasy.org/proteomics. The “Tools” option of the website was chosen, followed by the selection of “PeptideMass” function. For smaller-sized proteins (e.g. melittin, rat CGRP, cytochrome c and myoglobin) that were not reduced and alkylated, the amino acid sequence of each protein was inserted in the field. Trypsin was chosen as the enzyme for the database search, with no missed cleavages being allowed. For larger-sized proteins that were chemically reduced and alkylated, the option “cysteines treated with
iodoacetamide” was chosen. The possible surrogate peptides resulted from the digestion of each protein were displayed. Due to the possibility of chosen surrogate peptide in the target standard protein being present in other target proteins or sample matrix (in this case, rat plasma), each surrogate peptide was checked via BLASTP searches to ensure that each selected proteotypic peptide corresponded to a single gene product only.

Due to the limitation in mass range of the API5500 (up to 1000 mAU for the linear ion trap and 1250 for MRM quantitative mode), the multiply charged parent ions (mainly +2 & +3) were utilized for peptides larger than 1000 Da. An enhanced product ion (EPI) scan was then utilized, for up to five parent ions simultaneously. The EPI scan enabled the identification of the product ions with highest intensity and suitable collision energy to construct a quantitative (MRM) method.

Figure 5-1: Schematic for the method development of test proteins and peptides in rat plasma.

5.2.3 Characterization of performance of IMEPP tips
5.2.3.1 Plasma loading experiment for IMEPP tips
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The rat plasma sample was spiked with a mixture of four target proteins (i.e. melittin, rat CGRP, cytochrome c and myoglobin) to achieve a final concentration of 100 µg/mL for each protein. Different amount of rat plasma ranging from 1 – 60 µL (1, 2.5, 5, 10, 20, 40, 60 µL) were made up to a total volume of 170 µL with the digestion buffer and digested using IMEPP tips. The digestion protocols used for commercially available tryptic digest MonoTip® Trypsin and IMEPP tips were described previously in Section 2.3.9. For liquid phase digestion, trypsin (10 µg) was added to each sample solution and the solution was incubated at 37 °C for 24 h. The proteolysis was terminated by a pH decrease after the addition of formic acid to a final concentration of 0.1% (1 µL of 10% formic acid in water). The resultant peptide solutions were made up to a total volume of 200 µL using mobile phase A (5% acetonitrile/ 95% water with 0.1% formic acid) and were injected onto an AB SCIEX Triple Quadrupole™ 5500 mass spectrometer using the multiple reaction monitoring (MRM) mode. Extracted ion chromatograms (EIC) corresponding to tryptic digested peptides of target proteins were recorded and areas of the eluting peaks were computed.

5.2.3.2 Time course assay
Rat plasma spiked with four target proteins (100 µg/mL for each protein) was used in this assay. A 10 µL sample of the plasma was diluted with 160 µL of digestion buffer. The protein sample solution was digested using IMEPP tips and MonoTip® Trypsin for a duration of 30 min. Different digestion durations of 30 min, 1 h, 4 h and 24 h were set up for liquid phase digestion of the protein sample, while keeping the amount of trypsin (10 µg) constant. The digested samples were diluted 1:20 using the digestion buffer and analyzed with the LC-MS/MS system.

5.2.3.3 Sample clean-up and enrichment functionality assay
Chapter 5

The rat plasma spiked with four target proteins (100 µg/mL for each protein) was again used in this assay. A 10 µL sample of the plasma was diluted with 160 µL of digestion buffer, one with and one without 20% acetonitrile. Then, the plasma samples were digested with IMEPP tip and MonoTip® Trypsin. The digestion tips using 100% aqueous buffer were eluted with 50 µL of 100% acetonitrile containing 0.1% formic acid. Sample solutions digested in digestion buffer with and without 20% acetonitrile as well as the eluted samples from the aqueous-digestion-tips were analyzed using the LC-MS/MS system.

5.2.3.4 Volume test

The rat plasma (10 µL) spiked with four target proteins (100 µg/mL for each protein) was diluted with 60 µL and 160 µL of digestion buffer. Then, plasma samples with different total volumes (70 µL and 170 µL) were digested with IMEPP tips and analyzed using the LC-MS/MS system.

5.2.4 Sample preparation for quantitative analysis in rat plasma

For quantitative analysis, the rat plasma sample was spiked with a mixture of four target proteins (i.e. melittin, rat CGRP, cytochrome c and myoglobin) to achieve final concentrations of 1.5625, 3.125, 6.25, 12.5, 25 and 50 µg/mL for each protein in plasma. To each protein mixture with different concentration, 10 µL of plasma was added to 60 µL of buffer (100 mM Tris-HCl/ 10 mM CaCl2/ 20% acetonitrile) and the sample digested using IMEPP tips. The digested sample solutions were back-diluted to a volume of 200 µL using mobile phase A (5% acetonitrile/ 95% water with 0.1% formic acid).

For larger-sized proteins that need to be chemically treated, such as transferrin, BSA and hIgG, the rat plasma samples were spiked with a mixture of three target proteins to achieve final concentrations of 1.5625, 3.125, 6.25, 12.5, 25 and 50 µg/mL for each protein in plasma. The high-molecular weight proteins
spiked in plasma (10 µL) were diluted with 15 µL of Tris-HCl, pH 8.8 containing 10 mM of CaCl₂ and 20% acetonitrile, and were further diluted with 25 µL of 6 M (GdnHCl). Samples were reduced and alkylated. Alkylated protein samples were desalinated with 420 µL of the same buffer. Aliquots of 100 µL of the reduced and alkylated protein solutions were pipetted with 20 cycles of pipetting procedure and the tip was soaked in the sample solution for 30 min. The resultant peptide solutions were diluted with 100 µL buffer and analyzed by the AB SCIEX Triple Quadrupole™ 5500 mass spectrometer using the MRM mode. A calibration curve was constructed based on the peak areas recorded against the concentration of proteins spiked in rat plasma.

5.3 Results and discussion

5.3.1 Application of IMEPP tips to the digestion of model proteins spiked in standard buffer and rat plasma

The high complexity of plasma makes the digestion of proteins using the immobilized enzyme method very challenging. Tens of thousands of proteins are present in plasma and are dispersed over an extremely wide concentration range. Several different proteins have often been used in model test systems that include a proteolytic digestion step. In this study, six proteins with molecular masses ranging from 2.8 kDa to 150 kDa were used as the model proteins. Under optimized conditions, the excellent proteolytic activity of the IMEPP tips on these proteins was demonstrated in Chapter 4. Due to the low target substrate levels that are present in plasma, trypsin autolysis becomes a significant competing reaction which reduces substrate conversion, and the reaction is substrate-limited. In addition, the non-specific adsorption of proteins may also block the active sites on the IMEPP tips and decrease the tryptic digest activity. The newly developed
method, involving the immobilization of enzyme on a large through-pore organic polymer monolith support using higher enzyme concentration, enhanced the hydrolysis rate and enabling rapid diffusion, should promote efficient digestion of low concentration substrates. This is the first time a rapid digestion of proteins spiked in plasma using the IMEPP tips developed has been performed.

The high efficiency digestion of the small protein rat CGRP using the IMEPP tip is shown in Figure 5-2. The large protein peak was missing after the digestion. The IMEPP tip also showed very high proteolytic activity even when it was used to digest the protein spiked in rat plasma sample (Figure 5-2). No significant difference was found between the digestion using soluble trypsin and IMEPP tips (Figure 5-2). Figure 5-3 shows base peak chromatograms (BPC) of cytochrome c digest prepared using 24 h digestion with soluble trypsin, with 15 pipetting cycles (~ 6 min) for digestion of cytochrome c in digestion buffer and rat plasma using IMEPP tips at a concentration of 0.1 mg/mL (Figure 5-3). No marked difference between the digestions using soluble trypsin, IMEPP tip and digestion in rat plasma using IMEPP tip was observed (Figure 5-3). However, some cytochrome c remained undigested when in-solution digestion was used (Figure 5-3). By contrast, the IMEPP tip exhibited excellent performance, with complete digestion of cytochrome c and much faster digestion in comparison with the conventional in-solution digestion (Figure 5-3). It is also worth mentioning that the liquid phase digestion of protein spiked in rat plasma would result in undesirable formation of additional peptides due to enzyme autodigestion, which may lead to ionization suppression or interference in the interpretation of data (Figures 5-2 and 5-3). Meanwhile, it has been demonstrated that using tip-based protein digestion, the problem of enzyme autodigestion was eliminated (Figures 5-2 and 5-3).
Figure 5-2: Chromatograms of a tryptic digest of rat CGRP (0.005 mg/mL) using the IMEPP tip and soluble trypsin in both standard buffer and rat plasma. Conditions: HPLC column, Dionex Acclaim® PolarAdvantge C16 (2.1 x 100 mm, 3 µm); temperature, 30 °C; flow-rate, 0.2 mL/min; injection volume, 5 µL; mobile phases: A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile; a linear gradient of B% from 20% to 55% in 35 min.
Figure 5-3: Chromatograms of a tryptic digest of cytochrome c (0.1 mg/mL) using the IMEPP tip and soluble trypsin in both standard buffer and rat plasma. Conditions are the same as described in Figure 5-2.
Cytochrome c and other protein standards were chosen because they are inexpensive, stable and easy to prepare due to the lack of cysteines which simplifies the digestion protocols. Therefore, any specific treatment prior to digestion was not necessary when small proteins such as rat CGRP or cytochrome c were used. On the other hand, the three-dimensional protein structure of large proteins, such as BSA, was stabilized by chemical interactions such as disulfide bonding, and RP or hydrophilic interactions between the polypeptide chains. In the case of digesting BSA with the IMEPP tip, pre-treatments such as chemical and thermal were required. Without any specific pre-treatment prior to digestion, the IMEPP tip would be unable to catalyze digestion, since the active site of the trypsin did not come into contact with the peptide chain within shorter digestion times [4]. Therefore, the process of chemical reduction and alkylation are generally employed to enhance exposure sites of the protein which were previously inaccessible for proteolysis [11]. In this study, the salts present in the protein solution after this treatment were diluted 10 times so that the proteolytic activity of the immobilized enzyme would not be compromised. Figure 5-4 shows the LC-MS separation of the digestion of denatured BSA with the IMEPP tip. The digested peptide was obtained with 15 pipetting cycles at 37 °C. The peptide profile digested with the developed tip was the same as that processed with the conventional method.

The individual digests of the proteins in standard buffer and those that were spiked in rat plasma are shown in Figures 5-5 and 5-6. The digestion was both very fast and efficient and no real difference was observed between mass spectra obtained from the in-solution digestion (not shown) and the digestion using IMEPP tips for the digestion of rat CGRP, cytochrome c and BSA. In addition, the IMEPP tips were also used for the digestion of standard proteins, melittin and
Figure 5-4: Chromatograms of a tryptic digest of BSA (0.5 mg/mL) using the IMEPP tip and soluble trypsin in both standard buffer and rat plasma. Conditions are the same as described in Figure 5-2 except a linear gradient of B% from 10% to 50% B in 35 min.
Figure 5-5: ESI-TOF MS spectra of peptides obtained by digestion of six proteins using IMEPP tips. Conditions: direct infusion flow-rate, 180 µL/hr; acquisition time, 2 min; capillary voltage, 4.5 kV; end plate offset, 500 V; nebulizer, 0.3 bar; drying gas, 4 L/min; temperature, 180 °C. Asterisks define peaks of positively identified peptides after protein digestion.
Figure 5-6: ESI-TOF MS spectra of peptides obtained by digestion of four proteins spiked in rat plasma using IMEPP tips. Conditions are the same as described in Figure 5-5.
myoglobin, by direct infusion ESI-TOF MS method (Figure 5-5). The mass spectra of all samples were then compared against the protein digestion database (protein prospector, http://prospector.ucsf.edu/) to identify the digest and determine the sequence coverage. Asterisks were not included in the digestion of hIgG in Figure 5-6 because this protein contains six different components. It would be difficult to differentiate the peptides resulting from the digestion of these components using asterisks. Table 5.1 summarizes the results of highest sequence coverage obtained for each digested protein. An ideal coverage of 100% was achieved for the low-molecular mass peptide rat CGRP using soluble enzyme and the IMEPP tip. In addition, the sequence coverages of the proteins digested using IMEPP tips were above 88% in standard buffer and also above 84% for proteins spiked in rat plasma (Table 5.1).

A myoglobin trypsin digest always results in relatively few peptides and exhibits large protein peaks at high concentration (0.5 mg/mL), as shown in Figure 5-7 (a). This is because myoglobin contains a heme group which is hard to digest. The early studies of Russell et al. showed that enzymatic digestion in mixed organic-aqueous solvent systems were highly efficient in terms of both the rate of digestion and amino acid sequence coverage [12]. They reported that trypsin maintained proteolytic activity in a variety of solvents (e.g. methanol, acetone, 2-propanol and acetonitrile) using up to 80% organic solvent [12]. Several authors have also confirmed that the activity of immobilized trypsin is not affected by organic solvents, with the usage of up to 100% acetonitrile [13,14]. Gordon and David also reported an optimum of 45% acetonitrile used to achieve the highest sequence coverage of the digestion [14]. Therefore, a digestion buffer containing 45% or 80% acetonitrile in 50 mM ammonium acetate, pH 8.75 was used for myoglobin digestion in order to improve the digestion efficiency. Surprisingly, the
Table 5.1: Results of sequence coverage for the digestion of proteins and spiked samples using IMEPP tips.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (Da)</th>
<th>Concentration (mg/mL)</th>
<th>Sequence Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Soluble trypsin</td>
</tr>
<tr>
<td>Melittin</td>
<td>2848</td>
<td>0.005</td>
<td>100</td>
</tr>
<tr>
<td>Rat CGRP</td>
<td>3806</td>
<td>0.005</td>
<td>100</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>11702</td>
<td>0.01</td>
<td>97</td>
</tr>
<tr>
<td>myoglobin</td>
<td>16951</td>
<td>0.1</td>
<td>95</td>
</tr>
<tr>
<td>BSA</td>
<td>69294</td>
<td>0.5</td>
<td>92</td>
</tr>
</tbody>
</table>
Figure 5-7: ESI-TOF MS spectra of peptides obtained by digestion of myoglobin (0.5 mg/mL) using IMEPP tips in (a) 20% acetonitrile (sequence coverage: 97%), (b) 45% acetonitrile (sequence coverage: 88%) in digestion buffer.
sequence coverage obtained from the digestion of myoglobin using 45% acetonitrile was lower than using 20% acetonitrile [Figure 5-7 (b)].

Several reports have suggested that using high organic solvents might increase the proteolytic activity of the trypsin, since increasing the amount of organic solvent also increases the solubility of the substrate, which changes the conformation of substrate or enzyme itself, providing more accessible active sites for the substrate [12]. One thing that is known with certainty is that increasing the amount of acetonitrile also increases the eluting strength of the solution, enabling it to release more digested peptides from the monolith support and thus allowing more digested fragments to be detected. A similar value of sequence coverage was obtained despite using 45% acetonitrile in the digestion buffer for BSA digestion. Using 80% acetonitrile in the digestion buffer was also tried for the digestion of BSA. However, a higher percentage of acetonitrile resulted in the precipitation of the protein which blocked the IMEPP tip. Furthermore, it is important to note that the lowest concentration of acetonitrile in the digestion buffer is always preferable to facilitate the subsequent gradient separation of peptides in reversed-phase chromatography (e.g. LC-MS). Therefore, 20% acetonitrile was employed for the digestion of high-molecular weight protein.

The efficiency of the trypsin-immobilized organic polymer pipette tip was also evaluated with high-molecular weight proteins, such as human polyclonal IgG. The rapid growth of antibody drugs and drug candidates in the biopharmaceutical industry has created a demand for automated proteolytic digestion to assist in pharmaceutical stability studies, to identity assays and to facilitate quality control of these therapeutic proteins [15]. Therefore, efficient and rapid digestion is necessary for studying this extensive and complex protein. This protein contains four types of heavy chains (Igg-1, Igg-2, Igg-3 and Igg-4) and two
Figure 5-8: Chromatograms of a tryptic digest of hIgG (0.5 mg/mL) using the IMEPP tip and soluble trypsin in both standard buffer and rat plasma. Conditions are the same as described in Figure 5-4.
types of light chains (Igg-kappa and Igg-lambda). Again, this high-molecular weight protein has to be reduced and alkylated prior to the in-solution and IMEPP tip digestion. The peptide chromatogram of the digestions is shown in Figure 5-8. The mass spectra of the digests of hIgG using soluble trypsin and the IMEPP tip in both standard buffer and rat plasma are shown in Figures 5-5 and 5-6. Results characterizing the digestion of hIgG chains obtained using both soluble trypsin and the IMEPP tip are summarized in Table 5.2. Similar values of sequence coverage were observed for both the trypsin-immobilized pipette tip and use of the soluble enzyme. However, lower sequence coverage was observed for the digestion of protein spiked in rat plasma. This might be due to the high protein components which were present in rat plasma, blocking the majority of active sites on the trypsin-immobilized monolith support, thereby resulting in a loss of activity of the IMEPP tip during the digestion of heavy chains (i.e. Igg-1) [16]. However, this method works well for the light chains (i.e. Igg-kappa and Igg-lambda), yielding sequence coverage similar to that of the in-solution digestion, albeit with an additional advantage of very short digestion time (~ 6 min). In summary, these results suggest that the developed trypsin-immobilized monolithic polymer tips can be used for fast and efficient digestion of both low- and high-molecular weight proteins, thus indicating that this tip can be used for proteome studies.

5.3.2 Design of MRM assays for target proteins

The application of MRM to peptide analysis has created a new breakthrough for protein analysis in complex biological matrices. Due to the multiple fragment steps in MRM, higher selectivity is achieved. In this chapter, MRM methods were developed in order to identify the peptides after protein digestion, which can be used to interpret the digestion efficiency. Specifically, a range of different assays were developed including plasma loading capacity, time course assay, sample
Table 5.2: Comparison of sequence coverage identification of hIgG using soluble trypsin, IMEPP tips and IMEPP tips for spiked samples.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (Da)</th>
<th>Sequence Coverage (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Soluble trypsin</td>
<td>IMEPP (0.5mg/mL)</td>
<td>IMEPP (Spiked sample 0.5 mg/mL)</td>
</tr>
<tr>
<td>lgg -1</td>
<td>36106</td>
<td>51</td>
<td>31</td>
<td>5</td>
</tr>
<tr>
<td>lgg -2</td>
<td>35901</td>
<td>45</td>
<td>37</td>
<td>22</td>
</tr>
<tr>
<td>lgg -3</td>
<td>41287</td>
<td>39</td>
<td>36</td>
<td>32</td>
</tr>
<tr>
<td>lgg -4</td>
<td>35941</td>
<td>34</td>
<td>42</td>
<td>27</td>
</tr>
<tr>
<td>lgg -kappa</td>
<td>11609</td>
<td>80</td>
<td>68</td>
<td>61</td>
</tr>
<tr>
<td>lgg -lambda</td>
<td>11237</td>
<td>66</td>
<td>75</td>
<td>75</td>
</tr>
</tbody>
</table>
clean-up and effect of digestion volume for the evaluation of the digestion efficiency of the developed IMEPP tips in comparison with commercially available tryptic digest tips and liquid phase digestion.

To develop a targeted analysis method, proteotypic peptides from each protein were selected for detailed analysis and MRM transition design. The molecular weight of cytochrome c is 11701.55 Da; its singly charged molecular ion could not be observed by the LC-MS/MS because it is above the mass detection range of the instrument. Therefore, surrogate peptides obtained from the tryptic digest of this protein had to be used to represent the amount of the protein present in the sample matrix. An example of in silico digest of cytochrome c from the equine heart is given in Figure 5-9. The surrogate peptides at different charged states were calculated using the in-house Peptide Mass Calculator. The entire surrogate peptides were BLAST searched against the sample matrix (in this case, rat plasma) to look for uniqueness in these surrogate peptides. For each protein, two or more proteotypic peptides were selected for further analysis. Care was taken where possible to ensure the selected peptides did not contain cysteine or methionine residues and were between 8 and 15 amino acids in length [17]. From Figure 5-10, it can be observed that the surrogate peptide with amino acid sequence EETLMYEYLENPK only existed in equine heart cytochrome c and not in any rat genome sequences. This therefore can potentially be used as a surrogate peptide for quantification of equine heart cytochrome c in rat plasma.

The unique surrogate peptides of cytochrome c were selected as the first precursor ions in the product ion scan mode. The precursor ions were set into the EPI mode (normally up to five ions) in order to identify the most intense fragment ions with suitable collision energy to construct a quantitative (MRM) method (Figure 5-11). For each peptide, two or more transitions were selected from the
Figure 5-9: A typical result of *in silico* digest of cytochrome c.

Figure 5-10: BLAST search result of horse heart cytochrome c against rat genome.
Figure 5-11: EPI experiment spectra from cytochrome c.
remaining transitions based on the most intense y ions in the MS/MS spectra, with preference given to product ions with m/z values above the precursor ion to increase MRM specificity. In this case, the major fragment ions produced from the doubly-charged ion (m/z = 748.35) are m/z = 892.5, 763.4 and 600.3 (Figure 5-12). The fragment ions were checked using “MS-product” in the protein prospector to ensure that they were from the precursor ion. Table 5.3 shows that the fragment ions m/z = 892.5, 763.4 and 600.3 corresponded to y7, y6, and y5 ions of the surrogate peptide. To decrease the MS duty cycle while ensuring a high number of MS/MS scans over peak, only the most intense MRM transition for each peptide was kept in the final method, and the MS/MS conditions for these MRMs were optimized. Therefore, the most intense fragment m/z 892.5 was selected as the transition for one of the surrogate peptides for the quantification of cytochrome c for different assays and quantitative analysis (Figure 5-12). The same procedure was carried out for all the standard proteins (melittin, rat CGRP, myoglobin, BSA, transferrin, hIgG). Details of the conditions used for the analysis of each peptide, including optimized transition parameters and collision energies for targeted proteins are shown in Tables 5.4 and 5.5. In total, the list indexes eight proteins, with a total number of 22 surrogate peptides. Figure 5-13 shows a typical LC-MS/MS chromatogram with MRM transitions of protein mixture (melittin, rat CGRP, cytochrome c and myoglobin).

5.3.3 Characterization of the digestion performances of IMEPP tips in Pfizer UK

To evaluate the digestion performances of IMEPP tips, the following assays were considered in particular: the plasma loading capacity, digestion efficiency over different durations, sample enrichment functionality and the optimum sample
Figure 5-12: MRM methods workflow by selecting the most intense fragment ions from the product ion of cytochrome c. (A) shows the single product ion m/z 748.35 from the EPI experiment spectra. (B) shows the MS/MS fragment ions of the selected product ion.
Table 5.3: Theoretical peak table generated from the surrogate peptide m/z 748.35 of cytochrome c.

<table>
<thead>
<tr>
<th>P</th>
<th>129.1022</th>
<th>K</th>
<th>382.2029+2</th>
<th>y6+2</th>
<th>705.3124</th>
<th>a6</th>
<th>1138.4973</th>
<th>b9</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>136.0757</td>
<td>Y</td>
<td>445.2293</td>
<td>a4</td>
<td>733.3073</td>
<td>b6</td>
<td>1224.5453</td>
<td>a10</td>
</tr>
<tr>
<td>y1+2</td>
<td>147.1128</td>
<td>V</td>
<td>446.7242+2</td>
<td>y7+2</td>
<td>748.3529+2</td>
<td>y10</td>
<td>1237.6133</td>
<td>y10</td>
</tr>
<tr>
<td>K</td>
<td>179.6079+2</td>
<td>y3+2</td>
<td>473.2242</td>
<td>b4</td>
<td>763.3985</td>
<td>y6</td>
<td>1252.5402</td>
<td>b10</td>
</tr>
<tr>
<td>L</td>
<td>231.0975</td>
<td>a2</td>
<td>487.2511</td>
<td>y4</td>
<td>868.3757</td>
<td>a7</td>
<td>1321.5980</td>
<td>a11</td>
</tr>
<tr>
<td>N</td>
<td>244.1292+2</td>
<td>y4+2</td>
<td>512.2444+2</td>
<td>y8+2</td>
<td>892.4411</td>
<td>y7</td>
<td>1349.5930</td>
<td>b11</td>
</tr>
<tr>
<td>K</td>
<td>244.1656</td>
<td>y2</td>
<td>568.7864+2</td>
<td>y9+2</td>
<td>896.3706</td>
<td>b7</td>
<td>1366.6559</td>
<td>y11</td>
</tr>
<tr>
<td>E</td>
<td>259.0925</td>
<td>b2</td>
<td>576.2698</td>
<td>a5</td>
<td>981.4598</td>
<td>a8</td>
<td>1495.6985</td>
<td>MH</td>
</tr>
<tr>
<td>M</td>
<td>300.6712+2</td>
<td>y5+2</td>
<td>600.3352</td>
<td>y5</td>
<td>1009.4547</td>
<td>b8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>y2+2</td>
<td>332.1452</td>
<td>a3</td>
<td>604.2647</td>
<td>b5</td>
<td>1023.4816</td>
<td>y8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>358.2085</td>
<td>y3</td>
<td>619.3103+2</td>
<td>y10+2</td>
<td>1110.5024</td>
<td>a9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>360.1401</td>
<td>b3</td>
<td>683.8316+2</td>
<td>y11+2</td>
<td>1136.5656</td>
<td>y9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.4: Details of surrogate peptides, Q1, Q3 mass and specific collision energy for each target protein.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide Sequence</th>
<th>Peptide mass</th>
<th>Charge state</th>
<th>Q1</th>
<th>Q3</th>
<th>Collision Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melittin</td>
<td>VLTTGLPALISWIK</td>
<td>1511.9</td>
<td>2+</td>
<td>756.8</td>
<td>927.7</td>
<td>30</td>
</tr>
<tr>
<td>Rat CGRP</td>
<td>DNFVPTNVGSEAF</td>
<td>1396.6</td>
<td>2+</td>
<td>698.8</td>
<td>476.3</td>
<td>20</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>GLSDGKWQQVLNWG</td>
<td>1815.9</td>
<td>2+</td>
<td>908.4</td>
<td>390.3</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>VEADIAKGHEQVLIR</td>
<td>1606.9</td>
<td>2+</td>
<td>803.9</td>
<td>814.5</td>
<td>45</td>
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<tr>
<td></td>
<td>HGTIYTLGILGILK</td>
<td>1378.8</td>
<td>2+</td>
<td>689.8</td>
<td>885.5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>LFTGHPETLEK</td>
<td>1271.7</td>
<td>2+</td>
<td>636.3</td>
<td>716.3</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>HPGDFGADAQGAMTK</td>
<td>1501.7</td>
<td>2+</td>
<td>751.8</td>
<td>742.8</td>
<td>37</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>EETLMEYLENPK</td>
<td>1495.7</td>
<td>2+</td>
<td>748.3</td>
<td>892.4</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>TGGQPFTYTDANK</td>
<td>1470.7</td>
<td>2+</td>
<td>735.8</td>
<td>358.2</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>TGPNLHGLFGR</td>
<td>1168.6</td>
<td>2+</td>
<td>584.8</td>
<td>549.3</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>EDLIAYLK</td>
<td>963.5</td>
<td>2+</td>
<td>482.8</td>
<td>494.3</td>
<td>20</td>
</tr>
</tbody>
</table>
Table 5.5: Details of surrogate peptides, Q1, Q3 mass and specific collision energy for each target protein that was chemically treated.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide Sequence</th>
<th>Peptide mass</th>
<th>Charge state</th>
<th>Q1</th>
<th>Q3</th>
<th>Collision Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>QTALVELLK</td>
<td>1014.6</td>
<td>2+</td>
<td>507.8</td>
<td>785.5</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>LVNELTEFAK</td>
<td>1163.6</td>
<td>2+</td>
<td>582.3</td>
<td>951.5</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>DAFLGSFLYEYSR</td>
<td>1567.7</td>
<td>3+</td>
<td>523.3</td>
<td>717.3</td>
<td>25</td>
</tr>
<tr>
<td>Transferrin</td>
<td>YLGEYVK</td>
<td>1000.5</td>
<td>2+</td>
<td>500.8</td>
<td>724.3</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>SVIPSDGPSVACVK</td>
<td>1415.7</td>
<td>2+</td>
<td>708.4</td>
<td>558.8</td>
<td>35</td>
</tr>
<tr>
<td>IgG-Kappa</td>
<td>SGTASVVCLNNFYPR</td>
<td>1797.9</td>
<td>2+</td>
<td>899.0</td>
<td>435.3</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>TVAAPSVFIFPSPDEQLK</td>
<td>1946.0</td>
<td>3+</td>
<td>649.3</td>
<td>913.4</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>VYACEVTHQGLSSPVTK</td>
<td>1875.9</td>
<td>3+</td>
<td>626.0</td>
<td>807.3</td>
<td>25</td>
</tr>
<tr>
<td>IgG1</td>
<td>FNWYVDGVEVHNK</td>
<td>1677.8</td>
<td>3+</td>
<td>559.9</td>
<td>708.8</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>GPSVFPLAPSSK</td>
<td>1186.6</td>
<td>2+</td>
<td>593.8</td>
<td>699.5</td>
<td>30</td>
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</tbody>
</table>
Figure 5-13: LC-MS/MS chromatogram corresponding to the MRM transitions associated with specific peptides for each targeted protein.
volume for digestion. All the assays were completed using the MRM methods developed previously.

5.3.3.1 Plasma loading capacity of IMEPP tips

The plasma loading experiment was designed to determine the maximum amount of plasma that can be used to yield the highest efficiency for digestion. This is particularly important since, if a lower amount of plasma sample is used, the sensitivity of detection will decrease; if too much plasma is used, it will block the tips and also decrease the tryptic digestion efficiency (due to the relative amount of protein present in the sample). This plasma loading experiment was also used to compare the sample capacity when liquid phase digestion and tip-based technology (i.e. MonoTip® Trypsin and the IMEPP tip) were used. Figure 5-14 shows one of the examples of plasma loading capacity for liquid phase digestion compared to the two different tip-based technologies. Liquid phase digestion always gives lower plasma capacity, in this case 5-10 µL of plasma, due to the limited amount of trypsin used for digestion in a bid to minimize auto-digestion that can potentially interfere with the sensitivity of detection.

Immobilized enzyme technology especially for tip-based methods, offers the advantages of high plasma loading capacity of up to 40 µL of plasma, fast digestion rate, and the ability to use a high concentration of enzyme for digestion without compromising the detection. This is consistent with all the other tested proteins. The present study also showed that the developed IMEPP tips based on a porous polymer monolith exhibited higher capacity and efficiency than the commercially available tryptic digest tips based on a silica monolith acting as enzyme support. Enzyme immobilization on a porous polymer monolith offers distinct advantages over the silica monolith in terms of flexibility of synthesis, greater biocompatibility over a wide pH range and also versatile functionality.
Figure 5-14: Plasma loading capacity for liquid phase digestion, MonoTip® Trypsin and IMEPP tips for the digestion of cytochrome c spiked in different amount of rat plasma.
modification. The highly porous surface facilitates the flow-through of the sample solutions and moreover, the stability of the polymer support for trypsin immobilization means that polymer monolith will not shrink even with up to 40 µL of plasma used for the digestion.

5.3.3.2 Time course study

A time course study was set up in order to investigate the efficiency of digestion for the IMEPP tip for different durations compared to the commercially available enzyme-tip and traditional in-solution digest. Figure 5-15 shows clear predominance of the newly developed IMEPP tips compared to the commercially available product and liquid phase digest over a 30 min digestion period. When compared to the in-solution digest analyses, the 30 min IMEPP tip digest yielded results similar to those of the 24 h solution digest.

Figure 5-15: Digestion time course of cytochrome c with tip-based digestion (30 min) compared to commercially available MonoTip® Trypsin and in-solution digestion (30 min, 1 h, 4 h and 24 h).
5.3.3.3 Sample clean-up and enrichment functionality assay

Given the very small sizes of the samples handled in proteomics, miniature immobilized enzyme reactors placed in capillaries or microfluidic devices have been developed. However, the identification of proteins remains a challenge as the proteins are present in solutions at very low concentrations. The other significant challenge is that matrix interferences such as salts, buffers and detergents must be eliminated prior to the LC-MS/MS analyses. In order to obtain reliable analytical results using these techniques, sample pre-treatment and pre-concentration using solid-phase extraction (SPE) can be employed [18]. The compounds of interest are first absorbed onto the surface of porous materials and later released by elution with a strong solvent in a concentrated band. Custom-designed SPE devices have also been used to eliminate interfering compounds. A dual-function microanalytical device with the integration of SPE and enzymatic digestion for peptide mapping has been developed by Peterson et al. [13]. The samples were pre-concentrated in the first segment of the device, then eluted and digested in the second segment.

The immobilized enzyme polymer monolith contains hydrophobic functionality which can be used for SPE. Therefore, the effects of sample enrichment functionality on IMEPP tips were further studied, as shown in Figure 5-16. The recoveries rapidly decreased for the digestion in 100% aqueous buffer. In addition, the protein solution eluted from the aqueous digestion tip also showed a significant drop in recovery. Therefore, the sample enrichment functionality of the IMEPP tips was not very efficient. Digestion using buffer with 20% acetonitrile performed better than that obtained using an aqueous. The low recovery was possibly due to the properties of the monolith used. In this case a higher amount of cross-linking monomer was used to obtain a highly porous material. The
functional monomer was employed at a lower concentration of hydrophobic monomer, resulting in a loss of the hydrophobic functionality. It is recommended that a formulation should be used with a higher amount of functional monomer or cation-exchange monomer for future studies. However, as discussed in Section 5.3.1, the tips still provide great potential for better sample clean-up and higher sensitivity of detection without the problem of enzyme autodigestion.

Figure 5-16: Peak area of surrogate peptide of cytochrome c digested using MonoTip® Trypsin and IMEPP tips using buffer/20% acetonitrile, aqueous and the eluted samples from the aqueous digestion-tips.
5.3.3.4 Volume test

The volume test was employed to compare different dilutions for the same amount of plasma used for digestion to suit different purposes. For example, a volume just enough for a tip was used to have a fair comparison so that all the solution has the same residency digestion time on the stationary phase. If the total digestion volume used is 170 µL, it is expected to take 3-4 pipetting cycles for all solution to pass through the enzyme immobilized surface. Alternatively, we can pass all the solution through the immobilized bed if a smaller digestion volume of 70 µL used. One concern is that the concentration of plasma in solution might block the tip if a lower digestion volume used (also because of the lower dilution factor). Optimization steps will therefore be expected if different digestion volumes need to be used. In this case, 10 µL of plasma was used and diluted with 170 µL digestion buffer compared to 70 µL of digestion buffer for digestion in tips. Both of the sample solutions were then back-diluted to 200 µL using mobile phase A after digestion using IMEPP tips.

Figure 5-17 shows the recoveries for the surrogate peptides of four different proteins after digestion using an IMEPP tip for digestion volumes of 170 µL and 70 µL. It appears that the overall recoveries of surrogate peptides are higher using the 70 µL digestion volume. The most significant results can be seen for surrogate peptides MELITTIN_A, RAT CGRP_B and CYTO_F (Figure 5-17). However, the recoveries for the peptides from myoglobin were relatively low. This is expected due to the presence of heme groups which are hard to digest. As discussed earlier, the higher digestion efficiency when using a lower digestion volume could be due to increased exposure of target proteins to the enzyme immobilized solid support. Therefore, a digestion volume for tip digestion of 70 µL was chosen for further studies.
Chapter 5

Figure 5-17: Recoveries of surrogate peptides from four different proteins digested on IMEPP tips using different volumes for digestion.
5.3.4 **Quantitative analysis of target proteins using LC-MS/MRM method after digestion using IMEPP tips**

To demonstrate the capability of the developed IMEPP tips to accurately quantify proteins and peptides in biological samples, quantitative MRM assays were specifically designed for tryptic digested peptides using IMEPP tips for seven targeted proteins spiked in rat plasma. MRM methods have been developed in order to study four simple proteins (i.e. melittin, rat CGRP, cytochrome c and myoglobin) and three larger proteins that had to be chemically reduced and alkylated [i.e. BSA, transferrin and hIgG components (Igg-1 and Igg-kappa)]. The two different groups of proteins were spiked into rat plasma at varying concentrations and digested using IMEPP tips. The multiplexing capability of LC-QqQ-MS platforms for measuring peptides in complex digests is substantial, proving an opportunity to measure large panels of proteins accurately in each run. The protein levels were monitored to test the sensitivity and linearity of digested surrogate peptides using the IMEPP tips, as selected through the LC-MS/MRM method. Quantitative analysis information on six different proteins and the IgG components at six different concentration levels are reported in Table 5.6. Results show that the ratio of protein concentration and peak area correlation was linear at all concentration values studied, with correlation coefficient ($R^2$) values of at least 0.9884. Based on the results presented here, numerous targeted proteins ranging from low-molecular weight to those of high-molecular weight can be rapidly and efficiently digested and also quantitatively analyzed using peptide MRMs. It is also worth mentioning that the quantitative analysis of proteins digested using developed IMEPP tips demonstrates relatively good linearity over a wide range without the need for an additional internal standard to be used.

**Table 5.6: Quantitative information of the targeted proteins.**
<table>
<thead>
<tr>
<th>Proteins</th>
<th>Plasma used (µL)</th>
<th>Initial conc. (µg/mL)</th>
<th>Analysis Conc. (ng/mL)</th>
<th>Correlation coefficients (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melittin</td>
<td>10</td>
<td>0.01-10</td>
<td>0.5 - 500</td>
<td>0.9993</td>
</tr>
<tr>
<td>Rat CGRP</td>
<td>10</td>
<td>0.01-10</td>
<td>0.5 - 500</td>
<td>0.9970</td>
</tr>
<tr>
<td>Cyto c</td>
<td>5</td>
<td>1.56 – 50</td>
<td>40 - 1250</td>
<td>0.9987</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>5</td>
<td>1.56 – 50</td>
<td>40 - 1250</td>
<td>0.9985</td>
</tr>
<tr>
<td>BSA</td>
<td>1</td>
<td>1.56 – 50</td>
<td>8 - 250</td>
<td>0.9925</td>
</tr>
<tr>
<td>Transferrin</td>
<td>1</td>
<td>1.56 – 50</td>
<td>8 - 250</td>
<td>0.9898</td>
</tr>
<tr>
<td>Igg-1</td>
<td>1</td>
<td>1.56 – 50</td>
<td>8 - 250</td>
<td>0.9884</td>
</tr>
<tr>
<td>Igg-kappa</td>
<td>1</td>
<td>1.56 – 50</td>
<td>8 - 250</td>
<td>0.9958</td>
</tr>
</tbody>
</table>

5.3.5 Operational stability, storage stability and reusability

The stability of the IMEPP tips was studied. Several identical IMEPP tips were prepared and tested for the digestion of denatured BSA. The IMEPP tip used for digestion of denatured BSA after 3 months storage gave a sequence coverage of over 80%. The IMEPP tip was also tested for reusability after digestion of the cytochrome c spiked in plasma. A sequence coverage of over 80% could be achieved. Figure 5-18 shows the SEM images of the morphology of the monolith after the photografting of reactive functional monomer, enzyme immobilization and protein digestion. A modified monolith with the desired functional groups was successfully prepared without altering the basic morphology of the monolith. However, the proteolytic activity of the IMEPP tip decreased after digestion of large proteins spiked in rat plasma. This might be due to non-specific protein adsorption which blocked the active sites on the monolith pores resulting in a loss of activity. Nevertheless, it is important to note that the reusability of pipette tips is not crucial in the present case since they were designed to be disposable. In addition, the reusability of tips may not be desirable in biological applications where it is important to avoid any possible chances of carry-over.
Figure 5-18: SEM images of porous polymer monoliths inside a PP tip after (a) VAL photografting, enzyme immobilization and used for protein digestion and (b) the magnified part.
5.4 Conclusions

In this chapter, a miniaturized platform enzymatic reactor for application in bioanalysis is presented, based on a pipette tip format. The IMEPP tip showed high hydrolytic activity and long term stability for the digestion of low- and high-molecular weight proteins. Sequence coverages obtained with the IMEPP tip in 20 pipetting cycles, which is equivalent to a short total digestion time of 6 min, was comparable to those yielded by the conventional in-solution tryptic digestion for 24 h. Furthermore, the IMEPP tip was also successfully applied to the digestion of proteins spiked in rat plasma, demonstrating its potential to be used in proteomic studies, as an alternative to commercially available tryptic digest tips. High plasma loading capacity and good calibration curve linearity of the tips were also demonstrated, thus indicating a wide dynamic range for quantitative analysis. These evaluations were conducted in an industrial bioanalytical laboratory (Pfizer Global Research and Development, Sandwich, UK) and demonstrated the possibilities of high-throughput sample preparation for pharmaceutical and pharmacokinetic studies, leading to faster and safer discovery of new drugs to treat diseases. The developed IMEPP tip is highly suited for MS-based proteomic and peptidomic analyses, for which the amount of sample is often limited.

5.5 References

Chapter 5

Chapter 6

Optimization of Permeability and Format of IMEPP Tips for Proteomic Analysis

6.1 Introduction

In the pharmaceutical industry, the availability of highly developed tools for analytical procedures such as separation and detection instrumentation has led to an increase in the number of new drugs. With the advancement in analytical instrumentation, sample preparation has become a critical component in the analytical procedure but at the same time it has also become a bottleneck which limits high-throughput analysis. Because of this, much effort has been devoted to developing high-throughput sample preparation methods for toxicological and pharmacokinetic studies. An ideal sample preparation method should be easy to learn, environmentally friendly, economical, and involve minimal manual handling steps. Furthermore, semi- or fully-automated analytical techniques are required to cope with the growing number of samples to be analyzed in the pharmaceutical industry [1]. Recent developments in sample handling techniques are directed towards miniaturization, automation and on-line coupling of sample preparation units and detection systems to speed up the whole analytical process. The more rapidly these measurements can be done, the faster is the progress of the drug toward regulatory approval and the more economically viable the drug becomes for the consumers [2].

Tip-based technology has become increasingly popular in the development of high-throughput enzymatic digestion method for proteomic studies [3]. This miniaturized bioanalytical format is based on the stationary phase fixed inside a
pipette tip. The sample preparation is achieved by repeated aspirating and dispensing cycles using a manual micropipettor [4]. A 96-tip liquid handling robotic device has been introduced which allows 96 samples to be prepared simultaneously in only several minutes [5]. Compared with conventional liquid phase digestion, the tip-based digestion is easier, faster, and less expensive.

There are several commercially available enzyme tips, including DigesTip® Trypsin from ProteoGen Bio (Pisa, Toscana, Italy) and MonoTip® Trypsin from GL Sciences (Tokyo, Japan). More recently, a novel miniaturized SPE device, called microextraction by packed sorbent (MEPS) was introduced by Abdel-Rehim [6]. This MEPS device was demonstrated to be compatible with the majority of the commercially available autosamplers, as it allows sample purification, concentration and direct injection into GC or LC systems without modification of the chromatograph. Furthermore, this technique can be easily interfaced to GC- and LC-MS to provide a completely automated MEPS/LC-MS or MEPS/GC-MS system. The principles and application details of MEPS have been documented in a review by Abdel-Rehim [6]. This technique could be of interest in pharmaceutical and pharmacokinetic studies if the MEPS sorbent bed is immobilized with enzyme and integrated into a liquid handling syringe which allows protein digestion, sample clean-up and sample injection in a single step.

Since the introduction of monolithic stationary phases in the 1990s by Svec and Tanaka, monolithic materials have been employed in enzyme immobilization to achieve fast digestion steps from several hours to a few minutes or even seconds [7,8]. At present, a wide variety of monomers are used for the preparation of polymeric monolithic stationary phases. The diversity of monomers provides the possibility of synthesizing customized polymer monolith with a desired porosity, selectivity, pore diameter and functionality, according to the needs of specific applications [9]. In the previous chapters, butyl methacrylate monoliths have been
prepared in situ in 75 µm i.d. fused-silica capillaries and used for the HPLC separation of β-blockers and acidic compounds. The impact of different polymerization parameters (monomer content, porogenic solvent and cross-linker) on the porous properties, and hence on the separation selectivity for some acidic drugs and β-blockers, was studied. Recent developments and applications of monolith-based immobilized enzyme reactors for proteome analysis have been summarized in a review by Ma et al. [10].

This chapter describes the effects of the chaotropic agents, urea and guanidine hydrochloride (GdnHCl), disulfide bond reducing reagents dithiothreitol (DTT) and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), as well as different buffer solutions containing calcium chloride in the protein-containing solution used for digestion. The digestion efficiencies were evaluated using a Mascot database search of peptide fragment fingerprint (PFF) analysis. In addition, the IMEPP tip was prepared in different formats including an empty monotip and a specialized glass tube. Potentially, higher sample loading capacity and digestion efficiencies could be achieved. The influences of different polymer monolith formulations prepared in situ in PP tips on the permeability and digestion efficiencies were also investigated in detail.

6.2 Experimental

The general experimental details are described in Chapter 2. Detailed conditions are elaborated in each of the figure captions.

6.2.1 Comparison of protein digestion conditions

A comparative study of protein digestion in different buffer solution (ammonium acetate and Tris-HCl) with and without acetonitrile and calcium chloride was performed with 0.1 mg/mL cytochrome c solution. The effects of the chaotropic
agents, urea and guanidine hydrochloride (GdnHCl), disulfide bond reducing reagents DTT and TCEP were evaluated with the digestion of 0.5 mg/mL BSA solution. The composition of the solutions and reagents for the digestion are listed in Table 6.1. The sample preparation details are summarized in Sections 5.2.1 and 5.2.2. After the digestion of proteins using soluble trypsin and IMEPP tips, the digests were collected and analyzed via a LC-MS/MS method.

6.2.2 Protein identification using public protein sequence database

Peptide fragment fingerprints (PMFs) LC-MS/MS analyses for protein identification were performed with the Bruker Compass DataAnalysis 4.0 software using Mascot software (Matrix Science Ltd., UK, http://www.matrixscience.com website) and the public database SwissProt. Protein identification based on LC-ESI-MS/MS experiments was achieved by conversion of raw data to mgf-files. The mgf-files were searched using Mascot against SwissProt database with mammalia taxonomy. For proteins that were not chemically treated, such as cytochrome c, trypsin was selected as the digestion enzyme and 3 missed cleavage sites were allowed (precursor mass tolerance of 0.02 Da, fragment mass tolerance of 0.05 Da). The deaminated, Gln → pyro-Glu (N-term Q) and methionine oxidation were entered as variable modifications. Peptide charge was set to 1+. All peptide mass values were monoisotopic. Data format was set to Mascot generic and the instrument used was ESI-QUAD-TOF. A decoy setting was selected before the database search began. For proteins such as BSA that have already been treated with denaturants, all the parameters were the same except that carbamidomethyl cysteine was entered as a fixed modification and ammonia-loss, deaminated, Gln → pyro-Glu (N-term Q) and methionine oxidation were entered as variable modifications.
### Table 6.1: Buffers and denaturants used for protein digestion.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Buffer</th>
<th>In Solution</th>
<th>IMEPP tip</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>50 mM ammonium acetate + 20% ACN</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mM ammonium acetate + 10 mM calcium chloride + 20% ACN</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mM TRIS-HCl + 20% ACN</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mM TRIS-HCl + 10 mM calcium chloride + 20% ACN</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 mM TRIS-HCl + 10 mM calcium chloride + 20% ACN</td>
<td>x</td>
<td></td>
</tr>
</tbody>
</table>

**Denaturant**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Denaturant</th>
<th>In Solution</th>
<th>IMEPP tip</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>Urea + DTT + IAA</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>GdnHCl + TCEP + IAA</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>
6.2.3 *Preparation of highly permeable monoliths*

Surface modification in the pipette tip was done using single-step photografting according to the procedure described in Chapter 2. Nine different compositions of monoliths were prepared *in situ* in polypropylene pipette tips and all the compositions investigated are listed in Table 6.2.

6.2.4 *Manipulations of polymer monoliths in situ in polypropylene pipette tips*

Several types of IMEPP tips have been proposed, as shown in Figure 6-2. Tips (a) to (e) are Finntip-200 extended pipette tips and (f) is a Finntip-1000 purchased from Thermo Scientific. The tip (g) is called MonoTip empty tip 200 µL, purchased from GL Sciences (Tokyo, Japan). A piece of capillary tubing was inserted into the filled tip as a template to create a main channel through the immobilized bed. After polymerization, it was removed to increase the permeability of the solid support. There were two models of through-channel pipette tips prepared, as shown in Figure 6-3. Both models of through-channel pipette tips were filled with 40 µL of polymerization mixture and exposed under UV.

6.3 *Results and discussion*

6.3.1 *Optimization of protein digestion conditions*

Cytochrome c is a small protein (11.7 kDa) which is frequently used for evaluating enzymatic digestion efficiencies. Cytochrome c contains 105 amino acids and 21 tryptic cleavage sites (i.e. Arg and Lys residues). The peptides resulted from the digestion can be easily identified using either direct infusion or LC-MS methods. In Chapter 4, it was described how cytochrome c was used to evaluate the optimum amount of VAL needed for immobilization and the effects of trypsin concentration
Table 6.2: Compositions of the polymerization mixtures (%, w/w) used for the preparation of polymer monoliths *in situ* in pipette tips.

<table>
<thead>
<tr>
<th>Monomers / Initiator / Porogens (wt%)</th>
<th>S2M 70%</th>
<th>S2M 75%</th>
<th>S1 70%</th>
<th>S1 75%</th>
<th>Tetruglycol thermal</th>
<th>Tetruglycol UV</th>
<th>LP1</th>
<th>LPM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyl methacrylate</td>
<td>16</td>
<td>12</td>
<td>10</td>
<td>18</td>
<td>15</td>
<td>19</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td>Ethylene glycol dimethacrylate</td>
<td>24</td>
<td>18</td>
<td>15</td>
<td>12</td>
<td>10</td>
<td>21</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Tetra(ethylene glycol diacrylate)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>21</td>
<td></td>
<td></td>
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<tr>
<td>Vinylazlactone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>1-Propanol</td>
<td>42</td>
<td>49</td>
<td>52.5</td>
<td>42</td>
<td>45</td>
<td>40</td>
<td>40</td>
<td></td>
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<tr>
<td>1,4-Butanediol</td>
<td>12</td>
<td>14</td>
<td>15</td>
<td>21</td>
<td>22.5</td>
<td>20</td>
<td>20</td>
<td></td>
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<tr>
<td>1-decanol</td>
<td></td>
<td></td>
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<td></td>
</tr>
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<td>6</td>
<td>7</td>
<td>7.5</td>
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<td>7.5</td>
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<tr>
<td>Methanol</td>
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<td></td>
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<td></td>
<td></td>
<td>52.5</td>
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<td>Hexane</td>
<td></td>
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<td></td>
<td></td>
<td>22.5</td>
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<tr>
<td>2,2-dimethoxy-2-phenylacetophenone</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Azobisisobutyronitrile</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Median pore diameter, nm</td>
<td>1297</td>
<td>1514</td>
<td>1901</td>
<td>1231</td>
<td>1499</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Porosity, %</td>
<td>62</td>
<td>72</td>
<td>77</td>
<td>70</td>
<td>78</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Figure 6-2: Several types of IMEPP tips were prepared. (a) Tip filled with 20 µL of polymerization mixture; (b) the tip filled with 20 µL of polymerization mixture and exposed under UV horizontally for 2.5 to 6 min and vertically from 5 to 8 min; (c) the tip filled with 8 µL of polymerization mixture; (d) tip filled with 20 µL of polymerization mixture, and a larger “converter” was attached to it to allow the use of larger volume autopipette in order to give higher pipetting pressure; (e) top part of the tip filled with 20 µL of polymerization mixture (f) 1 mL-tip filled with 20 µL of polymerization mixture; (g) MonoTip empty tip 200 µL. All the polymerizations were conducted with the sharp end of the tip pointing downwards for 40 min and then with the sharp end up for 25 min, unless otherwise stated.
Figure 6-3: Two models of through-channel IMEPP tips: (a) monolithic bed in the middle section of the tip, (b) monolithic bed at the end of the tip. The tips were exposed horizontally under UV for 30 min.
on immobilization. It was also mentioned in Chapter 5 that cytochrome c was used to establish several tests in an industrial bioanalytical lab (Pfizer, UK) including plasma loading capacity, optimum volume for digestion, time course assay, sample clean-up and enrichment assay, and finally the qualitative and quantitative bioanalysis.

There were two different protein digestion conditions that were used for qualitative and quantitative analysis in Chapters 4 and 5. The reagents used in the Pfizer Analytical Research Center (PARC, University of Tasmania, Australia) were described in the experimental work by Krenkova et al. [7]. The standard buffer used to make protein solution is 50 mM ammonium acetate at pH 8.75 containing 20% acetonitrile. However, a different standard buffer was used in the Pfizer Bioanalytical Laboratory (Sandwich, United Kingdom). A 100 mM Tris-HCl buffer was used to prepare protein solution for digestion. Calcium chloride was also commonly added to the buffer solution because it has been reported that the calcium chloride can provide increased resistance to changes in conditions, such as pH or temperature [11]. The functions of calcium in stabilizing the enzyme conformation, reducing activation energy and maintaining the molecular folding of the denatured enzyme have also been reported [12]. As higher sequence coverage is normally achieved in the presence of an optimum amount of acetonitrile, acetonitrile was also added to all the protein solutions.

The enzyme activity assays for different buffers were conducted in solution, and the assumption made that the data should be similar to that generated from the immobilized enzyme in pipette tips. The sequence coverage of in-solution digestion of cytochrome c using different buffers for the preparation of protein solutions are summarized in Table 6.3. There was no significant difference in the sequence coverage when different buffers were used. However, the ammonium
acetate buffer gave slightly higher sequence coverage in most cases than when Tris-HCl buffer was used. The addition of calcium chloride to the digestion buffer did not lead to any significant increase in enzyme activity (Table 6.3); instead, it resulted in the undesirable formation of a high salt peak on the chromatogram, which might cause ion suppression in the MS analysis and interfere with data analysis [Figure 6-4(b)]. Further, the high salt content of the Tris-HCl buffer led to a reduction in the intensity of most of the peptide peaks and as a result less peptides were identified [Figures 6-4 (a, c, d)]. It was interesting to discover that the use of Tris-HCl buffer resulted in much higher intensity of some of the peptide peaks (Figure 6-5) for the cytochrome c digest. This could be attributed to the fact that the Tris-HCl buffer could create some specific cleavage sites for trypsin. Therefore, the use of ammonium acetate buffer was preferred because it did not require desalting steps.

For an optimum digestion of large and folded proteins, specific treatment prior to digestion was necessary to reduce the disulfide bonds. These proteins have to be treated by denaturing agents, such as urea or GdnHCl. Most enzymes used for proteomic studies have shown low tolerance against denaturants. However, immobilized enzyme has been shown to be more stable in the presence of a high concentration of denaturing agents [7]. In addition, the disulfide bridge was reduced using DTT or TCEP and the thiol group subsequently alkylated with iodoacetamide (IAA) to enhance exposure of sites of the protein previously inaccessible for proteolysis. Therefore, a comparative study was established to test the digestion efficiencies of various combinations of denaturing, reducing and alkylating agents.

The results of digestion of BSA by soluble trypsin and the IMEPP tip are shown in Table 6.3. The sample pre-treated with a combination of urea, DTT and
Table 6.3: Results of the digestion of cytochrome c and BSA using different buffers and denaturants.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>In Solution</th>
<th>IMEPP tip</th>
<th>Sequence coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mM ammonium acetate + 20% ACN</td>
<td>76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mM ammonium acetate + 10 mM calcium chloride + 20% ACN</td>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mM TRIS-HCl + 20% ACN</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mM TRIS-HCl + 10 mM calcium chloride + 20% CAN</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mM TRIS-HCl + 10 mM calcium chloride + 20% ACN</td>
<td>68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturant</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>BSA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea + DTT + IAA</td>
<td>75</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>GdnHCl + TCEP + IAA</td>
<td>71</td>
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</tbody>
</table>
Figure 6-4: Direct infusion mass spectra of cytochrome c digested in different buffer solutions. (a) 50 mM ammonium acetate + 20% acetonitrile, (b) 50 mM ammonium acetate + 10 mM calcium chloride + 20% acetonitrile, (c) 50 mM Tris-HCl + 20% acetonitrile and (d) 100 mM Tris-HCl + 10 mM calcium chloride + 20% acetonitrile.
Figure 6-5: Cytochrome c (0.1 mg/mL) after in-solution digestion in buffers (a) 50 mM Tris-HCl +20% acetonitrile and (b) 50 mM ammonium acetate +20% acetonitrile. Top: base peak chromatograms. Middle: mass spectrometry (MS) survey scans. Bottom: tandem mass spectra (MS/MS) of the precursor ion at m/z 585.1 corresponding to peptide TGPNLHGLFGRK, amino acids 29-39.
IAA yielded slightly higher sequence coverages for both in-solution digestion and the IMEPP tip.

The present results are in agreement with the findings of Spross and Sinz who reported that urea was a better denaturant than GdnHCl when DTT was used as the reducing agent and IAA was used as the alkylating agent [9]. Although the use of GdnHCl as denaturant and TCEP as reducing agent resulted in a lower sequence coverage, it was found that the LC-chromatograms of the digestion gave a cleaner separation profile and resulted in higher intensities of some of the peptide peaks (Figure 6-6). Therefore, the combination of urea, DTT and IAA was probably better suited for qualitative analysis whereas GdnHCl, TCEP and IAA could be used for quantitative analysis of proteins.

6.3.2 Development of a highly permeable IMEPP tip

Despite the high loadability of the IMEPP tips for the protein digestion in rat plasma of up to 40 µL, they were limited by high backpressure caused by the large amount of sorbent used. Most of the pipetting tasks have to be performed by attaching the IMEPP tips to a curved syringe, as shown in Figure 2-4, to give a higher pipetting pressure. Therefore, different formulations of monolith and physical formats of the polymer monoliths in situ in polypropylene pipette tips were prepared in terms of the positions and the amount of polymerization mixture used, in order to give the monolith support to a higher permeability.

6.3.2.1 Preparation of highly permeable monoliths

There are many parameters that influence the porous properties of monoliths, including the type and concentration of the porogenic solvent(s), the polymerization temperature and time, and the percentage of cross-linker and initiator. Based on the preliminary experiments reported in chapter 3, monomer mixtures consisting of BMA and EDMA, and a porogenic solvent mixture
Figure 6-6: Separation of peptides resulting from BSA digestion (0.5 mg/mL) using the IMEPP tip and soluble enzyme by the combining different denaturants and reducing agents. Other conditions are the same as in Figure 5-4.
containing 1-propanol and 1,4-butanediol, were used for the preparation of the IMEPP tips. Monolith formulations with the monomer Tetra(ethylene glycol) diacrylate were also investigated because they had been prepared in a capillary and reported with large pore sizes (~7 µm). Yu et al. reported that the polymerization mixture containing BMA and EDMA with a binary porogenic solvent of hexane and methanol yielded a monolith with large pore sizes (~8 µm) [13]. The porogenic solvent is the most important parameter for controlling the porous structure. Therefore, the ratio of monomer mixture to porogenic solvent in the polymerization mixture was slightly modified in order to increase the extent of through-pores and to facilitate low backpressure during enzyme digestion in the IMEPP tip. Compositions of the polymerization mixture are shown in Table 6.2. The monolith S2M, HEMA, tetruglycol thermal and tetruglycol UV were prepared from a polymerization mixture containing 40% (w/w) of the monomer mixture and 60% (w/w) of porogenic solvent. To improve the through-pores, the composition of porogenic solvent in the polymerization mixture was increased from 60% (w/w) to 70% (w/w) in S2M 70% and S1 70%, and increased from 60% (w/w) to 75% (w/w) in S2M 75% and S1 75%, respectively. The pore diameters and total porosities of the prepared monoliths were investigated through mercury porosimetry.

From Table 6.2, it can be observed that the composition of had an influence on the porous properties and morphologies of the monolithic support. The pore diameters of the monoliths S2M, S2M 70% and S2M 75% increased slightly as the proportion of porogenic solvent in the polymerization mixture increased. In most cases, the total porosities also increased as the proportion of porogenic solvent increased (Table 6.2). Hence, increasing the porogen-to-monomer ratio is a straightforward method to increase the pore size and porosities, and consequently, to increase permeability during operations. However, this approach may also
decrease the rigidity of the macroporous polymer [14]. Figure 6-7 displays SEM images of the monolithic structures of the monoliths formed in the tips. Although the UV-initiated polymerization is usually performed within a short period compared to thermal initiation, the SEM images clearly showed the uniform porous structure across the monolithic support of the pipette tip. The difference in the pore diameters of S1 70% compared to S2M was not significant. The S1 75% and S2M 75% monoliths prepared within the pipette tips were very porous, but both monoliths collapsed after being washed with acetone. In addition, four of the monoliths studied, including tetruglycol thermal, tetraglycol UV, LP1 and LPM1 formed an emulsion in the tip. Therefore, they were not further studied. The permeabilities of S2M and S2M 70% were further investigated by preparation in polypropylene pipette tips.

6.3.2.2 Manipulation of IMEPP tip formats

Different formats of macroscopic monoliths are commonly prepared, such as disks, rods (or columns) and polypropylene tips. They are often commercially available and produced by several companies such as BIA separations (Ljubljana, Slovenia) under the trade name CIM® Disk, or as separation columns (ProSwift™, Dionex Corporation, USA). Monoliths in microscopic formats, such as capillary columns, silica steel tubing and microfluidic chips have also been widely developed [14].

Based on the preliminary experiments studied in the pipette tip format, further adjustments were made in order to achieve more permeable tips that were compatible with autopipettes. Figure 6-8 shows different models of pipette tips prepared with the S2M monolith and Table 6.4 summarizes the physical properties of these tips. Tip model (b) was prepared by using a shorter UV exposure period. A 2.5 min exposure was not sufficient for the polymerization to occur. Consequently, the monolithic support collapsed when it was washed with acetone.
Figure 6-7: SEM images of porous polymer monoliths inside the PP tips.
after polymerization. UV initiation for 3 min resulted in partial polymerization of the polymerization mixture, leaving a large void between pores and therefore the tip was only partially compatible with the autopipette. As soon as the polymerization exceeded 4 min, the monoliths were completely polymerized and required higher pipetting pressure (Table 6.4).

The IMEPP tip that was prepared by polymerization using 8.5 min exposure was fully compatible with the autopipette (Table 6.4). Close examination of SEM images revealed that the polymer monolith was not completely attached to the tip wall. However, the polymer monolith was physically stable in the pipette during use (Figure 6-9). Meanwhile, the polymer monolith prepared in models (c, d, e, f) allowed only partial compatibility with autopipettes due to the limited backpressure experienced for suction operations (Table 6.4). The preparation of IMEPP tips in the different formats did not compromise the immobilized enzyme activities. At least 74% sequence coverage was achieved for the digestion of cytochrome c (0.1 mg/mL) using these different IMEPP tip formats.

Tip model (e) was initially intended to simulate a commercially available enzyme tip, namely MonoTip® Trypsin. This tip model yielded a larger internal i.d. of about 5 mm. A shorter monolith support prepared in the pipette tips could give higher permeability (Figure 6-10). The monoliths S2M and S2M 70% were further prepared in this tip model. The tip with monolith S2M only showed partial compatibility with the autopipette, similar to that prepared in the Finntip-200 format. A highly permeable tip was achieved by filling 15 µL of S2M 70% into a MonoTip and polymerizing under UV for 20 min. This monolith provided a permeable through-pore size which could hold the polymer monolith in place while the solution was aspirated or expelled in and out using autopipette. Although this may also be achieved using 10 µL of S2M mixture in the MonoTip, a
Figure 6-8: Several models of IMEPP tips were prepared. The labels (a) to (f) are indicated in Figure 6-2, (g) through-channel IMEPP tips.
Table 6.4: Physical properties of monoliths prepared in different formats.

<table>
<thead>
<tr>
<th>Tips model</th>
<th>Amount of polymerization mixture (µL)</th>
<th>Polymerization position</th>
<th>Polymerization time (min)</th>
<th>Stationary phase</th>
<th>Compatibility with autopipette (200uL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (a)</td>
<td>20</td>
<td>vertical</td>
<td>30</td>
<td>ok</td>
<td>no</td>
</tr>
<tr>
<td>2 (b)</td>
<td>20</td>
<td>horizontal</td>
<td>2.5</td>
<td>collapsed</td>
<td>NA</td>
</tr>
<tr>
<td>3 (b)</td>
<td>20</td>
<td>horizontal</td>
<td>3</td>
<td>ok</td>
<td>partial</td>
</tr>
<tr>
<td>4 (b)</td>
<td>20</td>
<td>horizontal</td>
<td>4</td>
<td>ok</td>
<td>no</td>
</tr>
<tr>
<td>5 (b)</td>
<td>20</td>
<td>horizontal</td>
<td>5</td>
<td>ok</td>
<td>no</td>
</tr>
<tr>
<td>7 (b)</td>
<td>20</td>
<td>horizontal</td>
<td>6</td>
<td>ok</td>
<td>no</td>
</tr>
<tr>
<td>8 (b)</td>
<td>20</td>
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<td>5</td>
<td>collapsed</td>
<td>no</td>
</tr>
<tr>
<td>9 (b)</td>
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<td>6</td>
<td>collapsed</td>
<td>no</td>
</tr>
<tr>
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<td>vertical</td>
<td>7</td>
<td>collapsed</td>
<td>no</td>
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<tr>
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<tr>
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<td>vertical</td>
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<td>Partial</td>
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<tr>
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<td>30</td>
<td>ok</td>
<td>Partial</td>
</tr>
<tr>
<td>16 (f)</td>
<td>20</td>
<td>vertical</td>
<td>30</td>
<td>ok</td>
<td>Partial</td>
</tr>
</tbody>
</table>

* Partial compatibility meant that the solution could be aspirated but not expelled in and out of the tips or vice versa.
Figure 6-9: SEM images of polymer monolith in pipette tip after the tip was exposed vertically for 8.5 min under UV.

Figure 6-10: Photo of (a) MonoTip empty tip and (b) MonoTip with polymer monolith.
larger monolith support in this case would be advantageous for enzyme immobilization. Sequence coverage of 80% was obtained using this S2M 70% MonoTip for the digestion of cytochrome c.

A main channel was created through the immobilized bed by inserting a piece of capillary tubing into a tip filled with polymerization mixture and then removed after polymerization. This method of creating a flow channel in the monolith was first demonstrated by Hsu et al. to fabricate disposable plastic microtips, namely EasyTip, for SPE as an off-line sample preparation of biological samples [15]. Most importantly, the through-channel created within the immobilized bed did not affect the functionality of the EasyTip. The recovery percentage of the tryptic digest samples loaded onto the EasyTip was nearly 100% [15]. The same technique was in applied to the IMEPP tips. Up to 40 µL (~6 mg) of polymerization mixture was used in the through-channel IMEPP tips to increase the loadability since this system was not restricted by the pipetting backpressure [Figure 6-8 (g)]. Therefore, using this through-channel technique, the permeability problem was overcome easily. Figure 6-11 shows a SEM photograph of the monolithic structure with a through-channel. The monolith was attached to the tip wall and no cracks were observed.

The monolithic support in the through-channel IMEPP tips was immobilized with trypsin and the digestion efficiencies were assessed using standard procedures. Figure 6-12 demonstrates the excellent digestion efficiency of the through-channel IMEPP tips since no significant difference in sequence coverage was observed compared to in-solution digestion as well as the IMEPP tip without through-channel (Table 6.5). However, additional peptide peaks were observed between the in-solution and in-tip digestion (Figure 6-12). To further confirm that these peptide peaks resulted from the digestion, two different
Figure 6-11: Through-channel in IMEPP tip created by inserting a piece of capillary tubing into the tip filled with polymerization mixture, and then removed after polymerization.
In-solution digestion 0.1mg/mL cyto c  
S.C. = 81%

0.1 mg/mL cyto c by through channel tip (a)  
S.C. = 77%

0.1 mg/mL cyto c by through channel tip (b)  
S.C. = 79%

Figure 6-12: Separation of peptides resulting from cytochrome c digestion (0.1 mg/mL) using the soluble trypsin and through-channel IMEPP tips (a) and (b). Digestion and separation conditions are the same as in Figure 4-10.
Table 6.5: Results of the digestion of cytochrome c and BSA using soluble trypsin, IMEPP tip with and without through-channel.

<table>
<thead>
<tr>
<th></th>
<th>In Solution</th>
<th>IMEPP tip without through-channel</th>
<th>IMEPP tip with through-channel</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sequence coverage (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>81</td>
<td>82</td>
<td>77</td>
</tr>
<tr>
<td>BSA</td>
<td>75</td>
<td>66</td>
<td>67</td>
</tr>
</tbody>
</table>
surrogate peptides were extracted from the LC-MS chromatograms for comparison. Figure 6-13 shows two different surrogate peptides with amino acid sequences EETLMEYLENPK and TGQAPGFTYTDANK resulting from the tryptic digest of cytochrome c using in-solution digestion and through-channel tip digestion. The results clearly show that the intensity of surrogate peptides improved by 20 to 30 times for the through-channel mode.

Protein identification by Mascot software showed that the additional peptide peaks from the through-channel IMEPP tips digest were mainly trypsin residues from the immobilization process (Figure 6-14). This could be due to the self-digestion of immobilized trypsin which was not completely washed out and was trapped or absorbed on the polymer monolith during immobilization. Thus, during the pipetting process, the digested trypsin was eluted into the sample solution, and caused the additional peptides on the LC-MS chromatograms. Therefore, the through-channel IMEPP tips were washed carefully with water and digestion buffer before used. Figure 6-15 shows that there were slightly fewer peptides observed for the LC-MS chromatogram of the protein digested using through-channel IMEPP tip after being washed with water and digestion buffer. However, a significant amount of additional peptides remained in comparison to the in-solution digestion. The separation by LC-MS/MS was very sensitive and even a small amount of trypsin leaching from the polymer monolith might result in significant additional peaks. However, even when the tip was washed with the water and buffer, the digestion efficiency was not compromised as the enzyme was immobilized strongly on the solid support with covalent bonds. Through-channel IMEPP tips exhibited high activity for digestion of high proteolytic resistivity proteins, such as BSA, with similar sequence coverage obtained using in-solution digestion (Figure 6-16).
Figure 6-13: Extracted ion chromatograms of surrogate peptides with (a) amino acid sequences EETLMELYENPK and (b) TGQAPGFTYTDANK resulting from the tryptic digest of cytochrome c using in-solution and through-channel tips digestion.
As mentioned earlier, the ideal monolith morphology in pipette format should contain larger amount of monolith plug for enzyme immobilization to achieve higher digestion capacity while still permeable enough for the protein to flow convectively through the interstices and interact with enzyme-immobilized surface. In this case, the design of Figure 6-3 is preferable because this design fulfills the requirement of allowing large amount of monolith support to be used while maintaining high permeability because of the through-channel. Most importantly, this design is compatible with the autopipette. The design from Figure 6-3 does not significantly enhance sequence coverage of enzymatic digestion. However, preliminary results on showed that the intensity of surrogate peptides improved by 20 to 30 times digested using through-channel enzyme tip, which is extremely useful for quantitative bioanalysis (Figure 6-13). This result has to be confirmed using MRM quantitative method.

Figure 6-14: Database searched using Mascot software against SwissProt database after cytochrome c digest was analyzed by LC-MS/MS and exported as mgf-files.
Figure 6-15: Comparison of LC-MS chromatogram for the digestion of cytochrome c using through-channel IMEPP tip (a) before and after being washed with water and digestion buffer.
6.3.3 Preparation of immobilized enzyme polymer monolith in glass tube for microdigestion

MEPS is an automated version of SPE which was first developed by Abdel-Rehim in 2003. MEPS has been growing in popularity for the extraction and concentration of a wide range of analytes in different matrices (urine, plasma and blood) ever since it was made commercially available [6]. Most importantly, it has been used on-line with instruments for high-throughput sample preparation. One of the ultimate goals in this project is to develop miniaturization devices for protein digestion which meet the demands of automated high-throughput performance. Thus, the format of this device should be easily prepared, replaceable and easily integrated with the analysis without further modification.

A special glass tube (~30 µL) with a needle insert was investigated as a potential format for a high-throughput sample preparation device (Figure 6-17). In order to anchor the monolithic structure to the glass tube wall through covalent bonding, the glass tube was surface-modified using the same procedure described in Section 2.3.3. Following this, the enzyme-immobilized polymer monolith (20 µL or 3 mg) was synthesized in the glass tube using the standard procedures for preparing polymer monolith, photografting of VAL functionality and enzyme immobilization. The glass tube was positioned between the syringe barrel and needle while the protein digestion took place on the immobilized bed (Figure 6-18).

To evaluate the digestion efficiency of this new format, a protein solution containing 0.1 mg/mL of cytochrome c was used for the digestion and its performance was compared to that of a standard in-solution digestion. Protein solution was drawn through the immobilized bed within the glass tube and dispensed for about 20 cycles manually using the syringe attached. The sample was then analyzed directly with LC-MS/MS and identified by the Mascot software.
0.5 mg/mL BSA by In-solution digestion
S.C. = 72%

0.5 mg/mL BSA by through channel tip (a)
S.C. = 67%

Figure 6-16: Separation of peptides resulting from BSA digestion (0.5 mg/mL) using the through-channel IMEPP tip and soluble enzyme.
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Figure 6-17: Photograph of a glass tube with needle insert.

Figure 6-18: Glass tube with immobilized bed and attached to a 3 mL syringe.
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Sequence coverage of 74% was obtained for a 20 cycle digestion which required only five min for the whole digestion process.

6.4 Conclusions

In this chapter, the effects of using different buffer solutions for protein digestion were investigated. The combination of ammonium acetate and acetonitrile yielded better results compared to protein digestion in Tris-HCl buffer with the addition of calcium chloride. Also, the combination of urea (up to 8 M), DTT and IAA yielded better digestion efficiencies for the optimum digestion of large and folded proteins without damaging the trypsin activities. Butyl methacrylate-based monoliths were prepared using various compositions of the polymerization mixture in order to increase the through-pores of the immobilized bed. It was shown that increasing the porogen/monomer ratio led to increases in the pore sizes and total porosities of monoliths, and in turn this gave higher permeability for the suction operations. Several formats of IMEPP tips were also developed to work concurrently with an autopipette. It was found that an IMEPP tip prepared by polymerization of 8.5 min exposure under UV and the MonoTip prepared with S2M 70% were fully compatible with the autopipette. Both IMEPP tip formats gave higher permeability. An IMEPP tip with a through-channel was also used to demonstrate the potential of compatibility with an autopipette without compromising enzyme digestion efficiency. A new format for enzyme digestion that has been miniaturized to work with a syringe further expands the possibility for high-throughput sample preparation that can be performed either manually or in conjunction with an autosampler to allow protein digestion, sample clean-up and sample injection accomplished in a single step.
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6.5 References

Chapter 7

General Conclusions

The use of polymeric monolithic materials for bioanalysis has increased over the recent years. These materials have a number of desirable properties which include:

1. Continuous structure of interlaced through-pores and skeleton that provides high permeability to enable chromatographic separations at extremely high flow-rates.

2. Inexpensive, requiring no frits and easily prepared \textit{in situ} in different formats by thermal-, UV- and \(\gamma\)-radiation.

3. Porous properties can be controlled by varying the compositions of the polymerization mixture, tailored to suit separations of large and small molecules.

4. Can be modified to feature almost any functionality; ion-exchange, affinity, chiral, mixed-mode, restricted access, hydrophobic and hydrophilic, in order to suit the stationary phases for specific analytes.

5. Biocompatibility and high stability over a wide pH range, which is crucial when dealing with biological samples.

Firstly, the SPMA-based monoliths were prepared both in bulk and inside Teflon®-coated UV transparent fused-silica capillaries using photoinitiated free radical polymerization. The relationship between the porogenic solvent composition and cross-linker with regard to morphologies, pore size distributions and separation performance were investigated. It was shown that the pore sizes
and separation properties of monoliths could be controlled by varying of the porogenic solvent ratio between 1,4-butanediol and 1-propanol. Increasing the percentage of 1-propanol relative to that of 1,4-butanediol in the polymerization mixture diminished the pore size in the prepared SPMA monoliths. Among the first set of columns (S1-S7) prepared using higher functional monomer/cross-linker ratio, the best separation of a mixture of target analytes was achieved with the porogen ratio 1,4-butanediol:1-propanol:water 30:60:10 (% w/w/w) for the S1 column. This column exhibited good repeatability for run-to-run separation but showed non-robustness for column-to-column and batch-to-batch separations. In the second set of columns (S1M-S3M), the proportion of cross-linker (EDMA) in the monomer mixture was switched with that of the functional monomer (BMA), with the compositions BMA:SPMA:EDMA 16:0.4:23.6 (% w/w/w). The S2M column demonstrated the best separation performance with satisfactory column permeability suitable for an acceptable level of separation of acidic drugs and β-blockers. The S2M column exhibited minimal column-to-column and batch-to-batch differences in the majority of the investigated chromatographic parameters. Therefore, SPMA-based monolithic columns with a higher amount of cross-linker (SM-monoliths) exhibited better repeatability than those polymerized with less cross-linker (S-monoliths). Furthermore, the separation mechanism of the investigated compounds was shown to be based on a mixed-mode functionality of the SCX-RP monolith. The present work has demonstrated the high permeability (calculated using Darcy’s Law) of the developed monoliths. Although it was not thoroughly explored in this project, the developed monoliths could potentially be used for extremely high flow-rates separations. Thus, further studies are warranted. Moreover, as it has not been fully demonstrated in the current work that tailored monoliths could provide
separations of large and small molecules, future studies may focus on improving
the developed materials for the efficient separations of large and small molecules.

A novel trypsin-immobilized polypropylene pipette tip prepared with the
newly optimized porous polymer monolith was developed. Disposable pipette tips
were surface-modified before in situ synthesis of a plug of polymer monolith as an
immobilized bed. A single photografting step using a stock solution of
modification mixtures containing MMA:EDMA:BP resulted in better attachment of
the polymer monolith on the polypropylene surface. A fluorescence assay
indicated that UV light was capable of penetrating the polypropylene wall,
enabling surface grafting of reactive functionalities (VAL) for trypsin
immobilization. The concentrations of VAL in the grafting mixture as well as the
grafting time were optimized, and the optimum trypsin concentration for
immobilization was also determined. The first demonstration of proteolytic activity
for the immobilized enzyme polypropylene pipette tips was carried out using a
solution of cytochrome c. The preliminary results obtained were very promising as
very high sequence coverage was achieved within a very short digest time of only
a few minutes.

The digestion performances of the IMEPP tips were further characterized
and evaluated using MicrOTOF-Q quadrupole time-of-flight MS and a LC-MS/MS
system including an AB SCIEX Triple Quadrupole™ 5500 mass spectrometer for
the application of bioanalysis. The IMEPP tip showed a very good catalytic efficacy
for different proteolytic resistivity proteins, ranging from 2.8 kDa to high
molecular mass proteins, such as hIgG of 150 kDa. Direct comparison of the
digestions achieved with IMEPP tips and soluble trypsin clearly demonstrated the
advantages of the former as they afforded much higher digestion efficiencies
within short periods of time. Further, IMEPP tips were also successfully applied to
biological sample preparation for qualitative and quantitative analysis in an industrial bioanalytical laboratory (Pfizer Inc., Sandwich). The developed IMEPP tips exhibited high plasma loading capacity of up to 40 µL of plasma, which could be used to yield the highest digestion efficiency. A time course assay demonstrated that the 30 min IMEPP tip digestion yielded similar results to the 24 h in-solution digestion. Although IMEPP tips did not possess significant sample clean-up and enrichment functionalities, nonetheless IMEPP tips eliminated the enzyme autodigestion which would have led to ion suppression in the MS analysis. Calibration curves were established for different target proteins and demonstrated relatively good linearity over a wide range from 40 ng/mL to 1000 ng/mL. IMEPP tips showed long-term stability and reproducibility. The results obtained were superior to those of commercially available tryptic digest tips.

The author’s laboratory and the Pfizer bioanalytical laboratory used different digestion buffers and denaturants. Therefore, experiments were conducted to evaluate the digestion efficiencies of the two systems. Digestion buffer comprising 50 mM ammonium acetate, pH 8.75 containing 20% acetonitrile yielded better results compared to digestion in Tris-HCl buffer. In addition, the combination of urea, DTT and IAA gave better digestion of reduced and alkylated proteins. Additionally, the monolith plug in the pipette tip was varied to increase the extent of through-pores and to facilitate the suction operation during enzyme digestion in the IMEPP tip. A highly permeable tip was achieved by filling 15 µL of S2M 70% in empty MonoTip® Trypsin, with polymerization under UV for 20 min. Newly developed through-channel IMEPP tips could also be used to overcome the permeability problem. Most importantly, these modified IMEPP tips allowed compatibility with autopipettes without compromising digestion performance. Trypsin-immobilized polymer monolith was also prepared within a syringe-
compatible glass tube and the protein digestion efficiency was evaluated. Preliminary results showed that the enzyme on the immobilized bed maintained high proteolytic performance in this special format.

Applications of the robust and repeatable monolithic columns and IMEPP tips developed in this work can be expected to be further expanded in the future. Further studies to achieve this goal would include:

- Monolithic columns developed were prepared by copolymerization of BMA, EDMA and SPMA, with the sulfonate ions contributing to the cation-exchange functionalities of the polymer chains. However, the cation-exchange capacity was relatively low. One alternative approach is to introduce this functionality by post-photografting the monolithic support with a desired functional monomer. The mixed-mode monolith sorbent produced could be further tested for separation performance (e.g. HPLC, LC-MS) or used as the bulk material for solid-phase extraction of large and small molecules.

- The tip-shape enzyme reactor has the advantage of easy applicability. With further development, this highly efficient enzyme reactor based on the pipette tip format can be adopted readily in the current setup of any laboratory for manual operation using an autopipette or by integrating it with an automated 96-tip robotic device for high-throughput sample preparation of bio-substances in pharmaceutical and pharmacokinetic studies, enabling faster and safer discovery of new drugs.

- So far, the IMEPP tips have only been used in the digestion of standard proteins. These tips could be applied in a wider range of biological samples of pharmaceutical interest.
• Although this project has only demonstrated the immobilization of trypsin on polymer monoliths in pipette tips, immobilization of other enzymes (e.g. LysC and PNGase F) using these supports will also be feasible. It is also possible to prepare multiple enzyme reactors in a range of different formats with different parameters such as in situ in capillary, different internal diameter columns, microchips, syringe barrels, syringe filters and spin columns, leading to significant commercial potential. These designs also mean that they can be applicable to all types of bioanalytical problems.

• It is also possible to prepare the immobilized bed in 96-well plates. The lower part of each well can be removed. The proteins samples are digested while eluting through the 96 wells and are subsequently injected for sample analysis (Figure 7-2).

• A monolith in a glass tube could be fitted into a glass syringe and connected to an autosampler for on-line sample preparation prior to MS analysis. Further modification of the monolith functionalities may enable analysis of complex matrices, e.g. using two monolithic phases with different functionalities, thus providing the possibility of extraction, enrichment, digestion and injection in a single step for analysis (Figure 7-3).
Figure 7-2: 96-well plate with immobilized bed.

Figure 7-3: Glass tube with two layers of monolithic phases fitted with gas tight syringe.