

DEVELOPMENT OF ENZYMATIC MICROREACTORS FOR THE ANALYSIS OF PROTEINS VIA MASS SPECTROMETRY

by

Michelle Diane Douma

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Abstract

Many techniques exist for protein analysis including gel electrophoresis, immunoassays and mass spectrometry to name a few. Although each of these techniques offers different advantages there are challenges associated with protein analysis that present limitations and drawbacks for each technique. These include limited sample volumes, incompatible matrices, complex mixtures, limit of detection, and complicated and time consuming analysis techniques. The research described in this thesis deals with methods which address the above mentioned challenges. The fabrication of enzymatic microreactors and the development of an extraction technique to remove proteins from incompatible matrices are presented.

A low temperature solvent bonding technique was developed that can easily be modified to accommodate pH and temperature sensitive enzymes such as pepsin. Microfluidic chips fabricated with poly(methyl methacrylate) were constructed using this bonding method. The low temperature bonding method allows enzymes to be patterned on microfluidic devices prior to bonding with no negative impact on enzyme activity. Both peptic and tryptic reactors were fabricated using this technique. These devices have a lifetime of one month and can perform protein digestions in as little as 2.4 seconds.

Alternatively, enzymatic microreactors were developed using a novel enzyme immobilization method on porous polymer monolith columns. These columns have the advantage of having the dual functionality of an enzymatic microreactor and an electrospray emitter permitting on-line digestions to be performed. Furthermore, the new immobilization method allows columns to have a longer lifetime as this method permitted the regeneration of the columns with fresh enzyme once enzyme activity was lost.

To overcome the issues associated with incompatible matrices, an extraction technique using acetonitrile and octyl- β -*D*-glycopyranoside was developed to remove proteins from organic matrices. This was developed to detect the presence of prion proteins in biodiesel. Afterwards, the prion proteins were enzymatically digested and detected by mass spectrometry.

Finally both types of enzyme microreactors were applied to proteins that are difficult to digest with traditional in-solution digestions. The digestion of prion proteins and α -1-protease inhibitor were found to be more efficient and conducted in significantly less time demonstrating the potential use of these devices in clinical research.

Co-Authorship

This this project was conducted under the supervision of Dr. Richard Oleschuk. Parts of Chapter 2 were co-authored by Dr. Terry Koerner and Laurie Brown. Data from undergraduate students were included in this report, specifically data from Eliane Shver and Daniel Bodley is found in Chapter 2, data from Ian Goode and Kelly Cashion in Chapter 3 and data from Gina Kerr in Chapter 4. Also a portion of Chapter 4 has been published (Douma, M.D., et al. *Can. J. Chem.* **2008**, 86, 774-781).

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Statement of Originality

The work presented within this thesis is my own. Any published (or unpublished) ideas and/or techniques from the work of others are fully acknowledged in accordance with the standard referencing practices. This thesis presents the development of a novel low temperature polymer bonding method that is modifiable to allow the immobilization of both robust and temperature/pH sensitive enzymes. Also an original enzyme immobilization method for porous polymer monolith columns is introduced that allows the columns to be regenerated upon loss of enzyme activity. Finally, a method has been developed that allows for the extraction of prion proteins from biodiesel, an incompatible matrix for standard protein identification techniques. This extraction coupled with enzymatic digestion, permitted prion proteins to be identified with mass spectrometry.

(Michelle Diane Douma)

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Table of Contents

Abstract.....	ii
Co-Authorship	iv
Acknowledgements.....	v
Statement of Originality.....	vii
Table of Contents.....	viii
List of Figures.....	xi
List of Tables	xix
List of Abbreviations	xx
Chapter 1 Mass Spectrometry in Proteomics.....	1
Chapter 2 Non-Denaturing Low Temperature Bonding of a Patterned Poly(methyl methacrylate) Enzymatic Microreactor.....	12
2.1 Summary	12
2.2 Introduction.....	13
2.3 Experimental.....	16
2.3.1 Materials and Chemicals.....	16
2.3.2 Enzymatic Patterning on PMMA Substrates	17
2.3.3 WSC Immobilization Method.....	21
2.3.4 WSC/NHS Immobilization Method.....	21
2.3.5 Low Temperature Bonding with Immobilized Enzyme.....	22
2.3.6 Thermal Bonding of PMMA substrates	23
2.3.7 Measurement of Cover Plate Adhesion Strength.....	23
2.3.8 Nanoidentation Measurements on the surface of PMMA.....	24
2.3.9 On Chip Enzymatic Digests.....	26
2.3.10 MADLI-TOF-MS Analysis of Enzyme Digests	26
2.3.11 Data Analysis	27
2.4 Results and Discussion	28
2.4.1 Immobilization of Trypsin on PMMA substrates	28

2.4.2 Low Temperature Bonding of Pre-Patterned Enzyme Substrates.....	34
2.4.3 Channel Structure of PMMA Microfluidic Chips.....	43
2.5 Conclusions.....	45
Chapter 3 The Fabrication of Regenerable Porous Polymer Monolith Enzymatic Microreactors for On-line Digestions with ESI-MS	46
3.1 Summary	46
3.2 Introduction.....	47
3.3 Experimental.....	51
3.3.1 Materials and Chemicals.....	51
3.3.2 PPM Formation.....	52
3.3.3 Enzyme Immobilization on PPM Columns	54
3.3.4 Quantification of Enzyme on the Surface.....	56
3.3.5 Enzymatic Digestions	57
3.3.6 Protein Separations	59
3.3.7 Regeneration of Enzymatic Columns	61
3.3.8 Data Analysis	62
3.4 Results and Discussion	63
3.4.1 PPM Formation.....	63
3.4.2 Immobilization of Enzymes on PPM Columns.....	65
3.4.3 Quantification of Enzyme on PPM Surface.....	70
3.4.4 Enzyme Longevity	70
3.4.5 Regeneration of Enzymatic Columns	73
3.4.6 Enzymatic Digestions of Various Proteins	77
3.4.7 On-line Enzymatic Digestions	81
3.4.8 Separation of a Protein Mixture.....	83
3.5 Conclusions.....	86
Chapter 4 Mass Spectrometric Detection of Proteins in Non-Aqueous Media – The Case of Prion Proteins in Biodiesel	88
4.1 Summary	88
4.2 Introduction.....	88
4.3 Experimental.....	91
4.3.1 Chemicals and Materials.....	91

4.3.2 Filtration and Whole Protein Extraction	92
4.3.3 Tryptic Protein Digestion.....	94
4.3.4 Microwave-Assisted Digestion of PrP	94
4.3.5 Mass Spectrometric Analysis of Whole Proteins.....	95
4.3.6 Data Analysis	95
4.4 Results and Discussion	96
4.4.1 PrP Characterization	96
4.4.2 Filtration of Spiked Biodiesel, Extraction of Filter Discs and Protein Analysis.....	99
4.5 Conclusions.....	105
Chapter 5 Clinical Applications of Enzymatic Microreactors	107
5.1 Summary	107
5.2 Introduction.....	107
5.3 Experimental.....	111
5.3.1 Chemicals and Materials.....	111
5.3.2 Fabrication of PMMA Microfluidic Chips	113
5.3.3 Formation of PPM Columns	113
5.3.4 Enzyme Immobilization Method	114
5.3.5 In-Solution Enzyme Digestions	115
5.3.6 Enzymatic Digestions Using Tryptic Microreactors.....	115
5.3.7 MALDI-TOF-MS Analysis of Enzymatic Digests	116
5.3.8 Data Analysis	116
5.4 Results and Discussion	117
5.4.1 In-Solution Digest of Antitrypsin	117
5.4.2 Tryptic Digest of Antitrypsin Using a Microfluidic Chip.....	118
5.4.3 Comparison of Antitrypsin and Oxidized Antitrypsin	119
5.4.4 In-Solution Digestion of PrP.....	122
5.4.5 Tryptic Digest of PrP Using a PPM Column	123
5.5 Conclusions.....	124
Chapter 6 Conclusions and Future Work.....	126
References.....	131
Appendix A Explanation of Sequence Coverage.....	136

List of Figures

Figure 1.1	Schematic of ESI-MS.	4
Figure 1.2	Schematic of MALDI-MS.	6
Figure 1.3	Peptide fragmentation notation.	9
Figure 2.1	Schematic for fabrication of PMMA microfluidic chips using a 150 μm diameter fused silica capillary. An 80 mm channel is defined by the placement of the capillaries at the entrance and exit of the microfluidic device. This channel length defines the surface to be immobilized with enzyme when immobilization occurs post-bond. Fluidic sealing is ensured post-bonding by double stamping the areas that contain the capillaries.	18
Figure 2.2	Schematic for fabrication of PMMA microfluidic chips using a combination of a 150 μm diameter fused silica capillary and 50 μm stainless steel wire. An 80 mm channel length of PMMA is left exposed for immobilization of enzymes to the polymer surface. Fluidic sealing is ensured post-bonding by double stamping the areas that contain the capillaries.	19
Figure 2.3	A schematic of the PMMA microchip set-up used for enzyme immobilization procedures. A shows the set-up used for enzyme immobilization on 80 mm of the microchannel length, and B shows the PDMS reservoir used for enzyme immobilization on 10 mm of the microchannel length.	20
Figure 2.4	A schematic diagram of a nanoindentation curve with all necessary nomenclature for the determination of hardness and reduced modulus. A shows the indentation under maximum load. B shows an image of the nanoindentation produced at a 100 μN while testing surface hardness. C shows a typical nanoindentation curve detailing the values obtained from the unloading curve. ^{70, 71} h_f is defined as the final displacement.	25
Figure 2.5	A schematic diagram of the enzyme immobilization procedures performed on the surface of PMMA.	28

Figure 2.6 The change in percent sequence coverage of cytochrome c over time that is observed for an 80 mm solvent bonded tryptic microreactor fabricated using either Method I or Method II, as well as an 80 mm thermally bonded peptic microreactor fabricated using Method II. The channel dimensions of the microreactor are 20 μm deep and 100 μm wide for the tryptic microreactors and a 150 μm diameter for the peptic microreactors. The residence time of the protein in the tryptic microreactors was 19.2 seconds and for the peptic microreactors is 2.8 minutes. 31

Figure 2.7 The change in percent sequence coverage of cytochrome c over time that is observed for an 80 mm thermally bonded tryptic microreactor fabricated using Method II. The channel dimensions of the microreactor are 50 and 150 μm diameter for the tryptic microreactors that were immobilized with enzyme post-bond. The residence time of the protein in the 50 and 150 μm diameter microreactors is 1.3 and 2.8 minutes respectively. 35

Figure 2.8 MALDI-TOF mass spectrum of an on-chip tryptic digest of cytochrome c. Peptides are indicated with a *. A sequence coverage of 89% was obtained after low temperature solvent bonding of the microfluidic chip. The residence time of the protein in the microreactor was 2.4 seconds. The enzymatic microreactor bed had a width of 100 μm , a depth of 20 μm and a length of 10 mm. 36

Figure 2.9 MALDI-TOF mass spectrum of a peptic digest of cytochrome c performed on a microfluidic chip. Peptides are indicated with a *. A sequence coverage of 87 % and 0% are obtained prior to (A) and after (B) applying thermal bonding conditions of 105°C respectively. The sequence coverage of 9% obtained after thermal bonding is from the presence of a single peptide being observed. The enzymatic microreactor bed had a diameter of 150 μm with a length of 80 mm. 37

Figure 2.10 MALDI-TOF mass spectrum of an on-chip peptic digest of cytochrome c. Peptides are indicated with a *. A sequence coverage of 87% was obtained after low temperature solvent bonding of the microfluidic chip. The residence time of the protein in the microreactor was 21 seconds. The enzymatic microreactor bed had a diameter of 150 μm with a length of 10 mm. 39

Figure 2.11 The hardness of unmodified and acid modified PMMA that has been treated in a 97% methanol and 3% water solution for 1 minute at 35°C. 42

Figure 2.12	Photomicrographs of a cross-section of PMMA substrates embossed with a 150 μm diameter fused silica capillary before (1) and after (2) thermal bonding at 105°C. All embossing was with a force of 2000 N for 6000 seconds while the temperature was varied to be 105°C (A), 110°C (B) and 115°C (C). All photomicrographs are taken at a magnification of 100x.	44
Figure 2.13	Photomicrograph at a 100 x magnification of the channel produced in a PMMA substrate embossed with a 150 μm diameter fused silica capillary.	44
Figure 2.14	Photomicrographs at a 100 x magnification of the junction between the 150 μm and 50 μm channels (A) and the 50 μm channel produced in a PMMA substrate embossed with a combination of a 150 μm diameter fused silica capillary and a 50 μm stainless steel wire at 125°C using a force of 2000 N for 600 seconds. A photomicrograph (C) taken at a magnification of 500x of the cross-section of a 50 μm channel after thermal bonding of the PMMA substrate and cover plate at 105°C using a force of 500 N for 300 seconds.	45
Figure 3.1	Photomicrograph showing the mist from the capillary instead of the Taylor cone at a flow rate of 100 nL/min ⁹⁸	49
Figure 3.2	The walls of the capillary are treated with z-6030 to provide an anchoring site for the PPM formation.	52
Figure 3.3	Compounds used in PPM formation.	53
Figure 3.4	The capillary is filled with solution and then masked off to only expose a 2.5 cm region of the solution to UV irradiation. The PPM is then formed by irradiated the area with UV light for either 15 minutes if a wavelength of 365 nm was used or 5 minutes if a wavelength of 254 nm was used. Upon completion a 2.5 cm PPM column is fabricated.	54
Figure 3.5	The mechanism for PPM formation. The initiator is represented by I.	54
Figure 3.6	Scheme 1 shows the Schiff base method used to immobilize trypsin on PPM columns using GMA as a monomer which bears an epoxide group. Scheme 2 shows the novel enzyme immobilization method that has been developed to immobilize an enzyme on a PPM surface comprised of an ester group functionality.	55

Figure 3.7	A schematic of the on-line protein separation set-up (A) and a photograph of the PPM emitter positioned near the MS orifice (B).	60
Figure 3.8	The structure of insulin. Chain A is represented in blue while Chain B is in green. The two insulin chains are held together by intra-strand and inter-strand disulfide linkages indicated by the brown lines.	63
Figure 3.9	A scanning electron microscope image taken of a PPM filled capillary fabricated with a porogenic solvent composed of 50% methanol and 50% dodecanol at a magnification of 1000 x (left) and 10,000x (right).....	64
Figure 3.10	The MALDI-TOF spectrum of the trypsin control test, showing 0% sequence coverage which indicates that trypsin is not adsorbing to the PPM column.....	67
Figure 3.11	MALDI-TOF mass spectrum of cytochrome c digest performed on a PPM filled capillary treated with trypsin. A 77 % sequence coverage was obtained. Tryptic peptides are indicated with a star. Autodigestion peaks of trypsin are indicated with a T.	67
Figure 3.12	The MALDI-TOF spectrum of the pepsin control test, showing 63% sequence coverage which indicates that pepsin is adsorbing to the PPM column. The stars denote digested peptide fragments.....	68
Figure 3.13	MALDI-TOF mass spectrum of cytochrome c digest performed on a PPM filled capillary fabricated with a porogenic solvent of 50% methanol and 50% dodecanol and butyl acrylate as the monomer. A 74 % sequence coverage was obtained. Peptides are indicated with a star.	69
Figure 3.14	A calibration curve constructed via a bicinchoninic acid assay to determine the amount of trypsin on a PPM column.	70

Figure 3.15 Longevity data obtained using a MALDI-TOF-MS for tryptic and peptic PPM columns. All columns were fabricated to a length of 2.5 cm. Assuming a 60% porosity, the residence time for each column would be 8 seconds. For the tryptic and peptic columns the desired enzyme was attached by first forming a carboxylic acid group on the surface followed by a treatment with WSC/NHS which provides the correct functionality on the PPM surface for subsequent enzyme attachment via an enzyme's amine group. For the PPM columns that were adsorbed with pepsin, no pre-treatment of the PPM was done which results in no covalent attachment of pepsin on the PPM surface. 71

Figure 3.16 The MALDI-TOF spectrum of a cytochrome c digest performed on a peptic PPM column. A sequence coverage of 79% was obtained. Peptides are indicated with a *. 73

Figure 3.17 The MALDI-TOF spectrum of a cytochrome c digest after denaturation of the peptic column. No peptides were observed which indicates loss of enzymatic activity..... 74

Figure 3.18 The MALDI-TOF spectrum of a cytochrome c digest on a tryptic PPM column fabricated using the modified immobilization procedure which involved one hour of acid treatment rather than 24 hours, resulted in a 70% sequence coverage. The stars denote digested peptide fragments..... 75

Figure 3.19 The ESI-MS spectrum of a cytochrome c digest on a regenerated tryptic column resulted in a 78% sequence coverage. The stars denote digested peptide fragments..... 76

Figure 3.20 The MALDI-TOF spectrum of a cytochrome c digestion on a regenerated tryptic column resulted in a sequence coverage of 73%. The stars denote digested peptide fragments. . 76

Figure 3.21 The ESI-MS spectrum of an off-line peptic digest of cytochrome c performed a PPM peptic column resulted in a 76% sequence coverage. A PPM column was also used as an electrospray emitter. The stars denote digested peptide fragments..... 78

Figure 3.22 The ESI-MS spectrum of an off-line peptic digest of insulin performed a PPM peptic column resulted in a 68% sequence coverage. A PPM column was also used as an electrospray emitter. The stars denote digested peptide fragments..... 79

Figure 3.23	The ESI-MS spectrum of an off-line peptic digest of myoglobin performed a PPM peptic column resulted in a 50% sequence coverage. A PPM column was also used as an electrospray emitter. The stars denote peptide fragments.....	80
Figure 3.24	The ESI-MS spectrum of an off-line peptic digest of BSA performed a PPM peptic column resulted in a 10% sequence coverage. A PPM column was also used as an electrospray emitter. The stars denote digested peptide fragments.....	81
Figure 3.25	The ESI-MS spectrum of an on-line tryptic digest of cytochrome c on a PPM column resulted in a 38% sequence coverage. The enzymatic microreactor was coupled to another PPM column to be used as an electrospray emitter so that the required acidic conditions for electrospray could be obtained post-digestion. The stars denote digested peptides fragments.	82
Figure 3.26	The ESI-MS spectrum of an on-line peptic digest of cytochrome c on a PPM column resulted in a 25% sequence coverage. The enzymatic microreactor was also used as a nanoelectrospray emitter. The stars denote digested peptide fragments.....	83
Figure 3.27	The ESI-MS spectrum of insulin obtained from separating a mixture of three proteins on a 6 cm C ₈ bead column using a PPM column as nanoelectrospray emitter.....	85
Figure 3.28	The ESI-MS spectrum of cytochrome c obtained from separating a mixture of three proteins on a 6 cm C ₈ bead column using a PPM column as nanoelectrospray emitter.....	85
Figure 3.29	The ESI-MS spectrum of myoglobin obtained from separating a mixture of three proteins on a 6 cm C ₈ bead column using a PPM column as nanoelectrospray emitter.....	86
Figure 4.1	Filtration apparatus used to remove proteins from sample solutions. 3M Empore C ₈ or C ₁₈ extraction disks were used for the filtration. Following the filtration the proteins were extracted from the disk and analyzed via MALDI-MS.....	93
Figure 4.2	Sequence of murine PrP. All methionies (M) are oxidized and a disulfide linkage exists between Cys157 and Cys192. The theoretical average molecular weight is 23 115 Da.	96
Figure 4.3	MALDI-TOF mass spectrum of PrP after (ZipTip) purification. Below m/z 11,000 there are a several protein fragments or contaminating proteins observable.	97

Figure 4.4	Electrospray MS/MS spectrum of the peptide with sequence YPGQGSPGGNR with m/z for $[M+2H]^{2+} = 545.3$.	98
Figure 4.5	MALDI-TOF mass spectrum of a microwave-assisted acid (TFA) digest of PrP. The resulting peptides specific for PrP are shown in the insert in the upper right corner. All the arrows indicate a loss of a glycine residue (57 Da) which is typical for TFA digests. ¹³⁸ Peaks below m/z 2600 are probably from digested contaminant protein fragments.	99
Figure 4.6	MALDI-TOF mass spectra of a PrP spiked water sample to test extraction efficiency. (A) 50 mL of water containing PrP (60 nM) (B) Extract from C ₁₈ extraction disk after filtration of water containing PrP (30 nM). Extraction was performed in a 1:1 solution of ACN and 0.5 % <i>n</i> -OGP for 20 mins. (C) Filtrate from filtration of 50 mL of water containing PrP (60 nM). For all three spectra a novel sensitive three layer matrix/sample preparation method was employed. ¹⁹	100
Figure 4.7	MALDI-TOF mass spectrum of the extract of PrP from a C ₁₈ extraction disk used to filter 25 mL of isooctane containing PrP (60 nM). Extraction performed in a 1:1 solution of ACN and 0.5% <i>n</i> -OGP for 30 minutes.	101
Figure 4.8	MALDI-TOF mass spectrum of the extract of PrP from a C ₈ extraction disk used to filter 25 mL of biodiesel containing PrP (60 nM). Extraction performed in a 1:1 solution of ACN and 0.5% <i>n</i> -OGP for 20 minutes.	102
Figure 4.9	MALDI-TOF mass spectrum of a BSA digest with trypsin performed directly on a C ₁₈ extraction disk after filtration of 1 L of biodiesel spiked with 4 µg of BSA. BSA peptides are marked with an * and trypsin autolysis peaks are indicated with a T.	103
Figure 4.10	MALDI-TOF mass spectrum achieved by post source decay to sequence the 1089 peptide achieved from a 2 hr tryptic digest performed on a piece of C ₈ extraction been used to filter 25 mL of biodiesel containing PrP (60 nM). Sample was passed through a C ₁₈ ZipTip prior to analysis and eluted with HCCA matrix solution.	104
Figure 5.1	MALDI-TOF mass spectrum of a 2 hour in-solution tryptic digest of antitrypsin. A 28% sequence coverage was obtained. Peptides are indicated with a * and autolysis trypsin peaks are indicated with a T.	118

Figure 5.2 MALDI-TOF mass spectrum of an on-chip tryptic digest of antitrypsin. A 35% sequence coverage was obtained. The residence time of the microfluidic device was 2.8 minutes. Peptides are denoted with a *..... 119

Figure 5.3 MALDI-TOF mass spectrum of a 2 hour in-solution tryptic digest of PrP. Two peptides were observed the larger of which provides unambiguous identification of PrP. 123

Figure 5.4 MALDI-TOF mass spectrum of a PPM tryptic digest of PrP. Three peptides were obtained, two of which can be used to identify of PrP. The residence time of the PPM column was 8 seconds. 124

Figure A. 1 MALDI-TOF mass spectrum of an on-chip tryptic digest of cytochrome c (A). Peptides are indicated with a *. A sequence coverage of 89% was obtained after low temperature solvent bonding of the microfluidic chip. The residence time of the protein in the microreactor was 2.4 seconds. The enzymatic microreactor bed had a width of 100 μm , a depth of 20 μm and a length of 10 mm. A sequence coverage plot of the above mass spectrum is shown in B.....138

List of Tables

Table 2.1 Cytochrome c peptide fragments observed from an on chip tryptic digests performed over a period of a month on a microreactor with an 80 mm channel bed immobilized with trypsin using Method II. The residence time of the protein in the microreactor was 19.2 seconds. An * indicates that the peptide was observed in the mass spectrum. The maximum allowed missed cleavages was set to three, but several peptides with fewer missed cleavages were also observed.....	33
Table 2.2 Cytochrome c peptide fragments observed from on chip pepsin digests performed over a period of a month on a microreactor with an 80 mm channel bed immobilized with pepsin using Method II. The residence time of the protein in the microreactor was 2.8 minutes. An * indicates that the peptide was observed in the MALDI mass spectrum. The maximum allowed missed cleavages was set to three, but several peptides with fewer missed cleavages were also observed.....	40
Table 3.1 Gradient elution profile for a sample containing 10 µM of insulin, cytochrome c and myoglobin. The flow rate was 300 nL/min with an injection volume of 1500 nL.	61
Table 5.1 Comparison of tryptic in-solution and on-chip digestions of antitrypsin. Peptides containing methionine residues are indicated in red as they are potential sites of inactivation. The residence time of the microfluidic chips was 2.8 minutes while the in-solution digestion was 2 hours.....	121

List of Abbreviations

μ -TAS - micro total analysis systems
2DGE - two-dimensional gel electrophoresis
ACN - acetonitrile
AFM - atomic force microscopy
BCA - bicinchoninic acid
BSA - bovine serum albumin
BSE - bovine spongiform encephalopathy
C-terminus - carboxyl terminus
CD - circular dichroism
CDI - 1,1'-carbonyldiimidazole
CID - collision-induced dissociation
DHB - 2,5-dihydroxybenzoic acid
DIC - disseminated intravascular coagulation
EDMA - ethylene dimethacrylate
ESI-MS - electrospray ionization mass spectrometry
FTIR - fourier-transform infrared
GMA - glycidyl methacrylate
HCCA - α -cyano-4-hydroxycinnamic acid
H-D - hydrogen-deuterium
ISD - in source decay
LC - liquid chromatography
MALDI-MS - matrix-assisted laser desorption/ionization mass spectrometry
MALDI-TOF - matrix-assisted laser desorption/ionization time of flight
MeOH - methanol
MS - mass spectrometry
MS/MS - tandem mass spectrometry
MSF - microstructured fibers
N-terminus - amino terminus
NCS - N-chlorosuccinimide
NHS - N-hydroxysuccinimide
n-OGP - octyl- β -D-glycopyranoside
PMMA - poly(methyl methacrylate)
PPM - porous polymer monolith
PrP - prion protein
PrP^C - normal cellular prion protein
PrP^{Sc} - infectious scrapie prion protein
PSD - post source decay
PU - polyurethane
PVDF - polyvinylidene difluoride

Q-TOF - quadrupole time of flight
RP-HPLC - reverse phase high performance liquid chromatography
SA - sinapinic acid
SDS-PAGE - proteins from sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM - scanning electron microscope
SRM - specified risk material
TFA - trifluoroacetic acid
TSE - transmissible spongiform encephalopathy
WSC - N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide
Z-6030 - 3-(trimethoxysilyl)propyl methacrylate

Chapter 1

Mass Spectrometry in Proteomics

Proteomics is the large-scale study of proteins, specifically their structure and function, and how biological or environmental factors impact both expression and function. Research in this field is of utmost importance since proteins are ultimately linked with various diseases, and a better understanding of the fundamental chemistries associated with proteins might lead to the development of new or improved disease treatments. A major concern in proteomics is protein expression, however this is a very complex area of study. As the body ages or is subjected to biological or environmental factors that induce stress on protein structures, causing the native protein structure to no longer be the most stable and lowest energy conformation, proteins often respond by changing structurally. Consequently, protein expression is dynamic, and this poses a challenge to researchers since the small changes in protein structure could lead to large effects in function are often not easy to identify, due in part to the overwhelming body of information that must be thoroughly understood as a pre-requisite. For this reason, the identification of biomarkers, which are signs of particular diseases, has been a focus of interest since a disease that is detected in its early stages has a higher probability of successful treatment.

There are many directions of study that are currently being pursued in proteomics. Separation techniques are under constant refinement since proteins are found in complex mixtures. Due to the fact that proteins are occasionally found in media that are not compatible with available analysis techniques (such as plasma),¹ purification techniques must be developed. Methods for protein quantification include protein tagging and chemical modification.² Structural information is continually sought since information about intact proteins can provide valuable insight into protein function while a protein's sequence can provide a means of unambiguous

identification.³ The study of proteins focuses not only on a protein's conformation, but also on modifications such as phosphorylation and glycosylation which can also result in a significant impact on protein function.

Bottom-up or top-down analysis are two complementary techniques that can be used for proteomic analysis. Bottom-up analysis involves taking a mixture of proteins and digesting the entire mixture to yield peptides of all corresponding proteins present in the original solution. The peptides are then analyzed based on their sequence to determine which proteins are present in the initial starting material. On the other hand, top-down analysis involves first analyzing the intact protein and then focusing on fragmentation to look at the amino acid sequence. This method allows all the necessary information about the protein to be discerned.

Traditionally, protein identification was through de novo sequencing. More specifically, the method of choice was Edman degradation which comprises a series of steps to chemically degrade either the protein or peptide fragments.^{4,5} Edman degradation allows for identification of the N-terminal residue and by analysis of the partial sequences generated by this technique the protein sequence can be derived or the fragments can be used for generating probes that are used in gene coding.⁶ Unfortunately, this technique is a slow process as every protein or peptide has to be sequenced separately. Despite this, the first two dimensional gel protein databases were developed using Edman degradation.⁷ Currently, large sequence databases have been developed which allow even small and partial sequences to be used for protein identification. Furthermore, mass spectrometers have helped to provide the data to make this type of identification possible.

Most of the advances that have been made in the field of proteomics can be attributed to the introduction of analysis techniques, specifically electrospray ionization mass spectrometry (ESI-MS)^{8,9} and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).¹⁰⁻¹² Both ESI-MS and MALDI-MS are soft ionization techniques meaning that little or no

fragmentation occurs during analysis, which is important for the study of intact proteins. For amino acid sequence analysis, however, fragmentation can be induced in ESI-MS and MALDI-MS by collision-induced dissociation (CID) in post source decay (PSD). Both techniques have their respective advantages, and both are essential in proteomics. As protein samples are often only present in small amounts, it is beneficial that both techniques only require microlitre volumes of samples, with MALDI-MS requiring smaller volumes than ESI-MS. MALDI-MS is generally preferred for simple peptide mixtures while ESI-MS integrated with liquid chromatography is the method of choice for the analysis of complex samples.

ESI-MS was first introduced by John Fenn and Masamichi Yamashita in 1984.^{8, 9} Analysis of samples involves passing a solution through a thin capillary. A voltage is applied to the capillary to ionize the sample. Charged droplets exit from the capillary and travel towards the orifice of the mass spectrometer (Figure 1.1). As the droplets travel through the air, solvent evaporation occurs. Eventually the Rayleigh limit is reached where the repulsion of the charge density of the droplet is greater than the surface tension of the droplet. This results in a Coulombic explosion, releasing smaller droplets.¹³ These small droplets then enter the mass spectrometer where they are analyzed according to their mass/charge ratio. A problem encountered with ESI-MS is the analysis of aqueous samples, which unfortunately is the basis of most protein solutions. Water does not quickly evaporate and as a result a stable signal is difficult to obtain with 100% aqueous samples.^{14, 15} Therefore, the addition of some organic content is often necessary for ESI-MS analysis.

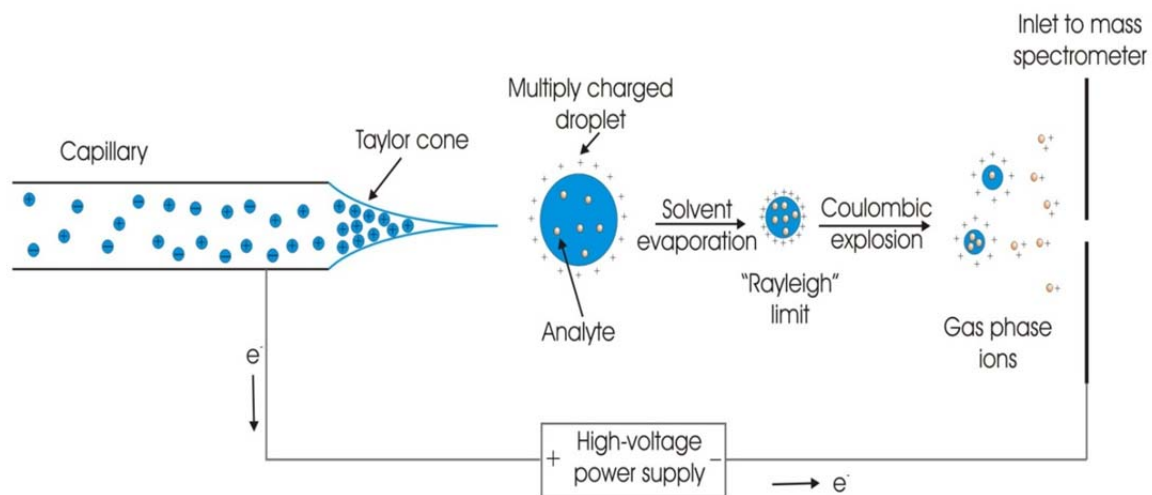


Figure 1.1 Schematic of ESI-MS (adapted from reference¹⁶).

Since ESI-MS analyzes liquid samples, on-line coupling to chromatographic techniques is possible. This is very important when complex mixtures are analyzed as it is often necessary to separate the analytes prior to analysis. With ESI-MS a chromatographic column can be attached before the capillary to separate the mixture. Furthermore, columns can also be used to preconcentrate the sample or to wash away interferences prior to analysis.¹⁷ Since ESI-MS analyzes liquid samples, all sample preparation steps can be performed on-line. The reduction of sample handling steps results in minimal sample loss. A disadvantage of ESI-MS is that the process is not ideal for high throughput analysis. A stable signal first has to be obtained which requires optimizing such parameters as solvent content, applied voltage, flow rate and, in certain cases, capillary position. Also, washing steps have to be performed between each sample. ESI-MS is optimal for smaller weight compounds of up to 2000 Da. Smaller analytes can be readily observed without the matrix interferences encountered with MALDI-MS. Higher molecular weight compounds can be readily analyzed through multicharging which results in a charge

envelope. Although the multiply charged ions that are often observed with ESI-MS are advantageous in analyzing larger analytes and identifying the mass of an unknown species the charge envelope can make the analysis of spectra more complicated if several analytes are present.

MALDI-MS offers a high throughput technique, with 100 samples that can be spotted per stainless steel target plate. Moreover, a wide mass range can be analyzed by MALDI-MS, but peaks in the mass range below 500 Da are difficult to discern due to the presence of matrix clusters. Sample preparation for MALDI analysis is the most critical step.¹⁸ The analyte solution is combined with a matrix and deposited onto a sample plate. Many methods for matrix sample preparation exist including dried droplet,¹⁹ two-layer¹⁸ and three-layer methods²⁰ with some common matrices being α -cyano-4-hydroxycinnamic acid (HCCA),^{18, 20} sinapinic acid (SA)¹⁸ and 2,5-dihydroxybenzoic acid (DHB).¹⁹ A homogenous crystallization of both matrix and analyte is optimal.²¹ The stainless steel target plate is placed into the MALDI-MS where a laser light is used to irradiate the matrix/analyte spot. The matrix absorbs the high energy of the laser so as to not destroy the analyte. The matrix is also responsible for incorporating a charge on the analyte. The process by which this happens is not well understood.²² The analyte is then desorbed from the steel target and the analytes are analyzed based on their mass/charge ratio (Figure 1.2).

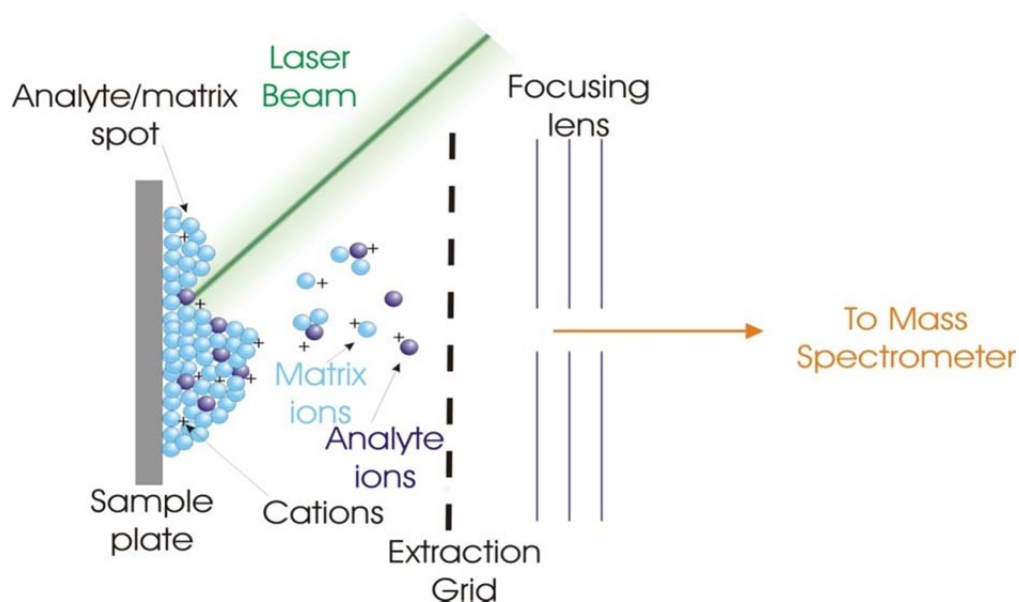


Figure 1.2 Schematic of MALDI-MS (adapted from reference²³).

MALDI-MS is relatively tolerant to salts and buffers which are commonly encountered in protein samples, especially after separation of proteins with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). With MALDI there are also only a few charge states observed for the ion which simplifies the spectra obtained. In the past, a limitation of MALDI was that it was considered to be an off-line technique. Commercially available MALDI spotters now allow MALDI-MS to be interfaced with separation techniques such as nano flow HPLC and nano liquid chromatography (LC).

With the advances in mass spectrometry, peptide mass fingerprint analysis is routinely used for high-throughput protein identification.²⁴ In this method the protein of interest, which may need to be purified prior to analysis, is either digested with an enzyme or chemically cleaved into peptides. For enzymatic digestions several different types of enzymes are available, each of which will produce different peptides of a given protein and have different advantages that suit certain forms of analysis better than others. For example, trypsin is a widely used enzyme that is very robust to analysis conditions and cleaves specifically at the C-terminus of lysine and

arginine residues except if followed by proline, and thus provides known peptide sequences. On the other hand, there are less robust enzymes such as pepsin that, due to their non-specific cleavage of proteins, result in less predictable peptide sequences. Despite the potential of non-specific cleavage sites, pepsin is routinely used in hydrogen-deuterium (H-D) exchange studies due to the fact that pepsin requires the acidic conditions compatible with this technique. Although pepsin will non-specifically cleave a given protein, it does preferentially cleave on the N-terminus of aromatic residues including tryptophan, tyrosine, and phenylalanine. Once this peptide mass fingerprint has been obtained it can be analyzed using mass spectrometry and compared to theoretical fingerprints of different proteins that are found in databases. From these databases the resulting peptides are matched to peptides from known proteins and a list of top matching proteins are given as potential matches. For unambiguous identification of a protein, research has shown that only a small number of accurate peptide masses are required.²⁴ Furthermore, only low picomole to high femtomole concentrations of proteins separated by gel electrophoresis are required for identification.²⁴ Several protein databases are available on-line for protein identification and include MOWSE,²⁴ ProfFound,²⁴ PeptIdent²⁴ and PeptideSearch.²⁴

As mentioned earlier for complex samples, pretreatment steps may be required to purify the sample prior to analysis to remove salts, buffers and detergents unless MADLI-MS is being used. Furthermore, separation of proteins in complex samples is often a prerequisite. There are two methods of choice for protein separation. The first involves two-dimensional gel electrophoresis (2DGE) to separate the proteins. After staining the selected bands are cut, enzymatically digested and then subsequently analyzed with MS. A disadvantage of 2DGE is its limited dynamic range;²⁵ however, advances in more sensitive staining methods,^{26, 27} large-format higher resolving gels²⁸ as well as fractionation of the sample before 2DGE separation²⁵ have helped minimize this drawback. The second method, liquid chromatography coupled with

MS/MS, is the current method of choice. Nonetheless, there are disadvantages associated with LC-MS/MS including the lack of sufficient peak capacity in one-dimensional separations and the large amounts of data that must be collected to use this technique.²⁵ The introduction of multi-dimensional chromatography such as two-dimensional (strong cation exchange/reverse phase)^{29,30} and three-dimensional (strong cation exchange/avidin/reverse phase)³¹ chromatographic separations have helped to increase peak capacity.

Although mass spectrometry allows for high-throughput analysis and requires minimal sample volumes it is not always the ideal choice for protein analysis as there are more sensitive and quantitative techniques that can be complementary to mass spectrometry, such as immunoassays (specifically, Western blot). Therefore, although mass spectrometry can provide initial evidence/identification of a protein to obtain the best detection limits and quantitative information, the method of choice is Western blot. Western blot is a very specific detection technique as it relies on the use of antibodies which are specific to a given protein. However, Western blot does have its limitations including the fact that it is a more complex and expensive technique. Furthermore, Western blot analysis is only a semi-quantitative technique and it is required to have the necessary antibody for the protein of interest in order for this technique to be feasible.

For specific sequencing of peptides using tandem mass spectrometry (MS/MS), either in source decay (ISD) or post source decay (PSD) can be used. The latter involves collision-induced dissociation (CID) either through product ion scanning, precursor ion scanning, neutral loss scanning or multiple ion monitoring. All of the above-mentioned techniques fragment the given peptide into different ions, including N-terminal ions (a, b, and c fragments) and C-terminal fragment ions (x, y and z fragments) as shown in Figure 1.3. Loss of water and ammonia can also be observed as well as internal fragmentation and immonium ions. With MALDI-PSD the

resulting spectrum is complex and y and b fragments are predominantly produced. The complexity arises from the various internal fragments produced. This, coupled with incomplete peptide backbone fragmentation and low mass accuracy of the higher mass fragments, results in MALDI-PSD being uncommonly used for protein identification.²⁴ Product ion scanning is the most common technique used in proteomics for MS/MS.³² Regardless of the technique used, the resulting fragmentation can be compared against existing databases to aid in sequencing of the analyzed peptide and provide confirmation of a given peak or help analyze an unknown sequence. One method that allows for a quantitative comparison of analysis techniques is to calculate digestion efficiency in terms of sequence coverage. Sequence coverage is defined as the number of amino acid residues observed from the peptides divided by the total number of amino acid residues of the studied protein. A more thorough discussion of the use of sequence coverage can be found in Appendix A.

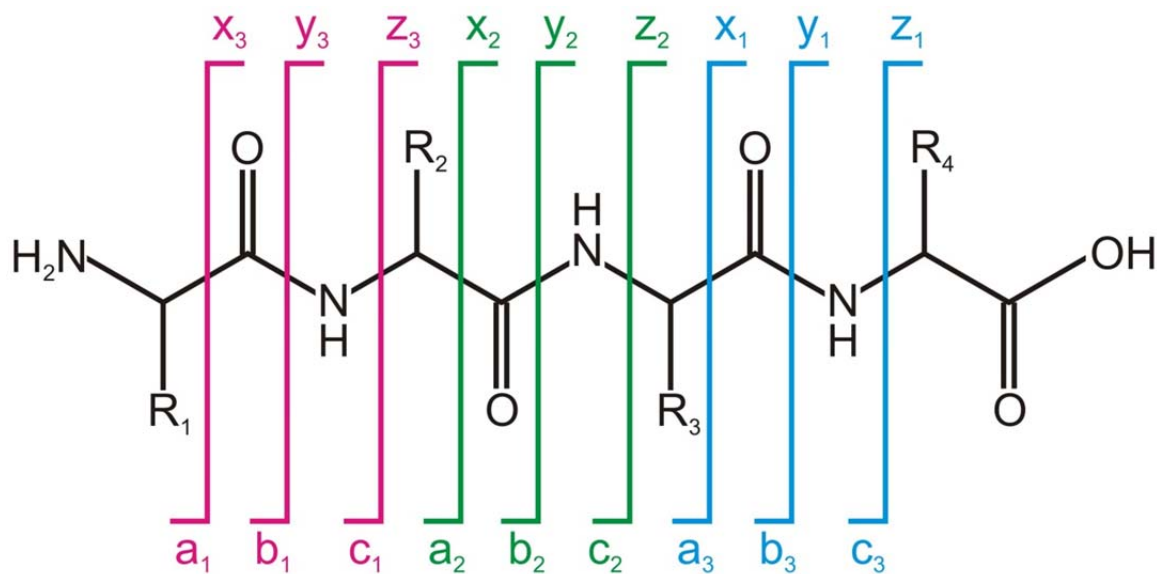


Figure 1.3 Peptide fragmentation notation (adapted from reference³³).

While peptide analysis is important for protein identification it is also important to obtain structural information about the given protein. H-D exchange is used to study higher order structures of proteins.³⁴ This technique is founded on the assumption that exchangeable hydrogen atoms in a protein will exchange at different rates which provides structural information about the protein. Specifically, two areas can be studied: solvent accessibility and hydrogen bonding. The first is based on the fact that hydrogen atoms exposed to solvent, i.e. those on the exterior of the protein, will exchange more quickly with deuterium atoms in comparison to those shielded from the solvent, i.e. those found in the interior of the protein. This information can then be used to provide information about the folding of the protein. Secondly, it has been observed that hydrogen atoms involved in hydrogen bonds will exchange more slowly in comparison to hydrogen atoms not involved in hydrogen bonding. As hydrogen bonding is involved in the formation of alpha helices and beta sheets, information about hydrogen bonding provides knowledge about the secondary and tertiary structure of the protein.

In addition to structural information, information about post-translational modifications of the protein is also important as they can result in conformational changes. Proteins produced from the RNA template in the ribosome are rarely biologically active/functional and generally require additional modifications to become active. Post-translational modifications are defined as chemical changes that occur after the translation process and include proteolytic cleavage, covalent modifications of amino acid residues via derivatizations and protein splicing. Some post-translational modifications have no apparent effect on the activity of the protein while others can alter the biological properties of the protein including solubility, stability, and/or specific activity. Moreover, sometimes these modifications may result in misfolding of the protein which could result in a lack of biological activity. Post-translational modifications cannot generally be analyzed via Edman degradation because either the modification has resulted in the protein chain

being unsuitable, for example an acylated N-terminus, or the conditions required for Edman degradation result in the protein reverting back to its unmodified structure, or the resulting peptides are too hydrophilic to be extracted or separated via chromatography after the phenylthiohydantoin derivative is formed.³⁵ Unlike Edman degradation, mass spectrometry does not suffer the aforementioned limitations and can be used to analyze post-translational modifications. Examples of post-translational modifications include N-glycosylation, O-glycosylation and phosphorylation, all of which can generally be determined by the molecular weight of the given peptide sequence. Some specific cases require tandem MS to locate the site of modification if more than one modifiable amino acid residue is located along the peptide chain.

This thesis describes the fabrication of enzymatic microreactors that can be used with either ESI-MS or MALDI-MS for the identification of proteins as well as the study of post-translational modifications. The devices include a polymer microfluidic chip and a porous polymer monolith column; the latter has dual functionality and can be used both as an enzymatic microreactor as well as a nanoelectrospray emitter. The application of the developed enzymatic microreactors has been demonstrated for standard proteins such as cytochrome c, bovine serum albumin, myoglobin and insulin as well as proteins that are traditionally “difficult” to digest and have clinical importance including recombinant prion protein and α -1-protease inhibitor also known as antitrypsin.

Chapter 2

Non-Denaturing Low Temperature Bonding of a Patterned Poly(methyl methacrylate) Enzymatic Microreactor

2.1 Summary

A low temperature solvent bonding system using methanol and water has been developed to bond poly(methyl methacrylate) (PMMA) microchips at 35°C. The substrate/cover plate adhesion strengths obtained with this bonding protocol peaked at 4000 kN/m² for unmodified PMMA substrates. Nanoindentation measurements performed using atomic force microscopy (AFM) revealed that only the first 30 nm of the PMMA surface showed a decreased hardness following surface modification and solvent treatment of the PMMA surface allowing the channel architecture to be maintained. The low temperature utilized for bonding enabled both a temperature robust and temperature labile enzyme to be facilely patterned prior to bonding with little-to-no loss in enzyme activity. Furthermore, the bonding methodology could be customized and used to fabricate an enzyme microreactor with pepsin (a pH, temperature and solvent sensitive enzyme).

The enzyme microreactor performance was characterized by the longevity of the microreactor, as well as the efficiency of the protein digest performed. The activity of an enzyme immobilized with *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (WSC) decreased over a period of days, whereas the enzyme immobilized with both WSC and *N*-hydroxysuccinimide (NHS) remained active even after a month of use. Even short 10 mm lengths of immobilized enzyme also provided high protein sequence coverage with short residence times.

2.2 Introduction

The development of microfluidics in the 1990s has led to the creation of micro total analysis systems (μ -TAS).³⁶⁻³⁸ The first devices fabricated were glass chips, on which electrophoretic separations were performed.^{39, 40} Since then, the field has grown rapidly and many common laboratory practices have been integrated on a microfluidic platform. Microfluidics offers many advantages over conventional bench-top chemistry. The devices have high throughput capabilities, require only minute amounts of sample, and analysis times are decreased.⁴¹

Initially, microfluidic devices were fabricated in glass or silicon because standard photolithography and chemical etching protocols had already been optimized for these materials in the microelectronics industry.⁴² Furthermore, the surface chemistry of glass and associated surface modifications has been well studied, allowing facile modification for specific applications. Glass devices are, however, relatively expensive to fabricate because each device requires a photolithographic step, so other substrate materials have been explored, with polymers receiving the most attention. Polymer microstructures can be fabricated inexpensively without the use of a clean room using high throughput techniques with methods such as injection molding,⁴³ laser ablation,⁴⁴ X-ray photolithography⁴⁵ and hot embossing.⁴⁶ After the microstructures have been fabricated, a cover plate must be bonded to the substrate to complete the capillary channel structure.⁴⁷ Several methods of bonding rigid polymers have been explored, including thermal bonding,^{48, 49} lamination,⁵⁰ adhesives,⁵¹ solvents⁵² and surface modifications.⁵³ Bonding of polymers is difficult because the changes to the surface must be closely controlled. Ideally, the bond must be able to withstand the requirements of the chemistry and attached instrumentation without delamination, the channel must not be clogged during bonding and the

channel integrity, shape and structure, and surface chemistry must not be altered.⁴⁷ To date, it has been difficult to find a bonding protocol that fulfills each of these requirements.

Recently there have been several groups that have published PMMA bonding methods that use temperatures lower than the glass transition temperature of PMMA. These include UV/ozone⁵⁴ (25, 50, 90 and 110 °C but prior to bonding polymer pieces were dried in a vacuum oven at 85 °C for 2 hours), carbon dioxide,⁵⁵ low molecular weight PMMA film⁵⁶ (95 °C for 20 min) or solvent bonding with such solvents as cyclopentanone⁵⁷ (75-85°C at 1-2N/cm² for 10-15 min), acetic acid⁵⁷ (75-85°C at 1-2N/cm² for 10-15 mins), isopropyl alcohol⁵⁸ (60°C at 1.3 bar for 10 min) or a mixture of 1,2-dichloroethane and ethanol.⁵⁹ Although all these techniques lower the temperature required for PMMA bonding, the temperature for all cases except that of 1,2-dichloroethane and ethanol or carbon dioxide assisted bonding can still be too high for non-denaturing protein conditions. Work by Lin *et al.*⁵⁹ using 1,2-dichloroethane and ethanol allows bonding of PMMA at room temperature, however these conditions have not been applied to microfluidic devices with enzyme immobilized on the surface. Another method using carbon dioxide assisted bonding was performed at 37°C and only showed a 10% reduction in bovine serum albumin content unlike thermal bonding which showed a 50% reduction.⁵⁵ ELISA was also used to determine the activity of immobilized lysozyme which was maintained by more than 95% using their method in comparison to less than 90% using thermal bonding.⁵⁵

Methods of fabrication of enzymatic microreactors include injection of a protein and enzyme solution^{60, 61} or immobilization of the enzyme on the microfluidic devices.⁶² Enzyme immobilization on a support has been shown to increase both the stability and activity of the enzyme.⁶³ Furthermore, minimal to no autolysis of trypsin is observed upon enzyme immobilization.⁶⁴ An important aspect of proteomics is the sequencing of proteins. Digests are performed to produce peptide fragments which are characteristic to a given protein and can be

used for subsequent protein identification.³ In-solution digests involving trypsin can suffer from long incubation times anywhere from 30 minutes to overnight. Digests performed in a microfluidic chip can be performed with reduced time and at room temperature to yield greater digestion efficiencies in comparison to results of digests performed in solution.⁶⁵ Methods of enzyme immobilization include enzyme entrapment on a membrane,⁶⁶ gel⁶⁷ or monolith.⁶⁸ Beads functionalized with enzyme can also be incorporated into the chip⁶⁹ or enzymes can be directly immobilized to the polymer channels.⁷⁰ A limitation with these techniques is that if a high bonding temperature is to be used to bond the cover plate, then certain enzymes have to be incorporated after bonding, as high temperatures would inactivate the enzyme.

In this work, PMMA enzyme microreactors have been fabricated with both pepsin and trypsin immobilized onto the channels of a simple microchip. A non-denaturing low temperature solvent bonding protocol was developed (below 37°C) to ensure that the enzyme is not denatured during bonding and remains active in the final device. By combining enzyme immobilization techniques with the new solvent assisted bonding protocol, enzymes can be immobilized on the chip in selected regions prior to polymer microchip fabrication to create patterned enzyme microreactors. Patterning prior to bonding eliminates the difficulties associated with patterning surface functionality following cover plate substrate adhesion. The solvent assisted bonding system has been characterized with tensile strengths and provided bond strengths that exceed those obtained with conventional thermal bonding. Nanoindentation measurements are used to probe the depth of material softening. Minimal softening ensures that channel architecture is preserved following bonding. Enzyme activity following bonding is characterized by digestion efficiencies of a model protein (cytochrome c). The necessity of low temperature solvent bonding conditions is demonstrated by fabricating an enzyme micro reactor with a thermally robust enzyme, trypsin, and a thermally labile and pH/solvent sensitive enzyme, pepsin using standard thermal bonding

conditions and low temperature solvent bonding conditions. Thermal bonding provides a functional trypsin microreactor however a similarly prepared pepsin micro reactor was nonfunctional. Aside from choosing to study both pepsin and trypsin due to their extreme sensitivity differences they were also chosen to illustrate the use advantages the fabricated PMMA chips can provide in protein research. Trypsin is frequently used in protein analysis as it provides specificity and thus it is easy to identify the peptides produces. Although pepsin at first glance seems undesirable to use due to its lack of specificity and sensitivity to environmental conditions it is nonetheless an important enzyme used in hydrogen/deuterium (H/D) exchange experiments. The use of pepsin in H/D exchange studies was introduced by Zhang *et al.*⁷¹ who showed that the acidic reaction conditions required for pepsin digestions were also amenable to quenching the H/D exchange reaction. Thus both trypsin and pepsin have desirable properties and are good complimentary enzymes in protein research.

The low temperature solvent bonding conditions enable a functional pepsin micro reactor to be fabricated and should provide a more universal bonding scheme as the solvent conditions can be easily tailored for the use with pH sensitive enzymes such as pepsin.

2.3 Experimental

2.3.1 Materials and Chemicals

Poly(methyl methacrylate) (PMMA) polymer sheets (150mm×150mm×1.5mm and 150mm×150mm×2mm) were purchased from Warehoused Plastic Sales Inc. (Toronto, ON, Canada) and cut to size before use. Fused silica capillary with a polyimide coating and an outer diameter of 150 µm and an inner diameter of 75 µm was purchased from PolyMicro Technologies (Phoenix, AZ, USA). Stainless steel wire with a diameter of 50 µm was purchased from Alta Aesar (Ward Hill, MA, USA). NHS and trifluoroacetic acid (TFA) were purchased from Aldrich

Chemicals (Milwaukee, WI, USA). WSC and α -cyano-4-hydroxycinnamic acid (HCCA) were acquired from Fluka (Buchs, Switzerland). Trypsin (bovine, 13000 units/mg), pepsin (porcine, 3600 units/mg) and cytochrome c (horse heart) were obtained from Sigma (Oakville, ON, Canada). TRIS·HCl, 2-propanol (ACS reagent grade), acetonitrile (ACN) (HPLC grade), and methanol (ACS reagent grade, Ultrapure grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sulfuric acid (ACS reagent grade), phosphoric acid (ACS reagent grade) and glacial acetic acid (ACS reagent grade) were purchased from Fisher Scientific (Nepean, ON, Canada). Ammonium bicarbonate, 2,5-dihydroxybenzoic acid (DHB), sodium chloride (ACS reagent grade), magnesium dichloride hexahydrate and calcium chloride dihydrate (ACS reagent) were acquired from Sigma (Oakville, ON, Canada). Deionized water (18.2 M Ω) was purified using a MilliQ apparatus (Millipore, Bedford, MA, USA).

2.3.2 Enzymatic Patterning on PMMA Substrates

PMMA substrates (1.5mm thick) were embossed using an electroplated nickel master with dimensions 100mm×100mm (Tecan, Dorset, UK) with a single straight channel design measuring 85 mm long, 100 μ m wide and 20 μ m deep. Briefly, PMMA and nickel master were brought into conformal contact, aligned and placed in a HEX-01 hot embosser Jenoptik Microtechnik (Jena, Germany). The embossing chamber was closed, evacuated and heated to 115°C and an embossing force 10 000 N applied for 10 minutes. Following stamping, the temperature of the tool and substrate was decreased to 85°C and then the chamber was brought to atmospheric pressure and opened. The nickel master and PMMA substrate were then carefully demolded by hand. The PMMA substrate and cover plates were cut to size, and 2mm holes were drilled in the cover plates in predefined locations to act as access reservoirs to the channels. As an alternative, larger channel diameters of 150 μ m could be fabricated by embossing a fused

silica capillary with an outer diameter of 150 μm into the PMMA substrate, this process is shown in Figure 2.1, using a temperature of 115°C with a force of 2000 N for 600 seconds. The capillary could then be removed to leave a 150 μm channel diameter in the PMMA.

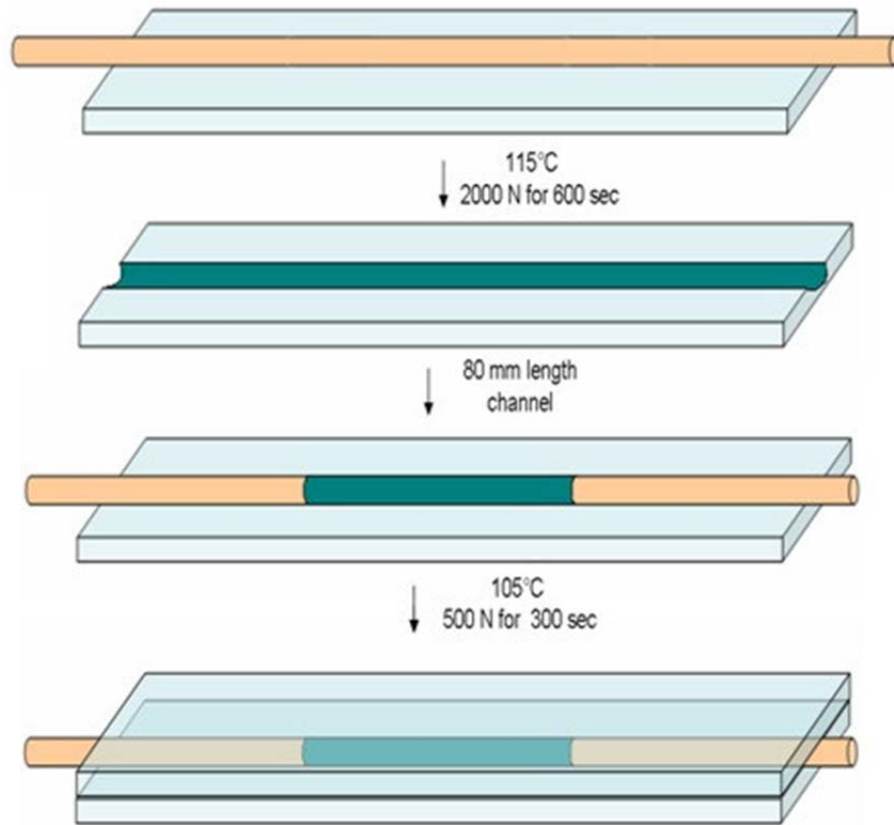


Figure 2.1 Schematic for fabrication of PMMA microfluidic chips using a 150 μm diameter fused silica capillary. An 80 mm channel is defined by the placement of the capillaries at the entrance and exit of the microfluidic device. This channel length defines the surface to be immobilized with enzyme when immobilization occurs post-bond. Fluidic sealing is ensured post-bonding by double stamping the areas that contain the capillaries.

Smaller channel diameter could also be fabricated using a similar method that involved threading a 50 μm stainless steel wire between two pieces of fused silica capillary with an outer

diameter of 150 μm and an inner diameter of 75 μm . The two pieces of capillary were then pulled toward each end of the wire leaving an 80 mm length of wire exposed between the two pieces. This wire/capillary apparatus was then embossed into a PMMA substrate at 125°C using a force of 2000 N for 600 seconds. Once the capillary and wire were removed a 150 μm channel diameter was left at both ends of the substrate with a 50 μm channel diameter in the center of the PMMA as shown in Figure 2.2. The smaller diameter channels were solely used to perform a longevity experiment on tryptic microreactors where the immobilization procedure was done post thermal bonding.

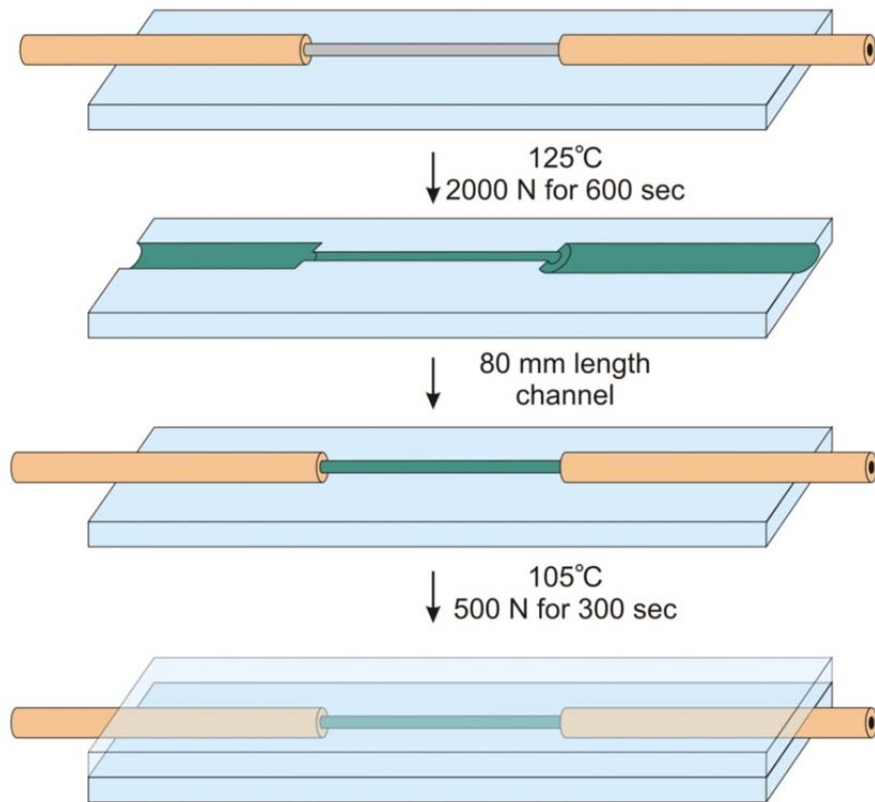


Figure 2.2 Schematic for fabrication of PMMA microfluidic chips using a combination of a 150 μm diameter fused silica capillary and 50 μm stainless steel wire. An 80 mm channel length of PMMA is left exposed for immobilization of enzymes to the polymer surface. Fluidic sealing is ensured post-bonding by double stamping the areas that contain the capillaries.

In both these two aforementioned cases the cover plate did not have the requirement of having access holes because fused silica capillary pieces with an outer diameter of 150 μm and an inner diameter of 75 μm could be placed at the entrance and exit of the channel to define a specific channel length while allowing the microfluidic chip to be connected to a syringe pump. The embossed PMMA substrates were immersed in 3M H_2SO_4 at 60°C for 20 minutes. The modified PMMA was then rinsed with copious amounts of water, followed by rinsing with 2-propanol and drying with a stream of nitrogen. PDMS pieces were cut to create solution reservoirs 2mm wide and 10mm or 80mm in length. These were positioned over the channel region and pressed against the PMMA substrate plates by hand until a watertight seal was formed (Figure 2.3). Two different methods of enzyme immobilization were used.

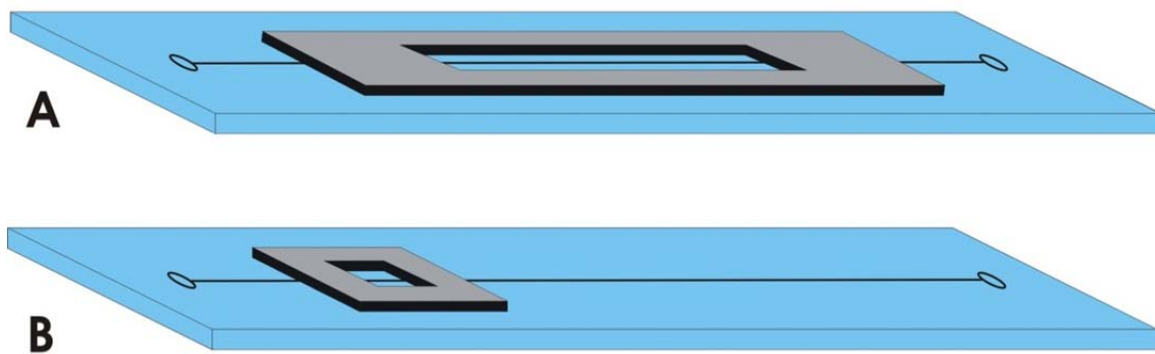


Figure 2.3 A schematic of the PMMA microchip set-up used for enzyme immobilization procedures. **A** shows the set-up used for enzyme immobilization on 80 mm of the microchannel length, and **B** shows the PDMS reservoir used for enzyme immobilization on 10 mm of the microchannel length.

2.3.3 WSC Immobilization Method

A solution containing 1.0 mg/mL of aqueous WSC was added to the PDMS reservoirs and allowed to react for 2 hours at room temperature. The PMMA was then rinsed with water and 2-propanol and dried with nitrogen. The PDMS pieces were replaced in the same positions on the surface of the PMMA, and a 10 mg/mL trypsin solution in 50 mM NH_4HCO_3 (pH 7.4), 10 mM NaCl and 10 mM MgCl_2 was added to the solution reservoirs. The reservoirs were covered with parafilm and stored at 4°C for 24 hours. After that time, the PDMS sample reservoirs were removed and the PMMA was rinsed gently with copious amounts of water to remove any adsorbed enzyme.

2.3.4 WSC/NHS Immobilization Method

For the second method, a solution of 1.0 mg/mL aqueous WSC and 0.1 mg/mL NHS was added to the PDMS solution reservoirs and reacted at room temperature for two hours. The PMMA substrates were then rinsed with water and 2-propanol and dried with nitrogen. The PDMS solution reservoirs were replaced and a 10 mg/mL trypsin solution in 50 mM NH_4HCO_3 (pH 7.4), 10 mM NaCl and 10 mM MgCl_2 or a 5mg/mL pepsin solution in 1% acetic acid was used to fill the reservoirs. The reservoirs were sealed with parafilm and stored at 4°C for 24 hours followed by gentle rinsing with water or 1% acetic acid (depending on the enzyme used) to remove adsorbed enzyme.

Alternatively for the microfluidic chips that were fabricated with capillaries the enzyme could be immobilized post bonding. In this case the PMMA could be treated with sulfuric acid post- or pre-bonding. For post-bonding treatment the acid step was omitted in the bonding protocol. Thus the first step of the immobilization procedure involved passing a solution of 1 M H_2SO_4 through the channel at a flow rate of 0.5 $\mu\text{L}/\text{min}$ using a syringe pump. The channel was subsequently rinsed with water at a flow rate of 5.0 $\mu\text{L}/\text{min}$. Next a solution of 1.0 mg/mL

aqueous WSC and 0.1 mg/mL NHS was passed through the microfluidic chip at a flow rate of 0.5 $\mu\text{L}/\text{min}$ for 2 hours. Next the channel was rinsed with water at a flow rate of 5.0 $\mu\text{L}/\text{min}$. A solution of either 10 mg/mL trypsin solution in 50 mM NH_4HCO_3 (pH 7.4), 10 mM NaCl and 10 mM MgCl_2 or 5 mg/mL of pepsin in 1% acetic acid was flushed through the microfluidic chip for 1 hour. The capillary ends were then sealed with parafilm and the microfluidic chip was stored at 4°C overnight. The resulting enzymatic microreactors were then either flushed with water or 1% acetic acid for trypsin or pepsin microreactors respectively.

2.3.5 Low Temperature Bonding with Immobilized Enzyme

PMMA cover plates were sonicated in a 1:1 2-propanol and water solution for 10 minutes and dried with nitrogen. For the tryptic microreactors, eight drops (approximately 250 μL) of a 97% methanol (ACS grade) and 3% water solution were evenly spread over the stamped PMMA substrate. The cover plate was aligned and placed in conformal contact with the substrate and placed within the embossing chamber. Alternatively, when peptic microreactors were prepared the conditions were modified to address the pH sensitivity of the enzyme (i.e. pepsin becomes irreversibly inactivated above a pH of 5). Solvent bonding conditions consisted of: 97% methanol and 3% of a 1M H_2SO_4 solution. The chamber was closed, a touch force of 250 N was applied for 30 seconds and the tool and substrate were brought to 35°C. A force of 3000 N was then applied for one minute. For the 100 μm wide x 20 μm deep channels, a one piece finger tight fitting (Upchurch Scientific, Oak Harbour, WA, USA) was connected to one of the access holes of the PMMA microchip with five minute epoxy (Lepage, Taylor, MI, USA). This step was unnecessary when the larger channel diameters of 150 μm were used. Capillaries lengths (~20 cm) were positioned at the entrance and exit of the channel to define the channel length, followed by bonding of the cover plate. For tryptic microreactors, the channels were rinsed with 50 mM

TRIS·HCl (pH 7.4) after bonding and stored at 4°C in a 50 mM TRIS·HCl (pH 7.4) and 10 mM CaCl₂ solution. To prevent drying of the enzyme during storage, the microchip access holes were sealed with parafilm. Prior to use, the PMMA microfluidic channels were rinsed with 50 mM TRIS·HCl (pH 7.4), followed by 50 mM NH₄HCO₃ (pH 7.4) at a flow rate of 5.0 μL/min for 10 minutes using a syringe pump (Harvard Apparatus, St. Laurent, PQ, Canada). With peptic microreactors, the channel was rinsed with 1% acetic acid solution and also stored at 4°C using the same solution.

2.3.6 Thermal Bonding of PMMA substrates

The temperature stability of immobilized pepsin and trypsin was tested using by thermal bonding enzyme immobilized substrates. Conditions consisted of: 105°C with 500 N of applied force for 300 seconds. The activity of the enzyme was ascertained by sequence coverage (see below) both before and after thermal bonding conditions were applied.

To fabricate enzymatic microreactors via thermal bonding and enzyme immobilization post bonding, the channel area where the entrance and exit capillaries are placed was double stamped at a temperature of 110°C with a force of 1000 N for 400 seconds to ensure fluidic sealing of the microfluidic chip.

2.3.7 Measurement of Cover Plate Adhesion Strength

The tensile strength of the bond between the sulfuric acid modified or unmodified PMMA substrates and unmodified cover plates was determined using an Instron 3369 tensile tester (Instron, Toronto, ON, CA). Two pieces of PMMA (70mm×20mm×1.5mm) were bonded with the aid of a solvent solution consisting of 97% methanol and 3% water. These PMMA substrates were bonded with known cross sectional areas ranging between 1.0 and 3.0 cm². The samples were clamped into the tensile tester with grips pulled away from each other at a rate of

1.3 mm/min, and set 114 mm apart initially.⁷² The force at which the bonded PMMA failed was measured. These values were divided by the cross sectional area to obtain the tensile strength of adhesion. Each value reported is the average of at least five samples per cross sectional area, and three cross sectional areas were tested for each method of bonding.

2.3.8 Nanoindentation Measurements on the surface of PMMA

PMMA samples were cut to 12 mm × 12 mm × 2 mm and left unmodified or treated with 3M sulfuric acid at 60°C for 20 minutes. Prior to nanoindentation experiments both acid modified and unmodified PMMA samples were immersed in 2mL of the solvent mixture (3% water, 97% methanol) at 35°C for 1 minute. Each sample was analyzed within 20 minutes of preparation. Nanoindentation experiments were performed using a Hysitron Triboindenter (Hysitron Inc., Minneapolis, MN, USA) equipped with a 1 μm 60 degree conospherical diamond tip probe. After the indentation the indents were imaged using a Quesant Q-SCOPE 250 atomic force microscope (AFM) intergrated within the Hysitron nanoindenter. The images were acquired in contact mode and the AFM tips had a nominal sharpness of approximately 20 nm.

The AFM was used to image the surface topography of the polymer after indentation to determine the integrity of both the polymer surface and the tip indentation. A typical nanoindentation data curve and an illustration of the indentation geometry with all pertinent parameters are given in Figure 2.4.

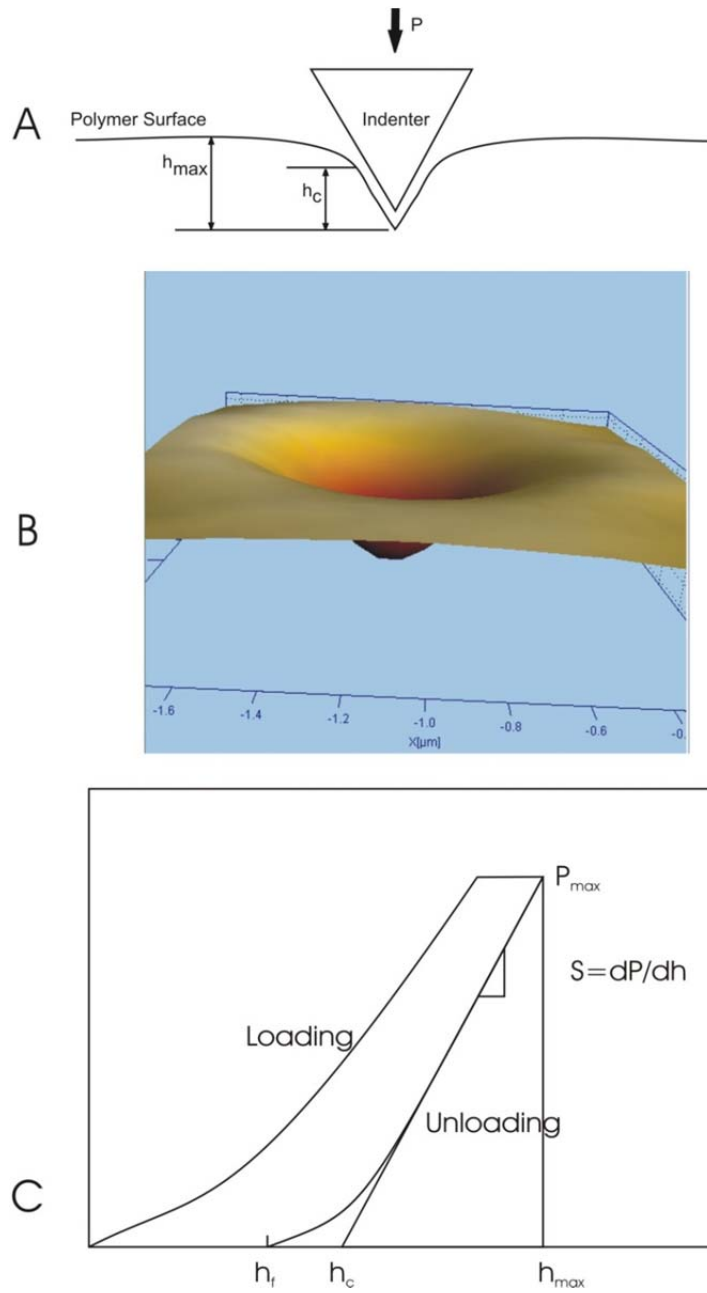


Figure 2.4 A schematic diagram of a nanoindentation curve with all necessary nomenclature for the determination of hardness and reduced modulus. **A** shows the indentation under maximum load. **B** shows an image of the nanoindentation produced at a $100\mu\text{N}$ while testing surface hardness. **C** shows a typical nanoindentation curve detailing the values obtained from the unloading curve.^{73,74} h_f is defined as the final displacement.

The Hysitron was calibrated using a fused quartz crystal. Loads ranging from 10 to 200 μN were performed on the PMMA samples. Each force was applied to the PMMA at least three times. The Young's modulus and the hardness of the PMMA samples were calculated using TriboScan 8.0 software developed by Hysitron.

2.3.9 On Chip Enzymatic Digests

Cytochrome c was chosen as a model protein to test the enzymatic microreactors as it is easy to digest and contains no disulfide linkages that require reduction/alkylation unfold the protein prior to enzymatic digestion. For tryptic microreactors, protein solutions (5 μM in 50 mM NH_4HCO_3 solution, pH 7.4) were prepared and diluted 3:2 with methanol (ACS grade) and pumped through the PMMA enzyme microreactors at a flow rate of 0.5 $\mu\text{L}/\text{min}$, producing an on chip residence time on chip of approximately 20 seconds for the smaller channel diameter (100 μm) and three minutes for the larger channel diameter (150 μm) when an 80 mm enzymatic channel bed was fabricated. When peptic microreactors were used, 5 μM cytochrome c in a 1% acetic acid solution was used and flowed through the chip at 0.5 $\mu\text{L}/\text{min}$ with a syringe pump. All digested solutions were collected and analyzed off-line by MALDI-TOF MS.

2.3.10 MADLI-TOF-MS Analysis of Enzyme Digests

A Voyager DE STR matrix-assisted laser desorption/ionization time of flight mass spectrometer (MALDI-TOF-MS) (Applied Biosystems, Foster City, CA, USA) was used to analyze all the collected protein digests except for the digest from the peptic microreactor fabricated using the low temperature bonding technique and the peptic microreactor longevity experiments. The protein digest from the low temperature bonded peptic microreactor was analyzed by a QSTAR XL Qq TOF MS instrument equipped with a MALDI source (Applied Biosystems, MDS SCIEX, Concorde, ON, Canada). For the longevity experiments of the

thermally bonded peptic microreactors a Voyager DE PRO MALDI time of flight mass spectrometer (Applied Biosystems, Foster City, CA, USA) was used. A two-layer HCCA matrix sample prep¹⁸ employed for analysis with the Voyager MALDI instrument, while a DHB dried drop method¹⁹ was used with the QSTAR MALDI. For analysis using the Voyager DE PRO MALDI a three layer DHB method was employed. The first layer consisted of 20 mg/mL DHB in 70 % ACN and 0.1 % TFA. The second layer was composed of 20 mg/mL DHB in 50% ACN and 2% phosphoric acid. The final layer was the sample solution being analyzed. A volume of 0.5 μ L of each solution was spotted on the MALDI target plate. The tryptic digest solutions were acidified with 1% trifluoroacetic acid prior to analysis. At least three spots were analyzed for each protein digest performed.

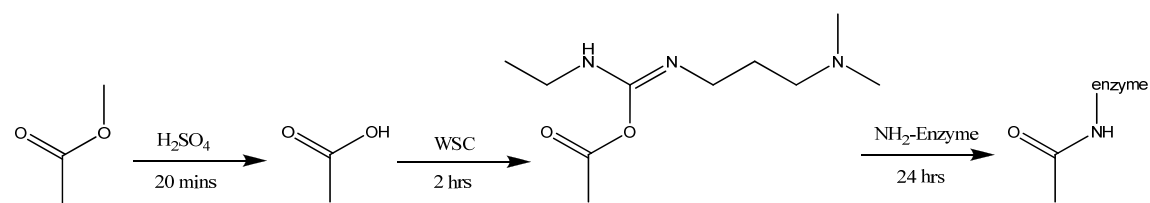
2.3.11 Data Analysis

The peptides obtained from on chip tryptic digests of cytochrome c were matched to theoretical peptide masses obtained from Protein Prospector (<http://www.prospector.ucsf.edu>) using a maximum of three missed cleavages and allowing for the variable modification of methionine oxidation. For tryptic digests the percent sequence coverage was determined by inputting the observed peptides into MASCOT peptide mass fingerprint. For peptic digests, the sequence coverage had to be calculated manually because pepsin can non-specifically cleave. Sequence coverage was calculated by dividing the total number of amino acid residues observed by the total number of amino acid residues in the protein. Thus both the theoretical peptide peaks obtained from Protein prospector and those experimentally determined by Bayraktar *et al.*⁷⁵ were used to calculate the sequence coverage.

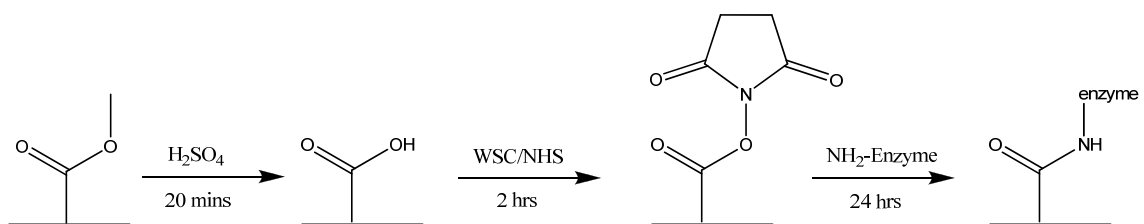
2.4 Results and Discussion

2.4.1 Immobilization of Trypsin on PMMA substrates

The patterning of reagents on a microfluidic substrate (pre-bond) greatly simplifies the fabrication procedure. In this study we demonstrate that through low temperature solvent assisted bonding, a temperature and pH sensitive enzyme can be patterned/immobilized to facilitate the formation of an enzymatic micro reactor. Different enzyme coupling strategies can be employed and in this study we employed two enzyme procedures that involve the activation of the surface of the polymer to facilitate enzyme attachment. Method I uses carbodiimide as the surface activator (Figure 2.5) while Method II uses the carbodiimide as well as N-hydroxysuccinimide to activate the surface (Figure 2.5).



Method I



Method II

Figure 2.5 A schematic diagram of the enzyme immobilization procedures performed on the surface of PMMA.

NHS has been shown to increase the yield and stability of the reaction intermediate, which can increase the lifetime of the enzyme microreactors.⁷⁶ This is a result of an increase in enzyme covalently attached to the surface as more sites are accessible for covalent attachment due to a decrease in hydrolysis of the intermediate. Both Methods I and II involve the reaction of the carboxylic acid surface termini with the activator to form an ester which readily reacts with amine groups on the protein to form an amide bond. The amount of carboxylic acid groups generated on the surface using the developed surface modification method has already been determined to be $240 \pm 70 \text{ nmol cm}^{-2}$.⁷⁷ This value was obtained using WSC which quantitatively and selectively binds to carboxylic acid groups.⁷⁸ Tryptic enzyme microreactors were prepared by pre-patterning trypsin on the PMMA surface followed by solvent bonding the embossed PMMA substrate and cover plate. Trypsin was immobilized within the microchannel using both Method I and II. MALDI-TOF MS analysis was performed on each of the cytochrome c samples collected from on-chip digestions. Cytochrome c digests were performed using a flow rate of $0.5 \mu\text{L/min}$ (19 second residence time) at 24 hour intervals for a period of one week to examine the activity of the enzyme over time. The efficiency of the digestion was determined by calculating the sequence coverage of the digested protein with a maximum of 3 allowed missed cleavages. As the number of missed cleavages were kept to a minimum the calculated sequence coverage results in a valid quantification of digestion efficiency. A more thorough discussion of sequence coverage can be found in Appendix A. The digests with both methods of immobilization yielded good sequence coverage on Day 1 (99% method II and 91% for method I respectively). These sequence coverages are slightly higher than that obtained from a 30 minute in-solution tryptic digestion of cytochrome where an 81% sequence coverage was obtained (data not shown). A slightly lower percent sequence coverage was obtained with microreactors prepared using Method I (Figure 2.5) which was expected since the WSC intermediate used in this method is less

stable against hydrolysis than the WSC-NHS intermediate in Method II.⁷⁶ However, after one week of use, the difference in digestion efficiency changes considerably. Method II chips continue to yield relatively high sequence coverage (77%), while the sequence coverage provided by chips prepared using Method I decrease to only 47% (Figure 2.6). No further testing of Method I chips was conducted beyond one week due to the significant decrease in sequence coverage after one week. This is hypothesized to be a result of less enzyme being covalently bound to the PMMA surface in Method I which would result in a more dramatic decrease in digestion efficiency as the enzyme starts to become inactive. Even following 4 weeks of storage and multiple uses the Method II type microreactors were still generating reasonable sequence coverage (59%).

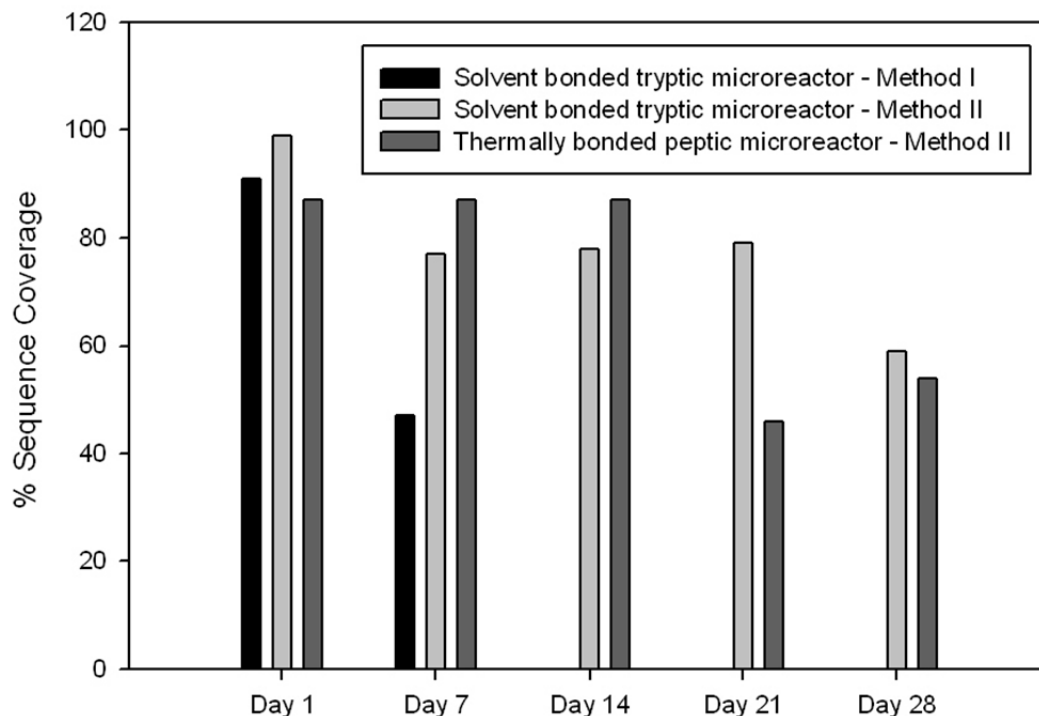


Figure 2.6 The change in percent sequence coverage of cytochrome c over time that is observed for an 80 mm solvent bonded tryptic microreactor fabricated using either Method I or Method II, as well as an 80 mm thermally bonded peptic microreactor fabricated using Method II. The channel dimensions of the microreactor are 20 μm deep and 100 μm wide for the tryptic microreactors and a 150 μm diameter for the peptic microreactors. The residence time of the protein in the tryptic microreactors was 19.2 seconds and for the peptic microreactors is 2.8 minutes.

The specific peptides observed during the longevity experiments are shown in

Table 2.1. Data show that the combination of WSC and NHS as surface activators greatly increases the stability and lifetime of the immobilized enzyme. As the enzyme activity is lost higher molecular weight peptides are not observed, after the first week of use. It is expected that peptides with higher m/z would be observed resulting from an increase in the number of missed cleavages as digestion efficiency is reduced. However, the reason why this is

probably not observed is that intensity of the peaks of higher molecular weight peptides is significantly lower than that of lower molecular weight peptides. As such even though these higher molecular weight peptides are expected, they are probably below the detection limit. To determine if the amount of use increases the rate of degradation of the enzyme, a second set of enzyme microreactors were fabricated using Method II. Based on the similar sequence coverage obtained for a used and unused microreactor, it appears that time since immobilization has more of an influence on enzyme activity longevity (under these conditions) rather than the number of protein digests performed on the chip. Furthermore, although only microliters of sample were required for analysis, the microfluidic chips were often run for 30-60 minutes each time to have extra volume to run replicates. The use the microreactors over the month would result in an overall use of the microreactor for approximately 6 hours and during this time no evidence of clogging of the channel was observed. Thus, proteins such as cytochrome c which can be more difficult to analyze in microfluidic devices due to the presence of the heme group which can lead to buildup on microfluidic channels⁷⁹ showed no deleterious effects.

Table 2.1 Cytochrome c peptide fragments observed from an on chip tryptic digests performed over a period of a month on a microreactor with an 80 mm channel bed immobilized with trypsin using Method II. The residence time of the protein in the microreactor was 19.2 seconds. An * indicates that the peptide was observed in the mass spectrum. The maximum allowed missed cleavages was set to three, but several peptides with fewer missed cleavages were also observed.

Peptide Fragments			Day				
<i>m/z</i>	<i>Amino Acid Sequence</i>	<i>Missed Cleavages</i>	<i>1</i>	<i>7</i>	<i>14</i>	<i>21</i>	<i>28</i>
795.4	MIFAGIK (Met-ox)	0		*	*	*	*
902.5	GDVEKGKK	2	*			*	*
923.5	MIFAGIKK (Met-ox)	1				*	
947.6	GKKIFVQK	2			*		*
1035.6	MIFAGIKKK	2					*
1168.6	TGPNLHGLFGR	0	*	*	*	*	*
1296.7	TGPLHGLFGRK	1	*	*	*	*	*
1350.7	TEREDLIAYLK	1	*	*	*	*	
1433.8	HKTGPNLHGLFGR	1	*	*	*	*	
1438.8	YIPGTMIFAGIK	1		*			
1470.7	TGQAPGFTYTDANK	0	*	*	*	*	
1478.8	KTEREDLIAYLK or TEREDLIAYLKK	2	*	*	*	*	*
1598.8	KTGQAPGFTYTDANK	1	*	*		*	
1606.9	KKTEREDLIAYLK or KTEREDLIAYLKK	3	*	*	*	*	
1634.4	CAQCHTVEK	0	*				
1639.8	EETLMEYLENPKK (Met-ox)	3	*		*	*	
1675.9	GGKHKTGPNLHGLFGR	2	*	*			
1695.0	KYIPGTMIFAGIKK or YIPGTMIFAGIKKK	3	*				
1712.8	TGQAPGFTYTDANKNK	1	*	*	*	*	
1840.9	KTGQAPGFTYTDANKNK	2	*	*	*	*	*
1876.6	CAQCHTVEKGGK	1		*	*		
1893.9	TEREDLIAYLKKATNE	3	*			*	
2209.1	GITWKEETLMEYLENPKK	2	*				
2249.8	IFVQKCAQCHTVEK	1	*				
2283.2	EETLMEYLENPKKYIPGTK	2		*			
2377.9	KIFVQKCAQCHTVEK	2	*				
2748.4	TGPNLHGLFGRKTGQAPGFTYTDANK	2	*				
2990.5	TGPNLHGLFGRKTGQAPGFTYTDANKNK	3		*			
% Sequence Coverage			99%	77%	78%	79%	59%

2.4.2 Low Temperature Bonding of Pre-Patterned Enzyme Substrates

To probe the effects of exposure to increased temperature during the bonding process trypsin was immobilized within the microchannels, after the device had been bonded, so the enzyme was not exposed to elevated temperatures prior to testing. The resulting enzyme microreactors were assessed for digestion efficiency and then subjected to thermal bonding conditions (105°C and 500 N of applied pressure for 300 sec). The exposed trypsin microreactors were then used to perform a digestion and the sequence coverage monitored. Not surprisingly, the trypsin microreactors did not show significant degradation in digestion efficiency since trypsin has been shown experimentally to maintain its activity up to 220°C.⁸⁰ In fact a slight increase in sequence coverage was observed with 64 % sequence coverage prior to, and 81% following the application of increased pressure and temperature. However, the increase in sequence coverage is due only to the cleavage of one additional peptide. Furthermore, the sequence coverage results obtained from immobilized trypsin exposed to thermal bonding temperatures coincides with data obtained from longevity experiments of trypsin immobilized post-bond to 150 μm channel diameter microreactors (Figure 2.7). This further proves that no degradation of the enzyme is observed upon exposure of immobilized trypsin to high temperatures.

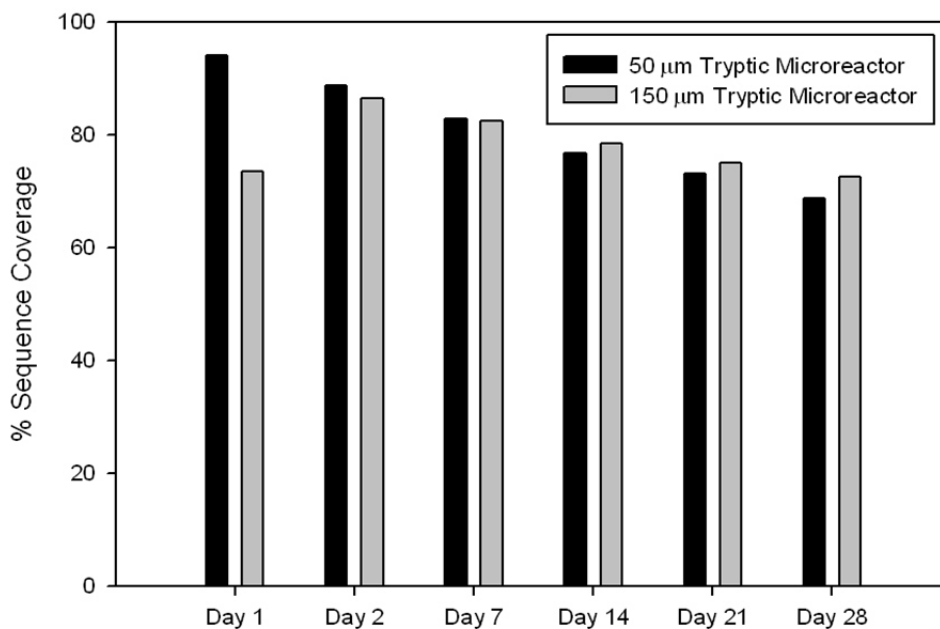


Figure 2.7 The change in percent sequence coverage of cytochrome c over time that is observed for an 80 mm thermally bonded tryptic microreactor fabricated using Method II. The channel dimensions of the microreactor are 50 and 150 μm diameter for the tryptic microreactors that were immobilized with enzyme post-bond. The residence time of the protein in the 50 and 150 μm diameter microreactors is 1.3 and 2.8 minutes respectively.

The ability to pattern the enzyme pre-substrate cover plate bonding is shown by immobilizing trypsin in only a 10 mm section of the 80 mm microchannel. Shortening the immobilized region reduces the microreactor residence time to 2.4 seconds however a 89% sequence coverage is still obtained for a cytochrome c digest (day 1) as shown in Figure 2.8. The high sequence coverage along with no intact protein being observed in the mass spectrum indicates that even with a very short residence time of 2.4 seconds cytochrome c is completely digested.

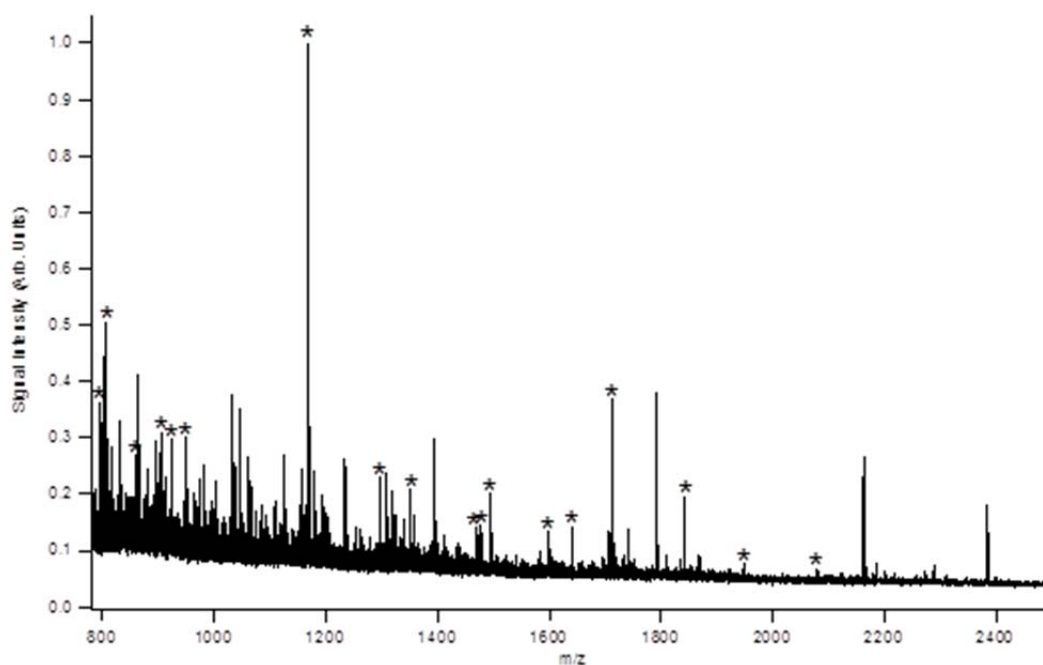


Figure 2.8 MALDI-TOF mass spectrum of an on-chip tryptic digest of cytochrome c. Peptides are indicated with a *. A sequence coverage of 89% was obtained after low temperature solvent bonding of the microfluidic chip. The residence time of the protein in the microreactor was 2.4 seconds. The enzymatic microreactor bed had a width of 100 μm , a depth of 20 μm and a length of 10 mm.

It has been shown that the temperature sensitivity of an enzyme decreases following immobilization (e.g. lipase loses its activity at 50°C while when immobilized its activity can be maintained in the temperature range of 30-60°C⁸¹) however, the temperature conditions required for bonding can still contribute to a significant loss in an enzyme's activity. As an example, microreactors containing immobilized pepsin (immobilization performed post bonding) showed a 87% sequence coverage initially however, following the application of thermal bonding conditions (Figure 2.9A) all peptic activity is lost (Figure 2.9B). This demonstrates the temperature fragility of pepsin when exposed to conditions required for thermal bonding and

shows that patterning pepsin pre thermal bonding would yield a microreactor with no enzymatic activity.

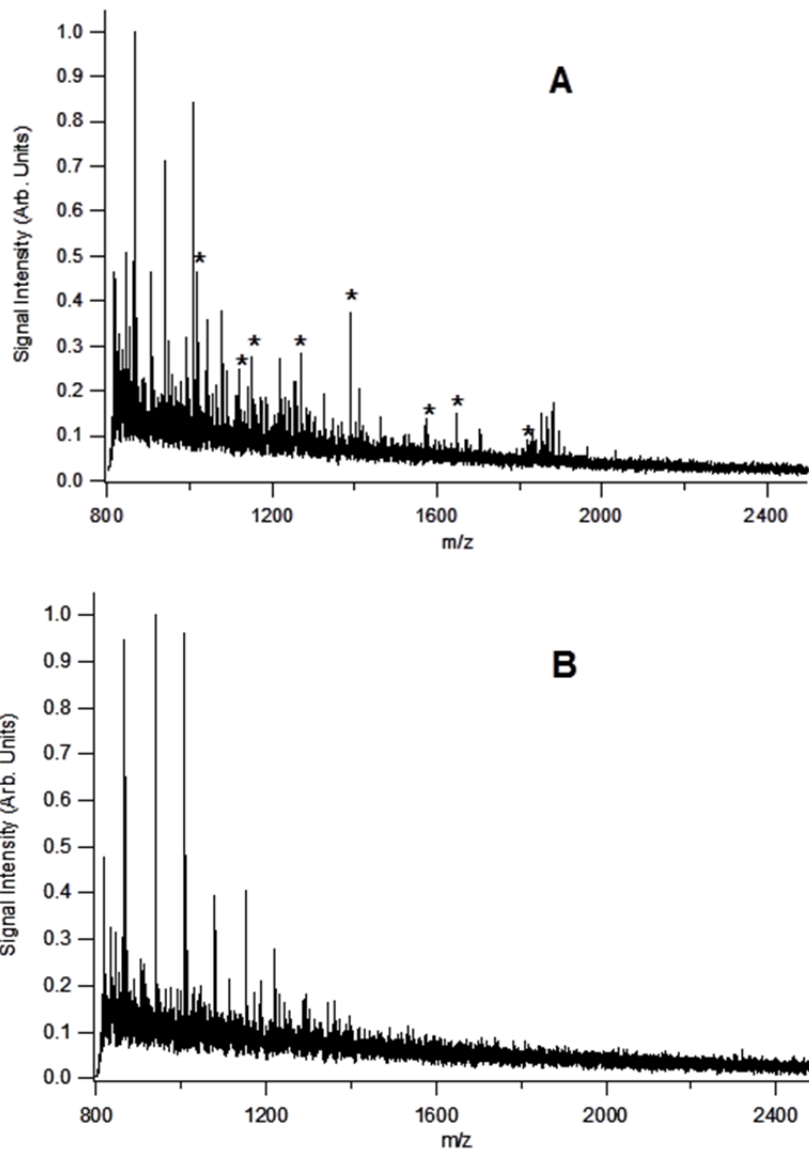


Figure 2.9 MALDI-TOF mass spectrum of a peptic digest of cytochrome c performed on a microfluidic chip. Peptides are indicated with a *. A sequence coverage of 87 % and 0% are obtained prior to (A) and after (B) applying thermal bonding conditions of 105°C respectively. The sequence coverage of 9% obtained after thermal bonding is from the presence of a single peptide being observed. The enzymatic microreactor bed had a diameter of 150 μm with a length of 80 mm. The residence time of the protein in the microreactor was 2.8 minutes.

A low temperature solvent assisted bonding method was developed to enable temperature sensitive enzymes (e.g. pepsin) to be patterned/immobilized on a PMMA substrate pre-bonding. In this case the bonding solvent must satisfy the following criteria. The solvent must enable to cover plate and substrate to be bond with sufficient strength that delamination does not occur and leakage from the micro channels is minimized. The solvent should not appreciably soften the substrate or cover plate surface so that micro channel integrity is preserved following bonding. The solvent should not permanently denature or deactivate the immobilized enzyme. We explored the use of a mixture of methanol and water as bonding solvents for PMMA since the solubility parameters of PMMA and methanol are 9.5 and 14.5 (cal/cm³)^{1/2}, respectively.⁸² The solubility parameter of the mixture of these two solvents should allow the controlled softening of only a few layers of polymer during bonding procedures. A large number of different bonding solvent compositions, temperature condition, applied forces and durations were explored using trial and error. We found a bonding solvent solution containing 97% methanol and 3% water produces a strong bond between two PMMA surfaces, when an embossing temperature of 35°C and 3000N of force is applied for one minute. When the substrate is immobilized with trypsin pre bond, a functioning trypsin microreactor results giving a sequence coverage of 99% for cytochrome c. Application of the same solvent bonding conditions to a pepsin immobilized substrate leads to no enzyme activity. Pepsin is pH labile and becomes irreversibly inactivated above a pH of 5. Acidification of the bonding solvent conditions with 3% of a 1 M sulphuric acid (i.e. 97% MeOH and 3% 1M H₂SO₄) was required to maintain enzyme activity. Sulfuric acid was chosen as it still allows for a strong bond while not disrupting the channel architecture of the micro reactor. HCl was employed but warping of the PMMA and destruction of the microchannel resulted. Furthermore even though pepsin has been reported to be temporarily inactivated at low organic

concentrations of a few percent,⁸³ the solvent bonding conditions used do not appear to permanently diminish the activity of immobilized pepsin. An 87% sequence coverage for cytochrome c is still obtained following low temperature solvent bonding (Figure 2.10).

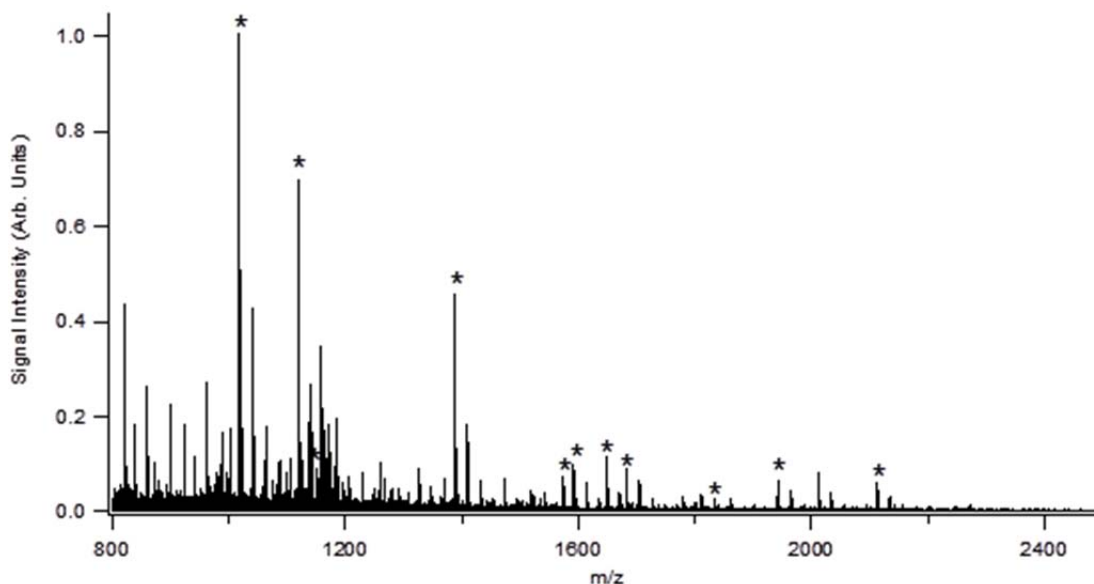


Figure 2.10 MALDI-TOF mass spectrum of an on-chip peptic digest of cytochrome c. Peptides are indicated with a *. A sequence coverage of 87% was obtained after low temperature solvent bonding of the microfluidic chip. The residence time of the protein in the microreactor was 21 seconds. The enzymatic microreactor bed had a diameter of 150 μm with a length of 10 mm.

Longevity experiments were performed on thermally bonded peptic microreactors where the immobilization of the enzyme was performed post bonding. The data for the longevity experiments is shown in Figure 2.6. The sequence coverage of the peptic microreactors (2.8 minute residence time) was stable for the first 2 weeks at 87% and then drops but remains at 54% after 4 weeks of testing. The specific peptides observed during the longevity studies are shown in Table 2.2.

Table 2.2 Cytochrome c peptide fragments observed from on chip pepsin digests performed over a period of a month on a microreactor with an 80 mm channel bed immobilized with pepsin using Method II. The residence time of the protein in the microreactor was 2.8 minutes. An * indicates that the peptide was observed in the MALDI mass spectrum. The maximum allowed missed cleavages was set to three, but several peptides with fewer missed cleavages were also observed.

Peptide Fragments		Day					
<i>m/z</i>	<i>Amino Acid Sequence</i>	1	2	7	14	21	28
960.5	NKNKGITW	*	*				*
1018.5	GRKTGQAPGF	*	*	*	*		*
1120.6	GDVEKGGKIF	*	*	*	*	*	
1150.6	IAYLKKATNE	*	*	*	*	*	*
1387.8	AGIKKKTEREDL	*	*	*	*	*	*
1571.9	AGIKKKTEREDLIA	*	*	*		*	*
1590.9	KGGKHKTGPNLHGLF	*	*	*	*		*
1647.9	IFAGIKKKTEREDL	*	*	*	*	*	*
1681.9	YLENPKKYIPGTKM	*	*	*			
1832.0	IFAGIKKKTEREDLIA	*	*	*		*	
1942.0	YLENPKKYIPGTKMIF	*	*	*	*	*	
2112.0	TYTDANKNKGITWKEETL	*	*	*	*		
% Sequence Coverage		87%	87%	87%	87%	46%	54%

Longevity experiments were also performed on tryptic microreactors fabricated by immobilizing enzymes to the PMMA surface post-bond. The data presented in Figure 2.7 shows that the enzyme activity, although decreasing, is maintained for a period of a month. This data is similar to the longevity data, in both initial sequence coverage and stability of the enzyme over time, obtained with tryptic microreactors where trypsin was immobilized pre-bond using the newly developed low temperature solvent bonding technique. This confirms that the solvent bonding process does not affect the activity of the enzyme which also validates that increased stability of immobilized enzymes to high concentrations of organic solvents. A trend that is observed is that although smaller diameter channels provide a higher initial sequence coverage,

the enzyme stability is greater for larger diameter channels as is observed in Figure 2.6 and Figure 2.7 which show that the enzyme activity decreases faster for smaller channel diameters which may be a result of autolysis of the enzyme when the enzyme is immobilized in close proximity.

A tensile tester was used to examine how much force is required to pull two pieces of PMMA apart post-bonding to determine the bond strengths associated with different bonding techniques. The bond strengths obtained for the solvent bonding protocol are greater than simple thermal bonding of PMMA substrates,⁸⁴ with solvent bonded unmodified PMMA having a tensile strength of $4.0 \times 10^3 \pm 0.8 \times 10^3$ kN/m². This tensile strength is such that a bonded substrate cannot be delaminated without causing damage to the chip. The acid modified PMMA microfluidic devices similar to those used for the enzyme immobilization experiments yielded a slightly lower tensile strength of $3.2 \times 10^3 \pm 0.6 \times 10^3$ kN/m² compared to that of unmodified PMMA which coincides with nanoindentation results (*vide supra*).

Nanoindentation experiments were performed to examine the relative effects of the acid treatment and solvent bonding conditions on the surface hardness of PMMA (Figure 2.4 and Figure 2.11). Unmodified PMMA showed a relatively constant hardness (0.4-1.3 GPa). Acid treatment and exposure to bonding solvent did not appreciably alter the hardness of PMMA below 60 nm which remains relatively constant at 0.3-0.5 GPa. This depth scale is significantly smaller than the depths associated with microfluidic applications/channels minimizing microchannel deformation associated with cover plate bonding.

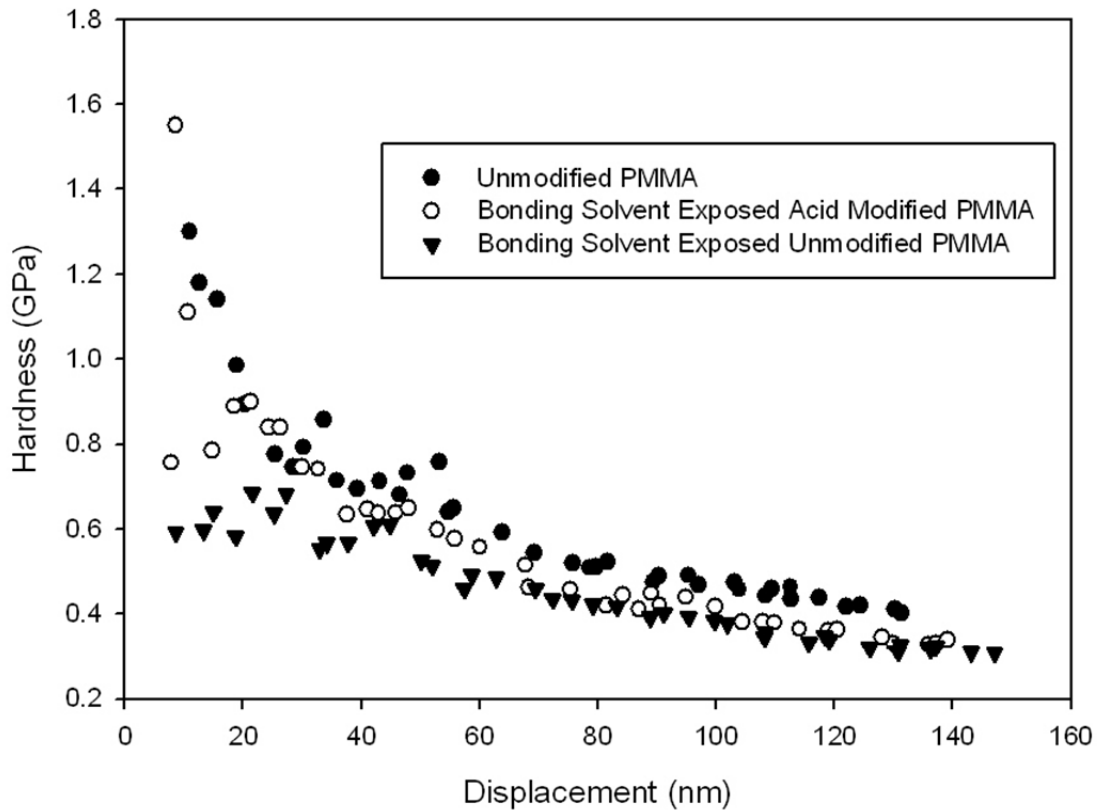


Figure 2.11 The hardness of unmodified and acid modified PMMA that has been treated in a 97% methanol and 3% water solution for 1 minute at 35°C.

The architecture/integrity of channels in microfluidic chips following solvent bonding was confirmed by a photo microscopy (data not shown). Unmodified PMMA that had been treated with a 97% MeOH/3% H₂O solution exhibited a decreased hardness of 0.6 GPa in comparison to unmodified PMMA or solvent exposed acid modified PMMA immediately at the surface but coincided with the other PMMA sample types at penetration depths greater than 50 nm. The decrease in hardness is as one would expect from the slight swelling of the surface due

to MeOH sorption resulting from similar solubility parameters. Exposure of the acid modified PMMA surface to the bonding solvent showed much more variability at the surface of the polymer with surface hardness readings typically between 0.7 and 1.6 GPa however the overall the hardness is slightly higher at the surface compared to the unmodified PMMA that had not been exposed to the bonding solvent. The reduction in softness for the solvent bonding solution exposed unmodified and acid modified PMMA presumably allows for an increase in the amount of intermixing of the surface layers of polymer during bonding, which may aid in the formation of the bond between the substrate and cover plate and explain the increased bond strength observed for solvent bonded PMMA. Furthermore the increased tensile strengths observed for the unmodified solvent exposed PMMA compared to the acid modified solvent exposed PMMA can also be attributed to the increased softness of the surface of the unmodified solvent exposed PMMA.

2.4.3 Channel Structure of PMMA Microfluidic Chips

Studies were conducted to determine which embossing conditions provide the best channel structure when using a 150 μm diameter fused silica capillary to fabricate a channel structure in PMMA. The photomicrographs taken of the fabricated thermally bonded microfluidic chips before and after bonding show that the optimal conditions for channel formation are when the fused silica capillary was embossed at 115°C for 600 seconds at a force of 2000 N. As shown in Figure 2.12, these conditions allowed for the entire capillary to be embossed into the substrate. This allowed for a more complete circular channel, in comparison to lower embossing temperatures which did not allow the capillary to depress completely into the PMMA substrate.

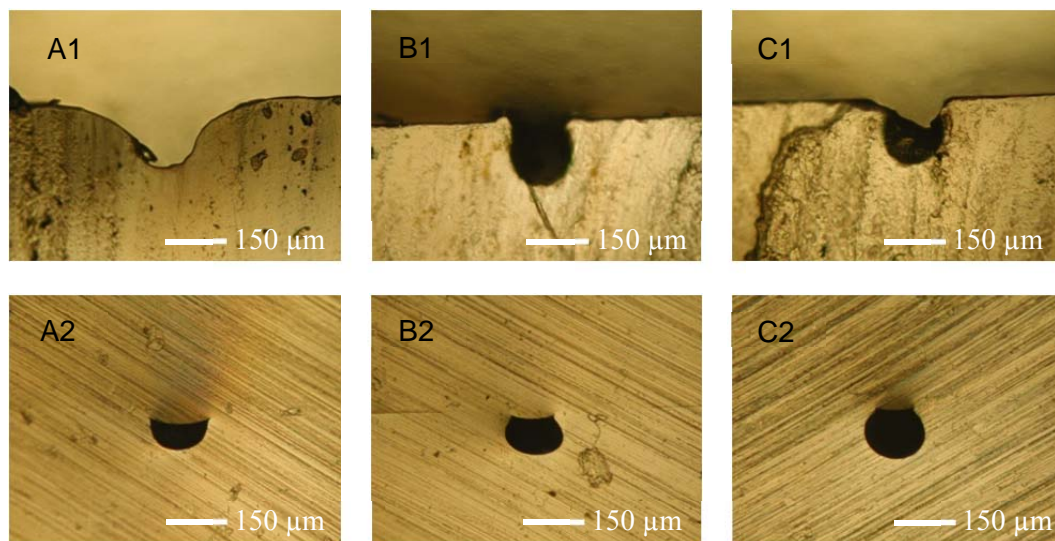


Figure 2.12 Photomicrographs of a cross-section of PMMA substrates embossed with a 150 μm diameter fused silica capillary before (1) and after (2) thermal bonding at 105°C. All embossing was with a force of 2000 N for 6000 seconds while the temperature was varied to be 105°C (A), 110°C (B) and 115°C (C). All photomicrographs are taken at a magnification of 100x.

The thermal bonding protocol at 105 °C maintains the channel structure as shown in Figure 2.12 and Figure 2.13. Microfluidic chips were also successfully fabricated with a smaller channel diameter of 50 μm as shown in Figure 2.14.

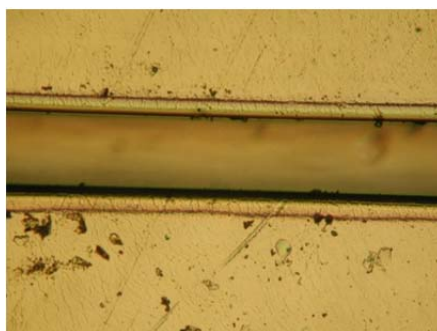


Figure 2.13 Photomicrograph at a 100 x magnification of the channel produced in a PMMA substrate embossed with a 150 μm diameter fused silica capillary.

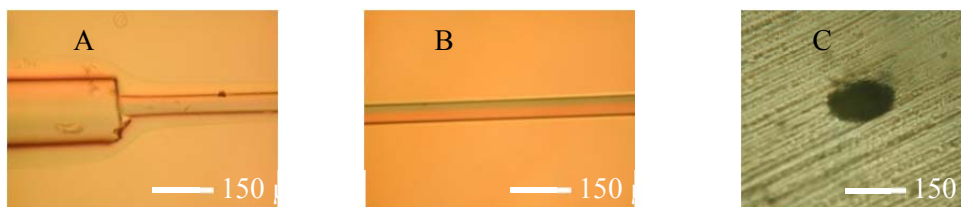


Figure 2.14 Photomicrographs at a 100 x magnification of the junction between the 150 μm and 50 μm channels (A) and the 50 μm channel produced in a PMMA substrate embossed with a combination of a 150 μm diameter fused silica capillary and a 50 μm stainless steel wire at 125°C using a force of 2000 N for 600 seconds. A photomicrograph (C) taken at a magnification of 500x of the cross-section of a 50 μm channel after thermal bonding of the PMMA substrate and cover plate at 105°C using a force of 500 N for 300 seconds.

2.5 Conclusions

It has been shown that the immobilization of enzyme on a PMMA microfluidic chip using WSC and NHS allows for a stable, effective enzyme microreactor to be fabricated. The ability to bond microchips at 35°C allows trypsin and pepsin to be pre-patterned on the microchip surface pre-bonding. This has been demonstrated for both trypsin and pepsin, the latter of which actually loses activity upon application of thermal bonding conditions of 105°C after pre-patterning. As such, the low temperature bonding method preserves channel architecture while maintaining enzyme activity by simply amending the pH conditions of the aqueous portion to match the optimal pH range of the chosen enzyme. This should allow these bonding conditions to be applicable to a wide range of enzymes. These results show promise for the facile fabrication of an integrated enzyme microreactor on chip as high sequence coverage is obtained for cytochrome c. This would allow protein identification to be performed quickly and easily, to aid in the efforts of medical and biological research.

Chapter 3

The Fabrication of Regenerable Porous Polymer Monolith Enzymatic Microreactors for On-line Digestions with ESI-MS

3.1 Summary

Fused-silica capillaries can be filled with porous polymer monolith (PPM) which can be used as a support to immobilize trypsin or pepsin. Unlike with microfluidic chips, various analysis steps cannot be performed on a single PPM-filled capillary, however, they can be coupled on-line to electrospray ionization mass spectrometry (ESI-MS). An enzyme immobilization method that allows enzymatic microreactors to be regenerated after the activity of immobilized enzyme has diminished has been developed. This method uses ester groups found in the PPM structure for the enzyme immobilization procedure. As there are always ester groups readily available if an acrylate monomer and crosslinker is chosen for PPM formation, the column can easily be regenerated. Both trypsin and pepsin, the latter being a temperature and pH sensitive enzyme, have been immobilized to the PPM surface. For tryptic microreactors, 33.1 mg of trypsin per gram of PPM was immobilized on the surface. The fabricated enzymatic microreactors have been used for both off-line and on-line digestions, with the pepsin column providing the most facile enzyme digestions as the acidic conditions necessary for peptic digestion of proteins are the same typically required for electrospray mass spectrometry analysis. An on-line tryptic digestion of cytochrome c yielded a sequence coverage of 38%. It has also been found that the addition of organic solvents does not hinder the digestion process but does facilitate generating a stable electrospray. Furthermore digestions of proteins without a denaturation step (i.e. non-reduced and non-alkylated) to cleave the disulfide linkages are still

possible on the PPM enzymatic columns; however, they provide a lower sequence coverage in comparison to proteins that have no disulfide linkages.

3.2 Introduction

Electrospray emitters are utilized to couple separation columns via a liquid junction to ESI-MS^{85, 86}, which permits nanoliquid chromatography (LC), capillary electrophoresis and electrochromatography to be interfaced with nanoelectrospray mass spectrometry. Pulled capillaries were introduced in the mid 1990s with spraying apertures down to 1-2 μm .⁸⁷ Small apertures are desirable as they provide higher ionization efficiency. The pulled capillaries can be positioned near the MS orifice to increase the number of ions entering the mass spectrometer, thereby resulting in an improved detection limit. The low flow rates also result in small sample consumption.

Although nanospray is more tolerant to interferences compared to conventional electrospray, sample pretreatment is often required prior to analysis.⁸⁸ As a result, separation, sample clean-up and/or preconcentration steps are applied for complex samples with low analyte concentrations. The removal of interferences is critical when small apertures are used since a problem commonly experienced with commercially available electrospray emitters is their tendency to clog. The use of C₁₈ beads in a packed column, held by a silica frit was the first procedure introduced to perform on-line sample clean-up.⁸⁹ The disadvantages of this technique are that bead packing is not always a trivial process and frits are needed to hold the beads in place. Depending on the analysis required, frits can become a hindrance since for chromatography they have shown to cause adsorption and band broadening.⁹⁰ Recently, our lab has demonstrated entrapping ODS-functionalized silica beads with PPM to fabricate a column that can be subsequently used as a nanoelectrospray emitter,⁹¹ a solid phase extraction column^{91, 92} and electrochromatography column.⁹²

PPM formation as described by Svec and Fréchet⁹³ has resulted in an alternative to packed columns. In this case, the PPM is directly attached to the wall of a fused-silica capillary and as a result no additional measures for polymer retention are needed. The polarity and the pore size of the PPM can be altered through choice of monomers⁹⁴ and porogenic solvent^{94, 95} respectively. When glycidyl methacrylate is used as a monomer, the use of longer chain alcohols results in smaller pore sizes as demonstrated by a higher back pressure and scanning electron microscope (SEM) images.⁹⁵ The hypothesis for the formation of smaller pore sizes is based on the solubility of the monomer in the porogenic solvent.⁹⁶ Glycidyl methacrylate is a non polar monomer and as such will be more soluble in non polar solvents (ie. higher chain alcohols). A denser polymer is formed when there is a higher solubility of the monomer in the porogenic solvent. As a result, smaller pores are formed in non polar solvents. With polar solvents, phase separation occurs quickly resulting in PPM with large pores where the porogenic solvent was present. Previous work by Yu *et al.*⁹⁴ has also shown that varying the amount of methanol in the porogenic solvent can effect pore sizes. By varying the methanol composition within any amount less than 60% can result in pore sizes of up to 8 μm , however higher percentages results in a large increase in pore sizes resulting in the loss of ability to finely tune the pore size. Fabricated PPM columns have been used for solid phase extraction,⁹⁷ reversed-phase chromatography,⁹⁸ ion exchange chromatography⁹⁹ and enzyme digestion.¹⁰⁰

Our lab has fabricated a nanospray emitter with PPM in a fused silica capillary^{17, 101} which provides a stable electrospray for a variety of flow rates in the range of 50-1000 nL/min. At flow rates of 100 nL/min or higher a single Taylor cone is observed. However, at lower flow rates a mist is sometimes observed (Figure 3.1) which is thought to be a result of multiple Taylor cones being emitted from the pores of the PPM. Minimal clogging is observed as a result of the multiple flow paths created from the pores generated within the PPM. Lectin affinity columns

have also been fabricated by immobilization of lectin on PPM and the columns utilized to perform solid phase extraction as well as to preconcentrate protein samples before mass spectrometry analysis.⁹⁵ PPM can also be formed within microfluidic chips which can then be used as emitters.^{102, 103}

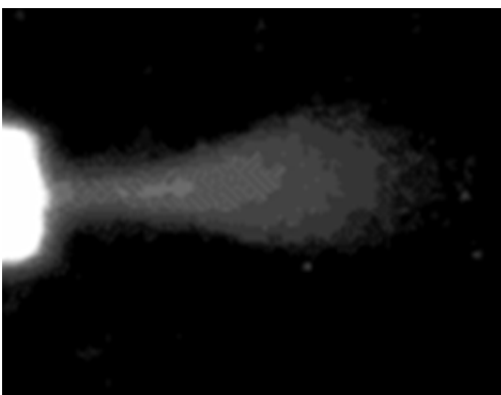


Figure 3.1 Photomicrograph showing the mist from the capillary instead of the Taylor cone at a flow rate of 100 nL/min¹⁰¹

Various methods have been developed to immobilize antibodies or proteins via an amine group to polymer surfaces generated from PPM formation with glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA). The monomer provides an epoxide end group which can be readily functionalized. Four main methods exist for protein or antibody immobilization which include the epoxide method,^{104, 105} 1,1'-carbonyldiimidazole (CDI) method,^{106, 107} Schiff base method¹⁰⁸ and the hydrazide method.¹⁰⁹ All methods take varying amounts of time anywhere from hours to days. However, each method results in different amounts of immobilization to the surface. The epoxide method provides the lowest amount of antfluoresceinisothiocyanate antibody on the PPM surface. In comparison to the epoxide method, the hydrazide method, the Schiff base method and the CDI method exhibited a 1.3-, 1.5- and 2.3-fold increase of antibody immobilized

to the PPM surface.¹¹⁰ Any of the methods described above can be used to immobilize trypsin on PPM.

Traditionally pepsin has not been as widely used as trypsin due to its drawback of unspecific cleavage sites, however it has becoming more popular in the literature due to it's potential to be used for on-line digests coupled to mass spectrometry analysis. Recently, PPM columns have been used as digestive microreactors with pepsin to digest various proteins.¹¹¹ In this work an efficient digestion has been provided for myoglobin, albumin and hemoglobin, however a separate nanoelectrospray emitter from New Objectives was used. Peptic columns have also been used to analyze membrane proteins with strong cation exchange and microflow reversed-phase liquid chromatography with electrospray ionization tandem mass spectrometry,¹¹² as well as for protein adducts of chemical warfare agents using a quadrupole time of flight (Q-TOF) hybrid tandem mass spectrometer.¹¹³ However, in all these cases the developed microreactors only serve as single functional device rather than the dual functionality proposed with using peptic columns as a digestion interface as well as a nanoelectrospray emitter that is not prone to clogging due to the multiple pores available.

A novel procedure to immobilize a variety of enzymes through their amine groups has been developed that does not rely on having a specific end group on the monomer used to fabricate the PPM. As long as there is an ester functional group somewhere within the PPM structure, the new immobilization procedure is successful. The immobilization mechanism is based on the acid hydrolysis of esters to form carboxylic acid groups on the surface which can then be used to covalently attach enzymes. Also as a result of the properties of the PPM columns as described above, such PPM columns can be used as enzymatic microreactors through enzyme immobilization and as well as function as electrospray emitters. Thus the generated enzymatic microreactors using a novel enzyme immobilization procedure will be used to perform an on-line

enzymatic digest. Furthermore, the newly developed enzyme immobilization procedure allows for the enzymatic column to be regenerated once the immobilized enzyme has lost its activity thereby extending the lifetime of the fabricated enzymatic column.

3.3 Experimental

3.3.1 Materials and Chemicals

UV transparent fused silica capillary with an outer diameter of 360 μm and an inner diameter of 75 μm was purchased from PolyMicro Technologies (Phoenix, AZ, USA). *N*-hydroxysuccinimide (NHS), and trifluoroacetic acid (TFA) were purchased from Aldrich Chemicals (Milwaukee, WI, USA). *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (WSC) and α -cyano-4-hydroxycinnamic acid (HCCA) were acquired from Fluka (Buchs, Switzerland). Trypsin (bovine, 13000 units/mg), pepsin (porcine, 3600 units/mg), serum albumin (bovine, >96%) (BSA), insulin (bovine pancreas), myoglobin (horse skeletal muscle), cytochrome c (horse heart), sodium bicarbonate, ammonium bicarbonate, 2,5-dihydroxybenzoic acid (DHB), sodium chloride (ACS reagent grade), magnesium dichloride hexahydrate (MgCl_2), calcium chloride dehydrate (CaCl_2) (ACS reagent grade), butyl acrylate, ethylene dimethacrylate (EDMA), glycidyl methacrylate (GMA), 3-(trimethoxysilyl)propyl methacrylate (z-6030), acetone (ACS reagent grade) 1-octanol, 1-decanol, 1-dodecanol, copper (II) sulfate, sodium carbonate, sodium tartrate, benzoin methyl ether, sodium cyanoborohydride, sodium periodate, biconchonic acid (BCA) and methyl α -D-mannopyranoside were obtained from Sigma (Oakville, ON, Canada). Acetonitrile (ACN) (HPLC grade), 2-propanol (ACS reagent grade), methanol (MeOH) (ACS reagent grade, Ultrapure grade) and TRIS·HCl, were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sodium hydroxide pellets (NaOH), sodium acetate, sulfuric acid (H_2SO_4) (ACS reagent grade), phosphoric acid (ACS reagent grade), hydrochloric acid (HCl) (Certified

ACS Plus) and glacial acetic acid (ACS reagent grade) were acquired from Fisher Scientific (Nepean, ON, Canada). All reagents were used without purification. Deionized water (18.2 MΩ) was purified using a MilliQ apparatus (Millipore, Bedford, MA, USA). C8 spherical reversed phase silica gel beads (3 μm particle size, 120 Å pore size) were purchased from SiliCycle (Quebec City, QC, Canada).

3.3.2 PPM Formation

UV transparent fused silica capillary was first pretreated by flushing the capillary with 1.0 M NaOH at a flow rate of 1 μL/min for 30 minutes using a syringe pump. The capillary was then rinsed with 0.10 M HCl at 1 μL/min for 30 minutes, followed by water for 30 minutes at 1 μL/min. The final step involved flushing the capillary overnight at a flow rate of 0.5 μL/min with a solution of 50% (v/v) Z-6030 in acetone as shown in Figure 3.2 to allow an anchoring site for the subsequent PPM formation.

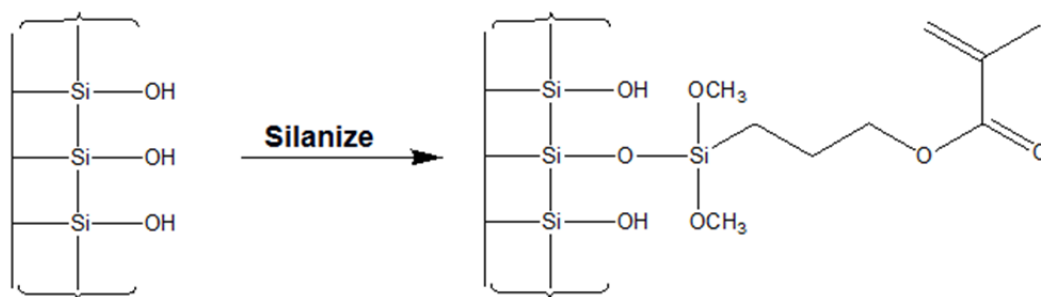


Figure 3.2 The walls of the capillary are treated with z-6030 to provide an anchoring site for the PPM formation.

A PPM solution was prepared by weight percent and consisted of the following: 39.6% monomer solution (23.6% GMA or butyl acrylate and 16% EDMA), 60% porogenic solvent and 0.4 % benzoin methyl ether as a photoinitiator (see Figure 3.3).

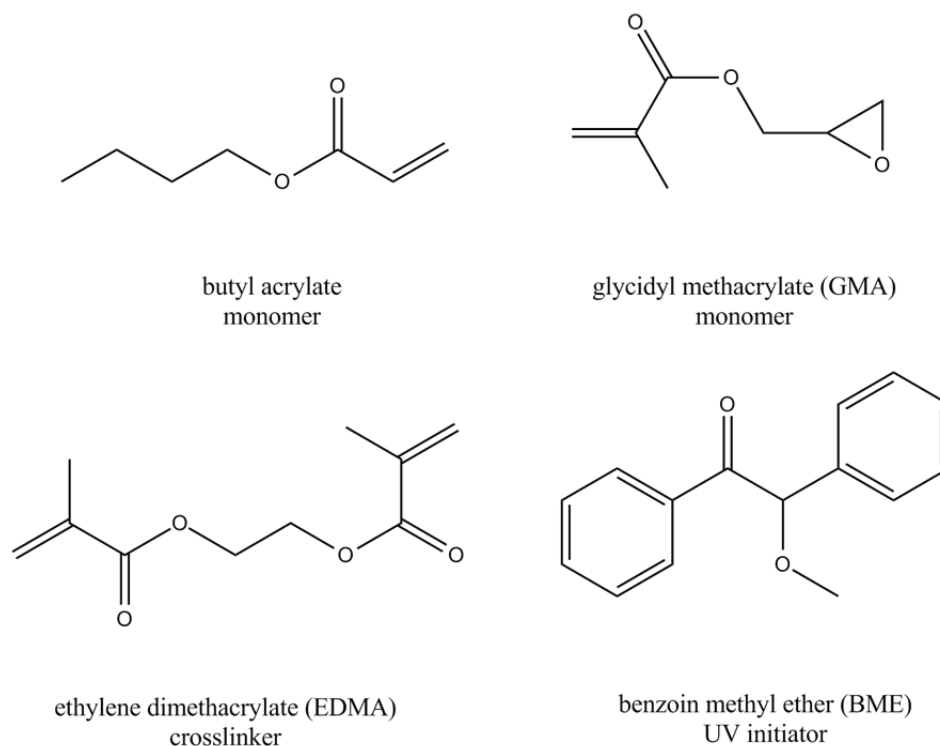


Figure 3.3 Compounds used in PPM formation.

Traditionally the porogenic solvent consisted of 25% MeOH and 75% dodecanol but columns were also fabricated by replacing the dodecanol with either octanol or decanol or even with a 1:1 mixture of MeOH to dodecanol. The resulting solution was then degassed with nitrogen for 5 minutes. The capillary was filled with PPM solution and the ends of the capillary were masked off to selectively pattern a 2.5 cm long PPM column as shown in Figure 3.4 and the mechanism for PPM formation is shown in Figure 3.5. The capillary was then either irradiated at 365 nm for 15 minutes or 254 nm for 5 minutes, both at distance of 4.5 cm. The resulting PPM column was then flushed at 5 $\mu\text{L}/\text{min}$ with water to remove any unpolymerized PPM solution from the masked region. The formation of the PPM was then confirmed under the microscope. The PPM columns were then either used as electrospray emitters or enzymatic microreactors when they were functionalized with enzymes.

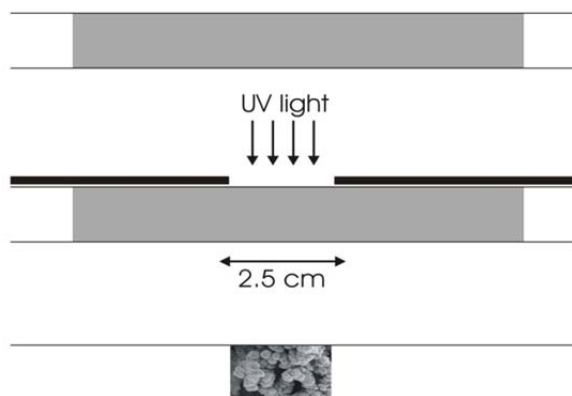


Figure 3.4 The capillary is filled with solution and then masked off to only expose a 2.5 cm region of the solution to UV irradiation. The PPM is then formed by irradiating the area with UV light for either 15 minutes if a wavelength of 365 nm was used or 5 minutes if a wavelength of 254 nm was used. Upon completion a 2.5 cm PPM column is fabricated.

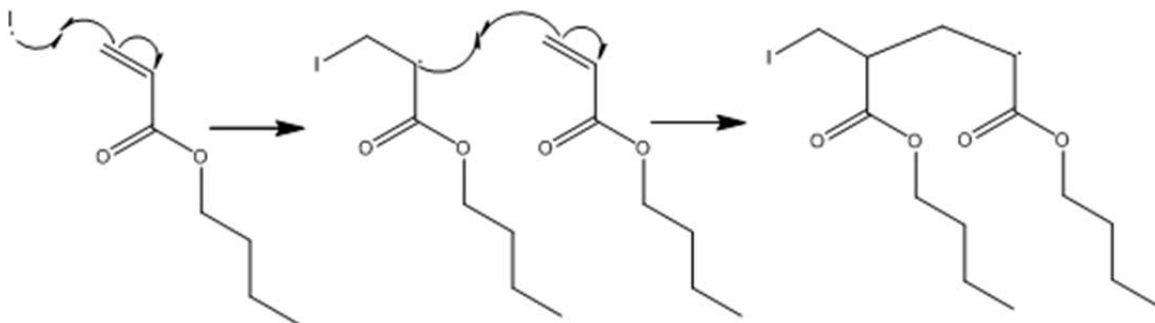


Figure 3.5 The mechanism for PPM formation. The initiator is represented by I.

3.3.3 Enzyme Immobilization on PPM Columns

For PPM columns fabricated from GMA/EDMA in a porogenic solvent of 25% MeOH and 75% dodecanol, the Schiff base mechanism¹⁰⁸ as shown in Figure 3.6 could be utilized to immobilize enzyme on the PPM surface. This method was only applicable to these specific

columns as an epoxide group was required for the mechanism. The PPM column was initially rinsed with water for 30 mins prior to the immobilization procedure. Then using a syringe pump the column was flushed with a 1 M H_2SO_4 solution overnight at a flow rate of $0.5 \mu\text{L}/\text{min}$. Following this step the column was rinsed with water for 30 mins. Next a 0.1 M sodium periodate solution was flushed through the PPM column for 2 hours at a flow rate of $0.5 \mu\text{L}/\text{min}$. Next a 5 mg/mL solution of trypsin in 0.1 M sodium acetate (pH 6.4) with 1 mM CaCl_2 , 1 mM MgCl_2 , 0.1 M methyl α -D-mannopyranoside and 50 mM sodium cyanoborohydride was prepared. This solution was then flushed through the PPM column overnight at a flow rate of $0.5 \mu\text{L}/\text{min}$. The final step of the immobilization procedure involved flushing the PPM column with a solution of 0.4 M TRIS-HCl (pH 7.2) and 50 mM sodium cyanoborohydride at a flow rate of $0.5 \mu\text{L}/\text{min}$ for 3 hours. This step was done to terminate any unreacted aldehydes. The resulting tryptic column was then rinsed with water, sealed and stored in the refrigerator at 4°C .

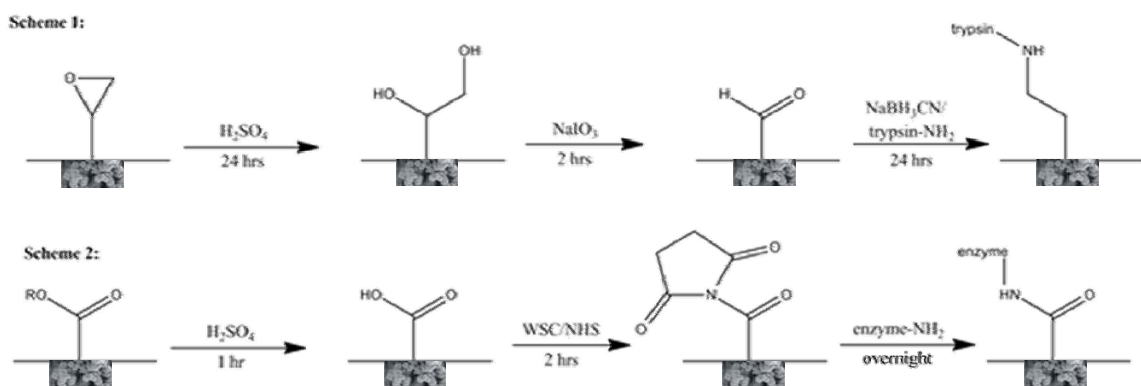


Figure 3.6 Scheme 1 shows the Schiff base method used to immobilize trypsin on PPM columns using GMA as a monomer which bears an epoxide group. Scheme 2 shows the novel enzyme immobilization method that has been developed to immobilize an enzyme on a PPM surface comprised of an ester group functionality.

Alternatively as shown in Figure 3.6, a novel immobilization method for PPM could be utilized on any PPM columns that had ester functional groups, including GMA and butyl acrylate. For this method, the first step involved flushing the column with a 1 M H₂SO₄ solution at a flow rate of 0.5 µL/min for 1 hour. The column was flushed with water for 1 hour and then a 1 mg/mL WSC and 0.1 mg/mL NHS solution was passed through the column at a flow rate of 0.5 µL/min for 2 hours. Once again the column was flushed with water for 1 hour followed by flushing with a 10 mg/mL trypsin solution in 50 mM ammonium bicarbonate, 10 mM magnesium chloride and 10 mM sodium chloride or a 5 mg/mL pepsin solution in 1% acetic acid overnight at a flow rate 0.5 µL/min. The final step involved removing any unreacted enzyme by either flushing the PPM column with a 50 mM ammonium bicarbonate solution for a tryptic column or a 1 % acetic acid solution for a peptic column. The columns were then sealed and stored in the refrigerator. This new method was tested using various iterations of the immobilization procedure to fabricate the enzymatic columns. The columns were then tested for enzymatic activity using a cytochrome c digest which was later analyzed on a QSTAR XL Qq TOF MS instrument equipped with a MALDI source (Applied Biosystems, MDS SCIEX, Concorde, ON, Canada) using a DHB dried drop method¹⁹ for the matrix.

3.3.4 Quantification of Enzyme on the Surface

The amount of trypsin immobilized on the PPM column was determined using a bicinchoninic acid protein assay.¹¹⁴ Two assay reagents were prepared. Reagent A consisted of 1 g bicinchoninic acid, 2 g sodium carbonate, 160 mg sodium tartrate, 400 mg sodium hydroxide and 950 mg sodium bicarbonate in 100 mL of water. Reagent B was an aqueous solution of copper sulfate (0.04 g/mL). The working reagent was prepared by mixing reagent A and B in a ratio of 100:2 (v/v). Instead of forming PPM in capillaries the PPM was formed in Pasteur pipettes so that it could be easily removed later for spectroscopic testing. As such the PPM was

not anchored to the glass surface as was done to fabricate enzymatic microreactors as the tapered pipette tip holds the PPM in place. In this case the PPM columns were formed by irradiation at 365 nm for 30 minutes. The solutions for washing and immobilizing trypsin to the surface were passed through the Pasteur pipette via nitrogen pressure. After the immobilization procedure was complete, the PPM columns were rinsed with 50 mM ammonium bicarbonate and then dried under nitrogen. The PPM was removed from the Pasteur pipette by back flushing the pipette with nitrogen from the nitrogen gas line. The PPM was then ground into a fine powder and dried under vacuum for a period of 24 hours. Next 50 mg of the dried PPM was added to 2 mL of the working reagent and allowed to react at room temperature for 2 hours. The resulting solution was then centrifuged for 5 minutes and then 1 mL of the supernatant was diluted with 2 mL of water. The absorption of the solution was measured at 560 nm using a USB-2000 CCD-based spectrophotometer (Ocean Optics, Dunedin, FL, USA) with a deuterium/tungsten-halogen light was used to take UV-Vis measurements for the bicinchoninic acid assay. A calibration curve of trypsin in solution was used to determine the amount of trypsin immobilized on the PPM surface. A solution of 50 mg of PPM that had not undergone the immobilization procedure in 2 mL of the working reagent was used as a blank.

3.3.5 Enzymatic Digestions

Enzymatic digest for tryptic columns were performed with a solution of 10 $\mu\text{g/mL}$ of protein in a 9:1 mixture of 50 mM ammonium bicarbonate and MeOH. For peptic digest a 10 $\mu\text{g/mL}$ protein solution was prepared in a 9:1 mixture of 1% acetic acid solution and MeOH to have the acidic environment required for the optimal activity of pepsin. All off-line digestions were done at a flow rate of 0.5 $\mu\text{L/min}$ using a syringe pump. For all protein digests, no reduction or alkylation of the protein was done prior to digest. The off-line digestion was collected in siliconized Eppendorf vials. After the digestion, PPM columns were flushed with either 50 mM

ammonium bicarbonate or 1% acetic acid depending on whether a tryptic or peptic column was used for the digestion.

The longevity experiments for both the peptic and tryptic microreactors were analyzed using a Voyager DE PROMALDI time of flight mass spectrometer (Applied Biosystems, Foster City, CA, USA). A three layer DHB method was used for the analysis. The first layer for the matrix method consisted of 20 mg/mL DHB in 70 % ACN and 0.1 % TFA. The second layer was composed of 20 mg/mL DHB in 50% ACN and 2% phosphoric acid. The third layer was the sample solution being analyzed. Each layer consisted of a volume of 0.5 μ L of each solution and was spotted on the MALDI target plate and allowed to dry before the next layer was applied. Prior to spotting the tryptic digest solutions on the MALDI target plate the solution were acidified with 1% trifluoroacetic acid. At least three spots were analyzed for each protein digest performed.

ESI-MS analysis of both on-line and off-line digestions were performed using a API3000 triple quadrupole mass spectrometer (MDS Sciex, Aurora, ON, Canada) in positive mode with an applied voltage of 3200V. For the on-line peptic digestions the solutions could be directly analyzed while the on-line tryptic digestions had to be acidified with 1.3% acetic acid using a syringe pump at 1 μ L/min prior to the solution entering an electrospray emitter. As a result of this, the peptic microreactors had a dual function as an emitter and a digest column. On the other hand, with trypsin the enzymatic microreactor had to be coupled to another PPM column that was solely used as an electrospray emitter. For both peptic and tryptic on-line digests, the protein solutions were prepared the same way as for the off-line digests and the protein solution was delivered continuously to the enzymatic column via an Eksigent nano-liquid chromatography (nano-LC) pump (Dublin, CA, USA) at a flow rate of 200nL/min. Off-line peptic digestions were analyzed via ESI-MS by taking the collected digestions solution obtained from a PPM enzyme

column and then directly infusing the peptide solution into the ESI-MS at a flow rate of 300 nL/min using a PPM column as an emitter.

3.3.6 Protein Separations

The column used for separation was a 6 cm 3 μ M particle size C8 reversed phase silica gel bead column that was fabricated by packing the beads up against a 1 cm PPM frit. A slurry of C8 beads was prepared by making a slurry of 6 mg of beads in ACN and the packing was done under pressure using a syringe pump placed vertically to have the assistance of gravity to facilitate packing. To ensure efficient packing, the final column was flushed using a HPLC pump. The set-up for the on-line separation involved a microtee joining the C8 separation column to a 2.5 cm long PPM column used as a nanoelectrospray emitter with voltage being applied at the bottom as shown in Figure 3.7A and Figure 3.7B. The separation column was hooked up to an Eksigent nano-LC pump. All mass spectra were obtained using a API3000 triple quadrupole mass spectrometer (MDS Sciex, Aurora, ON, Canada) in positive mode with an applied voltage of 3200V.

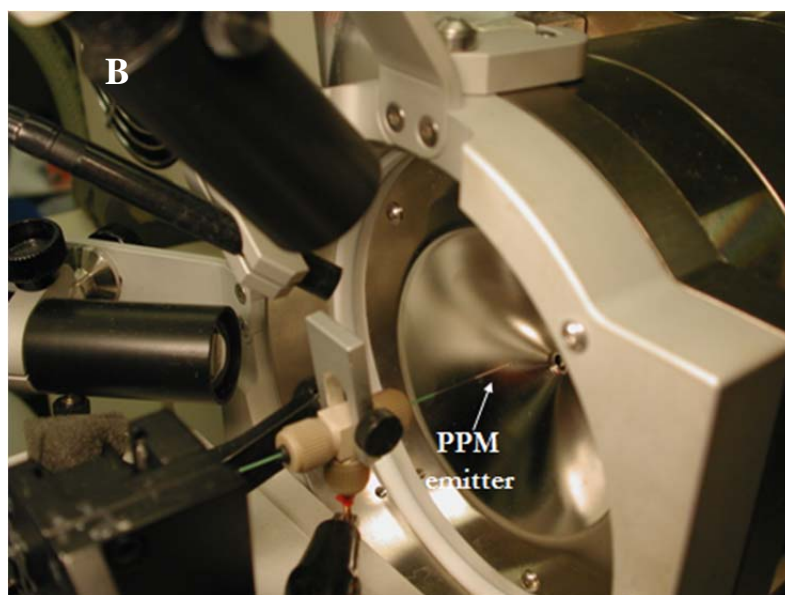
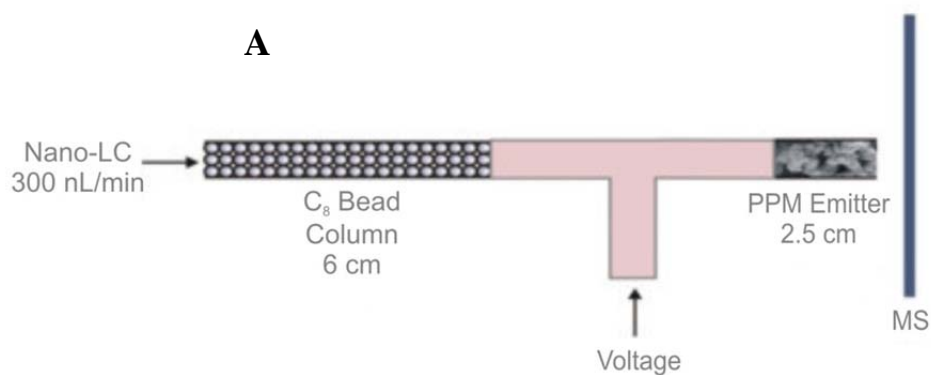


Figure 3.7 A schematic of the on-line protein separation set-up (A) and a photograph of the PPM emitter positioned near the MS orifice (B).

A protein solution of 10 μ M each of insulin, cytochrome c and myoglobin was prepared in water acidified with 1.25% formic acid. The flow rate of the nano-LC pump was 300 nL/min with an injection of 1500 nL. A gradient elution method was used for the separation and consisted of a mobile phase of water and ACN as the organic phase. The separation method for insulin, cytochrome c and myoglobin is shown in Table 3.1.

Table 3.1 Gradient elution profile for a sample containing 10 μ M of insulin, cytochrome c and myoglobin. The flow rate was 300 nL/min with an injection volume of 1500 nL.

Time (mins)	% H ₂ O	% ACN
0-2	96	4
2-12	50	50
13-18	10	90
18-20	96	4

3.3.7 Regeneration of Enzymatic Columns

A peptic PPM column was fabricated and tested for its activity via a cytochrome c digest analyzed by MALDI. For these experiments a QSTAR XL Qq TOF MS instrument equipped with a MALDI source (Applied Biosystems, MDS SCIEX, Concord, ON, Canada) was used. The matrix method employed was a DHB dried drop method.¹⁹ After the peptic column was deemed to be active, pepsin was denatured by flushing the peptic column with a 1.0 M NaOH solution at 5 μ L/min for 1 hour. Pepsin is irreversibly denatured under basic conditions. After this step the column was flushed with a 1% acetic acid solution overnight to remove the remaining basic solution. The column was then re-tested with a cytochrome c digest to confirm that the activity of pepsin had been destroyed. The column was then regenerated with trypsin by using the novel enzymatic immobilization procedure. The regenerated activity of the tryptic column was confirmed with a cytochrome c digest.

3.3.8 Data Analysis

The peptides obtained from tryptic digests, both off-line and on-line, of the various proteins studied were matched to theoretical peptide masses obtained from Protein Prospector (<http://www.prospector.ucsf.edu>) using a maximum of three missed cleavages and allowing for the variable modification of methionine oxidation. Different lists were generated based on whether or not the peptide analysis was done by MALDI-MS or ESI-MS. For tryptic digests MASCOT peptide mass fingerprint was used to calculate the percent sequence coverage obtained for the given protein. For peptic digests, the sequence coverage was calculated manually as pepsin can non-specifically cleave. Thus the sequence coverage was obtained by dividing the total number of amino acid residues observed by the total number of amino acid residues in the protein. For this calculation when MALDI-MS was used both the theoretical peptide peaks obtained from Protein prospector and those experimentally determined by Bayraktaret *al.*⁷⁵ were used to calculate the sequence coverage in this case. For ESI-MS the analysis was more complicated and although the theoretical peptide peaks from Protein prospector were used, all the possible non-specific cleaved peptide peaks were calculated manually by performing blank runs and then subtracting them from the mass spectra of the protein digest to obtain the peptide peaks. These peaks were then searched across the protein sequence for a match using the PAWS freeware. As a result of no proteins being denatured prior to analysis, insulin was the most complicated protein to be analyzed as the PAWS program could not account for the disulfide linkages binding insulin chains A and B together as shown in Figure 3.8. As such, the peptides around these disulfide linkages were calculated manually for the singly charged peptides up to a m/z of 1000 Da.



Figure 3.8 The structure of insulin. Chain A is represented in blue while Chain B is in green. The two insulin chains are held together by intra-strand and inter-strand disulfide linkages indicated by the brown lines.

3.4 Results and Discussion

3.4.1 PPM Formation

UV transparent capillaries with a 75 μm i.d. and 360 μm o.d. were pretreated with z-6030 (Figure 3.2) to provide an anchoring site for PPM formation. The solution used to fabricate PPM columns contains a mixture of porogenic solvent and monomer/crosslinker. The monomer solution used for PPM formation was composed of EDMA and GMA (Figure 3.3). The polymerization was UV-activated at a wavelength of 365 nm and involved using benzoin methyl ether as an initiator and proceeded via the mechanism shown in Figure 3.5. The resulting PPM solution was then passed through the capillary and a 2.5 cm section was irradiated with a UV lamp for 15 minutes as shown in Figure 3.4. The resulting PPM can be seen in a SEM image (Figure 3.9) with a resulting pore size of approximately 2 μm . The pore size was determined by measuring the pore size with respect to the scale bar displayed in the SEM image.

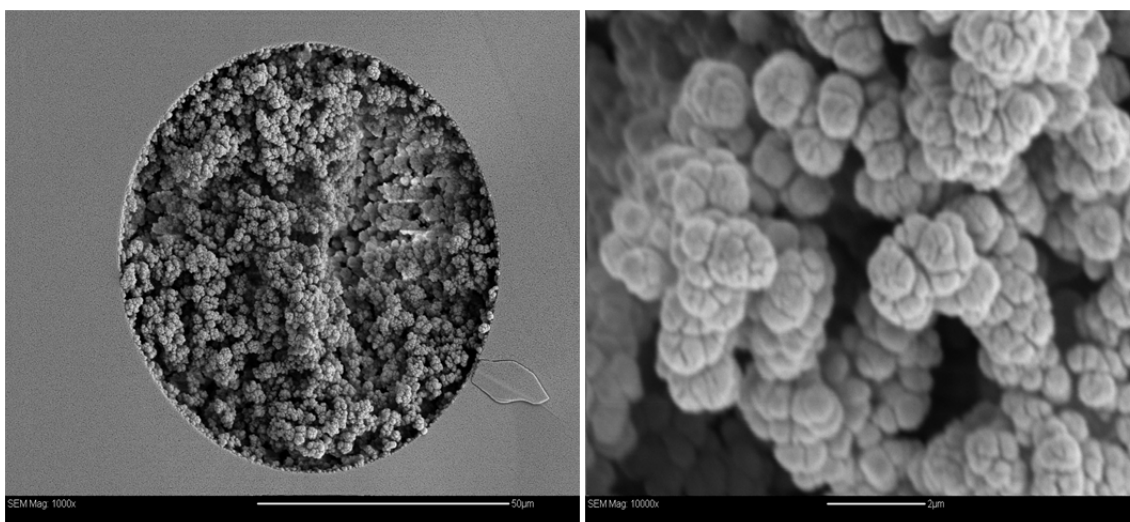


Figure 3.9 A scanning electron microscope image taken of a PPM filled capillary fabricated with a porogenic solvent composed of 50% methanol and 50% dodecanol at a magnification of 1000 x (left) and 10,000x (right).

Various compositions of porogenic solvent were tested. Varying the percentage of methanol, either 50% or 25%, as well as the use of either a short chain or long chain alcohol allows for the pore size to be controlled. Previous work from Bedair *et al.*⁹⁵ showed that a higher back pressure was obtained for a porogenic solution composed of dodecanol versus octanol which is an indication that smaller pore sizes are obtained with dodecanol. Small pore sizes are an important feature of the PPM column as they provide the largest surface-to-volume ratio which is key for their subsequent application as enzymatic columns. As discussed previously, the smaller pore sizes is thought to be a result of the solubility of the monomer in the porogenic solvent.⁹⁶ As GMA is non polar it will be more soluble in dodecanol than octanol. The work of Bedair *et al.*⁹⁵ involved immobilizing PPM with lectin using a Schiff base method, which proceeds through a similar mechanism to trypsin. PPM's prepared with a porogenic solvent composed of 25%

methanol and 75% dodecanol showed the most lectin immobilized to the surface and also offered the most stable electrospray when the PPM was used as an emitter. Consequently a porogenic solvent mixture was chosen to allow for the smallest pore size. To confirm that the smallest pore size would provide the most efficient digestion, PPM columns were fabricated using a porogenic solvent with 50% methanol with either 50% dodecanol, decanol or octanol. The highest sequence coverage for cytochrome c was obtained from columns fabricated with dodecanol as a porogenic solvent while columns fabricated with octanol provided the lowest sequence coverage. The sequence coverage for a cytochrome c digest for columns fabricated with octanol, decanol and dodecanol as a porogenic solvent was 46%, 48% and 71% respectively. This confirms that the highest sequence coverage is obtained when a PPM column with small pores is used. For the dual purpose of using PPM columns both as electrospray emitters and as enzymatic microreactors, the small pore size is essential for generating a stable electrospray as well as for achieving the largest surface-to-volume ratio. The latter is required for immobilizing a maximum amount of enzyme on the surface and also for increasing the likelihood of an efficient digestion of a protein as it passes through the column. The efficiency of the digestion is directly proportional to the ability of the protein to come into contact with the enzyme on the surface. Although an increased concentration of methanol has been shown to form larger pore sizes,⁹⁴ it was found that 50% methanol in comparison to 25% methanol provided the most reproducible PPM columns. Furthermore, the resulting columns provided the most efficient digestions as quantified by percent sequence coverage. As such PPM formation involved a 1:1 mixture of dodecanol and methanol as the porogenic solvent.

3.4.2 Immobilization of Enzymes on PPM Columns

Currently, the most widely used method for immobilization of enzymes on a PPM surface involves fabrication of a PPM column containing epoxide groups which is achieved by using a

monomer such as glycidyl methacrylate. The enzyme is then immobilized to the surface using a Schiff base method.¹⁰⁸ First the epoxide group is hydrolyzed to form a diol group followed by a subsequent oxidation using sodium periodate to form an aldehyde as shown in Figure 3.5. An enzyme of interest can then be attached to the surface via reductive amination. However, when this method was used, a digestion of cytochrome c on the tryptic PPM column yielded only a couple of peptides (28% sequence coverage) indicating that the amount of enzyme immobilized on the PPM surface was minimal.

A new method has been developed that involves PPM composed of acrylates. This method is much less time consuming, requires only one day versus two days, and also uses milder chemicals. The acrylate contains an ester group that can be hydrolyzed to form a carboxylic acid on the surface as shown in Figure 3.5. Subsequently the carboxylic acid group can be activated using WSC/NHS which is then followed by enzyme attachment via its amine group (Figure 3.5). Although only WSC could have been used, a combination of WSC and NHS is preferred as NHS has been shown to increase the yield and stability of the reaction intermediate, which in turn can increase the lifetime of the enzyme microreactors.⁷⁶ This effect has already been demonstrated in Chapter 2 with enzymatic microfluidic chips and as such was repeated for enzymatic PPM columns. To test this mechanism and to ensure that the enzyme was not merely adsorbing to the PPM surface, PPM columns were fabricated that omitted one or two steps of the immobilization procedure: enzyme treatment only, acid treatment followed by enzyme treatment and WSC/NHS treatment followed by enzyme treatment. For trypsin, none of these combinations yielded any tryptic peptides (see Figure 3.10) rather peptides were only observed when all three steps of the immobilization procedure were followed (see Figure 3.11).

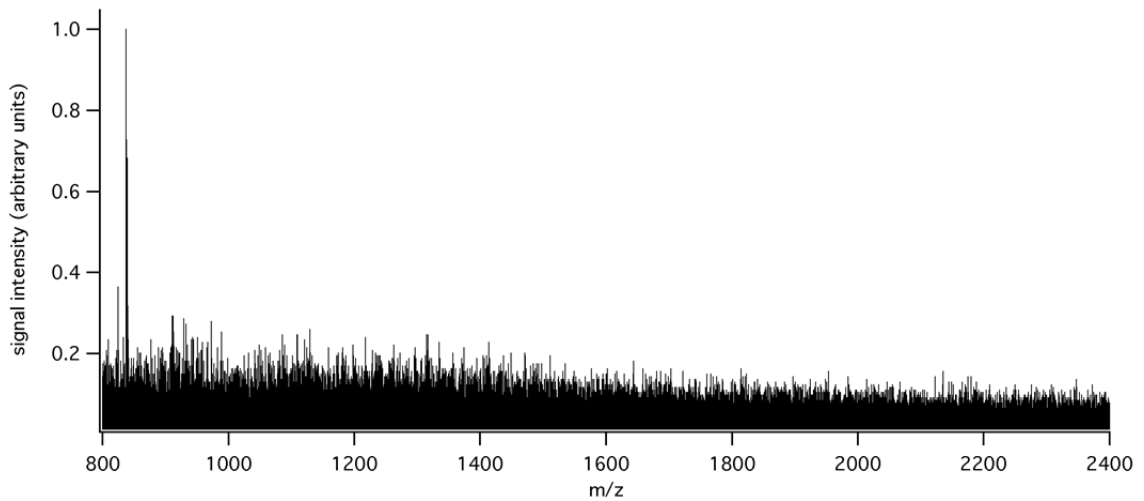


Figure 3.10 The MALDI-TOF spectrum of the trypsin control test, showing 0% sequence coverage which indicates that trypsin is not adsorbing to the PPM column.

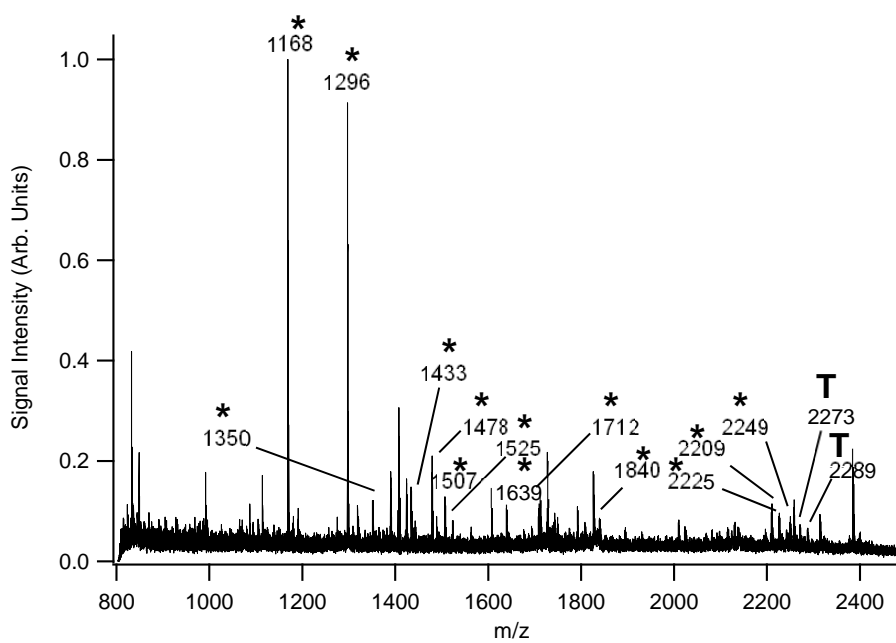


Figure 3.11 MALDI-TOF mass spectrum of cytochrome c digest performed on a PPM filled capillary treated with trypsin. A 77 % sequence coverage was obtained. Tryptic peptides are indicated with a star. Autodigestion peaks of trypsin are indicated with a T.

On the other hand, when the same experiments were repeated with pepsin it was observed that pepsin does adsorb to the surface as well as covalently attaching as shown in Figure 3.12. The differences regarding these two forms of treatment for pepsin was demonstrated in the longevity experiments which will be discussed in more detail, but show that the longevity of the enzyme is decreased when the enzyme is adsorbed to the surface rather than covalently attached.

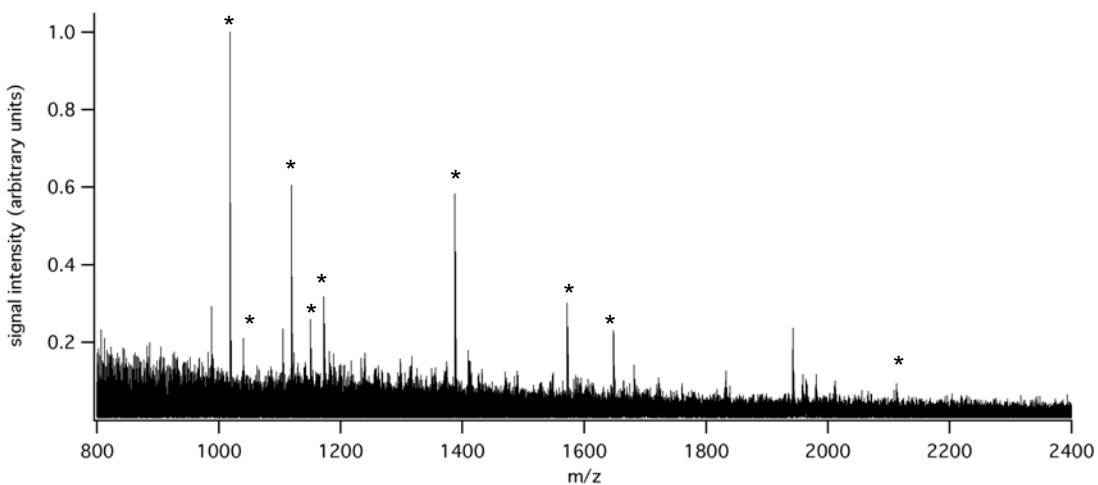


Figure 3.12 The MALDI-TOF spectrum of the pepsin control test, showing 63% sequence coverage which indicates that pepsin is adsorbing to the PPM column. The stars denote digested peptide fragments.

To further test the mechanism to see whether carboxylic acid groups were being formed from the ester groups, PPM was formed using butyl acrylate as a monomer with all the other reaction conditions staying the same. Butyl acrylate has no epoxide group and as a result the only method for covalent attachment of trypsin on the monomer is through formation of carboxylic acid groups on the surface. Results show that trypsin is covalently bound to the butyl acrylate surface as is indicated by the mass spectra in Figure 3.13 (it has already been demonstrated that trypsin cannot adsorb to the surface). These results suggest that any monomer with an ester group

could be used for the immobilization of trypsin. The variability in selection of monomer provides many possibilities in comparison to previously published protocols that rely on the presence of an epoxide group on the monomer for trypsin immobilization. Aside from the wide choice in monomer functionality, the new immobilization method has the advantage that it requires much less time, one day, compared to traditional methods such as the Schiff base method which take 2 days and the CDI method which takes approximately 6 days.

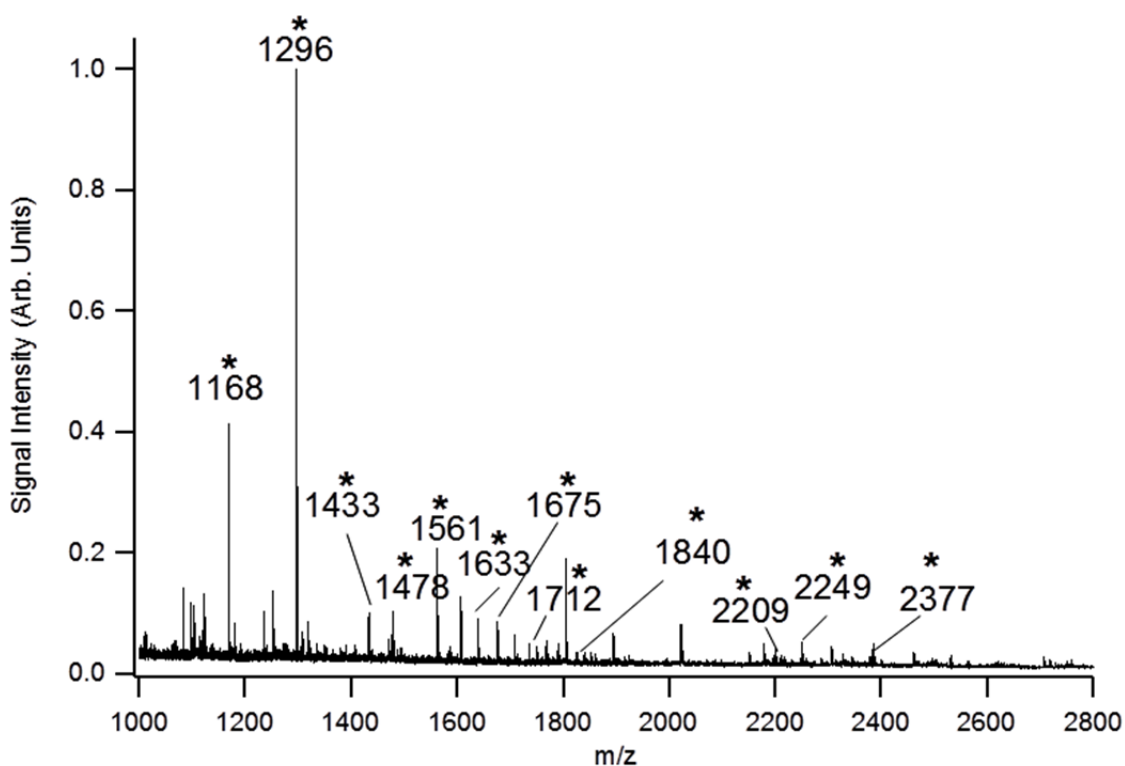


Figure 3.13 MALDI-TOF mass spectrum of cytochrome c digest performed on a PPM filled capillary fabricated with a porogenic solvent of 50% methanol and 50% dodecanol and butyl acrylate as the monomer. A 74 % sequence coverage was obtained. Peptides are indicated with a star.

3.4.3 Quantification of Enzyme on PPM Surface

A bicinchoninic acid assay was used to quantitatively determine the amount of trypsin immobilized on the surface of a PPM column. A redox reaction occurs in solution allowing the amount of enzyme to be calculated. As the trypsin is cleaved from the PPM surface it binds to copper (II) causing a reduction to copper (I) which results in a measurable colour change from green to purple which can be measured via an absorption spectra. This method was used to develop the calibration curve shown in Figure 3.14. From the calibration curve the amount of trypsin on a butyl acrylate/EDMA PPM column was determined to be 33.1 mg of trypsin per gram of PPM.

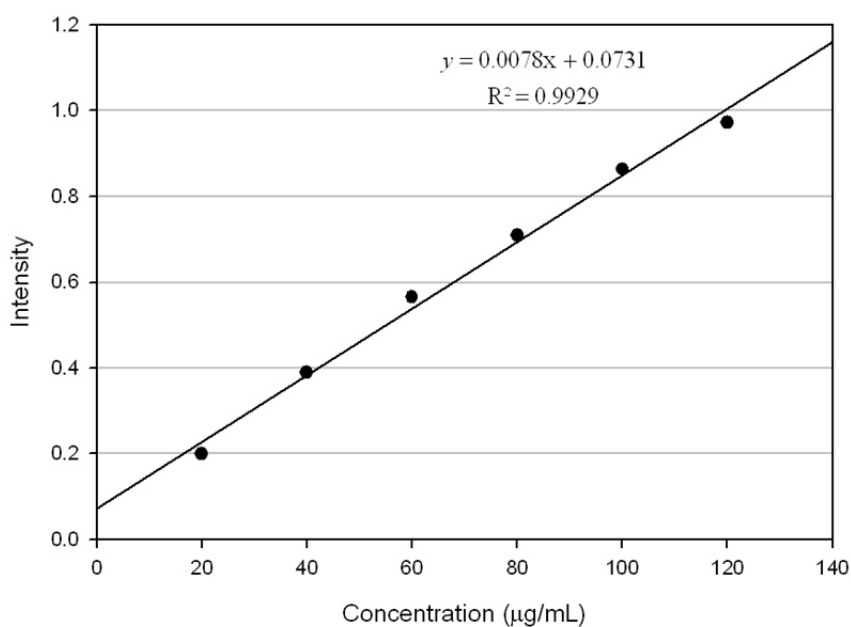


Figure 3.14 A calibration curve constructed via a bicinchoninic acid assay to determine the amount of trypsin on a PPM column.

3.4.4 Enzyme Longevity

Enzymatic digests of cytochrome c were performed for a period of one month on the fabricated enzymatic PPM columns to determine the longevity of the tryptic and peptic PPM

columns. Three different types of columns were tested: PPM columns with trypsin covalently attached, PPM columns with pepsin covalently attached and PPM columns with pepsin adsorbed to the surface. The results of these longevity experiments can be seen in Figure 3.15.

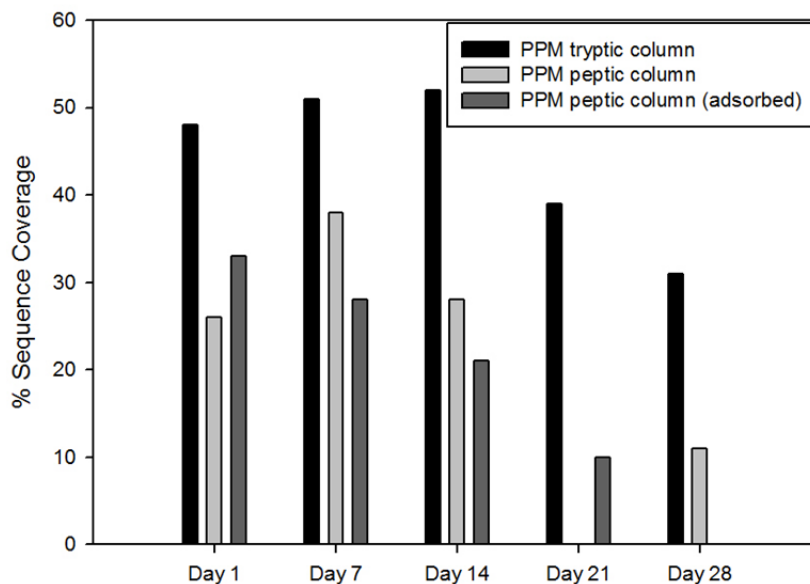


Figure 3.15 Longevity data obtained using a MALDI-TOF-MS for tryptic and peptic PPM columns. All columns were fabricated to a length of 2.5 cm. Assuming a 60% porosity, the residence time for each column would be 8 seconds. For the tryptic and peptic columns the desired enzyme was attached by first forming a carboxylic acid group on the surface followed by a treatment with WSC/NHS which provides the correct functionality on the PPM surface for subsequent enzyme attachment via an enzyme's amine group. For the PPM columns that were adsorbed with pepsin, no pre-treatment of the PPM was done which results in no covalent attachment of pepsin on the PPM surface.

Although the sequence coverage obtained for each digestion is significantly lower than was observed previously due to the sensitivity of the current MALDI-TOF-MS instrument being less in comparison to the previous MALDI-TOF-MS which went off-line due to instrument breakdown, several trends were observed. Firstly, the tryptic columns provide a higher sequence

coverage in comparison to peptic columns and still provided an efficient digestion after a month in comparison to columns covalently attached with pepsin, where the sequence coverage dropped to 15%. Furthermore, the columns adsorbed with pepsin provided a lower sequence coverage in comparison to columns covalently attached with pepsin which demonstrates the stability of the enzyme when it is covalently attached to a surface. The columns adsorbed with pepsin had no enzymatic activity after 1 month which was confirmed by data obtained on Day 28 and Day 35 (data not shown) while columns with covalently attached pepsin still provided digestion of cytochrome c, although the enzymatic efficiency was limited in that the sequence coverage was only 15%. It is important to note that there is no data point for Day 21 for the columns covalently attached with pepsin as the matrix did not result in good crystal formation on that given day which resulted in no signal being obtained. This is a common problem that can be encountered with the dried drop method in comparison to a two-layer matrix preparation and is a result of lack of homogeneity in crystal formation. Furthermore it was confirmed that was an anomaly for that day only, as digestions performed on the peptic columns resulted in the observance of peptides for both Day 28 and Day 35 (data not shown). These results show that although pepsin can be adsorbed to the PPM surface, however columns with covalently attached pepsin are more robust and provide a better digestion efficiency. Therefore the slight time gained in the fabrication of the columns adsorbed with pepsin does not outweigh the advantages obtained from columns with covalently attached pepsin. Also although tryptic columns can provide a more efficient digestion as well as provide specific cleavage sites, peptic columns are more suitable to be used for on-line digestions as the acidic conditions required for digestion are compatible with those of electrospray which is the reason why there is an emphasis on the fabrication and use of enzymatic peptic columns.

3.4.5 Regeneration of Enzymatic Columns

Regeneration of an enzymatic column was proven by first destroying the enzymatic activity of the column and then generating new sites on the PPM column for enzyme immobilization. As the immobilization method relies on generating carboxylic acid groups on the surface through hydrolysis of ester linkages, new functional groups can be generated due the ester groups that comprise the PPM composition, active enzyme can be immobilized to the column after enzyme activity has been lost without having to fabricate a new column. Thus using this immobilization method extends the normal longevity of an enzymatic microreactor.

The first immobilization procedure of the enzymatic PPM microreactor was done with pepsin as pepsin activity is easily irreversibly denatured as it is pH sensitive enzyme. The sequence coverage obtained for a cytochrome c digest performed on the fabricated peptic column was 79% as shown in the mass spectra (Figure 3.16).

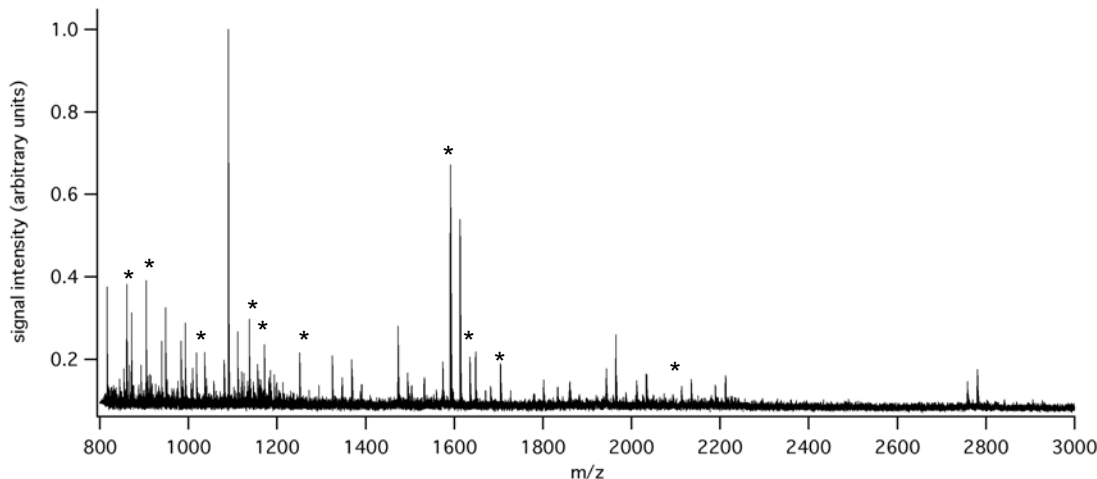


Figure 3.16 The MALDI-TOF spectrum of a cytochrome c digest performed on a peptic PPM column. A sequence coverage of 79% was obtained. Peptides are indicated with a *.

After the cytochrome c digestion confirmed the enzymatic activity of pepsin, pepsin was denatured using a basic solution that consisted of 1.0 M sodium hydroxide. As pepsin is

denatured above a pH of 5, the enzymatic activity of the column was lost after this process. The loss of enzymatic activity was confirmed by first flushing the column to remove any residual base and then attempting to perform a digestion of cytochrome c on the column. As expected no peptides were observed as shown in the mass spectra in Figure 3.17 which confirms the loss of peptic activity.

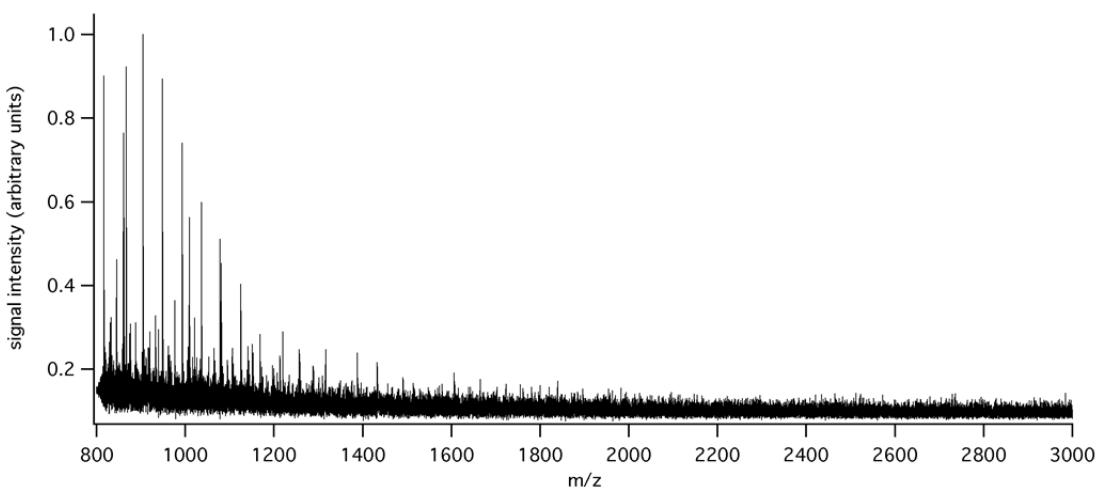


Figure 3.17 The MALDI-TOF spectrum of a cytochrome c digest after denaturation of the peptic column. No peptides were observed which indicates loss of enzymatic activity.

When the column is regenerated with enzyme it is hypothesized that not only will the old enzyme be cleaved off the PPM surface but that new sites in the backbone of the PPM can react via acid hydrolysis converting ester groups to carboxylic acid groups which will be subsequently used for enzyme attachment. However, the PPM column cannot be subjected to too harsh of an acid treatment or the polymer backbone will be weakened. To limit the hydrolysis, the column is only treated with acid for 1 hour instead of the initial 24 hour period that was used. When a 24 hour period was used the polymer backbone would breakdown which was confirmed by the low back pressure readings of the column after acid treatment. The reduced treatment time of 1 hour still provided enough active sites on the polymer to immobilize enzyme on the surface that

resulted in an efficient digestion. Initial testing of a one hour acid treatment resulted in a sequence coverage of 70 % for a cytochrome c digest on a tryptic column as shown in the MALDI-TOF-MS spectrum in Figure 3.18.

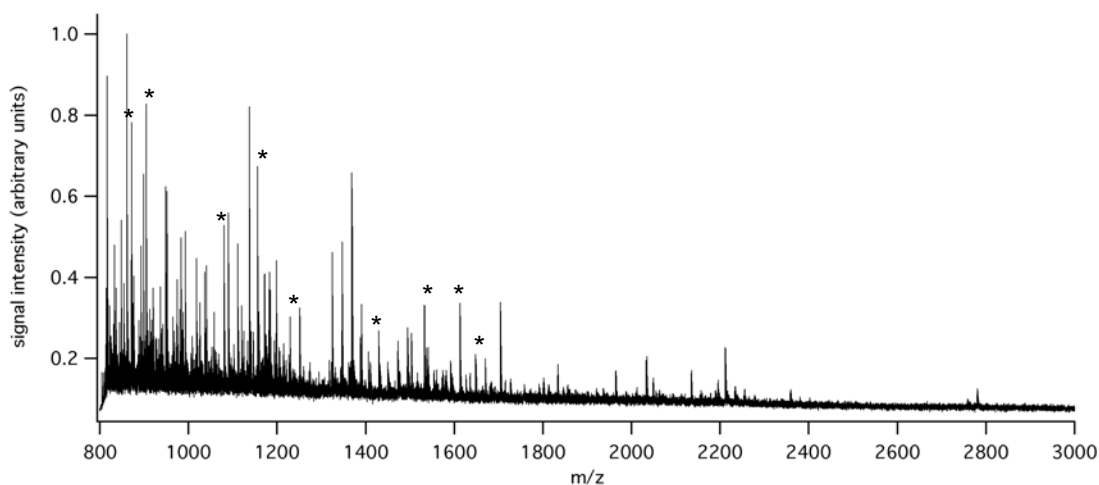


Figure 3.18 The MALDI-TOF spectrum of a cytochrome c digest on a tryptic PPM column fabricated using the modified immobilization procedure which involved one hour of acid treatment rather than 24 hours, resulted in a 70% sequence coverage. The stars denote digested peptide fragments.

As pepsin can be adsorbed to the surface, the column was instead regenerated with trypsin to demonstrate covalent attachment of the “new” enzyme on the surface. These new attachment sites were generated via carboxylic acid groups on the surface. When the column was treated with the immobilization method to attach trypsin on the surface, the resulting digest of cytochrome c gave a sequence coverage of 78% and 73% respectively as shown in Figure 3.19 (ESI-MS) and Figure 3.20 (MALDI-TOF-MS) confirming that the column was regenerated with enzyme.

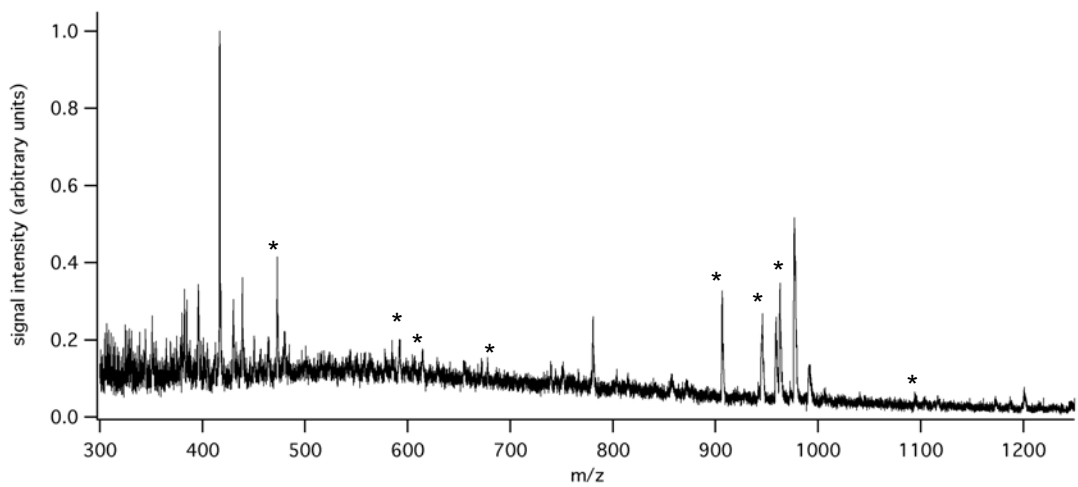


Figure 3.19 The ESI-MS spectrum of a cytochrome c digest on a regenerated tryptic column resulted in a 78% sequence coverage. The stars denote digested peptide fragments.

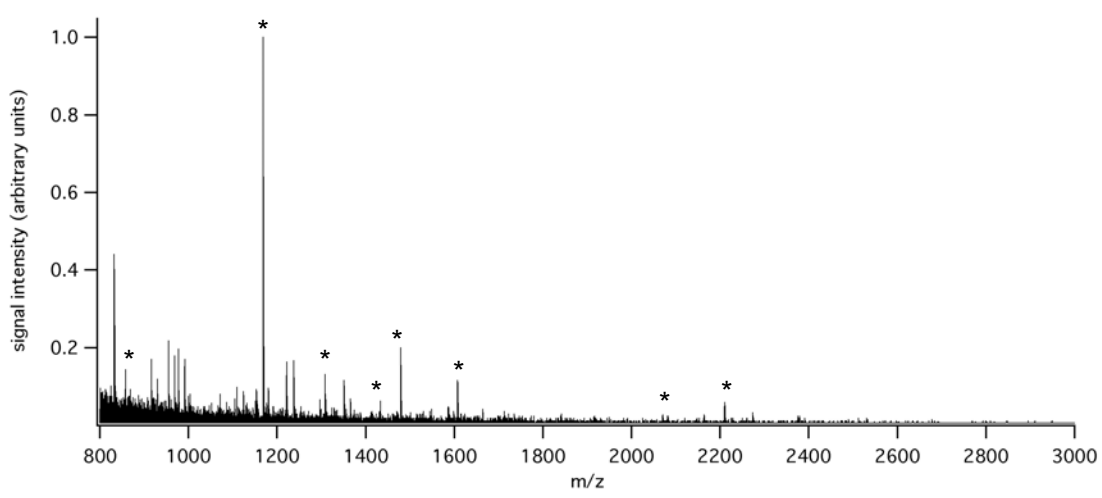


Figure 3.20 The MALDI-TOF spectrum of a cytochrome c digestion on a regenerated tryptic column resulted in a sequence coverage of 73%. The stars denote digested peptide fragments.

The results indicate that PPM columns can be regenerated with active enzyme on the surface which allows columns to be repeatedly used without refabricating the entire column, saving time and cost. Care just needs to be taken to ensure that the polymer backbone does not weaken. The column can withstand a few hours of acid treatment, whether the treatment is continuous or sequential, however after a 12 hour period the polymer will significantly weaken. Regeneration of the column provides yet another advantage to this newly developed immobilization procedure.

3.4.6 Enzymatic Digestions of Various Proteins

Traditionally PPM columns were fabricated using a porogenic solvent composition of 25% methanol and 75% dodecanol. Assuming a porosity of 60% for the fabricated columns, the residence time is approximately 8 seconds and provided sufficient time to provide an efficient digest of proteins including BSA, cytochrome c, insulin and myoglobin. Cytochrome c was always used as the benchmark protein as it is the standard protein used in literature and as a result of having no disulfide bonds no pretreatment steps are required. For other proteins although it will be demonstrated that they can still be digested on PPM enzymatic columns without any pretreatment steps, these steps would be required to provide the most efficient digestion. Reduction and alkylation are required to break disulfide linkages and prevent them from reforming which contribute towards the tertiary structure of a protein. Reduction of the protein is accomplished with either 2-mercaptoethanol or dithiothriitol and reduces the disulfide linkages to S-H bonds. After reduction, to prevent disulfide linkages from reforming an alkylating agent such as iodoacetamide is used. Cytochrome c contains no disulfide linkages. Rather the two cysteine residues found in cytochrome c are bonded to a heme group. The digestion of cytochrome c with a tryptic PPM column is 70% and 76% with a peptic PPM column as shown in Figure 3.18 and

Figure 3.21 respectively. This shows that columns with both trypsin and pepsin can provide an efficient digestion of standard proteins such as cytochrome c.

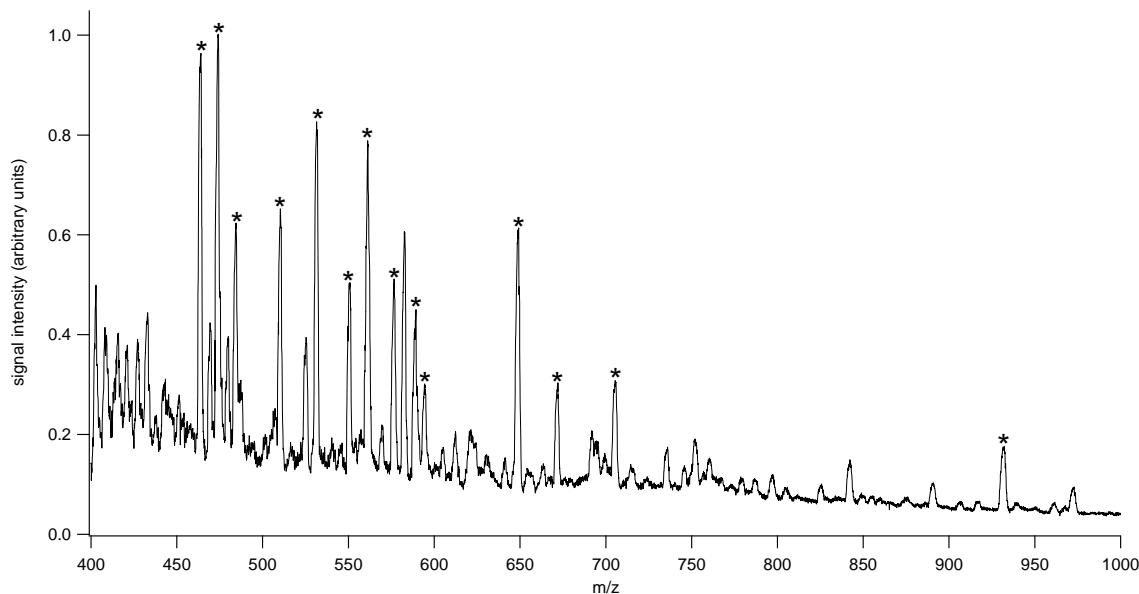


Figure 3.21 The ESI-MS spectrum of an off-line peptic digest of cytochrome c performed a PPM peptic column resulted in a 76% sequence coverage. A PPM column was also used as an electrospray emitter. The stars denote digested peptide fragments.

Analysis of other proteins using the fabricated enzymatic PPM columns focused on pepsin as an enzyme as it would allow for the enzymatic columns to be used directly for an on-line digest and an electrospray emitter. The other proteins analyzed on the peptic PPM column included insulin, myoglobin and BSA. As expected the sequence coverage obtained for each protein was dictated by the difficulty to digest a non-reduced protein. As mentioned earlier, cytochrome c is the easiest to digest of all four proteins as it has no disulfide linkages. The sequence coverage obtained for cytochrome c was 78%. This was followed by insulin where a 68% sequence coverage was obtained (see Figure 3.22). Insulin contains two intrastrand disulfide linkages holding chain A and chain B together as well as one additional interstrand disulfide

linkage as shown in Figure 3.8; however, as the protein is fairly small in mass it is fairly easy to digest. It is important to note that for insulin the peptides had to be calculated manually as the disulfide linkages were not reduced which resulted in peptides that contained both chains and thus could not be analyzed using pre-existing databases.

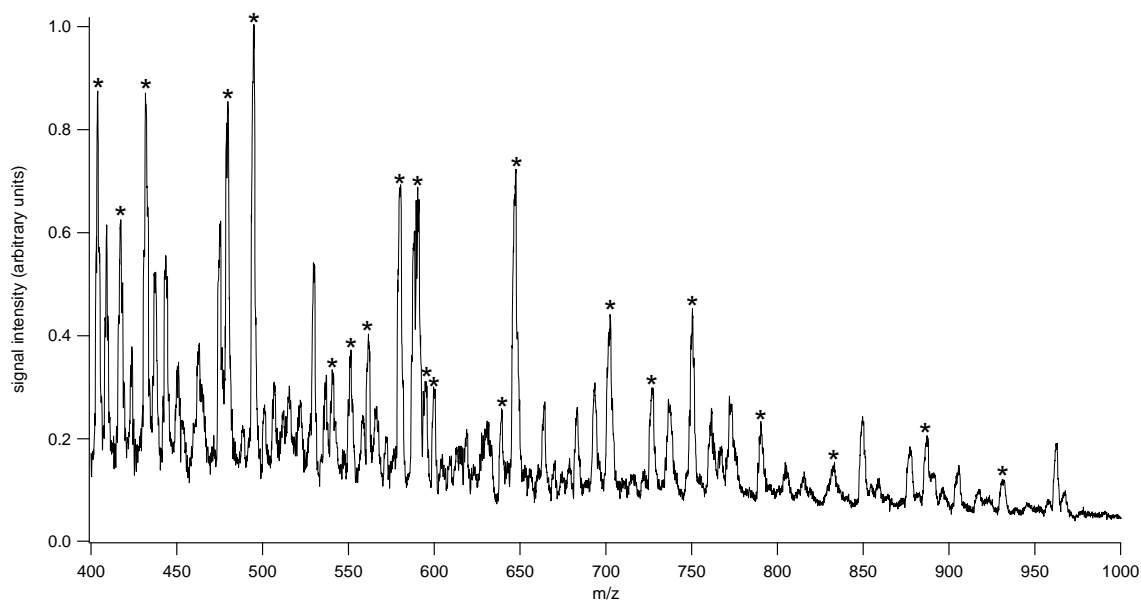


Figure 3.22 The ESI-MS spectrum of an off-line peptic digest of insulin performed a PPM peptic column resulted in a 68% sequence coverage. A PPM column was also used as an electrospray emitter. The stars denote digested peptide fragments.

The next highest sequence coverage was obtained from a peptic digest of myoglobin. Although myoglobin is a small protein and contains no disulfide linkages, it is known to be a more difficult protein to digest due to the presence of the heme group which is held in place by its tertiary structure and thus only a 50% sequence coverage was obtained (see Figure 3.23).

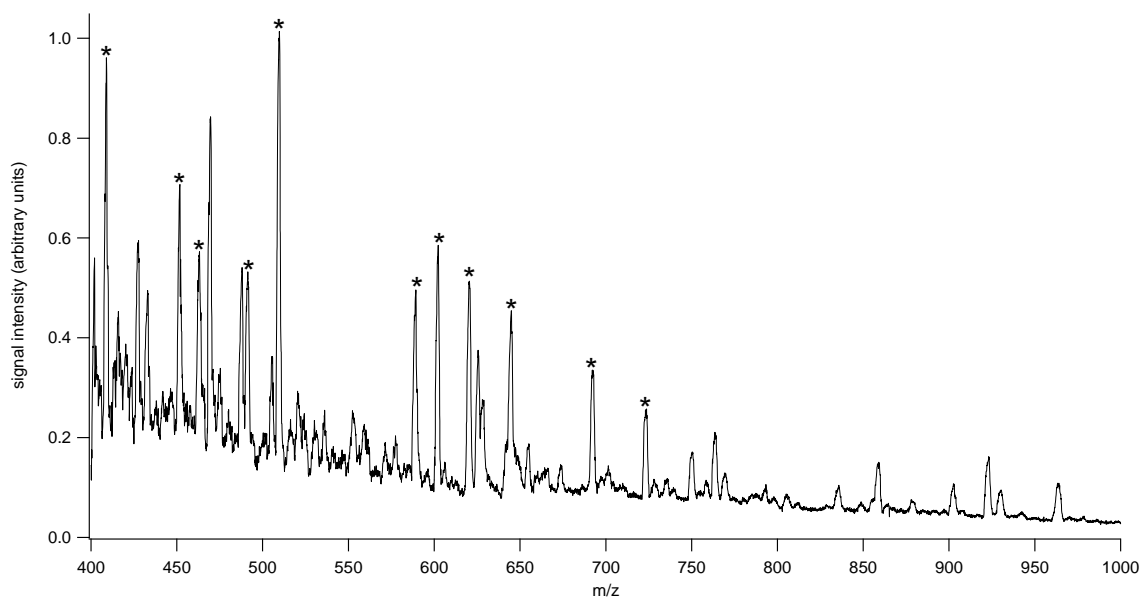


Figure 3.23 The ESI-MS spectrum of an off-line peptic digest of myoglobin performed a PPM peptic column resulted in a 50% sequence coverage. A PPM column was also used as an electrospray emitter. The stars denote peptide fragments.

Lastly, the most difficult protein to digest without a reduction/alkylation step is BSA which contains 17 disulfide linkages. However the efficiency of the peptic columns is demonstrated in this case because even without breaking apart the proteins 3-D conformation a sequence coverage of 10% is still obtained (see Figure 3.24). Had a reduction/alkylation step been used for BSA a much higher sequence coverage would have been obtained but omitting this step demonstrates the digestive strength of the PPM microreactors.

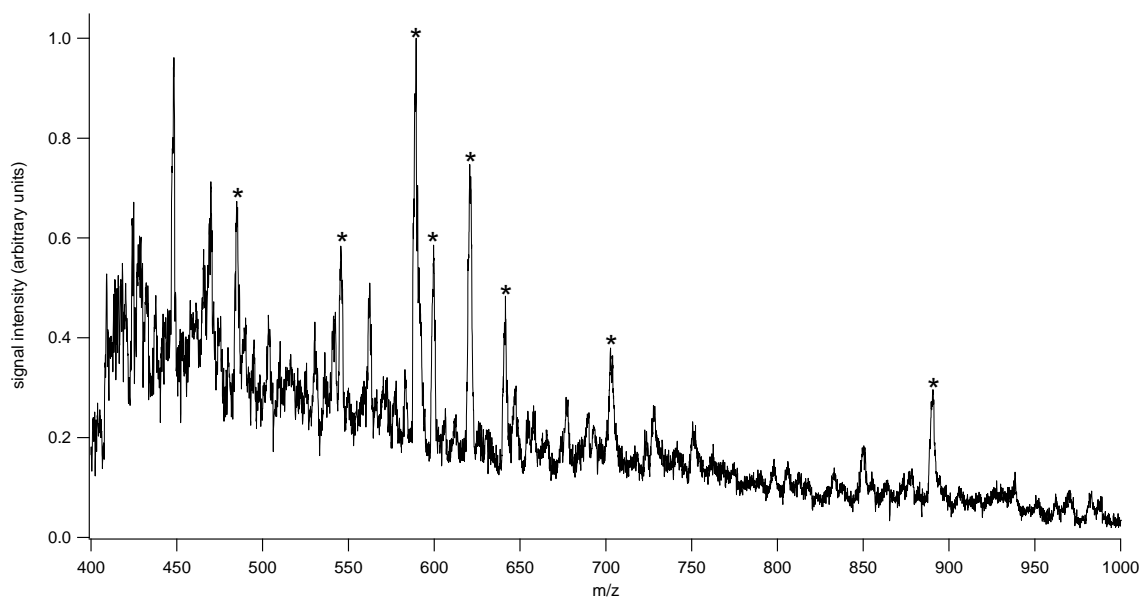


Figure 3.24 The ESI-MS spectrum of an off-line peptic digest of BSA performed a PPM peptic column resulted in a 10% sequence coverage. A PPM column was also used as an electrospray emitter. The stars denote digested peptide fragments.

3.4.7 On-line Enzymatic Digestions

It has been demonstrated that both the fabricated peptic and tryptic columns can be used for on-line digest when coupled with ESI-MS. As trypsin requires a neutral pH for optimal digestion a separate PPM emitter was required post digestion. The two PPM columns were coupled together using a micro tee which allowed the voltage to applied prior to the PPM emitter via liquid injection as well as a solution of 1.3% acetic acid was injected into the microtee using a syringe pump to provide the necessary acidic conditions for ionization. When an on-line tryptic digest was performed using ESI-MS a 38% sequence coverage was obtained for cytochrome c. The mass spectra of the digest can be seen in Figure 3.25.

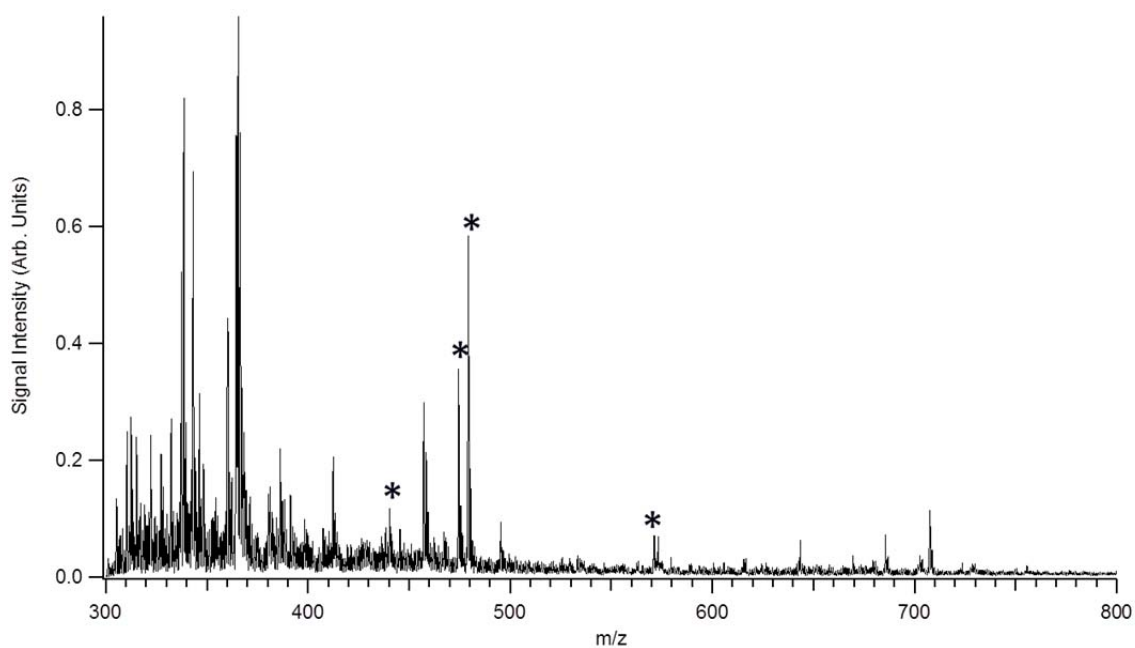


Figure 3.25 The ESI-MS spectrum of an on-line tryptic digest of cytochrome c on a PPM column resulted in a 38% sequence coverage. The enzymatic microreactor was coupled to another PPM column to be used as an electrospray emitter so that the required acidic conditions for electrospray could be obtained post-digestion. The stars denote digested peptides fragments.

On the other hand for the peptic columns, as pepsin already requires acidic conditions the enzymatic column could also be used directly as a nanoelectrospray emitter. When peptic columns were used for an on-line digest of cytochrome c a 25% sequence coverage was obtained. The mass spectrum for the on-line peptic digest of cytochrome c is shown in Figure 3.26.

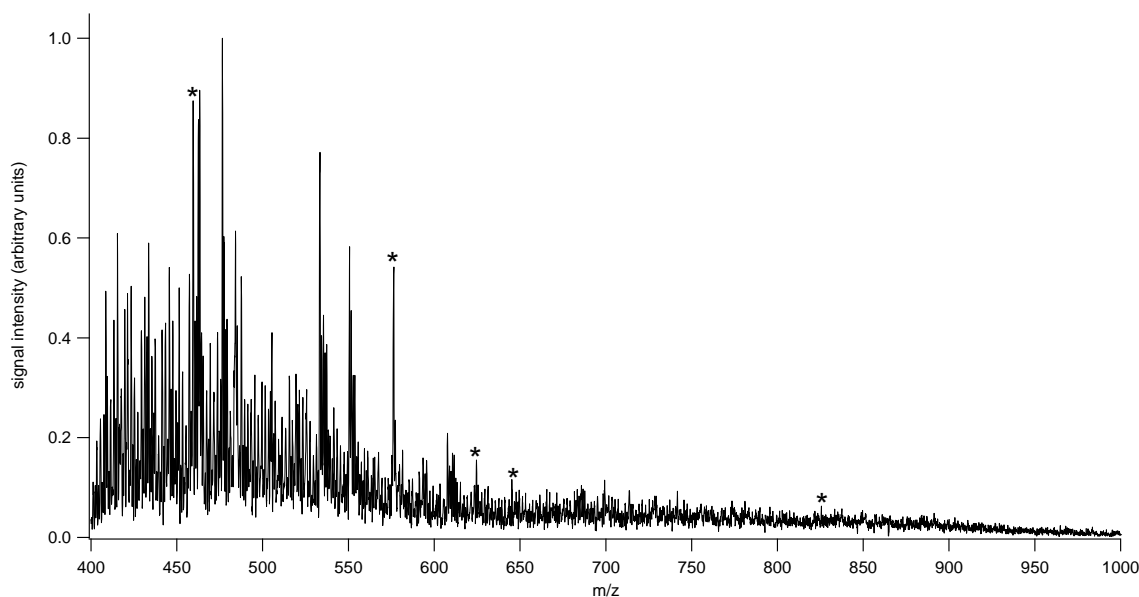


Figure 3.26 The ESI-MS spectrum of an on-line peptic digest of cytochrome c on a PPM column resulted in a 25% sequence coverage. The enzymatic microreactor was also used as a nanoelectrospray emitter. The stars denote digested peptide fragments.

One observation in comparison to off-line digest of cytochrome c was that for both the on-line tryptic and peptic digest the sequence coverage obtained was reduced however the on-line digests still provided a reasonable sequence coverage that could be used to help identify the protein of interest.

3.4.8 Separation of a Protein Mixture

It has previously been shown by Slysz *et al.*¹¹⁵ that a separation column can be attached prior to the column to separate protein mixtures. In this work an enzymatic columns of C₄ beads immobilized with trypsin on the surface was used to separate and digest four proteins, including cytochrome c, myoglobin, carbonic anhydrase and chicken ovalbumin. The optimal enzymatic conditions were found to be with a solution of 45% acetonitrile and 37°C. Although higher temperatures of 50-60°C have shown to produce more efficient digests,¹¹⁶ the autolysis of trypsin

is increased. No undigested protein was observed and 5 fmol, 12.5 fmol, 10 fmol and 200 fmol of carbonic anhydrase, myoglobin, chicken ovalbumin and cytochrome c could be detected, respectively.¹¹⁵ Cytochrome c was not tested for its limit of detection while the detection limit of myoglobin was found to be 5 fmol (85 pg)¹¹⁵. In this work by Slys *et al*¹¹⁵ it was also found that a minimum of 10 peptides is generally required for successful peptide mass fingerprinting which is usually achieved for 20 fmol of protein. A lower detection limit can be achieved using tandem MS since a single peptide unique to a protein could be sequenced for confirmation and afterwards be used for identification. Thus protein identification could be performed with only one peptide instead of 10 as long as that peptide is only found in the specific protein under investigation.

In conjunction with this work a collaborative project with Dr. David Schriemer (University of Calgary) was set up and as shown in insulin (Figure 3.27), cytochrome c (Figure 3.28) and myoglobin (Figure 3.29) can be efficiently separated on a C₈ column using the set-up shown in Figure 3.7 and the separation method shown in Table 3.1. The proteins were then analyzed via ESI-MS using a PPM column as an emitter. As the capabilities of a PPM column to be used as an emitter and an on-line enzymatic column have already been demonstrated, future work would entail coupling these two techniques together to perform a separation followed by a peptic digest on a PPM emitter thereby reducing the losses in separation efficiency due to dead volume, as the required electrospray conditions are already met when pepsin is used for digestions thereby limiting the need for a separate emitter post-digestion.

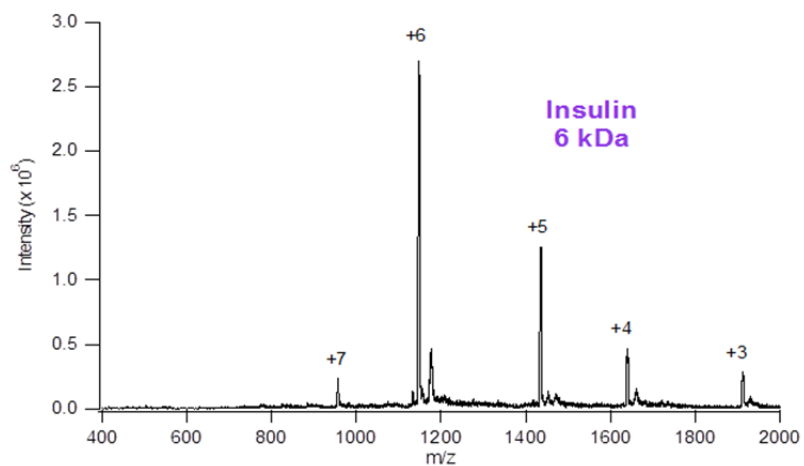


Figure 3.27 The ESI-MS spectrum of insulin obtained from separating a mixture of three proteins on a 6 cm C₈ bead column using a PPM column as nanoelectrospray emitter.

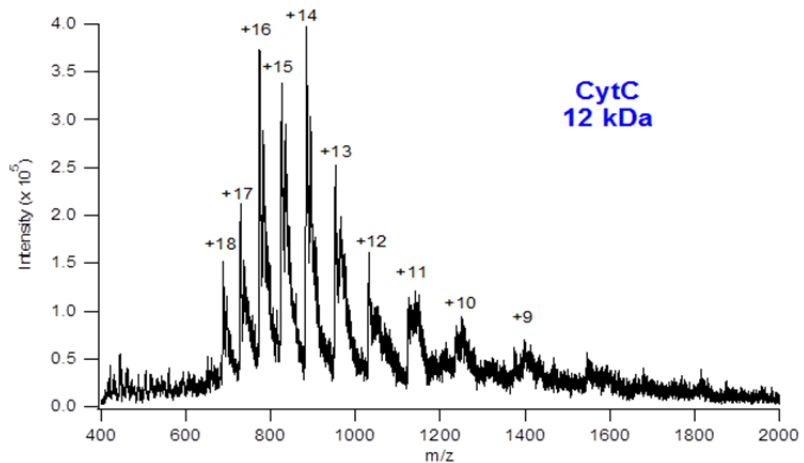


Figure 3.28 The ESI-MS spectrum of cytochrome c obtained from separating a mixture of three proteins on a 6 cm C₈ bead column using a PPM column as nanoelectrospray emitter.

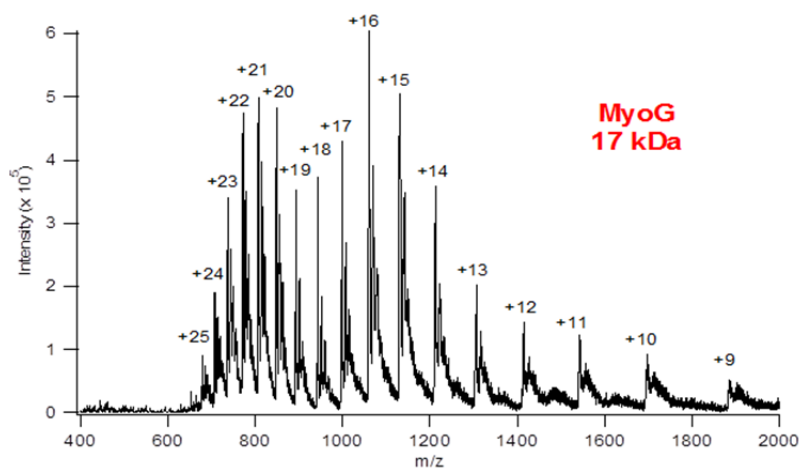


Figure 3.29 The ESI-MS spectrum of myoglobin obtained from separating a mixture of three proteins on a 6 cm C₈ bead column using a PPM column as nanoelectrospray emitter.

3.5 Conclusions

The new immobilization method that has been developed to covalently attach enzymes to PPM surfaces allows for an extended lifetime of the column as the column can be regenerated with active enzyme once the column no longer provides efficient digestions. This provides valuable time savings to the researcher as extra time is not required to fabricate an entire new column. Furthermore this immobilization protocol takes one day to complete in comparison to traditional techniques that require anywhere from 2-6 days. Other advantages of this immobilization method is that it is widely applicable to a variety of enzymes including robust enzymes such as trypsin and pH, temperature and solvent sensitive enzymes such as pepsin. It has been demonstrated that the fabricated enzymatic columns can be used both off-line and on-line to analyze a variety of proteins without a reduction/alkylation step. The protein studied included cytochrome c, insulin, myoglobin and BSA which provide a wide range of selection and as they are both small and large molecular weight proteins and some have multiple disulfide linkages while others have none. Although pepsin columns have been used for on-line digestions

previously, the use of the currently developed peptic columns are able to be used on-line with ESI-MS but also have a dual functionality of a digestion column and a electrospray emitter. Preliminary work on separating a protein mixture indicates that a PPM column can be used as an electrospray emitter for this process and thus in the future could easily be ammended to a digestive column. All the advantages of this new immobilization method make it quite feasible to use these columns on a regular basis for protein analysis as they have long lifetimes, can be regenerated with enzyme and are facile to make.

Chapter 4

Mass Spectrometric Detection of Proteins in Non-Aqueous Media – The Case of Prion Proteins in Biodiesel

4.1 Summary

Limitations in efficient extraction, minimization of media interferences, and suitable sample preparation methods pose significant challenges to the successful detection of protein traces in non-aqueous media. A filtration method is presented employing filter disks with embedded C₈-modified silica particles that allows the capture of proteins from non-aqueous sample volumes. The extraction process is followed by elution of the protein from the filter disk and by either mass spectrometric detection or tryptic digestion followed by peptide mapping and tandem mass spectrometry (MS/MS) fragmentation of protein-specific peptides. The method is applied to spiked biodiesel samples for the detection of prion proteins. The tryptic peptide with sequence YPGQGSPGGNR is specific for prion proteins and can be used for unambiguous identification. The developed extraction method has the potential application to be used for large-scale testing of protein impurities in non-aqueous media, for instance as a safety and quality control tool in the animal tallow-based biodiesel production process.

4.2 Introduction

Proteomics focuses on the identification and quantitation of protein species from a variety of biological matrices such as blood, urine, or cellular and tissue samples.¹¹⁷ As a result, most analysis and synthetic protocols have been developed and optimized to examine and determine proteins and peptides stemming from aqueous samples.¹¹⁸ Mass spectrometry (MS) has often become the detection method of choice because of its ability to provide precise molecular mass information for the proteins of interest. In combination with proteolytic digestion strategies,

peptide mapping and peptide fragmentation (MS/MS), mass spectrometry can provide unambiguous protein identification.¹¹⁷

With increasing interest in the detection of proteinaceous impurities in pharmaceutical production processes,¹¹⁹ advancements of biocatalysis in non-aqueous systems,¹²⁰ and the proliferation of fuels derived from biomass sources,¹²¹ there is an evolving need to develop novel protein detection and quantification methods that are effective for the associated non-aqueous matrices. An important example is the potential prion protein contamination in biodiesel produced from animal-based sources such as tallow.¹²¹ Transmissible spongiform encephalopathy (TSE), a fatal disease that affects the nervous system, has become a growing concern, especially in the cattle industry where the disease is referred to as bovine spongiform encephalopathy (BSE) or mad cow disease.¹²¹ It is hypothesized that prion proteins are the causative agent of TSE.¹²² A normal form of the protein (PrP^C) is thought to misfold into an amyloidogenic beta-sheet structure that has the ability to convert PrP^C into the infective prion protein (PrP^{Sc}).¹²² There is concern that biodiesel produced from specified risk materials (SRMs), including brain, inner organs or spinal cord, could be a source of infectious prion proteins.¹²¹ For inactivation of infectious proteins to occur, both high temperatures and high pressures are needed. Several recent reports have stated that rendering procedures and biodiesel production processes are safe and pose a negligible risk in terms of TSE infectivity, even under worst-case scenarios.¹²³⁻¹²⁵ Nevertheless, one report also points out that due to the variability of current rendering and biodiesel production processes, individual assessments for each specific case may be necessary.¹²³ Further scientific research into the matter is desirable, both to add additional levels of safety¹²¹ and to expand our knowledge of protein stability outside their natural environments, for example non-aqueous media.¹¹⁸

Bioassays are currently the preferred method for detection of TSE infectivity in biological tissues or organisms.¹²⁶ Samples are administered to test animals via injection or oral intake. After an incubation period or the onset of clinical signs of the disease, the host animal is euthanized and the brain is examined for TSE. A drawback to this testing method is the long incubation time of up to 30 months. The use of transgenic mice has shortened analysis times to 150-196 days.¹²⁷ However, biodiesel cannot be directly administered in such bioassays, due to its toxicity to the test animals. Furthermore, the average shelf life of biodiesel is in the range of 6-8 months¹²⁸ and thus any meaningful tests require detection methods with a shorter turnaround time. Other detection methodologies that rely on affinity interactions such as Western blot analysis can also be ruled out for direct biodiesel monitoring due to organic matrix interferences that would alter affinity binding either through solvent interactions or denaturation.¹²⁶ In addition, the presence of organic solvents can affect the activity of proteins. For instance, enzyme activity has been enhanced in the presence of organic solvents,¹²⁹ but over the time of several hours can also decrease enzyme activity.¹³⁰ Thus even if organic solvents were not an incompatible matrix for the desired analysis techniques, the tests could provide inconclusive results due to altered protein interactions in organic solvents.

The detection of prion proteins in biodiesel has been carried out using a combination of chloroform/methanol extraction, gel electrophoresis separation and immunoassay detection.¹²⁴ Here, we present a novel approach using biodiesel filtration through special filter disks combined with matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The use of a C₈-embedded membrane to filter and preconcentrate biodiesel samples is attractive due to its applicability for a wide variety of proteins and for different sample media, both aqueous and non-aqueous. Another feature is the ability to filter large sample volumes,¹³¹ a major advantage compared to tedious liquid-liquid extraction methods applicable only to small sample batches.

Previously membranes such as polyurethane (PU),¹³² poly(tetrafluoroethylene),¹³³ nitrocellulose,¹³⁴ polyethylene,¹³⁵ polyvinylidene difluoride (PVDF),¹³⁶ and nylon¹³⁷ have been used as sample supports for direct analysis of proteins by MALDI-MS. Proteins are adsorbed to the membrane surface, and washing steps can be incorporated without significant loss of protein. Depending on the nature and abundance of the proteins, it may be possible to perform MALDI-MS directly on the disk. This enables sensitive analysis methods optimized for aqueous samples to be employed (eg. MS and Western blot).

In the present study, we developed an extraction technique using a mixture of acetonitrile, non-ionic detergent and water that allows for PrP to be removed from an extraction filter disk containing C₁₈- or C₈-modified particles after filtration of a spiked biodiesel sample. This extraction procedure has proven effective for other proteins, including bovine serum albumin (BSA) and cytochrome c, and can also be applied for filtrations involving water or isooctane. Subsequent tryptic digestion of the extracted prion protein either on-disk or after re-suspension in water yields a peptide with a prion-specific sequence YPGQGSPGGNR, allowing unambiguous identification via MS/MS analysis. This method should be generally applicable for any large scale testing for proteinaceous impurities both in aqueous and non-aqueous media.

4.3 Experimental

4.3.1 Chemicals and Materials

Methanol, 2-propanol and acetonitrile (all ACS Reagent Grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Bovine serum albumin (BSA), horse heart cytochrome c, bovine trypsin (TPCK treated for deactivation of chymotrypsin activity), 2,2,4-trimethylpentane (isooctane) and octyl- β -D-glycopyranoside (*n*-OGP) were purchased from Sigma (Oakville, ON,

Canada). Ammonium bicarbonate (bioChemika Ultra) and α -cyano-4-hydroxycinnamic acid (HCCA) were obtained from Fluka (Buchs, Switzerland). Trifluoroacetic acid (TFA) was purchased from Aldrich Chemicals (Milwaukee, WI, USA). Glacial acetic acid (98%) was obtained from Fisher Scientific (Ottawa, ON, Canada). All chemicals were used without further purification.

Biodiesel from animal sources was donated from Environment Canada (Ottawa, ON, Canada). A refolded murine (of the rat and mouse family) prion protein (PrP) was expressed according to the protocol of Hornemann *et al.*¹³⁸ and received from the Saskatchewan Research Council (SRC, Saskatoon, SK, Canada). The PrP stock solution was received at a concentration of 0.725 mg/mL in *tris*-acetate buffer (pH 6.5) and was stored at -80°C. Refolded PrP, according to the protocol of Jackson *et al.*,¹³⁹ resulted in the β -folded murine prion protein (PrP^{*}) and which was received from the SRC as a precipitate and stored at -80°C. A MilliQ apparatus (Millipore, Bedford, MA, USA) was used to purify water (18.2 M Ω ·cm). 3M EmporeTM extraction disks (47 mm diameter) embedded with either octyl (C₈) or octadecyl (C₁₈) coated absorbent particles were purchased from Fisher Scientific (Ottawa, ON, Canada). C₁₈ and C₄ ZipTips were from Millipore (Bedford, MA, USA).

4.3.2 Filtration and Whole Protein Extraction

Filtrations were performed under vacuum by air aspiration using a regular laboratory filtration apparatus as shown in Figure 4.1. Either 3M Empore C₈ or C₁₈ extraction disks were used. Proteins studied by filtration included BSA as a surrogate and PrP. Prior to filtration, the medium of interest was spiked with protein to yield a final concentration of 60nM. To help the solubility of the PrP stock solution in biodiesel, a 1:10 solution of PrP stock and methanol was used for spiking. The biodiesel solution was continuously stirred or agitated during the filtration process to ensure uniform dispersion across the extraction disk. After filtration of PrP spiked

media such as water, isooctane or biodiesel, a piece of dry extraction disk, approximately 2 mm x 2 mm, was immersed in a 1:1 solution of acetonitrile and 0.5% *n*-OGP in deionised H₂O to remove the protein from the disk for subsequent MALDI analysis. The extraction was performed for a period of 20 to 30 minutes while sonicating. It should be noted that initial attempts to employ a Millipore filtration unit (Labscale TFF System, Model #XX42LSS11) failed. Unknown corrosive ingredients present in the biodiesel partially dissolved plastic parts of this equipment and rendered it unusable. As a consequence, any contact of biodiesel with plastic laboratory equipment was avoided.



Figure 4.1 Filtration apparatus used to remove proteins from sample solutions. 3M Empore C₈ or C₁₈ extraction disks were used for the filtration. Following the filtration the proteins were extracted from the disk and analyzed via MALDI-MS.

4.3.3 Tryptic Protein Digestion

Tryptic digests in solution were performed using a modified protocol from that of Qin *et al.*¹⁴⁰ A 1:2 solution of protein stock in 0.1 M NH_4HCO_3 was prepared to a total volume of 15 μL . A 1 μL aliquot of a 1 mg/mL solution of trypsin was added to the vial and the vial was incubated in a Fisher Scientific Dry Bath Incubator (Ottawa, Ontario) at 37°C for 2 hours. Tryptic digestions were also performed directly on extraction disks. Samples of biodiesel spiked with proteins were filtered through a C_8 or C_{18} extraction disk. After the disk was dried, a piece of extraction disk was cut and spotted with 1 μL of a 1:10 solution of trypsin (1 $\mu\text{g}/\mu\text{L}$) and 0.1 M NH_4HCO_3 . The piece of extraction disk was then placed into a weigh boat inside a plastic container. A wet sponge was also placed into the container to minimize evaporation. The container was placed on top of an incubator for 30 minutes at 37°C.

Another approach was to submerge pieces of extraction disks in a 1:10 solution of trypsin (1 $\mu\text{g}/\mu\text{L}$) and 0.1 M NH_4HCO_3 . The solution with the extraction disk was then incubated for 2 hours at 37°C and the supernatant analyzed for peptides.

4.3.4 Microwave-Assisted Digestion of PrP

Microwave-assisted acid digestion for PrP in solution was accomplished using a modified protocol adopted from Zhong *et al.*¹⁴¹ A 50 μL sample of PrP stock solution was concentrated under nitrogen to approximately 10 μL , and 5 μL of 98% TFA was added. The solution was then sealed with Teflon tape in a 1.5 mL propylene Eppendorf vial and microwaved on high for 10 minutes in a conventional microwave oven (max. 700 watts). A minimal volume (< 20 μL) was critical to withstand the vapour pressure created during the microwaving process. A container of water was placed beside the Eppendorf vial of protein and acid to absorb any excess microwave energy.¹⁴¹ After the TFA digest was performed, the microwave was unplugged and cooled to

room temperature before opening. The vial was then carefully opened in a fume hood and 0.1 M NH_4HCO_3 was added to neutralize the acid.

Sample preparation for mass spectrometry was either done with protein/peptide solutions, extracts or directly from extraction disks. In the latter case the small pieces of the disks were carefully glued onto MALDI target plates using 3M spray adhesive.

4.3.5 Mass Spectrometric Analysis of Whole Proteins

For exact molecular weight determination a 10 μL aliquot of the PrP stock solution was purified using a C_4 ZipTip and eluted with sinapic acid solution according to the protocol of Dai *et al.*¹⁸ For whole protein detection from C_8 or C_{18} extraction disks, small pieces of the extraction disk (~2 mm x 2 mm) were placed into an Eppendorf tube and extracted with a 1:1 solution of acetonitrile and 0.5% *n*-OGP. This solution was then further prepared, employing a novel sensitive three-layer matrix preparation method reported by Keller and Li.²⁰

MALDI-time of flight (TOF) analysis was performed on a Voyager DE STAR Biospectrometry workstation (Applied Biosystems, Framingham, MA) in positive linear mode. This instrument is equipped with a 337 nm nitrogen laser. Several hundred laser shots were summed for spectra collection. BSA and cytochrome c were used as standard proteins for calibration.

4.3.6 Data Analysis

Peptide mixtures resulting from protein digests, were first purified and concentrated employing a C_{18} ZipTip and further prepared according to the two-layer matrix preparation method of Dai *et al.*¹⁸ Peptide maps were obtained on the above mentioned Voyager instrument in positive reflectron mode. MS/MS analysis of specific peptides was done either with a post-source decay method on the Voyager instrument or from solution after purification with a C_{18} ZipTip and

elution with a 70% MeOH and 0.1% acetic acid solution, by employing a static nanospray setup on a QSTAR XL QqTOF mass spectrometer (Applied Biosystems/MDS-Sciex, Toronto, ON, Canada).

4.4 Results and Discussion

4.4.1 PrP Characterization

PrP was expressed according to the protocol by Hornemann *et al.*¹³⁸ The PrP sequence is shown in Figure 4.2 (209 amino acid residues). The addition of a serine residue at the C-terminus is intended to minimize proteolytic degradation and the protein contains one disulfide linkage between its two cysteine residues. From the above sequence the average mass of the protein, with all methionine residues oxidized as ensured by the protocol,¹³⁸ is calculated to be 23,115 Da.

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1   KKRPKPGGWN TGGSRYPGQG SPGGNRYPPQ GGGTWGQPHG GGWGQPHGGG 50
51  WGQPHGGGWG QPHGGGWGQG GGTHNQWNKP SKPKTNMKHM AGAAAAGAVV 100
101 GGLGGYMLGS AMSRPMMHFG NDWEDRYRE NMNRYPNQVY YRPVDQYNNQ 150
151 NNFVHDCCVNI TIKQHTVTTT TKGENFTETD IKMERVVEQ MCTTQYQKES 200
201 QAYYDGRRS

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Figure 4.2 Sequence of murine PrP. All methionines (**M**) are oxidized and a disulfide linkage exists between Cys157 and Cys192. The theoretical average molecular weight is 23 115 Da.

Figure 4.3 shows the mass spectrum of the whole murine PrP protein. The signals at m/z 23,113 and at m/z 11,553 result from the singly and doubly protonated ions of PrP, respectively. The observed signal at 23,113 Da is within 3 Da of the calculated m/z for the singly charged ion (m/z 23,116) and within the expected accuracy of the instrument at this mass range for external calibration (150 ppm or 3.5 Da). Several protein fragments or protein contaminants below m/z 11,000 are also observed.

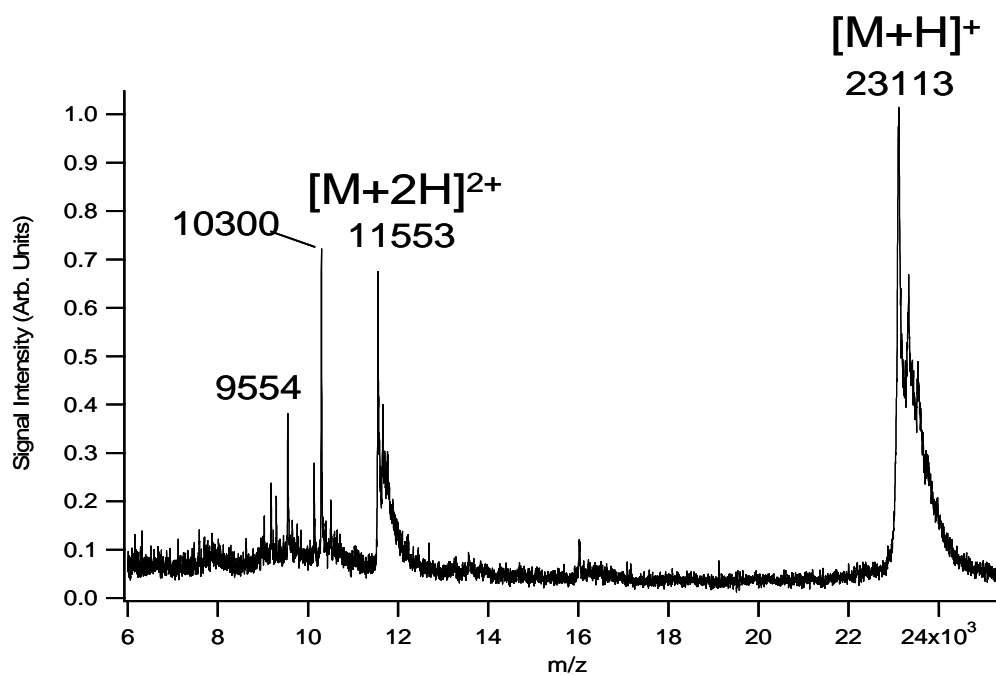


Figure 4.3 MALDI-TOF mass spectrum of PrP after (ZipTip) purification. Below m/z 11,000 there are a several protein fragments or contaminating proteins observable.

Tryptic digestion of PrP (both unfolded and refolded forms) directly from the stock solutions yielded two peptides with sequence YYR (127-129; m/z 501) and YPGQGSPGGNR (16-26; m/z 1089), respectively. The smaller peptide with sequence YYR has little diagnostic value due to its short sequence which is not specific for prion proteins. The YPGQGSPGGNR peptide (m/z 1089) is unique for prion proteins and is a conserved sequence found in more than 50 different species (MASCOT sequence search at www.matrixscience.com), such as bovine, deer and sheep. Since this sequence is specific to the prion protein it can be used as a biomarker for unambiguous identification of prion proteins.^{142, 143} The YPGQGSPGGNR peptide is located very close to the protein's N-terminus, even before the four octapeptide repeat units involved in copper binding.¹⁴⁰ The N-terminal region up to amino acid residue 121 is the so-called non-structured, flexible or "random coil-like" region of the protein and is not involved in alpha-helix

or beta-sheet formation.¹⁴⁴ Circular dichroism (CD) and fourier-transform infrared (FTIR) experiments have shown that PrP^C contains 40% alpha-helix and minimal beta-sheets, whereas PrP^{Sc} contains 30% alpha-helix and 45% beta-sheets.^{145, 146} The increased beta-sheets present in PrP^{Sc} presumably do not inhibit enzymatic digestion where this peptide is located. Figure 4.4 shows an electrospray MS/MS fragmentation spectrum of this peptide.

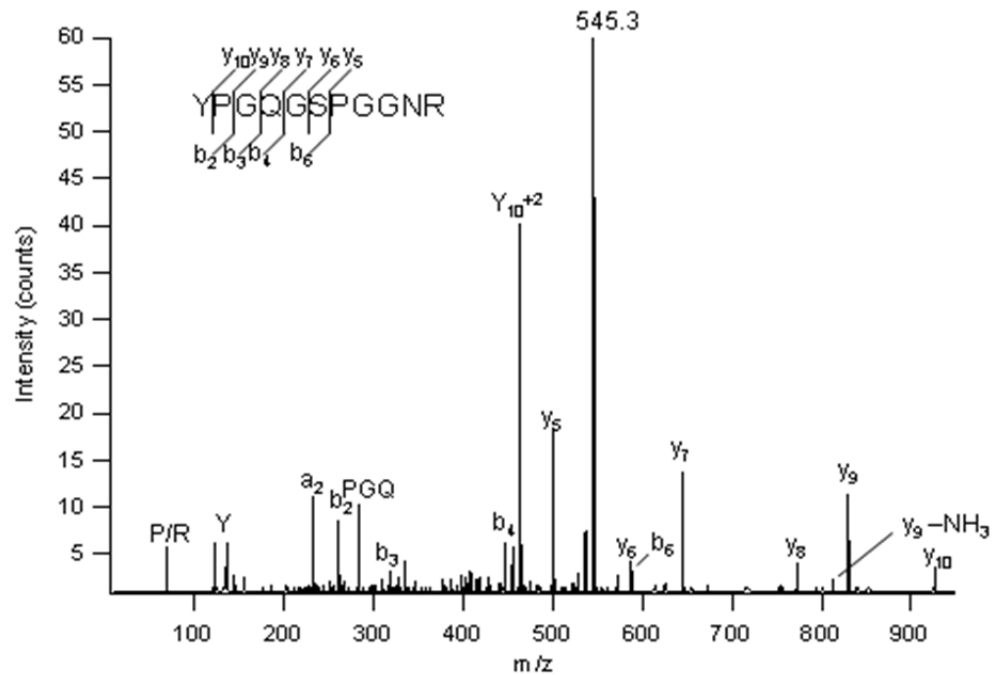


Figure 4.4 Electrospray MS/MS spectrum of the peptide with sequence YPGQGSPGGNR with m/z for $[M+2H]^{2+} = 545.3$.

Microwave-assisted acid digestion also yielded specific peptide signals for PrP in the mass range >3000 Da, as is shown in Figure 4.5. However, the spectra were generally dominated by several peptides in the mass range below 3000 Da that possibly originated from the protein contaminants in the PrP stock solutions. The observed peptides specific for PrP are in a mass range too high for efficient MS/MS fragmentation ($> m/z$ 3000) and thus have limited suitability for specific PrP detection in complex mixtures. In addition, the required protein amounts for

effective microwave-assisted digestion are in the microgram range,¹⁴¹ thus limiting this method's employability in sensitive protein detection. Microwave-assisted acid digestion experiments with filter disc pieces spiked with prion protein solution were not successful.

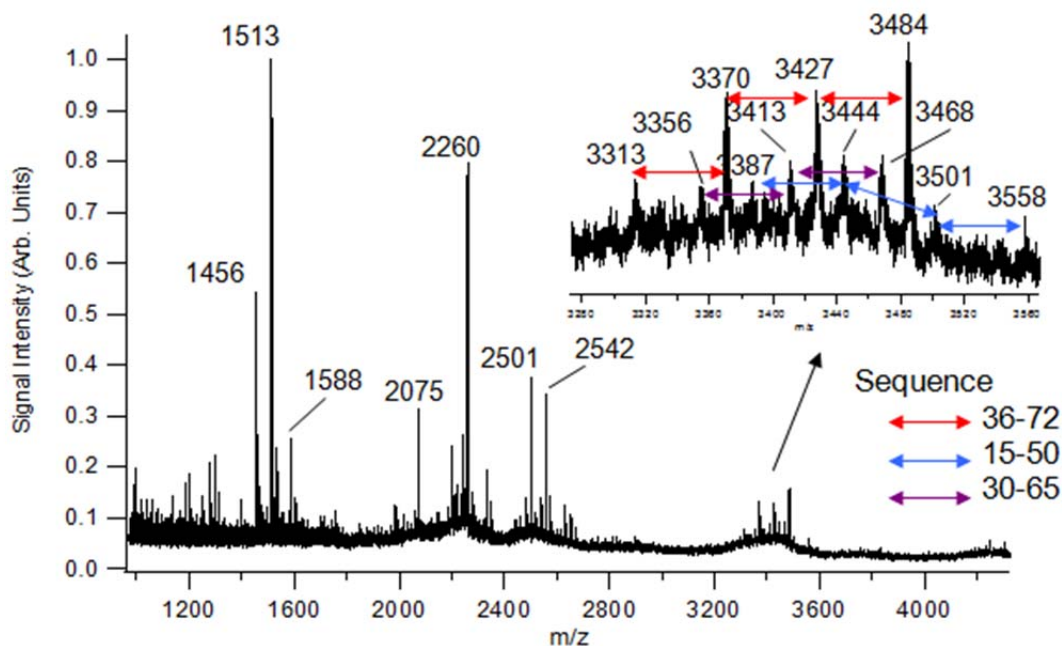


Figure 4.5 MALDI-TOF mass spectrum of a microwave-assisted acid (TFA) digest of PrP. The resulting peptides specific for PrP are shown in the insert in the upper right corner. All the arrows indicate a loss of a glycine residue (57 Da) which is typical for TFA digests.¹⁴¹ Peaks below m/z 2600 are probably from digested contaminant protein fragments.

4.4.2 Filtration of Spiked Biodiesel, Extraction of Filter Discs and Protein Analysis

Although direct analysis of protein on the extraction disk is possible, the analysis sensitivity is improved when protein is extracted back into solution. With the extract, a recently developed three-layer matrix preparation method for MALDI-MS can be applied which allows the detection of proteins in low nanomolar concentrations.²⁰ Furthermore, extracting the protein back into solution provides a medium that is compatible, after removal of acetonitrile, for other

analysis techniques including enzymatic digestion and immunoassay. After filtration of a protein spiked sample, the extraction method of 1:1 acetonitrile and *n*-OGP effectively removed the protein from the extraction disk.

To illustrate that the majority of the PrP was transferred to the extraction disk, a water-spiked sample was analyzed before and after filtration. PrP was detected in the water sample as well as after extraction from the disk, however PrP was not detected in the filtrate as shown in Figure 4.6.

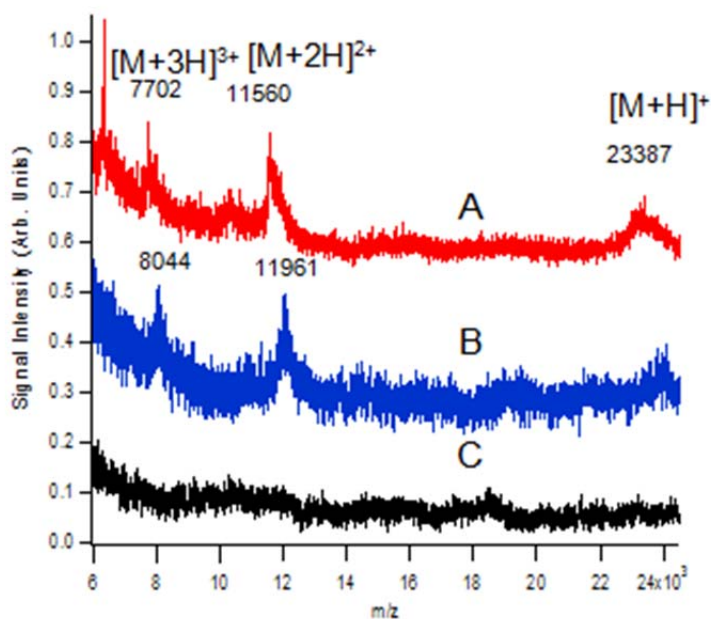


Figure 4.6 MALDI-TOF mass spectra of a PrP spiked water sample to test extraction efficiency. (A) 50 mL of water containing PrP (60 nM) (B) Extract from C₁₈ extraction disk after filtration of water containing PrP (30 nM). Extraction was performed in a 1:1 solution of ACN and 0.5 % *n*-OGP for 20 mins. (C) Filtrate from filtration of 50 mL of water containing PrP (60 nM). For all three spectra a novel sensitive three layer matrix/sample preparation method was employed.²⁰

The acetonitrile/*n*-OGP extraction procedure is applicable after the filtration of a variety of media including water, isooctane and biodiesel. The mass spectra of whole PrP from the extract of a C₈-disk used to filter 25 mL of a 60 nM PrP isooctane and biodiesel sample is shown in Figure 4.7 and Figure 4.8, respectively. Extraction disks with embedded C₈-modified silicon particles were found to be superior in terms of protein recovery for biodiesel filtrations and were thus used for all subsequent analysis. This observation is consistent with Engelhardt and Mueller's 1984 report where C₈-modified bonded phase showed optimal protein recovery in reverse phase high performance liquid chromatography (RP-HPLC) analysis.¹⁴⁷

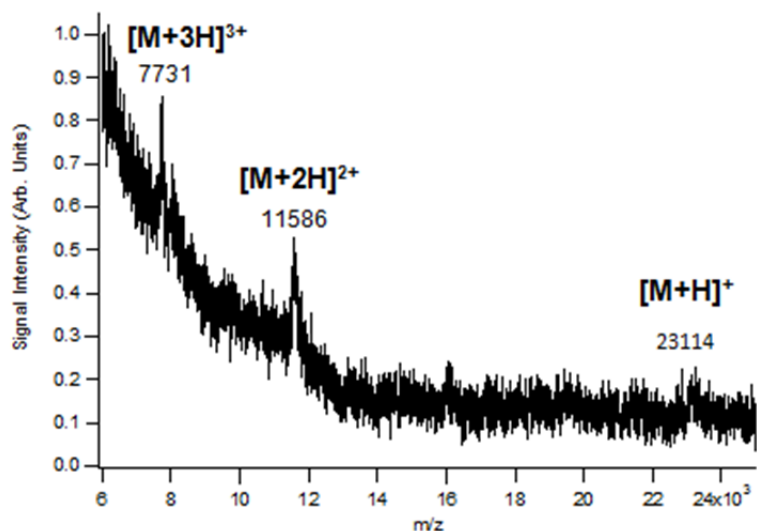


Figure 4.7 MALDI-TOF mass spectrum of the extract of PrP from a C₁₈ extraction disk used to filter 25 mL of isooctane containing PrP (60 nM). Extraction performed in a 1:1 solution of ACN and 0.5% *n*-OGP for 30 minutes.

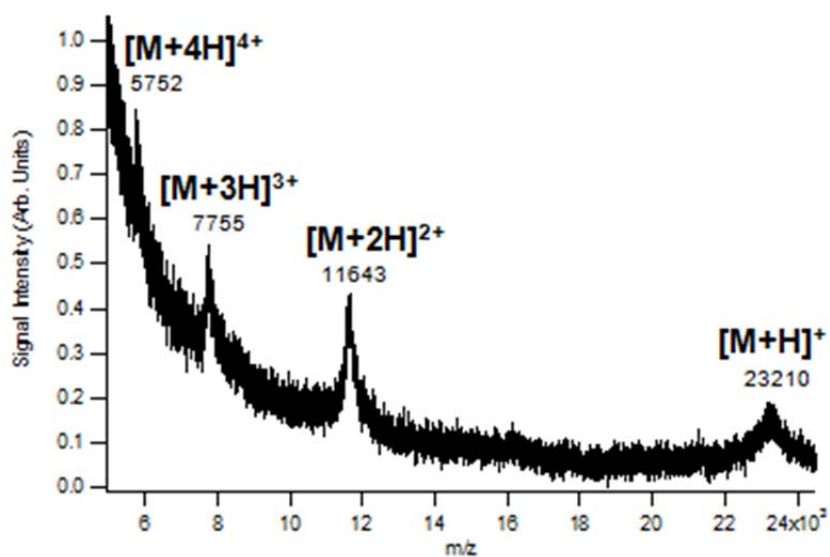


Figure 4.8 MALDI-TOF mass spectrum of the extract of PrP from a C₈ extraction disk used to filter 25 mL of biodiesel containing PrP (60 nM). Extraction performed in a 1:1 solution of ACN and 0.5% *n*-OGP for 20 minutes.

Difficulties were encountered during the filtration process with older batches of biodiesel that presumably became contaminated with bacterial growth over time. As a result, if the biodiesel was aged, a pre-filtration step had to be used to remove any particulate interference. For applicability of this technique to large scale analysis of the biodiesel production process, the biodiesel samples should be analyzed within a few months of production, due to their limited shelf life.¹²⁸

Digestion of proteins can be performed directly on the extraction disk after filtration of a protein spiked sample, and BSA, as shown in Figure 4.9, and cytochrome c were successfully analyzed in such a manner. For PrP, however, we found this approach not to be suitable, since this protein required a longer digestion time of more than 2 hours and thus the trypsin solution dried up before the enzymatic digestion was complete.

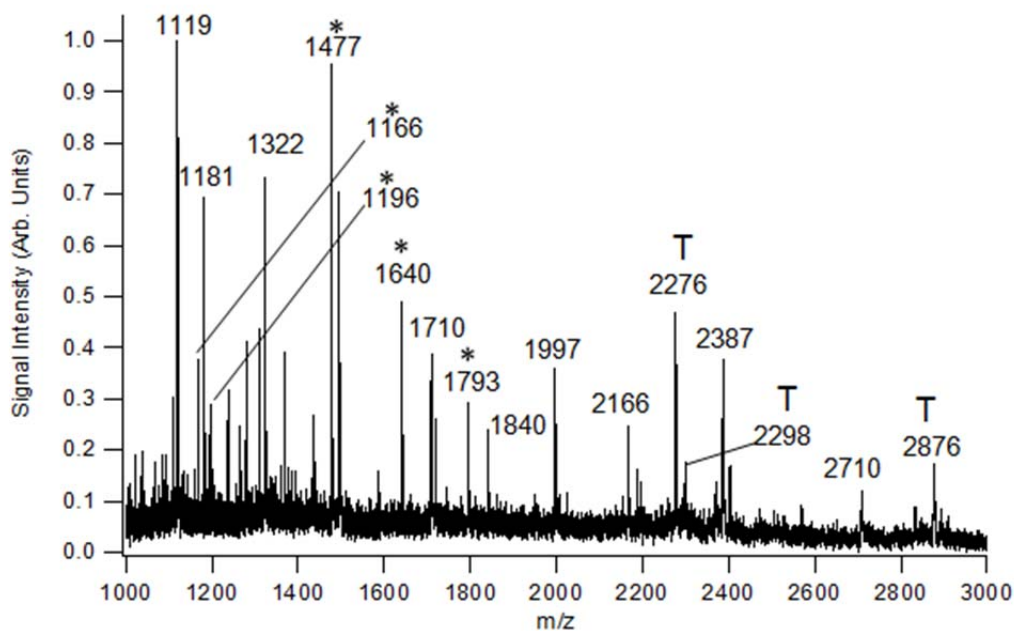


Figure 4.9 MALDI-TOF mass spectrum of a BSA digest with trypsin performed directly on a C₁₈ extraction disk after filtration of 1 L of biodiesel spiked with 4 µg of BSA. BSA peptides are marked with an * and trypsin autolysis peaks are indicated with a T.

To circumvent this problem, after filtration a small piece of the extraction disk was immersed directly in a trypsin solution, and the resulting peptide mixture contained both the m/z 501 and 1089 peptides. The MS/MS of the 1089 peptide obtained from a tryptic digest of a piece of extraction disk used to filter 25 mL of a 60 nM PrP-biodiesel sample is shown in Figure 4.10. In this case, MALDI post source decay was chosen for peptide fragmentation (as compared with nano-ESI employed for peptide marker screening in Figure 4.4) because of better sensitivity and compatibility with impurities. As can be seen from Figure 4.10, this approach allows also for unambiguous PrP identification. These results confirm that PrP can be effectively extracted from biodiesel and unambiguously identified by MS/MS.

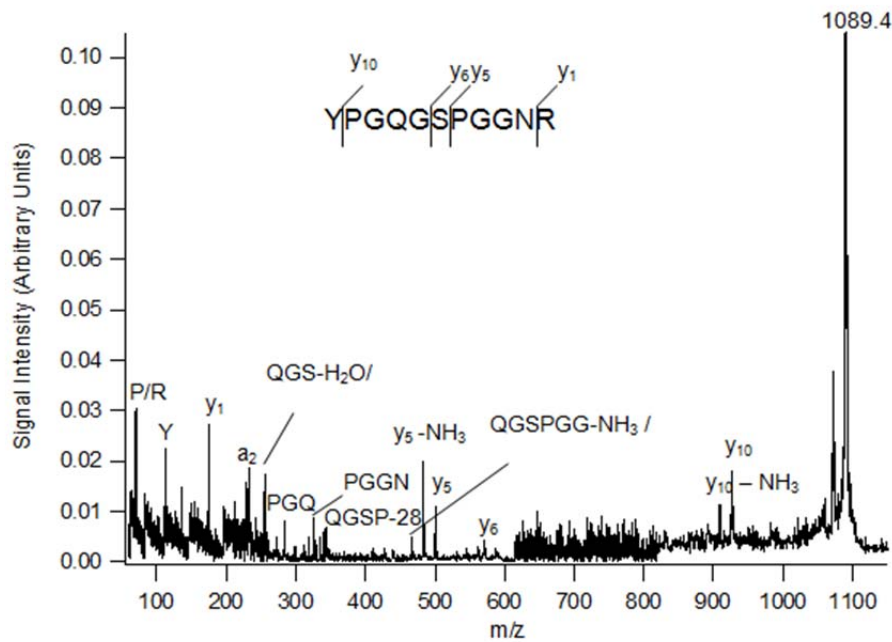


Figure 4.10 MALDI-TOF mass spectrum achieved by post source decay to sequence the 1089 peptide achieved from a 2 hr tryptic digest performed on a piece of C₈ extraction been used to filter 25 mL of biodiesel containing PrP (60 nM). Sample was passed through a C₁₈ ZipTip prior to analysis and eluted with HCCA matrix solution.

A 25 mL sample with 60 nmol/L PrP contains a total amount of ~60 μ g PrP. The effective area of the filter disk is approximately 960 mm² since only about 35 mm of its total 47 mm diameter is exposed to filtration solution. Assuming uniform distribution and complete capture of PrP on the extraction disk after the biodiesel filtration, approximately 250 ng (~10 picomole) of PrP is expected on a 2 mm x 2 mm (4 mm²) piece of extraction disk. Considering potential losses during the filtration process and irreversible protein adsorption to glass surfaces as well as complications incurred by the complex biodiesel sample matrix, it is clear that this approach cannot match the 20-30 attomole detection limit achievable by direct analysis of a pure PrP standard.¹⁴⁸ Nevertheless, the spiked amount of PrP is comparable to traditional initial

extraction experiments performed for BSA as a carrier protein in lipid media ($\sim 1 \mu\text{g}$ BSA/mL).¹²⁴ The question if such a detection limit is adequate for “real” samples cannot be readily answered, since each application’s requirements will differ and need individual comprehensive assessments. For applications where a more detailed method development is warranted and affordable, application of stable-isotope labeled standards and testing of dilution series will eventually establish the linear dynamic range and absolute detection limits. In this respect, it should be noted that the extraction disks and extracts obtained from the developed method allow transfer of the captured proteins to aqueous media and potentially allow employment of more sensitive and quantitative immunoassays (e.g. Western blot) and can also be used in bioassays for testing of infectivity.

4.5 Conclusions

The detection and characterization of proteinaceous impurities in non-aqueous media is a new emerging field and currently faces enormous challenges due to the lack of adequate methods. In the present study extraction disks with embedded C₈-modified silica particles were successfully employed to extract proteins from both aqueous and non-aqueous media. The ruggedness is demonstrated through the application of this method for a “difficult” protein (*i.e.* prion protein) in a corrosive medium (*i.e.* biodiesel), and thus this approach should be applicable to a wide range of potential samples where proteinaceous impurities are of interest. Mass spectrometry has proven to be an efficient detection tool for extracted proteins and in combination with enzymatic digestion, peptide extraction and MS/MS analysis allowed for unambiguous identification of the proteins. Although mass spectrometry is an invaluable tool in the method development process, current achievable detection limits might not always be sufficient in real world applications. However, the presented protein extraction method allows for recovery of the extracted proteins into aqueous media that also allow for ultrasensitive detection

methods such as immunoassays. Due to the ruggedness and simplicity of the general principle of this method it is anticipated that it should be easily adaptable to large-scale production processes in pharmaceutical or other industries, and it represents a realistic alternative to small-batch liquid-liquid extraction methods.

Chapter 5

Clinical Applications of Enzymatic Microreactors

5.1 Summary

In proteomics, real world samples often cannot be handled directly as they require either pretreatment or post treatment steps to make the detection of analytes possible. Specifically with proteins some of the challenges include limited samples volumes, complex protein mixtures, and incompatible matrices for the required analysis techniques. It has been demonstrated that the use of enzymatic microreactors can facilitate the analysis of protein samples that are traditionally difficult to analyze. In fact it has been shown that aside from reducing the digestion times from hours to seconds and providing a higher sequence coverage in comparison to digestions performed in-solution, concentration and purification steps have been eliminated. The two proteins studied were α -1-protease inhibitor (antitrypsin) and prion protein (PrP). With antitrypsin the sequence coverage was increased from 28% to 35% and information regarding oxidized methionine residues was readily obtained which was of interest as methionine oxidation has been linked to inactivation of the protein. With PrP an additional peptide was observed that can be used to identify the protein and purification and concentration steps prior to analysis were removed. These preliminary results show that enzymatic microreactors may have the possibility of being used for clinical research.

5.2 Introduction

The use of enzymatic microreactors, specifically microfluidic chips and porous polymer monoliths (PPM), has been established using a wide variety of standard proteins which are pure and relatively easy to digest including cytochrome c, insulin, bovine serum albumin (BSA) and myoglobin. Applicability of these devices to biological samples that are not always found in an

aqueous environment will be demonstrated using two proteins that are linked to diseases that are of public concern. These include prion protein, which is linked to transmissible spongiform encephalopathy (TSE) and antitrypsin, which can result in sepsis. These applications will show the use of enzymatic microreactors to provide insight into medical issues as well as aid in the digestion of proteins that are typically more resistant to tryptic digestion than standard proteins.

Protein misfolding can cause a large variety of diseases and may occur as a result of inherited or acquired gene variations, or abnormal amino acid modifications.¹⁴⁹ The consequences of the variations or modifications depend on a variety of factors such as environmental conditions, type of protein, interacting genetic factors, degradation processes and activity of folding.¹⁴⁹ Diseases that result from protein misfolding are referred to as conformational diseases.¹⁵⁰

An example of a disease acquired as a result of protein misfolding in the endoplasmic reticulum is hereditary emphysema.¹⁴⁹ This condition results from a deficiency in antitrypsin. In certain individuals, an accumulation of this protein can also result in liver damage. Antitrypsin is a major plasma serine protease inhibitor which is secreted for regulation of the proteolytic activity of certain enzymes and has more than 90 different variants.¹⁴⁹ The protein, discussed by Laurell and Eriksson in 1963,¹⁵¹ has a molecular weight of 52 kDa and has 394 amino acid residues with 3 asparagine-linked carbohydrate side chains. Antitrypsin is synthesized in the liver and secreted into the plasma. A deficiency of antitrypsin in the lungs results in proteolytic damage of the connective tissue by neutrophil elastase which is released during inflammation.¹⁵² The regulation of neutrophil elastase is important as dysregulation is implicated in several conditions including sepsis,¹⁵³ inflammatory thrombosis,¹⁵⁴ atherosclerosis,¹⁵⁵ myocardial infarction and coronary disease,¹⁵⁶ immune complex-mediated pulmonary hemorrhage,¹⁵⁷ vascular endothelial damage,¹⁵⁸ and persistent intravascular coagulation.¹⁵⁹ Increased levels of neutrophil elastase result in

disseminated intravascular coagulation (DIC) which is a disorder of the coagulation/fibrinolytic system.¹⁶⁰

Studies have been conducted on a variant of antitrypsin that has glutamic acid 342 replaced by a lysine residue, referred to as a Z mutation, which results in retarded protein folding and has been linked to protein aggregation.^{152, 161} Environmental factors such as smoking also lead to inactivation of antitrypsin. Currently methionine residues have been reported as being susceptible to oxidation from hydrogen peroxide found in cigarette smoke.¹⁶² Two of the nine methionine residues are readily oxidized including residue 358, known to cause loss of neutrophil elastase activity, and residue 351. The other seven methionine residues were 63, 220, 221, 226, 242, 374, and 385 were unaffected by oxidation processes.

The name of antitrypsin is deceiving since although it is a weak trypsin inhibitor it reacts to a greater extent with neutrophil elastase.¹⁶³ Inactivation of enzymes occurs through antitrypsin covalently and irreversibly binding to the enzyme. Much has yet to be discovered about the structure of antitrypsin to provide insight as to the result of environmental factors on the protein and how they translate into the body. Antitrypsin can be inactivated by both oxidation after a severe inflammatory response¹⁵² or bacterial proteases during infections.¹⁵³ Through use of trypsin digests on enzyme microreactors a more efficient digest can result and may provide insight into modifications of the amino acid sequence including oxidation sites and/or proteolytic degradation by elastase. There are two sites of antitrypsin that are susceptible to enzymatic attack resulting in antitrypsin inactivation.¹⁶⁴ There is an amino-terminal end of 20 amino acid residues and a 16 amino acid loop situated near the carboxy terminus that contains that reactive center responsible for binding of neutrophil elastase. The reactive center consists of the methionine 358 residue. The loop holds the antitrypsin protein in a stressed metastable form.¹⁶⁴ Upon cleavage of the loop, the two ends of the protein irreversibly separate to form a more stable relaxed structure

which results in proteolytic degradation of the protein. Therefore, while the majority of antitrypsin forms an irreversible covalently bound complex with neutrophil elastase, a small portion during the inhibitory mechanism does get proteolytically degraded.

Another example of a disease related to protein misfolding is TSE. This is a fatal disease that attacks the nervous system. It is believed that the prion protein, defined as a proteinacious infectious particle without nucleic acid,¹⁶⁵ causes TSE through a misfolding process. Specifically, the theory is that the normal form of the protein (PrP^C) misfolds into an amyloidogenic beta-sheet structure that then converts PrP^C into the infectious form (PrP^{Sc}).¹²² PrP^{Sc} is partially protease resistant and as a result cannot be easily identified by enzymatic digestion. Proteinase K digestion of PrP^C results in complete digestion, whereas with PrP^{Sc} a truncated version of the protein PrP 27-30 results with a mass of 27-30 kDa.¹⁶⁶ The limited proteolysis of PrP^{Sc} is used in many studies as it provides indication of the presence of PrP^{Sc} however, it is not always a feasible technique as it results in three sets of signals in most cycles of Edman degradation.¹²²

Previously, it had been widely accepted that the specific amino acid of a given protein results in one biologically active structure of that given protein.¹²² However, PrP appears to defy this rule. In fact, one primary structure of PrP results in two different possible conformations, PrP^C and PrP^{Sc}.¹²² Optical spectroscopy studies of these two PrP forms revealed that they were indeed different.¹⁴⁵ Studies using fourier-transform infrared (FTIR) and circular dichroism (CD) revealed that PrP^C consists of about 40% α -helix and minimal β -sheet, while on the other hand PrP^{Sc} contains about 30% α -helix and 45% β -sheet.^{145, 146} Therefore, although these two proteins have the identical amino acid sequence they have remarkably different structures.

The method of choice to test for TSE infectivity in biological tissues or organisms is to perform a bioassay.¹⁶⁷ First, a sample is administered to a test animal either by injection or intake. After the incubation period is over or the animal shows clinical signs of the disease, the

animal is euthanized and then the brain is analyzed for the presence of TSE. One of the drawbacks of using bioassays is that they require long incubation periods, for example 30 months is required for testing on cattle.¹⁶⁸ If transgenic mice are used instead the incubation period is shortened to 150-196 days.¹²⁷ Even with the reduced incubation period bioassays are still a lengthy procedure. Other detection techniques used for testing for TSE include Western blot analysis, which is much quicker than bioassays but it is still a intensive laboratory technique. Although not as sensitive a technique as Western blot analysis, mass spectrometry (MS) analysis can be used for identification of prion proteins post enzyme digestion. With MS, specific amino acid sequences can be used as biomarkers unique to prion proteins.^{142, 143} Unfortunately, the presence of a given biomarker does not distinguish between the two types of prion proteins. However, this coupled with the fact the infective PrP^{Sc} is partially protease resistant¹⁶⁶ can provide a rapid preliminary affirmation for the presence of TSE infectivity.

To demonstrate the use of mass spectrometry in clinical applications, tryptic microreactors, including PPM columns and microfluidic chips, that were developed to provide rapid analysis of traditional protein samples such as cytochrome c, were used to digest both antitrypsin and PrP, both of which are proteins that are difficult to digest using a standard in-solution digest.

5.3 Experimental

5.3.1 Chemicals and Materials

Poly(methyl methacrylate) (PMMA) polymer sheets (150mm×150mm×1.5mm) were purchased from Warehoused Plastic Sales Inc. (Toronto, ON, Canada) and cut to size before use. Fused silica capillary with a polyimide coating and an outer diameter of 150 µm and an inner diameter of 75 µm and UV transparent fused silica capillary with an outer diameter of 360µm and

an inner diameter of 75 μm were purchased from PolyMicro Technologies (Phoenix, AZ, USA). NHS and trifluoroacetic acid (TFA) were purchased from Aldrich Chemicals (Milwaukee, WI, USA). WSC and α -cyano-4-hydroxycinnamic acid (HCCA) were acquired from Fluka (Buchs, Switzerland). Trypsin (bovine, 13000 units/mg) was obtained from Sigma (Oakville, ON, Canada). TRIS·HCl, 2-propanol (ACS reagent grade), acetonitrile (ACN) (HPLC grade), and methanol (ACS reagent grade, Ultrapure grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sulfuric acid (ACS reagent grade) and glacial acetic acid (ACS reagent grade) were purchased from Fisher Scientific (Nepean, ON, Canada). Ammonium bicarbonate, sodium chloride (ACS reagent grade), ethylene dimethacrylate (EDMA), glycidyl methacrylate (GMA), 3-(trimethoxysilyl)propyl methacrylate (z-6030), benzoin methyl ether, acetone (ACS reagent grade), 1-dodecanol, magnesium dichloride hexahydrate and calcium chloride dihydrate (ACS reagent) were acquired from Sigma (Oakville, ON, Canada). All reagents were used without further purification. Deionized water (18.2 M Ω) was purified using a MilliQ apparatus (Millipore, Bedford, MA, USA). C₁₈ ZipTips were from Millipore (Bedford, MA, USA).

A refolded murine (of the rat and mouse family) prion protein (PrP) that had been expressed according to the protocol of Hornemann *et al.*¹³⁸ was received from the Saskatchewan Research Council (SRC, Saskatoon, SK, Canada). The PrP stock solution was received at a concentration of 0.725 mg/mL in *tris*-acetate buffer (pH 6.5) and was stored at -80°C. Antitrypsin and oxidized antitrypsin samples were received from Dr. John Samis from the Biochemistry Department at Queen's University (Queen's University, Kingston, ON, Canada). These samples were prepared from commercially available antitrypsin and oxidation was completed using *N*-chlorosuccinimide (NCS) prior to receipt of the samples. Both antitrypsin stock solutions were at a concentration of 2 mg/mL and were stored at -80°C.

5.3.2 Fabrication of PMMA Microfluidic Chips

PMMA substrates (1.5mm thick) were embossed with a fused silica capillary with an outer diameter of 150 μm using a HEX-01 hot embosser Jenoptik Microtechnik (Jena, Germany). The capillary was embossed into the PMMA at a temperature of 115°C with a force of 2000 N for 600 seconds to create a 150 μm channel. Prior to removing the PMMA from the embosser, the temperature of the tool and substrate was decreased to 85°C and the chamber was brought to atmospheric pressure. To define a region for enzyme immobilization fused silica capillary pieces with an outer diameter of 150 μm and an inner diameter of 75 μm were placed at the entrance and exit of the channel to define an 80 mm. The PMMA cover plate was then bonded to the embossed substrate plate at a temperature of 105°C with 500 N of applied force for 300 seconds. To ensure fluidic sealing of the microfluidic chip the area where the entrance and exit capillaries are placed was double stamped at a temperature of 110°C with a force of 1000 N for 400 seconds.

5.3.3 Formation of PPM Columns

Prior to PPM formation UV transparent fused silica capillary had to be pretreated. First the capillary was flushed with 1.0 M NaOH at a flow rate of 1 $\mu\text{L}/\text{min}$ for 30 minutes using a syringe pump (Harvard Apparatus, St. Laurent, PQ, Canada). The capillary was then rinsed with 0.10 M HCl at 1 $\mu\text{L}/\text{min}$ for 30 minutes, followed by water for 30 minutes at 1 $\mu\text{L}/\text{min}$. The final step involved flushing the capillary overnight at a flow rate of 0.5 $\mu\text{L}/\text{min}$ with a solution of 50% (v/v) Z-6030 in acetone to allow an anchoring site for the subsequent PPM formation.

A PPM solution was prepared by weight percent with the following compounds: 39.6% monomer solution (23.6% GMA and 16% EDMA), 60% porogenic solvent and 0.4 % benzoin methyl ether as a photoinitiator. The porogenic solvent consisted of a 1:1 mixture of MeOH and dodecanol. The PPM solution was then degassed with nitrogen for 5 minutes. The capillary was filled with PPM solution and to selectively pattern a 2.5 cm long PPM column the ends of the

capillary were masked. The capillary was then irradiated at 365 nm for 15 minutes at distance of 4.5 cm. The resulting PPM column was then flushed at 5 $\mu\text{L}/\text{min}$ with water to remove any unpolymerized PPM solution. The formation of the PPM was then confirmed under the microscope.

5.3.4 Enzyme Immobilization Method

For trypsin immobilization on PMMA microfluidic chips a solution of 1 M H_2SO_4 was flushed through the channel at a flow rate of 0.5 $\mu\text{L}/\text{min}$ using a syringe pump. The channel was rinsed with water at a flow rate of 5.0 $\mu\text{L}/\text{min}$. Next a solution of 1.0 mg/mL aqueous WSC and 0.1 mg/mL *N*-hydroxysuccinimide (NHS) was passed through the microfluidic chip at a flow rate of 0.5 $\mu\text{L}/\text{min}$ for 2 hours. The channel was again rinsed with water at a flow rate of 5.0 $\mu\text{L}/\text{min}$. A solution of 10 mg/mL trypsin solution in 50 mM NH_4HCO_3 (pH 7.4), 10 mM NaCl and 10 mM MgCl_2 was flushed through the microfluidic chip for 1 hour. The capillary ends were then sealed with parafilm and the microfluidic chip was stored at 4°C overnight. Afterwards the channel was flushed with 50 mM NH_4HCO_3 (pH 7.4) to remove adsorbed enzyme at a flow rate of 5.0 $\mu\text{L}/\text{min}$. The tryptic microfluidic chips were stored at 4°C in the refrigerator with a 50 mM TRIS·HCl (pH 7.4) and 10 mM CaCl_2 solution in the channel. During storage the capillary ends were sealed with parafilm. Prior to use, the PMMA microfluidic channels were rinsed with 50 mM NH_4HCO_3 (pH 7.4) at a flow rate of 5.0 $\mu\text{L}/\text{min}$ for 10 minutes using a syringe pump.

For immobilization of trypsin on PPM columns, the column was flushed with a 1 M H_2SO_4 solution at a flow rate of 0.5 $\mu\text{L}/\text{min}$ for 1 hour. The column was flushed with water for 1 hour and then a 1 mg/mL WSC and 0.1 mg/mL NHS solution was passed through the column at a flow rate of 0.5 $\mu\text{L}/\text{min}$ for 2 hours. The column was then flushed with water for 1 hour followed by flushing with a 10 mg/mL trypsin solution in 50 mM NH_4HCO_3 (pH 7.4), 10 mM MgCl_2 and 10 mM NaCl. The final step involved removing any unreacted enzyme by flushing the PPM

column with a solution of 50 mM NH_4HCO_3 (pH 7.4). The columns were then sealed with parafilm and stored at 4°C in the refrigerator.

5.3.5 In-Solution Enzyme Digestions

In-solution tryptic digestion of recombinant PrP was performed using a modified protocol from that of Qin *et al.*¹⁴⁰ A 1:2 solution of PrP stock in 0.1 M NH_4HCO_3 was prepared to a total volume of 15 μL . A 1 μL aliquot of a 1 mg/mL solution of trypsin was added to the vial and the vial was incubated in a Fisher Scientific Dry Bath Incubator (Ottawa, Ontario) at 37°C for 2 hours.

For an in-solution tryptic digestion of antitrypsin, 20 μL of antitrypsin in 100 μL of 0.1 M NH_4HCO_3 was incubated at 90°C for 15 minutes to denature the protein. Without the denaturing step no digestion of the protein would occur. Afterwards the solution was cooled and 5 μL of a 1 mg/mL trypsin stock solution was added to the vial containing the denatured antitrypsin. Digestions over a time of 30, 60, 90, 120 and 180 minutes as well as 24 hours were performed.

5.3.6 Enzymatic Digestions Using Tryptic Microreactors

Prior to digestion the sample of antitrypsin which consisted of 10 μL of a 2 mg/mL stock solution in 40 μL of 0.1 M NH_4HCO_3 was denatured in an incubator at 90°C for 15 minutes. After the sample had cooled, 400 μL of 50 mM NH_4HCO_3 and 300 μL of MeOH were added to the denatured antitrypsin sample. The resulting solution was then passed through a tryptic microfluidic chip with a syringe pump. The flow rate was 0.5 $\mu\text{L}/\text{min}$ resulting in a residence time of 2.8 minutes. The sample solution was collected for subsequent MS analysis.

For PrP digestion on a tryptic PPM column no pretreatment steps were necessary. A sample volume of 10 μL of the PrP stock solution was added to 990 μL of 50 mM NH_4HCO_3 and 600 μL of MeOH. The resulting solution was then passed through a tryptic PPM column at flow

rate of 0.5 μ L/min using a syringe pump. This flow rate gave a residence time of 8 seconds. The sample solution was collected for MS analysis. Unlike with in-solution digests of PrP, the sample solution did not have to be purified and concentrated with a C₁₈ ZipTip when an enzymatic microreactor was used.

All digested solutions were collected and analyzed off-line using an Applied Biosystems Voyager DE STR matrix-assisted laser desorption/ionization time of flight mass spectrometer (MALDI-TOF MS) (Framingham, MA, USA).

5.3.7 MALDI-TOF-MS Analysis of Enzymatic Digests

All digestions were analyzed using a Voyager DE STR matrix-assisted laser desorption/ionization time of flight mass spectrometer (MALDI-TOF MS) (Applied Biosystems, Foster City, CA, USA). A two-layer HCCA matrix¹⁸ was used for the analysis. The tryptic digest solutions were acidified with 1% trifluoroacetic acid prior to analysis and a minimum of three spots were analyzed for each protein digest performed.

For in-solution digests of PrP, the sample had to be purified and concentrated prior to MS analysis using a C₁₈ ZipTip. This step was able to be omitted when the digestion of PrP was performed using a tryptic enzymatic microreactor.

5.3.8 Data Analysis

The peptides obtained from tryptic digests of both PrP and antitrypsin were matched to theoretical peptide masses obtained from Protein Prospector (<http://www.prospector.ucsf.edu>) with a maximum of two missed cleavages and allowing for the variable modification of methionine oxidation. For antitrypsin the percent sequence coverage was calculated manually as the first 24 amino acids at the start of the amino acid sequence had been cleaved off that and this would not be accounted for if databases had been used to calculate sequence coverage. Sequence

coverage was calculated by taking the number of observable amino acid residues from the peptides identified by MS divided by the total number of amino acids in the protein.

5.4 Results and Discussion

5.4.1 In-Solution Digest of Antitrypsin

In-solution tryptic digests of antitrypsin, a weak protease inhibitor belonging to the serine protease inhibitor family has been performed. Initial digestions proved that antitrypsin had to first be denatured at 90°C for 15 minutes prior to digestion for any peptides to be seen. This was the expected result because if the protein is not denatured prior to digestion it would still be in its active form and thus could covalently bond to trypsin, and irreversibly inactivate it. Thus, the denaturing step proved to be a crucial step for a successful digestion.

Optimization of the in-solution digest of antitrypsin was performed by testing the digestions efficiency at different time periods measured by percent sequence coverage. A sequence coverage of 16%, 21% and 28% was obtained for in-solution tryptic digests carried out for 30, 60 and 90 minutes, respectively. All digestions were analyzed via MALDI-TOF-MS. A representative mass spectrum of a 2 hour tryptic digestion of antitrypsin is shown in Figure 5.1. Attempts to increase the sequence coverage by extending the digestion time resulted in no change in the sequence coverage. Specifically, a 24 hour in-solution tryptic digest of antitrypsin still yielded a 28% sequence coverage, the same sequence coverage that was obtained after a 2 hour digest. As a result, it was concluded that a 2 hour in-solution tryptic digestion of antitrypsin was optimum as it provided the highest sequence coverage in the least amount of time.

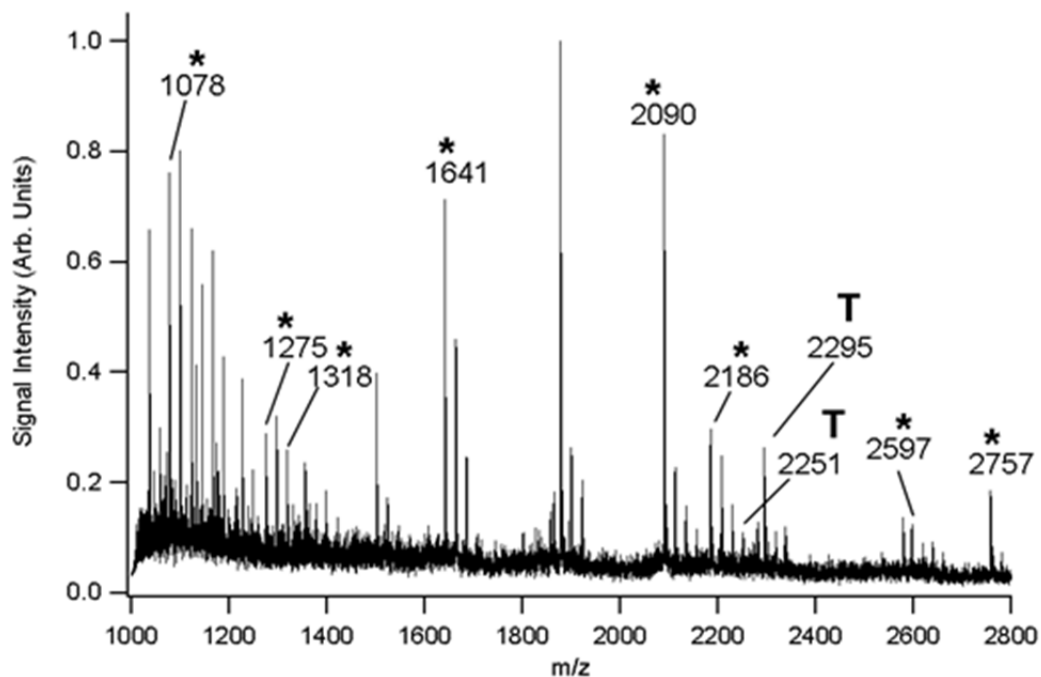


Figure 5.1 MALDI-TOF mass spectrum of a 2 hour in-solution tryptic digest of antitrypsin. A 28% sequence coverage was obtained. Peptides are indicated with a * and autolysis trypsin peaks are indicated with a T.

5.4.2 Tryptic Digest of Antitrypsin Using a Microfluidic Chip

Similar to the in-solution digest of antitrypsin, the protein had to be denatured prior to digestion on the fabricated tryptic microfluidic chip. After denaturing antitrypsin at 90°C for 15 minutes, the protein was flushed through the enzymatic microreactor at a flow rate of 0.5 $\mu\text{L}/\text{min}$ resulting in a 2.8 minute residence. A mass spectrum of the digestion is shown in Figure 5.2. The sequence coverage obtained for a 2.8 minute digestion on a microfluidic device surpassed that of even a 24 hour in-solution digest. With a 35% sequence coverage, the digestion of antitrypsin was higher than that of all the in-solution digestions. Furthermore, no inactivation of trypsin was observed over time as demonstrated by a 42 % sequence coverage obtained from a tryptic microfluidic chip that was 6 days old and had previously been used to perform an antitrypsin

digest. This indicates that antitrypsin has a negligible effect on enzyme activity and thus minimal to no inactivation occurred. These experiments demonstrate the advantages of using enzymatic microreactors as not only is the digestion time reduced from hours to minutes but the digestion efficiency is also increased.

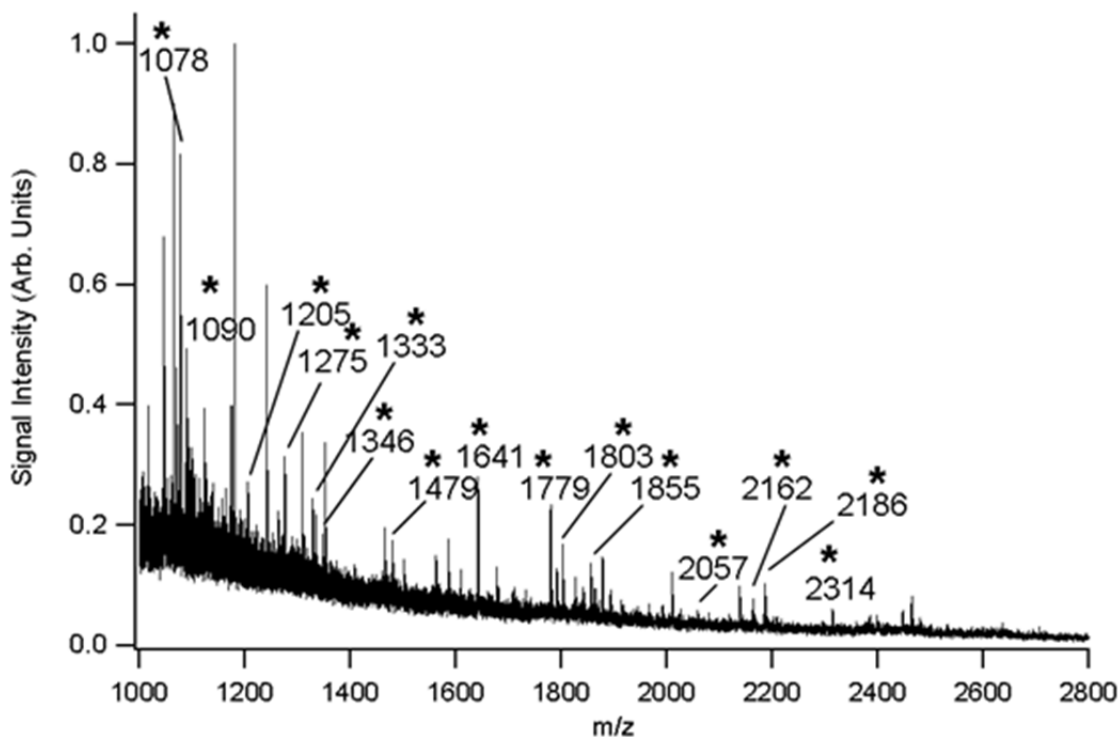


Figure 5.2 MALDI-TOF mass spectrum of an on-chip tryptic digest of antitrypsin. A 35% sequence coverage was obtained. The residence time of the microfluidic device was 2.8 minutes. Peptides are denoted with a *.

5.4.3 Comparison of Antitrypsin and Oxidized Antitrypsin

A deficiency of antitrypsin in the body results in conditions such as pulmonary emphysema, adult respiratory syndrome, cystic fibrosis and rheumatoid arthritis. As there are several variants of antitrypsin, there are many possible reasons why antitrypsin becomes inactivated. The analysis of tryptic peptide fragments can help to distinguish between various modifications of the protein

such as methionine oxidation, glycosylation or protein degradation. Previous research involving HPLC-MS analysis of peptides resulting from a cyanogen bromide (CNBr) digestion have shown that oxidation of methionine 358 and 351 result in the inactivation of antitrypsin.¹⁶² MALDI-TOF-MS analysis was performed to characterize peptide fragments obtained by tryptic digests obtained from both antitrypsin and oxidized antitrypsin samples. An in-solution digest of antitrypsin results in only one peptide fragment that contained methionine and thus does not easily permit the distinction between the two forms of the protein. However, an on-chip digest of antitrypsin produces more peptides containing methionine residues which are potential sites of inactivation. A comparison of peptides obtained from an in-solution tryptic digest and an on-chip tryptic digest of antitrypsin are shown in Table 5.1.

Table 5.1 Comparison of tryptic in-solution and on-chip digestions of antitrypsin. Peptides containing methionine residues are indicated in red as they are potential sites of inactivation. The residence time of the microfluidic chips was 2.8 minutes while the in-solution digestion was 2 hours.

Peptide Fragments		Type of Digest		
<i>m/z</i>	<i>Amino Acid Sequence</i>	<i>In-Solution Digest</i>	<i>On-Chip Digest</i>	
			<i>Day 1</i>	<i>Day 6</i>
1078.5281	FLENEDRR	*	*	*
1090.5686	WERPFEVK		*	*
1205.6782	LVDKFLEDVK		*	
1275.6850	GKWERPFEVK	*	*	*
1318.6764	LGMFNIQHCK	*		
1333.7731	LVDKFLEDVKK		*	
1346.6826	RLGMFNIQHCK		*	*
1479.7443	QINDYVEKGTQGK		*	*
1641.8641	ITPNLAFAFSLYR	*	*	*
1779.7687	TDTSHHDQDHPFNK		*	*
1803.9605	LQHLENELTHDIITK		*	*
1855.9781	FNKPFVFLMIEQNTK		*	*
1891.8561	DTEEDDFHVDQVTTVK			*
2057.9456	LYHSEAVTVNFGDTEEAK		*	*
2090.0963	ELDRDTVFALVNYIFFK	*		
2162.1345	VFSNGADLSGVTEEAPLKLSK		*	*
2186.0406	K/ LYHSEAVTVNFGDTEEAK/K	*	*	*
2314.1356	K LYHSEAVTVNFGDTEEAKK		*	
2597.3939	VFSNGADLSGVTEEAPLKLSKAVHK	*		
2757.5231	IVDLVKELDRDTVFALVNYIFFK	*		
% Sequence Coverage		28%	35%	42%

When oxidized samples of antitrypsin were digested on a tryptic microfluidic chip peptide peaks at *m/z* 1206, containing oxidized methionine 226 and *m/z* 1964 containing methionine residues 220, 221 and 226, two of which are oxidized, were observed. Furthermore, peptides observed at *m/z* 1346, 1490 and 2376 all contain methionine residue 250 with amino acid

sequences 247-257, 247-258 and 248-267, respectively. Of these three peptides, m/z 1346 and 2376 have non-oxidized methionine 250 while m/z 1490 has the methionine residue 250 oxidized. Also peptides containing methionine 374 in both its non-oxidized and oxidized forms were observed with peptides peaks at m/z 1855 (amino acid sequence 366-380) and 1871 (amino acid sequence 366-380).

As oxidized methionine residues are readily distinguished with this technique, the use of enzymatic microreactors show promise as a viable technique to test for oxidation sites once the digestion conditions have been optimized.

5.4.4 In-Solution Digestion of PrP

A two hour in-solution digest of PrP yielded two peptides as shown in the mass spectrum in Figure 5.3. The smaller peptide has the sequence YYR and consists of amino acid residues 127-129 with a m/z of 501. This peptide is too small to have any diagnostic value. However, a larger peptide with the sequence YPGQGSPGGNR was also observed. This peptide consists of amino acid residues 16-26 and has an m/z of 1089. The YPGQGSPGGNR peptide is unique to prion proteins and is a conserved sequence found in more than 50 different species including bovine, deer and sheep. As this sequence is specific to prion proteins it can be used as a biomarker for unambiguous identification of PrP.^{142, 143} Also, since the N-terminal region up to amino acid residue 121 comprises the “random coil-like” part of the protein and is not involved in either alpha-helix or beta-sheet formation¹⁴⁴ this peptide sequence should be able to be observed in both PrP^C and PrP^{Sc}.

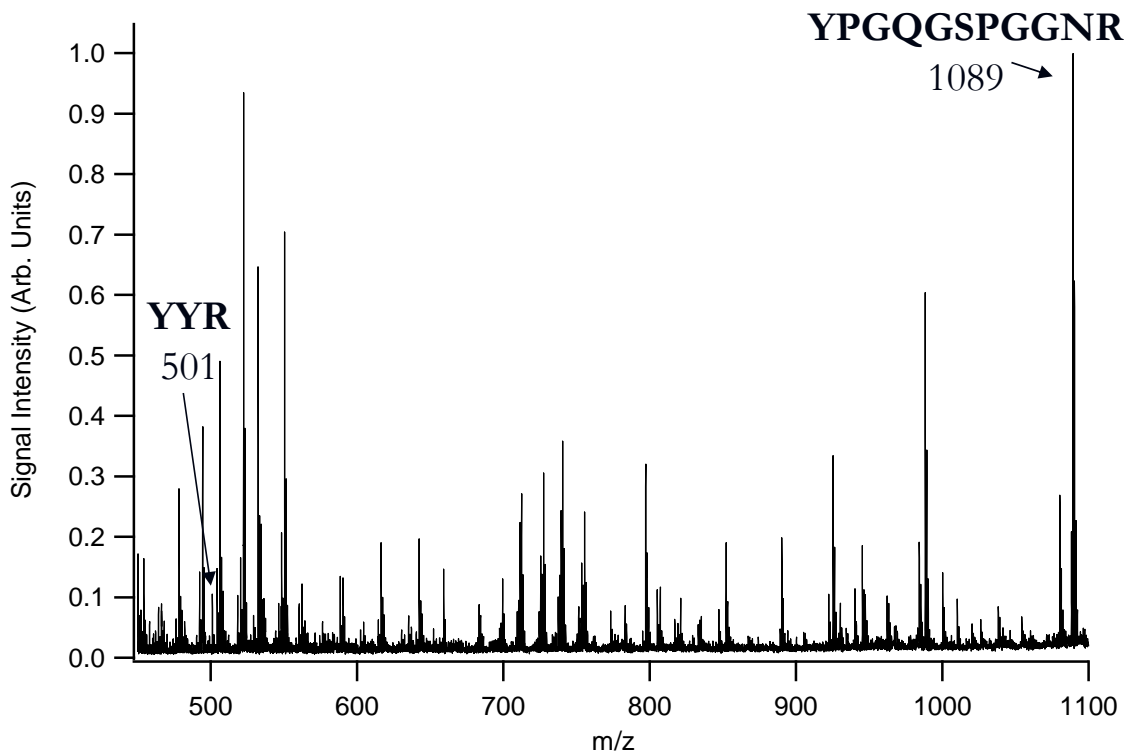


Figure 5.3 MALDI-TOF mass spectrum of a 2 hour in-solution tryptic digest of PrP. Two peptides were observed the larger of which provides unambiguous identification of PrP.

5.4.5 Tryptic Digest of PrP Using a PPM Column

Digestion times for PrP were able to be reduced from 2 hours to 8 seconds by the introduction of tryptic PPM columns. Not only was the time required for digestion significantly reduced but digestions performed on a tryptic PPM column also yielded an additional peptide as shown in Figure 5.4 that can be used to help identify PrP. Also of importance was that unlike with in-solution digests of PrP, when enzymatic microreactors were used no purification or concentration step using C₁₈ ZipTips was required before analysis. Thus not only is the digestion time reduced but sample handling steps are also eliminated making this a much simpler and faster digestion technique.

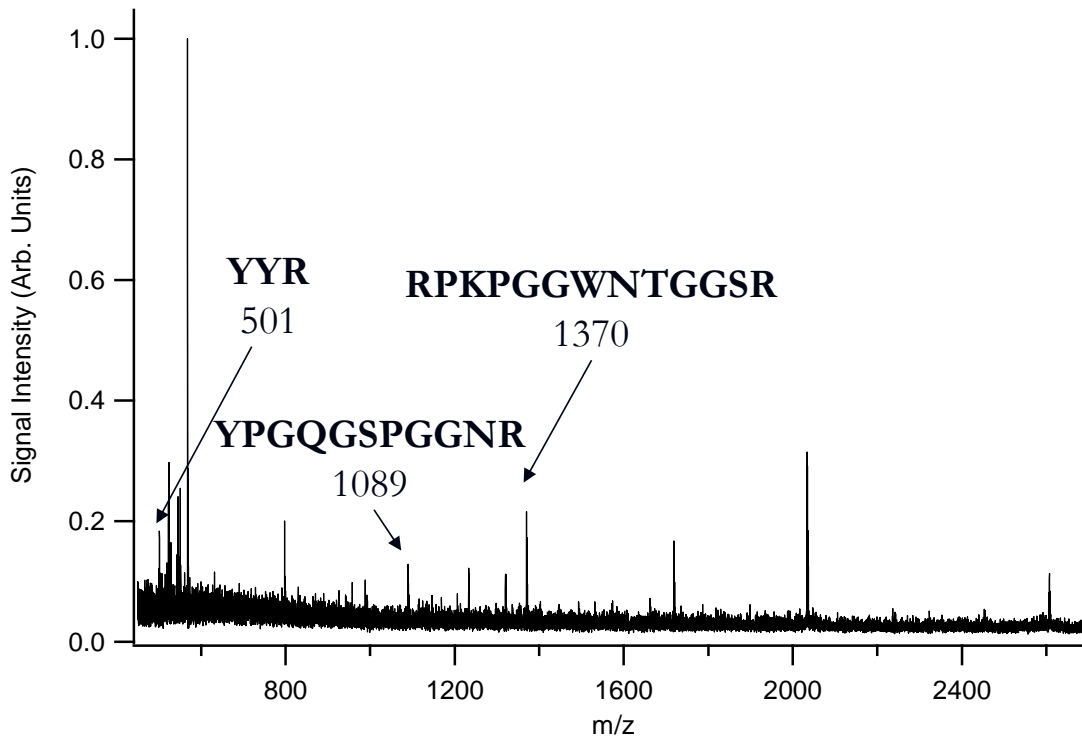


Figure 5.4 MALDI-TOF mass spectrum of a PPM tryptic digest of PrP. Three peptides were obtained, two of which can be used to identify of PrP. The residence time of the PPM column was 8 seconds.

5.5 Conclusions

Enzymatic microreactors including microfluidic chips and PPM columns have shown promise in providing more information about proteins that are linked to various diseases. Specifically it was possible to distinguish between the oxidized and non-oxidized forms of antitrypsin when digestions were performed with a tryptic microfluidic chip. Aside from providing a higher sequence coverage when microfluidic chips were used, 35% in comparison to 28%, the time of digestion was reduced from 2 hours to 2.8 minutes. Future work would entail testing antitrypsin samples taken from ICU patients with DIC and compare them to healthy

individuals to see if oxidation of methionine residues is evident in patients with DIC. In addition to using enzymatic microreactors with antitrypsin, PrP samples were also studied using tryptic PPM columns. In comparison to in-solution digests an additional peptide was observed that can be used to identify PrP and the digestion time was reduced from 2 hours to 8 seconds.

Furthermore, when tryptic PPM columns were used no purification or concentration steps of the sample were required prior to MS analysis. The application of enzymatic microreactors to protein samples that are traditionally “difficult” to digest demonstrates their potential to be used for clinical research.

Chapter 6

Conclusions and Future Work

As technology is rapidly advancing, researchers are striving to use new sensitive techniques to find out answers to questions that were previously unexplainable. With current techniques an abundance of information has been gained in the area of proteomics but there still exists a lot of unknown information. Although there are many sensitive techniques available for testing of proteins samples it is often a challenge to deal with protein samples that are a mixture of components and/or have limited sample volume available for testing. Herein, work has been presented that allows for analysis of small sample volumes as well as offers the capability of analyzing proteins that are difficult to study using traditional techniques or are in an incompatible matrix for protein testing.

Enzymatic microreactors have been fabricated using an inexpensive polymeric material, poly(methyl methacrylate) (PMMA) that can be patterned using relatively low temperatures and without the use of clean room. PMMA microfluidic devices have been fabricated using a fused silica capillary to emboss linear channel structures in the substrate. This also permits patterned modifications on the polymer surface through the positioning of the capillaries. By selectively positioning the capillary pieces, the polymer surface can be selectively exposed to surface modifications such as the immobilization of enzymes on the surface. This fabrication technique allows for temperature sensitive enzymes to be patterned on the microfluidic surface post bonding. However, if a more complicated channel pattern is desired, a nickel master may be used in combination with a low temperature solvent bonding method. Modification of a low temperature solvent bonding protocol enables a variety of enzymes (e.g. temperature, pH and

solvent sensitive enzymes) to be immobilized to the surface. Immobilized enzymes are more resistant to normal deactivation that can occur in the presence of organic solvents. Furthermore, a slight pH modification in the solvent bonding method allows for pepsin, a pH sensitive enzyme, to be immobilized on the surface prior to bonding the substrate/cover plate. The fabricated PMMA microreactors have been shown to maintain enzymatic activity for at least a month with the ability to still yield positive identification of a protein. These devices also have the capability of reducing digestion times from hours to a mere 2.4 seconds. Future work to explore the full potential for the chip-based enzymatic microreactors would be to develop separation and sample clean-up capabilities directly on the microfluidic device.

Enzymatic microreactors were also fabricated using porous polymer monoliths (PPMs) as a substrate in fused silica capillaries. These devices are advantageous because they have the ability to be directly coupled to mass spectrometry as emitters to perform nano-electrospray (nano-ESI). A novel immobilization procedure was developed that not only allows for a variety of enzymes to be immobilized to the PPM surface, but also allows more enzymes to be re-immobilized to the surface. Thus, as the enzymatic microreactors have a lifetime of about a month as a result of the enzyme activity decreasing over time, the enzymatic capabilities of the device can be regenerated by re-immobilizing enzymes to the surface. This is a result of the generation of new sites of attachment on the PPM surface. These PPM enzymatic microreactors have been utilized for both off-line and on-line enzymatic digestion with nanoelectrospray mass spectrometry (nano-ESI-MS). As enzymatic microreactors they offer a large surface-to-volume ratio that provides efficient digestions of proteins in seconds while using a minimum sample volume. As nano-ESI emitters they offer the capability of providing a stable electrospray without suffering from the problem of clogging that is associated with traditional tapered emitters. Preliminary work has been done to couple a PPM emitter with a separation column, however this

could easily be transformed in future work to have an all-encompassing device capable of separation and digestion while at the same time acting as a nano-ESI emitter.

Unfortunately proteins are not always in a compatible matrix for analysis. A filtration technique has been developed that allows proteins to be removed from an organic sample matrix that is not suitable for analysis of infectious proteins by routine animal or immunoassay testing. The filtration method not only allows direct analysis of proteins on the filtration disk via matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), but also subsequent on-disk enzymatic digestion for standard proteins or for more complicated proteins extraction into a compatible solvent for further testing. The developed filtration/extraction method has been applied to study the safety of biodiesel derived from animal sources, specifically detecting the presence of prion proteins (PrP) in a biodiesel sample. Enzymatic digestion employed to the filtration disk post-filtration allows the PrP protein to be unambiguously identified via MALDI-MS. Although the use MALDI-MS for analysis in this study does not provide the necessary sensitivity to ensure the safety of biodiesel for human use, the extracted protein sample is amenable to more sensitive techniques such as immunoassay testing. Future work should focus on exploring the full potential of this extraction technique for dealing with incompatible matrices and applying the cleaned-up sample to immunoassay techniques. This technique opens up a possibility of using animal waste material as an alternate fuel source rather than wasting its potential.

The use of enzymatic microreactors has been established using a wide variety of standard proteins which are pure and relatively easy to digest including cytochrome c, bovine serum albumin (BSA) and myoglobin. However sometimes in proteomics, proteins are encountered that are difficult to digest via standard digestion techniques. This limits the amount of information that

can be gained. Using the developed enzymatic microreactors, either the PMMA microfluidic chip or PPM column, non-standard proteins that are difficult to digest using standard in-solution digestion techniques were digested. The two specific proteins that were studied include PrP which is linked to bovine spongiform encephalopathy (BSE) and antitrypsin which when defective or present in deficient quantities can result in numerous medical complications as a result of uncontrolled neutrophil elastase. In both cases the enzymatic microreactors were able to provide more information about the studied proteins due to increased digestion efficiency.

The future generation of enzymatic microreactors has been developed using silica-based microstructured fibers (MSF). MSFs offer the advantage of having multiple channels that are capable of electrospray which eliminates clogging problems associated with commercially available tapered emitters. These multiple channels also provide a high surface-to-volume ratio for enzymatic digestion. Also MSFs provide a very stable electrospray over a wide range of flow rates while at the same time offer the capability of maintaining a stable electrospray in both organic and aqueous conditions. It has always been challenging for researchers to have a stable electrospray with high aqueous conditions which can limit the ability to perform on-line digests as enzymes have better activity in aqueous conditions and high organic contact can actually denature enzymes. Thus these MSFs are a breakthrough in providing the most efficient on-line digestions as optimal digestion conditions can be employed. Preliminary results using a Schiff base immobilization procedure to develop a tryptic microreactor show a sequence coverage of 70% for cytochrome c in a residence time of 60 milliseconds.

The development of multiple types of enzymatic microreactors provides the opportunity for a wide range of testing for previously “difficult to digest” samples or non-robust enzymes whether it be a result of sample matrix, protein structure or protein sensitivity. For example a

higher sequence coverage can be obtained for the studied protein, purification steps prior to analysis can be eliminated and the digestion time can be reduced from hours to as little as 8 seconds. These enzymatic microreactors can be fabricated using robust enzymes such as trypsin or pH and temperatures sensitive enzymes such as pepsin. Although future work needs to be conducted to demonstrate the full potential of the developed enzymatic microreactors, prion proteins were successfully identified using these devices and antitrypsin was characterized for oxidation of methionine residues. Additional research into the oxidation of methionine residues for antitrypsin samples obtained from both healthy and sick individuals using these devices could provide further insight into the causes of disseminated intravascular coagulation.

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Appendix A

Explanation of Sequence Coverage

Sequence coverage has been chosen to quantitatively assess enzymatic microreactors both in terms of longevity and digestion efficiency. Sequence coverage is defined as the number of number of observable amino acids divided by the total number of amino acids in a protein. Based on this definition if the term is used improperly it can result in misleading results for quantitative measurements. For example, the purpose of using sequence coverage for measuring digestion efficiency is to determine the number of amino acid residues represented by the observed peptides, however, if the number of missed cleavages is not set to a low value one would always obtain a high sequence coverage regardless of the experimental conditions and thus the results would be meaningless for quantitative purposes. However, if a researcher was interested in gaining all the possible information about a given protein then they would want to consider all peptide peaks and missed cleavages would not be taken into consideration.

Missed cleavages represent the number of sites where the enzyme could have cleaved the amino acid sequence but did not. Trypsin which is frequently used in this project because of its specificity, cleaves the peptide bond on the C-terminus side of lysine and arginine amino acid residues unless they are followed by proline. Along the amino acid sequence if a lysine or arginine residue is found that is not followed by proline and the amino acid chain is found to be intact at this location then this would be counted as a peptide with a missed cleavage. It is possible to have multiple missed cleaves in a given peptide. As a result if one allows for a very

high number of missed cleavages the protein would not have to be digested at all to get a 100% sequence coverage. For example, cytochrome c which is a relatively small protein with 105 amino acid residues has 21 possible cleavages sites for a tryptic digestion. Consequently, if the number of missed cleavages was set to 21 or higher a 100% sequence coverage could be given for the intact protein yet no digestion took place.

As a result to obtain meaningful data that permits quantitative assessment of the developed enzymatic microreactors the number of missed cleaves was kept constant at a low value of either 2 or 3. This allows for a couple of missed sites but would still represent a reasonable digestion by the tested microreactor. By setting stringent values for the number of missed cleavages of a given protein sequence, sequence coverage can be used to compare the longevity of the enzymatic microreactor and the digestion efficiency. A high sequence coverage with minimal missed cleavages indicates that the enzyme is performing efficiently and cleaving the majority of sites along the amino acid chain. Furthermore, measuring the change in sequence coverage over time, provided that the number of missed cleavages is kept constant, provides a way to monitor the loss of enzyme activity thus allowing a lifetime to be determined for the enzymatic microreactor.

A representative mass spectrum and sequence coverage plot of a cytochrome c digest performed on a tryptic microfluidic chip is shown in Figure A. 1.

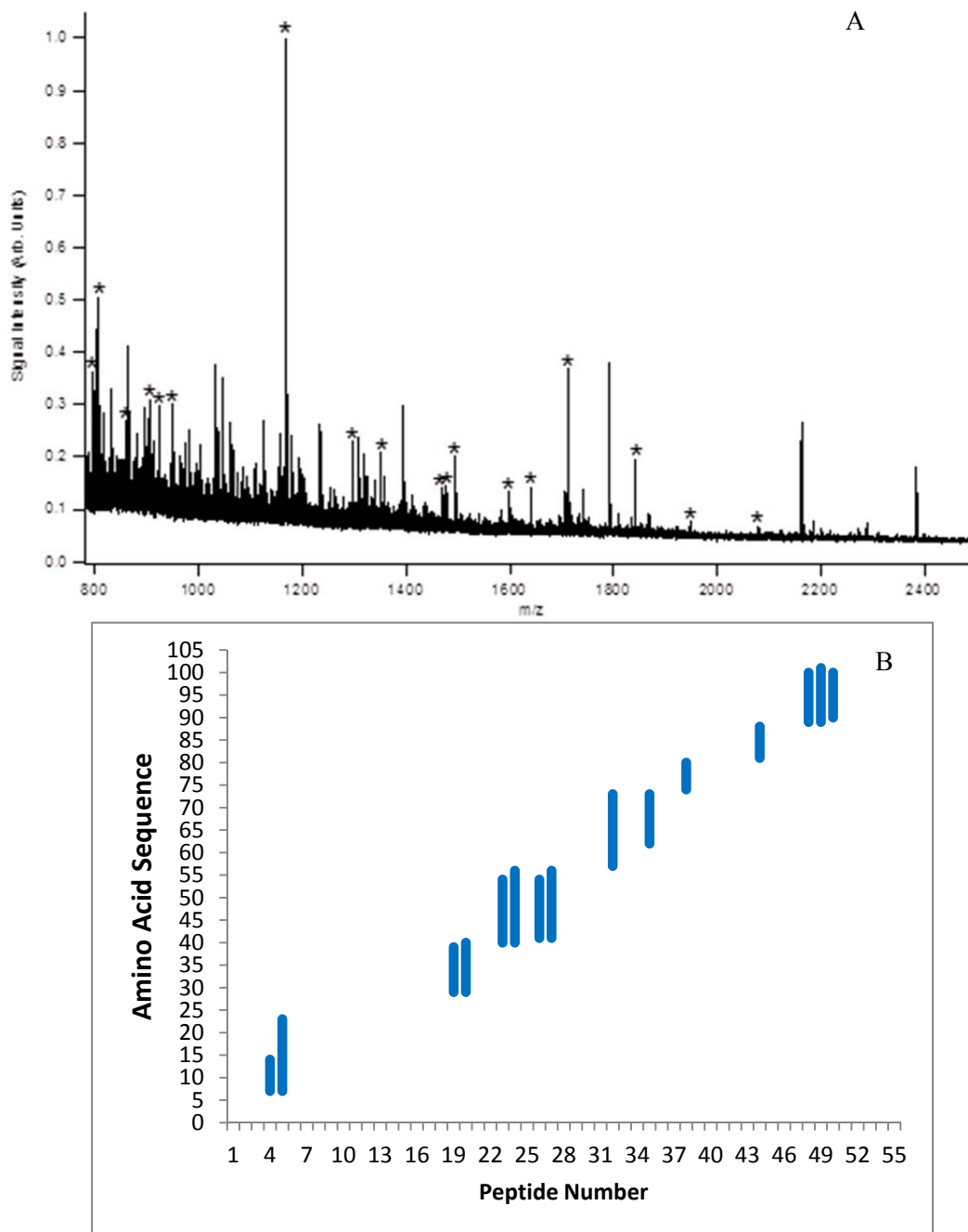


Figure A. 1 MALDI-TOF mass spectrum of an on-chip tryptic digest of cytochrome c (A). Peptides are indicated with a *. A sequence coverage of 89% was obtained after low temperature solvent bonding of the microfluidic chip. The residence time of the protein in the microreactor was 2.4 seconds. The enzymatic microreactor bed had a width of 100 μm , a depth of 20 μm and a length of 10 mm. A sequence coverage plot of the above mass spectrum is shown in B.