THE UNIVERSITY OF HULL

Toward a Microfluidic System for Proteomics

being a Thesis Submitted for the Degree of Doctor of Philosophy

The University of Hull

By
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Abstract

There has been much interest in the application of microfluidic systems to proteomics in recent years. This is because of the many unique advantages afforded by the reduced dimensions of microfluidic systems compared to classical methods. The reduction in the reaction vessel dimensions leads to a high degree of control, higher sensitivity, better selectivity and reduced analysis time.

Initial experimental results have shown the possibility for developing a novel approach for proteomic analysis. A highly efficient protein digestion microdevice was fabricated using commercially available immobilized trypsin on agarose beads, packed into a silica capillary and connected directly to an electrospray mass spectrometer *via* a 'microtight T' connector, from which aqueous acetic acid (0.2 %) was pumped to the mass spectrometer ion source. Six proteins with molecular masses ranging from 2846 to 77703 Da were digested separately, each within eight minutes using this system. In a second set of experiments a short monolithic separation column was fabricated and placed after the immobilized trypsin capillary. This system demonstrated partial separation of the tryptic peptides generated, and detection limits in the pmol range were obtained by utilization of this separation column.

The methodology was then transferred to a glass microchip and a novel system was fabricated. The system consists of an on-chip protein digestion channel and on-chip monolithic ion exchange separation column. The digestion system performance was evaluated using single proteins and mixtures of protein. Significant reduction in the digestion time was observed in comparison to the traditional digestion method and a complete digestion was obtained for cytochrome C in less than a minute while a protein mixture was digested within five minutes.

The on-chip separation column was initially evaluated using the tryptic digest of cytochrome C. High column efficiency was obtained as indicated from the peak width at half height measurement data (varying between 19.0 to 24.9 s). A

detection limit of 3.7 pmol was obtained; lower detection limits may be obtained if a nano-electrospray source were to be used. The separation column was also evaluated by separating a tryptic digest of a mixture of four proteins. Results showed at least 40 % improvement in the sequence coverage due to the separation achieved. In another experiment, the system was successfully used for protein digestion and a first dimension separation followed by an off-line second dimension separation. This was demonstrated using a tryptic digest of BSA and a number of additional peptides were identified as a result of the first dimension separation carried out using the microfluidic system. The system was also tested with a biological sample and initial results gave positive indications; however, this has to be confirmed after optimization of the protein extraction method. The developed system can be used along with the commercially available on-chip reverse phase columns to perform on-chip protein digestion and two dimensional separation of the generated peptides in a high throughput system for the analysis of biological samples.

Another novel microfluidic system was also fabricated. The system consists of an on-chip protein digestion channel and on-chip monolithic reverse phase separation column. The microfluidic system was evaluated using cytochrome C, BSA and a mixture of four proteins. The separation and digestion were completed within one hour. However, the column performance was lower than that of the on-chip monolithic ion exchange column previously fabricated as indicated from the peak width at half height measurement data (varying between 30.0 to 115.0 s).

Declaration

The work described in this thesis was carried out in the Department of Chemistry, The University of Hull under the supervision of Dr. K. J. Welham and Dr. P. Watts between September 2004 and September 2007. Except where indicated by references, this work is original and has not been submitted for any other degree.

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Commonly used abbreviations

- Two dimensional polyacrylamide gel electrophoresis. (2D-PAGE)
- Mass spectrometer. (MS)
- Bioinformatics. (BInfo.)
- Matrix assisted laser desorption ionization. (MALDI)
- Time of flight. (TOF)
- Peptide mass finger print. (PMF)
- Scanning electron microscope. (SEM)
- Collision induced dissociation. (CID)
- Liquid chromatography. (LC)
- Electrospray ionization. (ESI)
- Quadrupole ion trap. (QIT)
- Post source decay. (PSD)
- Quadrupole. (Q)
- Triple quadrupole. (Tri.Q)
- Fourier transform ion cyclotron resonance. (FT-ICR)
- Ion exchange. (IE)
- Reverse phase. (RP)
- Capillary electrophoresis. (CE)
- Capillary electrochromatography. (CEC)
- Multidimensional protein identification technology. (Mud PIT)
- Infrared multiphoton dissociation. (IRMPD)
- Electron capture dissociation. (ECD)
- Solid phase extraction. (SPE)
- On-chip capillary electrophoresis. (Chip-CE)
- Micellar electrokinetic chromatography. (MEKC)
- High performance liquid chromatography. (HPLC)
- Strong cation exchange. (SCX)

1.0 Introduction

1.1 Proteomics.

One of the major challenges in biochemical science is to understand how a biological process occurs at the molecular level and how these processes are altered in disease states. [1] This makes proteomics currently one of the most active fields of research worldwide. Proteomics is the science concerned with characterization of the total protein expressed by a living cell and how this expression changes under the influence of biological or environmental perturbation, [2,3] for example, when a cell or tissue is exposed to a certain type of drug. In many cases, a comparison between normal and diseased cell or tissue is carried out to find which proteins are expressed differently. Finding any post-translational modification of a protein is another objective which proteomic studies try to achieve. [2,4] Determining the interaction between different proteins present in a living system is also an important objective of some proteomic studies. [5] Usually, proteins carry out their function in close association with other proteins present in the cell. Therefore, determining the interaction between these proteins becomes important.

Proteomics is considered a very challenging science. This is because of the very large number of proteins with various molecular masses present in different concentrations in a single sample. Additionally their structure and concentration may be affected by biological or environmental perturbation.[3,6]

Proteins are complex biological molecules which consist of 19 types of α-amino acids and imino proline linked together to form long chains of polypeptides. The order in which amino acids link together varies from one protein to another. This is known as the amino acid sequence; it is also described as the primary structure of a protein. The local regions of regular structure (*i.e.* α-helices, β-strand and β-turns) are known as secondary structure and the three dimensional folded structure of a protein is known as tertiary structure. [7]

The two ends of a protein are different; one end has a free α- amino group and is termed the N-terminus, whereas the other end has a free carboxylic acid group and is termed the C-terminus. In some cases some modification may occur at one or both terminii. Acetylation is the most frequent modification at the N-terminus, while amidation is the most frequent one at the C-terminus. Another important modification that occurs in some peptides involves the amino acid cysteine; two molecules of cysteine react to give a disulfide bridge. [7]

Over the past ten years proteomics has developed rapidly and several methods of analysis now exist to identify proteins present in a sample. These methods vary in their strengths and limitations. Generally, methods in proteomics are based on two strategies: bottom up or top down. The first one is based upon analytical measurement for a set of peptides generated from digestion of a single protein or a mixture of proteins, while the second strategy is based upon analytical measurement directly from the intact proteins.

1.2 Classical method.

The classical method is based on the bottom up strategy and it refers here to the most commonly used method in proteomics. This method is based on the use of two dimensional polyacrylamide gel electrophoresis (2D-PAGE), mass spectrometry (MS) and bioinformatics (BInfo.).

Initially, the protein content of a cell or tissue is solubilized. In most cases purification of the protein fraction is required while in some instances pre-fractionation may also be required before applying a sample to the 2D-PAGE gel. Once the separation is carried out by 2D-PAGE, detection is performed using staining. Coomassie blue and silver staining are most commonly used. Once detected, selected spots are excised and digested using trypsin in most cases.[8] Digestion is a chemical reaction in which enzymes or some chemicals are used to cleave proteins into peptides. Protein digestion is a very important step in most current proteomics methods. This is because proteins cannot be easily eluted from gel and in some cases, large proteins do not possess a single molecular mass

due to their heterogeneous nature. [9] Additionally, digestion of proteins generates peptides with molecular masses well suited for detection with a mass spectrometer.[2] Moreover, the data obtained from MS analysis of peptides can be taken directly for comparison to the protein sequence derived from a protein and/ or nucleotide-sequence database.

Protein digestion is most commonly carried out using enzymes. This is because most enzymes are very specific and protein cleavages are carried out at specific sites. Several enzymes are in use, for example, trypsin, chymotrypsin and the endoproteinases Glu C, Lys C and Asp N. However, trypsin is the most widely used, because of its availability and specificity. Trypsin cleaves proteins at the C-terminal side of lysine and arginine residues, unless either of these is followed by a proline. The dual specificity of this enzyme means that it will cut proteins much more frequently than other enzymes that cut at only one amino acid residue. Glu C is another enzyme which is less frequently used; it cleaves at the C terminal side of glutamate residues. The different cleavage specificity of this enzyme compared to trypsin makes it a useful tool to provide complementary information to that from trypsin digestion. Other enzymes are much less frequently used, either because they generate fewer peptides (*e.g.* Lys C) or they generate small peptides due to high cleavage frequency (*e.g.* chymotrypsin).[2] Chemical cleavage methods are much less frequently used in proteomics. Cyanogen bromide is the most commonly used chemical reagent in protein digestion, cleaving at methionine residues. Usually, large peptides are generated due to the relative infrequency of methionine residues in most proteins and these large peptides do not always yield useful sequence data. The peptides generated from the digestion process are then extracted from the gel. [10] A mass spectrum is obtained using a matrix assisted laser desorption ionization mass spectrometer (MALDI) for the tryptic digest. This is known as a peptide mass fingerprint (PMF) mass spectrum. The spectrum contains ions for the molecular masses of the peptides produced. The PMF spectrum is specific in that if a protein is digested by a certain protease, it will produce specific peptides; hence, the mass spectrum obtained for this digest will act as a fingerprint for the protein. The PMF mass spectrum is then

searched against a database of known protein sequence to try and find a match and thus identify the protein. If the protein is not identified then further studies using a tandem mass spectrometric (MS/MS) method are required to obtain sequence identification from individual peptides within the PMF mixture. This is carried out in a process known as collision induced dissociation (CID). In this process peptide ions collide with neutral gas atoms and the kinetic energy absorbed induces fragmentation of the peptide into fragment ions and neutral fragments. The fragment ions are then analysed and a product ion spectrum is produced. The data in the product ion spectrum allows the sequence of the peptide to be deduced. Tandem mass spectrometry experiments can be carried out by a range of instruments, although commonly an advanced MALDI with hyphenated time of flight mass analyser (MALDI-TOF-TOF) instrument or a liquid chromatograph interfaced to electrospray ionization with a quadrupole ion trap mass spectrometer (LC-ESI-QIT-MS) are used. The LC-ESI-QIT-MS technique is usually more sensitive and can separate the peptides by LC prior to MS/MS analysis. [8] Figure 1 illustrates the commonly used steps in classical proteomics methodology. The key techniques in classical proteomics (2D-PAGE gel, MS and BInfo.) are discussed below.

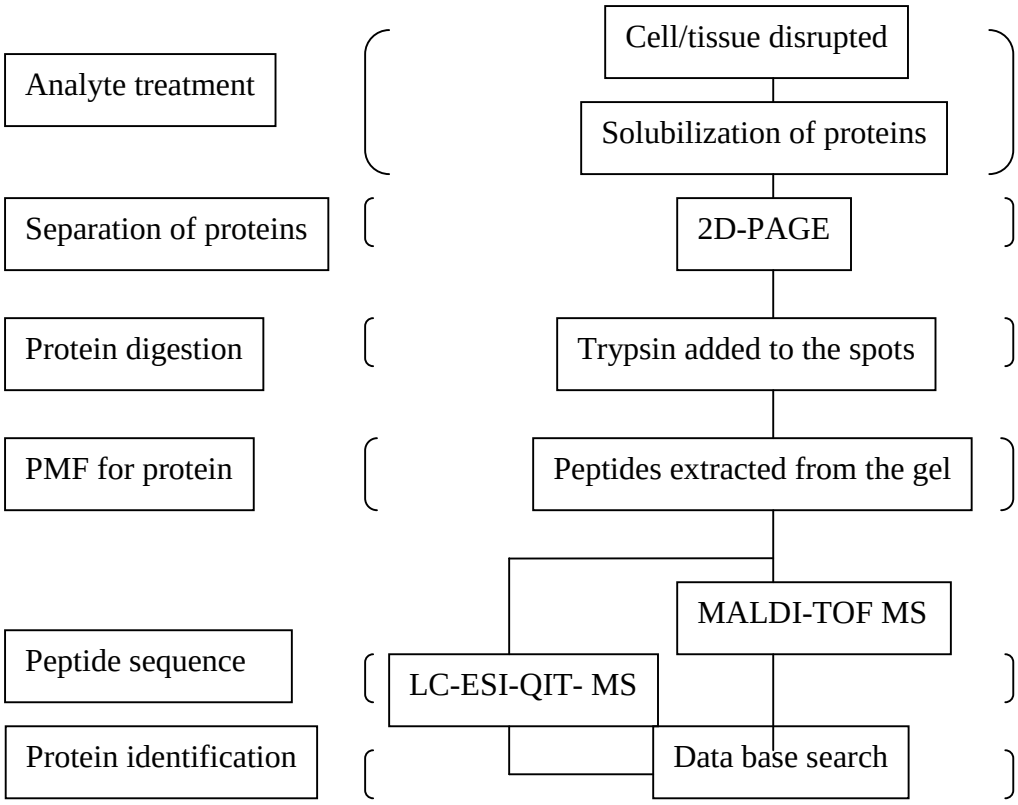


Figure 1: Procedure for classical proteomics.

1.2.1 Two dimensional polyacrylamide gel electrophoresis.

The 2D-PAGE technique was introduced in 1975. However, it was not well established until the 1990s due to the difficulties of the experimental setup and also due to the lack of techniques capable of analysing 2D-PAGE separated proteins. The introduction of immobilized pH gradient gels for isoelectric focusing (IEF) solved the most important experimental difficulty, which was reproducibility of the pH gradient. The introduction of mass spectrometric techniques which are capable of analysing thermally labile molecules solved the problem associated with the analysis of proteins separated by 2D-PAGE. [11,12]

The 2D-PAGE gel is capable of separating thousands of proteins. This high resolving power is due to the fact that the separation is based on two independent characteristics of proteins. The first separation is carried out based on the charge of proteins while the second separation is based on the molecular mass of proteins. Another important advantage of 2D-PAGE is that it provides an image "snapshot" such that the investigator can compare changes in the proteome based

on changes in the patterns of spots on the gel. Additionally, the gel can be stored for a long time at room temperature (even for months) and the separated proteins can be identified later by MS. Moreover, the experimental setup is not expensive and it is well suited for individual laboratories. Despite these advantages, 2D-PAGE has several important drawbacks, the most important being the limited capability of 2D-PAGE in detecting certain types of protein, for example, low abundance proteins which play, in many cases, key rolls in cellular processes. Very small and very large proteins are generally not observed under standard conditions. Membrane proteins are another group of proteins that cannot be detected easily in 2D-PAGE due to the low solubility of these proteins. Moreover, poor reproducibility is another drawback often observed in 2D-PAGE. Additionally, the technique is labour intensive and the throughput is low. For a high throughput analysis, separation and identification should be carried out in a flow system or using a robotic system; neither is easily implemented with the 2D-PAGE technique.[11]

To overcome some of the drawbacks of 2D-PAGE, Ünlü *et al.* [13] used fluorescent tagging of two samples with two different dyes, running them on the same 2D-PAGE gel. The amine reactive dyes were designed to ensure that proteins common to both samples have the same relative mobility regardless of the dye used to tag them. The technique is called differential in-gel electrophoresis (DIGE) and it is especially useful in cases where there is a need to compare several 2D-PAGE gels. However, the technique lacks a method for normalization of the background intensity and hence can be misleading for assessing changes in protein quantity, [14] which is based on intensity of the stain.

1.2.2 Mass spectrometry.

Mass spectrometry is the second main technique in the classical proteomics method. MS is an instrumental analytical technique in which qualitative and quantitative information about the protein samples is obtained. This is achieved by the formation of gaseous ions from protein samples and subsequent separation of these ions according to their mass to charge ratio. The signal from the

separated ions is then detected by a suitable detector and amplified. The resulting mass spectrum is a graph of relative abundance of the ions versus mass to charge ratio. [15,16] Figure 2 shows the main parts of a mass spectrometer.

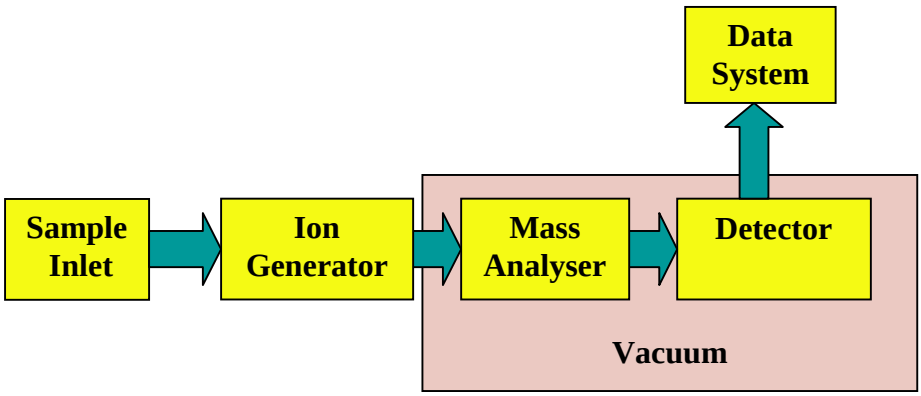


Figure 2: Main components of a mass spectrometer.

The discovery of MS goes back to the early years of the previous century. Sir Joseph John Thomson pointed out that it may be possible to use MS for molecular structural elucidation "The positive rays thus seem to promise to furnish a method of investigating the structure of the molecule". [17] However, the analytical applications for MS started nearly half a century later. [18]

Initially different types of molecule were analysed with a trend towards the analysis of complex molecules. Proteins and biological molecules remained a challenge because they decomposed at elevated temperatures. By the 1980s a new era for MS was established with the use of desorption techniques as ion production methods. These techniques provided valuable tools for the analysis of proteins and other thermally labile molecules. As a result, there has been a tremendous upsurge in the application of mass spectrometry to the analysis of proteins and other biological molecules over the last 20 years. [18]

Several ion generation techniques can be used for analysis of proteins and other biological molecules. These ion generation techniques can be divided into two categories: the first of these is sudden energy [19] or desorption [20] techniques, which involve very rapid vaporization of the analyte usually by striking analyte

molecules with energetic particles. [19] This rapid energy input causes vaporization of the analyte before decomposition can occur. [21] Some examples of this technique are: desorption chemical ionization, secondary ion mass spectrometry,[22] fast atom bombardment,[23] plasma desorption, [24] low energy particle impact, [21] low energy particle impact with liquid surface, [21] flow FAB [25] and matrix assisted laser desorption ionization (MALDI). [26]

The second category is nebulization ionization, [21] also known as spray techniques. [19] Nebulization ionization methods use strong electrostatic fields to extract ions from the analyte. These methods include electrohydrodynamic ionization,[27] field desorption, [28] thermospray, [29] atmospheric pressure chemical ionization [30] (APCI) and electrospray ionization [28] (ESI). Table 1 summarizes these ion generation techniques.

Type of ionization techniques	**Sub category**
Sudden energy	Desorption chemical ionization
	Secondary ion mass spectrometry (SIMS)
	Fast atomic bombardment (FAB)
	Plasma desorption
	Low energy particle impact
	Low energy particle impact with liquid surface
	Flow FAB
	Matrix assisted laser desorption ionization (MALDI)
	Electrohydrodynamic
Nebulization (Spray)	Field desorption
	Thermospray
	Electrospray (ESI)
	Atmospheric pressure chemical ionization (APIC)

Table 1: Ion generation techniques for thermally labile molecules.

In proteomics, the most common ion generation techniques used are MALDI and ESI, consequently these are discussed in greater detail below.

1.2.2.1 Matrix assisted laser desorption ionization method.

MALDI was introduced by Tanaka and Karas and Hillenkamp in 1988. [31] The sample is mixed with an excess of saturated matrix solution and approximately 1 to 2 µl of the mixture is then applied to the target plate. The target plate is then left at room temperature to dry. A vacuum lock is used to insert the plate into the source region of the mass spectrometer. The laser fires a beam of light to the target. The sample-matrix absorbs the energy, evaporates and the charged ions formed are accelerated toward the detector.

1.2.2.1.1 Mechanisms for ion formation in MALDI.

Ion formation in MALDI is a very complex process and it is one of the current active areas of research. [32] However, the currently accepted mechanism is based on two stages of ionization: primary and secondary. [33] The primary ionization stage refers to ions generated directly from the sample-matrix mixture, while the secondary ionization stage refers to ions which are not directly derived from the primary process and detected by the mass detector. The secondary ionization results from ion-molecule reactions in the desorption plume, where individual molecules transfer from the condensed phase to the gas phase. [33]

Two different models have been suggested for primary ionization: the cluster model[34] and photo-excitation model. [35] The principle difference between the two models is in the role of the matrix in the ionization process. The cluster model suggests that the matrix does not play a key role in the ionization processes, while the photo-excitation model is based on the idea that the matrix is crucially involved in the primary ion formation mechanism. [33]

The cluster model was introduced by Karas *et al*.[36] and it is based on the observation that singly charged ions in MALDI mass spectra are predominant. The model assumes that large biomolecules (*e.g.* proteins) are present in the form

of multiply protonated precursors in the low pH media of the matrix (analogous to ESI-MS). These biomolecules are liberated within clusters of matrix upon laser irradiation. The clusters would then lose neutral matrix in the plume region and decrease in size down to the embedded analyte ion. The generation of a substantial number of electrons by photo-ionization in the plume region will then reduce the charge state of the embedded analyte ion and the majority of the analyte ions would be neutralized. However, some analyte ions, "lucky survivors," will survive as singly charged ions which are detected in MALDI mass spectra.

The initial high ion velocity of the analyte ions is found to be matrix dependent and not so dependent on the analytes mass or charge; the velocity is also not affected by the laser fluence. This can be explained in that clusters are formed at the first stage of the MALDI process. These clusters contain the analyte ions and matrix molecules and since the concentration of the matrix is much higher than the concentration of analyte, the initial velocity is affected by the type of matrix rather than the type of analytes. [34] Several experimental [37,38] and simulation [39] studies showed formation of clusters in the plume, giving strong support to the cluster model. Although the number of clusters in the plume is small, the MALDI ion yield is small too. Additionally, in a separate study carried out using organic indicator dyes with different types of matrices covering a wide pH range (2 to 9), MALDI experiments were carried out to see if these dyes maintained their charge status when they were mixed with matrix crystals and dried. [40] Results showed that the colour of these dyes was maintained. This made it clear that the analytes are maintaining their solution charge states. Moreover, the study showed that the crystals typically contained between 0.3 and 3 % solvent. This means that the association of counter ion pairs is reduced by keeping ions solvated. However, the cluster model needs further investigation and experimental evidence. [34] It must be shown that large biomolecules are present in multiply protonated forms in the low pH media of the matrix. Additionally, it has been argued that photo-ionization is not a viable mechanism for the production of electrons in the plume. [41] Despite these points, the model is still valid and some

ions in MALDI are possibly generated by this mechanism. However, it cannot be considered as the only mechanism of ion formation and surely a range of scenarios needs to be considered to explain and predict the mass spectra generated by the MALDI ionization process. [41]

The second model assumes that the analyte ionization in MALDI is controlled thermodynamically and it is the result of secondary ion-molecule reactions in the plume. When a laser pulse irradiates the surface of the matrix-analyte sample, a small, hot and very rapidly expanding plume is generated. The primary ions are generated from this process, and an ion-molecule reaction in the plume then converts these primary ions into secondary products which are detected by the mass spectrometer. [42] Several mechanisms are proposed for the ion formation in the primary stage. The most important ones are based on energy pooling and multi-photon ionization. In the former mechanism two or more excited matrix molecules pool their energy to yield one matrix radical or one highly excited matrix molecule.[42] Several studies have clearly indicated the validity of this mechanism, and one has importantly proposed a successful quantitative model for MALDI ionization based on this mechanism. [43] The latter mechanism is based on the idea of two photon excitation of a single matrix molecule. This mechanism is valid if the ionization potential for matrix molecules is not far above the two photon energy, which was found to be the case with some types of matrix molecules. [42]

Secondary ions are formed as a result of proton transfer reactions, cationization reactions and electron transfer reactions, within the plume. [41]

The important points to draw from these proposed mechanisms are:

1) Ion formation in MALDI is a complex process and surely more than one mechanism is involved in the process.

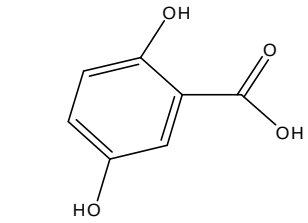
2) The matrix, which is a small organic chromophore, should absorb energy at the wavelength of the laser.

3) The type of matrix used influences the extent of fragmentation of the sample ions. The type of matrix also affects the mass resolution due to its effect on the initial velocity of the generated ions. Thus it is important to identify the most suitable matrix for a given application.
4) Most of the ions generated by MALDI are singly charged.

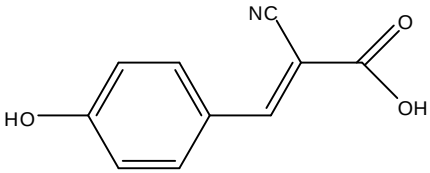
1.2.2.1.2 MALDI instrumentation.

The laser source is the main instrumental part in MALDI. Ultraviolet (UV) excitation sources are the most common laser type used in MALDI. The other, less commonly used, is an infrared (IR) excitation source. Nitrogen laser (337 nm) is the most commonly used; however, Er:YAG laser and various excimer laser lines are also frequently used. The pulse widths vary between 1 ns up to 100 ns. However, the pulse widths have little or no influence on the mass spectra obtained. [44]

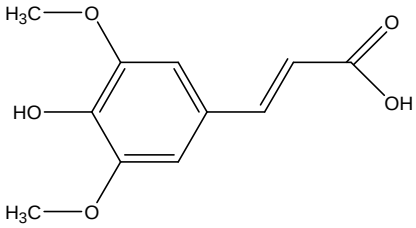
The type of matrix plays a very important role in MALDI experiments, as has been discussed earlier. Several matrices are commonly used. Figure 3 gives the structures and names of some of commonly used matrices.



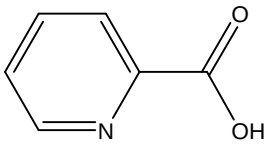
2,5-Dihydroxybenzoic acid



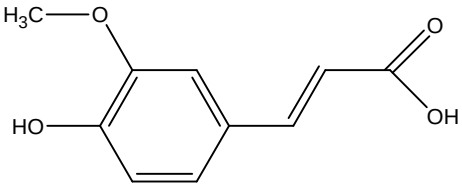
α-cyano-4-hydroxycinnamic



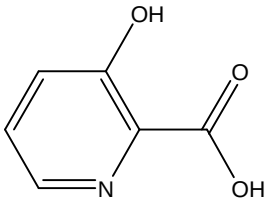
3,5-Dimethoxy-4-hydroxycinnamic acid



Picolinic Acid



4-Hydroxy-3-methoxycinnamic acid



3-Hydroxypicolinic acid

Figure 3: Chemical structures and names of some commonly used matrices in MALDI.

MALDI is predominantly coupled to a time of flight (TOF) mass analyser. This is because of the compatibility of the two, with both being pulsed rather than continuous techniques. In TOF, an electric field accelerates all ions into a field-free drift region with a kinetic energy of eV, where e is ion charge and V is applied voltage. Since the ion kinetic energy is 0.5 mv^2, where m is the mass of an ion and v is ion velocity, lighter ions have a higher velocity than heavier ions and reach the detector at the end of the drift region sooner. Two important technical developments in TOF increased the resolution of the instrument considerably, namely, electrostatic reflectors and delayed extraction (Figure 4). [45]

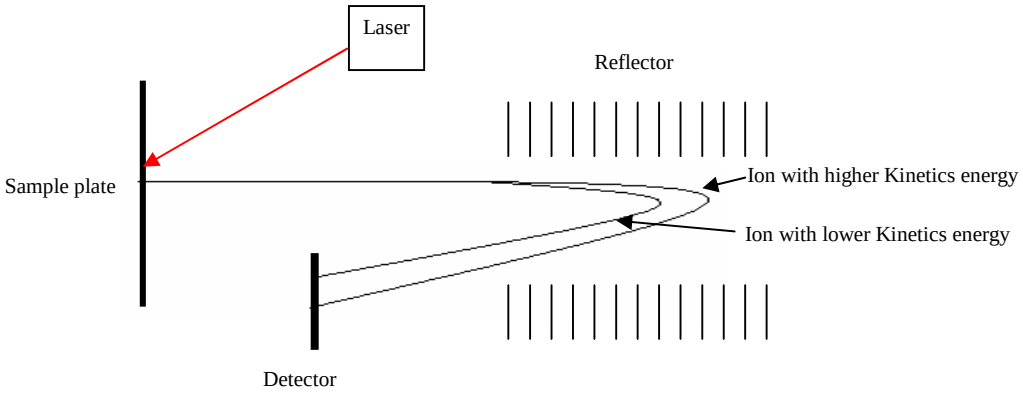


Figure 4: Schematic presentation of MALDI-TOF instrument.

The laser pulse produces a plume of materials, which contains neutral and ionic particles. These particles are subjected to a strong electrostatic field, which causes multiple collisions of ions present in the plume with the neutral particles. Hence, ions gain further energy, which increases the velocity of some ions of the same mass. This reduces the resolution of the mass analyser to a large extent. To overcome this problem, a reflector, an 'ion mirror' is placed at the end of the flight tube. [46] This increases the time of flight and allow ions of the same mass to be re-focused and compensates for the small differences in the kinetic energy of the same mass ions. The reflector has an electric field gradient and hence ions of the same mass but with higher kinetic energy will penetrate deeper than ions with lower kinetic energy. Thus, higher kinetic energy ions travel further before reversal of the direction of travel. This allows all ions with the same mass to reach the detector at the same time. The second important development to overcome the differences in the kinetic energy of ions with the same masses in the plume is the delayed extraction technique. Here the high voltage pulse is applied with a time delay (0.1- 0.5 μs) after the initial laser pulse [45] (*i.e.* the plume process occurs tens of micrometres from the solid sample surface).[46]

The reflector also allows peptide sequence information to be obtained by post source decay (PSD). The kinetic energy gained from collisions of ions present in the plume with the neutral particles can be released by undergoing fragmentation

while traversing the field-free drift region of the instrument. The fragments produced have the same velocity as the precursor ions. However, because their mass is less than the mass of the precursor ion, fragment ions are separated as a function of their kinetic energy by the time dispersions induced by the reflector and thus reach the detector earlier. [47] An ion gate is usually used to isolate the precursor ion and its fragments. It consists of an array of alternately charged wires. A voltage is applied such that all ions are deflected by a large enough angle so that no ions, except the precursor and its fragments, can reach the detector. [45]

1.2.2.2 Electrospray ionization method.

Electrospray ionization mass spectrometry was introduced by Yamashita and Fenn in 1984. [28] The technique quickly became very popular, and is now considered one of the most important techniques for the analysis of proteins and other biological compounds. Electrospray can be defined as a technique in which ions in solution transfer to a vapour phase under the influence of an applied electrical field. [48] In electrospray, the sample is introduced to the ionization source *via* a short stainless steel capillary tube.

1.2.2.2.1 Mechanisms for ion formation in ESI.

The introduction of a liquid analyte to a mass spectrometer requires solvent removal without loss of the analyte. This is because the evaporation of the solvent in the mass analyser under the influence of the vacuum will cause a large increase in the pressure and stop the analyser from functioning.

When a positive voltage is applied to a capillary and a solution of ionic molecules passes through the capillary, the ionic molecules in the solution will drift toward the surface of the solution and the negative ions will drift away from it. The accumulation of positive charge on the surface of the solution will lead to destabilization of the surface. The surface will be drawn out forming a cone shape. This is known as a Taylor cone. As a result of the high potential, the surface constrains positive ions within the droplets. [49] An identical process occurs

when a negative potential is applied but in this case negative ions will be constrained within the droplets. Another mechanism suggests that a conventional electrochemical reaction is taking place at the liquid-metal interface of the capillary. Positive ions are supplied to the solution either as a result of oxidation occurring at the metal surface or by converting negative ions in the solution to neutral molecules. [50] The occurrence of oxidation processes at the capillary tip has been reported. [51] Once the charged droplets are formed, the solvent in the droplets starts to evaporate and the droplet radius shrinks until it reaches the Rayleigh disintegration limit, where the droplets undergo fission into smaller droplets. The Rayleigh disintegration limits can be calculated from the Rayleigh equation:

Equation 1 $$Q^2 = 64\ \Pi^2 \epsilon_o \gamma R^3$$

where Q is the charge that is sufficient to overcome the surface tension, γ is the surface tension that held the droplet together, ϵ_o is the permittivity of a vacuum and R is the radius of the droplets. It is important to note that uneven fission takes place. Even fission will lead to a formation of two droplets of similar size and charge. Uneven fission will lead to the formation of offspring droplets not only with a smaller diameter but also with a higher charge to mass ratio. [52]

Three different mechanisms are proposed for the process of formation of gas phase ions. In the first (proposed by Dole *et al.*)[50], the evaporation of the solvent is a continuous process until the formation of an extremely small droplet which contains only one ion. Evaporation of the solvent from this droplet will lead to conversion of the droplet to a gas phase ion. The second, suggested by Iribarne and Thomson, [52,53] proposes that the emission of ions comes from very small droplets which are highly charged. The main difference between this mechanism and the earlier one is that this model does not require the production of very small droplets containing only one ion. This means that, when emission of ions occurs, the droplets may contain other solutes such as charge paired electrolytes. However, a mass spectrum obtained from sodium chloride solution did not show the presence of any paired sodium chloride. This experimental data gives strong

support to the Dole mechanism. [52] The third mechanism was proposed by Siu *et al.* [54] Siu suggested that the ions are emitted at the needle tip directly from the solution. However, this model has been criticized by a number of scientists. [15]

The important points to draw from these suggested mechanisms are:

1) Formation of charged droplets is essential. Every effort should be made to enhance this process. Solvents with low surface tension are favoured.

2) Electrospray is a soft ionization technique. Although the desolvation process is an energetic process, it transfers little (if any) internal energy to the ions. [55] As a result, fragmentation occurs rarely and in most cases only molecular ions are observed.

3) Electrospray works well with ionic or ionizable molecules. Neutral molecules cannot be analysed so easily.

1.2.2.2.2 ESI instrumentation.

Several designs have been implemented for electrospray ionization instrumentation. One current design consists of a metal capillary (0.1 to 0.2 mm i.d. and 0.2 to 0.5 mm o.d.) which is raised to a potential of 2 to 5 kV relative to a counter electrode located 1 to 5 cm away. This results in the formation of an electric field, the strength of which E_c can be calculated using the equation given by Loep:

Equation 2 $$\mathbf{E_c = (2V_c/r_c) \ln(4d/r_c)}$$

where V_c is the applied potential, r_c is the outer diameter of the capillary and d is the distance from the counter electrode. [56] When a solution of an analyte is introduced to the capillary, a fine spray of charged droplets emerges from the tip of the capillary. The droplets then migrate toward an orifice in the counter electrode through a stream of inert gas (Sheath gas). [57] If required, a solvent with a low surface tension may be added from a tube coaxial to the capillary (Figure 5). This is especially important when the analyte is dissolved in aqueous media. Water has a high surface tension which reduces the ionization process. Adding solvent with a low surface tension, such as methanol, will reduce the surface tension of the droplets and enhance the ionization process. [58]

When a gas enters the vacuum region in a mass analyser, it expands, and strong cooling takes place. This results in the formation of clusters if water or solvent vapour is still present. These clusters may be massive and may fall outside the mass range of the mass analyser. To avoid such problems, auxiliary gas is used. This gas is usually nitrogen and is supplied through an outer coaxial channel to the electrospray capillary, it is used to enhance desolvation and to assist in breaking up ion clusters. An alternative method used by some instrument manufacturers is to use a heated curtain of nitrogen gas perpendicular to the analyte flow, which sweeps water and solvent vapour away whilst allowing ions to penetrate towards the sampling orifice under the influence of the electric field.[59]

The use of auxiliary gas is especially important when high flow rates are used, for example in the case of conventional liquid chromatography-MS. Flow rates up to 1 ml min^{-1} can be handled. Finally, if any clusters are formed, they break down when they enter the vacuum area due to the collision with the nitrogen gas molecules. A mild collision is sufficient to break hydrogen bonds. [60, 61]

The sensitivity of electrospray can be improved by the use of a nano-electrospray ion source. Nano-electrospray is similar to a conventional electrospray ion source but differs in that (i) a gold coated glass capillary with an orifice of 1-2 μm is used to spray the analyte (ii) very low flow rates (about 20 nL/min) are used and (iii) very small droplets are produced. The sensitivity is enhanced by the formation of small droplets (higher surface to volume ratio). This is because when small drops form, 100 % ionization takes place and as a result the mass spectrometer becomes mass sensitive. The technique is quite insensitive to solvent composition, it is reported that sample in 0.1 M salt content can be sprayed without sheath flow. It is also reported that pure aqueous solutions can be sprayed successfully. [62]

Several mass analysers can be used with an ESI source, the most common being the quadrupole ion trap (QIT) and triple quadrupole (Tri.Q). [63] In the quadrupole

ion trap mass analyser, the molecules are vaporized in the trap volume, ionized, stored and then released according to their m/z values toward the detector. The ion trap can be used in conjunction with an external ionization source as a mass analyser only. This is more common these days. [64]

Tri. Q. is a combination of three sets of linear quadrupoles. A linear quadrupole consists of four parallel metal rods. A d.c. voltage U and a radiofrequency voltage ($V_o \cos \omega t$) are applied between adjacent rods. For given voltages, only ions of a certain mass to charge ratio pass through the rods and all other ions are thrown out of their original path. A mass spectrum is obtained by monitoring the ions passing through the quadrupole filter as the voltages on the rods are varied.[65]

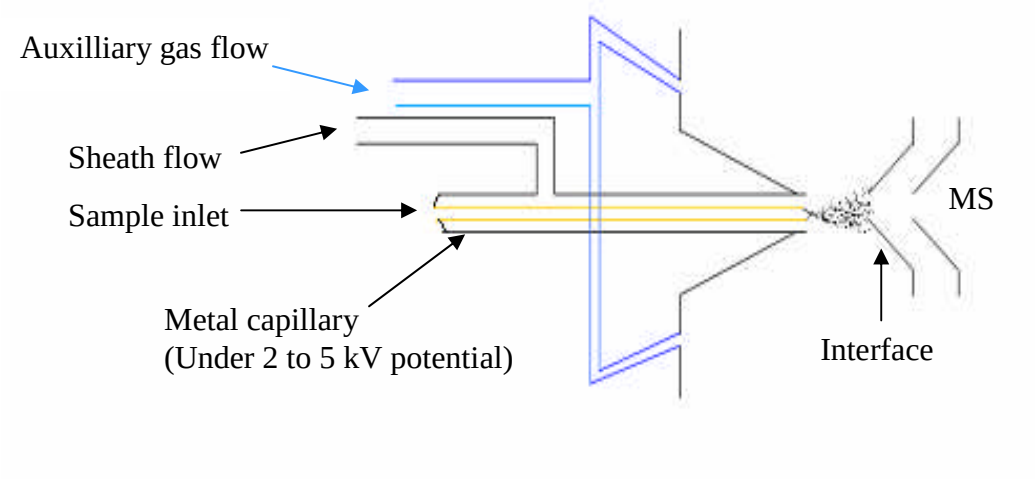


Figure 5: Schematic presentation of ESI instrument.

1.2.2.3 Comparison between MALDI and ESI as ion generation techniques in proteomics analysis.

ESI and MALDI are both considered soft ionization techniques and in both techniques the relationship between the amount of sample present and signal intensity is not very well understood. [66] This makes quantitative analysis in both techniques very difficult. Despite these common features between MALDI and ESI, the two techniques have several important differences. In MALDI a solid sample is used while in ESI a sample solution is used. [67] This difference in the physical states of the sample used has an important implication. ESI can easily be

coupled to a separation system like liquid chromatography, while such coupling is not straight forward with MALDI. [67] However, the ability to use solid samples for analysis directly in MALDI has unique advantages in some types of analysis such as imaging mass spectrometry in which MALDI is exclusively used. [68] Additionally, MALDI in most cases generates singly charged ions while ESI usually generates multiply charged ions. This makes interpretation of the MALDI spectrum much easier than the ESI spectrum, however, multiply charged ions are much easier to fragment, hence MS/MS spectra generated from ESI using collision induced dissociation (CID) are much more informative than those produced by MALDI. [69] Moreover, ESI is a continuous ionization technique, whereas MALDI is a pulsed ionization technique. This has an important implication on the ability to interface these two ionization techniques with different types of mass analyser. MALDI can be easily interfaced to mass analyzers which are capable of collecting ions for subsequent m/z separation, for example, QIT and FT-ICR, or are capable of measuring a complete mass spectrum for each event like TOF, whereas, ESI can be interfaced easily with Tri. Q, QIT and FT-ICR. [63] The use of certain types of mass analyser with these two ion generation techniques, has important effects on the performance of the mass spectrometers as an integrated system. For example, the mass resolution of MALDI-TOF is much higher then ESI-QIT. However, this has no direct relation with ESI as an ion generation technique but is rather due to the performance of the mass analyser used. Additionally, differences in the ability to couple these two techniques to various mass analysers have made several types of mass spectrometer configuration dominant in the field of proteomics. Some of these are: MALDI-TOF, ESI- Tri. Q and ESI-QIT. Recently several hybrid mass analyser systems have been introduced such as ESI-Q-TOF, MALDI-Q-TOF, MALDI-TOF-TOF and ESI-QIT-FT-ICR.

MALDI-TOF is commonly used for identification of proteins separated from gel. It is sensitive, high resolution (can resolve m/z = 1000.0 from 1000.1) and a wide dynamic range instrument. However, the instrument is not very well suited for complex mixtures of peptides. This is due to the difficulty of coupling the

instrument to a separation system like chromatography. Additionally, peptide sequencing using MALDI-TOF is not very reliable. ESI-Tri.Q and ESI-QIT are usually used to separate complex peptide mixtures as both can be interfaced to chromatographic systems. Moreover, peptide sequencing information obtained by these instruments is much more reliable. Usually ESI-QIT gives much more complete fragmentation than ESI-Tri.Q. However, both instruments are low resolution instruments (can resolve m/z = 1000 from 1001). [70]

Person *et al.* [71] carried out a comparison between MALDI-TOF and ESI-QIT by obtaining mass spectrometric data using these two instruments for peptide mixtures generated from proteins extracted from cells and separated using one or two dimensional gel electrophoresis. Results showed that shorter peptides cannot be detected by MALDI-TOF due to the signal present from the matrix. In contrast, the ESI-QIT is optimized for peptides between m/z 300 to 2000 Da. They also found that MALDI-TOF peptides are longer on average than those that are detected by ESI-QIT; thus, the amino acid coverage is slightly greater for MALDI. However, Cohen *et al.* [72] performed detailed studies on the effect of the matrix solvent system and the rate of matrix crystal growth on the mass spectra obtained using MALDI. The study was conducted using 4-hydroxy-α-cyanocinnamic acid as a common matrix used in proteomic studies. They found that analysis of high mass peptides (>2 kDa) is best performed using matrix solutions that have a pH <1.8 while analysis of small peptides (<2 kDa) is best carried out using matrix solutions that do not have acids added. In other studies, it was observed also, that the slow crystallisation procedure (hours) gave a preference for the ionization of high mass peptides, while this is not the case if a fast crystallisation method is used. These studies suggest that mass discrimination observed in MALDI is strongly influenced in certain cases by the sample preparation method rather than being a feature related to the nature of the ionization method. [72]

Stapels *et al*. [73] used peptides generated from protein digestion of an *Escherichia coli* sample in solution with trypsin. The generated peptides were separated using

HPLC and subsequently analysed using off-line MALDI-TOF-TOF or using on-line ESI-Q-TOF. The total number of peptides identified using these two systems was 667, of which 265 were identified in both of the systems while 130 were uniquely identified by ESI-Q-TOF and 272 were identified only by MALDI-TOF-TOF. These data indicate clearly that the majority of peptides were identified by only one technique or other but not both. The lower number of peptides identified uniquely by ESI-Q-TOF is partly due to the method of separation in which a lower percentage of trifluoroacetic acid was used to reduce the signal suppression effects, while it was possible to use a higher percentage of the acid with MALDI-TOF-TOF due to the separation being carried out off-line. Bodnar *et al.* [74] used a single HPLC and a splitter to split the effluent coming from the nano-LC column into two fractions, one for on-line LC-ESI, while the other was mixed with a matrix solution and deposited onto the MALDI target plate to obtain MS using an off-line MALDI instrument. The same mass analyser was used for both (Q-TOF) and a tryptic digest of a preparation of mammalian mitochondrial ribosomes was used to compare the mass spectrum obtained using these two instruments. Other studies have been conducted to compare MALDI and ESI. [75,76] Several important points can be drawn from these studies. First, it is observed that ESI identified more hydrophobic peptides than MALDI.[73, 77] This is because both the charges and the hydrophobic amino acids tend to occupy the outer surface of the droplets in ESI. [77] Second, ESI favours ionization of peptides that contain aliphatic amino acids and amino acids with hydroxyl moieties. [73] In contrast, regardless of the method of sample preparation, MALDI tends to preferentially ionize peptides containing basic and aromatic amino acids. The latter was explained by photoexcitation of these moieties during ionization.[75,76] However, ESI and MALDI have a tendency to ionize peptides which do not contain methionine. [73] Moreover, in all cases and regardless of the type of ionization method used, the sequence coverage is rarely 100 %. Hence, complete sequence coverage in most cases is not possible. [73]

Krutchinsky *et al.* [78] innovated a switchable MALDI and ESI ion source coupled to a Q-TOF mass analyser. The switching is carried out in 30 seconds and the

performance of the two ion sources is comparable. Using this approach it was found that the interpretation of singly charged peptides generated from MALDI has the advantage of being relatively easy to carry out compared to the multiply charged ions generated by ESI. However, ESI generates much more informative spectra due to the presence of these multiply charged peptides, which are generally easier to fragment.

In another attempt, Zhang *et al.* [79] analysed mammalian cells using MALDI-TOF-TOF, MALDI-FT-ICR and LC-ESI-QIT. The objective was to identify several proteins present in this complex extract. The first two techniques did not give any satisfactory data for the tryptic digest of the sample. This is due to the complexity of the mixture and also weak fragmentation, which did not allow confident identification of the proteins present. Using the same tryptic digest, several proteins (about 30) were confidently identified using LC-ESI-QIT. The work clearly demonstrates the importance of having a separation system prior to mass spectrometry. This is especially important when a complex mixture is present. It also demonstrates the need for new fragmentation techniques which may provide rich fragmentation to confidently identify proteins present in a sample.

FT-ICR-MS uses high magnetic fields to trap ions generated from ESI or MALDI and using cyclotron resonance, these ions are detected. FT-ICR-MS is considered the top end of mass analysers available to date. This is due to its very high resolving power which can exceed 1,000,000 (*i.e.* separate m/z 1000.000 from 1000.001). However, the high cost of the instrument restricts its use to a limited number of research groups. FT-ICR can be coupled to both ESI and MALDI; however, the mass accuracy obtained using ESI as an ion source is higher than MALDI. This is due to the multiply charged ions generated by ESI being in the low m/z region and having higher cyclotron frequencies than singly charged equivalents generated by MALDI. [80] The resolving power and the dynamic range can be improved further by coupling FT-ICR to an external ion trapping system such as QIT. [45]

The results of the reports discussed above clearly indicate the complementary nature of MALDI and ESI as ion generation techniques. [73,71] Ideally, both techniques should be used to obtain higher sequence coverage and increase the level of confidence in the result obtained. Moreover, despite the noticeable progress in mass spectrometry, there is still a need to have a separation system prior to mass analysis. This is due not only to the complexity of proteomic samples but also to the nature of the ion generation technique, which leads to considerable signal suppression if a complex mixture is present. Hence, developments in the field of separation science will have an important effect on proteomics.

1.2.3 Bioinformatics.

The third key technique in the classical method is bioinformatics (BInfo.). Binfo. is the science which deals with all kinds of activities (storage, retrieval and analysis) related to biomolecules structure such as amino acid sequences, secondary structure, tertiary structure and three dimensional modelling. The information is collected in huge databases, some of the most important are listed in Table 2. [81] These data are used to derive structural information about the different types of proteins present in the analyte. However, some information about the proteins can be obtained directly from the gel. The position of the protein spot in the gel can be used to predict the molecular mass and the isoelectric point *(pI)* value of that protein. [11] Several commercial software packages are available to aid and assist this process. Additionally databases are available for different gel snap shots for comparison purposes. [45] The main difficulty with this technique is that the spot patterns are highly dependent on sample preparation method. Additionally the technique does not provide any structural information about the protein of interest. However, the information can be used to support the data derived from mass spectra obtained from the protein of interest.

Site	Address
Swiss Institute of Bioinformatics	http://www.expasy.ch
European Bioinformatics Institute	http://www.ebi.ac.uk
Research Collaboratory for Structural Bioinformatics	http://www.rcsb.org
Protein Information Resource	http://pir.georgetown.edu

Table 2: List of some databases and their web addresses in bioinformatics.

Structural information can be derived from either PMF spectra or *via* amino acid sequencing using tandem mass spectrometry. [82] The molecular mass of peptides generated from digestion of a protein (PMF spectrum) is compared with the theoretical molecular mass of peptides that are produced by digestion of each protein in a database. [82] Proteins which contain peptide molecular masses that match the m/z value in the mass spectrum are considered a match. A list of proteins is generated with matching scores. Higher scores are assigned for the protein which has more peptides that match the m/z value in the spectrum. The higher the measured mass accuracy, the lower the number of peptides that exactly match that mass in the database and hence, a smaller list of matching proteins is generated. [83] Several problems are associated with this method of identification: firstly, the protein to be identified should be available in the search engine, any new or modified protein can not be identified using PMF. [84] Secondly, large proteins give rise to greater numbers of peptides and hence have an increased probability of matching. [85] Additionally, smaller peptides in the database have a greater number of matches compared to large peptides. However, the latest software uses an extensive probability based scoring system to eliminate most of these problems. [85] The method is good for high throughput analysis and relatively easy to use. However, the method is not very successful when it is used with protein mixtures. [84] Additionally, closely related proteins are difficult to distinguish due to the similarity in peptide masses generated from the digestion process of these proteins. Moreover, information can be obtained but the modification has to be explicitly defined in the search criteria. [84] The other method for identifying a protein is based on the mass spectrum generated from

the fragmentation process of a peptide using CID (tandem mass spectrum). Collisional induced dissociation (CID) generates fragments primarily at amide bonds (forming b and y ions in Figure 6) along the backbone, generating a ladder of sequence ions in a predictable manner. [63] Thus, the sequence from the mass spectrum can be compared and matched to the sequence obtained from the database or the sequence can be derived directly from the spectrum. The latter is known as the *de novo* peptide sequencing method. [86]

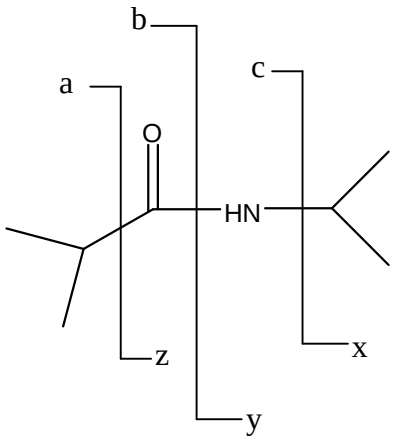


Figure 6: Nomenclature for fragmentation of peptide ions.

Tandem mass spectrometry generates a mass spectrum in which each peptide represents a unique piece of information. Therefore, matching one or more peptide mass spectra in the same protein provides a high level of confidence. Consequently, fewer peptides are required for identification. Moreover, post-translation modifications can be found and identified. However, the method is much slower than the previous PMF method and in many cases the CID process generates incomplete sequences which makes data interpretation more difficult.

1.3 Shotgun.

Several alternative methods have been proposed to overcome the disadvantages associated with the classic proteomic method. One of most promising is shotgun proteomics. In this method, separation of proteins using 2D-PAGE gel, which is considered a key step in the classical method, is removed and the complete

protein mixture is digested without any prior protein separation step. Instead of protein separation, the generated complex mixture of peptides is separated, using two dimensional liquid chromatography (2D-LC). Analysis is then carried out using a tandem mass spectrometer. Finally, protein identification is carried out by a sophisticated mathematical algorithm. The resultant peptides sequence data is then compared against the protein database (Figure 7). [87]

Several modifications have been added to the technique, for example, isotope-coded affinity tagging (ICAT). [88] This is to overcome the difficulties of quantitative analysis in shotgun proteomics. The tagging is carried out on cysteine residues of proteins with heavy or light stable isotopic labels (one for each sample). The two samples are then mixed, digested and analysed by mass spectrometry. The ratio of the signal intensities from the pair of analytes accurately indicates the abundance ratio for the two analytes. Several isotope tagging techniques have been described targeting different chemical groups present in proteins, such as amino groups, [89] and phosphate ester groups.[66]

Shotgun proteomics possesses several advantages compared to the classical proteomic method. Firstly, the reproducibility is much higher than the classic proteomic method. This is mainly due to the elimination of the 2D-PAGE gel. Secondly, it is a much less laborious and demanding technique. Despite these advantages, there are a few disadvantages. For example, quantification is very difficult, the possibility of missing any post-translation modification is high and the lengthy time required for the digestion is similar to the classical method.

The key techniques of shotgun proteomics are: two dimensional liquid chromatography, mass spectrometry and bioinformatics. The following sections will discuss these key techniques in relation to the shotgun method.

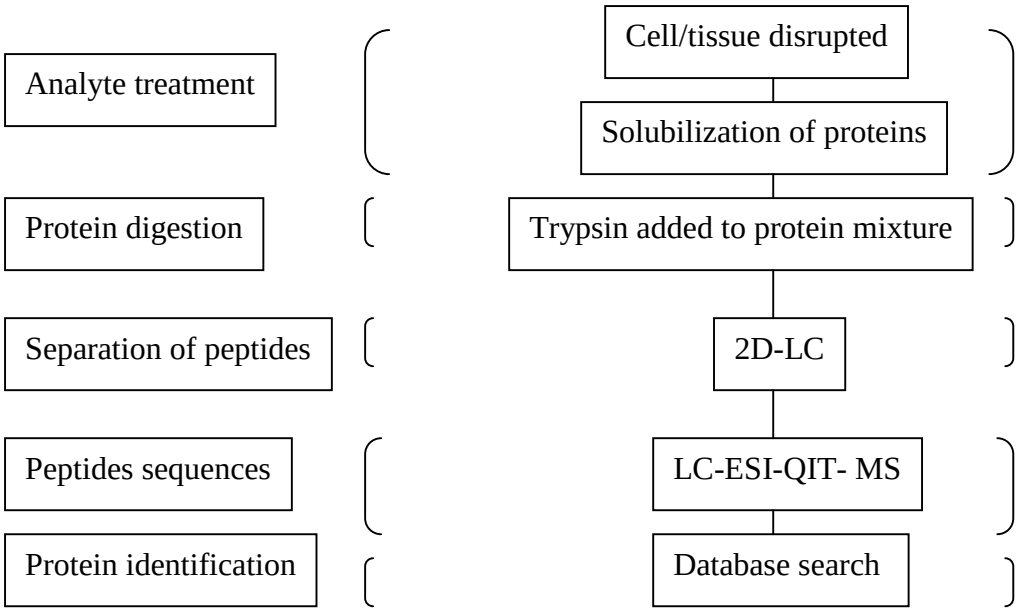


Figure 7: Procedure for shotgun proteomics.

1.3.1 Two dimensional liquid chromatography.

1.3.1.1 Brief introduction to chromatographic theory.

Separation of any mixture in liquid chromatography is a result of two different processes which take place in the column. The first process is the migration of the sample components through the stationary phase, which is essentially the result of two forces, *i.e.*, movements driven by the mobile phase and retardation resulting from the stationary phase. The second process is the band broadening process as the samples move through the stationary phase. Both processes are described mathematically, the first process described by the Purnell equation [90] and the second by the van Deemter equation. [91]

1.3.1.1.1 Purnell Equation

The resolution is defined as the difference in retention time of the two peaks divided by the average peak width at the baseline.

Equation 3 $\mathbf{Resolution = (t_{R2}-t_{R1})/ (0.5 \times (w_{2+}w_1))}$

where t_{R2} is the retention time of the second peak, t_{R1} is the retention time of the first peak, w_2 and $w_1$is the peak width at baseline of the second peak and first

peak respectively (baseline width formed by the tangents of the peak intersecting the baseline). [92]

Purnell [90] was able to relate the resolution to three main chromatographic parameters. These are the separation factors, α, the capacity factor, k' and the number of theoretical plates, N.

Equation 4

$$\textbf{Resolution} = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k'}{1+k'}\right)$$

For most cases resolution = 0.8 is adequate unless the relative intensity of the peaks is larger than 16:1. For preparative chromatography, resolution = 0.6, cutting and collecting the fraction on either side of the center of the peaks would furnish a fraction of about 90 % purity. If the resolution is equal to one then 98 % purity is obtained. For quantification the resolution should be greater than 1.0 and preferably 1.5. [93]

According to the Purnell equation, three factors determine the resolution. These factors are interrelated, changing one of them may significantly alter the others.

Capacity factors measure the retardation of the compound in terms of the number of column void volumes it takes to elute the center of the peak.

Equation 5 $\qquad k'= (Vr-Vo)/Vo$

where Vr is the volume of mobile phase required to elute a particular component from the column. Vo is the volume of mobile phase required to elute unretained component from the column.

The resolution does not increase proportionally to k', but to k'/(1+k'), which means that increasing k' from 0 to 1 would increase the resolution from 0 to 0.5 A further ten fold increase in k' value would increase the resolution from 0.5 to 0.91.

The most important factor affecting k' is the composition of the mobile phase. In reverse phase chromatography, the mobile phase usually consists of an organic solvent and water. As the percentage of the organic solvent increases, the strength of the mobile phase increases. The strength of the mobile phase should be such that the k' value will be approximately >1 and <20 and preferably < 10. A longer k' value will increase the retention time of analysis and may lead to a very broad peak that is difficult to detect. Ideally k' value should be equal to two, this will give a minimum time of analysis. [94]

A theoretical plate number, N is the most common performance indicator of a column. There is a tendency to equate the column efficiency value with the quality of the column. The column efficiency measurement is based on a Gaussian distribution of the sample population. This result is a Gaussian peak. The general formula for calculating N is:

Equation 6 $\mathbf{N= [16 (Vr/W)^2]}$

There are several factors affecting the column efficiency. These factors are: linear mobile phase velocity, eluent composition and the solute chosen for measurement.

The third factor which effects the separation is separation ratio, α, which is defined as the ability of the column to discriminate between two components and selectively retard them. It is measured as a ratio between the k' values of the two components.

Equation 7 $\mathbf{\alpha = k'_2/k'_1}$

If the separation ratio is 1.4 this means that the column selectively retard one component by 40% with the reference to the other. In general the larger the value of α the easier the separation, usually a value of α between 1.1 and 1.4 is sufficient.

1.3.1.1.2 van Deemter Equation

Martin and Synge introduced the Plate theory of chromatography.[95] According to this theory, the chromatographic column consists of a large number of elements or plates. Each plate has a definite height and solute molecules spend a definite time in each plate. It is assumed that the solute is in equilibrium with mobile phase and stationary phase all the time.

As the number of the plates, N, increases, the efficiency of the column increases. The value N can be obtained by relating t_R to the peak width (see equation 6)
N can also be related to the height of the plate as

Equation 8 $\mathbf{N = L/H}$

where L is the length of the column.
Although, the plate theory gives a way to measure the column efficiency, it does not provide information on how to improve the efficiency of the column.

The van Deemter equation [91] states the parameter which affects the efficiency of the column.

Equation 9 $\mathbf{H= A+ B/u+ (Cm+Cs)u}$

where

Equation 10 $\mathbf{A= 2\lambda dp}$
Equation 11 $\mathbf{B= 2\gamma Dm (1+\xi k')}$
Equation 12 $\mathbf{Cm= f1(k')/Dm\ dp^2}$
Equation 13 $\mathbf{Cs= f2(k')\ d^2f.u/Ds}$

where dp is particle diameter, λ is a constant, γ is constant, Dm is diffusibility of the solute in the mobile phase, ξ is longitudinal diffusion in stationary phase, u is linear mobile phase velocity, f1(k') is a function of k', Ds is diffusibility of the solute in the stationary phase, f2(k') is a function of k' and d^2f is film thickness of stationary phase.

The first term in the equation is known as the eddy factor (A). Sample molecules take different paths through the column as they migrate. As the homogeneity of the packing increases the eddy diffusion factor decreases. λ is a measure of homogeneity. The use of small particle size stationary phase will reduce the A term.

The second term (B) is the longitudinal diffusion of solute molecules. This arises from the tendency of solute molecules to migrate away from the concentrated center of the band toward the diluted region on either side. This type of diffusion take place in both the mobile phase and stationary phase. Longitudinal diffusion decreases as the flow rate increases.

The third term (C) in van Deemter's equation arises from the time required for the solute molecules to equilibrate between the mobile phase and stationary phase. The C term can be reduced by reducing the viscosity of the stationary phase or by increasing the temperature. These two factors increase the diffusion coefficient of the solute molecules in the stationary phase and hence improve the mass transfer process of the solute molecules between the stationary and mobile phase. [96]

The equation also shows that optimization of the flow rate is required as it is inversely proportional to plate height in term B while it is directly proportional in term C.

1.3.1.2 Brief introduction to two dimensional liquid chromatography.

The tryptic cleavage of proteins generates multiple peptides per protein. If the number of proteins in the analyte is several thousand, then the number of peptides will exceed hundreds of thousands. To date, no single separation method possesses a resolving power high enough to separate such a complex mixture. The alternative is to use a multidimensional separation method. In multidimensional separation the analyte is first subjected to separation *via* one method and then the separated components are subjected to another independent

separation method. In theory there is no limitation to the number of independent separation methods that can be coupled. However, due to practical constraints, the majority of separations reported to date are in two dimensions (2D). [97] However, 2D separation systems possess a peak capacity (maximum number of peaks that can be separated within the gradient time) approximately equal to the product of the peak capacity of both dimensions.[97] Hence, this generates a technique with resolving power capable of handling complex analytes such as a tryptic digest of a protein mixture.

The concept of multidimensional separation methods was suggested by J. C. Jidding.[98] According to Jidding any successful two dimensional separation should be based on two different chemical or physical mechanisms (*i.e.* two dimensions should be orthogonal) and no resolution gained in the first dimension should be lost in the second subsequent dimension. For example, if an ion exchange column is used as the first dimension, reverse phase column as a second dimension, peaks are eluted from the first column using salt gradient in the mobile phase. This salt gradient will not affect the elution order in the reverse phase column, while when organic solvent is used to elute the peaks from the reverse phase, the components present in the ion exchange column will not be affected (Figure 8).

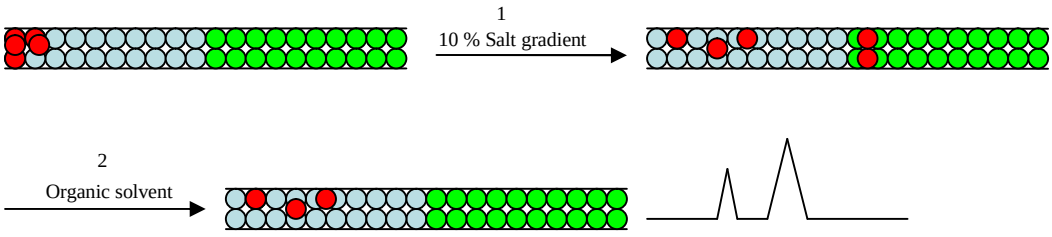


Figure 8: Schematic illustration of 2D separation using ion exchange and reverse phase columns.

2D separation methods can be divided into two types; the 2D in space and 2D in time. In the former, the separation is carried out on the same platform for example, the 2D-PAGE gel. Generally, all 2D planar surfaces are considered as 2D in space. In the latter, the separation is usually carried out in two different platforms, often on two different LC columns. Individual fractions separated by the first column are subsequently subjected to separation by the second column.[97]

2D in space and 2D in time can also be divided further into on-line and off-line types. In off-line techniques analytes can be collected after the first separation and then subjected to the second dimension, while in the on-line technique, the second dimension is carried out directly following the first dimension separation.

The on-line 2D-LC can be further divided into three types: heart cutting, column switching and directly coupled column. Heart cutting 2D-LC separation is carried out like a standard 1D analysis. Then, a desired segment of the first column effluent is transferred *via* a switching valve to a second column for further separation. The major drawback of this type of 2D-LC is that only part of the

analysis is considered. Obviously this will not be of general use in the case of proteomics, where all the components present in the mixture are of great importance. The column switching 2D-LC is the second type of on-line 2D-LC. In this method, valves are also used to interface the first column with the second one. The use of valves to interface the two columns offers great flexibility and in principle any two separation modes can be used. However, the cost of such great flexibility is greater instrumental complexity. Typically the technique requires multiple LC pumps, automated switching valves and computer control.

Directly coupled column 2D-LC is the simpler form of the column switching valves, in its basic form two columns with orthogonal separation mechanism can be coupled directly in series with no switching valves in between. Figure 9 shows the different types of 2D separation schematically.

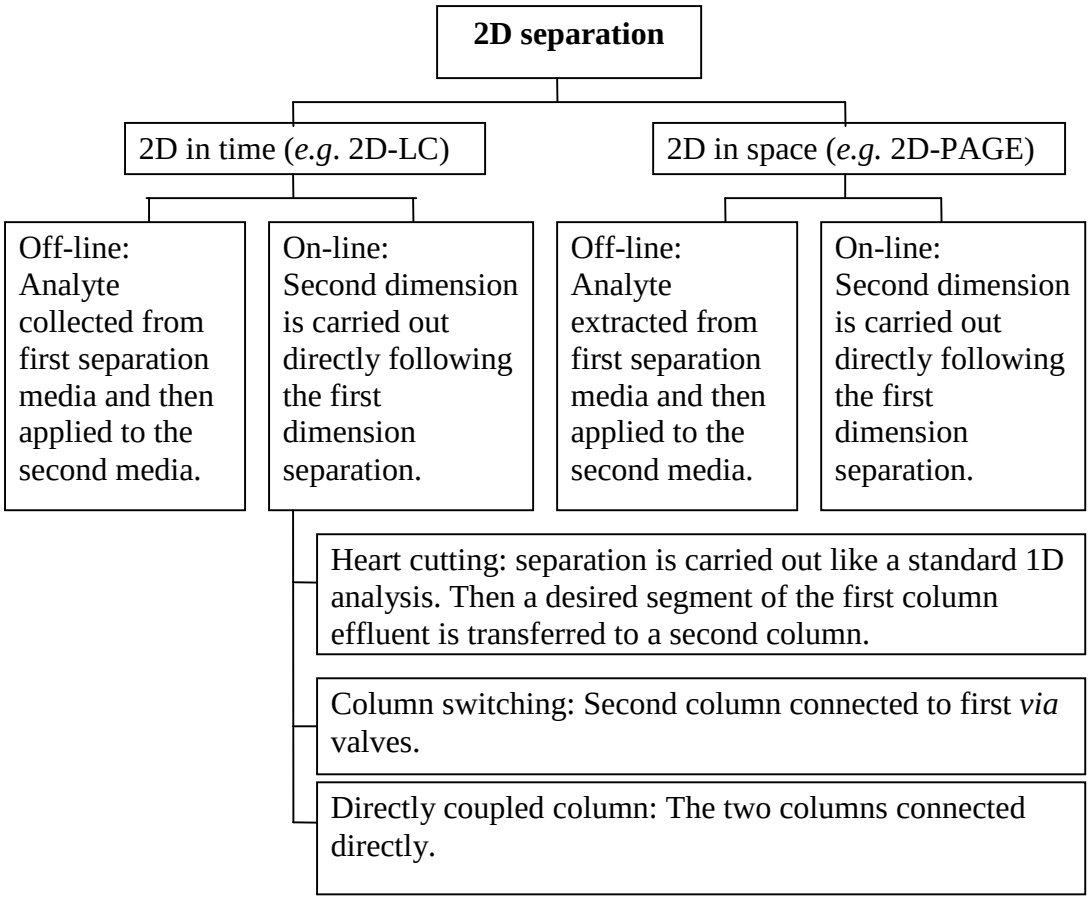


Figure 9: Schematic diagram showing different types of 2D separation.

Column performance is usually measured using theoretical plate number (N). However, equation 6 can only be used to calculate the theoretical plate number for isocratic systems and not for gradient systems as the increase in the mobile phase elution strength narrows the peak width. [99] In gradient systems (commonly used in 2D separation) usually peak width at half height along with resolution is used to evaluate column performance. [100,101]

Peak capacity (*P*) is another method for evaluating column performance if gradient elution is used. [102] Peak capacity is defined as the maximum number of peaks that can be separated within the gradient time (tg). Peak capacity practically, can be calculated using the following equation:

Equation 14 $$P = 1 + (tg / w_{13.4\%})$$

where tg is the total gradient time, $w_{13.4\%}$ is the average peak width at 13.4 % of peak height.

1.3.1.3 Two dimensional separation in proteomics.

A considerable number of publications in the field of peptide separation using different types of off-line and on-line 2D chromatographic systems have been published. These include ion exchange (IE)-reverse phase (RP), [103,104,105] size exclusion (SEC)-RP, [106,107] IE-SEC, [108] normal phase (NP)-RP, [109] and RP-RP. [110,111] Additionally, chromatographic and non chromatographic separation techniques have been coupled and used for peptide separation, for example, LC-capillary electrophoresis (CE) [112] systems or IEF-RP systems. [113] Other systems are also implemented which are based on non chromatographic separations. [114] However, the predominant method in proteomics is based on directly coupled column 2D-LC systems using ion exchange as the first separation dimension and reverse phase as the second dimension. [87]

Ion chromatography can be defined as “the reversible interchange of ions between a solid phase and a solution phase”. [115] The mechanism can be represented by the following equation:

Equation 15

$$M^-A^+ + B^+ \rightleftharpoons M^-B^+ + A^+$$

where M^-A^+ is the ion exchanger carrying cation A^+, B^+ is the solute ion.

The interaction between the charged molecules in ion exchange chromatography is based on electrostatic interaction. Electrostatic interaction is a strong and long distance interaction, compared to the hydrophobic interactions, which are the principal interaction in reverse phase chromatography. The electrostatic interaction is reduced when water is used as a solvent, due to its high dielectric constant and this explains its high solubilizing power for ions. However, the interaction is still strong and dominates the behaviour of charged molecules in this media. [115]

Ion exchange chromatography for the separation of tryptic digest of proteins is usually carried out using strong cation exchange (SCX) in which the ion exchanger is usually SO_3^{2-}. This is because at pH 3 or lower SO_3^{2-} remains negatively charged and the peptide carboxyls are protonated. The SCX separation primarily depends on the number of basic residues (including the N terminus) present in the peptides.

The mobile phase used in ion exchange chromatography has to contain salt that is capable of eluting the peptides from the column. Usually a salt gradient is used so that peptides elute from the column according to their charge density; the higher the charge density, the longer the retention time of the eluted peptides. If ESI-MS is used as a detector then it is important to ensure that the salt used is volatile. Limited types of salts can be used, such as ammonium fumarate, ammonium acetate and ammonium citrate. [116]

Reverse phase chromatography usually takes place in the form of ion pairing chromatography. In this type of chromatography, an acidic mobile phase is used and a negatively charged ion pairing agent is added to the mobile phase. This ion

pairing agent then pairs with peptides to increase their hydrophobicity and hence make their interaction with the non polar stationary phase stronger. [117] Several ion pairing agents are available which are compatible with mass spectrometry, for example, heptafluorobutyric acid (HFBA), trifluoroacetic acid (TFA), formic acid (FA) and acetic acid (AC). As ion pairing agents HFBA and TFA are considered the strongest, while acetic acid is the weakest. However, HFBA and TFA usually cause signal suppression and therefore are usually used at very low concentrations (0.02 or 0.05 %) while FA causes less signal suppression and can be used in higher concentrations. [118]

Using ion exchange as the first dimension and reverse phase as the second dimension chromatography, Link *et al.* [119] were able to identify more then 100 proteins in a single run. In their novel approach two different phases were packed in a single column. This single bi-phase column consists of ion exchange and reverse phase resin. A complex peptide mixture is applied to the ion exchange resin. Then, peptides are eluted by a step gradient of increasing KCl salt concentration. Each step releases a group of peptides which are then separated using reverse phase chromatography. The technique is now known as multidimensional protein identification technology (Mud PIT). In another experiment, they used ammonium acetate salt solution, which is compatible with ESI-MS, instead of KCl salt solution and the results were comparable. [118]

In another paper, Cagney *et al.* [120] used Mud PIT to profile the protein produced from eight human tissues: brain, heart, liver, lung, muscle, pancreas, spleen, and testis.

Membrane proteins are very hydrophobic proteins and usually very difficult to analyse using the classical method, due to the low solubility of these proteins in aqueous solvents. Fischer *et al.* [121] resolved the problem of solubility to a great extent by optimizing the sample pre-treatment and analysis methods. The shotgun method was used to identify peptides generated from the digestion process which

was carried out in organic solvents to increase the yield of hydrophobic peptides. Half of the proteins predicted in the sample were successfully identified.

Shotgun proteomics is now widely applied in different fields of proteomics and is regarded as an alternative method for resolving some of the problems associated with the classic method. [122,123 ,124]

The high resolving power of Mud PIT is mainly due to the separation being based on two distinctive physical properties of peptides namely the charge of the peptides and the hydrophobicity. Additionally the direct coupling of this chromatographic system to ESI-MS adds a third separation dimension, which is based on the mass of the peptides.

1.3.2 Mass Spectrometry.

There are two main differences between shotgun and classical proteomics with regard to the use of mass spectrometry in the analytical process. First, in shotgun proteomics generally, ESI is much more frequently used as an ion source than MALDI. This is because ESI can be easily coupled to the LC system. [125] Several attempts have been made to couple LC to MALDI, [126,127,126] but they have suffered from technical problems such as low mass resolution, carry over effect and high sample consumption rate. [73] Secondly, tandem mass spectrometry is the main identification technique used in shotgun. This is necessary because it is not possible to obtain peptide mass fingerprints for a single protein since the separation is carried out for the peptides generated from digestion of the protein mixture. The principle technique in tandem mass spectrometry is collisonally induced dissociation (CID). However, in many cases incomplete sequence coverage is obtained and often information regarding post-translation modification is lost. [128] To overcome these limitations, other methods have been proposed and evaluated, such as infrared multi photon-dissociation (IRMPD), [129] blackbody infrared dissociation, [130] surface-induced dissociation, [130] and electron capture dissociation (ECD). [131]

Among these methods, IRMPD and ECD are gaining popularity [70] due to the advantages possessed by these two techniques. In IRMPD the internal energy of an ion is increased by photon absorption. The absorbed energy is sufficient for the ion to dissociate. The method is faster than CID because gas is not required, hence the pressure reduction time necessary for effective MS/MS measurements by FT-ICR is significantly reduced. Additionally, low mass fragments are easily observed which is not the case with CID. Thus, the method provides more extensive sequence information. Moreover, the dissociation efficiency of this technique is good and it can be easily implemented as a routine analytical technique. [130]

ECD is another promising dissociation technique. In this method, electrons generated from a hot filament combine with protons from peptides to form neutral hydrogen atoms. This process gives the peptide enough energy to fragment into smaller ions. ECD is the only activation method which can be described as non-ergodic, which means that the energy is not redistributed over the whole molecule and the weakest bond broken, but rather cleavage occurs also at strong bonds such as N-Cα bonds generating c and z fragments (Figure 6). Thus it can be used as a complementary technique to CID or IRMPD. Other unique features include preferential cleavage of disulfide bonds and retention of post-translational modifications, for example, phosphorylation and glycosylation. Thus, sites of modifications may be more easily identified. These features represent important advantages over CID, which usually results in the loss of such labile modifications.[70]

1.3.3 Bioinformatics.

In many cases, the ultimate objective of the analyst in proteomics is to identify the proteins that are present in the original samples. In the classical method, proteins are separated first prior to a digestion step. The peptides generated from the digestion process are directly related to that particular protein which was digested and when peptides are identified then they can be related to that particular protein. However, this direct relationship between peptides and protein

is lost at the digestion stage in shotgun proteomics. Hence, in shotgun proteomics, bioinformatics has to do two important tasks: the first is to identify the peptides present and the second is to correlate these identified peptides back to the protein from which they were produced in the digestion process. [132] The task is further complicated by the huge number of data sets obtained from a single shotgun experiment. [133] Therefore, bioinformatics is considered the most crucial part of shotgun proteomics and understanding the limitations of the statistical and computational analysis is essential. [132]

Three key data are used to identify a peptide: the enzyme used to digest the protein, m/z value and the MS/MS spectrum of the peptide. [84] However, several limitations are present which complicate the identification process. For example, some peptides are not efficiently ionized or fragmented due to their physical and chemical properties. Moreover, identical peptides are present in several proteins that are produced from different genes from the same gene family. [132] Additionally, two amino acids (lle/leu) have identical masses and several others have very similar masses so that typical ion trap mass analysers cannot always distinguish between them. [132]

In addition to these limitations, database search engines add other types of limitations and ambiguity. Search engines differ in their sizes. Hence, different search engine may generate different lists of possible matches. [132] Moreover, different algorithms are used in the search. Generally, the algorithms can be divided into two types: the first type correlates the experimental spectra against theoretically predicted spectra while the second type is based on probability that the fragment ions present in the spectrum could be from sequences within the database by random chance. [133] SEQUEST is the most popular software in the first type of database search engines. It uses a special function to assess the quality of the experimental spectra and it depends on comparison of spectral features between the experimental and theoretical spectra. Based on this comparison a score is given which reflects how close the experimental data set is to the theoretically predicted one. The second type of database search engine uses

probability based matching to derive the correct peptide sequence from a database. Usually, a score threshold is selected to increase the reliability of the results and reduce errors. MASCOT is a probability based search engine which is commonly used, although others are also available.[133]

The second stage of the bioinformatics in shotgun proteomics starts after peptides are identified. In this process, the identified peptides have to be correlated to the proteins from which they have been generated. Several programs are available for such a task. In these programs, first, the user specifies a set of criteria to remove false identification. Second, a number of proteins are retrieved corresponding to each peptide, then, shared peptides are apportioned among all common proteins using probability based scoring. Finally, a protein list is generated. [132] In these programs, unique peptides play key roles, since if only one unique peptide is correctly identified then a protein can be identified with high confidence. Therefore, not surprisingly, more than 30 % of the proteins identified in shotgun proteomics are based on identification of a single peptide.[132] However, distinguishing between different proteins with high similarity is difficult; this is especially the case when only a small fraction of the protein sequence is covered. Additionally, in some cases shared peptides are assigned to a particular protein randomly. This problem is further complicated when no statistical evaluation of the obtained results is carried out. [132]

In summary, shotgun proteomics is a very promising method. However, the data analysis should be carried out with transparency and should be statistically evaluated to estimate the reliability of the results obtained from the experiments.[132,133]

1.4 Top down.

The classical methodology and shotgun proteomics are both based on the bottom up strategy. Top down, is the other strategy which can be used in proteomics. Methods based on top down strategy aim to obtain primary structural information from the gas phase dissociation of intact protein ions without prior recourse to

extensive separation or digestion [134] (Figure 10). The driving force of the top down strategy is the ease of finding any post-translation modifications occurring in a certain protein. In methods based on bottom up strategy, the sequence coverage of a protein is rarely 100 %, so the chance of missing certain modifications is high, whilst in the methods based on the top down approach, the protein is completely sequenced and hence theoretically there is no chance of missing any modification. [70] However, the major difficulty with the top down strategy is in obtaining an accurate mass measurement of large protein fragments. This is because mass spectrometry is well suited for mass measurement of peptides with masses less then 2 kDa, while obtaining a mass measurement for large proteins introduces greater uncertainty in the measurement. However, in the modern mass spectrometer, the uncertainty is reduced considerably with regard to mass measurement of high molecular mass proteins, especially with the introduction of FT-ICR. [135] This has given a strong impetus for methods which are based on the second strategy (top down), to be tested and evaluated. Early work carried out by Smith *et al.* [136] using ESI equipped with Tri.Q mass analyser suggested that obtaining direct sequence information from MS/MS spectra of proteins is possible but limited by the resolving power associated with product ion measurement in these instruments. This problem can be significantly reduced by the use of high magnetic field FT-ICR- mass analysers, which possess high resolving power in addition to high mass accuracy. [137] Using this technology, McLafferty and his group [134] demonstrated the advantages associated with the top down strategy. In their work, the molecular mass of the protein (carbonic anhydrase) was correctly measured (29024.3 Da) using masses of complementary pairs of fragments obtained by several methods for collisional dissociation of the molecular ion of the protein.

Among various dissociation methods, ECD is one of the most promising fragmentation methods in top down analysis. [138] Fragmentation of pure, undigested protein up to 60 kDa using ESI-FT-ICR and ECD has been reported. [134] However, for proteins above 70 kDa, Forbes *et al.*[139] suggested the use of enzymatic methods to generate peptides between 10 to 40 kDa, this will maintain

analysis efficiency and high sequence coverage. Recently, McLafferty announced fragmentation of proteins above 200 kDa using a newly developed method called prefolding dissociation (PFD). [140]

A method based on the top down strategy in its extreme form targets the analysis of protein mixtures 'as is', *i.e.* loaded directly into the mass spectrometer with no prior preparation. [4] However, this is far from reality at this moment. Several methods have been introduced to reduce the complexity of the protein mixture and hence make the approach more reliable. Meng *et al.* [141] used acid labile surfactant with 1D-PAGE gel and reverse phase chromatography to fractionate the protein mixture extracted from *Saccharomyces cerevisiae* with subsequent analysis by ESI-FT-ICR-MS (or MALDI-TOF-MS) to identify the intact protein by measuring their molecular masses and obtaining sequence information from direct fragmentation of the intact proteins using a Q-FT-ICR-MS hybrid. Other separation methods are also used, such as ion exchange chromatography followed by reverse phase chromatography.[142] Results indicate that improving the separation reduces the signal suppression and hence improves the sensitivity. However, the real challenge is in developing ion activation and dissociation methods which can be used to derive structural information from intact proteins directly and to determine the mass values of the fragment and protein ions to within 1 Da. [138,141]

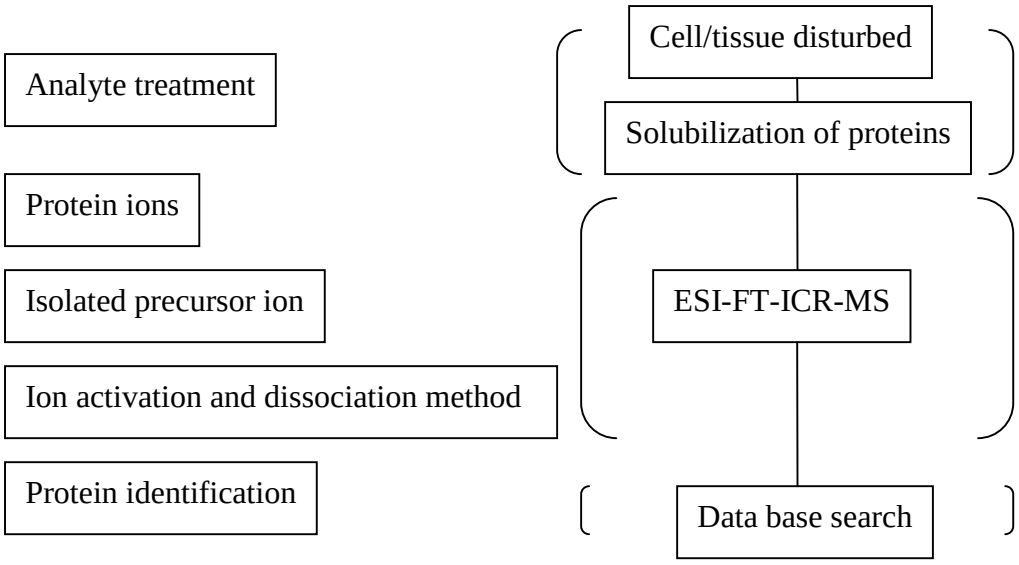


Figure 10: Procedure for top down proteomics.

In comparison to the shotgun and classical methods, the top down method is still in its early stages and considerable effort is required before it can be considered as an alternative method for proteomic analysis. [137] However, the top down method can be used as a complement to the other two methods. The structural information obtained from the methods based on bottom up strategy along with the molecular mass of intact protein or masses of complementary pairs of large fragments obtained from top down methods can increase the level of confidence in the results obtained to a great extent. [138] One of the earliest studies which used both methods in the analysis was conducted by VerBerkmoes *et al.* [143] for the analysis of the *shewanella onedenis* proteome. In their studies, 868 proteins were identified using the bottom up approach while only 22 were identified using the top down method. Tandem mass spectrometry was carried out for some unidentified proteins but the low sequence coverage prevented definitive identification for these proteins.

1.5 Microarray.

The great success of microarray technology in the analysis of DNA led several groups to transfer the technology to proteomic analysis. A microarray is a solid support in which an array of microspots are located, each microspot comprising a minute quantity of a specific binding agent (probe) that recognizes and binds to a

specific target molecule. [144] The technology does not require very high technical skills and can achieve high sample throughput. [145] The success of array technology in DNA analysis is due to the specific interaction present between the target molecule and the probe (*i.e.* key and lock), this one-to-one correspondence is not so readily available in proteins. [2]

Several microarray classes are available, varying from relatively nonspecific to highly specific ones, depending on the type of probes used in the array. The nonspecific arrays, which are based on ion exchange media, hydrophobic media and immobilized metal affinity ligands, can be used to fractionate the protein samples and once the proteins are digested then MS is used to identify the proteins present in each fraction. However, with these types of microarray, the main advantages which microarray possesses (*i.e.* low demand for technical skills and high sample throughput) are no longer relevant and the technology becomes similar to other proteomics methods. The technique is well suited to MALDI analysis and in its integrated version with MALDI, is known as SELDI (Surface enhanced laser desorption ionization). [146] Several protein profiling experiments of complex biological specimens and protein interaction studies have been carried out using the SELDI method.[147,148,149 ,150]

The specific class of microarray is based on the use of antibody, antigene or proteins. [151] Haab *et al.* [152] were the first to achieve protein screening using antibody microarrays with the kind of sensitivity and reproducibility that would be required to detect the vast majority of proteins present in a human cell lysate. In their work a comparative fluorescence assay using microarrays of antibodies was used.

Antibody based microarray depends on the immobilization of antibodies to the surface of the chip and the subsequent binding of proteins from a sample to the immobilized antibodies. However, proteins are extremely sensitive to the physical and chemical properties of the surface and the same surface may not be suitable for all proteins. [145] Additionally, the use of fluorescence detection does

not differentiate between background noise and a real signal. [145] Moreover, for an entire human proteome there is a need for at least one specific antibody directed against each human protein product. Thus, hundreds of thousands of antibodies will be required to carry out a protein profile of the entire proteome using microarray technology. The potential of this class of microarray technology is in finding a specific protein interaction or searching for some known protein in a cell or tissue sample. [153,154]

1.6 Imaging mass spectrometry.

In this technique a thin slice of tissue or cells (10 to 12 μm thickness) is attached on a sample plate and then the selected matrix is deposited or spotted on top of the sample. The sample plate in then introduced to the MALDI-TOF-MS and mass spectra obtained for different areas of attached tissue. Each spectrum represents molecules present in that area. Proteins of interest can be then be identified from the molecular ion observed. [155]

The technique can be used not only to give protein profiles but also to provide 'a map' of proteins in the cell or tissue. Using this technique Stoeckli *et al.* [156] identified a tumor biomarker in the proliferating area of the tumor. In another paper, Chaurand *et al.* [157] monitored over 400 proteins and localized three of them using the same technique. Schwartz *et al.* [158] reported the possibility of the use of this technique in protein profiling from brain tumors. The technique has also been used in other areas such as drug discovery, identification of biomarkers and lipid analysis.[159] However, several technical difficulties have to be addressed, the first of which is related to sampling and preparation techniques. It has been reported that ageing of tissue causes noticeable signal suppression even if the tissue is stored at -80 ºC; hence, a freshly prepared sample is needed for reliable analysis. [159] In practice this may not always be possible, especially when working in the clinical field. Additionally, low spectral quality can result from direct tissue sampling due to the nature of the tissue and high lipid content which limits access to the proteins present in the tissue. [159] Moreover, reproducibility is another difficulty associated with this technique. This is due to the quality of

spectra obtained, which is affected considerably by the sample preparation method such as tissue thickness and matrix deposition. [156] In this regard, sample automation techniques may be of great help in resolving these difficulties. The other technical difficulties are associated with MALDI as an ion generation technique. Tissues and cells are very complex samples and considerable numbers of chemicals are present, which causes considerable signal suppression. [155] Additionally, the sensitivity of MALDI-TOF as a technique reduces considerably as the molecular mass of the proteins present increases and therefore small proteins are more readily detected. However, one interesting solution has been proposed in which proteins contained in a thin tissue sample are transblotted through a membrane containing immobilized trypsin and the generated peptides are then captured by another membrane attached to the sample plate and covered with matrix. MALDI spectra and MS/MS can be obtained for the peptides generated and can be related to the proteins present in the tissue section which was sampled. [155] The key feature of this technique is that it allow digestion to take place whilst preserving the initial location. [160] Tissue imaging is a promising technique and the progress in top down methodologies will definitely improve this technique considerably as this will help in obtaining direct information from small and large proteins present in the tissue without the need for a digestion step.

1.7 Microfluidics.

Microfluidics refers to any devices where fluids can be driven in a network of micron sized channels etched into a solid substrate. [161] Several etching techniques are in use, the most common being photolithography and wet etching. Other techniques include radiation induced etching, [162] moulding, [162] direct laser techniques, [163] embossing [163] and ultrasonic machining. [164] The substrate of a microfluidic chip can be made from silicon, [165] glass, [163] plastic, [163] ceramic, [163] or even polymeric materials. [166] The choice of materials depends on the compatibility of the analytes, the availability and the cost of the material. [162, 163]

Hydrodynamic or electroosmotic flow (EOF) are used for moving the bulk liquid in the chip. Hydrodynamic pumping can be used with any type of chemicals.

However, it requires a pumping device, whereas EOF can be used only with ionic or polar chemicals but no external pumping device is required. Another advantage associated with EOF is the flat flow profile compared to the parabolic flow profile generated by hydrodynamic pumping. This flat flow profile reduces dispersion and improves resolution. [162]

1.7.1 Microfluidic based proteomics.

There has been great interest in the application of microfluidic systems to proteomics in recent years. [167] This is because of the many unique advantages afforded by the reduced dimensions compared to classical methods.

The principal features which make microfluidic systems attractive in proteomics are:

a) Fast diffusion rate.

Diffusion distance (d_{diff}) is governed by the following equation:

Equation 16 $$\mathbf{d_{diff} = \sqrt{Dt}}$$

where D is the diffusion constant (metre^2 s^{-1}) of the compound and t is time in seconds.

The gain in time when going from a millimetre range analytical process to a micrometre range is 10^6. This means that any analytical process governed by diffusion can be enhanced considerably when microfluidic systems are used. [168]

For separation methods where the mechanism of separation depends on a partitioning process *e.g.* chromatography, miniaturisation will enhance the analysis speed without loss of resolution. [169]

b) High surface to volume ratio.

The ratio between the inner surface area of the channel to the volume occupied by the solution is much higher in microfluidics compared to classical methods. For example, a typical surface area to volume ratio in a well from a 96 microtitre well plate is 500 m^{-1}, while the surface area to volume ratio in 50 x 50 μm (1 cm length) channel is 8 x 10^4m^{-1}. Hence, if an immobilized reactant is used, the

reaction will be much more efficient in microfluidics than in the classical method.[168]

These two features lead to several important advantages associated with microfluidic systems, such as high purity and yield in chemical application, small analyte volume and enhanced sensitivity with regard to analytical application. Moreover, microfluidics allows integration of different analytical processes in a single device and thus facilitates automation, which leads to high throughput, one of the important requirements for proteomics. [2]

The ultimate objective is to develop a microfluidic system in which cells are introduced to a chip, protein harvested, separated and digested, generated peptides separated and detected, finally proteins present in the cells are identified. This scheme is far off today. However, a number of components in the scheme have been developed. These are discussed below.

Several analytical techniques have been incorporated in microfluidic systems, for example, pre-concentration and separation, using various methodologies for immobilization most commonly using beads [170] and monolithic media. [171]

Two pumping techniques are used to pump beads into a microfluidic chip: hydrodynamic or electrokinetic. [172] The main difficulty with the use of beads in a microfluidic chip is finding a suitable way to keep them in place. Generally, two methods are used. The first is trapping the beads within a chamber. This is carried out either by forming a frit or by forming a restrictor at the end of the chamber (Figure 11) so that the beads are trapped. Usually a frit is more difficult as the formation of the frit requires heating and optimization of heating is difficult and requires well trained personnel. Care must be taken with this method of packing as it may not lead to even packing. The second method to place beads within a device is to immobilize the beads on the surface of the microfluidic chip using self assembly methods. This is achieved by modifying the surface of the microfluidic chip and preparing the beads with complementary materials, for

example, attaching a strand of DNA to the surface and a complementary strand to the beads. [173] Care should be taken with this method, as any change in the chemical medium, such as pH or ionic strength, may remove the bonding between the beads and modified surface of the channel. Another method to trap the beads is to use magnetic micro or nano particles. [174,175] These functionalized particles can be trapped in a channel simply by using an external magnet and allowing the fluid to pass through. This may be considered one of the most promising methods for trapping beads or particles in a microfluidic system due to its simplicity.

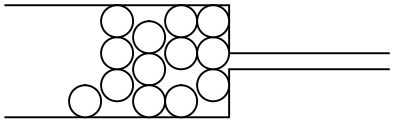


Figure 11: Schematic illustration of beads trapped inside a channel with a restrictor.

The other common technique used in a microfluidic chip is photoinitiated monolithic media. The main advantage of this technique is the possibility of controlling the location of the monolith inside the channel and implementing various analytical components in the specified parts on a microfluidic chip.[176]

A monolith can be defined as " a continuous unitary porous structure prepared by *in situ* polymerization". [177] Monoliths can be divided into two types according to the nature of the monolithic material used: organic based monoliths[178] and silica based monoliths. [179] Each has its own advantages and disadvantages. For on-chip proteomic analysis, organic based monoliths have several advantages, they are more compatible with biomolecules such as proteins than silica based monoliths.[180] Additionally, it has been shown that regeneration of the chip is possible when organic based monoliths are used. [181] This is a very important feature, especially during the optimization process of the monolith composition or if the monolith blocks the channel. Additionally, organic based monoliths are much easier to implement compared to silica

monoliths. [182] The discussion here will be focused on the organic based polymers; silica based monoliths have been reviewed in several other reports.[182,183,184]

Monoliths have been implemented on a microfluidic chip for various applications such as solid phase extractors, enzyme reactors and spray emitters. However, monoliths were originally developed as alternative separation media for HPLC and CEC. Therefore, the majority of applications are in these two fields. [176] The type of interaction between the monolith surface and the solutes is determined by the type of monomers used in the monolith formation. For example, if the monomer possesses hydrophobic properties then the monolith behaves like a reverse phase media. The monolithic mixture consists of monomer, crosslinker, initiator, microporogenic solvent and macroporogenic solvent. Usually the capillary or channel is filled with this mixture and then *in situ* polymerization is carried out using either thermal initiation or photoinitiation. The resultant monolith is highly porous. These pores inside the monolith can be divided into three types according to their size: micropores (<2 nm), mesopores (2-50 nm) and macropores (>50 nm). [185] If the monolith is used as a separation medium then the desired chromatographic interaction between the monolith and the molecules of interest occurs mainly in the micropores, as most of the surface area present in the monolith is due to these pores. However, for large molecules, the micropores are too small and these molecules cannot diffuse inside these pores. In this case, mesopores play the key role in providing the surface area for chromatographic interaction. [185] Macropores allow the bulk mobile phase to flow through and thus reduce the back pressure of the column. For a desired separation, a proper optimization is essential between the micropores, mespores and macropores. The pore sizes are affected by several factors which are discussed below:

a) Ratio between crosslinker and monomer.

Higher crosslinker to monomer ratio increases the mechanical strength. However, it also reduces the available surface area for chromatographic interaction. [186]

b) Ratio between micro and macro porogenic solvent.

Two types of solvent are usually used in forming the monolith: the microporogen solvent, which possesses higher solubility power to solubilise the polymer, and macroporogen solvent, which is a poor solvent for the polymer and reduces the solubility of the polymer in the mix. Increasing the ratio of microporogenic solvent to macroporogenic solvent leads to smaller pore sizes while decreasing the ratio produces larger pores. Thus, varying the percentage of the two porogens is a valuable tool to control the pore sizes without altering the composition of the polymers. [186]

c) Type of initiator.

Generally, the slower the rate of decomposition of the initiator, the larger the pore sizes. [187]

d) Method of initiation.

Thermal initiation is carried out at elevated temperature. Higher temperature increases the solvating power of the solvent system and hence phase separation occurs at a later stage, which results in a monolith with a smaller micropore diameter.[187] Photoinitiation is carried out at room temperature. Thus this usually produces monoliths with larger micropore sizes compared to thermally initiated monoliths.

1.7.2 Sample treatment.

Sample treatment here refers to all steps required to extract, purify and preconcentrate proteins or peptides. A complex mixture is usually used to solubilise proteins. Generally it contains chaotropic agents (*e.g.* Urea, Thiourea… *etc*.), detergents (*e.g.* Sodium dodecyl sulfate, Triton x100…*etc*.) and reductant (*e.g.* DTT…*etc*.), in addition to, isopropanol, glycerol and other chemicals. Once the proteins are solubilised, a purification step is required to remove salts, polysaccharides, lipids, DNA and RNA. Salts can be removed by dialysis or precipitation. The latter can also remove polysaccharides. Ultracentrifugation may be used to remove very large polysaccharides and lipids. High

concentrations of nucleic acids can be removed enzymatically by treating the analyte with DNase/ RNase. [46]

Few reports have been published in these fields (analyte treatment) incorporating microfluidic systems. These systems are mainly based on dialysis and solid phase extraction (SPE) concepts.

Dialysis is a technique where the sample is driven over a selectively permeable membrane in which non-permeating analytes pre-concentrate at the interface and are subsequently analysed.

Liu *et al.* [188] used a polymer membrane between two channels and compounds of interest were purified while the analyte flowed through the membrane. A similar design was used by Zhang *et al.* [189] where a polycarbonate membrane was inserted between two channels to selectively concentrate proteins. Song *et al.* [190] used laser induced polymerization and electrokinetic injection to concentrate protein samples with molecular mass larger than 5700 Da.

Solid phase extraction has been more commonly applied to microfluidic systems than dialysis. In these systems hydrophobic media are made and proteins or peptides are adsorbed on these media while the salts and hydrophilic materials pass through. This process cleans and pre-concentrates the analytes. Girault *et al.* [191] used on-chip analyte desalting. A polyamide chip was used and a PVDF membrane was placed between the inlet of the microchannel and the microfluidic connection. The results showed that no salt clusters were present in the spectrum. The method was also tolerant to high urea concentrations. Li *et al.* [192] used C_{18} beads to fill microfluidic channels which were than used for purification purposes. Salts and urea were removed from a standard peptide mixture or from in-gel digested protein. Using a similar method, Ramsey *et al.* [193] were able to detect samples at the 100 pmol level. The most promising way to perform protein purification in a microchannels was introduced by the Frechet group [194] in which monolithic material was polymerized directly in the microchannel. This

monolithic solid phase extractor works also as a pre-concentration device and thousand fold enhancements in concentration were observed.

Carlier *et al.* [195] also used an on-chip polymer monolithic phase which includes hydrophobic moieties as a sample purification system.

Yu *et al.* [196] used two types of monolithic media to concentrate the analytes, ion exchange and hydrophobic media. The system was found to be robust, reusable and concentrated peptides 1000 fold.

An elegant design was used by Schilling *et al.* [197] in which protein extraction was carried out. A solution containing cells and lysis buffer were flowed in the same channel. Cell lysis occured at the flow's interface. Cell debris did not diffuse in the lysis buffer, whereas proteins partitioned between the two flows. The lysis buffer then flows in to another microchannel where detection takes place. The device can also be used as a pre-fractionation device by adjusting the flow rate, high molecular mass compounds will have less time to diffuse to the buffer at high flow rate, and only low molecular mass compounds will be extracted in the buffer.

A compact disk has also been used for analyte clean-up and desalting prior to obtaining mass spectra from a MALDI-TOF-MS. [198] Up to 96 analytes could be treated at one time.

1.7.3 Protein digestion.

One of the most time consuming steps in proteomics is the in-gel digestion of proteins. On-chip digestion is an attractive alternative to the current in-gel digestion. This is due to the high efficiency and speed of the on-chip digestion which is due the high surface to volume ratio and fast diffusion rate in the chip. The three most common approaches which have been utilized to perform on-chip protein digestion, are: immobilization of trypsin to the inner surface of a channel (open channel), [199,200,201] packing a channel with beads [202,203,204] and using *in situ*

trypsin immobilization on a monolith. [205,206,207] The key point here is to increase the surface area so that larger amounts of trypsin can be immobilized, leading to higher digestion efficiency.

Ekstrom *et al.* [199] used a silicon chip to immobilize trypsin. The chip was treated with ethanol and hydrofluoric acid solution to increase the surface area further. Trypsin immobilization was carried out by coupling the enzyme to the wall of the silicon channels. Several proteins were digested separately in one minute.

Encapsulated sol gel trypsin has also been used, where a surface modified methylacrylate substrate was employed to trap trypsin within the sol gel. Cytochrome C was used as the protein model for digestion. [118,208]

Recently, Huang *et al.* [200] immobilized trypsin on the modified surface of a poly methyl methacrylate chip with zeolite nanoparticles. The system was tested with two proteins, cytochrome C and BSA and digestion was completed within 5 seconds. The system could be reused for up to a month if it is stored at 4 ºC.

Liu *et al.* [209] also reported protein digestion within five seconds. Natural polysaccarides, positively charged chitosan and negatively charged hyaluronic acid were multilayer assembled onto the surface of a poly(ethylene terephthalate) (PET) microfluidic chip to form a microstructured network for trypsin immobilization.

Plasma oxidation of poly dimethylsiloxane (PDMS) forms a layer of silanol groups. The silanol was then linked covalently to a trypsin-encapsulated sol gel matrix. BSA was digested in two seconds and sequence coverage, percentage of amino acids identified in a protein, of 27 % was obtained when the digest products were analysed. This very high speed of digestion was attributed to high trypsin loading and to the compatibility of the sol matrix environment with biomolecules, which minimizes denaturation of the encapsulated trypsin. [210]

Cross-linked polystyrene-divinylbenzene particles have been used to immobilize trypsin and are commercially available (Applied Biosystems) as a bulk medium and packed in a cartridge. A considerable number of reports have been published which utilize this solid support. [211,212,213] The high stability of the enzyme immobilization chemistry enables repeated use of the cartridge for hundreds of assays. However, it has not been used in a microfluidic system.

Trypsin has also been immobilized on glass beads. Bonneil *et al.* [202] used a 30 cm long fused silica capillary (530 μm i.d. x 800 μm o.d.) packed with commercially available trypsin immobilized on controlled pore glass beads to digest β-casein and insulin chain B at 37 ^{o}C. Separation of the generated peptides was carried out using capillary electrophoresis with diode array detection. This system was able to generate tryptic digests in 2.5 hours similar to that carried out by classical methods in 24 hours. However, the generated peptide maps were not very good when the length of the capillary was reduced to 15 cm.

Wang *et al.* [203] used commercially available agarose beads (40-60 μm) to pack a channel in a microfluidic system along with an on-chip-CE separation system directly interfaced to ESI-MS. At the end of the channel a flat bottom tip tapered to 10 μm was inserted and bonded with crystal bond. To stabilize the spray a make-up reservoir was connected to the tip and filled with separation buffer. The potential difference between the make up buffer and the emitter was set to 4 kV. Complete digestion of cytochrome C was obtained when the solution was pumped hydrodynamically at 1.0 μl min^{-1} and the sequence coverage exceeded 90 %. BSA was pumped at lower flow rate (0.5 μl min^{-1}) for complete digestion, and sequence coverage of 71 % was reported after the reduction and alkylation of cysteine residues.

Yue *et al.* [204] used the same type of beads in a weir in a microfluidic chip to digest β-casein. However, results showed incomplete digestion of the protein, which may be due to the larger bead size used (50-145 μm) compared to (40-60 μm) that used by Wang *et al.* [203] Slentz *et al.* [214] used silica based particles to

immobilize trypsin. Incomplete digestion was obtained for BSA when infused through the beads.

Freije *et al.* [215] compared the efficiencies of the trypsin immobilized on agarose beads, sepharose beads and poroszyme beads. Results showed that the efficiency of trypsin immobilized on agarose beads is less compared to the other two. The difference in efficiency is increased significantly when the trypsin beads are modified by an acetylation reaction.

Several factors affect the efficiency of the digestion process using trypsin immobilized on beads, for example, the size of the beads. Smaller beads increase the available surface area for protein digestion. The pore size of the beads also plays an important role in the efficiency of the digestion process. A suitable pore size is required so that proteins can diffuse inside these pores to reach the active site. However, some protein molecules will move around the beads rather than inside the pores and this will reduce the digestion efficiency. In monolithic media this cannot happen as the monolith is one large piece (*i.e.* no external surface) and hence it is expected that the efficiency of digestion will increase. However, the efficiency depends on several other factors such as the quantity of the immobilized trypsin, the surface area available for the interaction and the residence time. [3] Additionally, because there is 'no external surface in the monolith' the flow is governed by conduction and not diffusion. Hence the digestion process should be faster.

Trypsin immobilization using monolithic media was first proposed by Petro *et al.*[216] The immobilization was carried out on poly(glycidyl methacrylate -co-ethylene dimethacrylate). High activity of the immobilized trypsin was observed in comparison to trypsin immobilized on micrometre size beads made from the same materials. Several other studies have reported using trypsin immobilized on monolithic media. Anders *et al.* [217] used an activated capillary for the preparation of a porous polymer monolithic trypsin microreactor. The monomers making up the monolith were mixed with trypsin and introduced into the column for

polymerization and immobilization. MALDI-TOF-MS was used for the analysis and sequence coverage of 32.2 % was obtained for BSA, 47.1 % for cytochrome C and 29.4 % for myoglobin.

However, the most comprehensive studies were conducted by Peterson *et al.* [205] They immobilized trypsin on a 10 cm methacrylate monolithic column *via* azalactone functionality. This allows the trypsin to react *via* the amino and thiol groups present in the protein, hence a higher concentration of trypsin is immobilized. The immobilized trypsin column was coupled to a MALDI-TOF-MS. Solutions of proteins of interest were pumped through the monolith at a flow rate of 0.5 μl min^{-1}. Results showed that the sequence coverage varies from 100 % for small proteins (melittin) to 21.9 % for large proteins (holo-transferrin). However, when the monolithic column was coupled to ESI-Tri.Q-MS, the sequence coverage was reduced noticeably, with the lowest being (12.5 %) for holo-transferrin. This was due to the use of ammonium acetate buffer which is a very good buffer for ESI due its volatility but because of its low pH, it is not optimal for tryptic digestion to take place. Similar sequence coverage was obtained for myoglobin with this immobilized trypsin when it was moulded in microfluidic chips (67% compared to 65.3% in capillary). In both cases, the protein was not completely digested due to the short residence time available for enzymatic reaction to take place.

Recently, Feng *et al.* [218] used trypsin immobilized on a monolithic support coupled with an on-line LC-MS system for analysis of the total cell lysate of *Saccharomyces cerevisiae*. This is the first report on the use of trypsin immobilized on a monolithic support for digestion of such a complex mixture.

Trypsin adsorption on a poly vinylidene fluoride membrane which was fixed on-chip was also used to achieve a high enzyme concentration and hence fast protein digestion. Digestion of cytochrome C was achieved within 10 minutes using this system. [219]

1.7.4 Peptide separation.

The use of electroosmotic flow for pumping a sample into a mass spectrometer and the desired features associated with this flow driven technique, made capillary electrophoresis (CE) apparently the most suitable method for on-chip separation (Chip-CE) prior to the sample introduction to a mass spectrometer. The first peptide separation on a Chip-CE interfaced with an ESI-MS was achieved by Li *et al.* [220] In this work two designs were compared, the first based on a nanospray emitter directly coupled to a microfluidic chip *via* a low dead volume junction which was achieved by straight cutting the inlet of the capillary and inserting it into the hole on the microfluidic chip. This design was compared with the second more conventional one in which a sheath flow Chip-CE was interfaced with an ESI-MS. The separation efficiency of the first design was modest (N = 500-3500), while for the second design it was higher (N = 26000-58000) for mixtures of different peptide standards. By coating the channel with a quaternary amine reagent and using a gold coated electrospray emitter the first design sensitivity was increased ten fold and the detection limit was improved (3.2 to 43.5 nM for different peptides). [221]

A second design was proposed by Zhang *et al.* [222] in which a sleeve was fabricated in the chip to establish a chip capillary connection. This design was compared with pneumatically assisted electrospray from a chip. A tryptic digest of cytochrome C was separated using these two designs. Although the capillary coupling gave higher separation efficiency, the design yielded a dead volume at the capillary chip junction, degrading the separation performance.

Several other designs have been used and a considerable number of reports have been published in this field with various on-chip electro-driven separation techniques such as CE, [223] micellar electrokinetic chromatography (MEKC), [224,225] capillary electrochromatography (CEC), [181,226,227,228,229] and IEF. [230,231]

A few reports have been published in which hydrodynamic pumping systems have been used for on-chip peptide separation. The most successful ones are

based on a particle-type stationary phase 232,233 rather than a monolithic type stationary phase, which is much easier to implement for on-chip separation.234 Another important benefit of monolithic type stationary phases is the high porosity. This implies low back pressure, low flow resistance and consequently faster analysis, which is a basic requirement for efficient on-chip proteomic analyses. [185,235]

Ro *et al.* [101] reported microarray reverse phase monolithic columns in a cyclic olefin copolymer chip interfaced to MALDI-TOF-MS off-line. The system was evaluated using a mixture of digested proteins. The amino acid sequence coverage was doubled when obtained using this chip compared with MALDI-TOF alone. The chromatographic monolith was based on a methacrylate polymer. The same group reported the fabrication of a monolithic reverse phase column on-chip coupled to ESI-MS. [236] This on-chip column was than evaluated using a tryptic digest of BSA. However, the efficiency of these two monolithic columns was less than the efficiency of the on-chip particle based columns reported by Fortier *et al.* [233]

In another report, [237] a chip based reverse phase HPLC system was fabricated and used for peptide separation. A monolithic column of 1.7 cm in length was used and tested with a standard peptide mixture using isocratic elution. Good reproducibility but poor separation was observed.

In another attempt Xie *et al.* [238] fabricated a complete HPLC system on-chip including a gradient electrochemical pumping system. The column was made of 5 μm C_{18} stationary phase (1.2 cm length). The system was tested with a tryptic digest of BSA and compared with the Agilent 1100 series nanoflow HPLC system. A similar separation was observed. However, the peak shapes in the latter system were better which was attributed to the particle size used (5 μm compared to 3 μm). Additionally, obtaining reproducible data using the electrochemical pumping system at a very low flow (80 nl min^{-1}) rates may be difficult.

An elegant design introduced by Brivio *et al.* [239] used a glass microfluidic chip integrated within the MALDI-TOF analyte plate. The vacuum of the instrument was used to provide pressure driven flow, several organic and biological analytes were successfully analysed.

In another attempt, [240] open microchannels were used, so that the channels could be exposed to the MALDI laser beam. In this design, the matrix was added to the buffer and after separation, the solvent evaporated due to the heat generated by the electrophoretic separation, thus effectively freezing the analytes within the matrix. The chip was then transferred to the MALDI mass spectrometer for analysis.

1.7.5 Two dimensional separations.

The complexity of the proteomic analytes requires multiple dimensions for efficient separation of thousands of peptides present in a tryptic digest of proteins sample. [234] Chip based two dimensional separation is a very challenging task. To date, very few papers have been published. Ramsey's group [225] used CEC with CE mode as a multidimensional separation technique. However, this system cannot be considered as a truly multidimensional separation method. According to Jidding [98] any successful two dimensional separation should be based on two different chemical or physical mechanisms for each dimension, the mechanism of separation of CE is a part of CEC, as CEC is a mixed mode of separation of which CE is part.

In another attempt the same group used MEKC and CE. [241] Although this system is also not truly multidimensional, an impressive peak capacity of 4200 in less then 15 min was achieved. Herr *et al.* [242] coupled IEF with CE in an acrylic microfluidic chip. The peak capacity of this system was 1300 in 5 min. The common point about these systems is the use of laser induced fluorescence (LIF) detection which allows very high detection sampling rates. However, coupling any of the above systems to MS will lead to the reduction in separation performance and lower peak capacities due to the reduction in sampling rate. [168]

Additionally, step elution in these systems is not very convenient and therefore, the second dimension has to be very short, which limits the separation capability to a great extent. [243]

The chip-based HPLC from Agilent [232] has been used as a second separation dimension along with a cation exchange capillary column as first dimension. Off-line coupling of these two separation media was used in the analysis of a nuclear proteome and compared with one dimensional analysis using chip-based HPLC. Two hundred and six unique proteins were identified in the first case while only thirty four proteins were identified when only the chip based HPLC system was used for the analysis. [244]

1.7.6 Interfacing microfluidic chips to a mass spectrometer.

Initial work from Karger's group [245] in this area focused on the use of a microfluidic system as a high throughput sample delivery system. This is illustrated by interfacing multiple channels to a mass spectrometer using electroosmotic flow. The analytes were sprayed from the channels directly through the ESI interface into the mass spectrometer. In order to prevent the analysed solution from spreading, the outlet edge was coated with a hydrophobic reagent. However, the spray was not very stable due to the negligible electroosmotic flow resulting from the use of an acidic solution and from the low electric field. Hence a syringe pump was used to assist, at a flow rate of 100 to 200 nl min^{-1}. Ramsey *et al.* [246] reported that in order to get a stable electrospray, the application of a small pressure was required, as was mentioned previously by Karger's group; [245] however, the pressure could be induced electroosmotically. In their report (Ramsey *et al.*[246]) pressure induced electroosmotic flow was used and a stable Taylor cone at the end of the open channel was observed. This direct spray from a channel has been reported by other groups also. [247,248] However, several problems were associated with the interface, such as the formation of large liquid droplets at the surface of the chip which caused analyte dilution and band broadening. This reduced the sensitivity and precluded the use of such an interface for on-chip separation. [249] Several attempts have been made to reduce

the droplet size such as coating with hydrophobic agents, [245] derivatizing,[245] microfabrication of a pneumatic nebulizer integrated to the microfluidic chip [222] and using a hydrophobic substrate. [250]

Another way to interface microfluidic chips with a mass spectrometer was introduced by Figeys *et al.* [62] In this interface a silica capillary was connected to a microfluidic chip and used as an electrospray emitter. A 12 cm length of 75 μm i.d. x 150 μm o.d. fused silica tubing was used, with the inner surface derivatized with 3-aminopropyl silane. The pumping of the liquid was carried out electroosmotically by adjusting the electric field. Using a similar design, Lazar *et al.* [251] reported a detection limit in the sub-attomol range.

The connection of a microfluidic chip to a mass spectrometer *via* a capillary produces small, very well defined droplets which enhance the sensitivity and the resolution of the process. Another important advantage is that in this case any blocking occurring in the capillary can easily be overcome by replacing the capillary with a new one. It also helps in controlling the electrospray voltage at the point where liquid is emitted.

The disadvantage of this interface is the use of glue to connect the capillary to the chip, as this glue may cause analyte contamination or may itself block the capillary.[252] An elegant design introduced by Zhang *et al.* [252] removed the glueing problem by using a guide channel for connecting the capillary. The internal diameter of the guide channel was etched to match the outer diameter of the connection capillary. Another problem with this interface is the dead volume introduced due to the interfacing capillary between the separation chip and the MS.

The third interface is a microfabricated tapered electrospray tip, which is the most promising interface as it avoids all the problems associated with the first two types of interface. One of the earliest designs was proposed by Licklider *et al.* [253] In this design a silicon chip was used, in which a hollow needle structure of about

1 mm length was made beyond the edge of the chip. Stable spray was obtained when a high electric field (1 to 2 kV) was applied. Another design was proposed by Schultz *et al.* [254] in which a monolithic silicon substrate was used and a channel extended from the reservoir to the tip of the nozzle was etched. Dhalin *et al.* [255] fabricated an ESI tip on poly(dimethylsiloxane) (PDMS) chips. The tip was coated with conducting graphite powder, which allowed independent control of spray and separation potential (CE separation). Yin *et al.* [232] fabricated an ESI tip using laser ablation of a polyimide substrate. A conical tip was formed with dimensions from 35 to 100 μm o.d and up to 2 mm length. This type of tip is also now commercially available from Agilent laboratories.

1.7.7 Integrated microfluidic systems for proteomics.

Integrating different analytical steps of proteomic analysis in a single microfluidic platform is an important step towards automation. Although several separate steps have been reported, integrating does not simply mean summing up these steps in a single platform. Integrating also means compatibility between different techniques used in the microfluidic systems. To date few reports have been published in which dual function microfluidic systems have been used for proteomic analysis. Protein digestion is usually carried out in these microfluidic systems in addition to peptide separation or solid phase extraction.

Peptide separation is mainly carried out using Chip-CE. For example Wang *et al.*[203] used agarose beads for the digestion process, followed by Chip-CE separation for the generated peptides. Park *et al.* [256] performed on-chip digestion using adsorbed trypsin on a nitrocellulose membrane followed by Chip-CE separation. However, in their reports the focus was mainly on the digestion process and not on the separation efficiency of the system. Slentz *et al.* [214] incorporated protein digestion, immobilized metal affinity capture selection of histidine containing tryptic peptides and CEC. However, the device was used with fluorescence detection and not with mass spectrometry. Li *et al.* [192] designed a microfluidic system in which solid phase extraction and Chip-CE were incorporated. The microfluidic system was used for identification of proteins

present in human prostate cancer cells and 72 proteins were identified. In another design introduced by Peterson *et al.* [257] a dual function microfluidic chip was fabricated. Protein digestion and solid phase extraction were implemented using photoinitiated monoliths. Dodge *et al.* [258] fabricated PDMS based microfluidics in which digestion and CE separation is carried out in a single chip.

1.8 Conclusions.

A comprehensive analytical method for proteomics is proving to be very challenging and has not been achieved at this moment. None of the reported methods can be considered as ideal for all cases; each has its own strengths and limitations and ultimately is suitable only for particular applications. It is therefore useful to regard these different methods as complementary rather than competitive and in many cases, it may be required to use more than one method to achieve certain objectives. However, a clear determination of the objective can provide considerable aids for the analyst in selecting appropriate method(s) of analysis.

To date the most mature methods are the classical and shotgun methods. [3,259] Other methods are at various stages of development. However, in all these methods, mass spectrometry and bioinformatics are key techniques. Hence technology development in these two fields will have a direct impact on proteomics. [260] This is already observed in the case of the top down method, which gained a great momentum due to the development of the FT- ICR mass analyser. Another important field which will have a direct impact on proteomics is separation science. This is because the sensitivity of ESI and MALDI, the main ion sources used in proteomics, improves considerably when an efficient separation technique is used prior to the introduction of the analytes to these two ion sources.

1.9 Objective of the research.

Two criteria are of major concern in proteomics: high sensitivity and high throughput. [2] One of the problems associated with macro methods is the

mismatching between the proteomic sample and the technique used. For example, in the classical method, micrograms of sample are spread over a large 2D-PAGE gel. This spread may lead to sample contamination and loss, which increases further due to different handling stages.

Another difficulty associated with the macro methods is the mismatching between minute sample volume, the macro sample preparation and handling techniques and detection techniques that require minute sample volume. If the transition from micro to macro and then again to micro were replaced by a complete microsystem, high throughput analysis would be easier due to the homogeneity between various stages of analysis. The transition to the microsystem can be carried out using microfluidic systems designed specifically to handle proteomic samples. As discussed earlier mass spectrometry and bioinformatics are the key techniques in various methods developed in the field of proteomics. Different types of microfluidic systems can be made and interfaced to mass spectrometry and the desired analysis can be carried out.

The objective of this research is to explore the use of microfluidic systems in proteomics by developing an efficient on-chip digestion method integrated with an on-chip separation method and connected to an electrospray ionization mass spectrometer. The system can be highly sensitive due to the reduction of human interference and sample loss as all the digestion products will pass to the detector. The coupling of the microfluidic system, *via* capillary, with ESI-MS may also increase sample throughput. Figure 12 shows schematically the steps involved in the proposed methodology and Table 3 compares the proposed method with the most mature methods in proteomics (classical and shotgun).

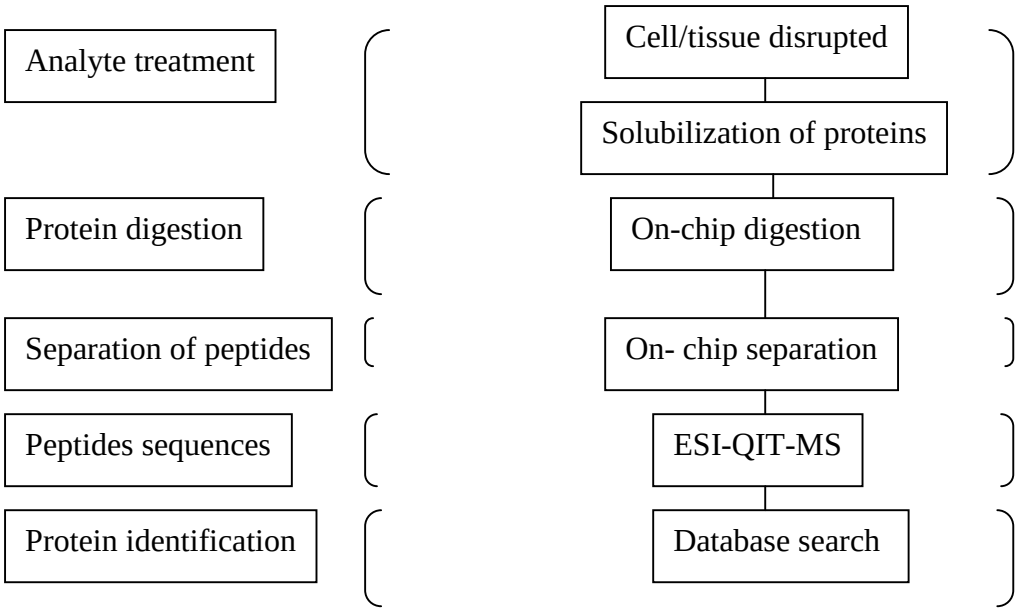


Figure 12: Procedure of the proposed proteomics methodology.

Analytical process	Technique used	Method		
		Classical	Shotgun	Proposed
Analyte treatment	Cell/tissue disrupted Solubilization of proteins	√	√	√
Protein separation	2D-PAGE	√	-	-
Protein digestion	In-gel In-solution On-chip	√ - -	- √ -	- - √
Separation of peptides	2D- LC On-chip	- -	√ -	- √
Peptides sequences	MALDI-TOF LC-ESI-QIT-MS Chip -ESI-QIT-MS	√ - -	- √ -	- - √
Protein identification	Database search	√	√	√

Table 3: Comparison between classical, shotgun and proposed proteomic method.

2.0 Protein digestion and peptide separation using a fabricated microdevice as a model for a microfluidic system

2.1 Introduction.

Preliminary studies and the optimization of various analytical parameters were first carried out using fused silica capillaries, which are commonly used as a model for channels within a microfluidic chip. [99,261] This is due to the similarities between the inner wall of the fused silica capillary and a glass microfluidic channel. However, the major difference is in the cross section, which is circular in the capillary while it is closer to rectangular in the microfluidic channel.

As discussed earlier (section 1.7.3), three methods have been commonly used in a microfluidic chip to immobilize trypsin: immobilization of trypsin to the inner surface of a channel (open channel), [199,200,201] packing a channel with beads [202,203,204] and using *in situ* trypsin immobilization on a monolith polymerized in a microfluidic channel.[205,206,207] The open channel method for trypsin immobilization has been reported to be the fastest and the most efficient; [200,210] however, it is difficult to integrate this method with other analytical steps in a single microfluidic system. This is because it requires special treatment for the inner surface of the part of the channel where digestion takes place. Such control is very difficult to implement and in all the reports in which the open channel method has been used for digestion, it was used in a microfluidic chip where no other process was taking place. The other two methods can be integrated much more easily with various analytical steps in a single microfluidic system. Table 4 compares two reports; in the first [203] trypsin immobilized on beads was used as the digestion media while in the second [205] trypsin immobilized on a photoinitiated monolith was used instead.

Trypsin immobilized media	Beads	Photoinitiated monolith
Type of device.	Glass chip (15 mm x 800 μm x 150 μm) on-line CE-ESI-MS.	Silica capillary (100 μm i.d., 10 cm. long)on- line ESI-MS.
Flow rate.	Between 1.0 to 0.5 μl min^{-1}.	0.5 μl min^{-1}.
Concentrations of the solution infused.	Cytochrome C 16 μM BSA 6 μM.	Cytochrome C 20 μM BSA 20 μM.
Residence time.	< 3.5 minutes.	< 1 minute.
Sequence coverage.	Cytochrome C 92% BSA 71%.	Cytochrome C 74 % BSA 18.5 %.
Number of peptide fragments identified	Cytochrome C 15 BSA 43	Cytochrome C 11 BSA 16
Complete digestion.	Yes.	No.

Table 4: Comparison between protein digestion efficiency of trypsin immobilized on two different media (beads [203] and monolith [205]).

The comparison here was carried out to conclude which of these two particular methods could be carried out easily and provide satisfactory results if the same experimental procedure was used. Two variables were considered, namely, the sequence coverage along with number of peptide fragments and the residence time required to obtain complete digestion of the protein sample. Sequence coverage can be defined as the percentage of amino acids identified in a protein. Sequence coverage along with the number of peptide fragments can be used as a variable to measure the digestion efficiency provided peptides with miscleavage were not used in calculating the sequence coverage. The second variable used to measure the efficiency is the residence time required to obtain complete digestion. Complete digestion is not easily confirmed as it depends on the detection system used. However, here it was assumed that the digestion was complete if no traces of intact protein could be seen in the detection system used. The second variable is somewhat complex, as one can increase the residence time and reduce the concentration of the injected protein or increase the concentration of the protein and reduce the residence time. Therefore, ideally the residence time and the amount of protein digested when the capillary or the channel is completely filled with the protein sample should be used as a measure of

digestion efficiency. However, due to the unknown volume occupied by the beads or the monolith, the exact volume of the channel or the capillary is often unknown. However, Peterson *et al* [205] did an estimation for the volume of the capillary after monolith formation by measuring the porosity of the monolith. According to these measurements, the volume was estimated to be 471 nl. This means that the amount of protein sample, required to fill the capillary, was 9.42 μmol (20 μM solution was used) and the residence time was 57 s. However, the digestion was incomplete and hence the exact amount of protein digested was unknown.

Based on these two variables and the available data from the two reports, the efficiency of the trypsin immobilized on agarose beads was higher than that of the trypsin immobilized on monolithic media in terms of sequence coverage and complete digestion. Although, residence time required to obtain the desired digestion was much longer in the former than in the latter, in practice increasing the residence time by reducing the flow rate is difficult as the flow rate was already low (0.5 μl min^{-1}). Additionally, trypsin immobilized on agarose beads is commercially available and can be used directly. Therefore, it was decided to use trypsin immobilized on agarose beads.

Electro-driven flow is the most commonly used separation technique in microfluidics. This is because electro-driven flow separation techniques are well matched with microfluidic chip devices for separation purposes, as no external pumping system is required. Additionally, these techniques have high resolving power compared to the techniques based on hydrodynamic pumping system, due to flat flow profiling. However, the problem with these techniques is in the compatibility with the principal detection technique used in proteomic analysis, mass spectrometry. Electro-driven flow separation requires addition of salts in the solution. This affects the sensitivity of ESI-MS considerably. Additionally, only volatile salts can be used when these techniques are interfaced to ESI-MS. Moreover, in some cases a high pH buffer is required to accelerate the electroosmotic flow, which once again affects the sensitivity of the ESI-MS

considerably. Furthermore, the complexity of the tryptic digest of proteins requires a multiple separation technique. However, since capillary electrophoresis relies on liquid phase separation, it is difficult to be adopted for stepwise elution in a multidimensional separation. [243]

The other principal difficulty in the interfacing of electro-driven flow separation techniques with MS is that none of the suggested designs reported to date fully utilize the potential power of separation of the electrophoretic techniques in terms of resolution and sensitivity, as these interfaces add a dead volume and in some cases cause flow disturbances. [168] HPLC is the only separation technique which can be interfaced to ESI-MS without any compromise on the sensitivity of ESI-MS. [262] Moreover, in most cases HPLC uses low pH mobile phases which are very well suited to coupling with ESI-MS. Additionally, the flow can easily be controlled without any need to have any make-up solution that may cause loss of resolution and without introducing any disturbance to the flow.

Beads were suggeted as a medium for the proposed digestion system, however, it was decided to exclude the use of particles as a medium for separation. This was due to the difficulties associated with obtaining a uniform packing with very small particles (3 to 5 μm in diameter). If the packing of the separation column is not uniform then voids are created in the separation column and the separation may be affected or lost depending on the volume of the voids in the column. Additionally packing a channel with small beads requires a very narrow restrictor to keep these particles in place in the microfluidic channel. Moreover, high back pressure is generated from packing a microfluidic channel with such small particles. These difficulties are not encountered with the proposed method of digestion as uneven packing will not affect the digestion process significantly and a very small restrictor is not required. Additionally, the back pressure generated from the use of large beads is negligible. The other alternative to particle type stationary phase is to use a monolithic type stationary phase where the column polymerizes *in situ* and no frit or restrictor is required. Moreover, the back

pressure generated from such a column is less than the back pressure generated from a column packed with particle type stationary phase.

2.2 Experimental.

2.2.1 Chemicals.

- Cytochrome C (horse) (Sigma chemical Co., St. Louis, MO - USA).
- Melittin (bee venom) (Sigma chemical Co., St. Louis, MO - USA).
- Ubiquitin (bovine) (Sigma chemical Co., St. Louis, MO - USA).
- Myoglobin (horse) (Sigma chemical Co., St. Louis, MO - USA).
- Bovine serum albumin (BSA) (bovine) (Sigma chemical Co., St. Louis, MO - USA).
- Apo-transferrin (bovine) (Sigma chemical Co., St. Louis, MO - USA).
- Dithiothreitol solution 1 M (DTT) (Sigma chemical Co., St. Louis, MO - USA).
- Trypsin gold (Promega Corporation, Madison - USA).
- Acetonitrile, HPLC grade (Fisher scientific equipments, Loughborough - UK).
- Glacial acetic acid (Fisher scientific equipments, Loughborough - UK).
- Ammonium acetate, HPLC grade (Fisons scientific equipments, Loughborough - UK).
- TPCK-treated trypsin, immobilized on 40 - 165 μm diameter 4 % crosslinked agarose beads (Pierce, Rockford, IL- USA).
- Benzoylperoxide (Sigma chemical Co., St. Louis, MO - USA).
- Styrene (Sigma chemical Co., St. Louis, MO - USA).
- Divinylbenzene (Sigma chemical Co., St. Louis, MO - USA).
- 1-Dodecanol (Sigma chemical Co., St. Louis, MO - USA).
- Decanol (Sigma chemical Co., St. Louis, MO - USA).
- Toluene, analytical grade (Sigma chemical Co., St. Louis, MO - USA).

2.2.2 Instrumentation.

- Electrospray mass spectrometer (ESI-MS): LCQ classic quadrupole ion trap mass spectrometer equipped with standard electrospray ion source, dual syringe pumps and controlled *via* PC based Xcalibur version 1.2 software. (Thermo Finnigan, LOC, San Jose - USA). An electrospray voltage of 4.0 kV and nitrogen sheath flow of 60 arbitrary units was employed. The temperature of the heated capillary was set to 200 ºC.
- Matrix assisted laser desorption ionization mass spectrometer (MALDI-TOF): Bruker Daltonics Reflex IV equipped with scout MTP and controlled by PC based Flex control version 1.2 (Bruker Daltonic, Bremen - Germany).
- pH Meter: fisherman hydrus 300 (Thermo Orion, Beverly, MA - USA).
- Scanning electron microscope (SEM) images were obtained using a Cambridge Instruments S360 (Leo Electron Optics, Thornwood - USA). The beam energy was set to 15kV with a probe current of 250 pA. The samples were attached to standard aluminium mounts using cyanoacrylate adhesive. A thin coating (2 nm) of 82 % / 18 % Au / Pd was subsequently applied using a Polaron sputter coater. The samples were spun at approximately 100 revolutions min^{-1} during sputtering to ensure an even coating.
- Baby bee syringe pump (Bioanalytical System Inc., West Lafayette, IN - USA).
- Column block heater (Jones Chromatography LTD, Wales - UK).
- Torr seal (Varian Ltd, Palo Alto, CA - USA).
- Microtight ‘T’ connector (Upchurch scientific, Inc., Oak Harbor, WA-USA).
- Inverted microscope wilovert standard HF40 (Hund, Weltzar-Germany).

2.2.3 Protein digestion.

All solutions were freshly prepared. Stock solutions were obtained by dissolving the protein (0.25 - 20.0 mg) in ammonium acetate solution (25 mM), which had been adjusted to pH 8.2 using dilute ammonium hydroxide solution. In the case

of ubiquitin and myoglobin other solutions were also prepared in which acetonitrile 50 % in ammonium acetate buffer (pH 8.2) was used to unfold these two proteins. Thermal protein unfolding was carried out using a column block heater. The thermostat was set to 90 ^{o}C. In the case of BSA and apo-transferrin, 60 µl of dithiothreitol 0.04 M was added to reduce disulfide linkages.

In the case of BSA and apo-transferrin, the tryptic digest was collected and stored for MALDI-TOF mass spectrometric analysis. To obtain a MALDI spectrum: 100 µl of a saturated solution of 2,5- dihydroxybenzoic acid dissolved in acetoinitrile 50 % (aqueous) was added to 10 µl of the analyte. The mixture was vortexed, and 1µl of the mixture was spotted onto a MALDI analyte plate. The analyte was analysed by acquiring data from 50-100 laser shots over the 1-3 kDa mass range, with the instrument operating in reflectron mode.

Mass spectrum generated for tryptic digest of a protein was compared with the theoretically predicted peptides using GPMAW 6.21 softwae (Lighthouse data, Odense - Denmark).

To obtain a spectrum for intact proteins, 100 µl of the stock solution was diluted into 300 µl with acetic acid solution (200 mM). Protein digestion was carried out by incubating protein solutions with trypsin gold (25:1 ratio) at 37 ^{o}C for 24 hours in ammonium acetate solution.

2.2.4 Procedure for preparing a digestion column.

The trypsin immobilized on agarose beads was initially washed as per the manufacturers' procedure. Briefly, 500 µl of ammonium acetate 25 mM (pH 8.2) was added to 100 µl of a bead suspension, mixed well and then centrifuged for 5 minutes. The supernatant was then discarded and the beads were washed twice more with ammonium acetate solution. The beads were then sieved through 67 µm pores and beads with diameter greater than 67 µm were packed into a fused silica capillary. To keep the beads inside the capillary, different types of frits and restrictors were tested and evaluated. These are described below:

a) Formamide 10 % in potassium silicate frit:

Formamide 10 % in potassium silicate solution (21 % SiO_2, 9 % K_2O) was stirred for 1 minute. The capillary (320 μm i.d. /420 μm o.d.) was then filled by syringe to 5 cm and placed in an oven at 100 ^{o}C for 30 minutes. The fritted capillary was then washed with methanol, dried by purging with nitrogen gas and left overnight to dry.[263]

b) Silica particles frit:

Silica particles (5 μm) were put into a fused silica capillary (320 μm i.d. /420 μm o.d.) then it was placed on a butane flame and the frit was formed (less than 1 cm in length) by fusion of the silica particles.

c) Monolithic frit:

Benzoylperoxide (20 mg), in a mixture of styrene (750 mg), divinylbenzene (1.25 g), 1-dodecanol (2.250 g or 1.950 g) and toluene (750 mg or 1050 mg) were stirred for about 5 min, purged with nitrogen for 10 min and sonicated for 10 min.[264] A silica capillary (320 μm i.d. /420 μm o.d.) was filled with the polymerization mixture to 5 cm by capillary action. The capillary was placed in a column block heater at 65 ^{o}C for 24 hours. The capillary was then flushed with acetonitrile followed by methanol 50 % in water to remove any remaining starting materials. The frit was then heated to 45 ^{o}C for 16 hours.

d) A fused silica restrictor:

A fused silica capillary 100 μm i.d. /300 μm o.d. was used as a restrictor by inserting it into a second capillary 320 μm i.d. /420 μm o.d. and sealed with torr seal glue.

The capillary (320 μm i.d. /420 μm o.d.) was then filled with beads to a length of 5 and 10 cm. The capillary was then connected to the MS *via* a low dead volume ‘microtight T’ connector (Figure 11).

2.2.5 Preparation of monolithic column.

In these experiments a monolithic column (prepared as the monolithic frit described in section 2.2.4) fabricated within a fused silica capillary, was placed after the 'T' connection and connected directly to the electrospray capillary by means of a low dead volume 'microtight' union. The proteins melittin, cytochrome C and BSA were run through the monolithic column after digestion through the immobilized enzyme beads packed in the capillary. The solvent used in this case was acetonitrile 20 % in aqueous acetic acid solution 0.2 %.

2.2 Results and discussion.

2.3.1 Fabrication of microdevice.

One of the major problems with on-line detection is the incompatibility between the chemical process conditions and the detection conditions. This incompatibility is also clearly apparent here, where the maximum trypsin activity is obtained at high pH buffer (pH 8.2), whereas the use of low pH buffer (below 7) is strongly recommended with positive mode ESI-MS as this enhances the sensitivity of ESI-MS considerably. Two solutions have been proposed. In the first one, known as the "wrong way around", [265] a buffer with pH above 7 was used for digestion and detection. However, this led to a reduction in the sensitivity of ESI-MS. The other solution uses ammonium acetate (pH 6.8) to carry out protein digestion and detection. The use of ammonium acetate buffer instead of ammonium carbonate resolves the problem of the buffer compatibility with ESI-MS as ammonium acetate is volatile and can be directly infused in ESI-MS. However, it is not optimal for tryptic digestion due to its lower pH range.[12] Here, an alternative solution was proposed using a low dead volume 'T' piece connector. Ammonium acetate was used as a digestion buffer solution but the pH was adjusted to 8.2 with dilute ammonium hydroxide solution to ensure optimum tryptic digestion. Acetic acid 200 mMol was pumped through the 'T' piece; the pH of the resulting digest solution was reduced to an acidic pH range, which is best suited for positive mode ESI-MS detection.

To test the performance of trypsin immobilized on agarose beads, a microdevice which consists of fused silica capillaries, a low dead volume 'T' connector and two syringe pumps was fabricated and directly interfaced to ESI-MS (Figure 13).

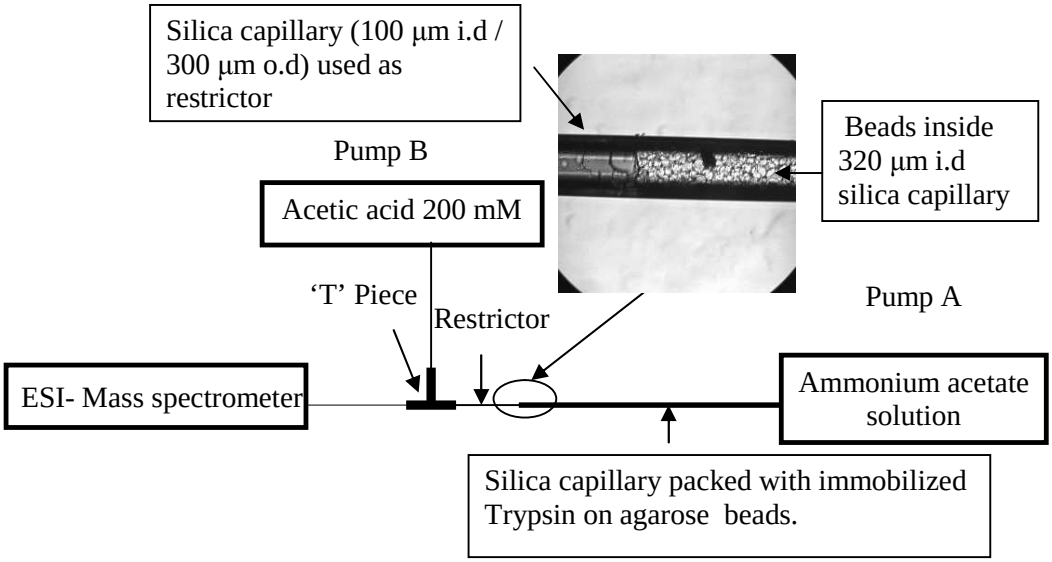


Figure 13: A schematic representation of the system used. Protein solutions were pumped through pump A and acetic acid solution 200 mM was pumped through pump B. The image shows a section of packed capillary (320 µm i.d.) and how the other capillary (100 µm i.d. /300 µm o.d.) restricted the beads.

To place beads inside a capillary, a frit or a restrictor has to be made at the end of the capillary, so that the beads can be trapped inside the capillary. The frit or restrictor should be made such that it is possible later on to implement it in the microfluidic chip. Several types of frits were tested, including: potassium silicate, silica particles and monolithic frit. The potassium silicate frit generated very high back pressure. It may be possible to optimize the pore size by changing the chemical composition; however, the optimization process is usually tedious and may take a long time. Therefore, silica particles were tested instead. This frit was made by inserting 5 µm silica particles inside a capillary and exposing the capillary to a butane flame. The resultant frit gave low back pressure but the

capillary broke down when it was connected to the 'T' connector. This is because the coating of the capillary was removed during the formation of the frit, making the capillary very fragile. Additionally, forming such a frit in a microfluidic chip is very difficult due to the use of the butane flame. A photoinitiated monolithic frit is a more realistic approach due to the possibility of controlling the location of such a frit on-chip and in the capillary. Initially, a thermally initiated polystyrene monolith was tested (5 cm). The result showed that the monolith initially did not generate high back pressure. However, after a few runs the back pressure increased. This was due to the movement of the beads inside the monolithic frit, as can be seen from the microscopic slide in Table 5.

The capillary restrictor was then tested. For this purpose a fused silica capillary (300 µm o.d. and 100 µm i.d.) was placed inside another capillary (420 µm o.d. and 320 µm i.d.) (Figure 14) and glued using Torr seal glue. The restrictor did not generate high back pressure and the beads were trapped inside the capillary. Another point, which made the use of the restrictor feasible, is the use of large beads (67 to 165 µm in diameter). This allows the use of a large restrictor (100 µm i.d. capillary) which lowers the back pressure. Moreover, the restrictor can be easily implemented in a microfluidic chip. This can be carried out by making a narrower channel at the end of the digestion channel. However, small beads can pass the restrictor and therefore, beads larger then 67 µm in diameter were used to pack the capillary. Table 5 summarizes the composition, advantages and disadvantages of the various frits and restrictors discussed above.

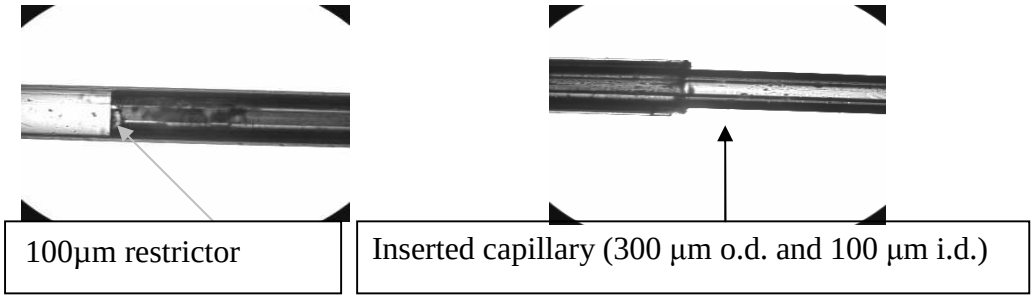


Figure 14: The two image shows a capillary (300 µm o.d. and 100 µm i.d.), inserted in another capillary (420 µm o.d. and 320 µm i.d.).

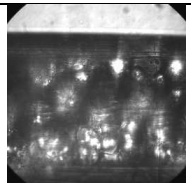
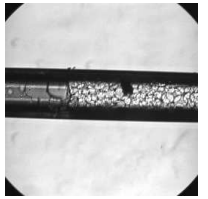
Frit type	Composition	Advantages	Disadvantages	Microscopic Slide
Potassium silicate	10 % formamide in potassium silicate solution	- Robust	- Produces relatively high pressure - Too dence	
Silica	Silica 5 µm particles	- Low back pressure. - Easy to make	- Fragilty of tubing when coating removed.	
Monolithic	Benzoylperoxide in a mixture of styrene, divinylbenzene, 1-dodecanol, and touluene.	- Low back pressure.	- Compressible, it blocks after a few runs.	
Restrictor	100 µm i.d. / 300 µm o.d.	- Easy to make - Robust. - Low back pressure.	- Small beads can pass through.	

Table 5: Types of frit and restrictor used, their advantages and disadvantages.

The performance of the trypsin immobilized on agarose beads was then evaluated using six different proteins ranging in molecular mass from 2846 to 77703 Da (Table 6). These six proteins were selected not only to cover a wide range of molecular masses but also, to cover a wider range of different protein structural properties. Melittin and cytochrome C are relatively easy to digest and they are commonly used as models in proteomics. [203,205] Ubiquitin is extremely compact and highly hydrogen bonded, it has a hydrophobic core and no disulphide bonds. The structure of ubiquitin is very stable to extremes of pH and heat and is largely unaffected by addition of guanidinium hydrochloride. It also resists digestion by trypsin, although this is known to occur at arginine-74 (Figure 15).[266] Myoglobin is also known to be resistant to proteolytic digestion. [267] BSA and apo-transferrin are both high molecular mass proteins and both contain disulfide bridges. [117, 268]

No.	Protein	Molecular mass (Da)
1	Melittin	2846
2	Ubiquitin	8560
3	Cytochrome C	12360
4	Myoglobin	16953
5	Bovine serum albumin (BSA)	69278
6	Apo-transferrin	77703

Table 6: Molecular mass of proteins used as a model.

Met-Gln-Ile-Phe-Val-Lys-Thr-Leu-Thr-Gly-Lys-Thr-Ile-Thr-Leu-Glu-Val-Glu-Pro-Ser-Asp-Thr-Ile-Glu-Asn-Val-Lys-Ala-Lys-Ile-Gln-Asp-Lys-Glu-Gly-Ile-Pro-Pro-Asp-Gln-Gln-Arg-Leu-Ile-Phe-Ala-Gly-Lys-Gln-Leu-Glu-Asp-Gly-Arg-Thr-Leu-Ser-Asp-Tyr-Asn-Ile-Gln-Lys-Glu-Ser-Thr-Leu-His-Leu-Val- Leu-Arg-Leu-**Arg**-Gly-Gly-

Figure 15: Ubiquitin aminoacid sequence showing arg-74 in bold.

These six proteins were passed separately through the trypsin immobilized on agarose beads packed in the fused silica capillary. Acetic acid 200 mM was pumped through the ‘T’ piece as described in the experimental section. This was

used to quench the digestion process and to improve the detection limit for ESI - MS by altering the pH of the solution from a basic pH to an acidic pH.

2.3.2 Digestion of cytochrome C and melittin.

Initially, a fused silica capillary packed to a 5 cm length with beads was tested using cytochrome C (1.7 μM) as a protein model diluted in ammonium acetate buffer. The pH of ammonium acetate solution was adjusted to 8.2 by ammonium hydroxide solution. The solution was injected at 1.0 μl min^{-1} and the result showed that cytochrome C was not digested. A stop flow experiment was carried out, and cytochrome C solution was incubated for 3 minutes in the capillary (*i.e.* 3 minutes residence time). The spectrum showed a few signals, which corresponded to tryptic digest of cytochrome C; however, most of the protein was still in its intact form. The residence time was then increased to 7 minutes. Although the intensity of the peaks corresponded to the peptides produced by tryptic digestion of the protein increased, the signals of the intact protein still predominated. However, when the residence time was increased to 14 minutes, traces of the signals corresponding to the intact protein were observed while signals corresponding to the peptides produced by tryptic digestion of cytochrome C predominated (Figure16). This indicated that the amount of trypsin available was not enough to digest the amount of cytochrome C passing the digestion channel. Therefore, a higher amount of trypsin was required so that most of the protein passing through the capillary could be digested.

The high signal intensity of lower charge state of the intact protein in Figure 16 compared to the mass spectrum obtained for the intact protein (Figure 17) was due to the stop flow mode experiment being carried out without flowing any acids after the ‘T connector’. This was carried out to distinguish easily between the signals due to a digestion process from the signals generated from the intact protein. When mass spectra are obtained for a protein in high pH media, the number of protons available are reduced and therefore, the lower charge state signal becomes more intense compared to the signal intensity of higher charge state.

Another capillary was packed to a length of 10 cm with the agarose beads and cytochrome C solution was pumped through at a flow rate of 3.0 µl min^{-1} while acetic acid was pumped at a flow rate of 2.0 µl min^{-1}. Complete digestion was obtained as no traces of intact protein were observed (Figure 18). This fast digestion (<3 minutes) compared to the earlier experiment in which silica capillary was packed to 5 cm and where complete digestion was obtained only after 14 minutes residence time indicates that the amount of trypsin, and to a less extent the residence time, plays a major role in the digestion process. If residence time was the principal factor then most of the cytochrome C should be digested when it was infused at a flow rate of 1.5 µl min^{-1} (residence time <3minutes) with the 5 cm packed capillary. However, this was not the case and only after 14 minutes residence time most of the protein was digested. Therefore, the enhancement in the rate of digestion observed when 10 cm digestion capillary used was mainly due to the increase in the amount of trypsin. A similar effect was observed by Bonneil *et al.* [202] when they doubled the length of the digestion capillary.

A sequence coverage of 72 % was obtained (Table 7), including the peptide with m/z 1017.2, which binds to the heme group and has m/z 817.6. [269] This data compares well with previously reported literature values for tryptic digestion of cytochrome C which vary between 11.7 % [205] to 92 % [203] using various methods of digestion.

The sequence coverage was calculated manually in comparison with the theoretical peptides masses of tryptic digest of cytochrome C within error of ±1 Da using GPMAW 6.21 software (Lighthouse data, Odense - Denmark).

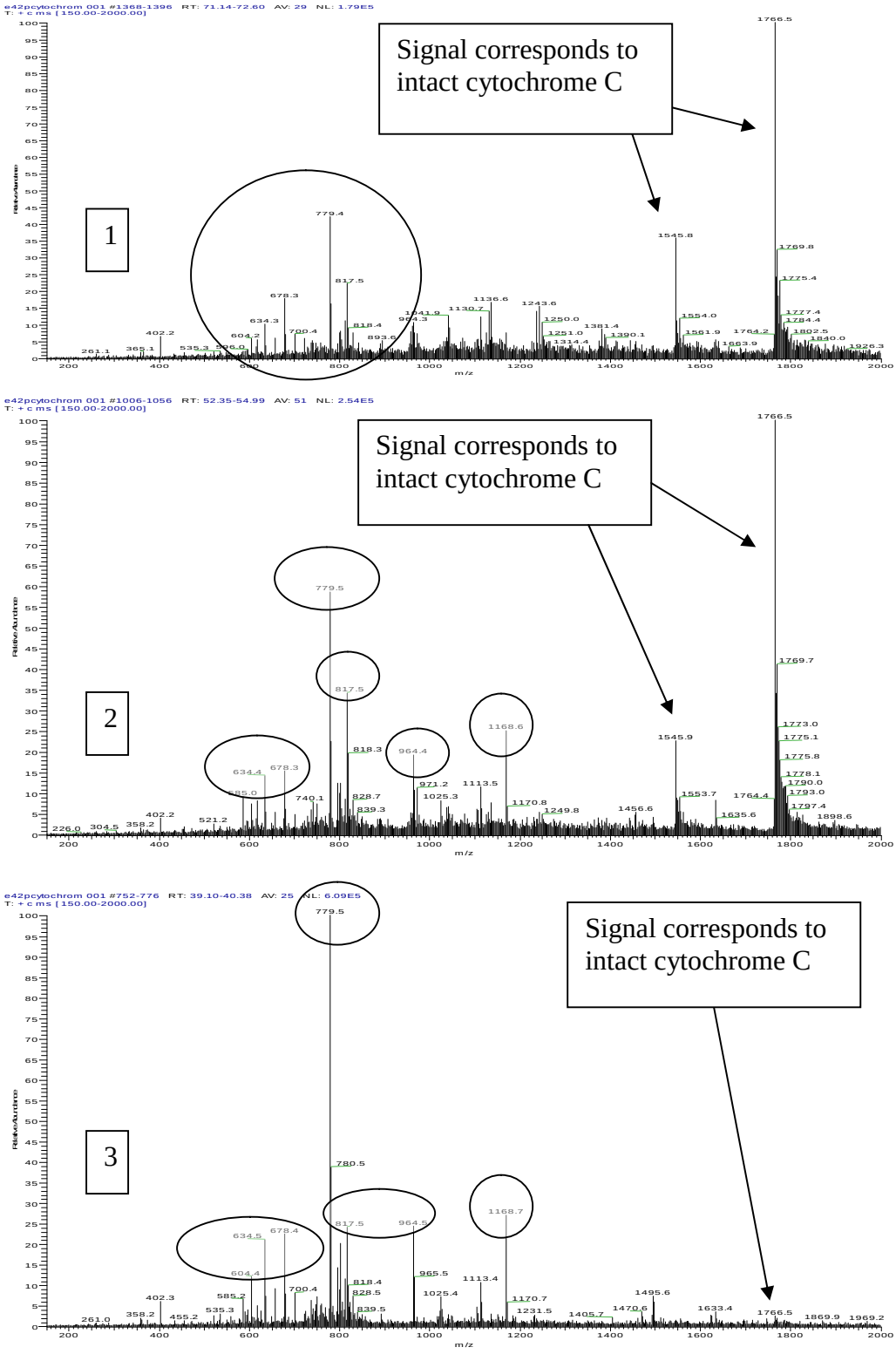


Figure 16: Mass spectrum of tryptic digest of cytochrome C, capillary was packed with immobilized trypsin on agarose beads to 5 cm. (1) Incubation time 3 minutes. (2) Incubation time 7 minutes. (3) Incubation 14 minutes. The circled signals correspond to tryptic digest of cytochrome C.

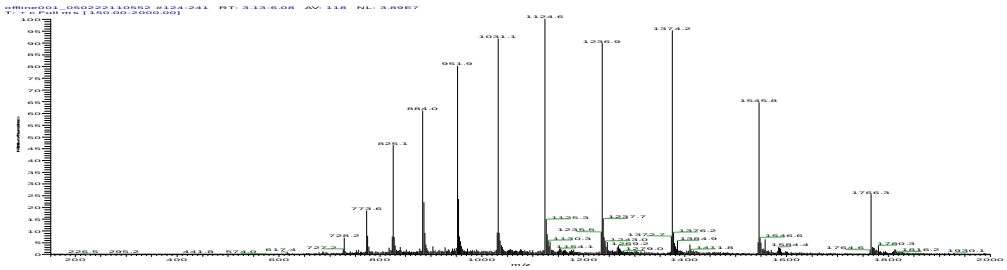


Figure 17: Mass spectrum of cytochrome C in acetic acid solution 200 mM.

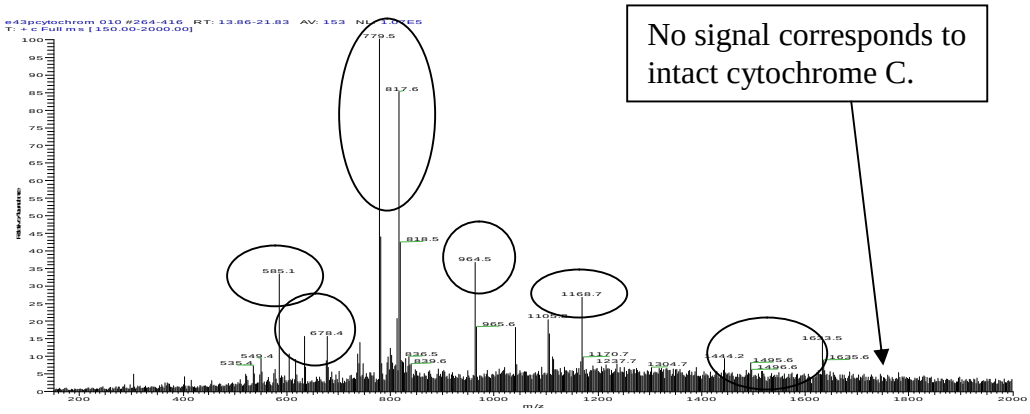


Figure 18: Mass spectrum of tryptic digest of cytochrome C, capillary was packed with immobilized trypsin on agarose beads to 10 cm. At flow rate of 3 µl min^{-1}. The circled signals correspond to tryptic digest of cytochrome C.

m/(z=1)	m/(z=2)	Sequence
634.8	317.9	Ile-Phe-Val-Gln-Lys-
1017.2	509.1	Cys-Ala-Gln-Cys-His-Thr-Val-Glu-Lys-
1168.7	585.1	Thr-Gly-Pro-Asn-Leu-His-Gly-Leu-Phe-Gly-Arg-
1471.6	736.3	Thr-Gly-Gln-Ala-Pro-Gly-Phe-Thr-Tyr-Thr-Asp-Ala-Asn-Lys-
1496.6	748.8	Glu-Glu-Thr-Leu-Met-Glu-Tyr-Leu-Glu-Asn-Pro-Lys-
678.4	339.9	Tyr-Ile-Pro-Gly-Thr-Lys-
779.5	390.5	Met-Ile-Phe-Ala-Gly-Ile-Lys-
964.5	483.1	Glu-Asp-Leu-Ile-Ala-Tyr-Leu-Lys-
Sequence coverage = 72%, number of peptide fragments identified = 8		

Table 7: Peptides generated from tryptic digestion of cytochrome C in microdevice.

Using the same conditions, melittin (13.0 μM) was also digested. Figure 19 shows the mass spectrum of intact mellitin and Figure 20 shows the mass spectrum of the tryptic digest of melittin using the fabricated device. It has been reported that melittin, when digested, generates three peptides m/z = 657.5, and 757 and 835.[203] The first two peptides are products of digestion while the third one is due to a missed cleavage. Using the first two peptides, 81 % sequence coverage was obtained (Table 8). This small protein was easily digested; however, smaller peptides generated from tryptic digest of melittin are hard to detect (Figure 21). This reduces the sequence coverage to 81 %. A peak with m/z value of 927.5 was also observed, which did not correspond to any known peptides.

Obtaining complete digestion for melittin and cytochrome C at 3.0 μl min^{-1} gave indications that there is a possibility of obtaining complete digestion for other proteins that are resistant to proteolytic digestion or high molecular mass proteins at lower flow rate. Therefore, it was decided to test this digestion capillary with other proteins and evaluate the result obtained before changing the length of the digestion capillary if required.

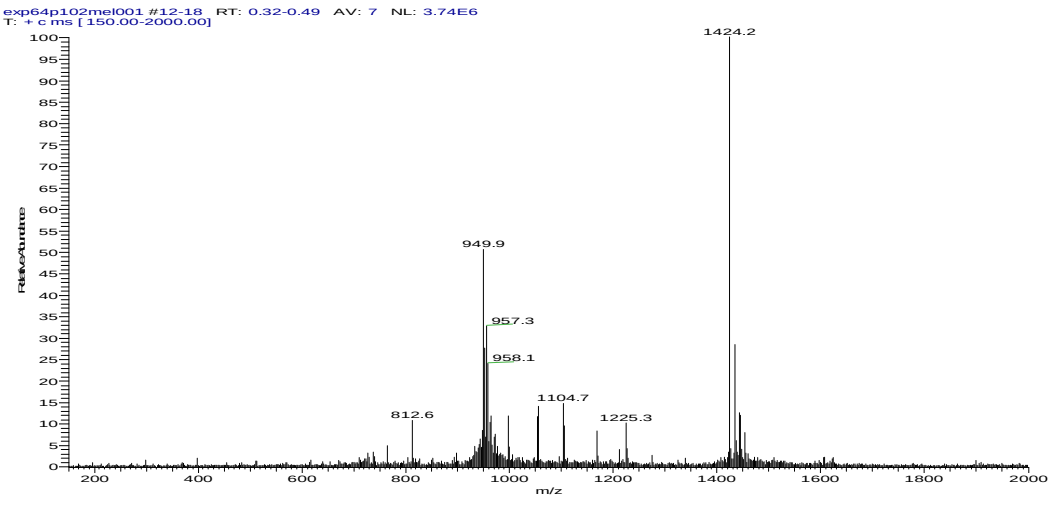


Figure 19: Mass spectrum of melittin in acetic acid solution 200 mM.

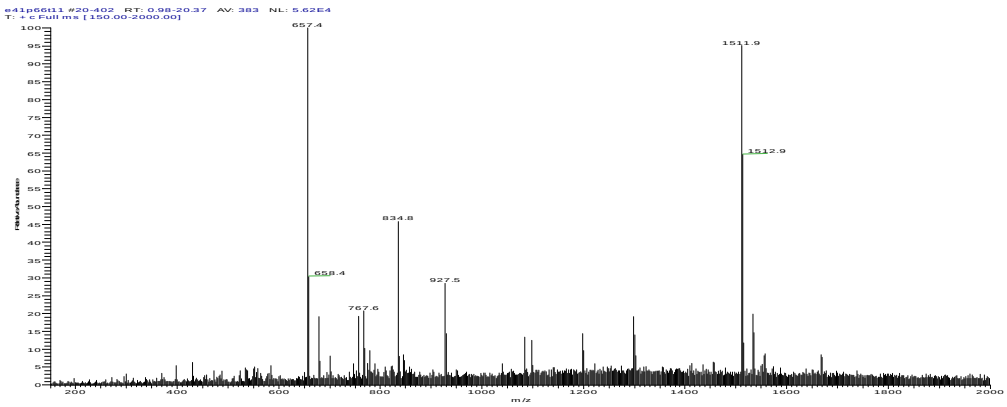


Figure 20: Mass spectrum of melittin solution when capillary was filled with immobilized trypsin on agarose beads to 10 cm. Flow rate 3 µl min^{-1}.

m/(z=1)	m/(z=2)	Sequence
657.4	329.42	Gly-Ile-Gly-Ala-Val-Leu-Lys-
1511.9	756.94	Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-
Sequence coverage = 81%, number of peptide fragments identified = 2		

Table 8: Peptide generated from tryptic digestion of melittin in microdevice.

Gly-Ile-Gly-Ala-Val-Leu-**Lys**-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-**Lys**-**Arg**-**Lys**-**Arg**-Gln-Gln-

Figure 21: Melittin aminoacid sequence. The Bold residues indicate trypsin cleavage sites.

2.3.3 Digestion of proteins resistant to proteolytic digestion.

In many cases, the native structure of a protein (*i.e.* three-dimensional structure) obstructs the digestion process and therefore, treatment of the protein sample is required prior to a digestion step. The process in which the three dimensional structure of a protein is converted to a more one-dimensional linear structure is called protein unfolding. This more linear structure makes the cleavage sites more accessible and hence improves the digestion process considerably. The method of protein unfolding varies depending on the type of protein but usually heating, surfactant or organic solvent are used. [266,270] It has been reported

"unfolding a protein in solution leads to the formation of higher charge states than the same protein in its native, tightly folded conformation". [271] This is because unfolding the protein makes the charge sites accessible and hence forms higher charge states. This was a basic criterion to see whether protein unfolding had occurred or not. Myoglobin is known to be resistant to proteolytic digestion. Two samples of myoglobin were prepared, the first was dissolved in ammonium acetate solution (pH 8.2) and diluted in acetic acid 200 mM, the second dissolved in acetonitrile 50 % in ammonium acetate solution (pH 8.2) and then diluted in acetic acid solution 200 mM. Mass spectra were obtained for both analytes. No significant differences were observed in the spectrum of intact protein (Figure 22). This is due to the use of acetic acid (200 mM) as diluents in both solutions prior to infusing the two solutions into ESI-MS. Low pH solution unfolds myoglobin. [266]

Four solutions of myoglobin (3.8 μM) were prepared as shown in Table 9 and were pumped separately through immobilized trypsin beads packed in the fused capillary at a flow rate of 1.0 μl min^{-1}. Mass spectra were obtained for each sample using ESI-MS. Analyte 1 and 2 did not show any digestion fragments whilst analytes 3 and 4 showed complete digestion and no traces of intact protein were present in their spectrum. Sequence coverage of 80 % was obtained and 10 peptide fragments were identified. A high intensity peak was observed at m/z 1135.7, which did not correspond to any expected peptide fragments. This may be due to the effect of the presence of organic solvents [267] or to the different degree of digestion due to the more limited time available in this procedure compared to the in-solution method of digestion. [272] Myoglobin is commonly used to test digestion system's performance. Typically the value of sequence coverage varies between 29 % [217] to about 80 %. [272] The results presented above suggest that the digestion process for myoglobin was enhanced in the presence of acetonitrile. It is also important to notice that no affect on trypsin activity was observed in all these cases. This indicates that trypsin is not sensitive toward acetonitrile or at least the active site is not affected. These findings are completely in agreement with other reports. [267]

Analyte	Buffer type	Unfolding method
1	Ammonium acetate Buffer pH 8.2	None.
2	Ammonium acetate Buffer pH 8.2	Heating at 90 ºC for 30 minutes.
3	Acetonitrile 50 % in ammonium acetate buffer pH 8.2	Organic solvent (acetonitrile)
4	Acetonitrile 50 % in ammonium acetate buffer pH 8.2	Organic solvent (acetonitrile) and heating at 90 ºC for 30 minutes.

Table 9: Types of buffer used and unfolding methods for digestion of myoglobin using the fabricated microdevice.

Park *et al.* [273] reported that a sequence coverage of 86 % was obtained, when myoglobin was unfolded thermally at 90 ºC for 20 minutes. We only obtained minimal evidence for digestion using thermal unfolding of myoglobin in contrast to Park *et al.* [273] However, the digestion method used here was different from their report. In their report, in-solution digestion was used and the myoglobin solution was incubated with trypsin for three hours at 37 ºC.

Ubiquitin (1.4 μM) was diluted in the same two diluents used in the case of myoglobin (ammonium acetate solution (pH 8.2) diluted in acetic acid 200 mM and ammonium acetate solution (pH 8.2) diluted in acetonitrile 50 %). Noticeable differences were observed between the spectra of the two ubiquitin solutions. The untreated ubiquitin solution spectrum showed two peaks, each one corresponding to a different protonation state of ubiquitin. The protonation states range from +5 to +6, whereas the protonation states for the treated ubiquitin solution range from +5 to +8 (Figure 23). The differences between the two spectra were due to the use of organic solvent. Ubiquitin is not affected by low pH solutions and therefore, when the two solutions were diluted with acetic acid (200 mM), the solution containing acetonitrile showed additional higher charge states. These were not shown in the aqueous solution.

Ubiquitin dissolved in aqueous solution was first digested using the microdevice system. Two peaks were observed at m/z 1690.0 and 1409.6, which correspond to cleavage at Arginine-74. Then the second ubiqutin sample (diluted in acetonitrile 50% in ammonium acetate buffer (pH 8.2)) was pumped through the microdevice. Other peaks corresponding to tryptic digestion were present in the spectrum in addition to the two peaks observed earlier, which indicated that more digestion had taken place. Sequence coverage of 80 % was obtained and 7 peptide fragments were identified.

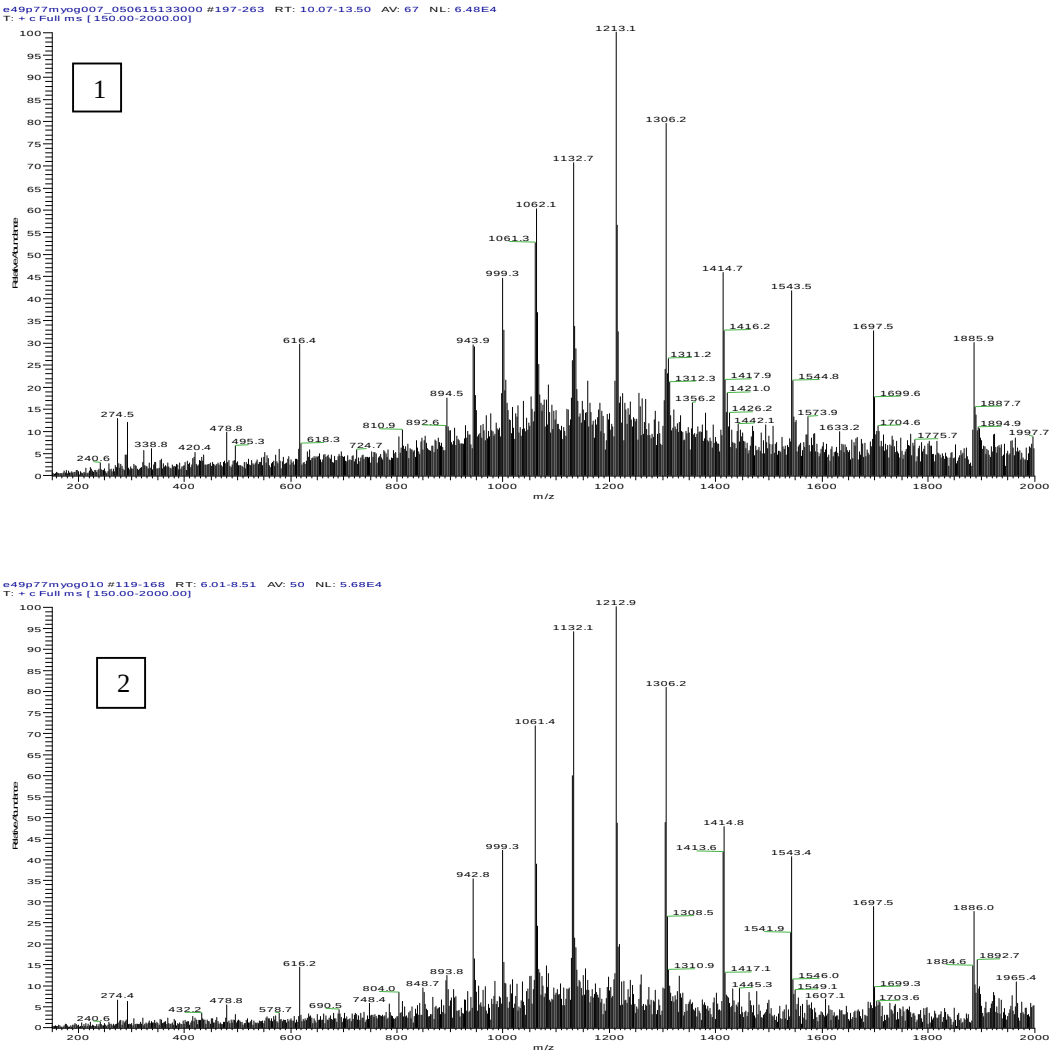


Figure 22: Mass spectrum of myoglobin dissolved in ammonium acetate solution and then diluted with acetic acid solution 200 mM (1), mass spectrum of myoglobin dissolved acetonitrile 50 in ammonium acetate solution and then diluted in acetic acid 200 mM (2).

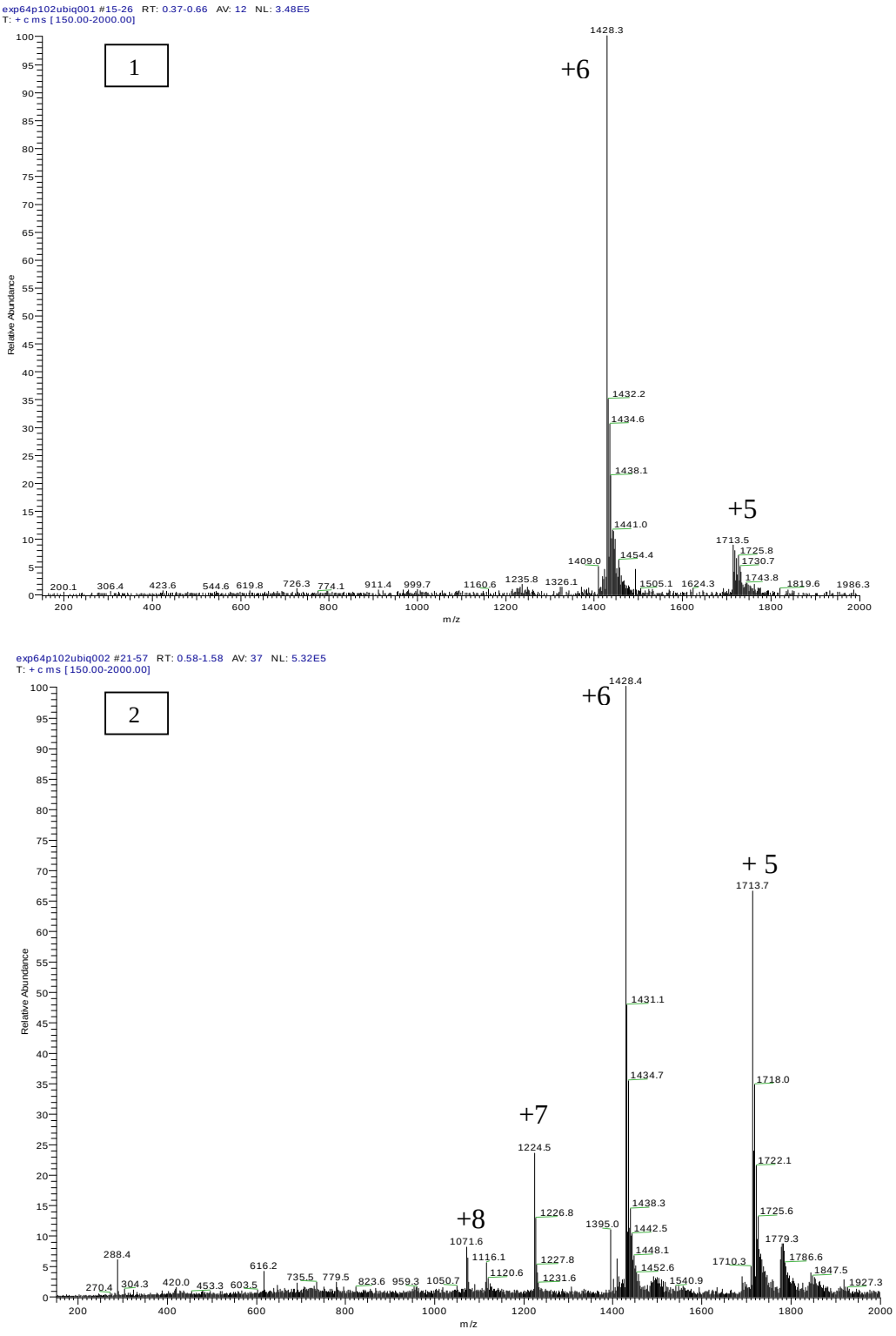


Figure 23: Mass spectrum of ubiquitin dissolved in ammonium acetate solution and then diluted with acetic acid solution 200 mM (1), mass spectrum of myoglobin dissolved acetonitrile 50 in ammonium acetate solution and then diluted in acetic acid 200 mM (2).

2.3.4 Digestion of high molecular mass proteins.

Initially, BSA was passed throught the digestion capillary at a flow rate of 1.0 µl min^{-1}. However, traces of digestion product were found in the spectrum, most of the protein was in its intact form. A disulfide bridge occurs between two cysteine residues present in different parts of the polypeptide chain due to the protein folding process. Treating the protein with a mild reducing agent such as DTT reduces the two cysteines and breaks the bridge. This helps to unfold the protein and hence make more digestion sites accessible. It also makes additional charge sites accessible and therefore, when BSA was treated with DTT, the most intense protonation state shifted from +39 to +44 (Figure 24). [274]

BSA is a protein commonly used as a model for digestion, usually it is treated with DTT. The sequence coverage here was 72 % (44 peptide fragments were identified) using the fabricated microdevice while literature reported values of 18.5 [205] to 80 % [203] using various methods of digestion. The digestion of BSA (1.1 µM) was carried out at a flow rate of 1.0 µl min^{-1}.

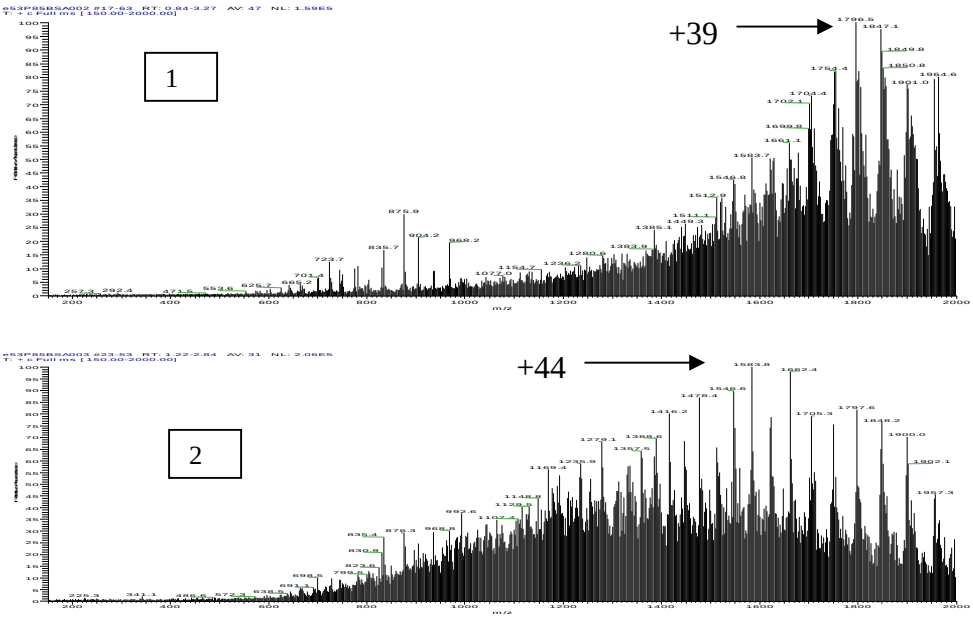


Figure 24: Mass spectrum of BSA in acetic acid solution 200 mM (1), mass spectrum of treated BSA with DTT in ammonium acetate solution, then diluted with acetic acid solution 200 mM (2).

Apo-transferrin (1.2 μM) is another high molecular mass protein although less commonly used as a model for digestion, and only one report used it (in a different form, holo-transferrin) with a sequence coverage of only 12 %. [205] However, digestion was carried out using on-line immobilized trypsin on a monolith interfaced directly to ESI-MS.

Apo-transferrin solution was passed through the microdevice and sequence coverage of 52 % was obtained and 32 peptide fragments were identified (after treating the solution with DTT, no tryptic digest was found without treating the solution with DTT). In comparison, the sequence coverage using spectra obtained from MALDI-TOF mass spectrometry was 58 % for BSA and 51 % for apo-transferrin based on PMF data. The lower sequence coverage obtained by MALDI in the case of BSA is most probably due to the formation of small peptides, which were below 1 kDa and were not detected in MALDI, where the scan was carried out between 1 to 3 kDa. For these last two proteins, MALDI spectra were also obtained to check for any traces of intact protein remaining, the results showed no such traces of intact protein in either case. Figure 25 shows the ESI mass spectra of the tryptic digest of myoglobin, ubiquitin, BSA and apo-transferrin obtained using the fabricated microdevice and Table 10 summarizes these results. The residence time is measured based on the volume of empty capillary and the flow rate used. Measuring the exact residence time was not possible due to the unknown volume occupied by the agarose beads when they are packed in the capillary. However, the residence time has to be less when the capillary is packed with the beads.

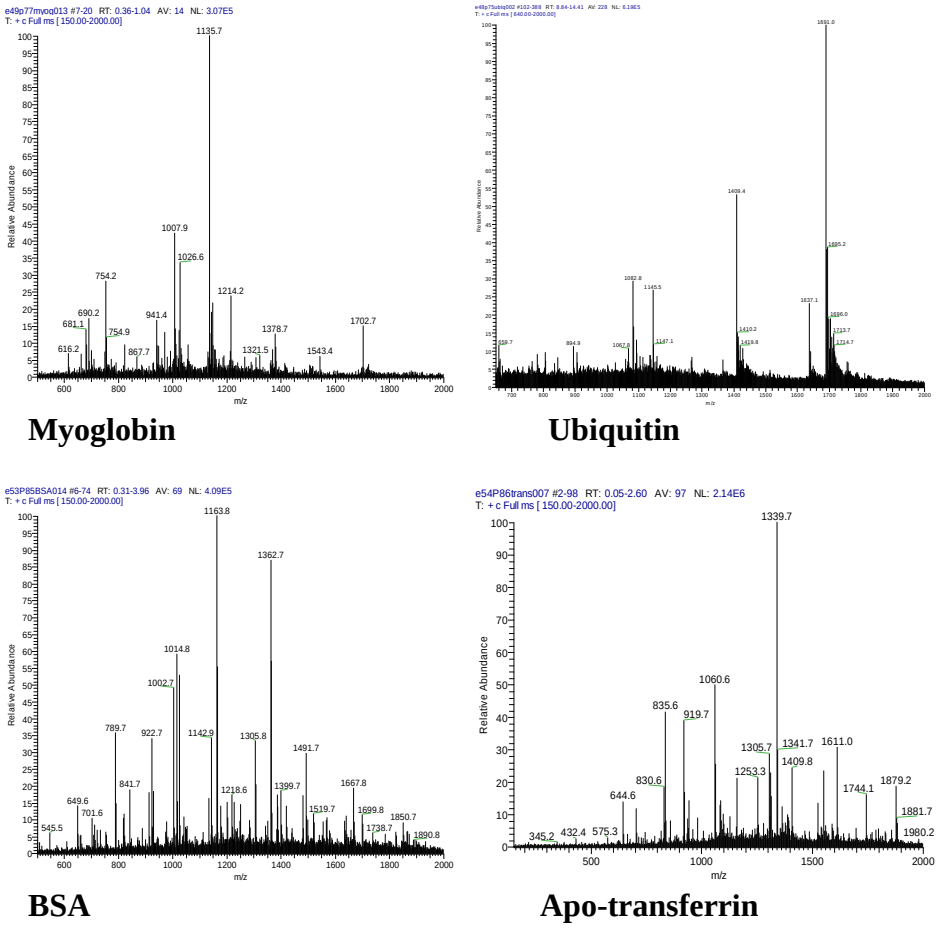


Figure 25: Mass spectra of peptides generated from digestion of four proteins separately using microdevice interfaced directly to the ESI-MS.

Protein	Flow rate in µl min^{-1}	Residence time in minutes	Sequence coverage %	Number of peptide fragments identified	Complete digestion
Melittin	3.0	< 3	82	2	Yes
Cytochrome C	3.0	< 3	72	8	Yes
Ubiquitin	1.0	< 8	80	7	Yes
Myoglobin	1.0	< 8	80	10	Yes
BSA (treated with DDT)	1.0	< 8	72	44	Yes
Apo-transferrin (treated with DDT	1.0	< 8	52	32	Yes

Table 10: Summarized result of on-line protein digestion using the microdevice interfaced to ESI-MS.

To compare the digestion efficiency of the trypsin immobilized on agarose beads with the in-solution trypsin, ten samples of protein solutions were prepared and digested using trypsin in ammonium acetate solution (pH 8.2) by placing each solution in an oven at 37 ºC for 24 hours. Two samples were prepared for ubiquitin and myoglobin; the first one was dissolved in an aqueous ammonium acetate buffer while the other in acetonitrile 50% in ammonium acetate solution.

Sequence coverage of 81 % (10 peptide fragments were identified) and 79 % (2 peptide fragments were identified) was obtained for melittin and cytochrome C respectively. However, the unknown peptide with m/z value of 927.5 which was observed when melittin was digested using the microdevice was also observed here. This indicates that the generation of this peptide is not due to the specific method used for digestion but rather to either nonspecific cleavage or due to the presence of some impurities in the melittin sample.

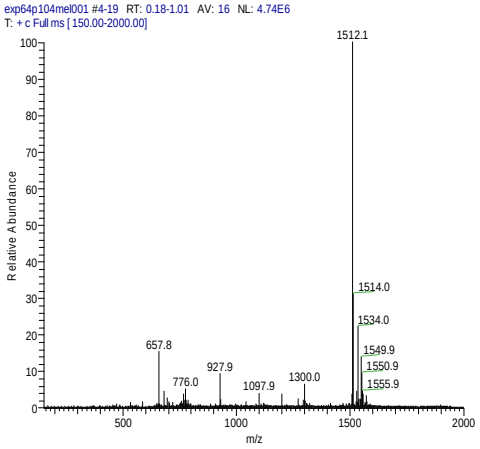
The mass spectrum of the tryptic digest of myoglobin carried out in 100 % aqueous solution showed traces of tryptic digest and most of the protein was still in its intact form. However, the protein (myoglobin) was completely digested when it was dissolved with the aid of acetonitrile. Additionally, the intense signal (m/z 1135.7) which was observed in the spectrum when the digestion was carried out in the microdevice and which did not correspond to any theoretically predicted peptides was not observed here. This means that this signal was due to the different degree of digestion due to the more limited time available in the procedure compared to the in-solution method of digestion. The mass spectrum of ubiquitin showed incomplete digestion of the protein when the digestion was carried out in aqueous buffer, while the mass spectrum of protein digest after treating the protein sample with acetonitrile 50 % in ammonium acetate solution (pH 8.2) showed a complete digestion of the protein. To study the effect of the acetonitrile on the digestion process further, digestion was carried out for ubiquitin and myoglobin separately, using acetonitrile 50 % in ammonium acetate solution. The two samples were placed in the oven at 37ºC. Digestion was quenched with acetic acid 200 mM after 1 hour. In both cases, the digestion was incomplete and most of the protein was in its intact form as can be observed from

the mass spectrum obtained. This means that the rapid digestion of these two proteins that took place in the fabricated microdevice was not due to acetonitrile use only, but also due to the digestion system used. Figure 26 shows the spectrum generated from tryptic digestion of the six proteins using in-solution digestion and Table 11 summarizes the results.

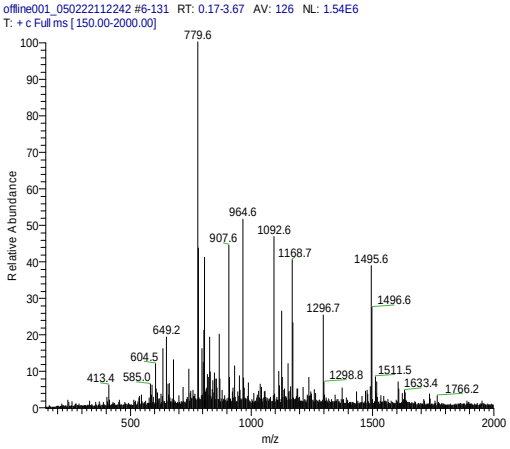
Russell *et al.* [267] did an extensive study on the use of organic solvents to aid the protein digestion process. They used 80 % acetonitrile solution to denature myoglobin. The results showed that complete digestion took place in 5 minutes when the reaction carried out at 37 ºC. The difference between these reported results and what has been reported here may be attributed to several factors such as the higher percentage of acetonitrile used, the different type of buffer used (25 mM ammonium carbonate) and finally, use of a water bath for incubation of the sample rather than the oven. A water bath provides a more efficient method for heat transfer to the solution and requires a shorter equilibration time.

The sequence coverage of BSA was 78 % (46 peptide fragments were identified) and that of apo-transferrin was 51% (29 peptide fragments were identified), when pre-treated with DTT. Complete digestion was obtained for both proteins when the digestion was carried out without treating the two proteins with DTT. This was due to the higher temperature used in the case of in-solution digestion (37 ºC), which is known to enhance the proteolitic activity of trypsin. However, the sequence coverage dropped to 24 % (17 peptide fragments were identified) and 23 % (19 peptide fragments were identified) for BSA and apo-transferrin respectively.

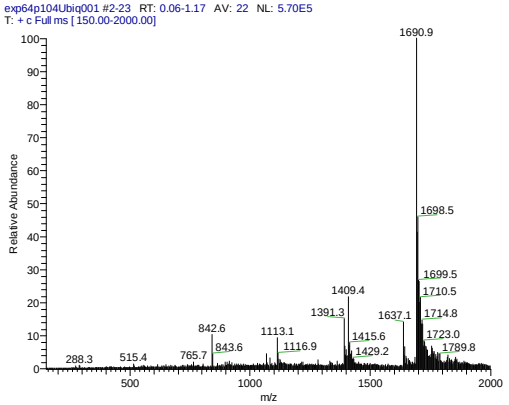
The results of tryptic digestion using the fabricated microdevice were compared with those from in-solution digestion method and presented in Table 12.



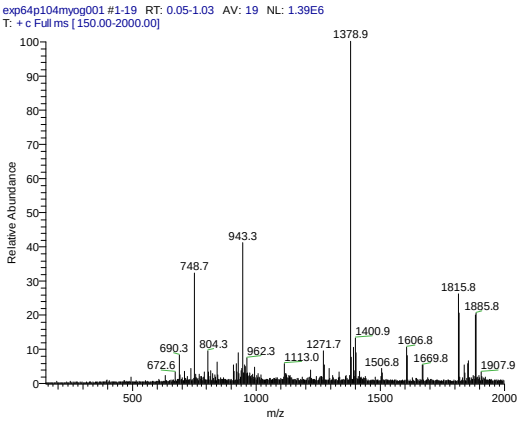
Melittin



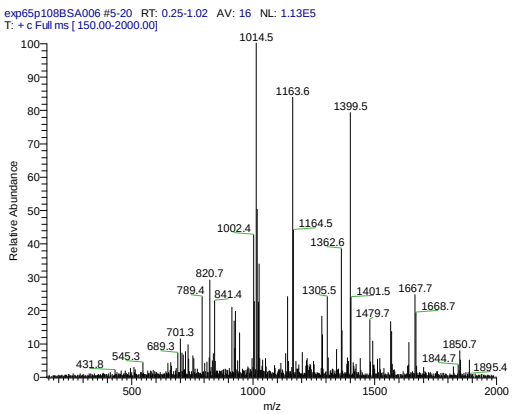
Cytochrome C



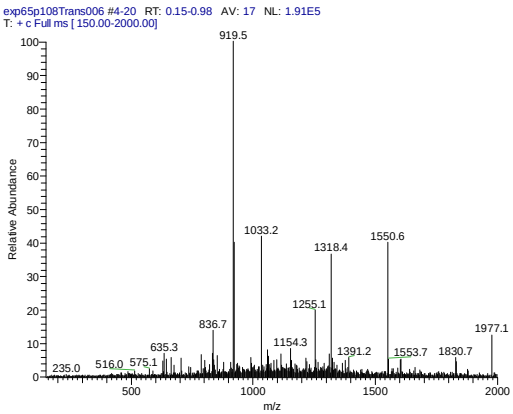
Ubiquitin



Myoglobin



BSA



apo-transferrin

Figure 26: Mass spectra of peptides generated by in-solution digestion of six proteins using trypsin in ammonium acetate buffer (pH 8.2). The reaction was quenched with acetic acid solution 200 mM.

Protein	Medium	Time (hours)	Sequence coverage	Number of peptide fragments identified	Complete digestion.
Melittin	Ammonium acetate solution (pH 8.2)	24	82	2	Yes
Cytochrome C	Ammonium acetate solution (pH 8.2)	24	79	10	Yes
Ubiquitin	Ammonium acetate solution (pH 8.2)	24	-	-	No
	Acetonitril 50 % in ammonium acetate solution (pH 8.2)	24	49	4	Yes
	Acetonitril 50 % in ammonium acetate solution (pH 8.2)	1	-	-	No
Myoglobin	Ammonium acetate solution (pH 8.2)	24	-	-	No
	Acetonitril 50 % in ammonium acetate solution (pH 8.2)	24	71	9	Yes
	Acetonitril 50 % in ammonium acetate solution (pH 8.2)	1	-	-	No
BSA (treated with DTT)	Ammonium acetate solution (pH 8.2)	24	78	46	Yes
BSA	Ammonium acetate solution (pH 8.2)	24	24	17	Yes
Apo-transferrien (treated with DTT)	Ammonium acetate solution (pH 8.2)	24	51	29	Yes
Apo-transferrien	Ammonium acetate solution (pH 8.2)	24	23	19	Yes

Table 11: Summarized results of in-solution protein digestion.

Protein	Digestion using in-solution method				Digestion using Microdevice			
	Time (hour)	Sequence coverage	Number of peptide fragments identified	Complete digestion	Time (minute)	Sequence coverage	Number of peptide fragments identified	Complete digestion
Melittin	24	82	2	Yes	< 3	82	2	Yes
Cytochrome C	24	79	10	Yes	< 3	72	8	Yes
Ubiquitin	24	49	4	Yes	< 8	80	7	Yes
	1	-	-	No				
Myoglobin	24	71	9	Yes	< 8	80	10	Yes
	1	-	-	No		-		
BSA (treated with DTT)	24	78	46	Yes	< 8	72	44	Yes
BSA	24	24	17	Yes	< 8	-	-	No
Apo-transferrin (treated with DTT)	24	51	29	Yes	< 8	52	32	Yes
Apo-transferrin	24	23	19	Yes	< 8	-	-	No

Table 12: Comparison between in-solution digestion and digestion using the fabricated microdevice.

2.3.5 Effect of the monolithic column on detection limits when connected to a microdevice.

A monolithic column was placed between the digestion capillary and the ESI-MS as shown in Figure 27. This was carried out to see if it was possible to carry out protein digestion and peptide separation in a single microdevice and the effect of such modification in the microdevice on the detection limits.

Initially, ammonium acetate solution (pH 8.2) was infused through the digestion capillary before connecting the capillary to the 'T-piece', in order to activate the trypsin immobilized on the agarose beads. Then the protein solution was infused in the digestion capillary. The dead volume of the connected capillaries was 1.0 μl and the volume of the digestion capillary was less than 8.0 μl. However, to ensure that the sample solution filled the digestion capillary, 9.0 μl of the sample was infused at a flow rate of 1.0 μl min^{-1} through pump A (see Figure 27). This was carried out only before starting the first injection of a new solution. Subsequent injections were made directly without the need to disconnect the digestion capillary.

After connecting the digestion capillary to the microdevice, the protein sample was pumped at 1 μl min^{-1} for one minute for solutions of melittin (13.0 μM), cytochrome C (1.7 μM) and BSA (1.1 μM). Pump A was then stopped (see Figure 27) and acetonitrile 20% in aqueous acetic acid solution 0.2% was pumped as a strong solvent to elute the peptides from the monolith.

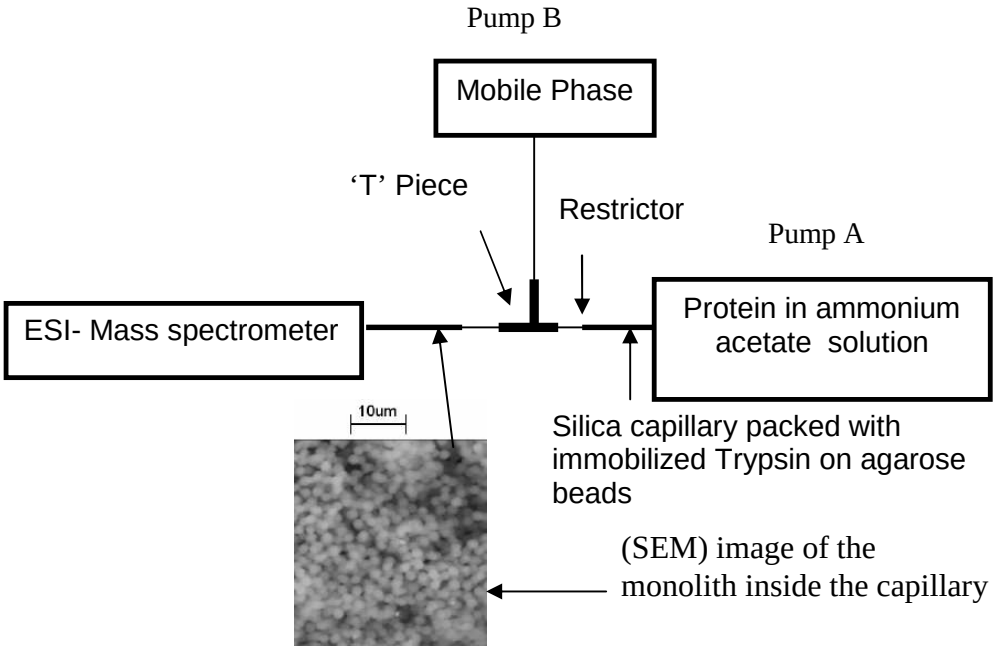


Figure 27: A schematic representation of the monolithic column used with the fabricated microdevice. Protein solutions were pumped from pump A in ammonium acetate solution. The proteins were digested on the immobilized trypsin column and the tryptic digests were pumped onto the monolithic column using acetonitrile 20% in aqueous acetic acid solution 0.2% from pump B prior to electrospray mass spectrometric analysis. The inset image shows an SEM image of the monolith structure inside the column.

It was observed that using this isocratic system a complete separation occurred for the peptides generated from digestion of small proteins like melittin, while a partial separation occurred for the peptides generated from digestion of cytochrome C. However, the separation was sufficient to detect a few peptides hardly identifiable without a separation step (Figure 28). High molecular mass proteins (BSA) did not show very good separation. This is expected as a gradient system is required for separation of such complex mixtures. However, some separation was observed and clear signal enhancement was also observed due to the partial separation. Furthermore, in all cases detection limits in a pmol range were obtained as calculated from the known concentrations and flow rates through the microdevice. This appears to be due to the use of the monolithic

column. When the monolithic column was used, the peptides adsorbed to the monolith and concentrated into a smaller volume due to the hydrophilicity of the buffer solution used in the digestion process. This appears to be a sample stacking effect on the head of the monolithic column which essentially provides sample pre-concentration. When a stronger elution solvent was then used (acetonitrile 20 % in aqueous acetic acid 0.2 %) the peptides started to partition between the monolith and the elution solvent. Effectively, this process constitutes a solid phase extraction of the peptides on the monolithic column prior to partial separation of the peptides and enhancement of the signal to noise ratio and hence an improvement in the detection limit. In addition, with some degree of peptide separation on the monolithic column there will be fewer species present in the electrospray droplets and therefore less competition for the available charge. This would reduce any sample suppression effects and may also account for some of the observed increase in sensitivity. The improvement in the detection limit observed here is very important as the concentrations of the proteins present in the biological samples are usually very low and hence the peptides produced from tryptic digestion of these proteins are also low. Therefore, such enhancement will increase the chances of detecting low abundance proteins.

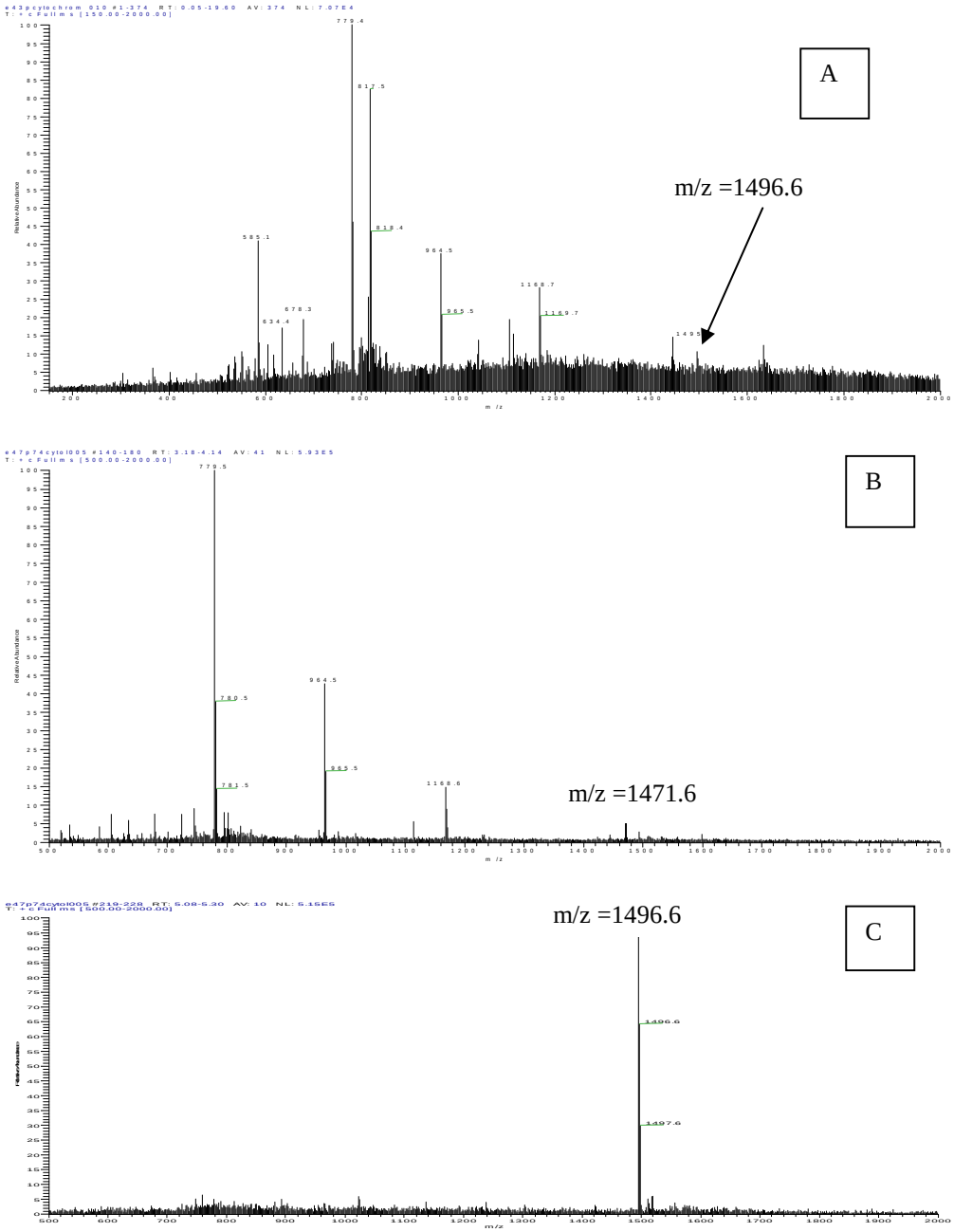


Figure 28: Mass spectra of peptides generated from digestion of cytochrome C. Mass spectrum A obtained without a separation step (1.7 µM). B and C obtained after connecting the monolithic column directly to the electrospray capillary (1.7 pmol). Mass spectrum B showing partial separation of several peptides (m/z = 779.5, 1168.7 and 964.5) it shows also presence of peptide (m/z = 1471.6) which cannot be easily detected in mass spectrum A. Mass spectrum C showing presence of completely separated peptides (m/z =1496.6) which can hardly be detected in mass spectrum A.

2.4 Conclusions.

A highly efficient microdevice has been fabricated for protein digestion. The microdevice has been evaluated using six different types of protein. The results showed that complete digestion took place within eight minutes with high sequence coverage varying between about 50 to 80 %. This shows clearly that trypsin immobilized on agarose beads is a very efficient medium for protein digestion when packed in this fabricated microdevice. The packing can be carried out easily with the use of a restrictor rather than frit to trap the beads inside the capillary. Two types of difficulties are commonly faced when directly interfacing protein digestion systems to a mass spectrometer. The first is with the use of ammonium carbonate buffer as a digestion media. Ammonium carbonate is not volatile and therefore cannot be directly infused into the mass spectrometer. The problem was resolved by the use of a mass spectrometer compatible buffer such as ammonium acetate with pH adjusted to maximize the activity of trypsin. The second is the need to reduce the pH of the solution infused to the mass spectrometer so that the sensitivity of the mass spectrometer is not affected by the use of high pH media. This problem was also resolved by the use of a low dead volume 'microtight T' connector and infusion of dilute acetic acid solution after the 'microtight T' connector so that the pH of the infused solution in the mass spectrometer is acidic.

The sample pre-treatment method is crucial and can affect the digestion process to a great extent, especially in the case of higher molecular mass proteins and proteolytically resistant proteins. The use of a monolithic column after the packed capillary provides some peptide separation capability and noticeable improvement in detection limits. The system provides high throughput protein digestion system, which could be used with appropriate instrumentation for shotgun proteomics.

3.0 Development of a microfluidic chip for protein digestion

3.1 Introduction.

The commercially available immobilized trypsin on agarose beads showed high efficiency in terms of sequence coverage and in terms of complete digestion when used in the fabricated microdevice. Therefore, it was decided to use these beads as a medium for on-chip digestion. According to the manufacturer, the trypsin immobilized on the agarose beads was treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) to inactivate any remaining chymotryptic activity. This increases the specificity of the cleavage as chymotrypsin cleaves at tryptophan and tyrosin if no proline follows them. The trypsin is also modified by acetylation of the amino groups of lysine residues to prevent autolysis and increase the rigidity of trypsin. TPCK treated trypsin cleaves at Lys-Pro and Arg-Pro bonds at a much slower rate than when these amino acids are not followed by proline. [275]

Wang *et al.* [203] used these beads in a microfluidic chip. Three proteins were used as models to evaluate the performance of on-chip digestion; mellitin, cytochrome C and BSA. However, digestion of the protein mixture was not examined. Moreover, some other factors were not tested, the most important being temperature. Increasing the temperature enhances the rate of the reaction. At the same time it may unfold some of the proteins which will have a positive effect on the digestion process of these proteins. It is found that the optimum temperature for in-solution digestion is 37 ^{o}C. Higher temperatures reduce trypsin activity due to denaturing of the active site of the trypsin. However, Gao *et al.* [219] reported that the digestion efficiency improved significantly when the temperature of the reaction increased to 50 ^{o}C. In their system they immobilized trypsin on a poly (vinylidene fluoride) membrane. Xi *et al.* [276] immobilized trypsin on silica beads coated with chitosan. The immobilization was carried out *via* (i) direct activation of an epoxy group, (ii) epoxy activation followed by diazatization activation or (iii) epoxy activation followed by introduction of glutaraldehyde (Figure 29). It was observed that the relative trypsin activity reached 100 % at 60 ºC in the first case (direct activation of an epoxy group) and in the second case the relative

activity was 100 % at about 50 ºC, while in the third case and with in-solution trypsin, the relative activity reached 100 % at 37 ºC. Direct activation of the epoxy group reduces the distance between the solid support and the trypsin molecules and makes the connection between the two shorter and stronger, thereby increasing the rigidity of the trypsin, while in the second and third cases the chain length increases and the rigidity decreases. This suggests that the immobilization process increases the rigidity of trypsin and this makes it more resistant to the unfolding process at higher temperature. This rigidity should also reduce the autodigestion processes. The idea of enhancement of trypsin activity and the reduction in the autodigestion process due to the increased rigidity of the trypsin structure is also supported by the enhancement that occurs in trypsin activity due to chemical modification. Chemical modifications are carried out by introducing small chemical groups such as methyl or acetyl to the chemical structure of trypsin. These added chemical groups increase the steric effect and hence increase the rigidity of the trypsin, leading to effects similar to immobilization. [215]

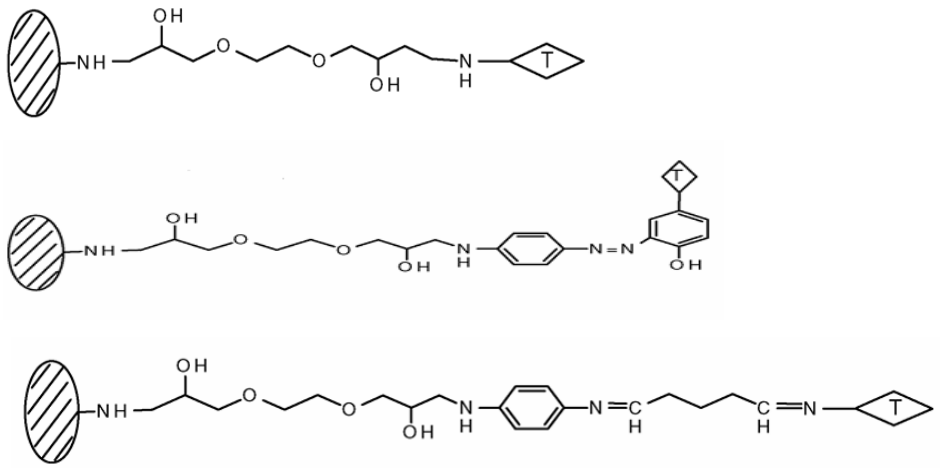


Figure 29: Trypsin immobilization carried out *via* direct activation of epoxy group, epoxy activation followed by diazatization activation or epoxy activation followed by introduction glutaraldehyde. [276]

Xi *et al.* [276] studied the effect of pH on trypsin immobilization. The results showed that in all cases studied the trypsin relative activity reaches 100 % at pH

around 8.0. However, the relative activity of immobilized trypsin is higher at lower pH than trypsin in-solution.

In this experiment a microfluidic channel was fabricated and packed with immobilized trypsin on agarose beads. The microfluidic chip was then evaluated using a single protein sample and mixed protein samples. Additionally, the effect of temperature was studied. Furthermore, sample carry over and stability of the immobilized trypsin on agarose beads packed in the microfluidic chip was investigated. The effect of pH was not studied as the result of the study carried out by Xi *et al.* [276] previously discussed.

3.2 Experimental.

3.2.1 Chemicals.

- Acetonitrile, HPLC grade (Fisher scientific equipments, Loughborough - UK).
- Glacial acetic acid (Fisher scientific equipments, Loughborough - UK).
- Ammonium acetate, HPLC grade (Fisons scientific equipments, Loughborough - UK).
- TPCK-treated trypsin, immobilized on 45 - 165 μm diameter 4 % crosslinked agarose beads (Pierce, Rockford, IL - USA).
- Proteins used described in section 2.2.1.

Agarose beads (100 μl suspension) were treated as described in section 2.2.4. However, the beads were sieved first through 67 μm pores. Beads smaller than 67 μm were collected separately, while beads larger than 67 μm were collected and sieved again through 106 μm. Beads with diameter between 67 to 106 μm were collected.

All solutions used were filtered through a 0.2 μm filter (Millipore, USA).

3.2.2 Instrumentation.

See section 2.2.2.

3.2.3 Protein digestion.

See section 2.2.3.

3.2.4 Microfluidic chip fabrications.

A glass chip (thickness 3 mm) was used and the channel etching was carried out using wet etching techniques with HF. Since channels of different dimension were required within the same chip, the top plate was used to etch the digestion channel, while the bottom glass plate was used to etch the channel specified for forming the chromatographic column. Two chips were used (figure 30), the first with the following dimensions for the digestion channel: 15 mm (length) x 800 μm (width) x 150 μm (depth), while for the second chip, the length was extended to 40 mm and the other dimensions remained the same. The bonding of the two plates was carried out thermally at 580 ºC. Three holes were made; the diameter of hole 1 was 1 mm while the diameter of holes 2 and 3 was 368 μm. A peak tube with 100 μm i.d. was fixed at hole 1 while a fused silica capillary with 50 μm i.d. was fixed at hole 2 and 3. The fixing was made using torr seal glue designed for high pressure applications.

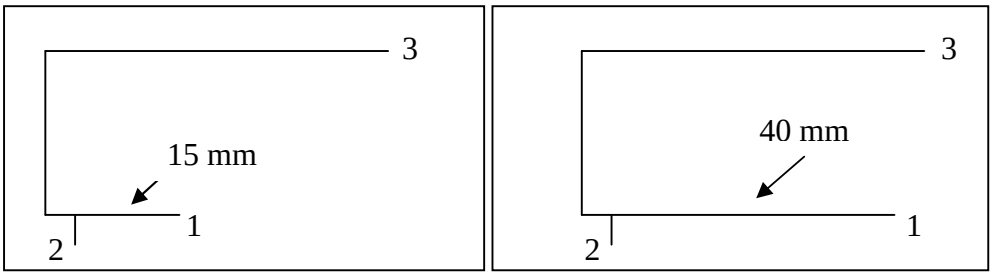


Figure 30: Schematic diagram for the two microfluidic chips used showing the three holes location.

3.2.5 Procedures for on-chip digestion.

A few microlitres of agarose beads suspension were placed inside hole 1. A small vacuum was applied at hole 2 using a glass syringe, while hole 3 was blocked. After filling the channel with beads, peek tubing was fixed in the 1 mm hole and

glued. The microfluidic chip was then connected to the ESI-MS *via* hole 2. Protein solutions were infused *via* hole 1 and formic acid was infused *via* hole 3. The channel was washed when the protein solution was changed with 100 μl of a mixture of ammonium acetate solution and formic acid 200 mM solution (1:1)

3.3 Results and discussion.

3.3.1 Microfluidic chip design.

The chip design was made taking into consideration that a separation column has to be made after the digestion step on the same chip. As discussed in the previous chapter (section 2.3.5), when the monolithic column was connected to the fabricated microdevice and before infusing the sample to the digestion capillary, the immobilized trypsin was activated by infusing ammonium acetate solution. The capillary was then disconnected from the microdevice and 9 μl of the sample was infused into the digestion capillary. The actual volume of the digestion capillary was less then 8 μl and the volume of the connected capillaries was 1 μl. However, to ensure that the sample filled the digestion capillary, 9 μl was infused. To reduce the sample loss and the dead volume, the volume of this channel was made to 1.7 μl as per the volume of the digestion channel used by Wang *et al.* [203] and the channel dimension was 15 mm (length) x 800 μm (width) x 150 μm (depth).

The method reported by Broadwell *et al.* [277] was used to calculate the area of the channel and it is illustrated in Figure 31. The calculation worksheet was obtained from the web site of the chemistry department of the University of Hull. [278]

The mask was made with the dimensions 15 mm (length) x 500 μm (width). If the depth of the channel is 150 μm, then the width will be 800 μm (500+150+150). This is due to the use of the wet etching technique for the fabrication of the microfluidic chip, which results in an isotropic etch, *i.e.* the glass is etched at an equal rate in all directions from the point exposed to a UV source. [277] This leads to the actual width of the channel increasing more than the width of the mask by a factor of twice the assigned depth. Additionally, it also means that the channel prepared with wet etching cannot have a depth greater than the width. It was

observed that some variation occurs in the dimension of the channel as can be seen from the SEM image in Figure 31, where the actual width was about 750 μm. This was due to the etching method used (photolithography). However, measuring the exact dimension for every chip made was not possible as the method used for measuring the exact width is destructive. Therefore, some variation may be observed if the chip is replaced by another one.

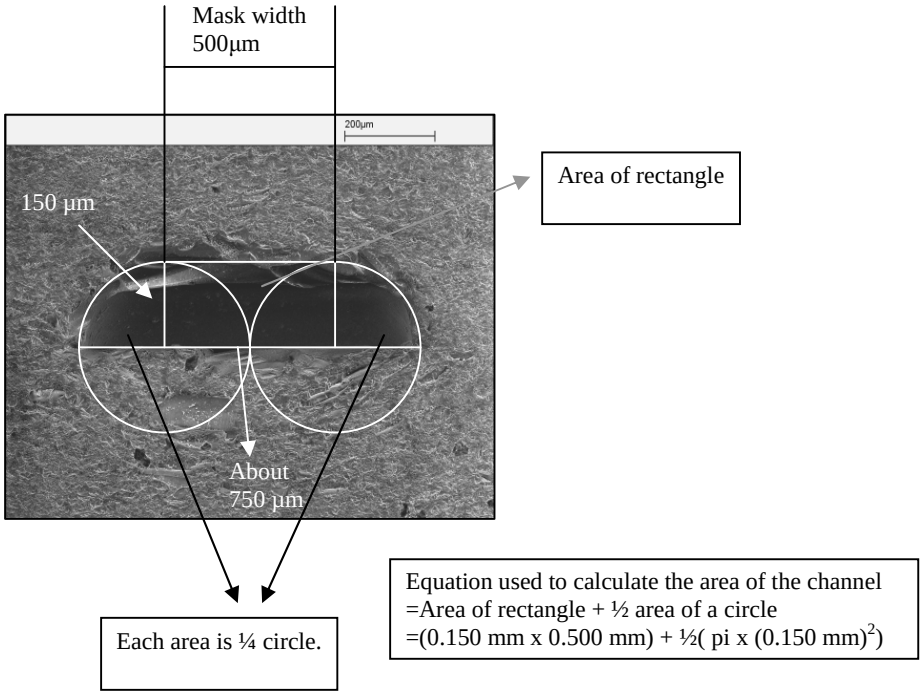


Figure 31: Illustration for the method used to calculate the volume of channels in microfluidic chips. The SEM image is for the cross section of the digestion channel.

3.3.2 Filling the channel with agarose beads.

Initially, beads with similar diameter to those used by Wang *et al.* were used here to fill the channel (40 to 67 μm). This is for two reasons: firstly, to ensure that the beads would enter the channel with no difficulties, and secondly, to enhance the protein digestion process by using smaller diameter beads, as this would increase the surface area available for the proteins to interact with the immobilized trypsin. However, it was observed that some beads passed through the separation channel (Figure 32), although, the depth of restrictor (separation channel) was

only 30 μm. However, it may be that some of the beads were less than 40 μm in diameter or the channel depth was larger than 30 μm. Therefore, to ensure that no such problem would occur, beads with a diameter between 67 and 106 μm were used to fill the channel.

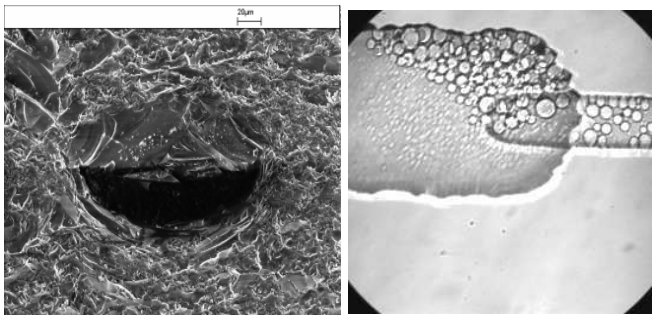


Figure 32: Left, SEM image for the cross section of the separation channel (restrictor). Right, microscopic slide for beads (40 to 67 μm in diameter) passing the restrictor in the microfluidic chip.

Filling the channel with beads was not very difficult; however, some experience is required. Removal of air bubbles is important and helps to ensure homogeneous packing (Figure 33). Additionally, filtering the washing solvent was also important to remove any particulates, which may block the channel. However, the most serious difficulty was in the hole drilling location. If the drilling is not done properly, pieces of glass can enter the channel and block it (Figure 33). Therefore, it was very important to ensure that the microfluidic chip was free from such a defect. The hole which was used to fill the beads was larger then the other two (1 mm compared to 368 μm). This was done to facilitate the filling process. The bead suspension was placed inside this hole and then vacuum was applied at the other end using a glass syringe. After filling the channel with beads, peek tubing was placed in the hole and glued. Figure 34 shows the different stages of filling the channel. After packing the channel with the beads, the chip was connected to the ESI-MS *via* hole 2 while formic acid 50 mM was infused through hole 3.

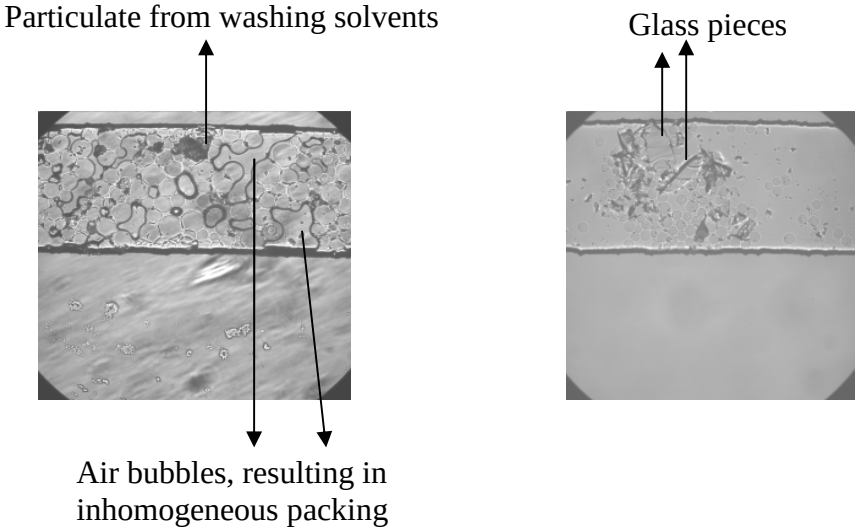


Figure 33: The first image shows the effect of presence of air bubble and particulate, while the second image shows improper drilling can leads to that some glass pieces to pass through to the channel.

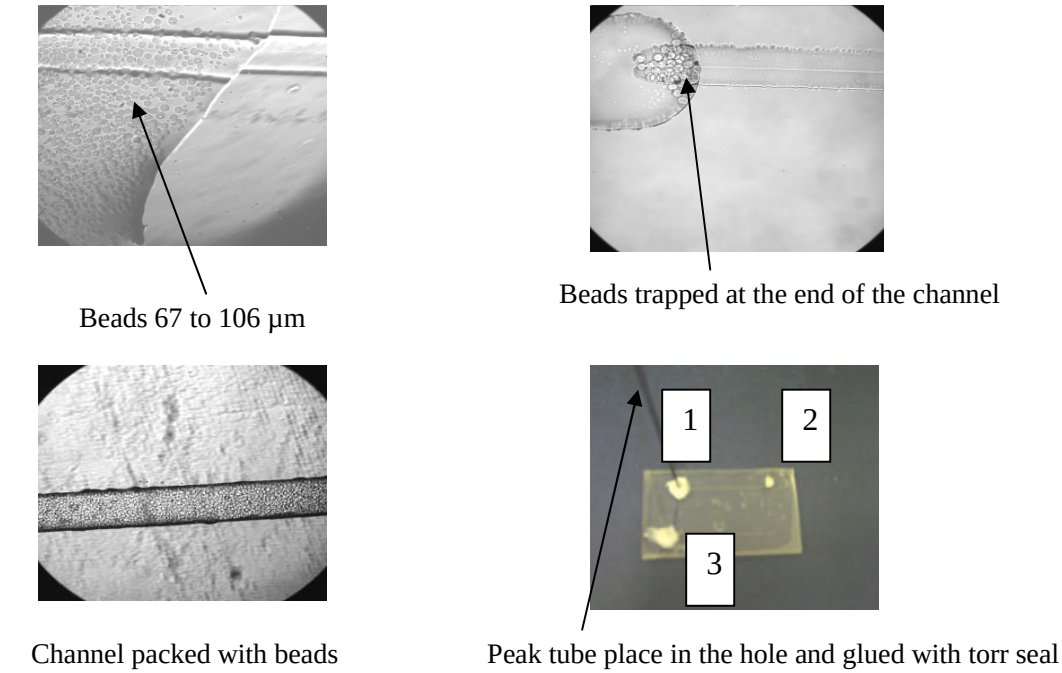


Figure 34: The top left image shows the size of the beads in comparison to the channel size. The top right image shows how the beads were trapped inside the channel. The bottom left image shows the channel homogeneously packed with the beads. The bottom right image shows the peek tube fixed at hole 1 after packing the channel with the beads.

3.3.3 Digestion of cytochrome C and melittin.

Initially a solution of melittin (13.0 μM) was infused at a flow rate of 3.0 μl min^{-1} No traces of intact protein were found in the spectrum and all the signals present were due to the digestion of melittin. A solution of cytochrome C (1.7 μM) was then infused through the digestion channel at a flow rate of 1.0 μl min^{-1}. Wang *et al.* was able to obtain complete digestion for cytochrome C when it was injected at this flow rate (1.0 μl min^{-1}). However, here the result showed that no digestion took place, as was observed from the generated mass spectrum. The flow rate was then reduced to 0.5 μl min^{-1}. However, still no signal from tryptic digestion of cytochrome C was observed. This may have been due to the larger beads compared to the one's used by Wang *et al.* As observed earlier, the amount of the trypsin present in the digestion channel plays a key role in the digestion process and when larger beads were used, the amount of immobilized trypsin was reduced due to the reduction in the surface area present.

A new chip was designed in which the total volume of the channel was extended to 4.4 μl. This was carried out by extending the length to 40 mm instead of 15 mm. The total volume of this channel was almost half of the volume of the digestion capillary used in the fabricated microdevice. Therefore, to compensate for the reduction in the volume of the channel and hence reduction in amount of the trypsin, smaller beads were used (<106 μm diametre compared to <165 μm diametre). This allows a larger number of beads to be packed, thereby increasing the amount of the trypsin in the microfluidic channel. The residence time required to obtain complete digestion for the larger proteins (BSA and apo-transferrin) was below 8.0 minutes in the microdevice (flow rate 1.0 μl min^{-1}). Since the volume of the digestion channel is almost half of the digestion capillary, at half flow rate, the residence time should be comparable.

Solutions of cytochrome C and melittin (1.7 μM and 13.0 μM respectively) were infused separately using a syringe pump at flow rate of 1.5 μl min^{-1}. The flow rate was assigned so that the residence time in this system (<3.0 minutes) was comparable to the residence time in the microdevice system previously used. The results showed that complete digestion took place, as no traces of intact protein

were detected in the eluent. A sequence coverage of 74 % (9 peptide fragments were identified) and 81 % (2 peptide fragments were identified) was obtained for cytochrome C and melittin respectively.

The results were comparable to those previously obtained using the microdevice or using in-solution digestion (72 % and 79 % respectively). The small variation in the sequence coverage of cytochrome C from one system to another was mainly due to detection of small peptides (consisting of five amino acids, *e.g.* m/z 604.3) in the case of in-solution digestion and in the case of on-chip digestion, whereas these peptides were not detected when digestion was carried out using the microdevice system.

3.3.4 Effect of temperature on the digestion efficiency of the microfluidic chip

It was not stightforward to study the effect of temperature on the protein digestion process when the microdevice was used, this was due to the difficulty in placing the microdevice in an oven. However, the microfluidic chip can be easily placed inside an oven and hence the effect of temperature on the protein digestion can be studied. The experiment was arranged such that the temperature of the top surface of the chip was 40 ºC. In order the achieve this, the temperature of the bottom surface was at 45 ºC, as this surface was in contact with the heating block.

A solution of cytochrome C (1.7 μM) was infused at 5 μl min^{-1} which reduced the residence time to less than one minute and still complete digestion was obtained while sequence coverage was not affected. The experiment was repeated for 3 days to see if any autolysis or nonspecific cleavage was occurring but no such effect was observed. When the same solution was infused at the same flow rate but at room temperature, a spectrum of intact cytochrome C was obtained with traces of tryptic digest products present.

The temperature was then increased to 55 ºC (top surface) and the same solution was infused. However, the sequence coverage was dropped to 42 % after 1 hour

due to missing of the peptides with m/z values of 964.5, 1471.6 and 1496.6, which indicates that trypsin efficiency dropped down. Based on this, it was decided to carry out the rest of the experiments at 40 ºC (top surface).

3.3.5 Digestion of proteins resistant to proteolytic digestion.

Myoglobin (3.8 μM) was then tested. Initially, it was dissolved in aqueous ammonium acetate buffer and was infused through the beads in the microfluidic chip. No digestion was observed, this is in line with the known properties of myoglobin as previously discussed in chapter two (section 2.3.3). However, when myoglobin was dissolved in acetonitrile 50 % in ammonium acetate buffer (pH 8.2) solution, complete digestion was observed and no traces of intact myoglobin were observed. In comparison with the data from the microdevice, the sequence coverage was lower (71 % compared to 80 % and 8 peptide fragments were identified compared to 10). A high intensity peak at m/z 1135.7 was also observed here, in addition to another intense peak at m/z 1022.1, neither of which corresponded to any expected peptide fragments. These results indicate again that organic solvents are an effective method for unfolding myoglobin. However, there is evidence of some missed cleavage products occurring.

Ubiquitin (1.4 μM) was also dissolved in acetonitrile ammonium acetate buffer and the results showed a complete digestion of the protein. The relative intensities of the peaks corresponding to cleavage at Arginine-74, were lower than observed in the microdevice and the relative signal intensity of the tryptic fragments was much higher; however, the sequence coverage remained the same (80%) and in both cases, 7 peptide fragments were identified.

3.3.6 Digestion of high molecular mass proteins.

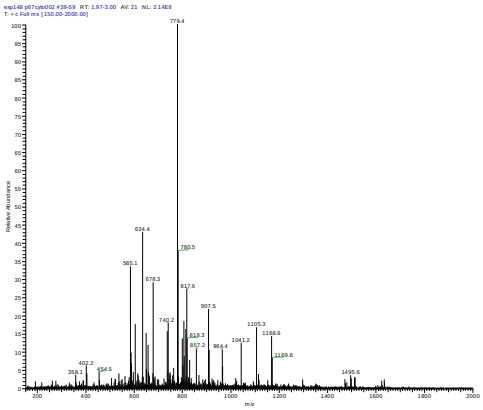
In the previous experiment (protein digestion using the microdevice system) the digestion of high molecular mass proteins (BSA and apo-transferrin) could not be achieved without sample pre-treatment (both protein samples were treated with DTT). In this experiment the digestion of these two proteins was carried out without any sample pre-treatment step. This is because it was observed that the efficiency of this system increased considerably when the digestion was

performed at 40 ºC as in the case of cytochrome C where the residence time required to obtain complete digestion dropped from three minutes to less than a minute.

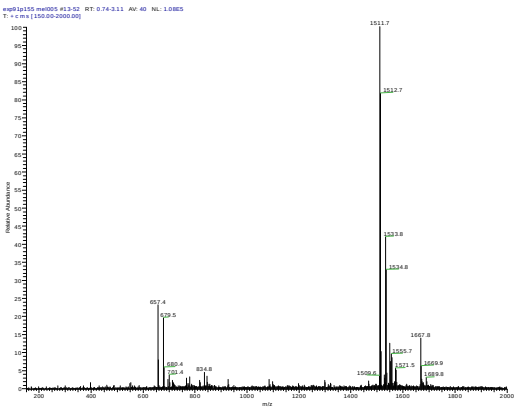
BSA and apo-transferrin solutions were infused separately at a flow rate of 1.0 µl min^{-1} and temperature was set at 40 ºC (1.1 µM and 1.2 µM respectively). The result showed that in both cases the proteins were completely digested as no traces of intact protein were observed. Sequence coverage of of 27 % was obtained for BSA (17 peptide fragments were identified) while 21 % was obtained for apo-transferrin (19 peptide fragments were identified). This is considered acceptable values since no sample pre-treatment was carried out in either case. Typical digestion values reported for BSA vary between 18.5 [205] to 80 % [203] while 12 % [205] sequence coverage has been reported for holo transferrin.

The noticeable enhancement in the digestion efficiency when the temperature increased to 40 ºC has been previously reported on many occasions. Higher temperature increases the average kinetic energy of the molecules and hence increases their collisions. Therefore, the more likely that the activation energy can be reached or exceeded. However, different proteins may be affected differently by the rise in temperature. This can be clearly seen in these experiments where the digestion of cytochrome C, ubiquitin, BSA and apo-trasferrin was enhanced, while no such effect was observed for myoglobin. This is mainly due to the native structure of myoglobin being unaffected by the increase in the temperature. Figure 35 shows the spectra generated from on-chip digestion at 40 ºC of the six proteins.

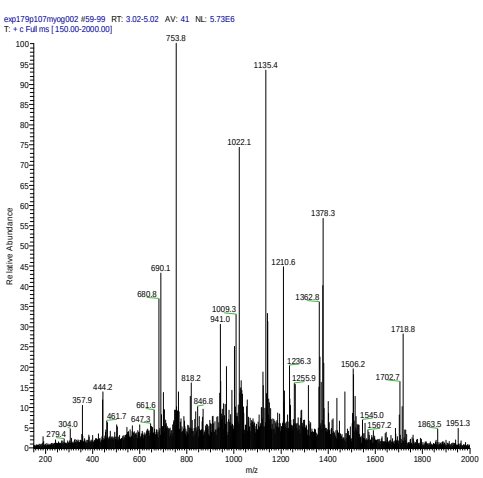
Table 13 compares results from in-solution, microdevice and on-chip digestion for six proteins in terms of the speed and completeness of digestion. The data clearly shows that the efficiency of the on-chip digestion is higher than the other two. The improvement observed in digestion efficiency for on-chip compared to the microdevice is mainly due to the higher temperature at which on-chip digestion was carried out.



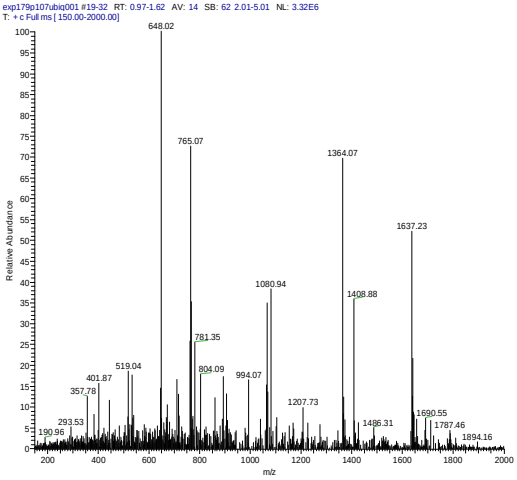
Cytochrome C



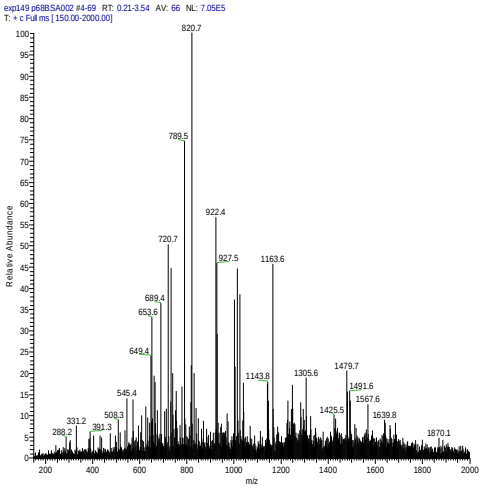
Melittin



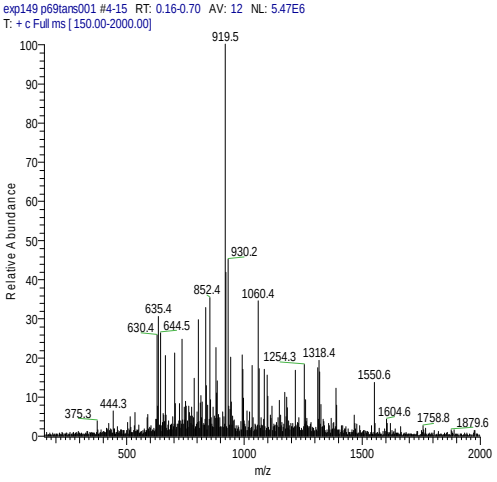
Myoglobin



Ubiquitin



BSA



Apo-transferrin

Figure 35: Mass spectra of peptides generated by digestion of six proteins using immobilized trypsin on agrose beads packed in the microfluidic channel interfaced directly to the ESI-MS. The digestion was carried out at 40 ºC.

Protein	Digestion using in-solution trypsin (37 ºC)				Digestion using microdevice at room temperature				Digestion using microfluidic chip (40 ºC)			
	Time (hour)	Sequence coverage	Number of peptide fragments identified	Complete digestion	Time (minute)	Sequence coverage	Number of peptide fragments identified	Complete digestion	Time (minute)	Sequence coverage	Number of peptide fragments identified	Complete digestion
Melittin	24	82	2	Yes	< 3	82	2	Yes	< 1	82	2	Yes
Cytochrome C	24	79	10	Yes	< 3	72	8	Yes	< 1	74	9	Yes
Myoglobin	24	71	9	Yes	< 8	80	10	Yes	< 5	72	8	Yes
Ubiquitin	24	49	4	Yes	< 8	80	7	Yes	< 5	80	7	Yes
BSA	24	24	17	Yes	< 8	-	-	No digestion	< 5	27	20	Yes
Apo-transferrin	24	23	19	Yes	< 8	-	-	No digestion	< 5	21	19	Yes

Table 13: Comparison between in-solution digestion, microdevice and microfluidic chip digestion system.

3.3.7 Digestion of protein mixtures.

The objective of this experiment was to see if the system can digest multiple proteins present in a single sample. This can be achieved by monitoring the unique peptides generated from digestion of each protein. If even a single unique peptide corresponding to the trypsin digestion of a certain protein is present in the spectrum and no traces of the intact protein is present then this can be taken as evidence that the system can digest multiple proteins in a single sample. However, this may not be straightforward; when a complex mixture of peptides is present in a single sample, then strongly ionizable peptides may suppress the signals of weakly ionizable peptides due to the limited charge available in the ESI technique. Thus, many unique peptides may not be detectable.

The other objective was to see how the sequence coverage is affected when a complex mixture of peptides are present. To achieve these two objectives, three samples were prepared. The first sample contained cytochrome C (8.6 µM) and melittin (70.2 µM), the second contained BSA (9.4 µM) in addition to cytochrome C (8.6 µM) and melittin (70.2 µM) while the third contained cytochrome C (7.8 µM), melittin (63.6 µM) and BSA (8.5 µM) in addition to apo-transferrin (8.0 µM). Unique peptides generated from each protein were monitored and the sequence coverage of cytochrome C in each case was calculated in comparison to the sequence coverage of the protein when digested alone. Figure 36 shows the spectrum of the tryptic digestion of the first sample (flow rate is 5 µl min^{-1}, residence time < 1.0 minute). The sample was relatively simple and contained only two small proteins. However, even with such a simple mixture of two proteins, the sequence coverage of cytochrome C dropped from 74 % to 50 %. This was due to missing two peptides (m/z 1471.6 and 1496.6). The net charge of these two peptides is the lowest among all the peptides present in the peptide mixture as calculated using GPMAW software and therefore, usually they give weak signals, as observed from the PMF spectrum of a tryptic digest of cytochrome C obtained earlier. Therefore, the loss of these signals was most probably due to the signal suppression of these two peptides as a result of the presence of the other peptides. This shows the importance of having a separation system prior to MS as even such simple peptide mixtures are strongly affected.

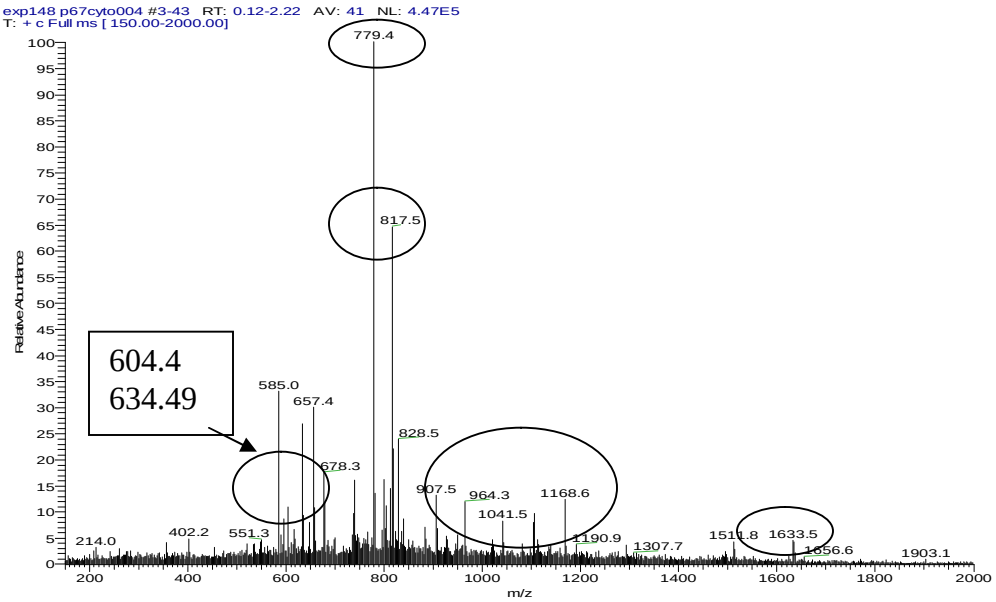


Figure 36: Mass spectrum of tryptic digestion of two proteins: cytochrome C and melittin. The circled signals corresponds to tryptic digest of cytochrome C.

The second mixture was also digested and the spectrum again shows the presence of some unique peptides related to the digested proteins (Table 14). However, no unique peptides are present which can be related to melittin. The two peptides generated from tryptic digest of melittin have m/z values of 657.8 and 1512.9. Other peptides with very similar m/z values are present in the tryptic digest of BSA (658.7 and 1512.8). This shows the significance of having a high resolution mass analyser as such an analyser could easily differentiate between these peptides. The fact that both melittin and BSA produces peptides of very similar mass means that these peptides cannot be considered unique to either protein. For this reason these peptides have been omitted from Table 14. The sequence coverage of cytochrome C was not affected and it was the same as in the first sample (50 %).

m/(z=1)	m/(z=2)	Sequence
Unique peptides generated from digestion of mellitin		
None		
Unique peptides generated from digestion of cytochrome C.		
634.8	317.9	Ile-Phe-Val-Gln-Lys-
1017.2	509.1	Cys-Ala-Gln-Cys-His-Thr-Val-Glu-Lys-
1168.7	585.1	Thr-Gly-Pro-Asn-Leu-His-Gly-Leu-Phe-Gly-Arg-
604.7	302.9	Gly-Ile-Thr-Trp-Lys-
678.4	339.9	Tyr-Ile-Pro-Gly-Thr-Lys-
779.5	390.5	Met-Ile-Phe-Ala-Gly-Ile-Lys-
Unique peptides generated from digestion of BSA		
1164.3	582.7	Leu-Val-Asn-Glu-Leu-Thr-Glu-Phe-Ala-Lys-
1348.5	674.7	Thr-Cys-Val-Ala-Asp-Glu-Ser-His-Ala-Gly-Cys-Glu-Lys-
1890.2	945.6	His-Pro-Tyr-Phe-Tyr-Ala-Pro-Glu-Leu-Leu-Tyr-Tyr-Ala-Asn-Lys-
923.1	462.0	Ala-Glu-Phe-Val-Glu-Val-Thr-Lys-
790.0	395.5	Leu-Val-Thr-Asp-Leu-Thr-Lys-
1576.7	788.9	Glu-Cys-Cys-His-Gly-Asp-Leu-Leu-Glu-Cys-Ala-Asp-Asp-Arg-
1015.2	508.1	Ser-His-Cys-Ile-Ala-Glu-Val-Glu-Lys-
1284.5	642.8	His-Pro-Glu-Tyr-Ala-Val-Ser-Val-Leu-Leu-Arg-
1497.6	749.3	Asp-Asp-Pro-His-Ala-Cys-Tyr-Ser-Thr-Val-Phe-Asp-Lys-
1011.1	506.0	Gln-Asn-Cys-Asp-Gln-Phe-Glu-Lys-
1480.7	740.9	Leu-Gly-Glu-Tyr-Gly-Phe-Gln-Asn-Ala-Leu-Ile-Val-Arg-
1023.2	512.1	Cys-Cys-Thr-Glu-Ser-Leu-Val-Asn-Arg-
1015.2	508.1	Gln-Thr-Ala-Leu-Val-Glu-Leu-Leu-Lys-
1003.2	502.1	Leu-Val-Val-Ser-Thr-Gln-Thr-Ala-Leu-Ala-

Table 14: Digestion of protein mixtures containing melittin, cytochrome C and BSA using microfluidic chip.

When the third sample was digested (melittin, cytochrome C, BSA and apo-transferrin), the generated spectrum showed unique peptides related to three proteins (cytochrome C, BSA and apo-transferrin), some of which are listed in Table 15. However, a further drop in the sequence coverage of cytochrome C was observed (42%). This is due to missing the peptide with m/z 964.5. Figure 37 shows three spectra; the first is the spectrum generated from digestion of cytochrome C alone, while the second one shows the spectrum generated from digestion of protein mixture 1 containing melittin and cytochrome C. The third shows the spectrum generated from digestion of protein mixture 3 containing melittin, cytochrome C, BSA and apo-transferrin. The data shows also how the sequence coverage of cytochrome C is affected as the mixtures get more complex.

m/(z=1)	m/(z=2)	Sequence
Unique peptides generated from digestion of melittin		
None		
Unique peptides generated from digestion of cytochrome C.		
1017.2	509.1	Cys-Ala-Gln-Cys-His-Thr-Val-Glu-Lys-
604.7	302.9	Gly-Ile-Thr-Trp-Lys-
678.8	339.9	Tyr-Ile-Pro-Gly-Thr-Lys-
779.5	390.5	Met-Ile-Phe-Ala-Gly-Ile-Lys-
Unique peptides generated from digestion of BSA		
923.1	462.0	Ala-Glu-Phe-Val-Glu-Val-Thr-Lys-
790.0	395.5	Leu-Val-Thr-Asp-Leu-Thr-Lys-
Unique peptides generated from digestion of apo-transferrin		
920.1	460.6	Gly-Tyr-Leu-Ala-Val-Ala-Val-Val-Lys-
837.0	419.4	Gln-Gln-Asp-Asp-Phe-Gly-Lys-

Table 15: Digestion of proteins mixture containing melittin, cytochrome C, BSA and apo-transferrin using microfluidic chip.

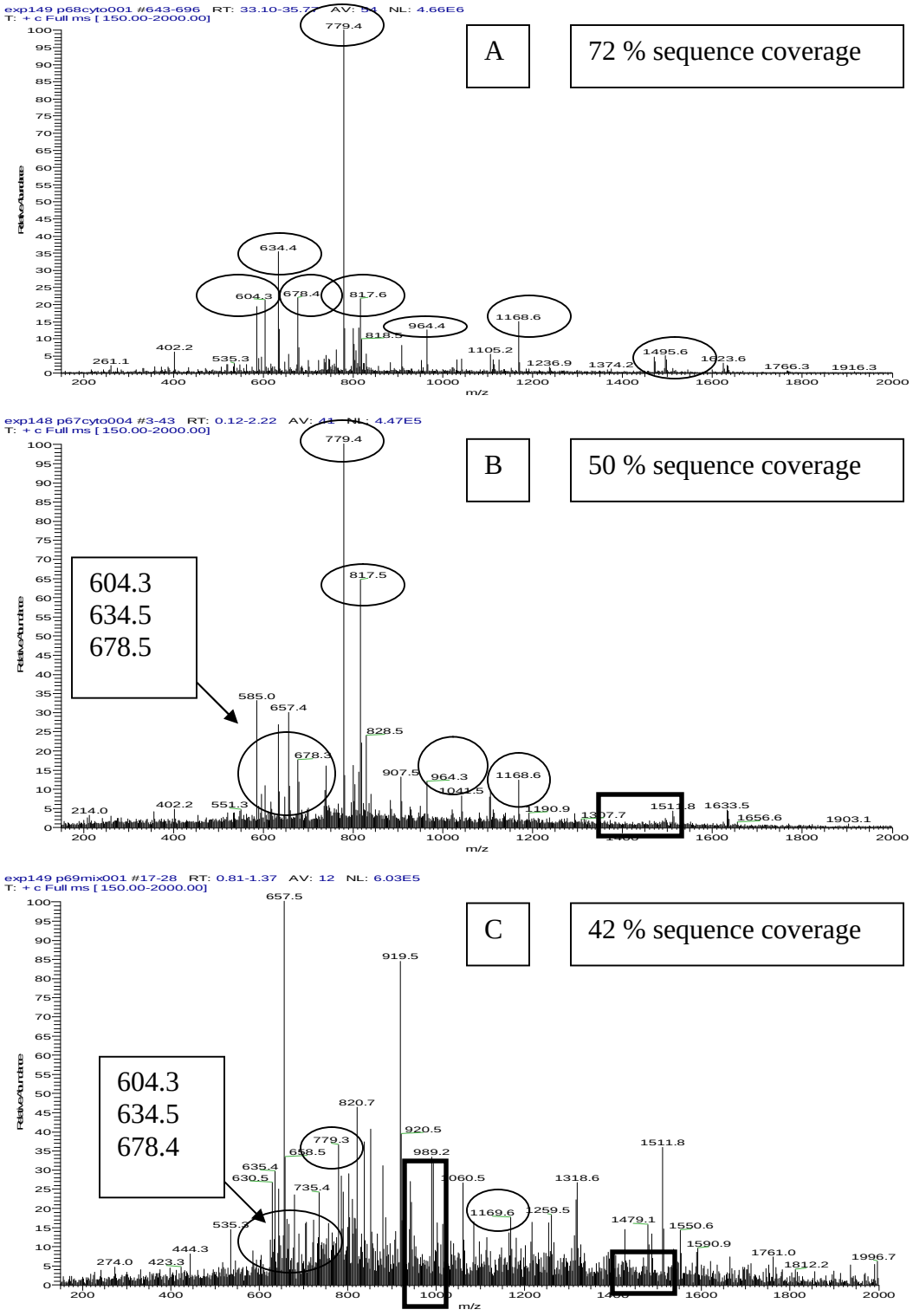


Figure 37: Mass spectra generated from tryptic digestion of cytochrome C and protein mixture samples 1 and 3. The rectangular area in the spectra B and C shows the missing peptides which were present in spectrum A.

3.3.8 Stability of the immobilized trypsin beads in the microfluidic chip.

The study was carried out using a 15 mm long channel packed with trypsin and the trypsin was activated using ammonium acetate solution (pH 8.2). A fresh solution of highly concentrated melittin sample (300 .0 μM) was infused at a flow rate of 5 μl min^{-1} (at higher flow rate, peaks corresponds to intact protein were detected). This test was repeated after 1 day, 7 days, 20 days and 35 days. The digestion was carried out at 40 ºC and the beads were washed after each test with 100 μl of 50 mM formic acid to inactivate the trypsin. They were stored at room temperature and it was ensured that the channel was completely filled with 50 mM of formic acid solution such that no air bubble was trapped.

The results showed that in all the tests, complete digestion was obtained and no traces of intact protein were present. However, it was observed also that the relative intensity of m/z 657.4 and 834.9 increased with time while m/z 1511.7 was still the highest intensity peak in all the spectra (Figure 38). Additionally, the intensity of a few other peaks increased noticeably after 7 days (m/z 927, 1083.7 and 1299.7).

In summary the changes observed in the spectrum even after 35 days cannot be considered significant and this indicates that the digestion system is stable for at least 35 days when stored at room temperature and the channel filled with 50 mM formic acid solution. This can be explained due to the immobilization process. Xi *et al.* [276] showed that the relative activity of immobilized trypsin decreased less than 10 % over 30 days.

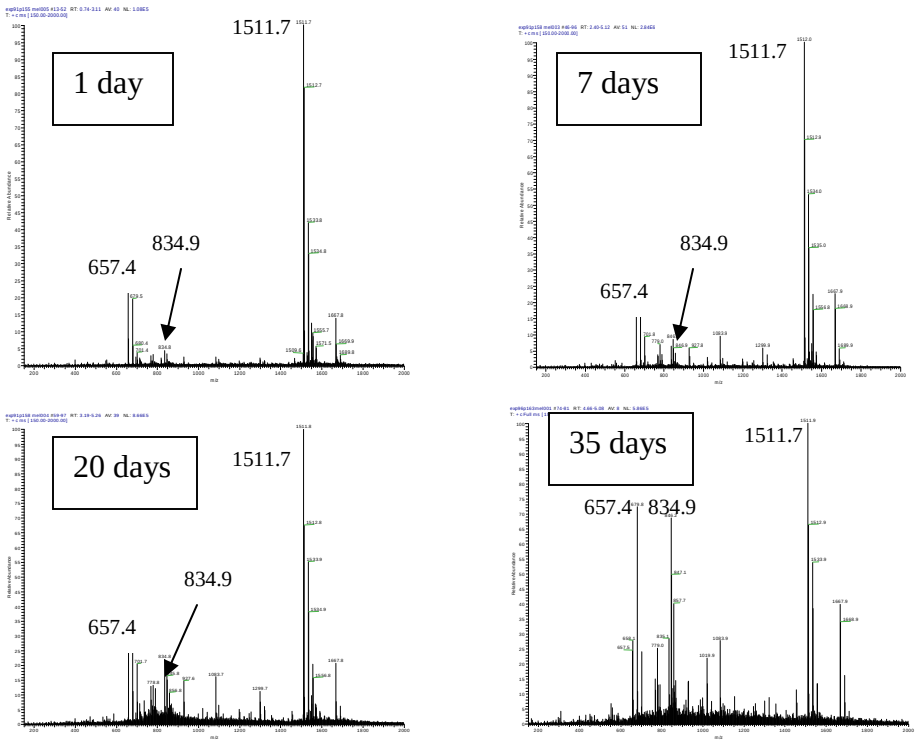


Figure 38: Mass spectra of tryptic digestion of melittin using microfluidic chip. The digestion was carried out after 1, 7, 20 and 35 days from filling the channel with the agarose beads

3.3.9 Sample carry over test.

To study sample carry over contamination or what is known as memory effect, [203] two samples were prepared, one for cytochrome C and other for melittin. Each sample was injected separately after the other three times and a washing step was carried out in between with 100 µl of a mixture of ammonium acetate and formic acid 200 mM (1:1) solution at 40 ºC. Memory effects were not observed and no cross contamination was found between successive runs. Wang *et al* [203] used 50 µl of the same solution but they used ammonium carbonate buffer instead of ammonium acetate buffer. However, the cleaning was carried out at room temperature.

3.3.10 System reusability.

One of the most important aspects of any on-chip process is reusability. This is especially important where glass chips are used, due to the high cost of fabrication

of chips made from glass. Therefore, it is important to consider the reusability aspect of the microfluidic chip digestion system used here.

The torr seal glue used to connect the peek tube in hole 1 can be removed using concentrated formic acid. Once the glue is removed, the peek type tube can be easily disconnected from the microfluidic chip. Then, applying a small pressure *via* the other hole (2) removes all the beads placed inside the channel. If any beads stick to the wall of the channel, which happened rarely, then concentrated formic acid can be used to dissolve the beads and then the channel can be washed with plenty of water to ensure the removal of all formic acid from the channel.

3.4 Conclusions.

On-chip digestion using trypsin immobilized on agarose beads packed in a microfluidic channel is a very efficient method of protein digestion in terms of sequence coverage and in terms of complete digestion. The work clearly demonstrated the efficiency of the digestion system used here and its capability to digest complex mixtures within five minutes. The high efficiency is due to the high surface area to volume ratio and also due to the use of heat (digestion was performed at 40ºC). The use of higher temperature enhances the digestion process significantly due to the increase in the proteolytic activity of the immobilized trypsin. Additionally, the system is very stable and can be used for at least 35 days without any significant loss in the efficiency of the system. Moreover, the system can be prepared easily and can be reused with no difficulties. Another advantage of the system is the low cost of the trypsin used, which was not more than 3 pounds per packing. However, proteins resistant to proteolytic digest showed a few missed cleavages, which may affect the identification of these proteins, particularly if present in mixed samples.

4.0 Development of a photoinitiated monolithic ion exchange column in a microfluidic chip for separation of tryptic digests of proteins

4.1 Introduction.

The previous chapter showed that immobilized enzyme in a microfluidic chip was a very efficient system for protein digestion. However, it also clearly demonstrated that there is a need to have a separation system between the digestion chip and the ESI-MS when mixtures are present. The objective of the next chapter is to explore the possibility of performing on-chip peptide separation using on-chip chromatographic techniques.

Various materials have been used as a substrate for microfluidic systems, for example, silicon [165], glass, plastic, ceramic [163] or even polymeric materials [166]. Generally, the choice of materials depends on the compatibility of the analytes, the availability and the cost of the material. [162] However, for on-chip monolithic columns, glass is one of the most appropriate materials due to its similarity to the inner wall of a fused silica capillary. This facilitates comparison between the capillary column and the on-chip column. Additionally, microfluidic chips made from glass can handle higher back pressure due to strong thermal bonding between the top and bottom glass plates, which many other substrates such as polydimethylsiloxane (PDMS) cannot handle. [214] Moreover, the monolith can easily be anchored to the inner wall of the glass channel after silanization of the channel wall.

Another important aspect for on-chip monolithic columns is the use of photoinitiation as a polymerization method rather than thermal initiation. Using photoinitiation, the monolith can be formed within a specified area in the channel of a chip by exposing that area to a light source and allowing the polymerization of the monolith to take place.

The third important aspect for on-chip monolithic columns is the back pressure generated by the monolith, which should be minimal, especially if the column is one of the components of an integrated system.

On-chip chromatographic separation has been carried out using reverse phase chromatography. [232,233,236] However, the complexity of the tryptic digest requires at least two dimensional chromatographic systems for efficient separation of the thousands of peptides which may be present in a single sample. [234]

The most commonly applied two-dimensional chromatographic separation in proteomics is based on the linear combination of a strong cation exchange column as a first dimension followed by a reverse phase column as a second dimension of separation. [110]

Separation of tryptic digests of proteins has not yet been reported successfully on-chip using cation exchange monolithic stationary phases. However, anion chromatography has been implemented on-chip using particles [279] and monoliths [280] (Figure 39). The former was used to separate inorganic anions and the result showed that the separation of the on-chip column was superior when compared to capillary separation. This is due to the reduced depth of the on-chip column compared to the capillary, which leads to a higher interaction with the analytes and hence better separation. [279] An on-chip monolithic anion exchange column has also been used to separate proteins and the result showed that the monolithic column generated very fast and efficient separations. A quartz chip was used with column length of 4.5 cm (width 40 μm). The back pressure generated was 2.2 MPa at a flow rate of 0.4 μl min^{-1}.

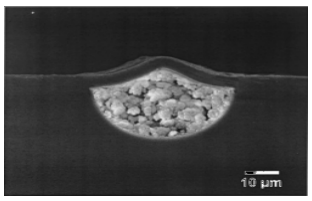


Figure 39: Monolith formation inside a microfluidic channel. [280]

For strong cation exchange media, sulphite groups should be introduced to the solid support, so it can act as a strong cation exchange column. Many approaches have been reported for introducing sulphite groups into the monolith backbone, among them, copolymerization [281] and grafting of a sulphite containing monomer. [282, 283] Peter *et al.*[284] used ethylene dimethacrylate, butyl methacrylate and 2- acrylamide-2-methyl-1-propanesulphonic acid (AMPS) to make a monolith for CEC. Butyl methacrylate provides the hydrophobicity required, while AMPS provides the charge (SO_3^{2-}) required for the electrophoretic separation. However, the presence of the hydrophobic site means that if the monolith is used as an ion exchange column, organic solvent has to be added to the mobile phase to suppress the hydrophobic interaction. Thus the column cannot be used in 2D separation if the second dimension separation is based on reverse phase. Recently, Gu *et al.* [285] prepared poly [2-acrylamido-2-methyl-1-propanesulphonic acid co-poly(ethylene glycol) diacrylate] as a monolithic ion exchange column. A peak capacity of 179 in a 60 minutes gradient was obtained. However, the use of AMPS as co-monomer adds hydrophobicity to the monolith. AMPS contains isobutyl groups, which adds four carbons to the monolith in addition to the two present from ethylene dimethacrylate. As a result the column cannot be used in conjunction with a reverse phase column in a two dimensional chromatographic system. [285] Ueki *et al.* [286] functionalized glycidyl methacrylate (GMA) with sodium sulphite, using ethylene dimethacrylate (EDMA) as a cross-linker and then showed the application of this column to the separation of inorganic cations. This direct sulphonation of the monomer does not add any hydrophobicity. Additionally, Wei *et al.*[287] functionalized EDMA-GMA and used it as a monolithic weak cation exchange column for protein separation and claimed “the modified column is really hydrophilic and shows no hydrophobic interaction with these proteins”. Moreover, in a separate report a monolithic column made of GMA monomer and EDMA as crosslinker was hydrolyzed and a sample of protein mixture was injected into this column. All proteins eluted at void volume suggesting a total lack of nonspecific interactions such as hydrophobic interactions.[281] This indicates that although the presence of an ethylene group in the structure of EDMA adds some hydrophobicity, the effect was not significant and therefore, even bulky molecules like proteins did not have any significant hydrophobic interaction. Furthermore analysis of cations from human saliva

samples was reported [286] with a direct injection of the sample and again all proteins eluted at void volume. It is also known that peptides require a more hydrophobic surface than proteins for interaction. [100] Usually ethylhexyl methacrylate (C_8) [236] or lauryl methacrylate (C_{12}) [100] are used for peptide separation while butyl methacrylate (C_4) [288] monolith has been used for protein separation in reverse phase chromatography. However, other reports suggest that EDMA as crosslinker may have some hydrophobic interaction.[285,289] The extent of the hydrophobic interaction would depend on the percentage of EDMA in the polymeric mixture and on the porosity of the monolith. In a separate study, butyl methacrylate was used as monomer along with EDMA as crosslinker. The study clearly demonstrated the effect of EDMA percentage in the monomer mixture and the porosity on the separation. The monolith exhibited a poor separation for all tested compounds when the percentage of EDMA was 39.5 % and the total porosity was 0.72. The performance of the column improved when the percentage of EDMA increased to 49.5 % and the porosity reduced to 0.65 (height equivalent to theoretical plate (HETP) was 990 μm for toluene). When the percentage of EDMA increased 54.4 % and the total porosity was reduced to 0.63, sharp peaks were obtained for all tested compounds (HETP was 40 μm for toluene). [290] The percentage of EDMA in the monomer mixture used by Ueki *et al.* [286] is 25 %. Currently, polysulphoethyl stationary phase is used as an ion exchange column and it has been found that it exhibits some hydrophobicity. [289] Based on these observations, it was decided to use the method described by Ueki *et al.* [286] to prepare monolithic ion exchange columns for peptide separation.

In this experiment the sulphonated GMA-EDMA monolith was first made in a silica capillary using thermal and photoinitiation. The optimized photoinitiated monolith was then fabricated in a microfluidic glass chip. The performance of the on-chip column was evaluated and compared to the capillary column.

4.2 Experimental.

4.2.1 Chemicals.

- Acetonitrile, HPLC grade (Fisher scientific equipments, Loughborough - UK).

- Formic acid (Fisher scientific equipments, Loughborough - UK).
- 3-(Trimethoxysilyl)propylmethacrylate (TSP), Sigma Chemical Co (St. Louis, MO - USA).
- Benzoylperoxide (BP), Sigma Chemical Co (St. Louis, MO - USA).
- 2,2-Dimethoxy-2-phenylacetophenone (DAP), Sigma Chemical Co (St. Louis, MO - USA).
- Glycidyl methacrylate (GMA), Sigma Chemical Co (St. Louis, MO, USA).
- Ethylene dimethacrylate (EDMA), Sigma Chemical Co (St. Louis, MO - USA).
- Propan-1-ol, Sigma Chemical Co (St. Louis, MO - USA).
- Butane-1.4-diol, Sigma Chemical Co (St. Louis, MO - USA).
- Ethanol, Sigma Chemical Co (St. Louis, MO - USA).
- Trypsin Gold, mass spectrometry grade, Promega (Madison, WI - USA).
- Teflon coated (UV transparent) fused silica capillary 100 μm i.d. /360 μm o.d. Polymicro Technologies, LLC (Arizona - USA). All chemicals were used as supplied, without further purification.
- Proteins used described in section 2.2.1.

4.2.2 Instrumentation.

- A binary gradient liquid chromatography (LC) pump (Series 200 LC Pump, Perkin Elmer, MA - USA) was connected *via* a guard column (Jupiter 5 μm, C18, 50mm, Phenomenex Ltd., Macclesfield Cheshire - UK) to a high pressure flow splitter (Upchurch scientific, WA - USA). The guard column was solely used to reduce pressure pulses and obtain a pulse free flow. The flow splitter was then connected to the injector (2 μL loop, Valco, Schenkon - Switzerland or 265 nl peek tubing, Upchurch scientific, WA - USA) from which the monolithic column was interfaced to an electrospray ionization mass spectrometer (ESI-MS), described earlier in section 2.2.2.

- Photoinitiation was carried out using a UV lamp (Spectraline, Indiana - USA) at a wavelength of 365 nm and a distance of 10 mm from the capillary. Peptide net charges were calculated using GPMAW 6.21 software (Lighthouse data, Odense - Denmark).
- Furnace (Carbolite CWF 1200, Oregon - USA).
- Other instruments, see section 2.2.2

4.2.3 Protein digestion.

The digestion was carried out either using the in-solution method (section 2.2.3) or using the on-chip method (section 3.2.5).

4.2.4 Preparation of monolithic columns.

The capillary was first silanized to facilitate anchoring of the monolith to the wall of the capillary. This was achieved by washing with acetone followed by water and then activation of the surface by aqueous sodium hydroxide solution (0.2 M) at 2.0 μL min^{-1} for 1 hour. The capillary was then washed with water, followed by aqueous hydrochloric acid solution (0.2 M) at 2.0 μL min^{-1} for 1 hour, flushed with water, then ethanol and finally silanized with a solution of TSP 20 % in ethanol (pH 5.0, adjusted with acetic acid) at 2.0 μL min^{-1} for 1 hour. The capillary was finally dried using nitrogen gas and left overnight. [257]

The monolith was prepared and sulphonated as described by Ueki *et al.* [14], however, BP or DAP were used as initiators instead of 2,2-azoisobutyronitrile (AIBN). GMA (0.9 mL, methacrylic monomer) and EDMA (0.3 mL, cross-linker) were dissolved into a ternary porogenic solvent, propan-1-ol (1.05 mL), butane-1.4-diol (0.6 mL), and water (0.15 mL). After purging with nitrogen for 3 min, initiator (12 mg, corresponding to 1 wt % of the amount of total monomers) was added to the monomer solution.

The UV transparent silica capillary 100 μm i.d. / 360 μm o.d. was filled by syringe with the polymerization mixture to a length of 15 cm. The capillary was then placed in a column block heater (Jones Chromatography Ltd - UK) at 60 ^{o}C for 24 hours or exposed to UV light for different times (Table 16). For the thermally initiated

monolith, the capillary containing the monolith was cut to a length of 5 cm, whereas for the UV photoinitiated monolith only a 5 cm length was exposed to the UV radiation with the rest of the capillary securely masked with photo-mask and black tape. The capillaries were then flushed with ethanol and water for 30 minutes each to remove any remaining starting materials. Sulphonation was carried out by passing aqueous Na_2SO_3 (1M) at a flow rate of 3 µL min^{-1} for 12 hours. The pH of the solution was adjusted to 11 with sodium hydroxide (0.2 M). Sulphonation was allowed to proceed at 75 °C in the column block heater. The monolith was then washed with nitric acid (10 mM) for 15 min at a flow rate of 2.0 µL min^{-1} and finally washed with water for 6 hours at the same flow rate. The type of initiator, duration and method of initiation are presented in Table 16 for the columns tested.

Column	Initiator	Duration	Method of initiation
1	BP	24 hours	Thermal
2	BP	15 hours	Photo
3	DAP	30 minutes	Photo
4	DAP	20 minutes	Photo
5	DAP	10 minutes	Photo
6	DAP	7 minutes	Photo
7	DAP	5 minutes	Photo
8	DAP	3 minutes	Photo

Table 16: Initiator, duration and method of initiation used in preparing various ion exchange monolithic columns.

The mobile phase and gradient conditions were as described below when the ion exchange monolithic column was used:

For separation of a tryptic digest of cytochrome C, mobile phase composition was A, aqueous formic acid (0.4 %, pH 2.6) and B, aqueous ammonium acetate (550 mM, pH 2.6, adjusted with formic acid). The gradient started at 100 % A for 3 minutes, then decreased to 0 % A over 10 minutes and was then left at 0 % A for 20 minutes. Solvent was then returned to 100 % A and equilibrated for 15 to 20

minutes. Flow rate was set between 1.4 and 1.8 μL min^{-1} within the accuracy of the pump and flow splitter used.

For the separation of a tryptic digest of BSA, mobile phase composition was A, aqueous formic acid (0.4%, pH 2.6) and B, aqueous ammonium acetate (550 mM, pH 2.6 adjusted with formic acid). The gradient started at 100 % A for 3 minutes then was decreased to 0 % A over 100 minutes and left at 0 % A for 20 minutes. Solvent was then returned to 100 % A and equilibrated for 15 to 20 minutes. Flow rate was set between 1.4 and 1.8 μL min^{-1}.

The hydrophobicity test of the monolith backbone (*i.e.* without sulphonation) was carried out using a tryptic digest of cytochrome C and aqueous formic acid (0.4%, pH 2.6) was used as the mobile phase. Flow rate was set between 1.4 and 1.8 μL min^{-1}.

4.2.5 On-chip monolithic column.

The glass microfluidic chip used had the following channel dimensions, 9 cm x 150 μm x 30 μm, (length, width and depth). The fabrication was carried out using a standard lithographic method and the bonding was carried out thermally by placing the chip in a furnace for four hours at 585 ºC. At each end of the channel, a hole was made with 360 μm diameter and two fused silica capillaries (50 μm i.d.) were inserted into the end of each hole to avoid any loss of separation due to void volumes.

To form a photoinitiated monolithic column only 5 cm of the column was exposed to the UV light source. The rest of the chip was completely masked with photo-resistant mask and sealed with black tape. The formed monolith was than sulphonated and washed as described earlier (section 4.2.4).

The mobile phase and gradient conditions were as described below when the on-chip ion exchange monolithic column was used:

For the separation of a tryptic digest of cytochrome C, mobile phase composition was A, formic acid 0.4 % (aqueous, pH 2.6) and B, ammonium acetate 550 mM

(pH 2.6 adjusted with formic acid). The gradient started with A 100 % for 3 minutes, then was decreased to 0 % A over 10 minutes and was left at 0 % A for 30 minutes. Solvent was then returned to 100 % A and equilibrated for 15 to 20 minutes. Flow rate was set between 1.4 and 1.8 μL min^{-1}. The separation was carried out at 25 °C, 40 °C and 65 °C.

For separation of the mixture of tryptic digest of melittin, cytochrome C, BSA and apo-transferrin, mobile phase composition was A, formic acid 0.4 % (aqueous, pH 2.6) and B, ammonium acetate 550 mM (pH 2.6 adjusted with formic acid). The gradient started with A 100 % for 3 minutes, then was decreased to 0% A over either 10, 20 or 60 minutes and left at 0 % A for 20 minutes. Solvent was then returned to 100 % A and equilibrated for 15 to 20 minutes. Flow rate was set between 1.4 and 1.8 μL min^{-1}. The same mixture was separated using a commercial column (Jupiter 4 μm Proteo 90A, 150 mm x 0.33 mm, Phenomenex). The mobile phase composition in this case was A, formic acid 0.1 % in water and B, formic acid 0.1 % in acetonitrile. The gradient started with A 100 % for 3 minutes, then was decreased to 0% A over 60 minutes. Solvent was then returned to 100 % A and equilibrates for 15 to 20 minutes. Flow rate was set at 12.0 μl min^{-1}. The separation was carried out at 25 °C.

4.2.6 Decomposition of the on-chip monolithic column.

The decomposition was carried by placing the chip in a furnace (Carbolite CWF 1200) at 520 ^{o}C at a ramp of 10 ^{o}C min^{-1} for 2 hours. The chip was then washed with sodium hydroxide for several hours (5 to 7 hours) and then with water for 12 hours, both at flow rate of 3.0 μl min^{-1}. [181]

4.3 Results and discussion

4.3.1 Separation of tryptic digestion of proteins using thermally initiated monolithic ion exchange column.

A special type of fused silica capillary was used, which was coated with light transparent polymers to allow photoinitiation polymerization to take place.

However, the polymer coating does not add much strength to the capillary and therefore, the capillary was fragile compared to the standard coating materials used.

The optimization of the ion exchange monolithic column was carried out using 100 μm i.d. capillary columns (5 cm in length). Flow rates in the range of microlitres per minute are required when such small dimensions are used. To generate flow rates in the microlitres per minute range, a flow splitter is required to be connected to the standard LC pump available. However, when the splitter was connected, it was observed that high pulses were generated. These pulses affect the separation considerably due to generation of turbulent flow in the separation column. To reduce the pulse effect, an analytical column was placed between the pump and the splitter. The analytical column absorbed the pulses generated from the pump and a relatively pulse free flow was obtained after the column. However, due to the use of a splitter the control on the flow rate was limited and maintaining a constant flow was very difficult, therefore variations in retention time were observed. The variations are not related to the column but rather to the flow, which varies between 1.4 and 1.8 μL min^{-1}.

Capillary silanization is an important step prior to formation of the monolith in the capillary. Usually silanization is carried out by reacting (3-trimethoxysilyl) propyl methacrylate to the inner wall of the fused silica capillary. This is to anchor the monolith to the inner wall of the capillary (Figure 40). [291] The methacrylate group introduced is now available and will participate in the polymerization process, thus the polymer is anchored to the inner wall of the tube.

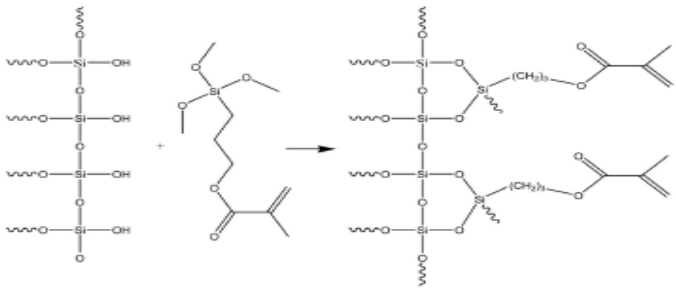


Figure 40: Reaction of (3-trimethoxysilyl propyl methacrylate) with the wall of silica capillary or glass surface. [291]

In order to study the effect of this process, two capillaries were filled with monolith; the first capillary was not silanized while the other capillary was silanized. After formation of the monolith in both capillaries, scanning electron microscope images were obtained for both capillaries. Figure 41 shows clearly the effect of silanization and how in the bottom image the monolith was anchored to the inner wall of the capillary while the top image shows clear gaps between the inner wall of the capillary and the monolith. The presences of such gaps will have a considerable effect on the separation and may cause peak broadening or a complete loss of separation.

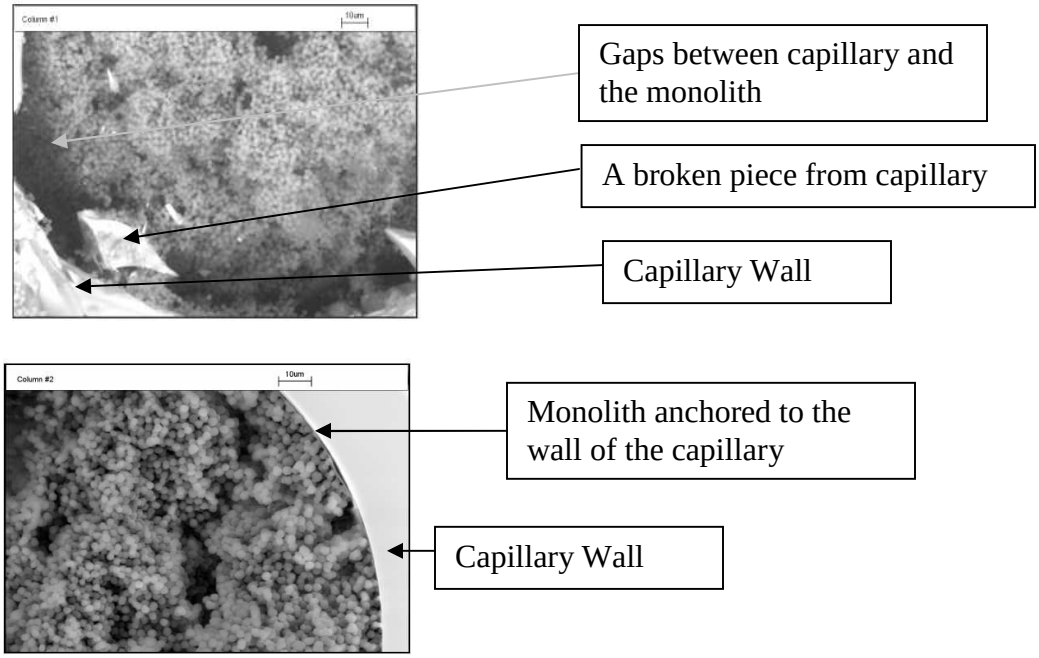


Figure 41: SEM images of silica fused capillary packed with monolith. Top for a capillary packed with monolith without silanization. Bottom for a capillary packed with monolith after silanization of the inner wall of the capillary.

The monolith formation reaction was carried out as described in the experimental section according to the method described by Ueki *et al.;* however, benzoyl peroxide was used as initiator instead of AIBN. This is because it was observed that in some cases voids are formed in the monolith [292] when AIBN is used as

photoinitiator. This may be due to the fast reaction and generation of nitrogen gas during the polymerization. When benzoyl peroxide (BP) was used as initiator, no such problem was observed (Figure 42). Therefore, it was decided here to use BP instead of AIBN. The sulphonation process was also carried out according to the same reference and using the equations illustrated in Figure 43.

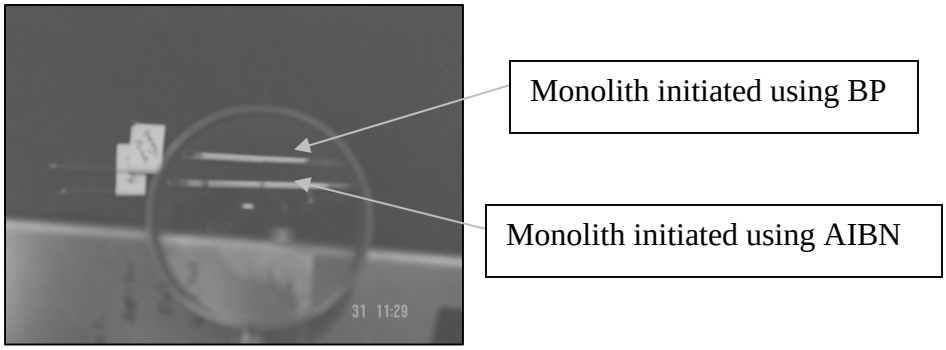


Figure 42: Two monolith columns the first on was initiated using BP which shows no void formed inside the monolith whereas the second one (AIBN was used as initiator) shows two clear two voids formed.

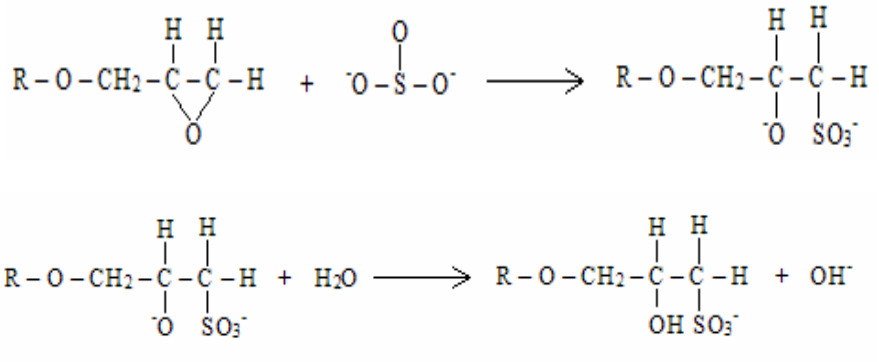
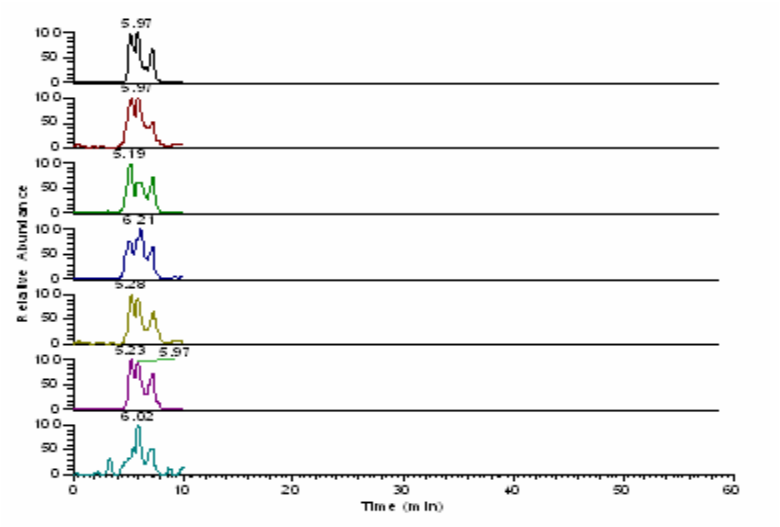


Figure 43: Sulphonation of GMA. [293]

Initially, the epoxy groups present in the monolith were hydrolysed and a tryptic digest of cytochrome C (27.0 pmol) was injected into the column. All peptides eluted at void volume confirming the previously reported result that minimal nonspecific interaction is present between the monolith and these peptides. Another column was then prepared in which sulphonation was carried out and 40 %

acetonitrile in 0.4% formic acid was used as mobile phase. The same tryptic digest was injected and no peak eluted from the column for more than 60 minutes. Then, the mobile phase was changed to 550 mM ammonium acetate in 0.4 % aqueous formic acid solution. All peptides eluted after about 7 minutes. This clearly indicated that the interaction between the peptides and the monolith is primarily based on ionic interaction. Figures 44 and 45 shows the two chromatograms.



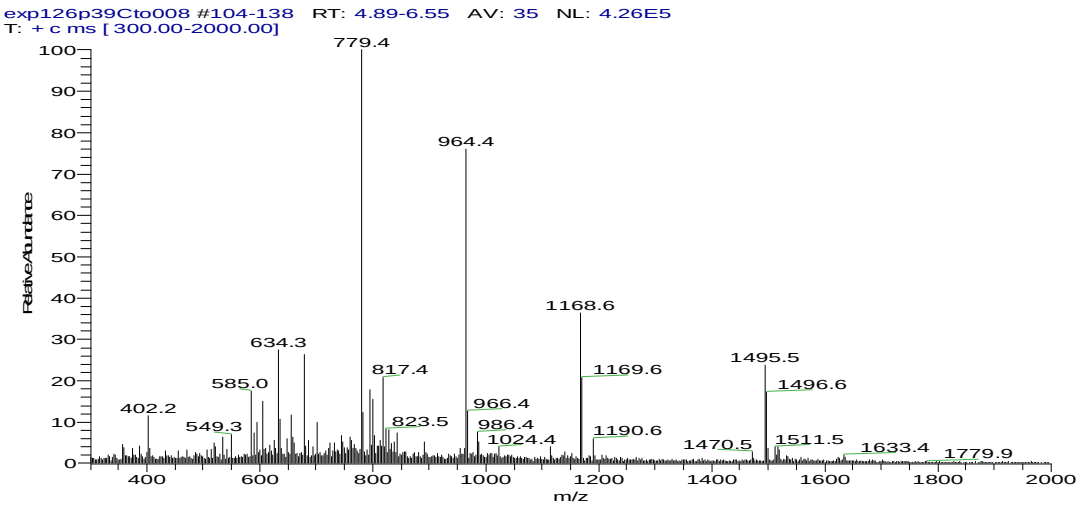


Figure 44: Chromatogram of tryptic digest of cytochrome C obtained using hydrolysed GMA- EDMA monolith. All peptides eluted at void volume. For other conditions, see experimental section.

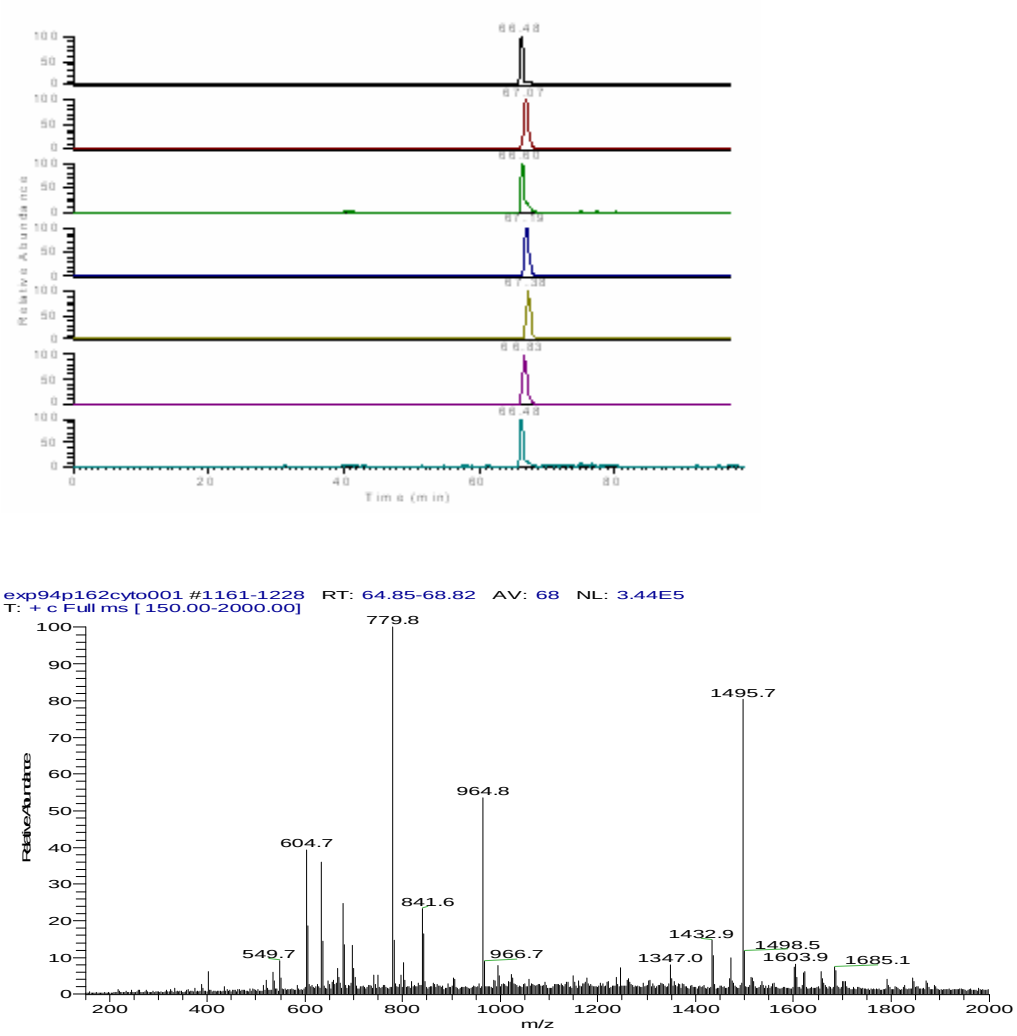


Figure 45 Chromatogram of tryptic digest of cytochrome C obtained using sulphonated GMA- EDMA monolith. For other conditions, see experimental section.

After this preliminary test of the column, the gradient chromatographic system was used to separate the peptides generated from tryptic digest of cytochrome C. The mobile phase consisted of 0.4 % formic acid in water (pH 2.6) and ammonium acetate 550 mM in aqueous solution (pH adjusted to 2.6 with formic acid). Using a 10 minutes gradient, separation of the cytochrome C tryptic digest mixture was achieved. The gradient started at 100 % aqueous formic acid (0.4 %, pH 2.6) for 3 minutes, then decreased to 0 % over 10 minutes during which aqueous ammonium acetate (550 mM, pH 2.6, adjusted with formic acid) reached 100 % and was left

then at 100 % for 20 minutes. Figure 46 shows separation of eight selected peptides.

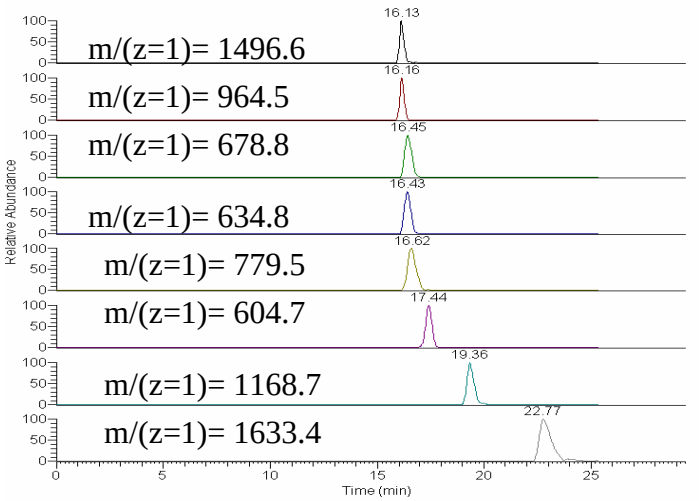


Figure 46: Separation of tryptic digest of cytochrome C using gradient elution and sulphonated GMA-EDMA monolith thermally prepared. For other conditions, see experimental section.

Peaks 1 and 2 eluted together; peaks 3, 4 and 5 were also eluted together. The last three peaks were resolved. This was confirmed with a resolution calculation (Table 17) carried out using equation 3 (Resolution = $(t_{R2}-t_{R1})/ (0.5 \times (w_2+w_1))$).

No.	Retention time (minutes)	Peak width at half height (s)	Resolution
1	16.16	18.8	0.1
2	16.18	18.8	0.1
3	16.43	25.0	0.0
4	16.45	25.0	0.0
5	16.62	25.0	0.2
6	17.44	22.5	1.1
7	19.36	26.3	2.9
8	22.77	37.5	3.8

Table 17: Peak width at half height and resolution calculations for chromatogram presented in Figure 46.

The elution order of the peptides was according to their net charges as shown in Table 18 where the net charge of each peptide is indicated. The first three peptides possess least net charge and they were eluted together. Then all peptides with net charge of 1.20 eluted together, with the exception of peptide 6, which eluted later. This peptide is the smallest one and hence the delay in the elution time can be related to the higher charge density for this peptide. Generally, the higher the net charge density, the larger the capacity factor. However, the selectivity factor for peptides possessing identical charge density would be one and hence no separation would be expected between these peptides (assuming no other types of interaction between the stationary phase and the peptides are present). The net charge density depends not only on the net charge of the molecule but also on the molecular size. The last two peptides eluted (6 and 7) possess the highest net charge density and hence their capacity factor was the largest. For the rest of the work five peptides were monitored: 1, 3, 6, 7 and 8 (Figure 47). The other peptides in the digest co-eluted with one or other of these peptides.

No.	m/(z=1)	Retention time (minutes)	Theoretical net charge at pH 2.6
1	1496.6	16.13	1.16
2	964.5	16.16	1.16
3	678.8	16.43	1.20
4	634.8	16.45	1.20
5	779.5	16.62	1.20
6	604.7	17.44	1.20
7	1168.7	19.36	2.25
8	1633.4	22.77	>2.25*

* The exact net charge is not possible to determine due to the present of heme group bonded to this peptide.

Table 18: Molecular mass, theoretical net charge at pH 2.6 of peptides generated from tryptic digest of cytochrome C and retention time of these peptides obtained from chromatogram in Figure 46.

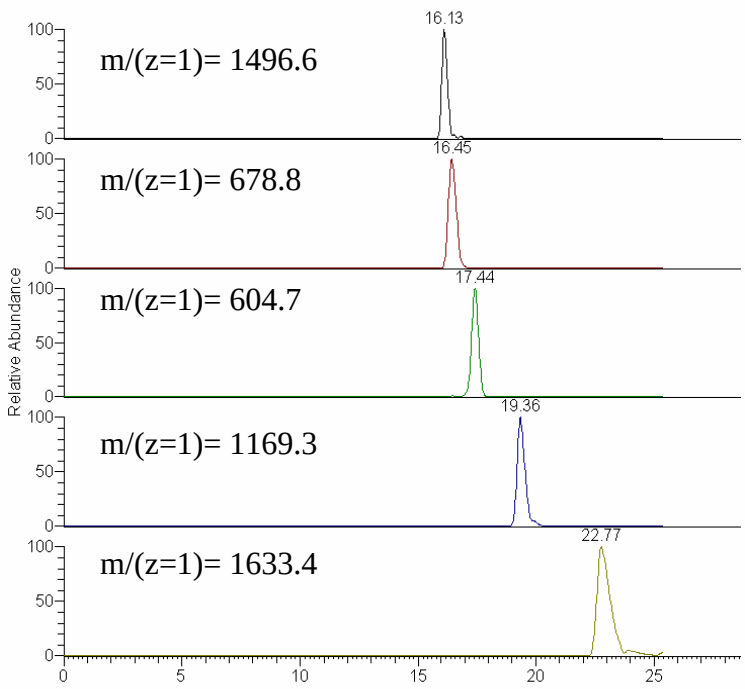


Figure 47: Chromatogram of five selected peptides for monitoring the performance of the monolithic ion exchange column.

The effect of salt concentration was also studied using 100, 250 and 550 mM of ammonium acetate solution. Increasing the salt concentration would increase the mobile phase strength and hence reduce the capacity factor, which may adversely affect the resolution. However, increasing the mobile phase strength would increase the number of theoretical plates. This has a positive affect on resolution. Therefore, optimizing the mobile phase strength was required. Table 19 shows the peak width at half height and the resolution at 550 mM. The resolution was highest at 100 mM. However, reproducibility was poor, with 250 mM salt concentration the last peak eluted was very broad and the retention time was extended (45.98 minutes). When 550 mM ammonium acetate was used, all the peaks eluted within 24 minutes. Increasing the salt concentration further would reduce the resolution between first two peaks considerably. The resolution between the first two components was less than one and ideally weaker mobile phase should be used. However, this would increase the analysis time. Therefore, it was decided to use 550 mM ammonium acetate solution (pH 2.6) as a mobile phase to at this preliminary stage. The average peak width at half height was 26 s (variation between 18.8 to 37.5 s). In comparison

to literature, it has been reported recently that a peak width of 16.2 s at base line was obtained using 5 % gradients.[285] However, the monolith possessed high hydrophobicity such that 40 % acetonitrile was required to elute all the peptides from the column. Therefore, the column was not suitable for use in two dimensional chromatographic separations if reverse phase was used as the second dimension. Additionally, phosphate buffer and a sodium chloride salt gradient was used to elute the peptides from the column. Neither phosphate buffer or sodium chloride can be used if the column is directly connected to ESI-MS. In another report, [294] a peak width between 10 to 15 s at half height was obtained using 75 μm i.d. and 7 cm length packed with particle type ion exchange resin. The narrower peak width obtained by this column may be attributed to the different stationary phase used and also to the smaller i.d. of the column in comparison to the 100 μm i.d. column used here. When a smaller i.d. column is used the radial diffusion decreases and hence this reduces band broadening.

The detection limit was found to be 3.7 pmol when tryptic digest of cytochrome C was used.

Peak	Retention time (minutes)	Peak width at half height (s)	Resolution
Buffer 550 mM			
1	16.13	18.8	0.4
2	16.45	25.0	0.4
3	17.44	22.5	1.5
4	19.36	26.3	2.9
5	22.77	37.5	3.8
Average peak width at half height = 26.0 s.			

Table 19: Peak width at half height and resolution calculations of chromatogram presented at Figure 47.

Using the same mobile phase, a tryptic digest of BSA (72.2 pmol) was separated using an extended gradient time of 100 minutes. Several peptides were detected

which were not detected earlier without separation (*e.g.* m/z 1519.7, 749.3, 979.1). Figure 48 shows the part of the generated chromatogram. A few peaks in the chromatogram eluted very closely. However, co-eluted peptides from the ion exchange column will possess very similar ionic properties, thus, signal suppression in ESI-MS will be much less provided the peptide concentration is not significantly different. This is in contrast to co-eluted peptides from a reverse phase column coupled to ESI-MS where the elution order depends on the hydrophobicity of the two peptides and their ionic properties may or may not be similar, which may lead to strong signal suppression effects. This is considered a unique feature of ion exchange chromatography compared to other types of liquid chromatography when ESI-MS is used as a detector for the chromatographic system.

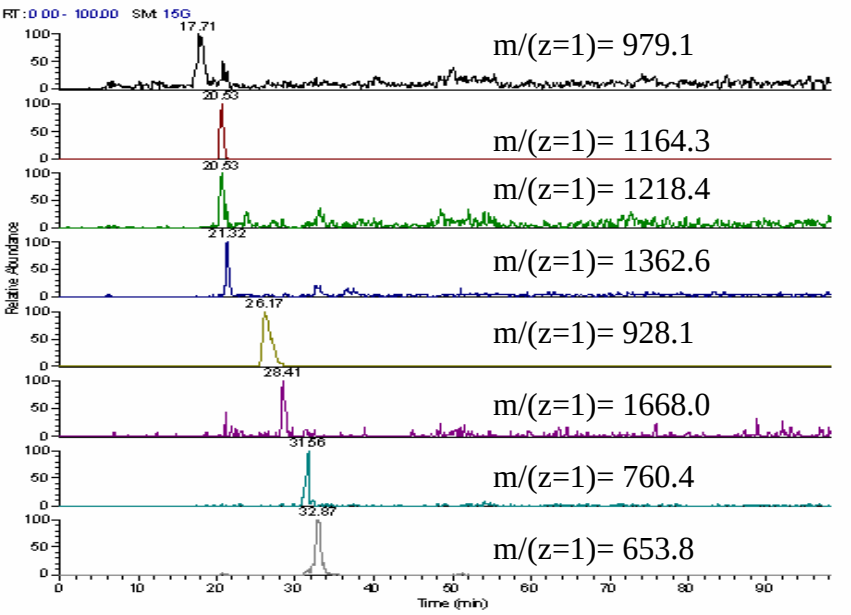


Figure 48: Separation of tryptic digest of BSA using sulphonated GMA-EDMA monolith. See experimental section for other conditions.

The data above confirm the suitability of the column for use as an ion exchange column for peptide separation. However, if the column is going to be fabricated on a microfluidic chip then the pressure generated from the column should not damage the microfluidic chip. It has been reported that the microfluidic glass chip can handle up to 12.5 MPa [278] which was also confirmed experimentally. However, as a

precautionary step the maximum pressure was set not to exceed 12.1 MPa. The back pressure generated from the column was found to be 0.83 MPa at flow rate of 1.0 μl min^{-1}, which means that this monolithic ion exchange column can be easily implemented on a microfluidic chip.

4.3.2 Separation of tryptic digestion of proteins using photoinitiated monolithic ion exchange column.

The next step was to prepare a photoinitiated monolithic ion exchange column, so that it is then possible to fabricate the column in the specified area of the microfluidic chip.

Initially, photoinitiation was carried out for 24 hours; however, the back pressure was high and the column could not be washed. An SEM image was obtained for this column and compared with an SEM image obtained for the thermally prepared monolithic column. Figure 49 shows clearly that the photoinitiated column has much fewer macropores and in some areas the pores are completely blocked.

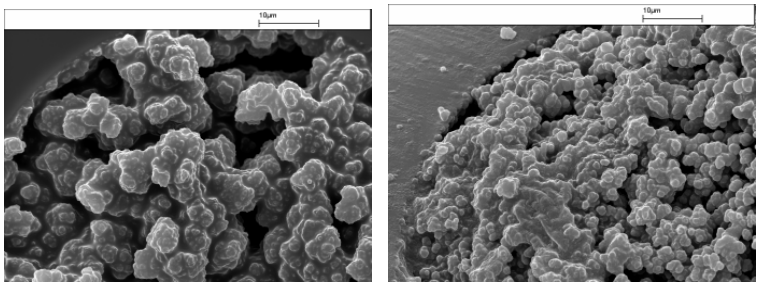


Figure 49: SEM image of monolith formed using thermal initiation (left) and SEM image of monolith formed using photoinitiation (24 hours).

Another photoinitiated monolithic column was then prepared with the initiation time reduced to 15 hours. The column was washable and after sulphonation the back pressure was found to be 2.1 MPa at a flow rate of 1.0 μl min^{-1}. It can be observed from the SEM image (Figure 50) that the morphology of this column is different from the previous two (presented in Figure 49). Compared to the thermally prepared monolith, where the globules are fused and the macropores are about 6 to

12 μm, the globules are less fused and smaller macropores are formed (about 2 to 10 μm). This may explain the increase in the back pressure observed.

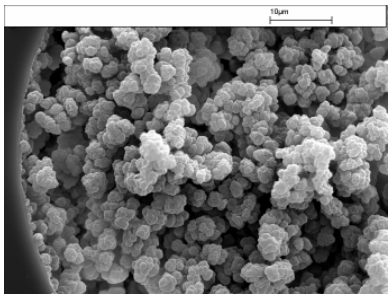


Figure 50: SEM image of monolith formed through UV initiation for 15 hours.

Tryptic digest of cytochrome C (27.0 pmol) was used to evaluate the column performance using the same experimental conditions used before. Figure 51 shows the chromatogram generated using this column.

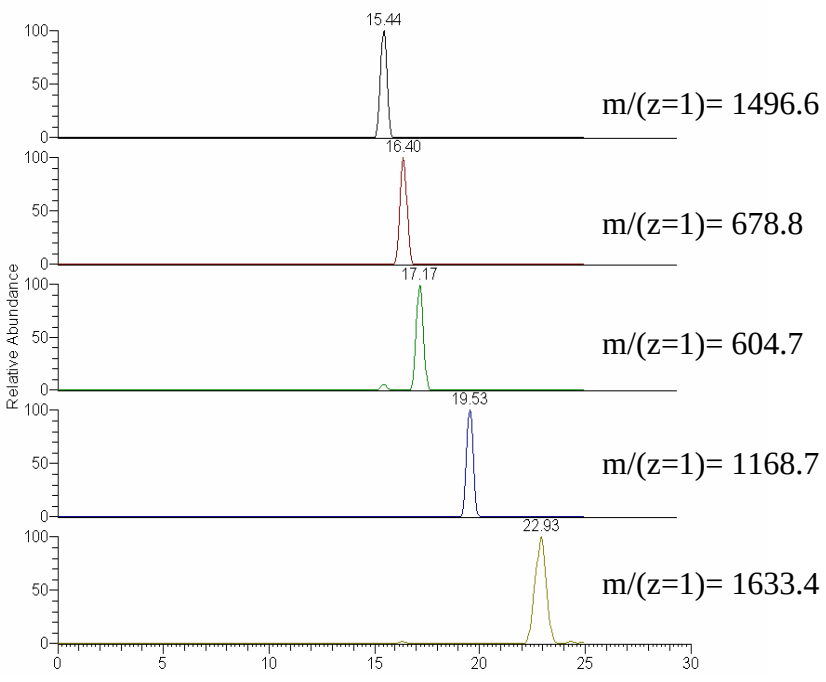


Figure 51: Separation of tryptic digest of cytochrome C using gradient elution and sulphonated GMA-EDMA photoinitiated monolith (BP initiator 15 hours). For other conditions, see experimental section.

Peak	Retention time (minutes)	Peak width at half height (s)	Resolution
Buffer 550 mM			
1	15.44	21.0	1.5
2	16.40	21.4	1.2
3	17.17	21.0	1.2
4	19.93	21.0	3.9
5	22.93	31.5	4.5
Average peak width at half height = 23.2 s.			

Table 20: Peak width at half height and resolution calculations of chromatogram presented in Figure 51.

The data presented in Table 20 shows clearly, that the performance of the photoinitiated monolithic column is better than that of the thermally prepared one, with improved resolution between the first two peaks. A comparison between a thermally initiated monolithic ion exchange column and a photoinitiated example has not been reported. However, such comparison has been carried out using monolithic reverse phase columns. The general observation is that the surface area of thermally prepared monolithic columns is twice that of photoinitiated monolithic columns [295] and the back pressure generated from the photoinitiated monolithic column is always higher than the back pressure generated from thermally initiated columns. [296,297] Therefore, the thermally prepared column performance is higher than that of the photoinitiated column.

The first observation indicates that the micropores formed in the monolith when photoinitiation is used are larger than those formed when thermal initiation is used. This is due to the low temperature at which photoinitiation is taking place, which leads to early phase separation and hence formation of larger micropores. [187] The higher back pressure observed in the photoinitiated monolithic column can be attributed to the fast polymerization in photoinitiation compared to thermally

initiated polymerization. This fast polymerization leads to the formation of a higher number of nucleation sites, which leads to a higher back pressure. However, here the performance of the photoinitiated monolithic ion exchange column was better than the thermally prepared one as the peaks width at half height and resolution calculation shows. This may be due to the electrostatic interaction, which is characterized by being a strong and long distance interaction; [115] thus, the capacity factor would be less affected by the change in the pore size in the case of ion exchange columns. Additionally, the larger micropores formed in the photoinitiated monolithic column may have compensated for any unfavourable effect on mass transfer and improved the column efficiency (*i.e.* strong interaction with the stationary phase may slow down mass transfer processes and hence increase plate height). For reverse phase monolithic columns, the mechanism of separation is based on hydrophobic interactions, which are weak interactions and hence when the size of micropores increases, the interaction between the solute molecules and the stationary phase decreases, leading to reduced capacity factors, which adversely affects the column performance. It may be argued that the thermally initiated monolithic ion exchange column was not optimized and hence the optimized column would have performed better than the photoinitiated monolith. However, Ueki *et al.* [286] has optimized the thermally initiated monolithic column. The use of slower initiator (BP was used here, is slower then AIBN used by Ueki *et al.* [286]) did not change the porosity of the column as the back pressure measurements indicated. GMA-EDMA polymerizes quickly and it has been reported close to 100 % conversion of the monomers in 10 hours when the initiation was carried out at 55 ^{o}C using AIBN as initiator.[186]

The higher back pressure observed in this column was due to the fast polymerization (preparation time is 15 hours) compared to the thermally prepared monolithic column (preparation time 24 hours). However, 8 and 12 hours photoinitiated monoliths did not anchor properly to the internal wall of the fused silica capillary and thus could not be used.

The main drawback of the method used for preparation is the lengthy time required for preparing the column. The time required for preparing a thermally initiated

monolithic ion exchange column was 42 hours, while for the photoinitiated column it was 33 hours. In order to reduce the preparation time a faster initiator was used and the performance of the generated columns were tested. It has been reported that 2,2-dimethoxy-2-phenylacetophenone (DAP), is a very fast photoinitiator and can be used with GMA-EDMA monoliths. [288] BP was replaced by DAP and several columns were prepared with different initiation times as was shown earlier in Table 16.

Columns 3 and 4 generated high back pressure and could not be washed. Columns 5, 6, 7 and 8 were washed and sulphonated. The back pressure was found to be 2.8, 2.3, 1.7, 1.0 MPa at flow rates of 1.0 μl min^{-1}. Since column 8 generated the lowest back pressures and the monolith was found to be properly formed as shown by SEM (Figure 52), the column was evaluated first using tryptic digest of cytochrome C (27.0 pmol)

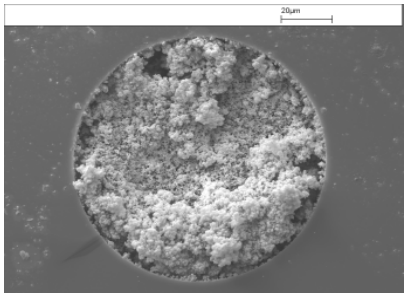


Figure 52: SEM image of monolith formed using photoinitiation for 3 minutes using DAP as initiator.

No separation was observed and all the peaks eluted at void volume except the peptide with m/z value of 1633.4, which was eluted later on, however, the peak shape was distorted. Column 7 was then tested; peak tailing was observed. This tailing was reduced considerably when the sample was diluted five times. Columns 6 and 5 gave good separation, however, the performance of column 5 was better as indicated by peak width and resolution calculations (Figure 53, Table 21).

The column performance improved as the initiation time increased. This was due to the increase in the density of the monolith. As a result, sulphite sites present in the monolith also increased and this increase the capacity factor and leads to improvement of the column performance. In column 8, few sulphite sites were present and therefore, only peptides with the highest ion charge density were retained. The sulphite site increased in column 7 due to the increase in the initiation time. However, the loading capacity was limited and therefore, when the initiation time was increased further, the loading capacity improved considerably. The resolution between the first and second peaks was improved when the initiation time was increased from 7 minutes to 10 minutes. The difference in charge density between these two peptides is small and hence the increase in the initiation time increased the selectivity and improved the resolution between peptides possessing similar net charge density.

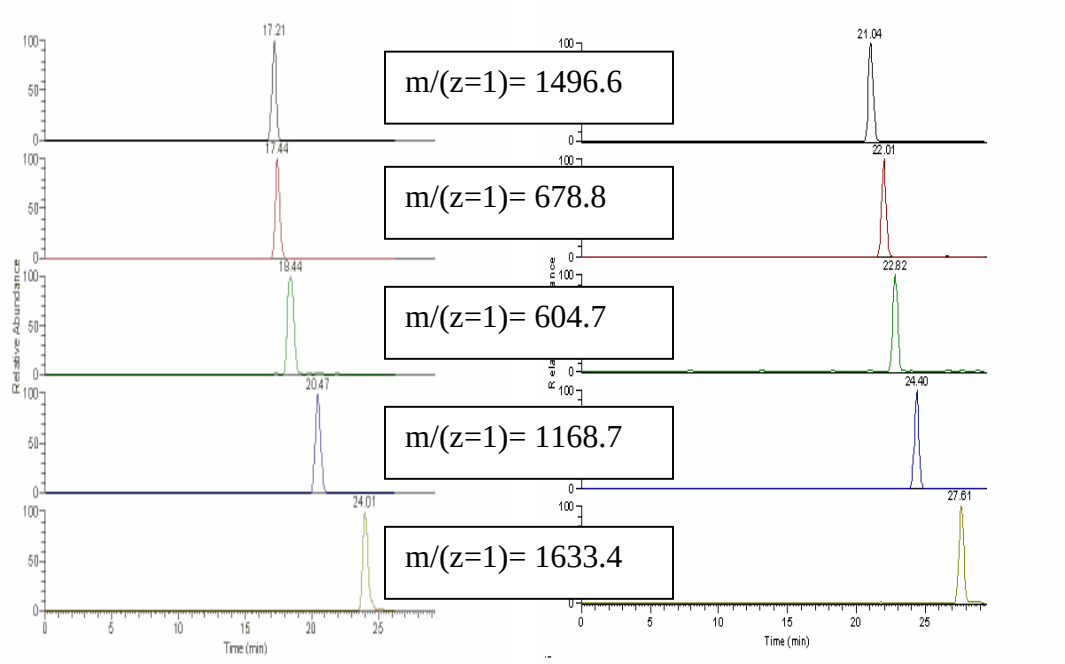


Figure 53: Separation of tryptic digest of cytochrome C using gradient elution and sulphonated GMA-EDMA photoinitiated monolith (Column 6, DAP initiator 7 minutes (left) and column 5, DAP initiator 10 minutes (right)). For other conditions, see experimental section.

Peak	Retention time (minutes)	Peak width at half height (s)	Resolution
Column 5			
1	17.21	25.0	0.3
2	17.44	23.7	0.3
3	18.44	32.5	1.3
4	20.47	27.9	2.5
5	24.01	27.9	4.6
Average peak width at half height = 27.4 s.			
Column 6			
1	21.60	23.0	1.3
2	22.36	23.0	1.2
3	23.13	23.0	1.2
4	25.17	24.9	2.4
5	28.70	24.9	4.9
Average peak width at half height = 23.8 s.			

Table 21: Peak width at half height and resolution calculations of chromatograms obtained using column 5 and 6 (Figure 53).

Relative standard deviation was also evaluated using column 6, four injections were made and no changes between the runs were made. The relative standard deviation of the retention time of the five peaks was found to be 1.66, 1.92, 1.32, 1.22 and 0.68 %. The relative standard deviation is less than 2 % in all cases, which is comparable to other published data. [285]

The column reproducibility was also checked. For this purpose, another column was prepared with BP initiator for 15 hours. The results showed that the columns are reproducible, as can be seen in Table 22. Although some variation in retention time and resolution was observed, this is partly related to the use of the flow splitter

and the difficulty in precisely maintaining the flow rates when switching from one column to another.

Retention time (minutes)		Resolution	
Column A	Column B	Column A	Column B
15.44	14.12	1.5	1.6
16.40	15.27	1.2	1.2
17.17	16.03	1.2	1.2
19.93	18.31	3.9	3.6
22.93	21.88	4.5	5.6

Table 22: Separation of tryptic digest of cytochrome C using two different monolithic ion exchange columns (both BP initiator for 15 hours). For other conditions, see experimental section.

The injection volume (2 μl) used here was relatively large compared to the dimension of the column used. A smaller injection loop was tested to see if this improves the column performance as it may improve the peak width. An injection loop of 265 nl was made from peak tubing and used for further studies. Smaller loop sizes were not possible to make due to the difficulty of fitting a shorter length of peek tubing to the injector. No significant improvement was observed in the peak widths when the smaller volume loop was used (Table 23 and Figure 54). This may be explained as follows: when the injection is made the peptides bind to sulphite groups present at the head of the column, while the diluent passes through the column. Therefore, when the concentration was kept constant the peak width was not affected even if a larger injection volume was used. However, the performance of the column was affected considerably by increasing the amount of the injected sample. A higher amount (270.0 pmol) overloads the column (Figure 55) and peptides with lower charge density showed excessive tailing. It is known that large injection volumes cause band broadening. However, the effect of large injection volume can be minimized if the initial mobile phase is a weak solvent as was the case here (100 % aqueous). The weak mobile phase causes sample accumulation at

the inlet of the column and leads to a similar effect to solid phase extraction. Using this technique it has been possible to inject 200 ml of sample in a column with 30 cm length and 0.4 cm in diameter. [92]

The type of solvent also has an important effect and if the diluent is a stronger solvent than the mobile phase, then this will cause band broadening. However, if the mobile phase is similar to the diluents then the effect of sample volume will be reduced. The diluent used here was very similar to the mobile phase used (both were dilute formic acid). [92]

Peak	Retention time (minutes)	Peak width at half height (s)	Resolution
Column 4, injection volume 2 µl			
1	17.21	25.0	0.3
2	17.44	23.7	0.3
3	18.44	32.5	1.3
4	20.47	27.9	2.5
5	24.01	27.9	4.6
Average peak width at half height = 27.4 s.			
Column 4, injection volume 265 nl			
1	19.05	24.8	0.3
2	19.30	24.8	0.3
3	20.54	27.3	1.5
4	22.48	29.0	2.2
5	26.54	29.0	4.8
Average peak width at half height = 27.0 s.			

Table 23: Peak width at half height and resolution calculations of chromatograms obtained using column 6 with different injection volume (2 µl and 265 nl).

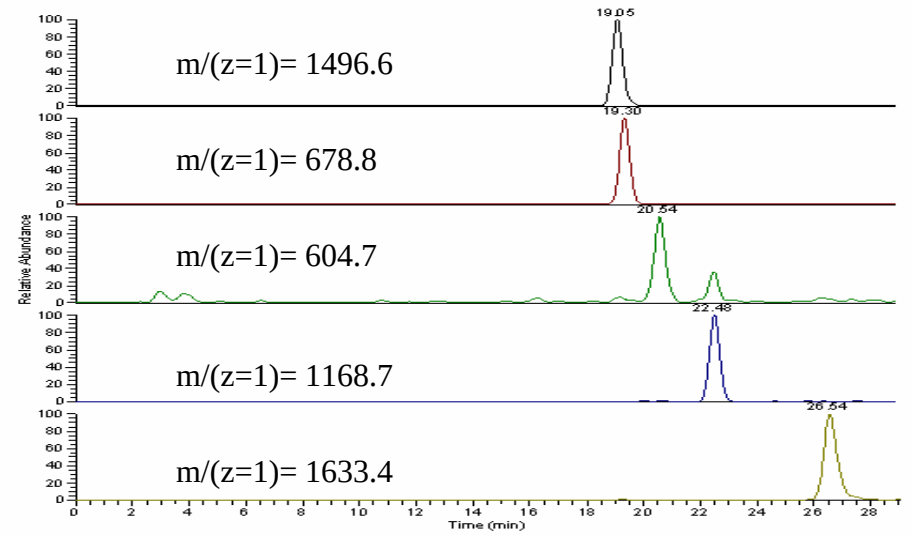


Figure 54: Separation of 27.0 pmol of tryptic digest of cytochrome C using 265 nl injection volume, gradient elution and sulphonated GMA-EDMA photoinitiated monolith (DAP initiator 7 minutes). For other conditions, see experimental section.

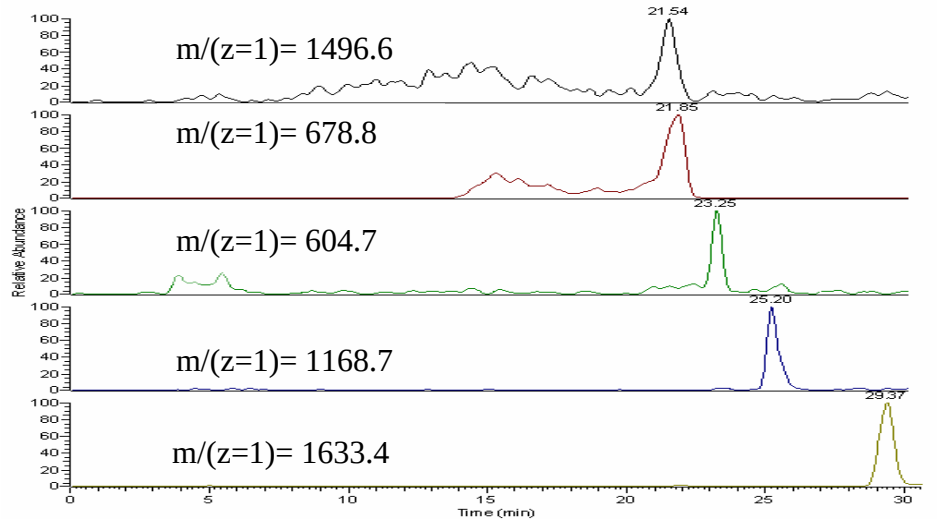


Figure 55: Separation of 270.0 pm of tryptic digest of cytochrome C using 265 nl injection volume, gradient elution and sulphonated GMA-EDMA photoinitiated monolith (DAP initiator 7 minute). For other conditions, see experimental section.

The back pressure of these columns was also measured. The results presented in Table 24 showed that the back pressure of the BP initiated column was slightly lower than the others. However, the back pressure of all photoinitiated columns was higher than that for the thermally initiated columns. This has been commonly observed and Geiser *et al.*[296] reported three-fold increase in the back pressure for the photoinitiated column compared to the same column thermally initiated. This is due to the faster initiation compared to the thermal initiation, which leads to an increase in the number of nucleation sites and hence higher back pressure. This also explains the higher back pressure generated from a faster photoinitiator (DAP) compared to a slower initiator (BP).

The column performance of the BP photoinitiated monolithic column was higher than the others. Additionally the back pressure was lower. Based on these experimental observations, it was decided to make this BP photoinitiated column on a microfluidic chip.

No.	Initiator	Initiation method	Duration	Back pressure (MPa, flow rate at 1.0 μl min^{-1})
1	BP	Thermal	24 hours	0.8
2	BP	Photo	15 hours	2.1
3	DAP	Photo	30 minutes	Very high, not washable
4	DAP	Photo	20 minutes	Very high, not washable
5	DAP	Photo	10 minutes	2.8
6	DAP	Photo	7 minutes	2.3
7	DAP	Photo	5 minutes	1.7
8	DAP	Photo	3 minutes	1.0

Table 24: Back pressure generated from each ion exchange monolitrhic column at flow rate of 1.0 μl min^{-1}.

4.3.3 Separation of tryptic digestion of proteins using on-chip photoinitiated monolithic ion exchange column.

Initially, the channel of the chip was silanized to ensure that the monolithic column when formed, would be anchored to the wall of the channel. Then the monolith solution was infused into the chip and a 5 cm length of the channel was exposed to photoinitiation for 15 hours. The formation of the monolith was confirmed using a light microscope (Figure 56) which showed also that the monolith was anchored to the inner wall of the channel. However, it was observed also that the monolith extended to 7 cm although the exposed area was only 5 cm. This may indicate that when the initiation process starts the polymerization process continues a certain distance into the masked region. This means that complete control of the dimensions of the column on-chip may be difficult and some expansion due to the polymerization process may occur. It was found after a few experiments, that proper masking of the chip by covering it completely with photo-mask and black tape and exposing only the specified area for preparation of on-chip columns was very important and helps in controlling the location of the monolith formation. It was also observed that neither black tape nor aluminum foil alone was sufficient to stop formation of the monolith throughout the channel.

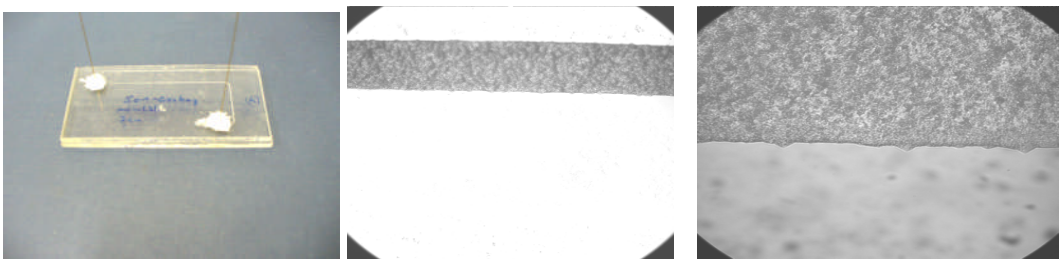


Figure 56: Left image for microfluidic chip with on-chip monolithic column. Center image is for a section of the on-chip monolithic column. Right image showing that the monolith is anchored to the internal wall of the channel and no significant void areas are observed.

The back pressure generated from the on-chip column (5.5 MPa at flow rate of 1.0 μl min^{-1}) was higher than the back pressure generated by the capillary column. The

increase in back pressure was not only due to the length of the column but also to the change in cross section which was approximately three times smaller compared to the cross section of the capillary column (Figure 57).

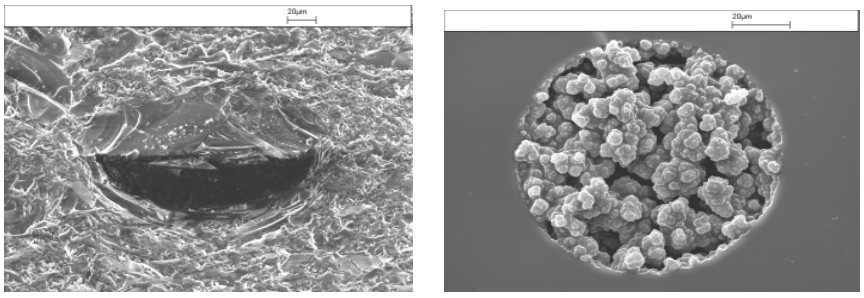


Figure 57: SEM images, left showing the cross section of the channel in the microfluidic and right showing cross section of a capillary 100 µm i.d.

The on-chip monolithic ion exchange column was compared with that formed in the capillary by monitoring the masses of the five peptides generated from tryptic digestion of cytochrome C (27.0 pmol) m/z 1496.6, 678.8, 604.7, 1168.7 and 817.5 in order of elution. It was observed that the total run time increased from less than 25 minutes for the capillary column, to more than 42 minutes for the on-chip column, which was due to column length and smaller cross section. However, the average peak width at half height also increased to 33.0 s (variation from 25.0 to 50.0 s). The temperature of the on-chip column was then increased to 40 ºC by placing the microfluidic chip in an oven. The average peak width at half height decreased to 32.0 s (variation from 22.9 to 40.6 s), and the run time reduced to about 38 minutes. Than the temperature was increased further to 65 ºC, the average peak width at half height decreased to 21.4 s (variation between 19.0 to 24.9 s) and the total run time to 25 minutes (Figure 58). In all cases resolution values above or close to one were obtained, which indicates that the overlap between the adjacent peaks was about 2 %. [298] Table 25 summarizes this data.

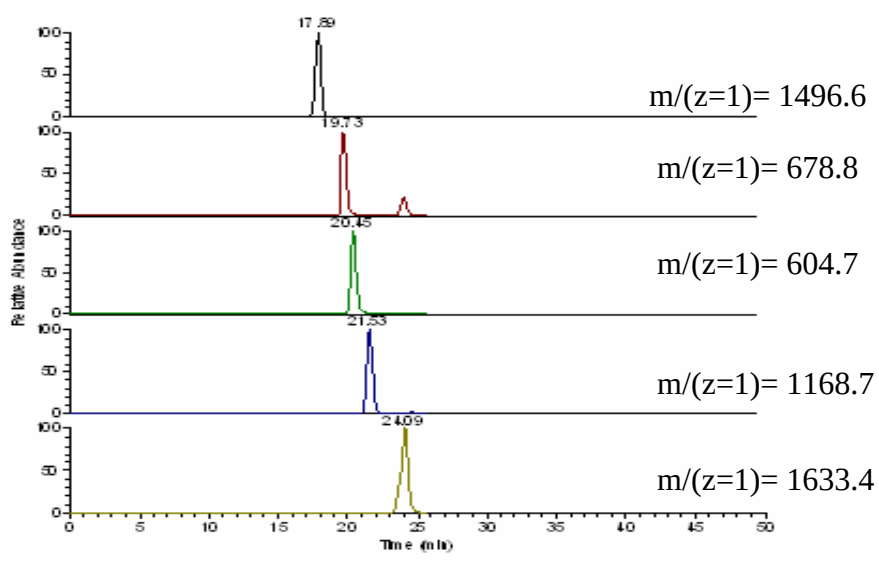


Figure 58: Separation of tryptic digest of cytochrome C using gradient elution and on-chip sulphonated GMA-EDMA photoinitiated monolith (BP initiator 15 hours) at 65 ºC. For other conditions, see experimental section.

Peak	25 ºC		40 ºC		65 ºC	
	Peak width at half height (s)	Resolution	Peak width at half height (s)	Resolution	Peak width at half height (s)	Resolution
1	33.3	2.5	26.5	2.7	19.0	2.5
2	33.3	2.0	22.9	1.3	19.0	0.9
3	25	2.8	35.3	1.3	22.0	0.9
4	25	2.8	35.3	1.6	22.0	1.3
5	50	4.0	40.56	2.9	24.9	3.4
	Average peak width at half height = 33		Average peak width at half height = 32		Average peak width at half height = 21.4	

Table 25: Effect of temperature on the peak width and resolution for separation of tryptic digest of cytochrome C using on-chip monolithic ion exchange column (BP initiator 15 hours). For other conditions, see experimental section.

In comparison with the separation in capillary, the dimensions of this column were considerably different and this was reflected in the separation performance. The smaller cross section and longer monolith formation in the on-chip column led to higher resolution in general. This was due to the reduction in the radial diffusion due to the smaller diameter. However, it also led to peak broadening due to the slow mass transfer as a result of strong interactions between the stationary phase and the sample. Therefore, to improve the peak width, separation was carried out at a higher temperature. This improved the mass transfer due to the higher diffusion rate of the sample in the stationary phase and as a result the peak width improved. Another source of dispersion in the on-chip column was the capillary connection between the chip and the electrospray ion source (50 μm i.d. and 8 cm length). If an on-chip ESI nozzle was used, this would also be expected to improve the peak widths.

To test the column further, a complex mixture was prepared containing peptides produced from tryptic digestion of melittin, cytochrome C, BSA and apo-transferrin. This mixture was then separated using this column. As previously discussed the pre digested protein mix produced the spectrum shown in Figure 37 (C) when infused directly with no prior separation. It was observed that the sequence coverage of cytochrome C dropped from 72 % when the protein was digested separately to 42 % in this mixture. This is due to the missing signals of the three peptides at m/z 1471.6, 1496.6 and 964.5. Additionally only four unique peptides from apo-transferrin were detectable (4.8 % sequence coverage). Although it was not expected to obtain a base line separation from such a complex mixture, the separation should improve the detection limits and hence the sequence coverage. Figure 59 shows the separation of this complex mixture using 10, 20 and 60 minute salt gradients. The separation improved when the gradient increased from 10 to 20 minutes. However, a considerable improvement in the separation was observed when the salt gradient was increased to 60 minutes.

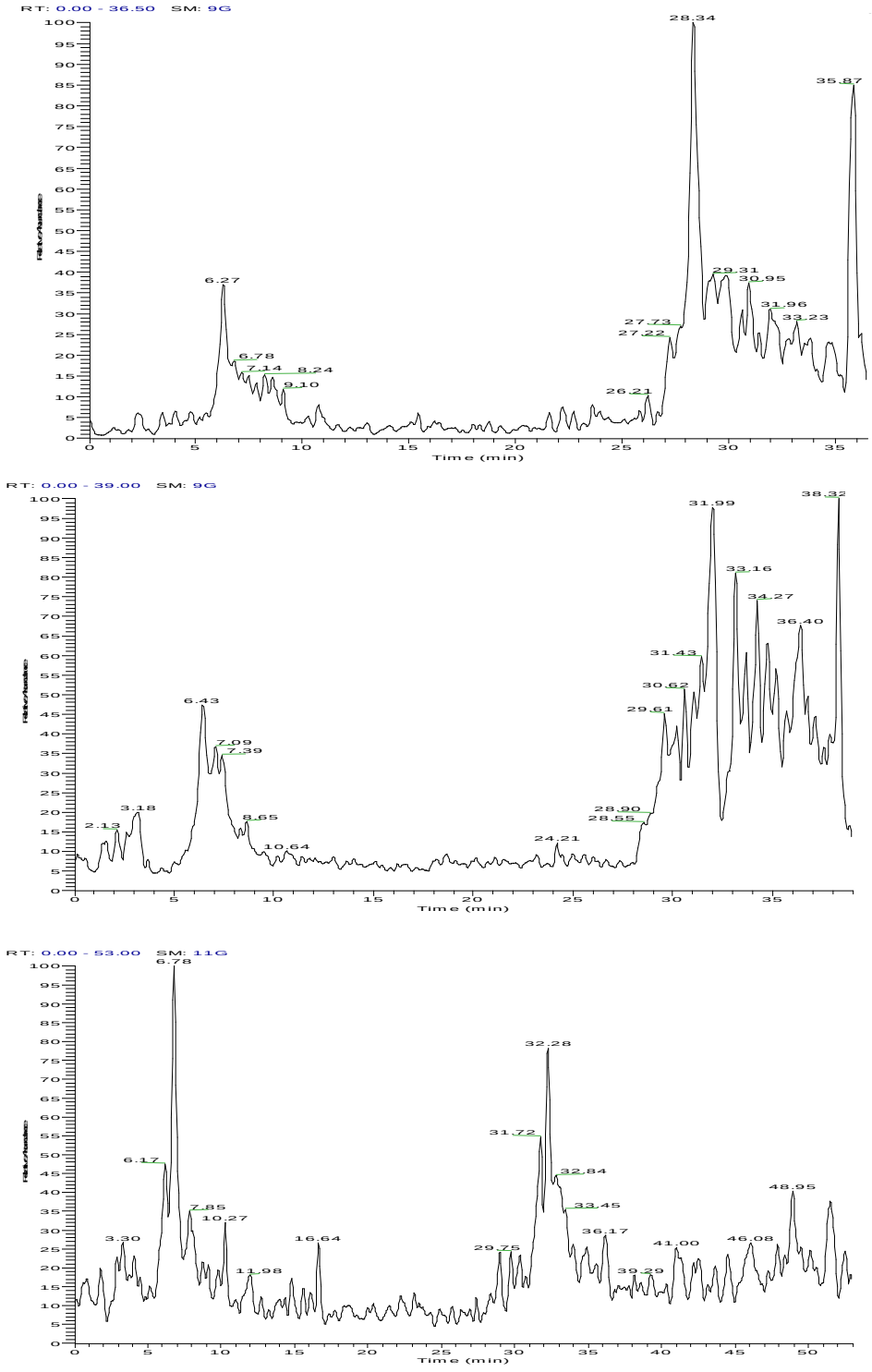


Figure 59: Total ion chromatogram traces for 10, 20 and 60 minute salt gradients to separate a tryptic digest of four protein mixture. For other conditions, see experimental section.

The injected amount of each protein was 1.4 nmol, 15.5 pmol, 17.1 pmol and 16.1 pmol for melittin, cytochrome C, BSA and apo-transferrin respectively. In each run the three missing peptides of cytochrome C were monitored. Since the three missing peptides possess low net charge they should be detected at the beginning of the run. Only m/z 1496.6 was detected in all three runs. However, two peaks were found in each run. The other tryptic peptide, corresponds in mass to a peptide also generated from tryptic digestion of BSA (m/z 1497.6); however, the net charge of this peptide is 2.11 and therefore, it eluted later on at t_R 27.42 minutes, while the one generated from tryptic digestion of cytochrome C possesses a net charge of 1.16 and eluted at t_R 8.19 minutes (Figure 60). The sequence coverage of cytochrome C improved to 59 %. Obviously, these peptides should be easily distinguishable using a higher resolution mass spectrometer as previously discussed. However, a mass difference of 1 amu is not always easily distinguishable in an instrument with low (unit mass) resolution.

Unique peptides generated from the tryptic digest of apo-transferrin within the mixture were also monitored. Figure 61 shows seven peptides generated from the tryptic digest of apo-transferrin and separated using a 60 minute salt gradient. These peaks were also detected in the 10 and 20 minute salt gradient experiment. This confirms that these signals are real signals as their m/z values and elution order is identical. The first two peaks appeared as split peaks. This is due to these two components having a weak interaction with the monolithic ion exchange column and eluting near the solvent front. The sequence coverage based on these seven unique peptides improved to 12 %. The same mixture was then run on a commercial reverse phase column, which is specifically designed for peptide separation (Figure 62). This was carried out to see if any of the peptides produced from tryptic digest of apo-transferrin was not detected due to signal suppression or poor separation in the on-chip monolithic ion exchange column. The results indicated no such problems occur. The signal intensity of some of the peaks (1 and 4) in the ion exchange column was higher than the signal intensity in the commercial column. This may be due to the enhanced sensitivity of the ion exchange column due to smaller i.d. of the column compared to the commercial reverse column. Reduction in column diameter enhances the sensitivity if the mass

of the injected sample remains the same. This is due to the reduction in the radial diffusion of the sample solution when injected in a smaller i.d. column. Additionally, a smaller diameter speeds up the diffusion process of the solute molecules into the stationary phase, which speeds up the mass transfer process and hence improves the column efficiency. [169]

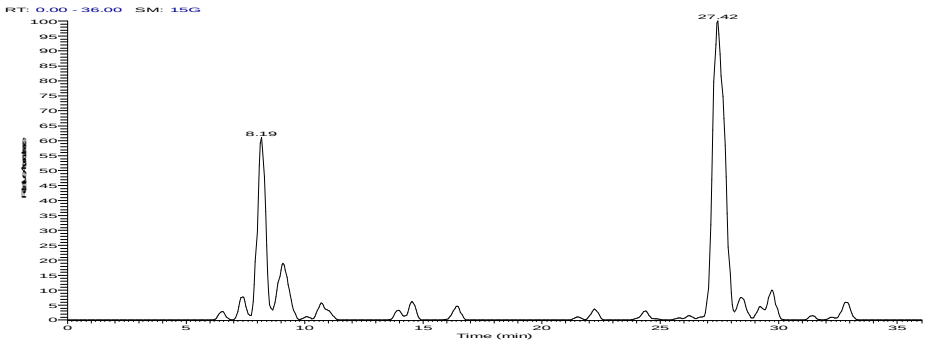


Figure 60: Two peptides with very similar masses (m/z = 1496.6 and 1497.6) are resolved when a mixture of trytptic digest of four proteins were injected. Separation was carried out using gradient elution and an on-chip sulphonated GMA-EDMA photoinitiated monolith (BP initiator 15 hours) at 65 ºC. For other conditions, see experimental section.

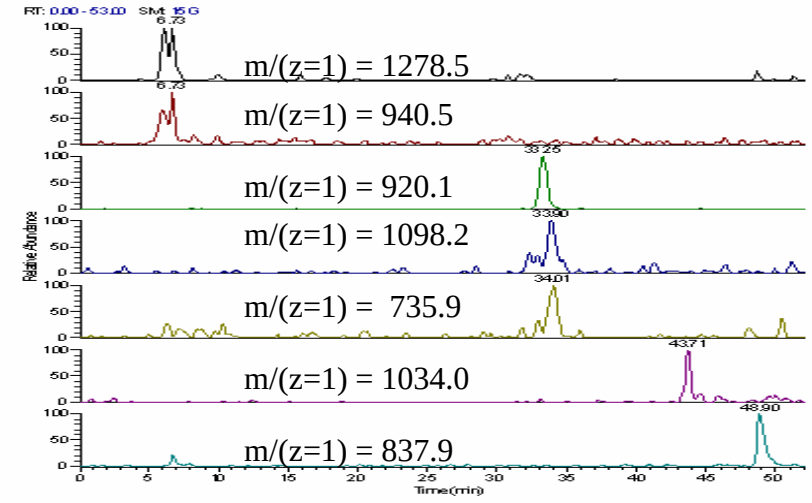


Figure 61: Separation of seven peptides generated from tryptic digest of apo-transferrin in a mixture of tryptic digest of melittin, cytochrome C and BSA using a 60 minutes salt gradient. For other conditions, see experimental section.

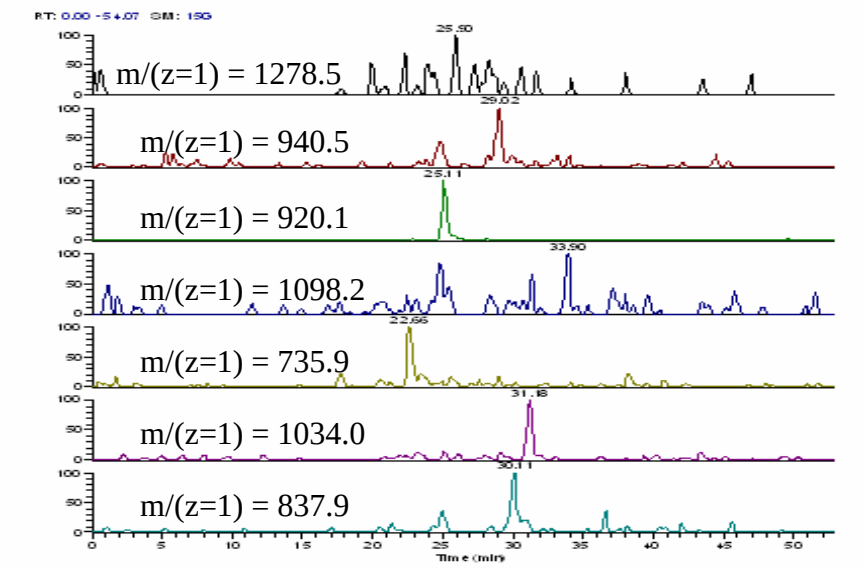


Figure 62: Separation of seven peptides generated from tryptic digest of apo-transferrin in a mixture of tryptic digest of melittin, cytochrome C and BSA, using commercial column and a 60 minutes solvent gradient. For other conditions, see experimental section.

The chip used for fabrication of the monolithic ion exchange column was exposed to a higher pressure (9.0 MPa) for 8 hours continuously by increasing the solvent flow rates. The results were very promising and the chip did not show any damage or leaks at this higher pressure. The pressure was then increased to 10.3 MPa for about two hours and again no leak was observed. This indicates that no problems would be anticipated in integrating the on-chip column with other analytical steps with regard to the back pressure.

A thermal decomposition protocol for removal of the polymer material from the channels of the microfluidic chip was tested. This is an important aspect for on-chip analysis, because in some cases the microfluidic chip may be blocked due to particulate impurities. Additionally, this is useful for proteomic analysis as it will eliminate problems with sample carryover. The protocol developed by Throckmorton *et al.* [181] was used here. However, the decomposition temperature was reduced to 520 ˚C instead of 550 ˚C as previously reported. The method worked well and did not generally affect the strength of the chip bonding, although in a few isolated cases the bonding was affected and the chip was damaged.

4.4 Conclusions.

Photoinitiation seems to have a positive affect on the performance of the monolithic ion exchange column due to the formation of the monolith at room temperature, which changes the morphology of the monolith. Additionally, the photoinitiation method described here reduces the column preparation time to 18 hours, compared to 42 hours which is required in the case of thermally prepared monolith, while maintaining the column efficiency. This can be carried out using DAP as initiator, which reduces the initiation time to 10 minutes. However, the optimum column for on-chip separation was found to be 15 hours photoinitiated using BP as initiator, which may be due to the slow initiation rate compared to the monolith initiated using DAP. The slow initiation rate reduces the nucleation rate and hence reduces the back pressure.

The monolithic column was then fabricated on-chip and used for separation of tryptic digests of proteins. The results showed that it is possible to make an on-chip

column comparable in chromatographic terms to fused silica capillary columns. However, due to the smaller cross section of the on-chip column a length less than 5 cm may be adequate.

An on-chip monolithic column possesses several advantages; for example, the column can be coupled to the on-chip digestion or coupled to on-chip reverse phase columns to perform the desired on-chip two dimensional chromatographic separation. Another important advantage is the possibility of reusability of the microfluidic chip. This is because the monolith is based on organic materials, which can be decomposed easily at higher temperature without affecting the microfluidic chip bonding. Additionally, the monolithic column is very cost effective and costs less than a pound per column. [286]

5.0 Development of a photoinitiated monolithic reverse phase column for separation of tryptic digests of proteins

5.1 Introduction.

Two types of reverse phase monolithic columns have been commonly used to separate tryptic digests of proteins. The first is based on polystyrene (PS) while the second is based on methacrylate. Moor *et al.* [264] used a PS-divinyl benzene (DVB) monolith prepared using toluene and dodecanol as porogenic solvents to separate a tryptic digest of cytochrome C. Premstaller *et al.* [299] used PS-DVB for separation of peptides generated from a tryptic digest of human transferrin using tetrahydrofuran and decanol as porogens. Tetrahydrofuran is a poorer solvent than toluene for PS; as a result the monolith formed has fewer micropores and more macropores which resulted in a faster mass transfer and hence higher column efficiency. [299] However, in both of these reports the monolith was polymerized using thermal initiation. To perform on-chip peptide separation using these monoliths, photoinitiation should be used instead of thermal initiation so that the monolith forms within a specified space in the channel of the microfluidic chip.

Several methacrylate based monolithic columns have been used for separation of tryptic digests of proteins. Ro *et al.* [100] used photo polymerized octylstyrene (OS) and divinylbenzene (DVB) and compared their chromatographic performance with lauryl methacrylate (LMA) and ethylene dimethacrylate (EDMA). The objective of the study was to increase the hydrophobicity of the monolithic column by using long chain hydrocarbons. Both columns showed good separation (tryptic digest of BSA was used as a model). The peak widths at half height varied between 27.0 s and 89.0 s while the resolution varied between 0.44 and 10.85 for the POS-DVB column. The peak widths at half height for the other column (LMA-EDMA) were between 29.0 and 91.0 s, while the resolution was between 0.20 and 8.20. This data indicates that the performance of the POS-DVB column is better than that of the LMA-EDMA column. However, no data on the back pressure generated was provided.

A few attempts have been made with varying degrees of success, to fabricate on-chip monolithic columns for peptide separations. Yu *et al.* [227] used a light transparent capillary as a model for an on-chip column and prepared a monolith consisting of a mixture of ethylene dimethacrylate and 2-acrylamide-2-methyl-1-propanesulfonic acid *via* photoinitiation. A standard mixture of four peptides was used to evaluate the column. However, the peptides could not be eluted from the column due to the strong coulombic interactions between the amino group of the peptides and the sulphonic functionalities of the monolith and good separation was obtained only after the addition of sodium octanesulphonic acid to the mobile phase as an ion pairing agent. On-chip polymerization was not carried out to evaluate on-chip peptide separation using this monolith.

Le Gac *et al.* [99] used a lauryl methacrylate (LMA) photoinitiated monolithic column and a tryptic digest of cytochrome C to evaluate the column performance. Good separation was obtained for the monolithic column. However, the separation was not successful when the monolith was polymerized on-chip, due to the length of the on-chip monolith (2.2 cm) compared to the length of the monolith in the capillary (15 cm) and due to the changes in the solvent composition which were made to increase the pore size and reduce the back pressure.

Recently, Liu *et al.* [236] reported separation of tryptic digests of proteins using ethylhexyl methacrylate (C_8) as a reverse phase monolithic column which was fabricated in a cycloolefin copolymer (COC). The peak widths at half height ranged from 26.4 to 135.0 s. Although the efficiency of this monolithic column was less than the efficiency of the on-chip particle based columns reported by Fortier *et al.*[233] the back pressure generated from this column was less than 6.9 MPa in comparison to 10 MPa generated from particle based columns with both at flow rates of 300 nl min^{-1}.

In this experiment the chemical composition and the experimental conditions for photoinitiated PS-DVB and LMA-EDMA monoliths were investigated and the monoliths produced were used for the separation of tryptic digests of cytochrome C as a model for separation of peptides generated from protein digestion. This was

carried out to find the suitability of the two columns for on-chip column formation in terms of chromatographic separation and the back pressure generated. The back pressure generated should not exceed 5.9 MPa at a flow rate of 1.0 µl min^{-1}. This is to ensure that the column can be easily fabricated on the microfluidic channel. The chromatographic performance of these two types of column was also compared with a commercial packed capillary column designed specifically for separation of tryptic digests of proteins (Jupiter 4 µm Proteo 90A, 150 mm x 0.33 mm, Phenomenex).

5.2 Experimental.

5.2.1 Chemicals.

- Acetonitrile, HPLC grade (Fisher scientific equipments, Loughborough - UK).
- Formic acid (Fisher scientific equipments, Loughborough - UK).
- Dithiothreitol (DTT), Sigma Chemical Co (St. Louis, MO - USA).
- 3-(Trimethoxysilyl)propylmethacrylate (TSP), Sigma Chemical Co (St. Louis, MO - USA).
- Benzoylperoxide (BP), Sigma Chemical Co (St. Louis, MO - USA).
- 2,2-Dimethoxy-2-phenylacetophenone (DAP), Sigma Chemical Co (St. Louis, MO - USA).
- Polystyrene (PS), Sigma Chemical Co (St. Louis, MO - USA).
- Divinylbenzene (DVB), Sigma Chemical Co (St. Louis, MO - USA).
- 1-Dodecanol, Sigma Chemical Co (St. Louis, MO - USA).
- Toluene ,Sigma Chemical Co (St. Louis, MO - USA).
- Butane-1.4-diol, Sigma Chemical Co (St. Louis, MO - USA).
- Propanol, Sigma Chemical Co (St. Louis, MO - USA).
- Trypsin Gold, mass spectrometry grade, Promega (Madison, WI - USA).
- Lauryl methacrylate (LMA), Sigma Chemical Co (St. Louis, MO-USA).
- Ethylene dimethacrylate (EDMA), Sigma Chemical Co (St. Louis, MO - USA).

- Proteins used described in section 2.2.1.

5.2.2 Instrumentation.

See section 4.2.2**.**

5.2.3 Protein digestion.

See section 4.2.3.

5.2.4 Preparation of monolithic column in fused silica capillary.

Silanization was carried out as described in section 4.2.4.

PS-DVB monolith mixture (PS monomer, DVB crosslinker, dodecanol macroporogens, toluene microporoge and BP initiator) was stirred for about 5 min, purged with nitrogen for 5 min and sonicated for 10 min. [264] A silica capillary 100 μm i.d. /360 μm o.d. was filled to 15 cm by syringe with the polymerization mixture. The capillary was placed in a column block heater (Jones Chromatography Ltd. - UK) at 65 ^{o}C for 36 hours or exposed to the UV light source for 24 hours. For the thermally initiated column, the capillary was cut to 5 cm whereas for photoinitiation only 5 cm of the capillary was exposed to the light source with the rest covered with thick black tape. The capillary was then flushed with acetonitrile to remove any remaining starting materials. The chemical composition of the monolith, method of polymerization and the duration of polymerization are listed in Table 26.

Column	Initiator	Monomer	Crosslinker	Macroporogens solvent	Microporogens solvent	Method of polymerization and duration (hours)
1	4.0 mg	750 mg	1.25 g	1.950 g (65%)	1.050 g (35%)	Thermal at 65 ^{o}C (36)
2	4.0 mg	750 mg	1.25 g	1.65 g (55%)	1.350 g (45%)	Photoinitiated (24)
3	4.0 mg	750 mg	1.25 g	1.50 g (50%)	1.50 g (50%)	Photoinitiated (24)
4	4.0 mg	750 mg	1.25 g	1.410 g (47%)	1.59 g (53%)	Photoinitiated (24)

Table 26: Chemical composition and experimental condition of the monolith prepared in 100 µm i.d. capillary.

LMA-EDMA monolithic column was prepared as follows: propanol (212 mg) and 1,4-butane-diol (53 mg) were mixed. To this mixture LMA(180 mg) and EDMA (120 mg) were added. Finally 3.0 mg of DAP was added to the solution and mixed well. A silica capillary 100 μm i.d. /360 μm o.d. was filled to 15 cm with the polymerization mixture by syringe. The capillary was then exposed to UV light for 20 minutes, and finally the capillary was flushed with acetonitrile to remove any remaining starting materials.

For PS-DVB and LMA-EDMA monolithic columns the following gradient elution was used A: 0.4 % formic acid (aqueous) B: 0.4 % formic acid in acetonitrile. A 100 % for 3 mins, A 70% for 30 minutes, A 70 % for 10 minutes and back to 100 % A over 2 minutes. Flow rate was set between 1.4 and 1.8 μL min^{-1}. Injection volume of 265 nl was used.

The commercial column used was a ((Jupiter 4 μm Proteo 90A, 150 mm x 0.33 mm, Phenomenex). The mobile phase composition in this case was A, formic acid 0.1 % in water and B, formic acid 0.1 % in acetonitrile. The gradient started with A 100 % for 3 minutes, then decreased to 50 % A for 40 minutes. Flow rate was set to 12.0 μl min^{-1}. Injection volume of 2 μl was used.

5.3 Results and discussion.

5.3.1 Photoinitiated polystyrene-divinylbenzene monolithic column in capillary

Two types of monolith were tested, PS-DVB and LMA-EDMA. The performance of the two columns was compared with a commercial column in term of peak width at half height and resolution.

The commercial column was first used to separate the tryptic digest of cytochrome C (27.0 pmol) (Figure 63). Eight peaks were selected, their width at half hight and the resolution calculations are listed in table 27. The average peak width at half height was found to be 38.2 s (variation from 32.1 to 42.9 s). The resolution calculation shows that only two peaks were completely resolved. Partial separation

occurs in most cases. However, no separation was found between peaks four and five.

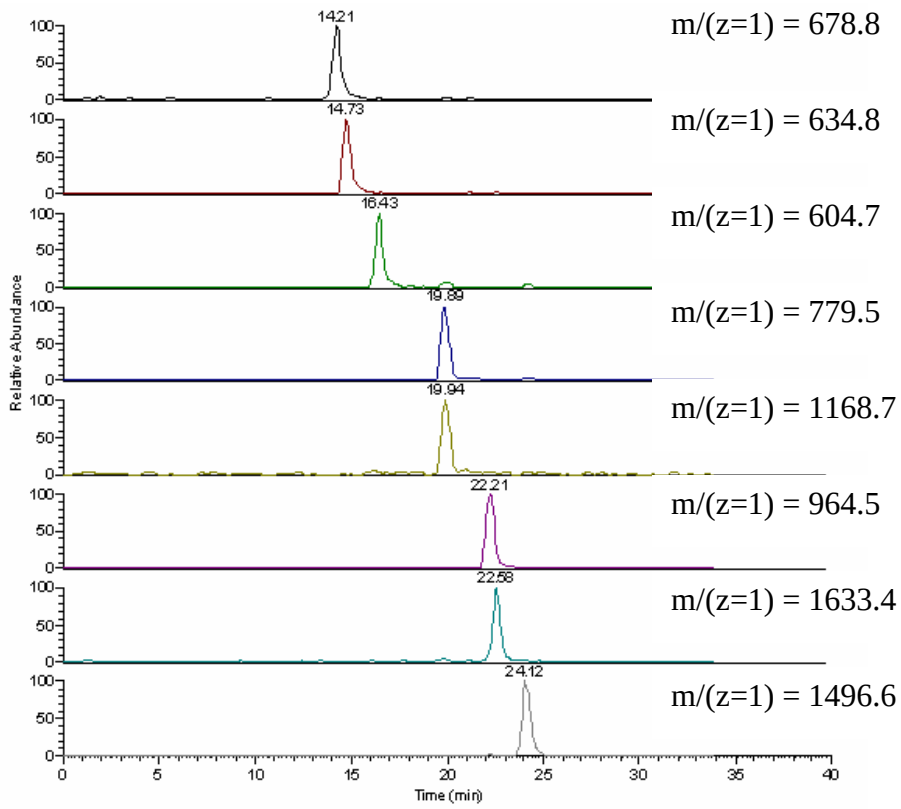


Figure 63: Separation of tryptic digest of cytochrome C using Jupiter 4 µm Proteo 90A, 150 mm x 0.33 mm, Phenomenex column. For other conditions, see experimental section.

No.	Retention time (minutes)	Peak width at half height (s)	Resolution
1	14.21	42.9	0.4
2	14.73	42.9	0.4
3	16.43	32.1	1.6
4	19.89	42.9	0.0
5	19.94	42.9	0.0
6	22.21	37.5	0.4
7	22.59	32.1	0.4
8	24.12	42.9	1.6
Average peak width at half height = 38.2 s.			

Table 27: Peak width at half width and resolution calculations of the chromatogram presented at Figure 63.

The performance of the PS-DVB monolithic column used by Moor *et al.* [264] and Premstaller *et al.* [299] was found to be higher than the performance of the columns prepared by Ro *et al*. [100] Therefore, it was decided to make a PS-DVB monolith first. However, the initiator AIBN was replaced by BP.

Initially, PS-DVB monolith was thermally polymerized using the method described by Moor *et al.* [264] However, the initiation time was extended to 36 hours, because BP is a slower initiator than AIBN and therefore, a longer initiation time is required to obtain a monolith with similar properties. [187]

Figure 64 shows the separation of the tryptic digest of cytochrome C (27.0 pmol) using this column. Average peak width of 39.2 s at half height was obtained (variation from 34.6 to 46.2 s). However, small peptides were not retained; this is due to the hydrophobicity of PS-DVB being insufficient to retain these peptides. To retain these small peptides, usually a modification of the PS-DVB monolith by surface alkylation is carried out or octylstyrene is used instead of styrene. [100] However, increasing the hydrophobicity of the column may have an adverse effect on the column efficiency and peaks corresponding to highly hydrophobic peptides may broaden. Additionally, small peptides are less important than larger ones for protein identification. This is because finding unique small peptides for identifying a protein is more difficult. Moreover, small peptides contribute less to total protein sequence coverage. Table 28 lists the peak width measurements at half height and resolution calculation. The resolution calculation shows that partial separation has been achieved between the rest of the peaks. The separation may improve further if the experimental conditions were optimized further.

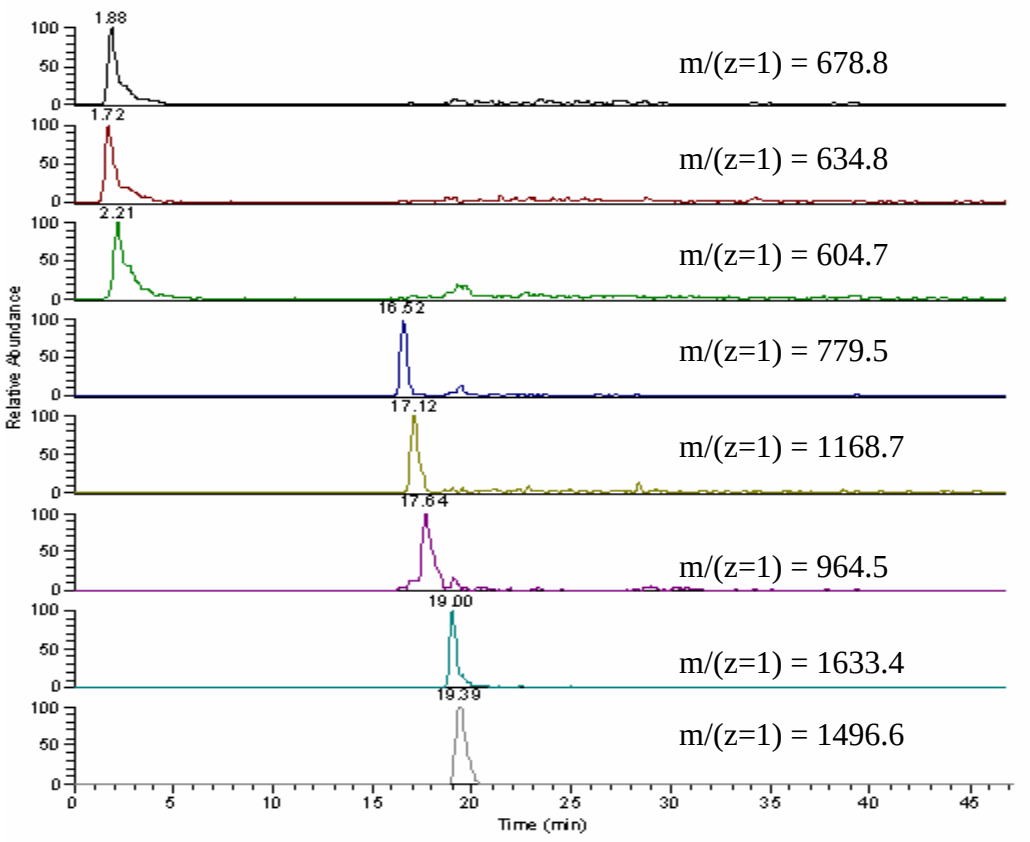


Figure 64: Separation of tryptic digest of cytochrome C using PS-DVB thermally initiated column. For other conditions, see experimental section.

No.	Retention time (minutes)	Peak width at half height (s)	Resolution
1	1.73	Unretained peaks	
2	1.88		
3	2.21		
4	16.52	34.6	0.6
5	17.12	34.6	0.6
6	17.64	46.2	0.5
7	19.00	34.6	0.3
8	19.39	46.2	0.3
Average peak width at half height = 39.2 s.			

Table 28: Peak width at half height and resolution calculations of the chromatogram presented at Figure 64.

Using the same chemical composition, the polymeric mixture was photoinitiated. It was observed that the back pressure was very low, indicating that the monolith may not be formed well. Moor *et al.* [264] reported that the porosity of the monolithic column was affected by the change in the percentage of toluene. The percentage of toluene was increased to 50 %. SEM was obtained for the thermally initiated and the photoinitiated (50 %) columns. SEM shows that the monolith was well formed and anchored to the inner wall of the capillary (Figure 65). However, when the photoinitiated monolithic column was used for separation of a tryptic digest of cytochrome C (27.0 pmol), it was found that the small peptides did not retain in the column while other peptides were only partially separated. However, the peak shape was not good and noticeable peak broadening was observed (Figure 66). The peak broadening observed here was due to the small micropores formed as a result of the high percentage of toluene (50%). This slows down the mass transfer process and hence causes band broadening. Therefore, it was decided to reduce the percentage of toluene to 45 %. Some difficulties in forming the monolith were observed. In some cases the monolith was leaking out from the capillary. This may be due to the large pores and low crosslinking, which resulted in weak monolith formation.

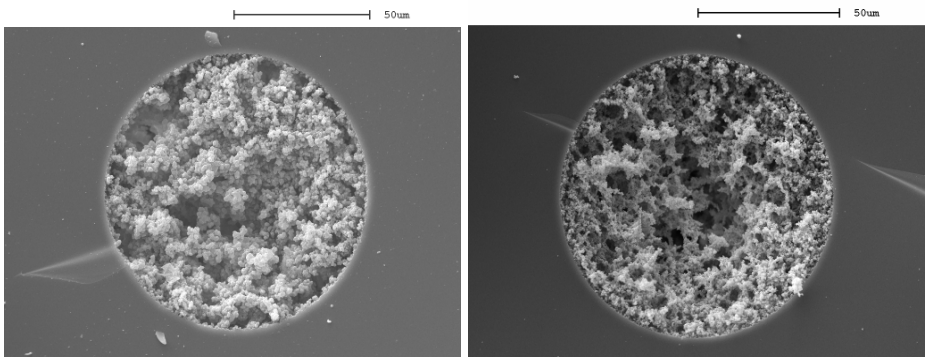


Figure 65: SEM images of thermally initiated PS-DVB monolith (35 % toluene) and photoinitiated PS-DVB monolith (50 % toluene).

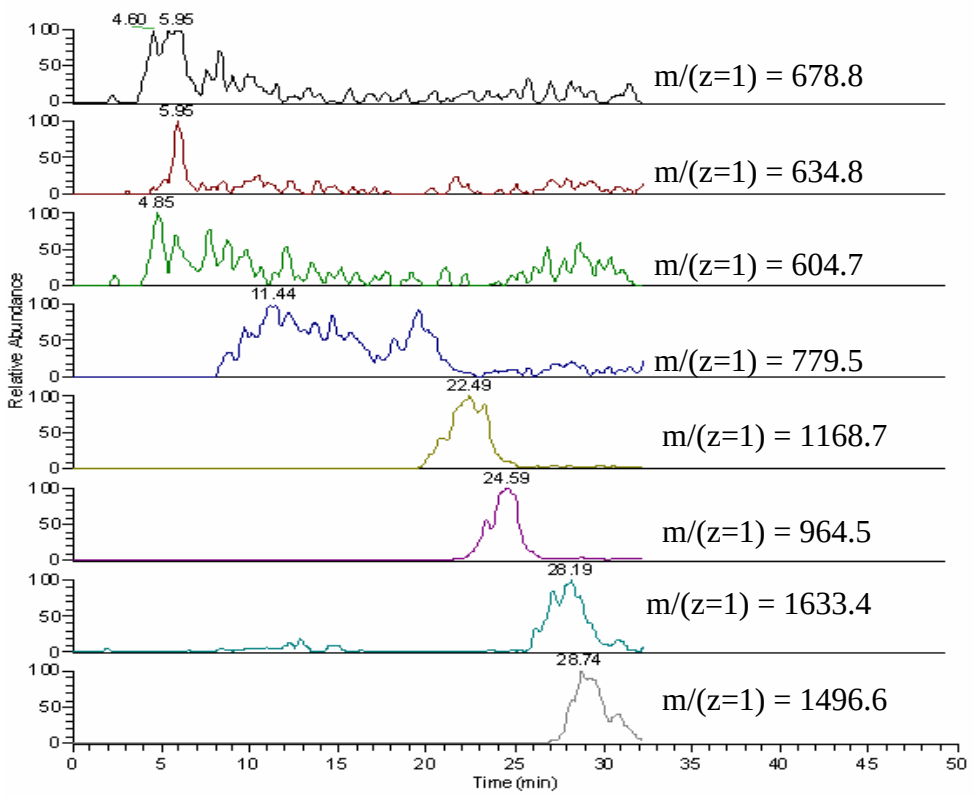


Figure 66: Separation of tryptic digest of cytochrome C using photoinitiated PS-DVB monolith (50 % toluene) column. For other conditions, see experimental section.

Another PS-DVB monolith was prepared using the method described by Premstaller *et al.* [299] The thermally polymerized column was prepared and tested using the same mixture. Average peak width at half height was found to be 55.3 s (variation between 50.0 to 75.0 s). The column performance may improve if further optimization is carried out on the monolith preparation method. However, this was not carried out and instead a photoinitiated monolithic column was prepared using the same method. The monolith was not properly formed and was not bonded to the inner surface of the capillary, causing it to leak out. The initiator was replaced by AIBN. This time the monolith formed well but similar observations were made and clear voids were apparent.

In summary, thermally polymerized PS-DVB monoliths have been used as chromatographic media and were prepared here also. However, the performance of

the column drops significantly when it is photoinitiated. This may be due to the physical changes in the monolith morphology on photoinitiation, as the initiation is carried out at room temperature. This changes the solvating power of the system and as a result the onset and rate of phase separation also change which leads to modifications of the separation properties. Additionally, it was observed that the monolith is very sensitive to small changes in the chemical composition, which is in agreement with literature reports, and there were difficulties in regenerating the monolith. [264] Despite these difficulties, it may be possible to generate photoinitiated PS-DVB monoliths comparable in chromatographic terms to the thermally initiated PS-DVB. This can be carried out by careful changes in the chemical composition of the monolith such as changing the monomer to crosslinker ratio to strengthen the monolith. However, this process would be very time consuming. Therefore, it was decided to concentrate on testing the other monolith.

5.3.2 Photoinitiated lauryl methacrylate-ethylene dimethacrylate monolith in capillary.

LMA-EDMA monolith was prepared by the method of Ro *et al.* [100] using photoinitiation. The column has been characterized and the loading capacity was found to be 11.3 μg when BSA was used as a sample while a detection limit of 1 fmol μl^{-1} was achieved.

The initiation time was found to be a useful factor for controlling the back pressure generated from the monolith and 20 minutes initiation time was found to be the optimum in terms of the back pressure, which was found to be 5.2 MPa at 1.0 μl min^{-1}. Figure 67 shows the separation of a tryptic digest of cytochrome C (27.0 pmol) using this 20 minutes photoinitiated monolithic column and Table 29 lists the peak width at half height and resolution calculations. The average peak width was found to be 43.5 s (variations from 20.6 to 75.5 s). The column performance was lower than that of the commercial reverse phase C18 particle based column. However the performance of this column was similar to the thermally initiated PS-DVB as the peak width and resolution measurement shows. The average peak width was larger than the peak width at half height of the thermally initiated PS-DVB, however, the resolution calculations shows that the performance of this column was

better as peak 6 was resolved here. The column may be improved further if longer initiation times were used; however, this would generate columns with higher back pressures.

The first three peptides which were not retained in the PS-DVB column were also not retained here. The three other peptides (m/z 779.5, 1168.5 and 964.5) eluted quickly compared to their elution time in the PS-DVB monolith, although the hydrophobicity of LMA is higher than that of PS. This is possibly due to the size of the micropores formed in this column compared to the micropore size formed in the thermally initiated PS-DVB monolith. However, this did not affect the resolution between these peaks. It was also observed that the selectivity of the columns varied slightly with respect to the elution times of the final two peaks, with that for the peptides of m/z 1496.6 eluting earlier than the other peak corresponding to m/z 1633.4. This was not the case for either the PS-DVB monolithic column or the C18 commercial column, where the peptide with m/z 1496.6 eluted later than m/z 1634. This was not expected as LMA-EDMA is closer in chemical structure to a C18 particle based column. A detection limit of 3.2 pmol of tryptic digest of cytochrome C was achieved. Lower detection limits have been reported by Ro *et al.* [100] However, the higher detection limits obtained here may be due to the use of a standard electrospray source whereas a nano-electrospray source was used by the Ro *et al*. [100] Relative standard deviation of retention time varies between 0.1 and 1.9 % (based on two successive runs) which indicates a good reproducibility. Similar relative standard deviation was obtained when a tryptic digest of BSA (72.2 pmol) was injected (Figure 68). However, a number of peptides did not interact strongly enough with the column and eluted quickly (within 10 minutes). This led to a limited resolution between these peptides. In comparison to the EDMA-LMA column reported by Ro *et al.* [100] where most of the peptides were retained on the column, the time of initiation was the same in both cases. However, Ro *et al.* [100] used five lamps in the initiation process whereas only a single lamp was used here; hence, the morphology and the pore size of the two monoliths would be expected to be different.

The chromatogram shows the peptide peaks that were earlier shown separated using the monolithic ion exchange column (Figure 46). Since the separation in reverse phase chromatography is based on the hydrophobic interaction, the last eluted peptide (m/z 1218.4) possesses the highest hydrophobicity. This peptide was also eluted from the ion exchange column, indicating that the hydrophobicity of the monolithic ion exchange column is minimal and that all peptides generated from a tryptic digest of BSA were eluted from the monolithic ion exchange column.

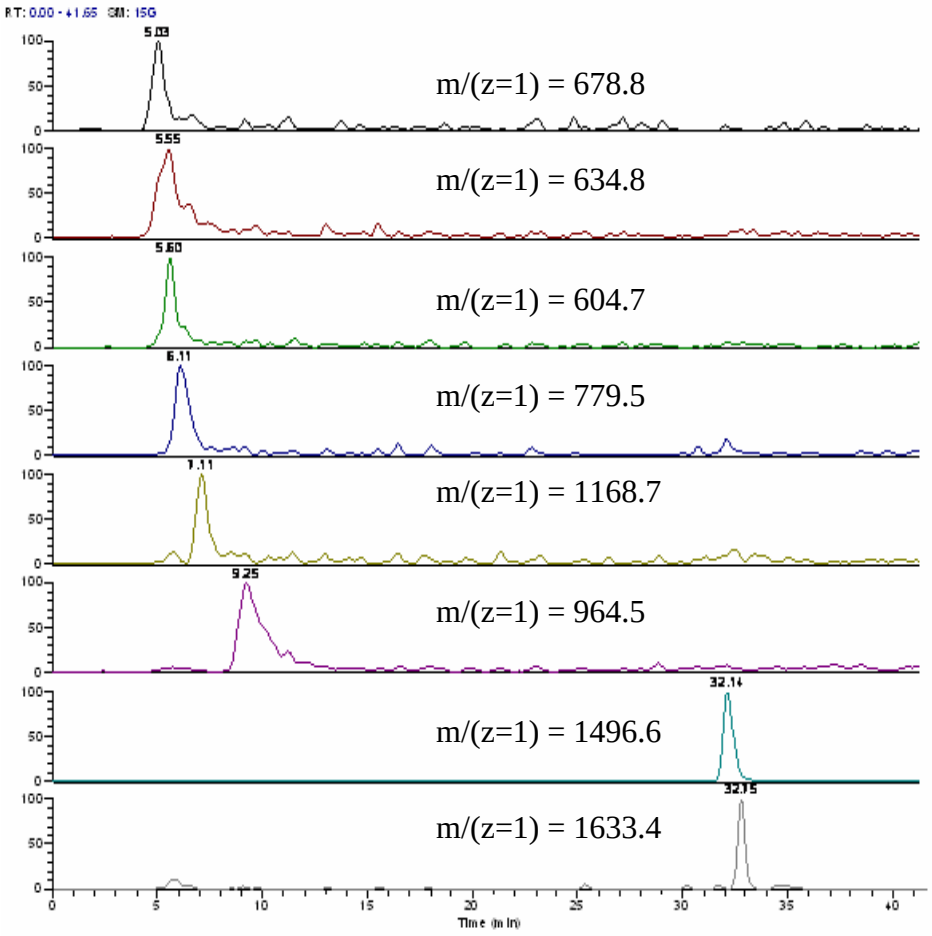


Figure 67: Separation of tryptic digest of cytochrome C using LMA-EDMA monolithic column. For other conditions, see experimental section.

No.	Retention time (minutes)	Peak width at half height (s)	Resolution
1	5.03	Unretained peaks	
2	5.55		
3	5.60		
4	6.11	46.9	0.6
5	7.11	37.5	0.6
6	9.25	75.5	1.1
7	32.14	37.5	0.6
8	32.75	20.6	0.6
Average peak width at half height = 43.5			

Table 29: Peak width at half width and resolution calculations of the chromatogram presented at Figure 67.

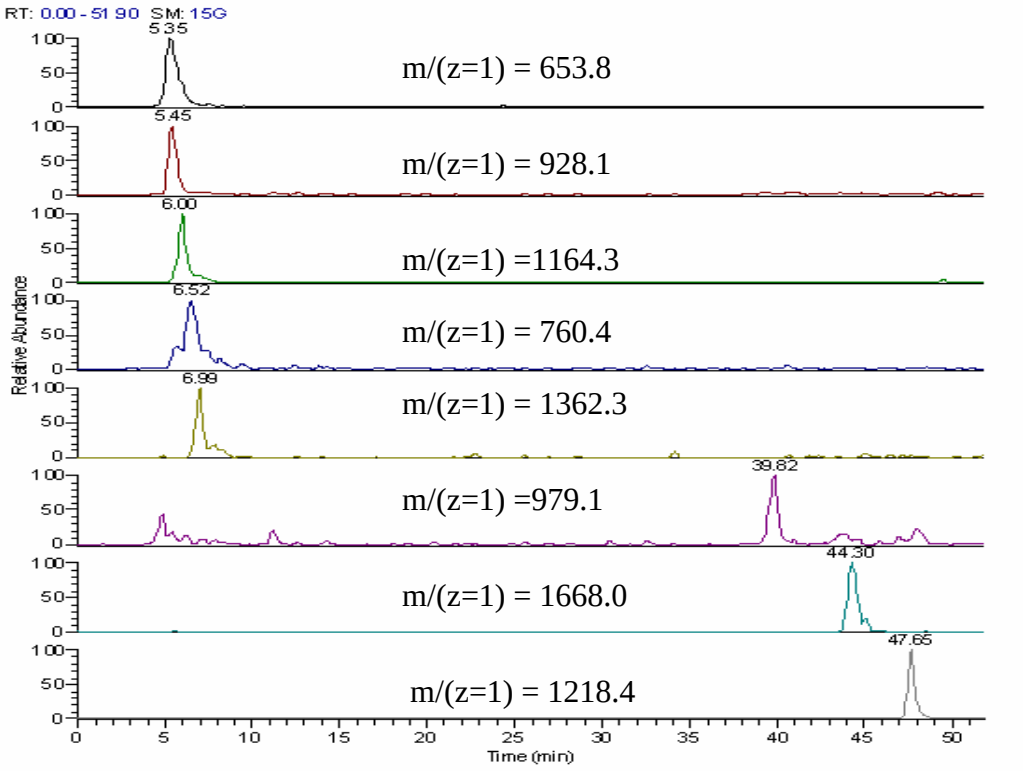


Figure 68: Part of the chromatogram showing separation of a tryptic digest of BSA using LMA-EDMA monolithic column. For other conditions, see experimental section.

5.4 Conclusions.

Thermally initiated monolithic reverse phase columns were easier to make and their performance was usually better than comparable photoinitiated reverse phase columns, however it was not possible to control the location of the column in a microfluidic chip if the columns are thermally initiated. Photoinitiated monolithic reverse phase columns were more difficult to make due to the initiation being carried out at room temperature and due to the fast initiation which increases the nucleation rate and hence increases the back pressure. Despite these difficulties it was possible to make a monolithic reverse phase column using LMA-EDMA with relatively low back pressure (5.2 MPa at flow rate of 1 µl min^{-1}), which could be handled by a microfluidic chip. However, due to this, some compromise on the performance of the column has to be made.

It is expected that the pressure generated from the monolithic column will increase further when fabricated in the channel of a microfluidic chip. This is due to the smaller dimensions of the channel on the microfluidic chip compared to the dimension of the silica capillary used here. However, the reduction in the cross section of the column may improve the column performance.

The back pressure generated from the ion exchange column is much lower and theoretically implementing both columns in a single channel of a microfluidic chip should be possible.

6.0 Integrated microfluidic systems for proteomics

6.1 Introduction.

In chapter two a microdevice was developed in which protein digestion with peptide separation was carried out. Results were very promising and a considerable improvement in the detection limits was observed, in addition to the speed at which the digestion was carried out. In this chapter the objective was to transfer the model to a microfluidic system. This could be carried out by integrating the efficient on-chip digestion method developed in chapter three with the on-chip cation exchange column developed in chapter four. A second design would consist of the reverse phase column developed in chapter five together with the digestion system. The two modes of chromatographic separation were selected to be carried out on-chip separately aiming to couple them later on to carry out a protein digestion with two dimensional on-chip chromatographic separations.

6.2 Experimental.

6.2.1 Chemicals.

For on-chip digestion, see section 3.2.1.

For on-chip monolithic exchange column formation, see section 4.2.1.

For monolithic reverse phase column formation, see section 5.2.1.

For treatment of biological sample

- Trichloroacetic acid (TCA) (Fisher scientific equipments, Loughborough - UK).
- Acetone (Fisher scientific equipments, Loughborough - UK).

6.2.2 Instrumentation.

See section 4.2.2.

6.2.3 Protein solution.

See section 2.2.3.

6.2.4 Microfluidic chip fabrication.

See section 3.2.4.

The design of the chip was as follows: The top plate was assigned for the chromatographic column, while the bottom plate was assigned for protein digestion. The two plates were then thermally bonded. Figure 69 shows the schematic diagram of the two plates and Figure 70 shows the schematic diagram of the chip after bonding and how the digestion channel and the chromatographic columns were formed in the channel.

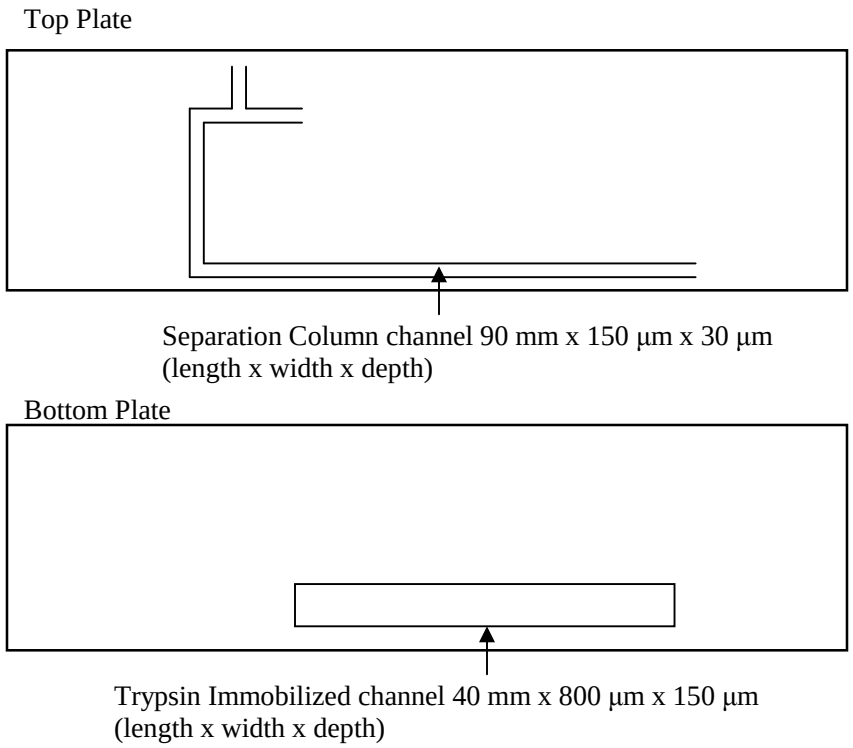


Figure 69: Schematic diagram of the two plates used to form the microfluidic chip (etched surface on top).

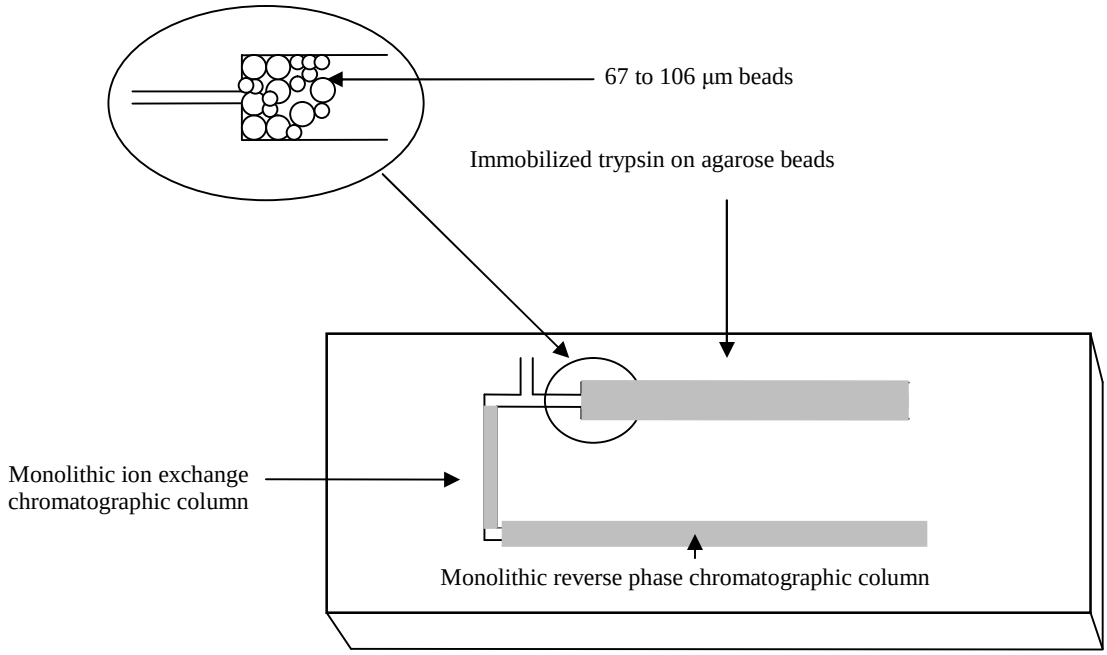


Figure 70: Schematic diagram of the microfluidic chip showing the two monolithic column and the beads inside the channel.

6.2.4.1 On-chip digestion and separation using a monolithic ion exchange column.

The monolithic ion exchange column developed previousely (section 4.3.3) was polymerized on-chip to a length of 4 cm (first image in Figure 71). After the formation of the column, the digestion channel was filled with agarose beads as described earlier (section 3.3.2). The temperature of the oven was set to 40 ºC (second image in Figure 71). Other chromatographic conditions for the separation of the tryptic digest of cytochrome C were as described in section 4.2.5.

Initially, ammonium acetate solution (pH 8.2) was infused through the digestion channel before connecting the chip to ESI-MS. This was carried out to activate the trypsin immobilized on the agarose beads. Then the protein solution was infused to the digestion channel. The dead volume of the connected capillaries was less than 1.0 μl and the volume of the digestion channel was less than 4.4 μl. To ensure that the sample solution filled the digestion channel, 5.0 μl of the sample was infused at a flow rate of 1.0 μl min^{-1} using a syringe pump connected *via* hole 1. This was

carried out only before starting the first injection of a new solution. Subsequent injections were made directly without the need to disconnect the chip.

After connecting the chip to the ESI-MS *via* hole 3, the protein sample was pumped at 1 µl min^{-1} for two minutes (27.2 pmol of cytochrome C injected). The syringe pump was then removed and the connection was blocked (third image Figure 71); this was to force the mobile phase to pass through the chromatographic column. The mobile phase was pumped *via* hole 2 and the back pressure generated from the column was found to be 3.7 MPa at a flow rate of 1.0 µl min^{-1}.

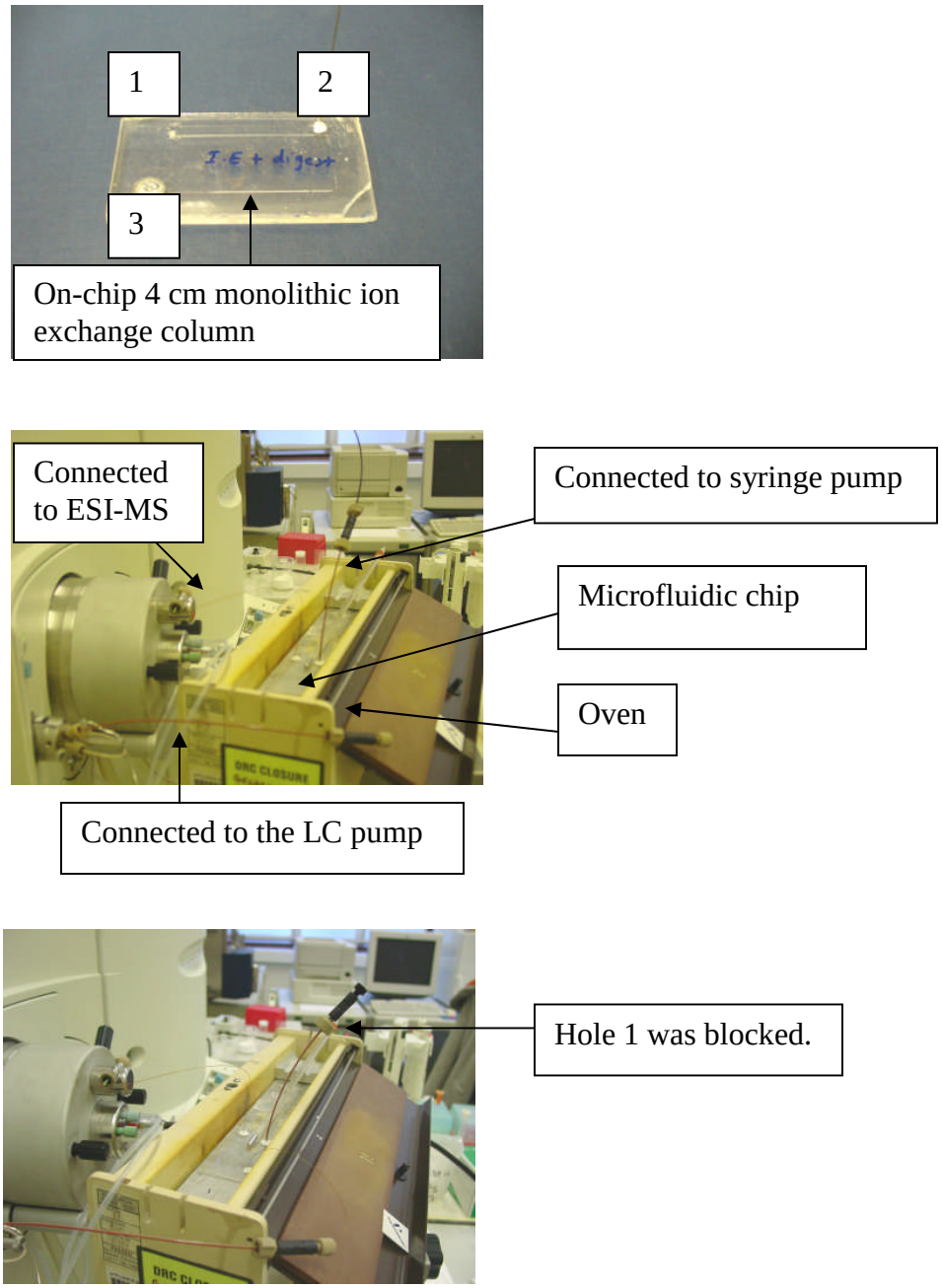


Figure 71: The first image shows the formation of the 4 cm monolithic ion exchange column on-chip. The second and third images show the connection of the chip to the ESI-MS and the overall experimental setup.

6.2.4.2 On-chip digestion and separation using a monolithic reverse phase column.

The EDMA-LMA monolithic column developed in chapter five (section 5.3.2) was polymerized on-chip to a length of 5 cm. A similar experimental set-up was used as described in section 6.2.4.1. However, the injection time was reduced to 20 s (34.0 pmol of cytochrome C injected, 90.7 pmol of BSA injected).

The concentration and the digestion method of cytochrome C, BSA and a mixture of four proteins (melittin, cytochrome C, BSA and apo-transferrin) was as described in sections 3.3.4, 3.3.6 and 3.3.7 respectively (for mixture 3, injected amount was 233.3 pmol, 2.6 pmol, 2.8 pmol, 2.7 pmol respectively). The chromatographic conditions for separation of cytochrome C and BSA were as in section 5.2.4 when EDMA-LMA was used as the reverse phase column. However, the separation was carried out at 40 ^{o}C. The separation conditions for the peptides generated from on-chip tryptic digestion of the four proteins were the same as those for separation of the tryptic digest of BSA.

6.2.4.3 On-chip digestion and separation using two dimensional chromatography

Off-line 2D chromatography was carried out as follows:

BSA (100 μM) was digested using on-chip digestion (agarose beads) and the generated peptides were fractionated using the on-chip monolithic ion exchange column.

The chip was placed in an oven where the temperature was set to 40 ^{o}C (with reference to the top surface). The injection was carried out for two minutes at a flow rate of 1.0 μl min^{-1} (200 pmol of BSA was injected). The first separation was carried out using ammonium acetate solution 3 % (pH 2.6 adjusted with formic acid) as the mobile phase, the second separation with 6 % solution while the third was with 10 % solution. Each fraction was collected for 10 minutes in a separate vial.

After these fractions were collected, another sample of the on-chip tryptic digest of BSA was collected without any separation *via* hole 2 and diluted five times. The four samples were than injected separately onto a PS-DVB monolithic reverse phase column (thermally initiated using BP).

The following gradient elution was used for the second dimension separation (PS-DVB monolithic column) solvent A: 0.4 % formic acid (aqueous) solvent B: 0.4 % formic acid in acetonitrile. A 100 % for 3 minutes, deceased to 70% A over 30 minutes, and then back to 100 % A over 2 minutes. Flow Rate was set between 1.4 and 1.8 μL min^{-1}. The equilibration time was 15 minutes.

6.2.5 Biological sample.

The protein sample was provided by Castle Hill hospital and was received after carrying out the extraction. The sample was extracted from breast cancer cell line. Cells were harvested using trypsin, washed with PBS and resuspended in 1ml of the extraction buffer. The sample was vortexed and incubated at 4 ^{o}C for 15 minutes on an end-over-end rotator. Cell debris were removed by centrifugation at 13000 r.p.m for 15 minutes and the supernatant was removed to a fresh tube.

The following protein extraction buffer was used: 8 M Urea, 2 % [3- [(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate CHAPS (detergent), 50 mM DTT, 0.2 % Bio-Lytes (pH 3-10), 1 % protease inhibitor mix and 0.002% bromophenol blue.

The protein concentration was determined using the 2D Quant Kit (Amersham Biosciences) and was calculated to be 1.61 mg/ml.

500 μl of TCA solution (5 g into 3.5 ml Water) was added to the protein sample. The sample was incubated at 4 ^{o}C for ten minutes and centrifuged at 14000 r.p.m. for 15 minutes. The supernatant was then removed and the whitish colour precipitate formed was washed with 200.0 μl acetone. The process was repeated twice. The protein was then dissolved in ammonium acetate buffer (pH 8.2) 40.0 μl.

On-chip digestion was carried out as described earlier (section 6.2.4.1) and 2.0 µl of sample was injected into the monolithic ion exchange column. The chromatographic conditions were the same as the one used for separation of the mixture of tryptic digest of melittin, cytochrome C, BSA and apo-transferrin in section 4.2.5.

For separation using the commercial column (Jupiter 4µm Proteo 90A, 150 mm x 0.3 mm, phenomenex), the tryptic digest of the biological sample wass collected *via* hole 2 of the microfluidic chip. The chromatographic conditions were as described in section 4.2.5.

6.2.6 Toward on-chip separation using two dimensional chromatography.

The monolithic ion exchange column (chapter four) was polymerized on-chip to a length of 2 cm. After the formation of the column, the EDMA-LMA monolithic column developed in chapter five was polymerized in the same chip to a length of 5 cm.

6.3 Results and discussion.

The previous chapters showed the possibility of performing on-chip digestion and on-chip separation using ion exchange chromatography using two separate microfluidic chips. Several monolithic reverse phase columns have been tested and it was found that EDMA-LMA is the most suitable one for an on-chip column. The theoretical calculation of the back pressure generated allows integration of these separated microfluidic chips into a single chip where protein digestion and peptide separation can be carried out. This will have many advantages; first, fast and efficient digestion of proteins; second, the direct transfer of the peptides generated from protein digestion process to the chromatographic on-chip column removing any handling process between the two stages and thus no sample loss or sample contamination will take place; third, the total analytical process can be automated once the protein is extracted from the sample. These advantages may increase the sample throughput and sensitivity of the method.

6.3.1 On-chip digestion and separation using ion exchange column.

After the formation of the ion exchange column on-chip and filling the chip with agarose beads to carry out on-chip digestion followed by on-chip separation, the chip was then directly connected to ESI-MS and cytochrome C was injected. The peptides generated from the on-chip tryptic digestion process were separated as shown in Figure 72. Avergae peak width at half height of was found to be 22.9 s (variation from 19.7 to 25.1 s).

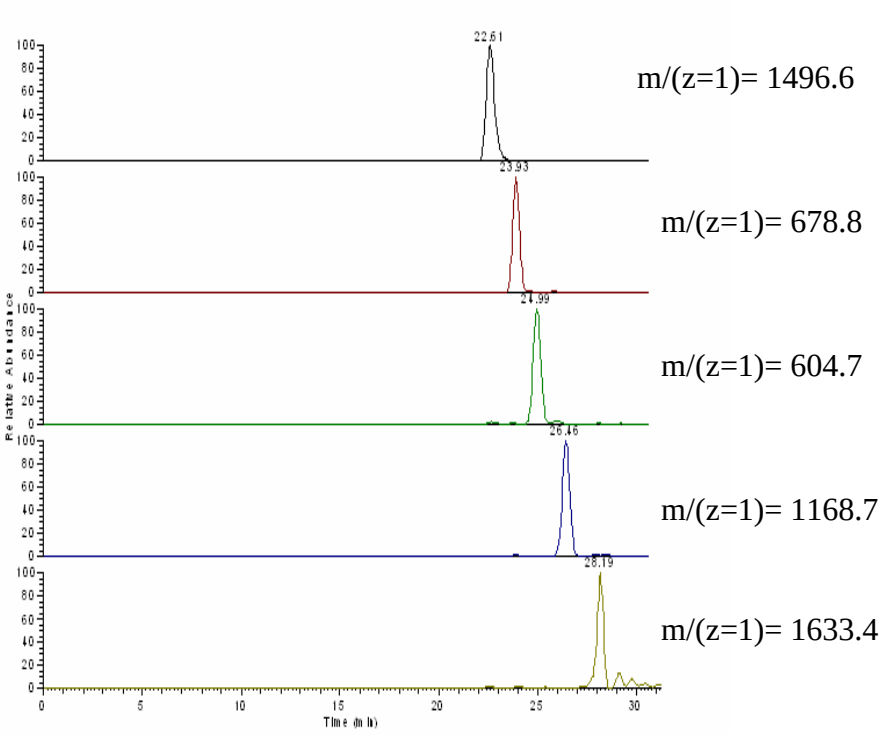


Figure 72: Separation of tryptic digest of cytochrome C separated using on-chip monolithic ion exchange column. For other conditions, see experimental section.

Table 30 compares the peak width and resolution of this column with the 7 cm on-chip ion exchange column previously made (chapter four, section 4.2.5) at the same temperature (40 ºC). The peak width at half height of the chromatogram generated using the 4 cm column was reduced considerably while the resolution was slightly affected. Despite this, it was expected that the peak width would be smaller due to the shorter column length and higher separation temperature. However, the band broadening here was due to some other factors, for example, the digestion solvent

(ammonium acetate solution) which was used here in comparison to formic acid 50 mM used for direct injection earlier. Formic acid was used as a mobile phase at the beginning of the run and thus charge states of peptides were not changed while ammonium acetate solution (pH 8.2) may change the charge state of the peptides and hence the interaction with ion exchange sites in the column decreases leading to diffusion and hence band broading. Similar effect commonly observed when solvent stronger then mobile phase is used as diluent.

Peak	On-chip column (4 cm) at 40 ºC		On-chip column (7 cm) at 40 ºC	
	Peak width at half height (s)	Resolution	Peak width at half height (s)	Resolution
1	22.3	1.6	26.5	2.7
2	19.7	1.3	22.9	1.3
3	25.1	1.3	35.3	1.3
4	25.1	1.8	35.3	1.6
5	22.3	2.3	40.6	2.9
	Average peak width at half height = 22.9		Average peak width at half height = 32.1	

Table 30: Comparison between 4 cm and 7 cm on-chip monolithic ion exchange column in terms of peak s width and resolution.

It was observed that leaking started from hole 2. This was not observed earlier. The glue used was made for high pressure applications and no such leak should be observed at the hole. If pressure builds up then the leak would be expected to start from the bonding of the two plates, as this is the weakest point. To investigate this further, several holes were made on a single piece of glass using the same drilling tool and the diameter of these holes was measured. The data indicated that some variations occurred, the diameter of these holes varied between 372 to 389 µm. This was because the drilling was carried out manually. Precise control of the drilling angle is difficult and depends on the experience of the driller. However, this would have an important impact on the back flow and may also increase the void volume.

Additionally, if the glue was not resistant to the mobile phase then with time the mobile phase would start leaking from the hole.

The glue was also tested. It consisted of two components, a base resin and a hardener. According to the manufacturer, the mixing ratio should be 2:1 by volume. It was found that any deviation from this ratio weakens the glue considerably.

Another problem was observed also, the breakage of glass pieces around the hole due to the drilling. These breakages make insertion of the capillary very difficult. To resolve this problem, a thinner glass was used (1 mm thickness), and during the drilling another piece of glass was placed at the bottom of the plate. Glass plate with 1 mm thickness was easier to drill and the breakages around the hole were reduced considerably. Despite these technical difficulties, the experiment showed that the design was successful and the digestion and the separation was achieved within 35 minutes.

6.3.2 On-chip digestion and separation using reverse phase column.

LMA-EDMA monolithic column was fabricated on-chip (5 cm length, Figure 73) and then the digestion channel was filled with the agarose beads. The chip was connected to the ESI-MS as described before. The back pressure generated was measured and found to be 6.6 MPa at a flow rate of 1.0 µl min^{-1}.

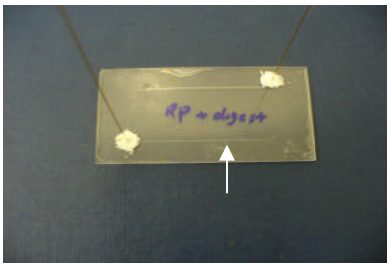


Figure 73: Image of on-chip monolithic reverse phase column indicated by the arrow sign.

The time of injection was used to control the injected amount of the tryptic digest of a protein as explained earlier (about 300 nl).

First, cytochrome C (34.0 pmol) was injected and the generated peptides were separated using the on-chip monolithic reverse phase column.

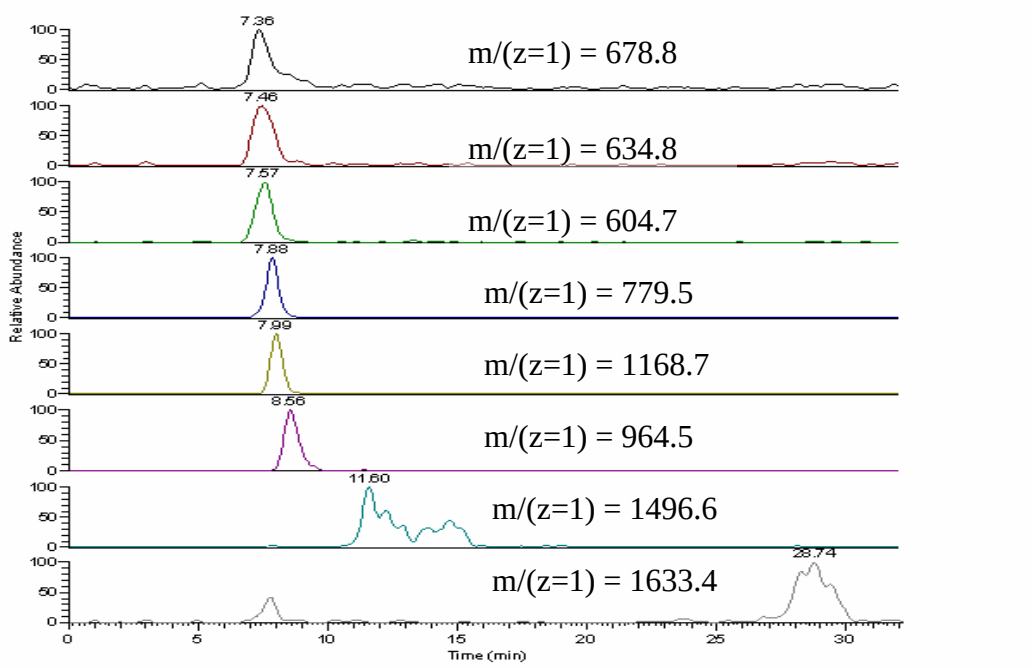


Figure 74: Separation of tryptic digest of cytochrome C separated using on-chip monolithic reverse phase column. For other conditions, see experimental section.

The separation is shown in Figure 74. In comparison to the capillary monolithic column, several differences were observed: first, the resolution between all the peaks were reduced, this is with the exception of the last two peaks. The loss of the resolution was due to the higher temperature at which the separation took place (40 ^{o}C compared to 25 ^{o}C when the separation was carried using the monolith in a capillary). Higher temperature enhances the mass transfer process, which reduces the band broadening as observed here. However, this may have a negative impact on the resolution as it reduces the fraction of the time the molecules spend in the stationary phase and hence reduces the capacity factor. Second, as could be observed, the last two peaks were distorted. These distorted peaks may be due to some miscleavage occurring during the digestion process and the generated miscleaved peptides being co-eluted with the last two peaks. However, this was not observed earlier. Additionally, the mass spectrum of the two distorted peaks

showed the presence of a single ion only. The other possibility was the loading capacity of this on-chip monolithic column which was less due to the smaller dimension of this on-chip column compared to the capillary column used in the previous chapter. However, the distortion of the last two peaks could not be completely explained by these facts and the presence of some contamination in the sample may be another factor, which also contributed to the formation of the distorted peaks.

To test the column further, BSA sample (90.7 pmol) was injected and the generated peptides were separated directly using the on-chip monolithic reverse phase column. Figure 75 shows part of the separation. No such distorted peaks were observed and the peak width measurement (made on 16 selected peaks) varied between 30.0 to 115.0 s. However, a number of peptides were unretained in the column and this limited the resolution between the peptides present in the mixture.

The column performance was comparable to the latest literature results [236] (see section 5.1). These reported results along with the experimental data obtained here demonstrate the difficulty in obtaining a highly efficient on-chip photoinitiated monolithic reverse phase column. This may be due to the use of photoinitiation as has been discussed ealier (sections 4.3.2 and 5.3.1). This is inaddition to the constraints on the density of the monolith formed, due to the higher pressure, resulting from denser monolith, damaging the microfluidic chips.

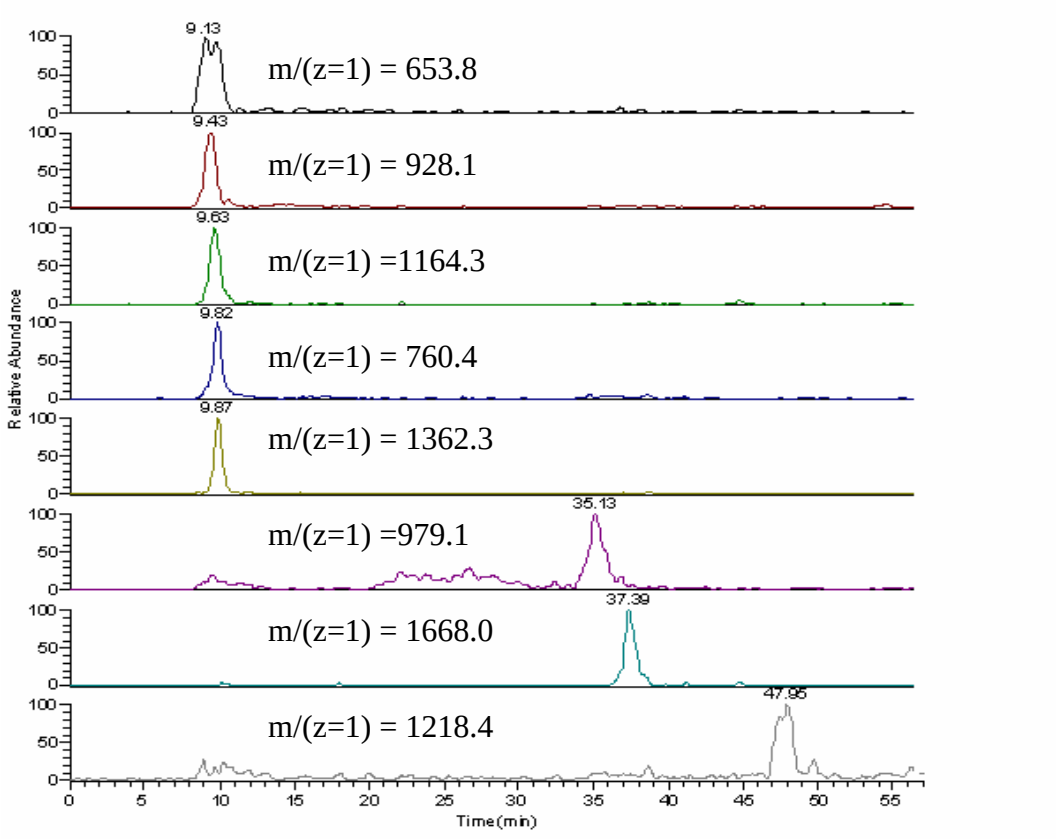


Figure 75: Separation of tryptic digest of BSA using on-chip monolithic reverse phase column. For other conditions, see experimental section.

Despite the limited resolution obtained with the reverse phase on-chip column, when the digested mixture of four proteins (melittin, cytochrome C, BSA and apo-transferrin, section 3.3.7) was injected. The results of this on-chip separation of the tryptic peptides showed that the sequence coverage for cytochrome C increased to 49 % (without separation the sequence coverage was 42 %). This was due to the detection of a peptide with m/z 964.3, which was not detected without separation (Figure 76).

Only one additional peptide generated from the tryptic digest of apo-transferrin was detected (Figure 77). In comparison, the separation of the same mixture carried out using a commercial reverse phase column (Figure 62) identified two additional peptides. However, the amount of the digest injected here was less due to the smaller injection volume.

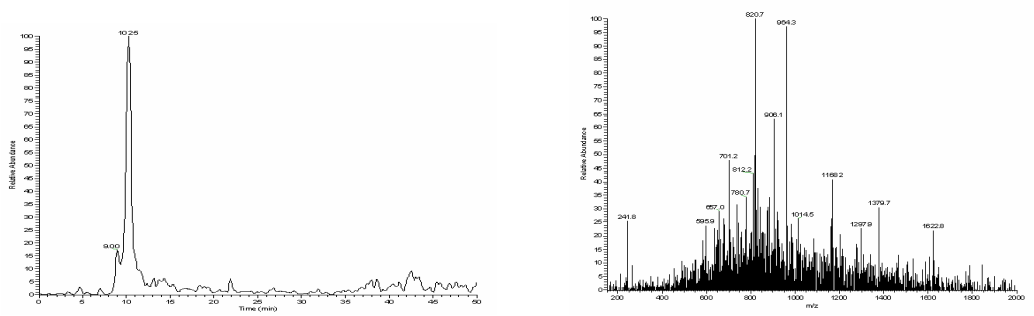


Figure 76: Detection of peptide (m/z 964.3) generated from tryptic digest of cytochrome C in a mixture of four proteins. This peptide was not detected without on-chip reverse phase separation. Separation was carried out using gradient elution and on-chip EDMA-LMA, photoinitiated monolith at 40 ^{o}C. For other conditions, see experimental section.

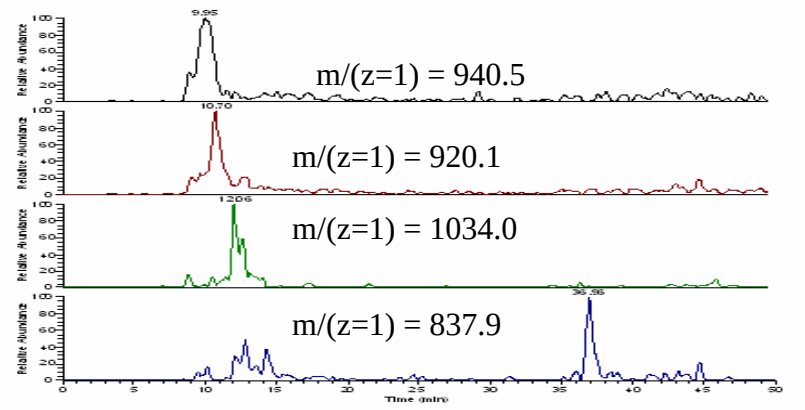


Figure 77: Separation of four peptides generated from tryptic digest of apo-transferrin in a mixture of melittin, cytochrome C and BSA, using on-chip digestion followed by on-chip separation using monolithic reverse phase column. For other conditions, see experimental section.

6.3.3 An off-line 2D experiment using the microfluidic system developed for proteomics.

The complexity of the tryptic digest of proteins requires a highly efficient separation system. A two dimensional chromatographic system may provide the desired capability. However, both separation dimensions should be highly efficient in order to obtain high peak capacity. The on-chip reverse phase column used here

provided limited resolution and a number of peptides were not retained in the column. This is comparable to the best results yet reported in the literature using on-chip monolithic reverse phase columns.[236] Therefore, for a real proteomic sample, this may not be of practical use and a higher efficiency reverse phase column would need to be used. However, the monolithic ion exchange column was efficient and comparable to the best on-chip column reported. Fortier *et al.* [233] used the Agilent HPLC-Chip to separate a tryptic digest of eight proteins. An average peak width of 20.4 s was obtained, while Xie *et al.* [238] demonstrated the use of an electrochemical pumping system on-chip with a particle based column. Peak widths between 45 s and 30 s were obtained. These data suggest the possibility to use of the monolithic ion exchange column in an off-line 2D chromatographic experiment with the above reported systems, as the peaks widths were comparable (varying between 19.0 to 24.9 s). To test the suitability of the column for such a 2D experiment, an off-line 2D experiment was carried out using a tryptic digest of BSA as a sample generated from on-chip digestion.

Initially the on-chip digestion was carried out at a flow rate of 1.0 µl min^{-1}, and the sample was collected from hole 2 and injected; after diluting the sample five times with 50 mM formic acid, in the reverse phase column. The separation was not optimized as shown in the Figure 78 and many peaks eluted together. The inset figure shows the mass spectrum of all peptides eluted between retention time of 12 to 25 minutes.

On-chip digestion followed by on-chip separation (ion exchange monolithic column) was carried out using three isocratic runs. In the first 3 % ammonium acetate solution was used while in the second and third, 6 % and 10 % were used respectively.

The first fraction eluted from the ion exchange column was then injected in the reverse phase column and the separation was carried out using the same conditions as in Figure 78. Figure 79 shows the chromatogram and mass spectrum for all peptides eluted between retention time 12 to 25 minutes from this fraction. Several peptides were detected (*e.g.* m/z 1163.5. 1014.5, 997.4, 820.5 and 740.6). All of

them can be found in the previous figure. The second fraction (Figure 80) when injected showed mainly one peptide with m/z 927; however, traces of another peptide (m/z 820.6) were also present. The third fraction (Figure 81) showed three peptides, m/z 820.6, 707.0 and 653.4. In these three fractions no peptide was eluted in two fractions, with the exception of peptide m/z 820.6 which was observed in the three fractions. However, the main peak of the peptide was in the third fraction. The intensity of the peak corresponding to this peptide was high. This implies the concentration of this peptide was high and therefore it could be seen in all the fractions.

The ultimate objective, in most cases, of any separation in proteomics is to enhance the detection limits so that more peptides can be detected. This could be seen in this 2D experiment. In the second fraction the peptide with m/z 927 could not be seen when the sample was separated using the reverse phase column only (1D separation).The same is true with regard to the third fraction, as it contains two peptides which could not be seen in the 1D experiments (m/z 653.4 and 707.0). The detection of these peptides were due to the enhancement of the separation, which resulted in reduction of signal suppression due to the limited number of peptides present in each fraction.

It could be observed generally that the noise levels in the last three runs were high and increased as the percentage of organic solvent increased. This could be due to the lower surface tension of the organic solvent used compared to water. As a result, smaller droplets were formed in the spray which increased the ionization efficiency. However, this also generated a higher noise level. Despite these observations, the experiment demonstrated the suitability for use of the on-chip digestion and peptide separation system in an off-line 2D separation system.

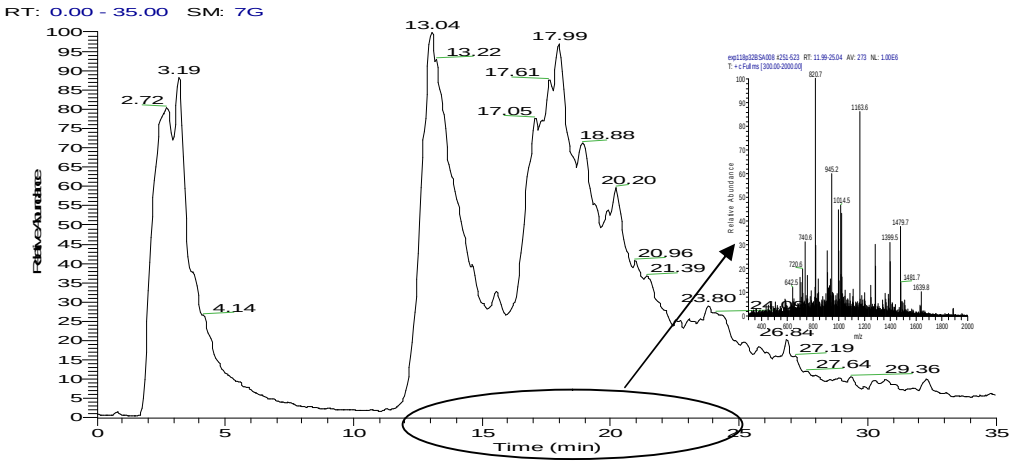


Figure 78: Total ion chromatogram for a tryptic digest of BSA. Separation was carried out using the PS-DVB monolith. For other conditions, see experimental section. The inset figure shows the m/z values of peptides eluted between t_R 12 to 22 minutes.

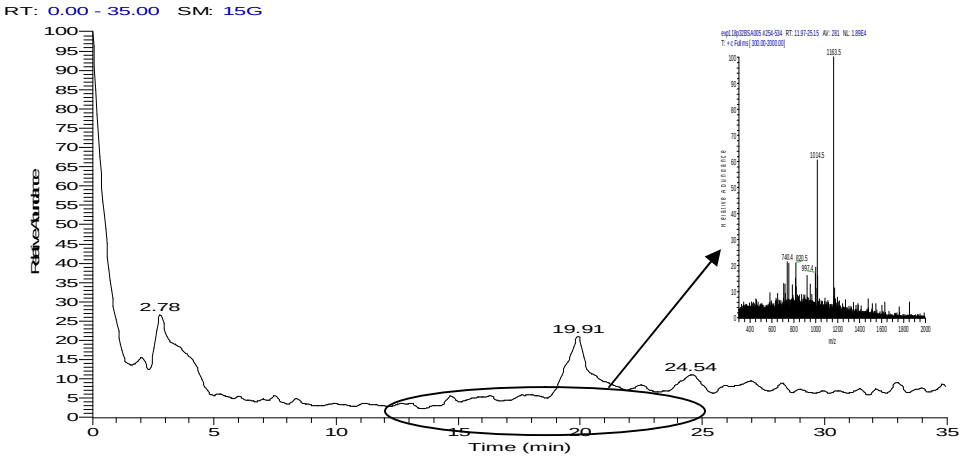


Figure 79: Total ion chromatogram for fraction one separated using the PS-DVB monolith. For other conditions, see experimental section. The inset figure shows m/z values of peptides eluted between t_R 12 to 25 minutes.

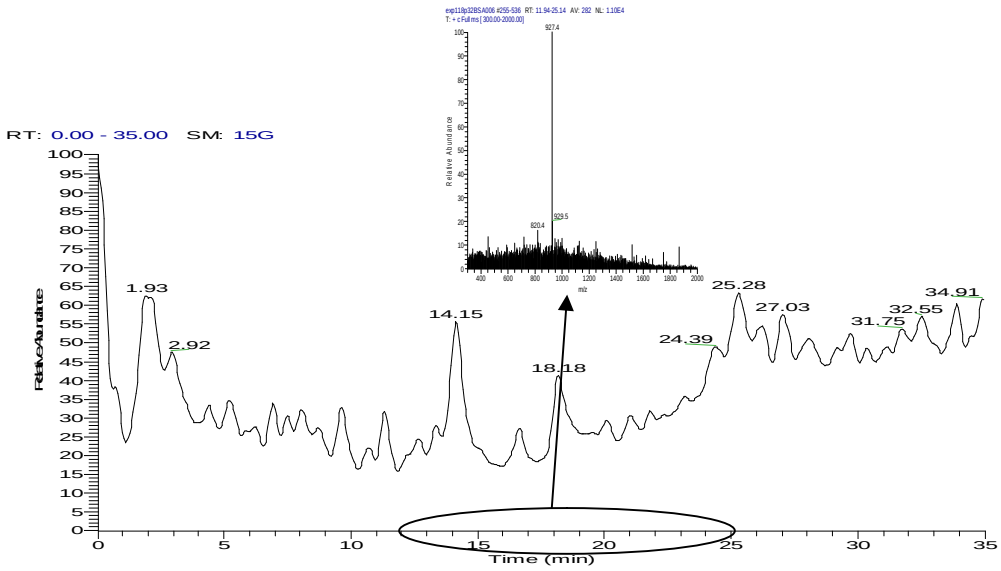


Figure 80: Total ion chromatogram for fraction two separated using the PS-DVB monolith. For other conditions, see experimental section. The inset figure shows m/z values of peptides eluted between t_R 12 to 25 minutes.

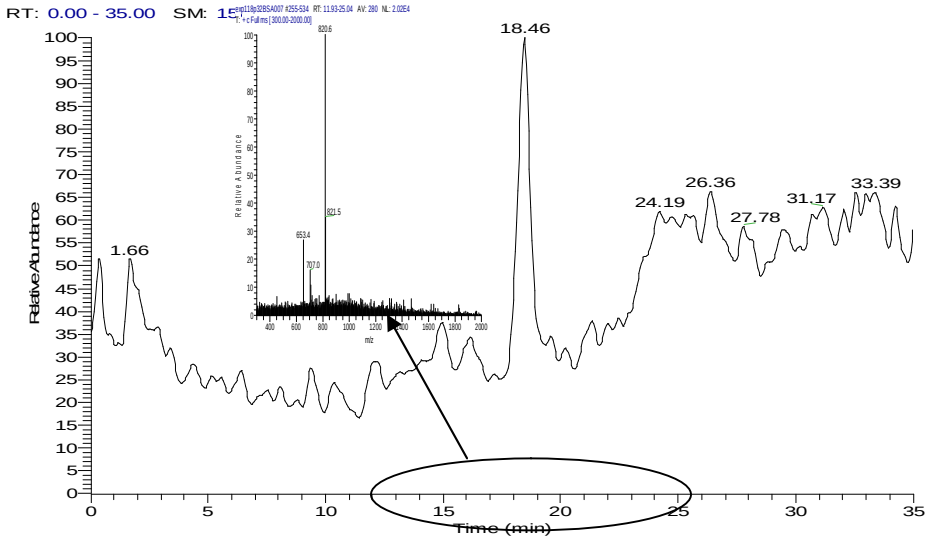


Figure 81: Total ion chromatogram for fraction three separated using the PS-DVB monolith. For other conditions, see experimental section. The inset figure shows m/z values of peptides eluted between t_R 12 to 25 minutes.

6.3.4 Testing the developed microfluidic chip for proteomics with a biological sample.

The microfluidic system developed for protein digestion and peptide separation using the monolithic ion exchange column was then tested with a biological sample.

The biological sample was received from Castle Hill hospital. The method of protein extraction was optimized for in-gel digestion and was not tested with in-solution digestion commonly used in shotgun type experiments. The sample was used here to assess the suitability of the method for use with the on-chip proteomic method and the type of problems that could arise.

After extraction of the proteins using TCA as described in the experimental section, the sample was dissolved in 40.0 μl ammonium acetate buffer (pH 8.2). The sample was digested on-chip and was collected *via* hole 2 in the microfluidic chip (about 10 μl).

In subsequent experiment, the chip was connected to the LC pump *via* hole 2 and the biological sample was pumped directly from the digestion channel into the on-chip ion exchange column at a flow rate of 1.0 μl min^{-1} for two minutes using a syringe pump connected to hole 1.

The chromatogram generated from the injection of the biological sample into the on-chip ion exchange column is presented in Figure 82. The m/z for the peak at retention time about eight minutes was found to be 616.1 and a smaller signal was also observed at m/z 1230.4. However, the peaks overloaded the column and reduced the intensity of the mass spectra of other peaks present in sample and therefore, these peaks could not be identified. A second sample was run after changing the mass scanning range to m/z 800-2000. The change was made so that the peak with m/z 616.1 could not be detected. However, the same component appeared again due to the other signal at m/z 1230.4 and the column was overloaded. The peak traces could be detected even after 30 minutes from injection.

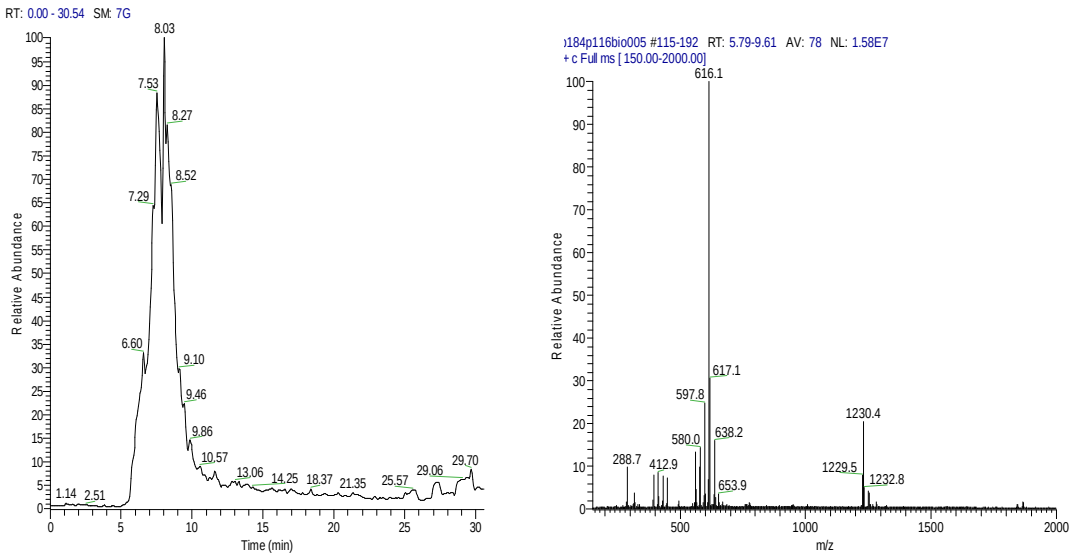


Figure 82: Left figure is for the total ion chromatogram generated from injecting the biological sample in the ion exchange column. For other conditions, see experimental section. Right figure show the mass spectrum of the peak at t_R 8.03 minuts.

The sample collected from on-chip digestion was then injected in the commercially available reverse phase column. The generated chromatogram showed several peaks; however, the highly intense peak m/z 1230.4 was also observed here and reduced the signal intensity of others. Although the mass spectra of other peaks were weak, the chromatogram was reproducible, indicating that these peaks were from the sample and not noise (Figure 83). This was also supported by the chromatogram obtained from running a blank sample. The loading capacity of the reverse phase column used was higher as the diameter of the column was larger. Therefore, the column was not overloaded to the same extent as the ion exchange column. The strong signal was due to the presence of CHAPS, which was used in protein extraction mixture. CHAPS is a non-ionic surfactant [300] and therefore, it eluted early in the ion exchange column while it was eluted later in the reverse phase column. Additionally, the molecular mass of the CHAPS is 614.9, which matches the m/z observed in the mass spectrum. The other peak observed at m/z 1230.4 was also due to CHAPS as it forms multimers extending to higher m/z values. [301] Moreover, it has been reported that CHAPS does not have a significant effect on trypsin digestion; however, it interferes with peptide separation and

should be removed prior to the analysis. [300] All this data suggests that the other peaks present in the chromatogram corresponded to peptides generated from the on-chip digestion process.

In summary, the generated data indicate that the biological protein sample was digested on-chip. However, due to the presence of CHAPS, the ion exchange column was overloaded and the peptide signals were strongly suppressed.

However, this has to be confirmed after optimizing the sample treatment steps for the on-chip proteomic system developed. Several protocols have been developed for in-solution digestion. [244,272,294,302,303] These can be tested and modified accordingly.

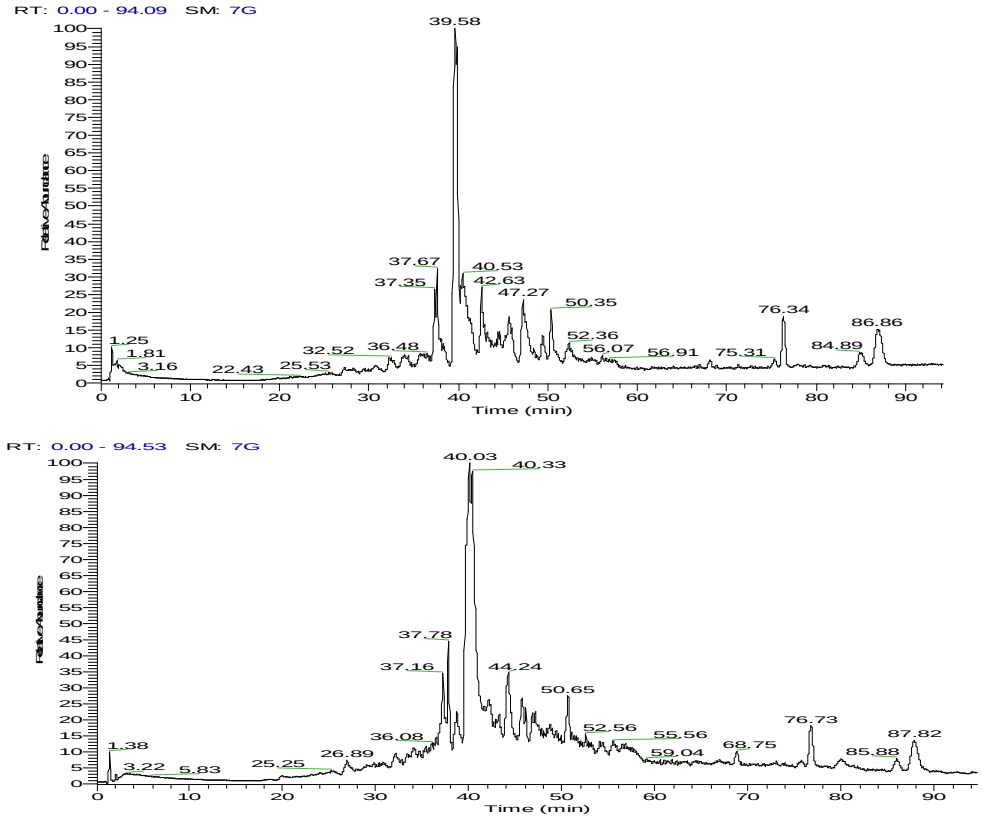


Figure 83: Top and down figures are for the total ion chromatogram of two runs of biological sample. For other conditions, see experimental section.

6.3.5 Toward on-chip separation using two dimensional chromatography.

The use of a monolith as the medium for on-chip separation was based on two main reasons. The first was due to the possibility of controlling the location of the two monoliths in the microfluidic chip when UV light was used as a method of initiation. The second was due to the low back pressure generated from these two columns compared to the back pressure generated from a particle based stationary phase.

To illustrate these two points, a 2D monolithic based column was fabricated on-chip. Initially, a monolithic ion exchange column was made. This was carried out carefully and 2 cm of the channel was exposed to photoinitiation. The rest of the channel was masked with a thick mask consisting of photo-mask and three layer of black tape. After formation of the monolith, it was treated as described earlier (chapter four).

The next step was making the monolithic reverse phase column. The difficulty here was how to control the exact location of the reverse phase column such that the reverse phase monolith would not form on a part of the ion exchange monolith. This is because if the reverse phase monolithic column is formed on the top of the ion exchange monolithic column, then this may cause several difficulties. First of all, the mixed part of the monolith will act like an ion exchange and reverse phase column. Hence neither salt gradient nor the organic solvent alone would elute the peptides. Secondly, the back pressure at that point would be very high and could block the column. However, it was also important to minimize the distance between the two monolithic columns, so that the dead volume would be minimal. It was decided to leave about half a centimeter between the two columns so that there would be enough space between the two columns, even if the reverse phase column was extended. Leaving a distance of 0.5 cm between the two columns was based on results from making several monolithic reverse phase columns in fused silica capillary and measuring the length of the monolithic columns formed under the mask.

Using the same masking procedures, the monolithic reverse phase column was fabricated on-chip. The void volume between the two columns was about 2.0 pl (Figure 84).

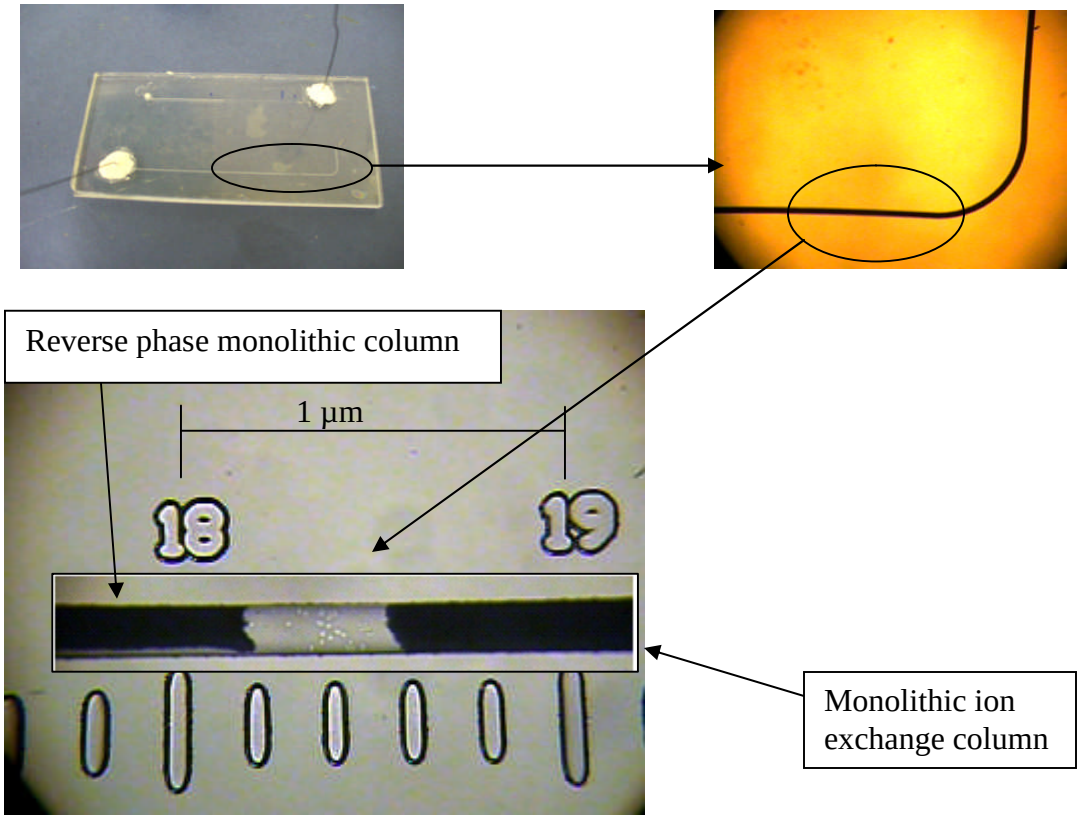


Figure 84: Top left image shows the microfluidic chip with two monolithic columns: ion exchange and reverse phase. Top right image shows part of the two on-chip columns. Bottom image shows the gap between the two columns.

The column was than washed with acetonitrile for 24 hours. Then the back pressure was measured using 30 % acetonitrile in water as mobile phase. It was found that the back pressure was 8.3 MPa at a flow rate of 400 nl min^{-1}. The back pressure generated from these two monolithic columns (total length of the two columns 7 cm) was lower than the back pressure generated from the single on-chip 4.5 cm particle based reverse phase column from Agilent, which was 10.0 MPa at a flow rate of 300 nl min^{-1}. [304]

The work above demonstrated that the back pressure generated for the two types of monolith on-chip was suitable for on-chip column fabrication and their locations can be controlled if proper masking is carried out.

If a higher efficiency photoinitiated monolithic reverse phase column could be made, then 2D separation on-chip could be carried out with little effort, even if the back pressure generated from the 2D separation system was higher (up to 12.1 MPa).

6.4 Conclusions.

Two novel microfluidic systems have been designed; in the first on-chip digestion and on-chip separation using ion exchange chromatography could be carried out while in the second, on-chip digestion and on-chip separation could be carried out using reverse phase chromatography. The integration of the digestion and separation steps in a single microfluidic system has many advantages, for example, shortening the total analysis time and reducing sample loss and risk of contamination. The first microfluidic system could be used for protein digestion and first dimension separation in a two dimensional separation experiment. This could be achieved by collecting fractions from the microfluidic system after the separation column and then a second separation could be carried out using standard liquid chromatographic techniques. This has been demonstrated using a tryptic digest of BSA.

The second system, in which reverse phase separation was carried out, provided some separation and improved the detection limits. However, the on-chip column provided limited resolution due to the low capacity factor of the chromatographic column fabricated.

Initial results from analysis of the biological sample indicated that the microfluidic system could be used for analysis of a real biological sample. However, the protein extraction method should be optimized for the system.

Fabricating the two monolithic columns on-chip was possible with good control of their location. The back pressure generated was well below the pressure generated by the particle based on-chip column reported and is within the range tolerated by well bonded glass chips.

7.0 Conclusions and further work

Mirofluidic systems are very promising techniques for proteomics. The features of microfluidics are a perfect match with the need of proteomics for a method of analysis that provides high throughput and high sensitivity. [2] The overall speed of analysis provided by microfluidic systems fits with proteomics demand for an analytical method with high sample throughputs. Reduction of human interference and hence reduction of contamination and sample loss, is another feature that helps in improving the sensitivity of the analytical methods.

The research presented here used these advantages to develop microfluidic systems for proteomics, which sped up the analysis time considerably and enhance the sensitivity.

Initially, a microdevice was fabricated as a model for the microfluidic system. This model consisted of a digestion capillary, a separation monolithic capillary column, and a 'T piece'. The model was very successful and six proteins with various molecular masses were digested within eight minutes. The simple isocratic separation system showed some separation and detection limits in the pmol range were obtained.

A microfluidic system was designed based on this successful model and the increase in the speed of analysis was clearly demonstrated by the significant reduction in the digestion time from 24 hours using the traditional method to less than 5 minutes. This was carried out with a single protein and mixtures of various proteins. Additionally, preliminary results from digestion of a biological sample gave promising indications.

The on-chip digestion was carried out using commercially available immobilized trypsin on agarose beads. This was found to be very cost effective and time saving and the system could be ready to use within one hour.

The generated peptides were then separated without any handling as all the digestion product was directly passed to the separation column from the digestion

channel. This prevented sample loss and contaminations. A quantity in the low pmol range could be detected. The detection limits could be improved further if a nano-electrospray was used instead of the standard ESI-MS used here.

The on-chip peptide separation was carried out using chromatographic rather than electrophoretic methods. This avoided the need for a special interface between the microfluidic system and the ESI-MS, which may reduce the sensitivity of the ESI-MS or may cause degradation of separation, which could reduce the sensitivity. For several years, it was believed that electrophoresis is the right separation method to be used for peptide separation in microfluidic systems interfaced to ESI-MS. [243] Several papers were presented in which electrophoresis was used as the method for peptide separation.[168] However, it has been realized lately that chromatographic separation systems are more appropriate. [243]

Most of the difficulties associated with packing the stationary phase in microfluidic systems can be resolved by the use of monolithic columns. These can be photoinitiated and hence their formation on-chip can be controlled. Additionally, monolithic columns generate low back pressure compared with particle based stationary phases. This is due to the macropores present in the monolith. Moreover, monolithic columns can be easily anchored to the inner wall of the channel, hence removing the need for a frit.

Separation of peptides carried out using the on-chip monolithic ion exchange column developed was very good. The column exhibited a performance comparable with the best published data in this field. The peak width measurements at half height using this column were between 19.0 to 24.9 s. This is in comparison to the few published data in this field where peptide separation was carried out using on-chip reverse phase columns and a hydrodynamic pumping system. [233, 238]

Another novel microfluidic system was also fabricated. In this system, separation was carried out using a monolithic reverse phase separation column. The microfluidic system was evaluated using cytochrome C, BSA and a mixture of four proteins. The digestion and separation were completed within an one hour. The

column performance based on peak width at half height measurements (varying between 30.0 to 115.0 s) was comparable to the best published data in the field of peptide separation using on-chip monolithic reverse phase columns and a hydrodynamic pumping system. However, the column performance was lower than the on-chip monolithic ion exchange column fabricated in the other microfluidic system. This was due to the adverse effect of the photoinitiation method on the performance of reverse phase columns.

An attempt has been made to develop an on-chip two dimensional separation system using ion exchange and reverse phase monolithic columns. The results showed that it was possible to have complete control of the location of the columns in the microfluidic chip using photoinitiation. Additionally, the back pressure generated by the two columns could be handled easily within the glass microfluidic chip. However, the main difficulties were in fabricating a photoinitiated reverse phase monolithic column comparable in performance to the monolithic ion exchange column.

Future work should focus on optimizing the protein extraction method, as already several methods have been reported for in-solution digestion followed by chromatographic separation. [302,244, 272,303,294] These methods should be tested and modified, if necessary, to suit the microfluidic system developed here. Additionally, the microfluidic system developed can be integrated with the few on-chip reverse phase columns (particle based stationary phase) that have been reported to provide a complete system for protein digestion and an off-line two dimensional separation for the generated peptides. [233, 238]

Future work with regard to the development of the fabricated microfluidic systems should be carried out in two directions. The first is developing on-chip monolithic columns for protein separation. Protein separation prior to digestion will enhance the digestion process and improve peptide separation. The other direction is to improve the interface to the ESI-MS system. This can be carried out by developing an on-chip nozzle for ESI-MS on the same chip. This would remove any separation

degradation which may be caused by the tubing between the chip and ESI-MS currently present which may adversely affect the detection limits.

The final area where research can be directed is in developing a microfluidic system for protein extraction. This is considered the most challenging part of the research theme. However, a few reports have shown the possibility of designing microfluidic systems that could be used for protein extraction. [188,194,305] These can be used as a starting point for the research and further development should be carried out. However, these areas go beyond the scope of the current research reported here.

8.0 References

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9.0 Appendices