

High-Throughput Peptide Mass Mapping using an Integrated Capillary Device Coupled to a Mass Spectrometer

Frantisek Svec, Department of Chemistry, University of California, Berkeley, California, USA.

This article describes the development of a simple enzymatic reactor and its interfacing with a mass spectrometer for high-throughput mass mapping of peptides. This procedure combines preconcentration and enzymatic digestion in the nano-electrospray emitter.

The recently decoded human genome is believed to be a massive source of information that will lead to improved diagnostics of diseases, earlier detection of genetic predispositions to diseases, gene therapy, rational drug design and pharmacogenomic "custom drugs". The upcoming "post-genomic" era will then target the gene expression network and the changes induced by effects such as disease, environment or drug treatment. In other words, knowledge of the exact composition of proteins within a living body and the changes in composition that reflect both healthy and diseased states will be used to determine the pharmacological action of potential drugs. The rapid acceleration of research in the area of proteomics requires the development of new technologies in the drug discovery process.

Peptide Mapping

Peptide mapping appears to be ideally suited for the identification of proteins and the determination of post-translational modifications. The protein of interest is digested by a proteolytic enzyme, most often trypsin, and the peptides in the resulting mixture are identified using mass spectrometry. This process produces a

peptide map that is unique for each protein and means that known proteins can be identified by searching an existing database. Most of the current tryptic digestions are performed in aqueous solutions or in gels. This procedure is labour intensive, can take several hours, and the desired mixture of peptides can potentially be contaminated with additional peptides originating from the autodigestion of trypsin itself, which can complicate the unambiguous assignment of the studied protein.

In contrast, immobilization of the enzyme onto a solid support increases its stability while it eliminates the danger of unwanted autoproteolysis because of site isolation of the biocatalyst molecules. Obviously, such immobilization may only occur on surfaces that are fully accessible to the enzyme and will not hinder subsequent access of substrate to the active sites. In addition, the larger the surface available on the support, the higher the amount of the accommodated enzyme. Therefore, porous supports are favoured and some conjugates of enzymes with porous polymer beads have already been commercialized for use in conjunction with electrospray ionization (ESI) mass spectrometry.

Recently, extensive efforts focused on the preparation and application of porous monoliths.¹ For example, a monolith comprising reactive functionalities is suitable as support for the immobilization of trypsin that then performs the rapid digestion of proteins. This article describes the development of a simple enzymatic reactor, which also functions as a microdevice that combines both preconcentration and enzymatic digestion in the nano-electrospray emitter, and its interfacing with a mass spectrometer.

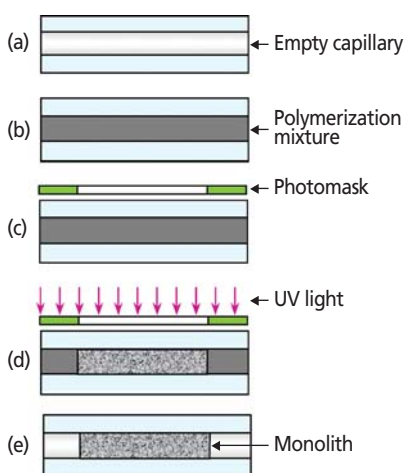
Preparation of Monolithic Support in Capillary

Most of the monolithic supports are prepared via thermally or redox-initiated free radical polymerizations. Because the ultimate goal is the fabrication of complex microfluidic devices, these polymerization modes are not best suited for the preparation of monoliths within a specific part of the device. Therefore, we developed the photolithographic-like technique involving photopolymerization through a mask. This simple approach shown schematically in Figure 1 enables the formation of reactive porous monoliths within a specific part of the microsystem.²

In previous studies, several groups used the glycidyl methacrylate-based monoliths with epoxide functionalities for enzyme immobilization. However, these supports react only slowly with proteins and extended reaction times of up to five days are required to achieve the desired activity. Multi-step reaction schemes involving the modification of epoxide groups with a diamine followed by activation using a dialdehyde accelerated the overall immobilization process but their multistep nature remains impractical. In contrast, the use of 2-vinyl-4,4-dimethylazlactone (VAL) **1** has enabled us to prepare very reactive monolithic materials first by copolymerizing this monomer with a hydrophilic protein-friendly monomer – 2-hydroxyethyl methacrylate **2** and a crosslinker – ethylene dimethacrylate (EDMA) **3**. The azlactone functionality of these monoliths reacts readily with nucleophiles such as the amino or thiol groups of proteins thereby enabling their rapid and efficient immobilization via a dipeptidic linker (Figure 2).

Figure 1: Scheme of the preparation technique producing a monolith with well-defined size and location.

(a) Empty capillary or ESI emitter; (b) capillary filled with the polymerization mixture consisting of monomers, porogenic solvents and a photo-initiator; (c) capillary with attached photomask; (d) irradiation of the capillary contents through the mask with UV light in the range of 220–330 nm for several minutes to fabricate the monolith; (e) removal of the unreacted polymerization mixture from the dark parts and washing with a solvent.



The rapid acceleration of research in the area of proteomics requires the development of new technologies in the drug discovery process.

Enzymatic Reactor

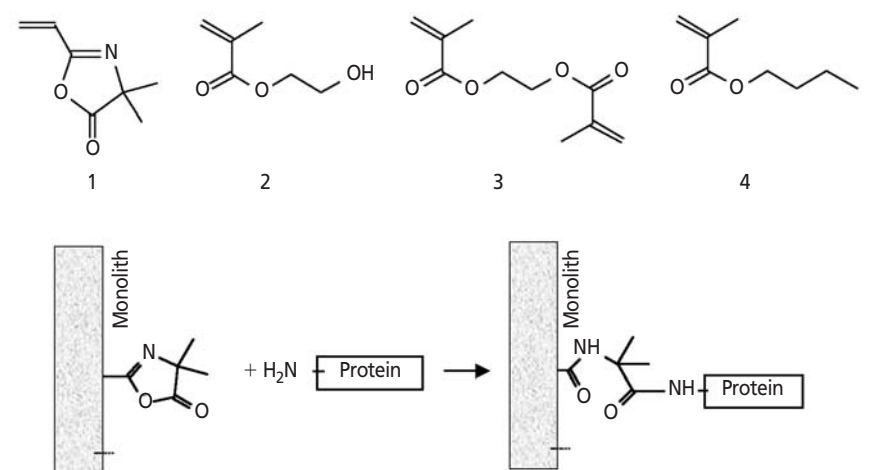
Because all the protein solutions must flow through the pores of the monolithic material during its use, it is essential to control the size of the pores that permeate it. Obviously, flow resistance is inversely proportional to pore size. The pore size of monoliths is best controlled by the composition of the porogenic system consisting of various mixtures of 1-decanol and cyclohexanol. A monolith with a pore size of about 1 μm , a pore volume of 1.33 mL/g and a specific surface area of 6.2 m^2/g was found optimal for immobilization of trypsin and subsequent proteolysis of proteins.³ The immobilization reaction, which involves pumping trypsin solution containing sodium sulphate, sodium carbonate and benzamide through the VAZ monolith located in a capillary, is rapid and is usually completed in 3 h. As an example, the conjugate prepared by pumping a 2 mg/mL trypsin solution for 3 h exhibits for N- α -benzoyl-L-arginine ethyl ester (BAAEE) a Michaelis constant K_M of 33.8 mmol/L and a maximum velocity V_{max} of 1.70 nmol/min . Figure 3 shows an ESI-TOF-MS spectrum of melittin digested on-line in this monolithic reactor enabling 100% sequence coverage. Note, that in contrast to digestion in solution, the residence time here is only 71 s.⁴ This demonstration confirmed that the concept

of rapid protein digestion using a monolithic immobilized enzyme reactor with mass transfer of substrate enhanced by convection is operative.

Fabrication of a Complex Multifunctional Device in an ESI Emitter

Although the copolymerization of reactive monomer proved successful, each change in the polymerization mixture would require a tedious reoptimization of both the chemistry and preparation procedure. To avoid this, we developed a new two-step approach to well-defined monoliths with a wide variety of surface chemistries based on photografting.^{2,5} The first step is the preparation of a “generic” monolith using the technique already shown in Figure 1. In our study, we used monoliths prepared by copolymerization of EDMA **3** and butyl methacrylate **4**. This first step is then followed by photografting of the pore surface with polymer chains formed from monomers with desired functionalities such as VAL **1**. The extent of grafting, which affects the activity of the immobilized trypsin microreactor, depends on both the time of irradiation and the intensity of the ultraviolet (UV) light. The former is easy to control by on/off switching and a simple filter can be used to modulate the light intensity at the

Figure 2: Structures of monomers used in this study: 2-vinyl-4,4-dimethylazlactone **1**; 2-hydroxyethyl methacrylate **2**; ethylene dimethacrylate **3**; and butyl methacrylate **4**. The reaction path shows immobilization of protein via its amine group using vinylazlactone functionality.



wavelength used to excite the benzophenone and trigger the grafting. The advantage of this technique is the separation of pore formation from formation of the surface chemistry.

This technique also enables the simple preparation of monoliths with co-contiguous chemistries also by using photomasks.⁶ For example, a monolith with two different functionalities is obtained in two simple steps: The first step consists of a polymerization that produces a hydrophobic "generic" monolith. In the second step, part of the capillary with the monolith inside is masked, the pores filled with VAL solution containing benzophenone, and the capillary is irradiated with UV to achieve grafting of the pore surface only in the unmasked part of the device. The reaction with trypsin immobilizes the protein covalently in the VAL-functionalized area. This path shown in Figure 4 leads to a dual function device that involves an unmodified hydrophobic part, which enables preconcentration via solid-phase extraction, and a microreactor with immobilized enzyme.

The simplicity of the *in situ* method allows the preparation of the monolith directly in the tip of a pre-formed nano-electrospray emitter. Micrographs in Figure 5 show that the monolith protrudes to the very end of the tip and is clearly visible within the orifice of the emitter with no dead volume.

The design of our dual-function microdevice consisting of a 20 mm long bioreactor and a 5 mm long concentrating section is well suited for the mapping of proteins present in dilute solutions through

proteolytic digestion followed by the identification of the resulting peptide masses using mass spectrometry. Because this capillary device is prepared directly in the nano-electrospray emitter, its interfacing with a mass spectrometer is a very simple process. To demonstrate the operational ability of our dual function device for this application, a 19 pmol/μL aqueous myoglobin solution was pumped through the monolith and digested. The peptides were collected in the short highly hydrophobic part and then released from the concentrating element using a 50 nL plug of a strong solvent, acetonitrile. Depending on the spatial location of the concentrator block within the emitter, pumping of the protein solution through the device may also occur in the opposite directions (i.e., first through the concentrator and then through the microreactor). Figure 5 shows the monitored total ion current signals for the dual function device in digestion-then-concentration configuration. The plots confirm the desirable signal increase observed after loading a higher sample volume.

The concentration step also has a significant effect on the quality of the mass spectrum. For example, Figure 6 shows the mass spectra for myoglobin digested with no preconcentration of the peptides and

compares this approach with that obtained after digestion of 15 μL of myoglobin solution, preconcentration of peptides, and finally their release in MS with 50 nL of acetonitrile. Clearly, as peptides from a larger volume of sample are loaded, their signal increases and the signal-to-noise (S/N) ratio improves. This enables positive detection of a larger number of peptides and a sequence coverage of 75% at higher detection confidence. In contrast, much lower sequence coverage of only 41% is observed in experiments with a single function device (enzyme reactor) operating with the dilute protein solution without the preconcentration step regardless of the volume of protein solution (Figure 7).

A similar system consisting of a bioreactor containing trypsin immobilized on a 25 mm long epoxy-functionalized silica monolith located in a 4.6 mm i.d. tube and a 25 × 4.6 mm i.d. column packed with C18 silica particles for peptide concentration coupled to an ion trap MS with ESI ion source has been reported very recently.⁷

Conclusion

The limited speed and extensive manual manipulation required by two-dimensional gel electrophoresis introduced by O'Farrell 29 years ago is unlikely to match the future needs of rapid screening techniques because of the slow speed, complex handling of the separations, and the limited options available for exact

Figure 3: ESI-TOF-MS spectra of melittin peptides obtained by digestion using monolithic immobilized enzyme microreactor. Conditions: Protein concentration of 20 pmol/μL in 10 mmol/L ammonium acetate buffer pH = 6.7, flow-rate through reactor 0.4 μL/min.

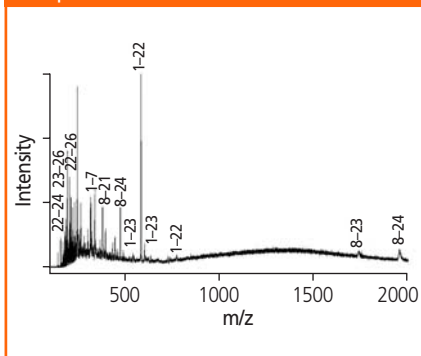


Figure 4: Scheme of the preparation technique leading to a monolith with dual chemistry. (f) Monolith is filled with functional monomer; (g) attachment of photomask that covers part of the "generic" monolith prepared according to Figure 1 followed by irradiation with UV light to create reactive chemistry only in a specific part of the monolith; (h) removal of unreacted monomer from the dark parts and washing with a solvent.

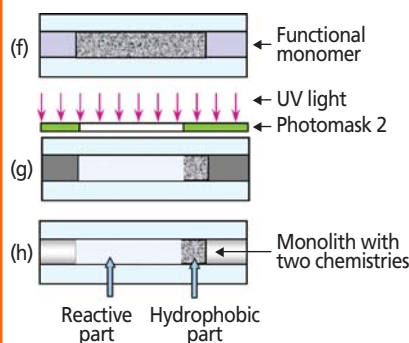


Figure 5: Optical micrograph of monolith prepared directly in a nanoESI emitter.

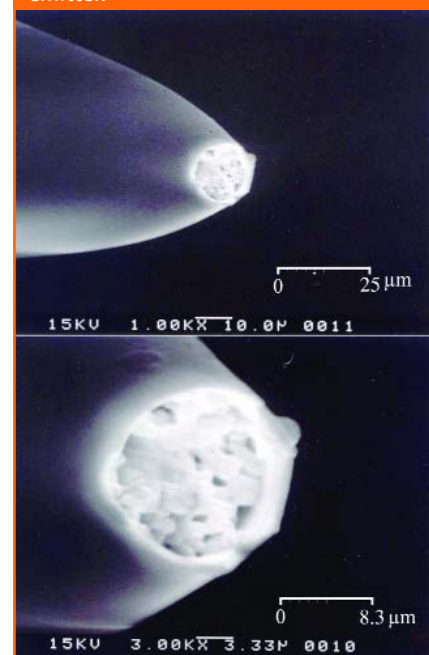


Figure 6: Total ion current monitoring of compounds eluted from a monolithic dual function device combining digestion and concentration for different loading volumes. Conditions: Flow-rate of 0.5 $\mu\text{L}/\text{min}$, room temperature; Loading: myoglobin solution 19 pmol/ μL ; loaded volumes 2 (1), 5 (2), 10 (3), 15 (4), and 20 μL (5). Elution: acetonitrile 50 nL.

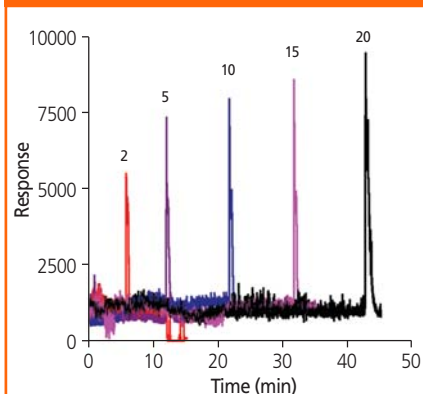
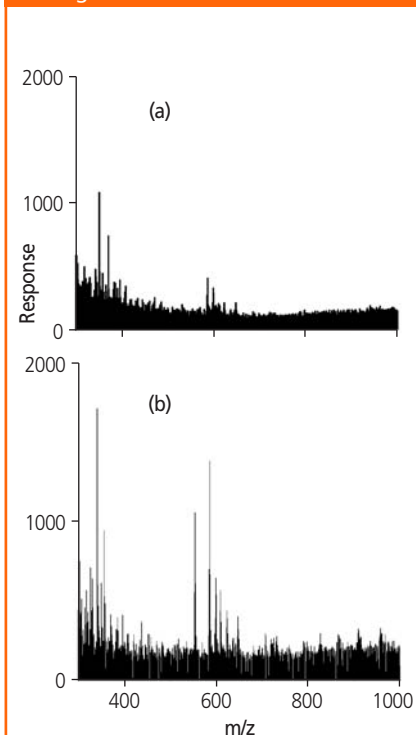


Figure 7: Mass spectra of compounds contained in peaks of total ion current chromatograms loaded on a single function (a) and dual function device after preconcentration of 15 μL myoglobin solution (b). For conditions see Figure 6.



quantification. New approaches to these types of separations must be studied. Microscale high performance liquid chromatography, electrochromatography and capillary electrophoresis are good contenders for this mission as they enable multidimensional separations while also providing better compatibility with mass spectrometry, currently the best and most sensitive detection method.

Obviously, much remains to be done in this area. Our approach is demonstrated here with a dual function monolith in a nano-electrospray emitter prepared using photopatterning. However, this method can easily be extended to the fabrication of even more complex structures and opens up new avenues for the development of a number of functional microdevices with tailored properties. For example, our current research focuses on the preparation of more complex monolithic devices comprising a preconcentration unit for proteins from dilute solutions with ion exchange functionalities from which the proteins are released in a gradient of salt in the mobile phase; a bioreactor operated in the solely aqueous medium at higher concentration of proteins and under the optimum pH conditions; a unit with hydrophobic functionalities for concentration and desalting of peptides; and finally, a microcolumn for HPLC separation of the peptides. Since this device will be prepared again directly in the nano-electrospray emitter, coupling it with a mass spectrometer will be very simple.

Acknowledgments

This work was in part supported by the Division of Materials Sciences and Engineering of the US Department of Energy under contract No. DE-AC03-76SF00098. Support of this research by a grant of the National Institute of General Medical Sciences, National Institutes of Health (GM-48364) is also gratefully acknowledged.

References

1. F. Svec, T.B. Tennikova and Z. Deyl, *Monolithic Materials: Preparation, Properties, and Applications* (Elsevier, Amsterdam, 2003).
2. T. Rohr et al., *Macromolecules*, **36**, 1677 (2003).
3. D.S. Peterson et al., *Anal. Chem.*, **74**, 4081 (2002).
4. D.S. Peterson et al., *J. Proteome Res.*, **1**, 563 (2002).
5. T. Rohr et al., *Adv. Funct. Mater.*, **13**, 265 (2003).
6. D.S. Peterson et al., *Anal. Chem.*, **75**, 5328 (2003).
7. E. Calleri et al., *J. Chromatogr. A*, **1045**, 99 (2004).

Frantisek Svec holds a PhD in polymer chemistry from the Institute of Chemical Technology in Prague. He then worked at the Institute of Macromolecular Chemistry of the Czechoslovak Academy of Sciences. He joined the faculty at Cornell University in 1992 and was appointed to the University of California, Berkeley, USA in 1997. He is also visiting professor of analytical chemistry at the University of Innsbruck, Austria and senior scientist in the Lawrence Berkeley National Laboratory. Dr Svec has authored over 300 scientific publications, 75 patents and edited several books. He currently serves as President of the California Separation Science Society. His research concerns macroporous polymers in different shapes and their use in numerous applications including gas and liquid chromatography, electrochromatography, enzyme immobilization and microfluidics.

Column Editor, **Robert Smits** is the Secretary-General of the Koninklijke Vlaamse Chemische Vereniging (Royal Flemish Chemical Society). He is Chairman of the biennial "International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analysers" and a member of the Editorial Advisory Board of *LC•GC Europe*.