

# Development of a trypsin-immobilized monolithic polymer with pipette-tip format for protein digestion

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## 1. Introduction

### The importance of protein digestion

- Proteomic research – primary structure of proteins, detection of post-translational modifications
- MS/MS-based peptide sequencing for protein identification
- Quality control (QC), quality assurance (QA) for the products in biotechnology, chemical synthesis and pharmaceutical industries<sup>1</sup>.

### Problems and challenges

- In-solution digestion**
- Time-consuming
  - Low efficiency
  - Enzyme autodigestion

### Enzyme-immobilized digestion

- Fast
- High efficiency
- High-throughput
- Eliminate autodigestion
- Reproducible and reusable

### Enzyme immobilization on porous polymer monolith

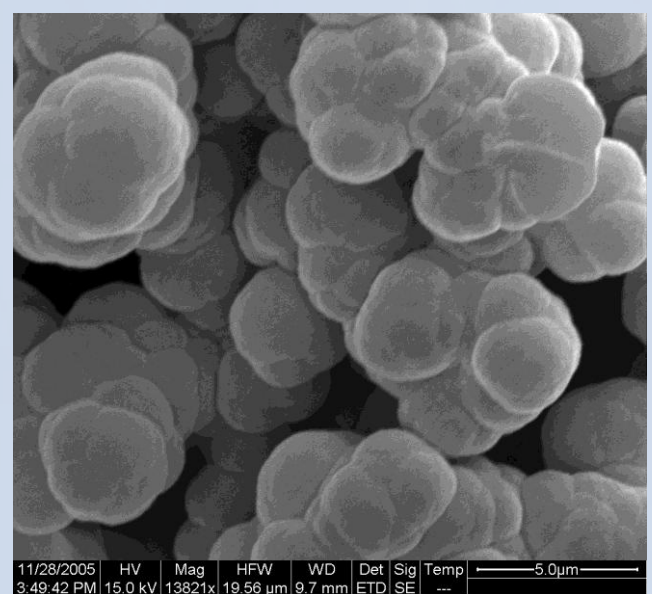


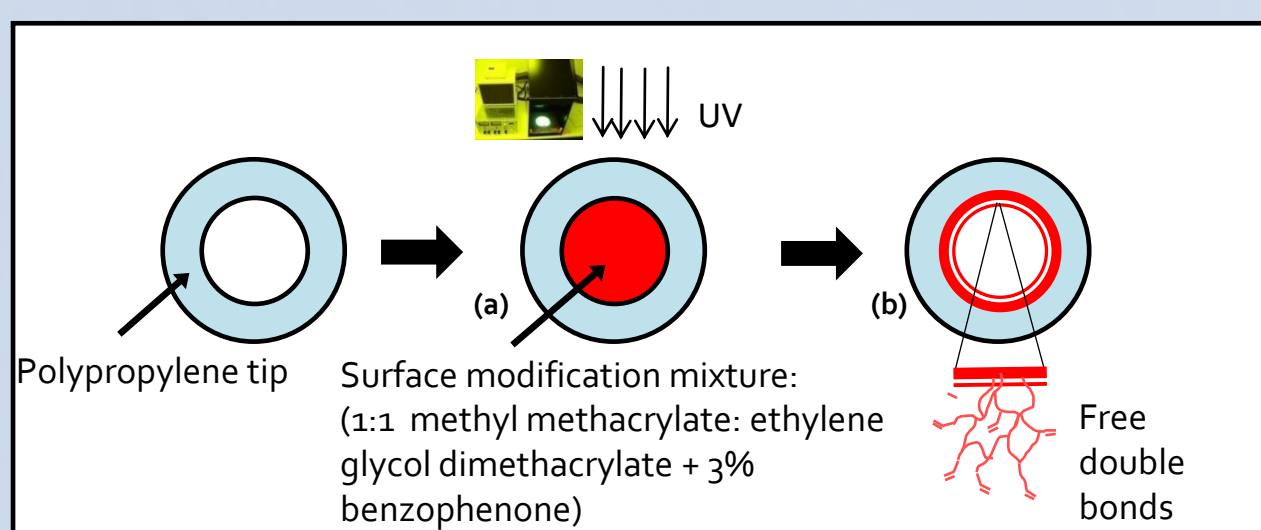
Figure 1. SEM image of organic polymer monolith at a magnification of 13000X.

The micrometer-sized pores and large surface area of monoliths could reduce the diffusion path length and provide low-pressure drop, leading to high digestion efficiency.

## 2. The Goal

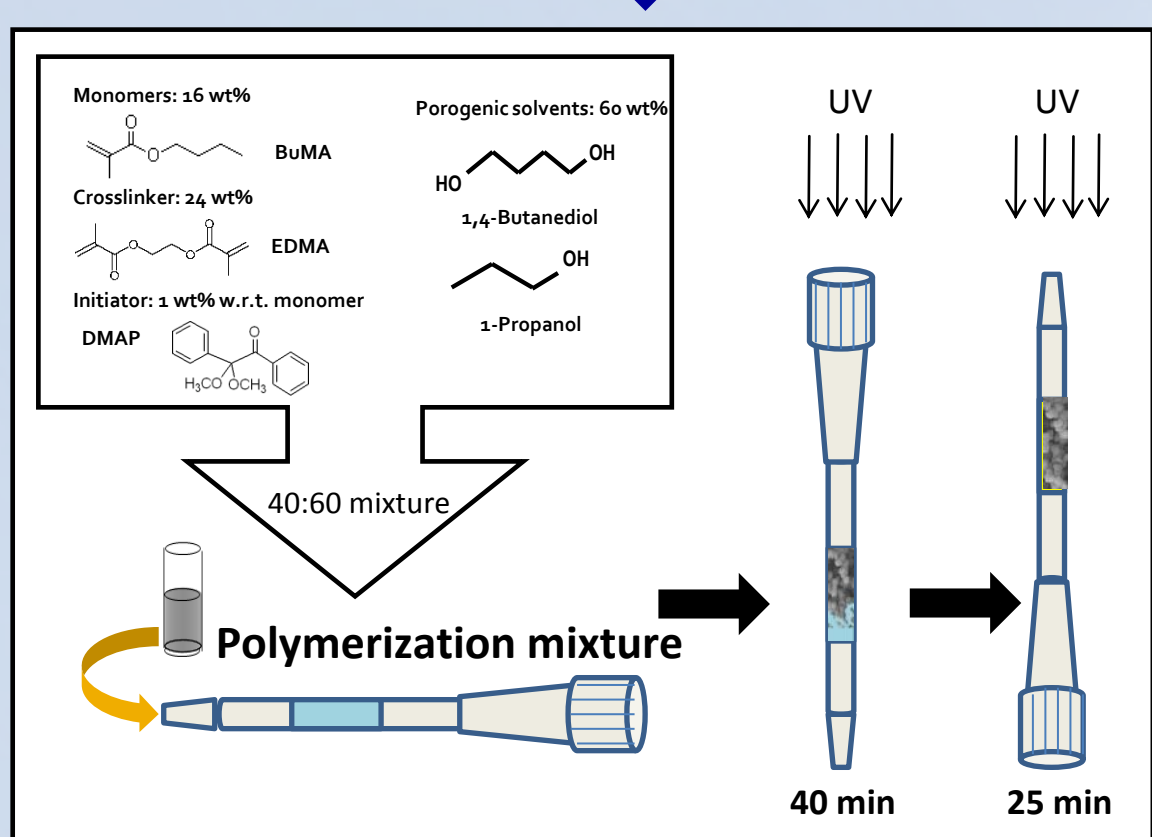
- Synthesis of monoliths *in situ* in polypropylene pipette tips.
- Surface modification of monolithic polymer via photografting to obtain reactive azlactone functionalities for trypsin immobilization.
- To perform digestion of model proteins and samples spiked in serum using the immobilized enzymatic polypropylene pipette tip (IMEPP).

## 3. Approach



### Surface modification of the PP tips

Figure 2. Schematic diagram of the photoinduced surface modification and preparation of a monolith in an empty pipette tip<sup>2</sup>.



### Preparation of polymer monolith *in situ* in PP tips

Figure 3. Schematic diagram showing *in situ* preparation of polymer monoliths in polypropylene pipette tips<sup>3</sup>.

### Photografting of reactive azlactone functionalities for trypsin immobilization

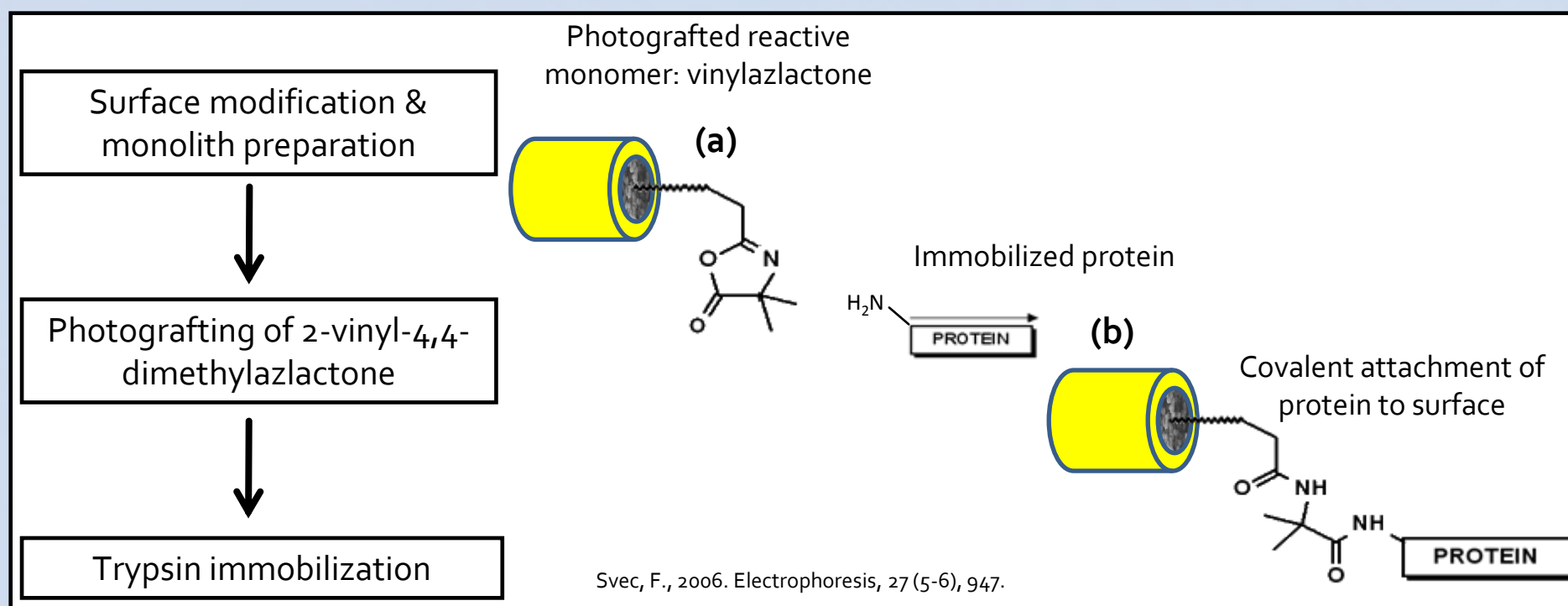


Figure 4. Schematic diagram of the photopatterning process<sup>2</sup>.

### Protein digestion steps

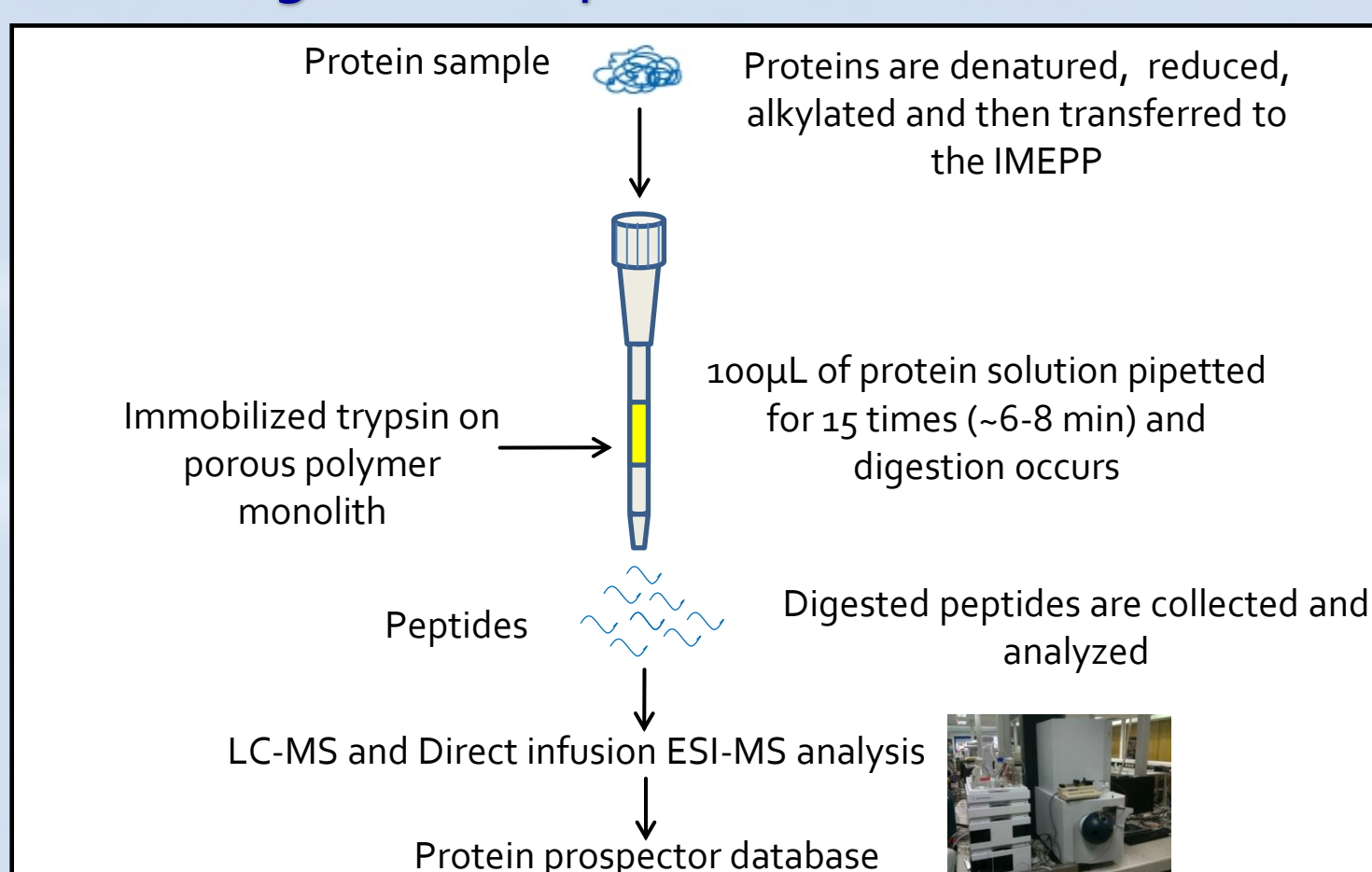


Figure 5. Schematic diagram of protein digestion.

## Instrumentation



Figure 6. OAI deep UV illumination system (Model LS30/5) fitted with a 500W HgXe-lamp for the surface modification and *in situ* preparation of the organic polymer monolith in the polypropylene pipette tips, and photografting of reactive vinylazlactone group for trypsin immobilization.

LC-MS separation was performed with an Agilent 1200 Series LC (Agilent Technologies, Palo Alto, CA) coupled to the micrOTOF-Q-MS from Bruker Daltonics (Bremen, Germany), operating at a resolution of 10000. Separation conditions: 2.1 x 100mm i.d. Dionex C16 column; injection volume, 5 µL. Mobile phases: A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile.

## 4. Results

### Surface modification of the PP tips

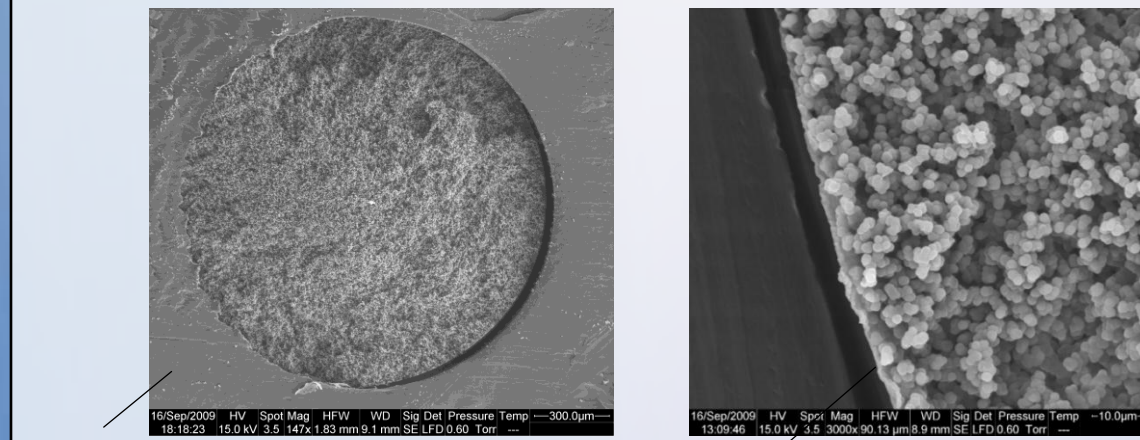


Figure 7. SEM images of porous polymer monoliths inside a PP tube **without** surface modification (a) and the magnified part (b).

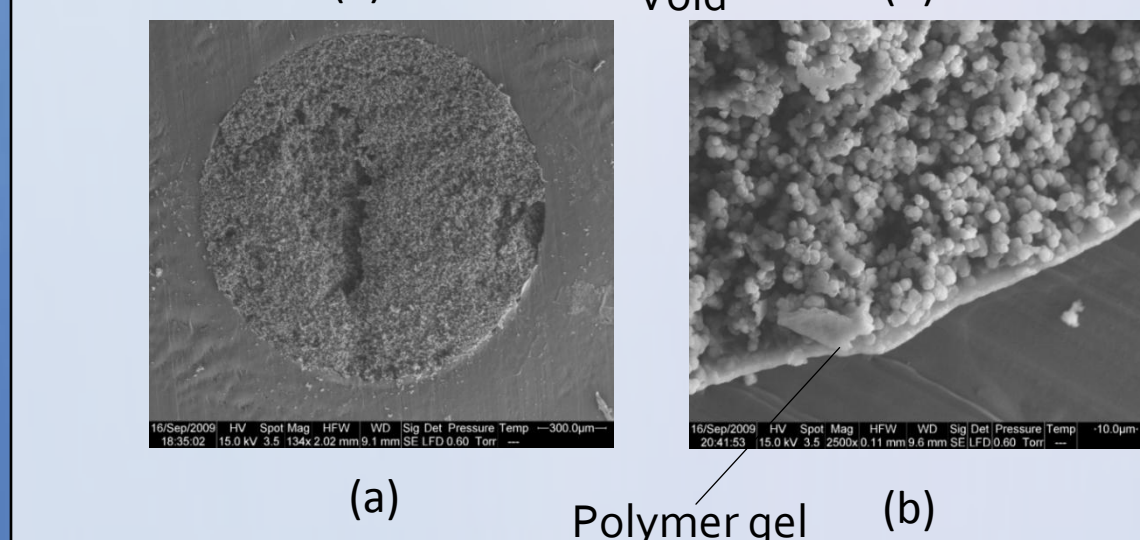


Figure 8. SEM images of single-step surface modification of PP tip **with** MMA/EDMA 1:1 with BP (3 wt. %) (a) and the magnified part (b).

### Effects of the VAL concentration and exposure time on IMEPP protein digestion of cytochrome c

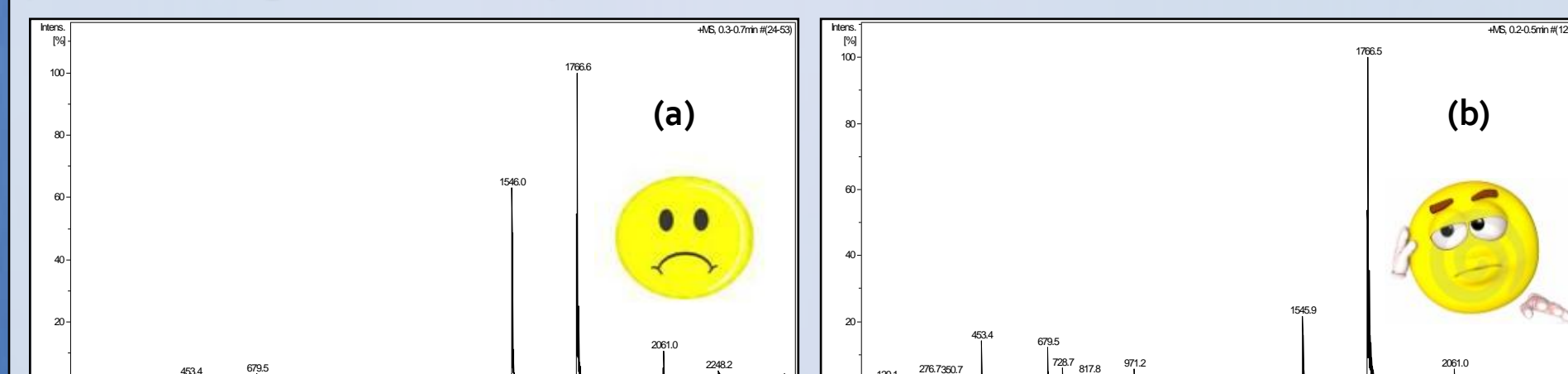


Figure 9. ESI-TOF mass spectrum obtained from cytochrome c digestion using pipette tips (a) **without** VAL photografting; (b) photografting under UV light at an exposure time of **2 min** with **15% VAL** in photografting mixture; (c) exposure time of **30 min** with **25% VAL** in photografting mixture.

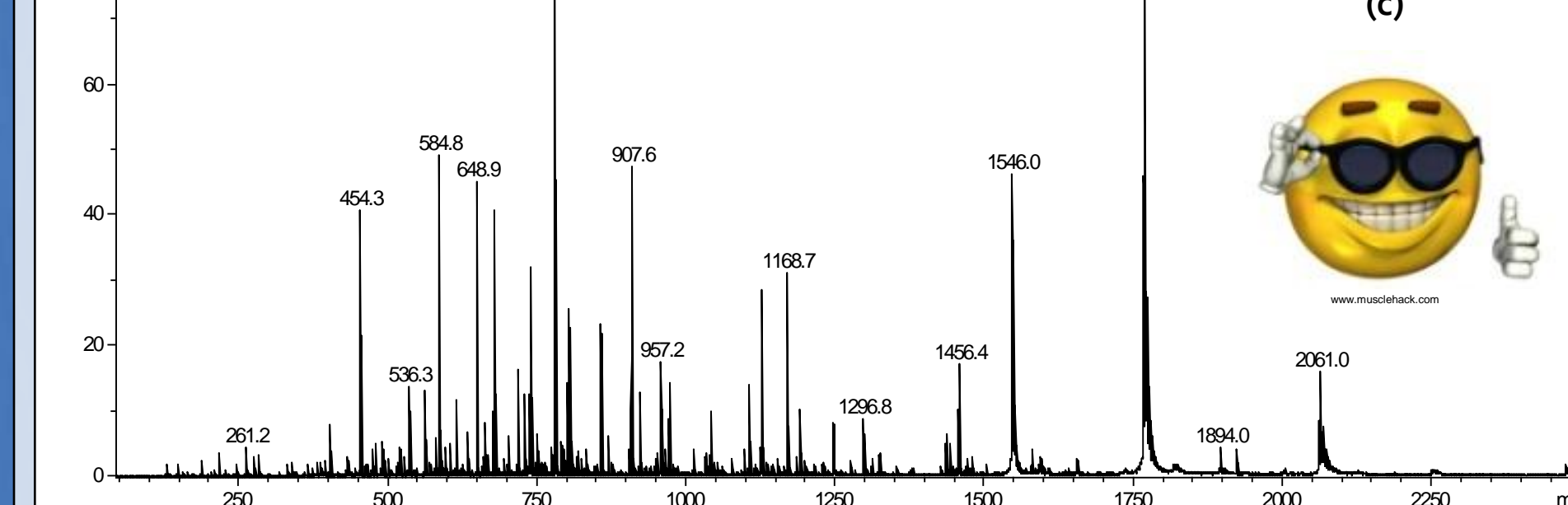
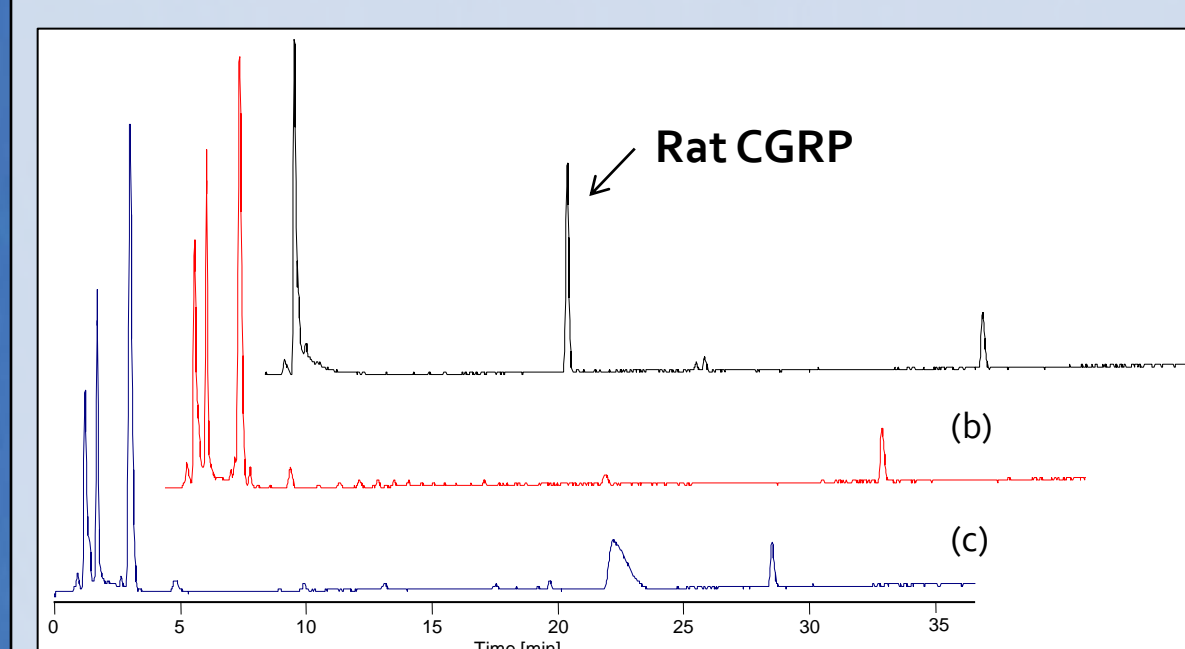


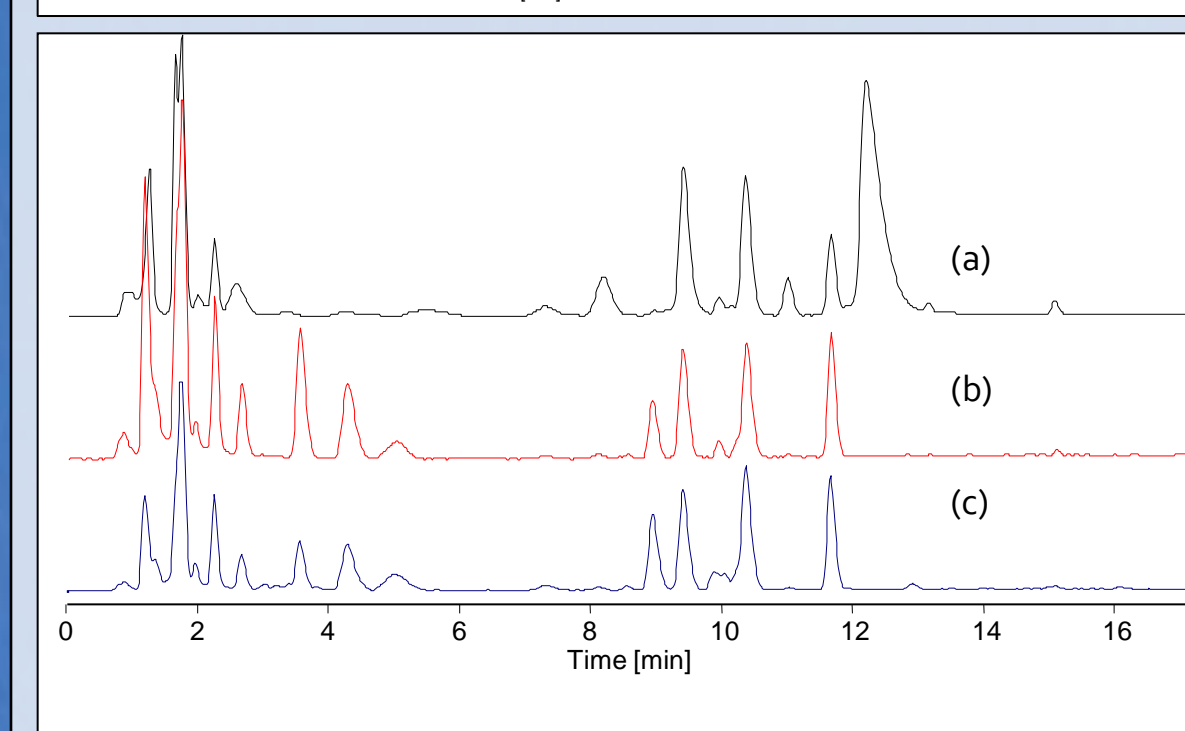
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### Protein digestion and LC-ESI-TOF MS



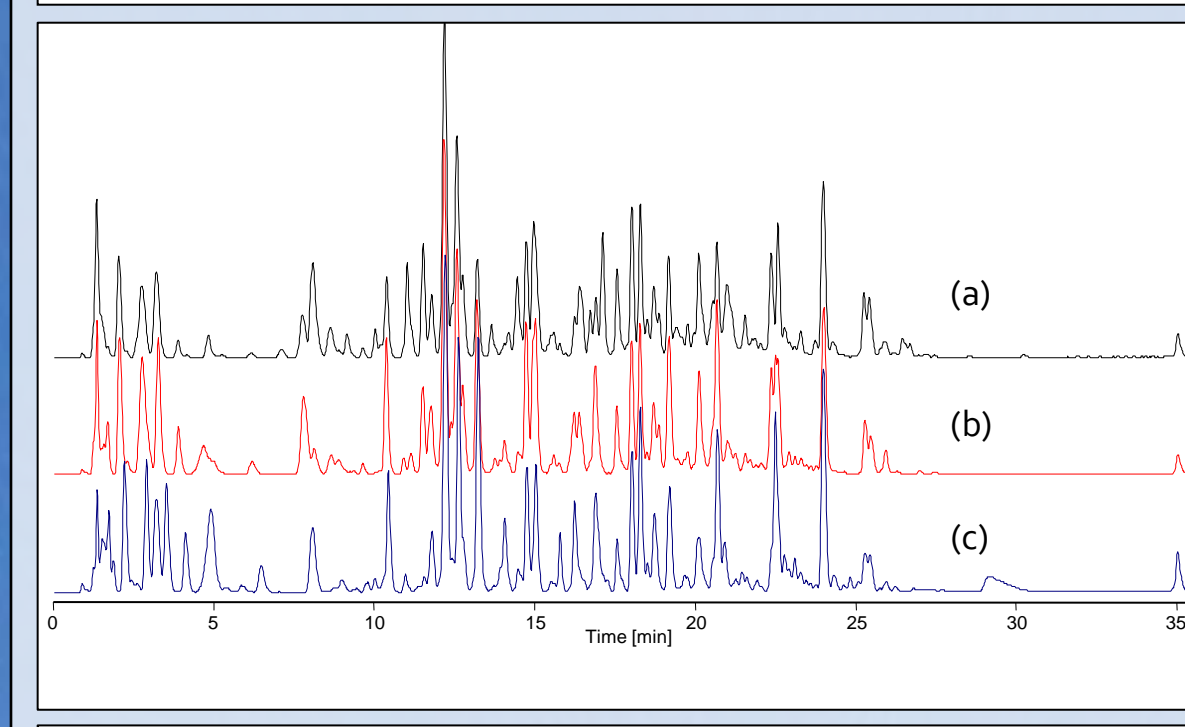
### Rat CGRP (0.005mg/mL)

Figure 10. Separation of peptides resulting from the Rat CGRP digestion using the IMEPP. Digestion condition as in Fig. 9. Separation conditions: Gradient: 0 min, 20% B and then ramp from 20% B to 55% B in 35 min; flow rate, 0.2 mL/min. BPC, base peak chromatogram.



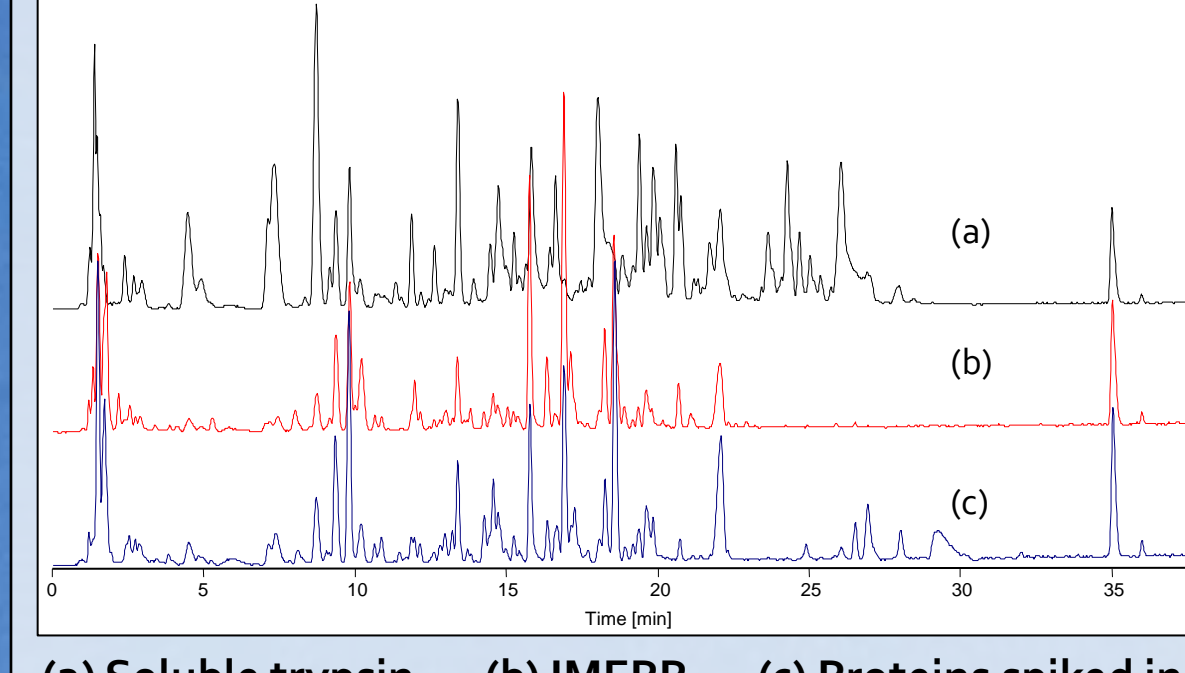
### Cytochrome C (0.01mg/mL)

Figure 11. Separation of peptides resulting from the cytochrome c digestion using the IMEPP and soluble enzyme. Digestion and separation conditions as in Fig. 10.



### BSA (0.5mg/mL)

Figure 12. Separation of peptides resulting from the BSA digestion using the IMEPP and soluble enzyme. Digestion and separation conditions as in Fig. 10. Gradient: 0 min, 10% B and then ramp from 10% B to 50% B in 35 min.



### hIgG (0.5mg/mL)

Figure 13. Separation of peptides resulting from the BSA digestion using the IMEPP and soluble enzyme. Digestion and separation conditions as in Fig. 12.

## Protein digestion and mass spectrometry

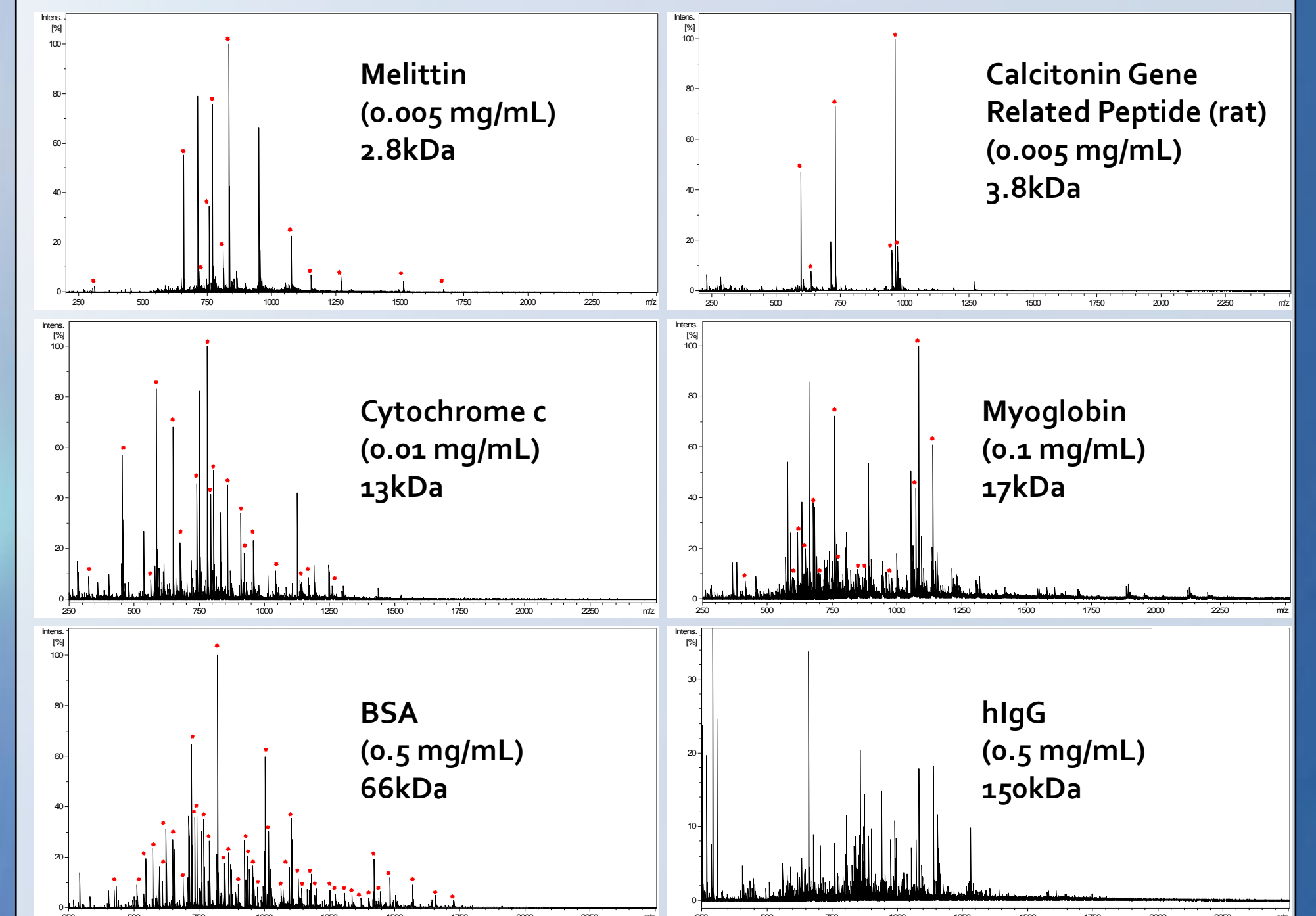


Figure 14. ESI-TOF MS spectra of peptides obtained by digestion of six proteins using IMEPP. Other conditions as in Fig. 9.

## Digestion of proteins spiked in rat serum

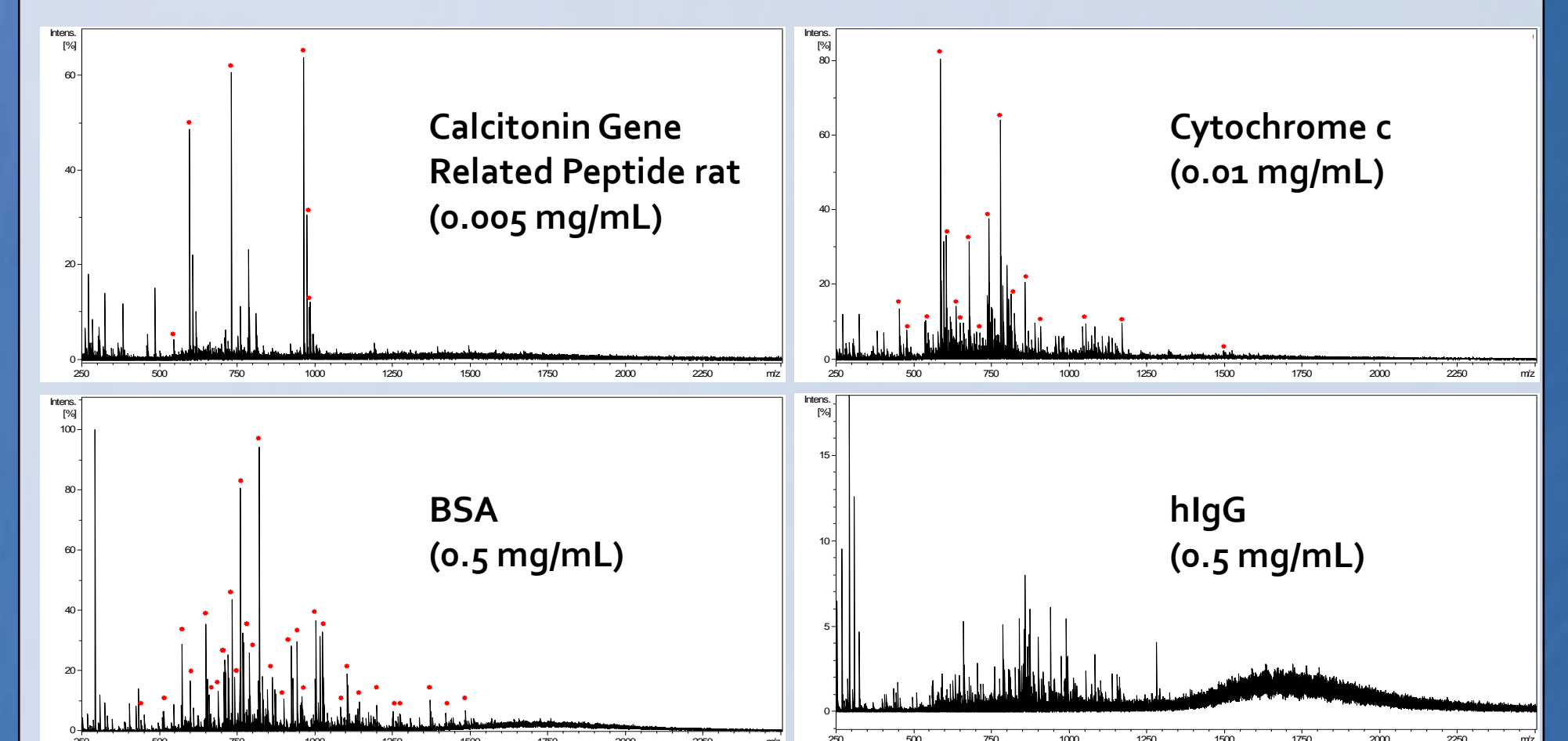


Figure 15. ESI-TOF MS spectra of peptides obtained by digestion of four proteins spiked in rat serum (~70µg proteins in 100 µL) using IMEPP. Other conditions as in Fig. 9.

Table 1. Results of sequence coverage for the digestion of proteins and samples spiked in rat serum using IMEPP.

Protein	MW (Da)	Concentration (mg/mL)	Sequence Coverage (%)		
			Soluble trypsin	IMEPP	IMEPP (Spiked sample)
Melittin	2848	0.005	100	100	100
Rat CGRP	3806	0.005	100	100	100
Cytochrome c	11702	0.01	97	95	87
myoglobin	16951	0.1	95	89	66
BSA	69294	0.5	92	88	84

Table 2. Comparison of sequence coverage identification of hIgG using soluble trypsin, IMEPP and IMEPP for sample spiked in rat serum.

Protein	MW (Da)	Sequence Coverage (%)		
		Soluble trypsin	IMEPP (0.5mg/mL)	IMEPP (Spiked sample 0.5 mg/mL)
IgG-1	36106	51	31	5
IgG-2	35901	45	37	22
IgG-3	41287	39	36	32
IgG-4	35941	34	42	27
IgG-kappa	11609	80	68	61
IgG-lambda	11237	66	75	75

## 5. Conclusions

- Monoliths can be synthesized *in situ* in polypropylene pipette tips. ✓
- Surface modification of monolithic polymer can be used to create reactive azlactone functionalities for trypsin immobilization. ✓
- These tips can be used to perform digestion of proteins and peptides in standard buffer and in rat serum. ✓

## 6. Future work

- LC-MS/MS separation to evaluate the efficiency and selectivity of the tips
- Characterization of the tips (e.g. amount of grafted VAL and enzyme immobilized)
- Digestion of bioanalytical samples.
- Integration of an automated 96-tip robotic device to allow enzymatic digestion of samples within a few minutes.



## References:

- J. Krenkova, N.A. Lacher, F. Svec, Anal. Chem. 81 (2009) 2004.
- T.B. Stachowiak, T. Rohr, E.F. Hilder, D.S. Peterson, M. Yi, F. Svec, J.M.J. Frechet, Electrophoresis 24 (2003) 3689.
- Z. Altun, A. Hjelmstroem, M. Abdel-Rehim, L.G. Blomberg, J. Sep. Sci. 30 (2007) 1964.