Analyst

COMMUNICATION

A mesofluidic platform integrating on-chip probe ultrasonication for multiple sample pretreatment involving denaturation, reduction, and digestion in protein identification assays by mass spectrometry†

Cite this: DOI: 10.1039/c3an02178e

Received 23rd November 2013
Accepted 3rd January 2014

DOI: 10.1039/c3an02178e

www.rsc.org/analyst

J. D. Nunes-Miranda,[abc] Cristina Núñez,[cd] Hugo M. Santos,[bc] G. Vale,[ce] Miguel Reboiro-Jato,[f] Florentino Fdez-Riverola,[f] Carlos Lodeiro,[c] Manuel Miró*[g] and J. L. Capelo*[c]

The integration of ultrasound (US)-assisted sample processing on-chip for automated high-throughput shotgun proteomic assays is herein presented for the first time. The proof of concept of this system was demonstrated with the analysis of three proteins and serum from patients with lymphoma or myeloma.

One of the most powerful tools to date in proteomics arises from the use of many different MS-based approaches for protein identification.[1] Over the past decade we have witnessed the development of a wealth of distinct strategies to (a) reduce the time needed to perform protein digestion and to (b) simplify sample handling for protein identification.[2] The use of external energy sources, such as heating,[3] ultrasonication,[4–6] infrared radiation,[7,8] high pressure[9] or spinning,[10] has been proven most appropriate for fast, efficient and reproducible sample treatment in protein identification assays from complex biological specimens.

Ultrasonic energy as a way to speed up the enzymatic digestion of protein cleavage from overnight (12 h) to less than 120 s was first reported in 2005 (ref. 5) and was validated on a short notice by different research groups.[11–14] Later, the use of ultrasonic energy was successfully extended to different steps of sample handling for protein identification, namely, protein solubilization/denaturation, protein reduction and protein alkylation.[15] Identification workflows circumventing desalting procedures using ultrasonication have been also described.[16]

Properties of physical and chemical reactions are dramatically modified under the effect of an ultrasonic field generated by an ultrasonic probe (High-Intensity Focused Ultrasound, HIFU).[17] Although the mechanism that is responsible for the enzymatic digestion enhancement using focused ultrasound is not completely understood, it appears to be related to the increase in mass transfer rates induced by the cavitation phenomena and heating from ultrasonication.[17]

The field of microfluidics has evolved tremendously over the past decade and attracted a great deal of attention in the bioanalytical arena[18] for expedient probing of single cells,[19] the manipulation, identification and separation of cells (*e.g.*, cancer cells),[20–22] the examination of protein structure and function,[23] the simplification of polymerase chain reaction (PCR) procedures,[24,25] and the exploration of aptamer interactions with proteins or small molecules.[26,27] Recent trends geared towards the integration of overall (bio)analytical protocols on-chip including electrophoretic and microsolid-phase extraction approaches for purification, enrichment and digestion of target species.[28,29] The third generation of flow injection, the so-called lab-on-a-valve (LOV) concept, opened up a host of prospects for microfluidic handling of biological specimens and simplification of analytical workflows exploiting automatic programmable flow.[30–32] Developmental milestones of LOV in bioanalytics over the past few years include the automation of nucleic acid assays, the miniaturization of affinity chromatographic separations of proteins and DNA, and the reliable and expeditious accommodation of enzymatic and cellular assays and immunoassays on-chip as pinpointed in recent comprehensive reviews.[33–35]

This paper introduces a novel methodology for automatic protein digestion on-chip in the homogeneous phase using an LOV configuration integrating probe sonication for expeditious protein reduction, alkylation and digestion for shotgun

[a]Department of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal
[b]Institute for Biotechnology and Bioengineering, Centre of Genomics and Biotechnology, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal
[c]REQUIMTE, Departamento de Química, Faculdade de Ciencias e Tecnologia, FCT, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal. E-mail: jlcm@fct.unl.pt
[d]Ecology Research Group, Department of Geographical and Life Sciences, Canterbury Christ Church University, CT1 1QU, Canterbury, UK
[e]Centro de Química Estrutural, Instituto Superior Técnico de Lisboa, Torre Sul, Lisbon, Portugal
[f]SING Group, Informatics Department, Higher Technical School of Computer Engineering, University of Vigo, Ourense, Spain
[g]FI-TRACE Group, Department of Chemistry, University of the Balearic Islands, Palma de Mallorca, Spain. E-mail: manuel.miro@uib.es

† Electronic supplementary information (ESI) available: Experimental section – chemicals and starting materials and instrumentation methods. See DOI: 10.1039/c3an02178e

proteomics. The mesochannel system is fabricated as a monolithic structure and mounted atop a conventional multiposition valve in sequential injection networks for facilitating the automation of wet chemical assays. In addition to compactness and portability, the main asset of LOV is its open architecture to accommodate reactions of diverging kinetics without platform reconfiguration. The permanent rigid position of the sample processing channels also ensures repeatability of mesofluidic manipulations. This provides robustness and reliability of operation, and makes the LOV system amenable to real life samples and peripheral instruments. A vast amount of effort has been directed over the past few years toward the simplification of proteolytic digestion using on-line or on-chip configurations[9,33–43] as well as the integration of probe sonication in microfluidic/mesofluidic devices.[44,45]

A syringe pump with programmable speed (Crison, Spain) equipped with a 500 μL gas tight glass syringe (Hamilton, Switzerland) was utilized as a liquid driver for mesofluidic operations. The dedicated LOV mesoconduit fabricated from chlorotrifluoroethylene (Kel-F) for chemical resistance encompasses eight integrated mesochannels (1.2 mm i.d./14.0 mm length), excepting the integrated reaction chamber with a nominal capacity of 600 μL (port 5) that was enlarged to house the tip of the sonication device (Dr Hielscher, model UTR200, Teltow, Germany). The mesofluidic platform was mounted atop of an eight-port multiposition selection valve (Valco Instruments, Houston, TX). All the modules of the SIA system (automatic burette, valve, fluorimeter) are connected to a computer *via* an RS-232C interface and controlled by the Autoanalysis Station 3.0 Software (SCIware, Palma, Spain) to address the peripheral ports of the unit (1–8), for sequential aspiration of the various constituents for the US-based protein digestion procedure. The flow network was built from a PTFE tubing of 0.5 mm i.d., excepting the tubing connecting the pump with the external carrier reservoir, which was made from 1.5 mm i.d. PTFE tubing. The holding coil (HC) has a capacity of 500 μL. The LOV assembly for automatic sample processing in shotgun proteomics is schematically illustrated in Fig. 1. The analytical procedure for automatic on-chip protein digestion exploiting US assisted LOV is listed in Table S1.†

To test the applicability of the LOV mesofluidic system for on-chip protein digestion, the following parameters were investigated in detail: (1) pH; (2) ammonium bicarbonate buffer/acetonitrile ratio; and (3) ultrasonication amplitude.

It is well known that enzymatic digestion needs to be carried out under well-controlled pH conditions, as trypsin exhibits maximum activity at a pH slightly above 7.[46] Therefore, the first approximation to this issue was to assay two different pHs, 7.3 and 7.8. To this end, samples were prepared in 12.5 mM or 100 mM AMBIC (with ACN at a 1 : 1 ratio) to obtain the digestion pHs of 7.3 and 7.8, respectively.

LOV assays for α-lacta, BSA and OVA were compared with the batchwise (off-line) counterparts (see the ESI†). Results showed that digestion of BSA at pH 7.8 yielded more peptides and a better sequence coverage in both off-line and on-line approaches (data not shown). At such pH, the proteins studied were correctly identified either by the off-line or by the

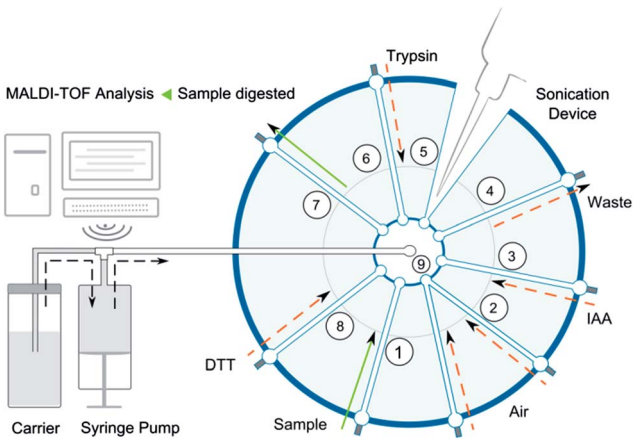

Fig. 1 Diagrammatic description of the lab-on-a-valve system used for automated US-assisted proteolytic digestion of proteins.

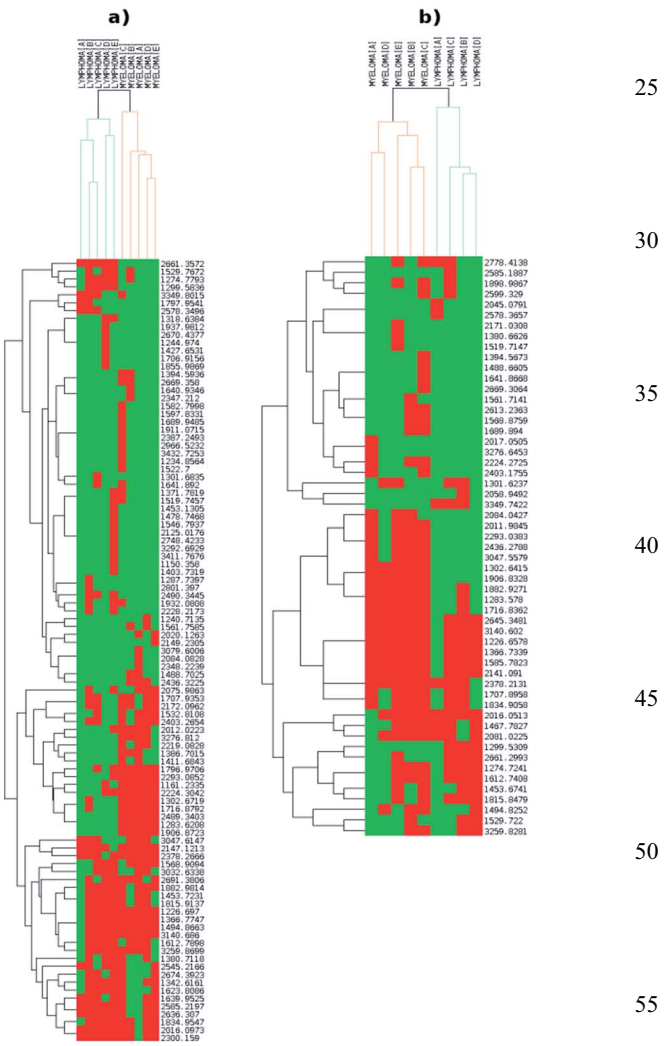

Fig. 2 Clustering analysis of spectra obtained for sera samples of ten patients, five with lymphoma and five with myeloma. (a) Off-line sample treatment; (b) lab-on-valve sample treatment.

LOV method, except ovalbumin. This protein was identified under the present experimental conditions only with the off-line method. A detailed comparison of the peptides identified for the three proteins by the two methods reveals that the common peptides formed were as follows: 86% for α-lacta, 50% for OVA and 40% for BSA (see Fig. S1†). Also the number of methylation reactions observed were similar, thus indicating that alkylation is not altered when the sample treatment is done in the LOV platform. The sequence coverage and the number of peptides identified for the three proteins were almost the same regardless of the method used (see Fig. S2†).

OVA was proven not to be completely dissolved in 100 mM AMBIC/ACN at a 1 : 1 volume ratio, leading to failure in its digestion and subsequent identification. This is most likely a consequence of the large amount of acetonitrile used for protein solubilization/denaturation, as large proteins tend to precipitate in solutions containing ACN concentrations equal or higher than 50%.[16,43] For this reason, we decided to increase the AMBIC/ACN ratio from 1 : 1 to 3 : 1. Ovalbumin was then entirely dissolved, which indeed contributed to the positive identification of the protein as shown in Fig. S1.† Therefore, the AMBIC/ACN ratio was affixed to 3 : 1 for further studies.

Ultrasonic amplitude is one of the core parameters that most significantly influence the efficiency of ultrasonication in liquid samples. Never before (to the best of our knowledge), ultrasonication had been assayed inside a chip microdevice. Previous experiments have established that the amplitude should be thoroughly optimized in protein cleavage assays.[47,48] If the amplitude is settled too low or too high the sequence coverage and the number of peptides matched are lower than those obtained when medium amplitude is chosen. In the first case because the cleavage is not boosted adequately while in the second case because the sample is degraded. Therefore, it was decided to assess the effects of the ultrasonic amplitude by varying it in the range spanning from 20% to 50%. BSA and α-lacta were selected for this set of experiments.

Fig. S2(A)† shows better sequence coverage for α-lacta protein using 50% amplitude than 30% amplitude but the difference is not significant. In addition, the number of peptides matched with both amplitudes is almost the same. However, for the case of BSA 30% is clearly the best amplitude as the number of peptides matched is considerably higher than with any of the other amplitudes. Therefore, it is considered that the sample treatment is not sample dependent. Accordingly, the amplitude of 30% was selected for further experiments.

To evaluate the applicability of the automated ultrasonic-based LOV method for identification of proteins in complex biological samples, a number of sera samples from two different groups of patients were digested. Sera from five patients with lymphoma and five patients with myeloma were used in a profiling-based approach as described in the ESI (see Table S2†). For control purposes the samples were off-line treated in the same manner as in the LOV platform, that is, using the same reagents (volumes and concentrations) and the same ultrasonic variables (time and amplitude).

Sera samples were first depleted from the most abundant proteins following a chemical sequential depletion method described in the ESI.† Once depletion was completed, the samples were reduced (20 mM DTT) and alkylated (150 mM IAA) using ultrasonic energy (30% UA and 1 min UT, for reduction and alkylation and 30% and 5 min for protein digestion) using the optimized protocol. Once the spectra of the ten samples were obtained in quintuplicate, the statistical treatment described in the ESI† was carried out.

Clustering analysis performed with the spectra is depicted in Fig. 2. With the batchwise (off-line) sample treatment it was possible to match all the samples to patients with either lymphoma or multiple myeloma (Fig. 2a). The same classification was almost obtained when the samples were treated on-line. Only one sample was not correctly classified and was deemed to be an outlier, as it was not classified within any group (Fig. 2b). A closer view of the MALDI spectra for this sample revealed a spectrum with fewer signals and with lower intensity that the ones obtained for the other sera. This is most likely due to a problem during crystallization in the MALDI plate rather than to the sample treatment. Due to this issue, Fig. 2b presents the clustering result without this sample (lymphoma [E]).

Conclusions

On-chip US assisted sample handling proposed in this work based on the LOV mesofluidic concept opens new avenues in proteomics. The conjunction of protein digestion and ultrasonication on-chip allows for automatic processing and fingerprinting of human serum of patients with myeloma and lymphoma taken as model samples and minimizes the risk of contamination and experimental errors thereby reducing the measurement uncertainty so as to improve the quality of proteomics data.

The optimal conditions for automatic protein digestion in the LOV platform involved an ammonium bicarbonate solution/acetonitrile ratio of 3 : 1, an ultrasonication amplitude of 30% and a digestion time under the effects of ultrasound of 5 min at pH 7.8. The sample treatment workflow in the LOV platform takes about 25 min per sample, which is due to the low velocity rates employed when loading/dispensing the solution volumes. This represents about 10 minutes more comparing with the off-LOV method. However, the off-LOV method requires laborious sample handling and the permanent assistance of a technician and is prone to sample contamination.

Future work will address protein quantification by 18-O labeling as well as protein identification by on-line coupling the LOV system to HPLC-MS/MS for shotgun proteomics.

Acknowledgements

J. D. Nunes-Miranda acknowledges the doctoral grant SRFH/BD/80496/2011. C. Núñez thanks Xunta de Galicia for her post-doctoral contract (I2C program). G. Vale and H. M. Santos, acknowledge the post-doctoral grants SFRH/BPD/73117/2010 and SRFH/BPD/73997/2010 respectively, provided by the

Portuguese Foundation of Science and Technology (Fundação para a Ciência e a Tecnologia-Ministério da Educação e Ciência). Manuel Miró acknowledges financial support from the Spanish Ministry of Economy and competitiveness through project CTM2010-17214. The authors are grateful to Dr V. Cerda for the loan of analytical instrumentation. Scientific Society ProteoMass is acknowledged for financial support. The authors also thank REQUIMTE-FCT PEst-C/EQB/LA0006/2013.

Notes and references

1 N. Blow, *Nat. Methods*, 2008, **5**, 741.
2 J. L. Capelo, R. Carreira, M. Diniz, L. Fernandes, M. Galesio, C. Lodeiro, H. M. Santos and G. Vale, *Anal. Chim. Acta*, 2009, **650**, 151.
3 J. Havlis, H. Thomas, M. Sebela and A. Shevchenko, *Anal. Chem.*, 2003, **75**, 1300.
4 M. Galesio, D. V. Vieira, R. Rial-Otero, C. Lodeiro, I. Moura and J. L. Capelo, *J. Proteome Res.*, 2008, **7**, 2097.
5 D. Lopez-Ferrer, J. L. Capelo and J. Vazquez, *J. Proteome Res.*, 2005, **4**, 1569.
6 R. Rial-Otero, R. J. Carreira, F. M. Cordeiro, A. J. Moro, L. Fernandes, I. Moura and J. L. Capelo, *J. Proteome Res.*, 2007, **6**, 909.
7 H. Bao, T. Liu, X. Chen and G. Chen, *J. Proteome Res.*, 2008, **7**, 5339.
8 S. Wang, T. Liu, L. Zhang and G. Chen, *J. Proteome Res.*, 2008, **7**, 5049.
9 D. Lopez-Ferrer, K. Petritis, N. M. Lourette, B. Clowers, K. K. Hixson, T. Heibeck, D. C. Prior, L. Pasa-Tolic, D. G. Camp, 2nd, M. E. Belov and R. D. Smith, *Anal. Chem.*, 2008, **80**, 8930.
10 S. P. Mirza, A. S. Greene and M. Olivier, *J. Proteome Res.*, 2008, **7**, 3042.
11 J. Klimek, J. S. Eddes, L. Hohmann, J. Jackson, A. Peterson, S. Letarte, P. R. Gafken, J. E. Katz, P. Mallick, H. Lee, A. Schmidt, R. Ossola, J. K. Eng, R. Aebersold and D. B. Martin, *J. Proteome Res.*, 2008, **7**, 96.
12 M. Carrera, B. Canas and J. M. Gallardo, *J. Proteomics*, 2012, **75**, 3211.
13 K. C. Hansen, L. Kiemele, O. Maller, J. O'Brien, A. Shankar, J. Fornetti and P. Schedin, *Mol. Cell. Proteomics*, 2009, **8**, 1648.
14 H. Lam, E. W. Deutsch, J. S. Eddes, J. K. Eng, N. King, S. E. Stein and R. Aebersold, *Proteomics*, 2007, **7**, 655.
15 F. M. Cordeiro, R. J. Carreira, R. Rial-Otero, M. G. Rivas, I. Moura and J. L. Capelo, *Rapid Commun. Mass Spectrom.*, 2007, **21**, 3269.
16 H. M. Santos, C. Mota, C. Lodeiro, I. Moura, I. Isaac and J. L. Capelo, *Talanta*, 2008, **77**, 870.
17 T. J. Mason, *Philos. Trans. R. Soc., A*, 1999, **357**, 355.
18 M. L. Kovarik, P. C. Gach, D. M. Ornoff, Y. Wang, J. Balowski, L. Farrag and N. L. Allbritton, *Anal. Chem.*, 2012, **84**, 516.
19 Y. Xu, K. Jang, T. Yamashita, Y. Tanaka, K. Mawatari and T. Kitamori, *Anal. Bioanal. Chem.*, 2012, **402**, 99.
20 J. Chen, J. Li and Y. Sun, *Lab Chip*, 2012, **12**, 1753.
21 J. Nilsson, M. Evander, B. Hammarstrom and T. Laurell, *Anal. Chim. Acta*, 2009, **649**, 141.
22 D. Pappas and K. Wang, *Anal. Chim. Acta*, 2007, **601**, 26.
23 M. Landreh, J. Astorga-Wells, J. Johansson, T. Bergman and H. Jornvall, *FEBS J.*, 2011, **278**, 3815.
24 F. Ahmad and S. A. Hashsham, *Anal. Chim. Acta*, 2012, **733**, 1.
25 Y. Zhang and P. Ozdemir, *Anal. Chim. Acta*, 2009, **638**, 115.
26 J. Hu and C. J. Easley, *Analyst*, 2011, **136**, 3461.
27 Y. Xu, X. Yang and E. Wang, *Anal. Chim. Acta*, 2010, **683**, 12.
28 B. C. Giordano, D. S. Burgi, S. J. Hart and A. Terray, *Anal. Chim. Acta*, 2012, **718**, 11.
29 C.-C. Lin, J.-L. Hsu and G.-B. Lee, *Microfluid. Nanofluid.*, 2011, **10**, 481.
30 M. Miró and E. H. Hansen, *Anal. Chim. Acta*, 2007, **600**, 46.
31 M. Miró and E. H. Hansen, *Anal. Chim. Acta*, 2012, **750**, 3.
32 J. Ruzicka, *Analyst*, 2000, **125**, 1053.
33 X. W. Chen and J. H. Wang, *Anal. Chim. Acta*, 2007, **602**, 173.
34 M. D. Luque de Castro, J. Ruiz-Jiménez and J. A. Pérez-Serradilla, *TrAC, Trends Anal. Chem.*, 2008, **27**, 118.
35 M. Miró, H. M. Oliveira and M. A. Segundo, *TrAC, Trends Anal. Chem.*, 2011, **30**, 153.
36 S. Abele, P. Smejkal, O. Yavorska, F. Foret and M. Macka, *Analyst*, 2010, **135**, 477.
37 J. Lee, H. K. Musyimi, S. A. Soper and K. K. Murray, *J. Am. Soc. Mass Spectrom.*, 2008, **19**, 964.
38 J. Lee, S. A. Soper and K. K. Murray, *Analyst*, 2009, **134**, 2426.
39 T. Liu, S. Wang and G. Chen, *Talanta*, 2009, **77**, 1767.
40 J. Ma, L. Zhang, Z. Liang, Y. Shan and Y. Zhang, *TrAC, Trends Anal. Chem.*, 2011, **30**, 691.
41 A. J. Percy and D. C. Schriemer, *Anal. Chim. Acta*, 2010, **657**, 53.
42 E. C. Stigter, G. J. de Jong and W. P. van Bennekom, *Anal. Bioanal. Chem.*, 2007, **389**, 1967.
43 C. Fernandez, H. M. Santos, C. Ruiz-Romero, F. J. Blanco and J. L. Capelo, *Electrophoresis*, 2011, **32**, 2966.
44 I. Iranmanesh, R. Barnkob, H. Bruus and M. Wiklund, *J. Micromech. Microeng.*, 2013, **23**(10), 105002.
45 J. D. Adams, C. L. Ebbesen, R. Barnkob, A. H. J. Yang, H. T. Soh and H. Bruus, *J. Micromech. Microeng.*, 2012, **22**(7), 075017.
46 T. Sipos and J. R. Merkel, *Biochemistry*, 1970, **9**, 2766.
47 L. Fernandez, H. M. Santos, J. D. Nunes-Miranda, C. Lodeiro and J. L. Capelo, *Journal of Integrated Omics*, 2011, **1**, 144.
48 H. M. Santos, R. Rial-Otero, L. Fernandes, G. Vale, M. G. Rivas, I. Moura and J. L. Capelo, *J. Proteome Res.*, 2007, **6**, 3393.

