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On-chip protein sample desalting and preparation for direct coupling with electrospray ionization mass spectrometry

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Abstract

A membrane-based desalting step integrated in a MS microchip is presented: drugs, peptides and proteins are adsorbed on a hydrophobic poly(vinylidene difluoride) membrane, which allows the washing out of salts. The integration with microfluidics permits a controlled elution of analytes from the membrane and their direct mass spectrometric analysis by electrospray ionisation MS. The desalting process is demonstrated with picomole amounts of propanolol, insulin and cytochrome *c*. Moreover, this stop-and-go desalting process is tolerant to high concentrations of urea, and to the presence of reductants such as dithiothreitol. This particular feature allowed the chemical tagging of cysteines in β-lactoglobulin A with iodoacetamide. Finally, the integration of chemical tagging, on-chip desalting and MS microchip paves the way for the development of high-throughput analytical procedure for structural proteomics.

Keywords: Chip technology; Membranes; Desalting methods; Proteins; Poly(vinylidene difluoride); Iodoacetamide; Lactoglobulin

1. Introduction

The development of soft ionization techniques, such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), has made possible the analysis of large biomolecules by mass spectrometry. However one of the main problems of ESI-MS for end-users is its incompatibility with many salts that prevent ionization of biomolecules [1]. Most often, peptides and proteins come with salts (mainly sodium or potassium chloride), detergents, denaturants or solubilizing agents. Several classical solutions exist to address this problem: dialysis, solid-phase extraction (SPE), reversed-phase high-performance liquid chromatography (HPLC) and size-exclusion chromatography, but they are not very well suited for the handling of small volumes (below 5 μl) and require dedicated apparatus or workstations. Several other methods are commercially available for the desalting of minute amounts of protein samples, such as C_{18} loaded pipette tips (Millipore ZipTips) or C_{18} loaded nano-spray capillaries (LC Packings); but these products are costly, or require numerous manual handling steps.

At the same time the last few years have seen the

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developments of new miniaturized microspray emitters for coupling with ESI-MS [2]. These microdevices can be made of different materials, such as glass [3–5], silicium [6,7] or polymers like poly(dimethylsiloxane) (PDMS) [8–10], polyethylene terephthalate [11], or polyimide [12] [13]. Their clear key advantages over glass nanocapillaries are the design flexibility, the possibility of mass production thanks to fabrication technologies developed in the microelectronics industry, and the relatively low cost (at least for those made of polymers). These microchips have been used for simple infusion of samples into the mass spectrometer and some of them have reached remarkable analytical performances [7]. Examples of chip capillary electrophoresis prior to mass analysis have also been presented [5,14–21].

A few microdevices have been developed for sample cleanup and desalting: Xu et al. [22] presented a microfabricated device comprising a dialysis membrane: the sample containing salts was counterflown against an ammonium acetate buffer and directly infused into the mass spectrometer. A dramatic increase in the signal quality was obtained for oligonucleotides as well as proteins. This approach has been pursued by Xiang et al. [23] with a dual microdialysis set-up to eliminate salts and low and high molecular mass species prior to ESI-MS. The integration of a membrane-based desalting step into a polyimide MS microchip has been presented previously [24]. Basically, a poly(vinylidene fluoride) (PVDF) membrane was used as a solid-phase for desalting and solvent exchange by passive adsorption of analytes onto the membrane, washing out of salts and subsequent elution and spray. In this scheme, all steps were done manually within the microchip reservoir with relatively high volumes of samples and eluants (10–50 μl). A good desalting of drugs, peptides and proteins was obtained for samples dissolved in phosphate-buffered saline (PBS) solutions.

One particular applications of this kind of integrated desalting is the combination of chemical reactions or tagging with mass spectrometry, which has proven in the past decade to be one of the most fruitful approaches to obtain rapidly information about protein stability through $H/^{2}H$ exchange [25–27], and accessibility or involvement in reactivity [28–30] or binding reactions [31] of specific amino acids. Though mass spectrometry clearly provides less structural information than crystallographic studies, it appears to be the best analytical compromise between the information obtained and the pace of characterization that is needed in structural proteomics [32] or target or drug screening [27]. Moreover, this approach is particularly well suited to studies where very limited amount of samples are available.

Herein we present the integration of the previously presented on-chip membrane-based desalting step with microfluidics. It allows the handling of lower volumes (typically less than 1 μl pumped at low flow-rates compared to 10–50 μl pipetted in the microchip reservoir and electroosmotically driven in [24]), and a better preconcentration of the samples. Moreover, the applicability of this desalting process was checked in the presence of chaotropes such as urea. Finally, an application of this on-chip desalting for specific chemical labeling of cysteines in β-lactoglobulin A in combination with mass spectrometric analysis is presented.

2. Experimental

2.1. Materials and chemicals

Urea, S-propanolol hydrochloride and cytochrome *c* from horse heart were obtained from Fluka (Buchs, Switzerland). PBS tablets (0.138 *M* NaCl, 0.0027 *M* KCl, 0.01 *M* phosphate, pH 7.4), 1,4-dithio-D,L-threitol (DTT), iodoacetamide, β-lactoglobulin A from bovine milk and insulin from bovine pancreas were from Sigma (St. Louis, MO, USA). Methanol (Merck, Darmstadt, Germany) and acetic acid (Fluka) were used without any further purification. Deionized water (18.2 MΩ) was produced using a Milli-Q system from Millipore (Bedford, MA, USA). Proteins were dissolved in deionized water to a 1 mg/ml concentration. The PVDF membrane is an Immobilon-P membrane from Millipore.

2.2. Chip fabrication and coupling with MS

The polyimide microchips are provided by DiagnoSwiss (Monthey, Switzerland). Their fabrication

has been described in detail elsewhere [33,34]. The microchannel is 120 μm wide, 35 μm deep and 1 cm long. The coupling of the microchip with the mass spectrometer is quite simple, and has been presented previously [11,13]: one extremity of the microchannel is cut in a tip shape, and placed in front of the heated capillary of the MS. The spraying voltage (typically between 2 and 2.8 kV) is applied through a microelectrode embedded within the microchannel, and the position of the chip is adjusted to obtain a stable spray. The microchip is coupled to a syringe pump (Cole Parmer 74900) thanks to a custom low dead-volume holder. The PVDF membrane is sandwiched between the inlet of the microchannel and the fluidic connection, as shown in Fig. 1. The mass spectrometer is a LCQ Duo Ion Trap (Finnigan, San Jose, CA, USA). The heated capillary is kept at 200 °C, and the spray voltage is adjusted in order to have a spray current between 50 and 200 nA. All spectra are acquired in the positive ion mode, in a full-scan mode. In each experiment, the ion transmission parameters are optimized automatically in order to improve the detection of the analyte of interest.

2.3. Desalting procedure

The membrane is first wet with methanol (0.2 μl/min during 5 min) and then rinsed with water (0.2 μl/min during 5 min), as recommended by the supplier. The sample is then adsorbed on the membrane by pumping the salty solution through the membrane at 0.2 μl/min to 5 μl/min. When the desired volume is pumped through the membrane, salts are washed out by pumping water (same duration and flow-rate compared to the retention step). The eluting and spraying solution (methanol–acetic acid–water, 50:1:49) is then pumped to elute and spray the retained drugs, peptides and proteins.

2.4. Alkylation of β-lactoglobulin A

β-Lactoglobulin A was incubated in PBS (pH 7.4) at a concentration of 2.7 μ*M* in the presence of 8 *M* urea and 4 m*M* DTT for 30 min at 45 °C. Just before incubation, the solution was gently flushed with nitrogen, in order to expel oxygen. Iodoacetamide was then added to a final concentration of 38 m*M*, as in a previous study [35]. The solution was flushed again with nitrogen, and the mixture was incubated at 45 °C in the dark for 30 min. DTT and iodoacetamide stock solutions were freshly prepared.

3. Results

3.1. Preconcentration of the sample

In order to investigate the preconcentration that can be obtained, propanolol dissolved in PBS to a concentration of 87 μ*M* was pumped at 0.5 μl/min during 30 min. After washing out of salts with deionized water, the spraying solution was applied at

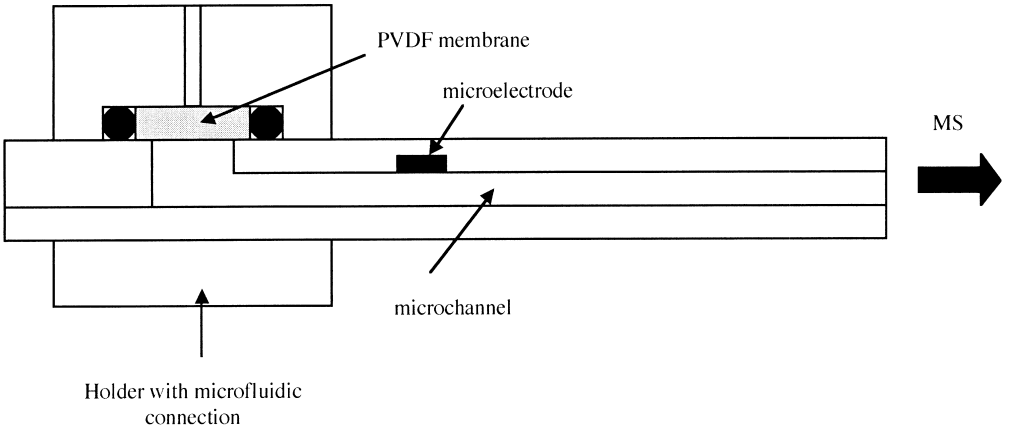

Fig. 1. Cross section of the microchip with microfluidic connection; the membrane piece is sandwiched between the microchannel reservoir and the fluidic connection.

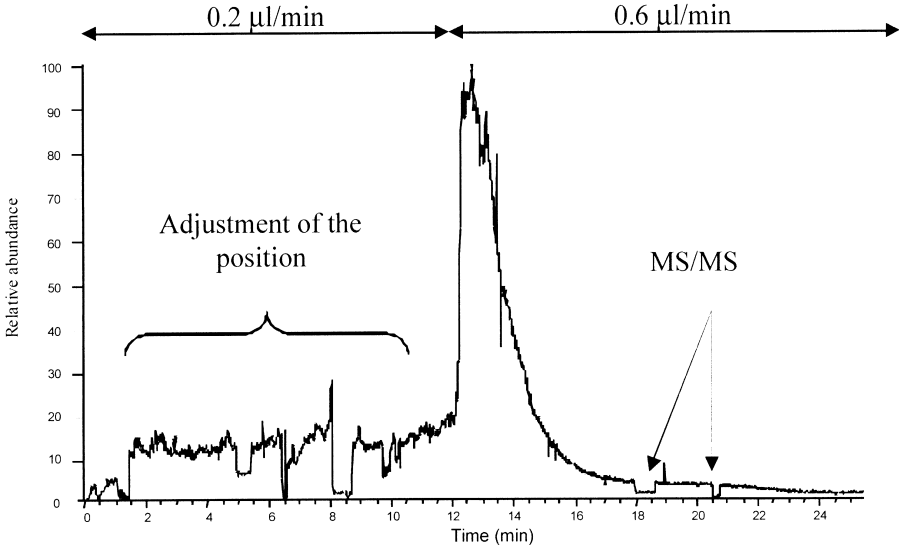

Fig. 2. Chromatogram of propanolol specific ion. 90% of propanolol was eluted within 2 min.

0.2 μl/min. The positioning of the chip was optimized and the flow-rate was then switched to 0.6 μl/min. Fig. 2 shows the elution profile of propanolol. Spectra were acquired in full-range mode without any averaging, and the chromatogram of specific ion m/z 260.3 was post processed. It can be observed that approximately 90% of propanolol is eluted within 2 min, which results in a preconcentration ratio of 12.5. The tailing that can be seen on the elution profile in Fig. 2 can be attributed to diffusive phenomena within the membrane, and desorption of the analytes into the reservoir, which represents a dead volume of 5 nl. This represents an improvement compared to the work described in Ref. [24] where eluant and spraying solution (typically 50 μl) was pipetted into the microchip reservoir and driven only by electroosmotic flow. Thus only the spraying voltage controlled the flow-rate, and the elution time could not be modulated at all. It can be noted that the elution behavior of the analyte depends on the eluant flow-rate, on the nature of the eluant, and on the concentration of organic solvent within the eluant. All three parameters can be optimized for each analyte of interest. For example to preconcentrate a very hydrophobic analyte, one can use acetonitrile instead of methanol, optimize the concentration of acetonitrile in order to recover the maximum amount of analyte from the membrane, and then increase the eluant flow-rate until the sampling rate of the mass spectrometer becomes the limiting factor. In principle, any volume can be pumped through the membrane to adsorb analytes of interest. The only limitation is the binding capacity of the membrane, which is 85 μg/cm^2 for insulin (information given by the supplier). For the 3 mm diameter pieces of membrane used on the microchip, this makes a binding capacity of 1 nmol for insulin. Given that a typical starting concentration is 50 μg/ml (which means that one has to pump ca. 115 μl through the membrane to obtain 1 nmol) and that elution takes place within 2 min at 0.6 μl/min as for propanolol, the maximum reachable preconcentration factor is ca. 95. This is obviously an upper estimation, because the usable area on the membrane is limited by the channel inlet dimensions rather than by the overall membrane dimensions, and because at this high concentration, some strong tailing is likely to occur, which will increase the elution volume.

3.2. Desalting of a mixture

A mixture of propanolol (50 μ*M*), insulin (50 μg/ml) and cytochrome *c* (50 μg/ml) dissolved in PBS was pumped through the microchip at 0.2 μl/min for 15 min. Salts were then washed out with water, and the eluting and spraying solution was

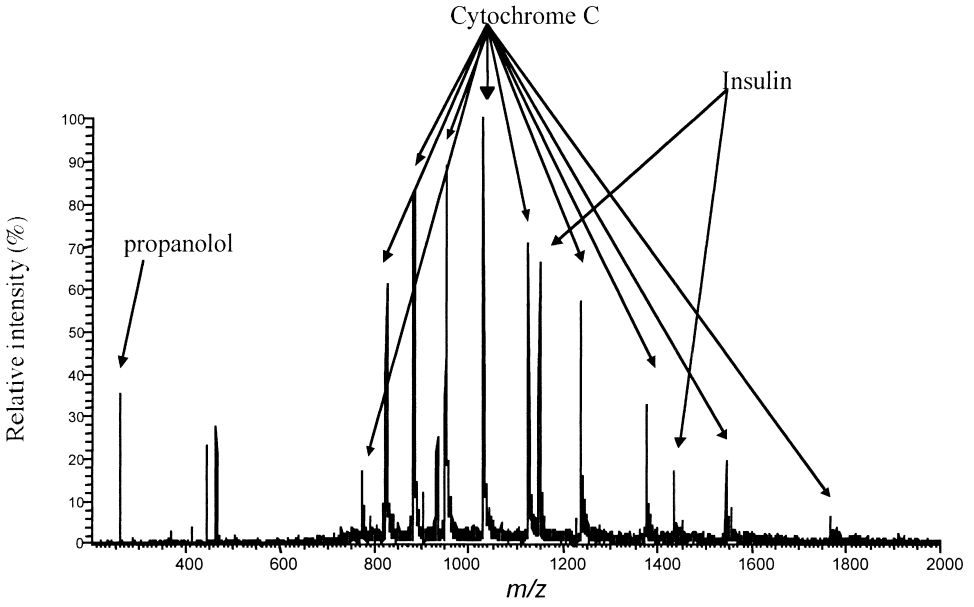

Fig. 3. Spectrum of a mixture of propanolol (50 μ*M*), insulin (50 μg/ml) and cytochrome *c* (50 μg/ml) dissolved in PBS after desalting.

applied at 0.6 μl/min. Fig. 3 shows the resulting spectrum: all analytes were recovered and no salt cluster can be observed. Extra peaks around *m*/*z* 450 are contaminants from a previous experiment. The noise level is comparable with that obtained for salt-free solutions.

3.3. Desalting in the presence of urea

Urea is used classically as a solubilizing agent for hydrophobic proteins [36] or as a denaturant. A mixture of propanolol (50 μ*M*), insulin (50 μg/ml) and cytochrome *c* (50 μg/ml) dissolved in PBS in the presence of 7 *M* urea was pumped through the microchip at 0.2 μl/min. The elution step was done at 0.6 μl/min. Fig. 4 shows the resulting spectrum, recorded without any averaging. Surprisingly, the presence of urea at high concentration does not prevent the adsorption of analytes on the PVDF membrane and does not add any spectral noise.

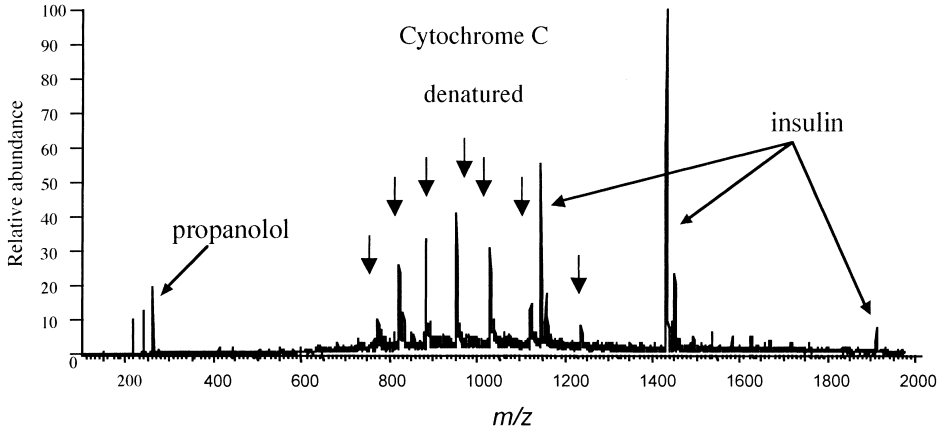

Fig. 4. Desalting of a mixture of propanolol, insulin and cytochrome *c* dissolved in PBS in the presence of 7 *M* urea.

Whereas the mechanism of action of urea as a protein denaturant is not fully understood, it is well established that urea disrupts intra-molecular hydrophobic interactions. But it does not seem that urea prevents interactions between hydrophobic analytes and the PVDF membrane.

Another effect complicates a direct quantitative comparison between spectra in Figs. 3 and 4: desorption of analytes from the membrane with methanol–water–acetic acid (50:49:1) results in differential elution of propanolol, cytochrome *c* and insulin, depending on their respective hydrophobicity. In the simple desalting experiment (without the urea treatment), propanolol elutes first, insulin second and cytochrome *c* comes third. This means that with that low percentage of methanol the three analytes cannot be observed at their maximum of elution within the same spectrum. After the urea treatment, elution behavior is slightly altered in the sense that elution times for insulin and cytochrome *c* are longer (ca. 1 min longer) so that intensities in the spectrum presented in Fig. 4 are not directly comparable to those in Fig. 3.

Interestingly, one can see from Figs. 3 and 4 that the cytochrome *c* peak distribution is shifted towards lower m/z ratios (higher charge states) when incubated in high concentrations of urea. It is now well established that the peak distribution in ESI mass spectra is strongly correlated with the folding state of the protein [37–43]. Common hypotheses to explain this phenomenon are a lower solvent accessibility of basic and acidic residues in well-packed folded proteins, and that unfolding stabilizes higher charge states by increasing the distance between charges of the same polarity. However in a recent study, Grandori [44] proposed a new hypothesis: it is consensual that carboxyl groups (resp. basic groups) of peptides and amino acids do not contribute to ESI mass spectra in the positive ion mode (resp. negative ion mode) due to neutralization by proton-transfer reactions at the surface of the ESI droplets. Grandori thus proposed that interactions between basic and acidic residues in folded proteins protect groups of opposite polarity to that being detected from neutralization during electrospray, reducing the final net charge compared to that of unfolded proteins. The net charge of the folded protein is thus the difference between the number of basic and acidic residues, rather than the number of basic (resp. acidic) groups when working in positive (resp. negative) ion mode. Whatever the precise mechanism may be, ESI-MS is now a well-established technique for monitoring changes in protein tertiary structure in solution. Here, one can see in Fig. 6b) that cytochrome *c* denatured in 7 *M* urea does not refold during washing out of salts with water and elution, as shown by the loss of higher peaks attributed to the native state of the protein [37].

3.4. Chemical tagging of cysteine residues

In order to show the interest of such an integrated desalting step, a specific chemical tagging of cysteine residues of β-lactoglobulin A was done with iodoacetamide. The scheme of the reaction is shown in Fig. 5. Alkylation of cysteines with iodoacetamide is very specific at near-neutral pH (7 to 8.5) and is a well-known procedure in preparation of samples for two-dimensional gel electrophoresis [45,46] to prevent formation of disulfides, or acrylamidation during migration within the gel; it has also been used to increase the information content of peptide mapping

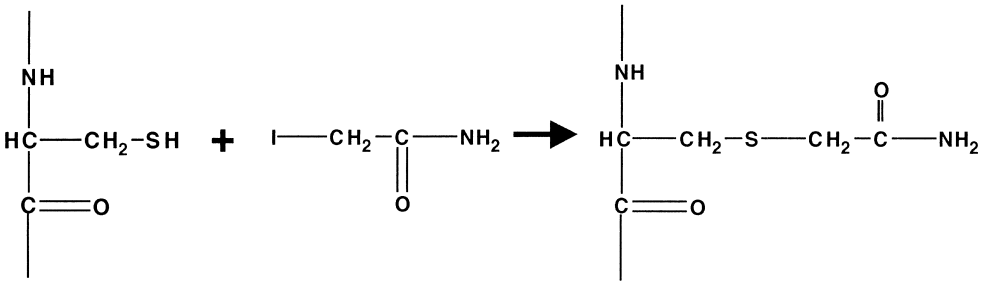

Fig. 5. Reaction of iodoacetamide with the cysteine moiety, leading to a mass shift of 57.

experiments [35], or to undergo quantitative comparison of two proteinaceous samples by the isotope-coded affinity tag (ICAT) approach [47,48], where iodoacetamide is linked to a biotin to allow isolation of labeled tryptic digested peptides. Here, the purpose was simply to count the total number of cysteines in β-lactoglobulin A by fully denaturating in 8 *M* urea followed by DTT reduction and alkyla-

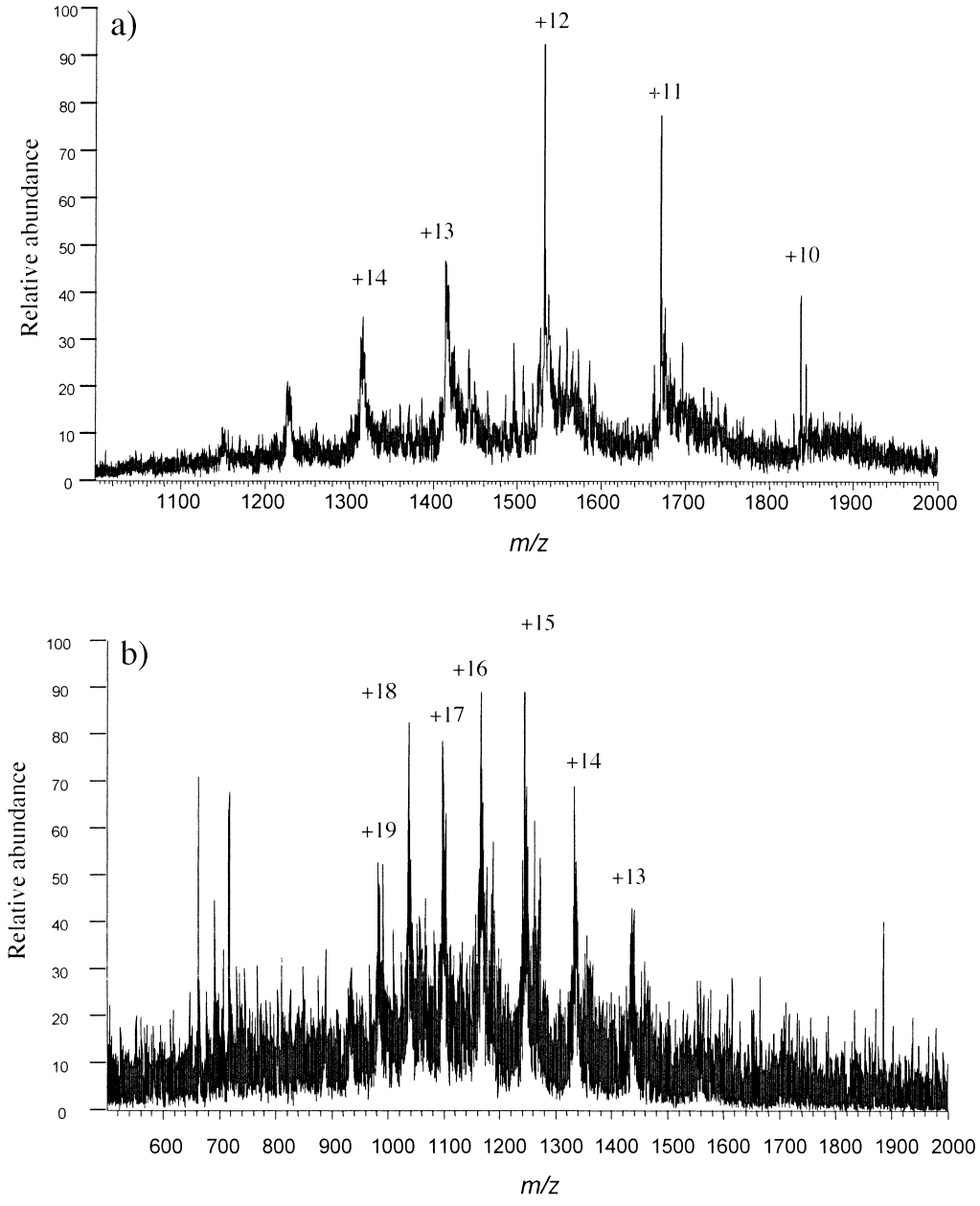

Fig. 6. (a) Spectrum of β-lactoglobulin A infused at 200 nl/min at a concentration of 2.7 μ*M*; full-scale intensity is 2.25. 10^5 counts. (b) Spectrum of alkylated β-lactoglobulin A eluting from the membrane after desalting; full-scale intensity is 3.44. 10^6 counts. Both spectra were acquired without averaging.

tion by iodoacetamide. Fig. 6a shows the spectrum of β-lactoglobulin A infused from the chip at a concentration of 2.7 μ*M* in MeOH–CH_3COOH–water (50:1:49) at 200 nl/min. The molecular mass obtained from the spectrum is 18 363.8±6.1. Fig. 6b shows the spectrum of β-lactoglobulin A eluting from the membrane after denaturation, reduction by DTT and alkylation by iodoacetamide; desalting was done by pumping 50 μl of the incubated sample at 5 μl/min and washing out of salts with the same volume of water at the same flow-rate. The resulting molecular mass is 18 650.6±2.9. The mass shift corresponds exactly to the tagging of five cysteine moieties by iodoacetamide ($\Delta m = 57$).

4. Conclusion

The integration of a membrane-based desalting step with microfluidics was developed. This process takes advantage of the high surface-to-volume ratio of a hydrophobic PVDF membrane as well as its ease of integration in the microchip. The combination of microfluidics with a membrane solid-phase allows a controlled elution of analytes that can then be sprayed and analyzed by mass spectrometry. The desalting of drugs, peptides and proteins in the presence of PBS was demonstrated; it results in spectra with no salt clusters and noise levels comparable to salt-free solutions. The desalting process presented herein is also tolerant to high concentration of urea (up to 8 *M*), and thiourea (data not shown), and can be used in the presence of DTT or β-mercaptoethanol. This tolerance to a wide range of co-solvents was exemplified by the alkylation of β-lactoglobulin A.

In this first approach, relatively large amounts of peptides and proteins were used: 12 pmol of cytochrome *c*, 28 pmol of insulin and 150 pmol of propanolol in the simple desalting experiments; 135 pmol of β-lactoglobulin A were used for the chemical tagging experiment. Moreover, the influence of co-solvents on the recovery of analytes from the membrane was not studied, and the optimization of elution conditions to reach a true separation between differentially eluted analytes is still under investigation. However, it must be stated that when used in the pmole range, every tested analyte could be detected in the eluting solution: glutamine, human Angiotensin II, myoglobin . . . (data not shown), and that this concentration range is comparable to that reached classically in desalting prior to mass spectrometry (see for example Ref. [49]). Here emphasis was put on the possibility to carry out chemical reactions in conditions incompatible with electrospray ionization (neutral pH, aqueous buffer, high salt and co-solvent concentrations), and then go directly to the mass spectrometric analysis through the desalting step. Moreover, miniaturization allows the manipulation of minute amounts of samples, and the accessibility to medium or high-throughput characterization through integration of the different steps of sample preparation on the same microdevice and automation.

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References

[1] T.L. Constantopoulos, G.S. Jackson, C.G. Enke, J. Am. Soc. Mass Spectrom. 10 (1999) 625.
[2] P.A. Limbach, Z.J. Meng, Analyst 127 (2002) 693.
[3] Q. Xue, F. Foret, Y.M. Dunayevskiy, P.M. Zavracky, N.E. McGruer, B.L. Karger, Anal. Chem. 69 (1997) 426.
[4] R.S. Ramsey, J.M. Ramsey, Anal. Chem. 69 (1997) 1174.
[5] Y.Z. Deng, N.W. Zhang, J. Henion, Anal. Chem. 73 (2001) 1432.
[6] L. Licklider, X.Q. Wang, A. Desai, Y.C. Tai, T.D. Lee, Anal. Chem. 72 (2000) 367.
[7] G.A. Schultz, T.N. Corso, S.J. Prosser, S. Zhang, Anal. Chem. 72 (2000) 4058.
[8] J.S. Kim, D.R. Knapp, Electrophoresis 22 (2001) 3993.

[9] J.S. Kim, D.R. Knapp, J. Am. Soc. Mass. Spectrom. 12 (2001) 463.
[10] J.S. Kim, D.R. Knapp, J. Chromatogr. A 924 (2001) 137.
[11] T.C. Rohner, J.S. Rossier, H.H. Girault, Anal. Chem. 73 (2001) 5353.
[12] J.S. Rossier, N. Youhnovski, N. Lion, E. Damoc, F. Reymond, H.H. Girault, M. Przybylski, Angew. Chem. Int. Ed. Engl. 42 (2003) 53.
[13] V. Gobry, J. van Oostrum, M. Martinelli, T. Rohner, J.S. Rossier, H.H. Girault, Proteomics 2 (2002) 405.
[14] T. Wachs, J. Henion, Anal. Chem. 73 (2001) 632.
[15] Y. Deng, J. Henion, J. Li, P. Thibault, C. Wang, D.J. Harrison, Anal. Chem. 73 (2001) 639.
[16] J. Kameoka, H.G. Craighead, H.W. Zhang, J. Henion, Anal. Chem. 73 (2001) 1935.
[17] J.J. Li, P. Thibault, N.H. Bings, C.D. Skinner, C. Wang, C. Colyer, J. Harrison, Anal. Chem. 71 (1999) 3036.
[18] B. Zhang, F. Foret, B.L. Karger, Anal. Chem. 72 (2000) 1015.
[19] B. Zhang, H. Liu, B.L. Karger, F. Foret, Anal. Chem. 71 (1999) 3258.
[20] B. Zhang, F. Foret, B.L. Karger, Anal. Chem. 73 (2001) 2675.
[21] J. Li, J.F. Kelly, I. Chernushevich, D.J. Harrison, P. Thibault, Anal. Chem. 72 (2000) 599.
[22] N. Xu, Y. Lin, S.A. Hofstadler, D. Matson, C.J. Call, R.D. Smith, Anal. Chem. 70 (1998) 3553.
[23] F. Xiang, Y. Lin, J. Wen, D.W. Matson, R.D. Smith, Anal. Chem. 71 (1999) 1485.
[24] N. Lion, V. Gobry, H. Jensen, J. Rossier, H.H. Girault, Electrophoresis 23 (2002) 3583.
[25] S. Ghaemmaghami, M.C. Fitzgerald, T.G. Oas, Proc. Natl. Acad. Sci. USA 97 (2000) 8296.
[26] V. Katta, B.T. Chait, J. Am. Chem. Soc. 115 (1993) 6317.
[27] D.M. Rosenbaum, S. Roy, M.H. Hecht, J. Am. Chem. Soc. 121 (1999) 9509.
[28] D. Suckau, M. Mak, M. Przybylski, Proc. Natl. Acad. Sci. USA 89 (1992) 5630.
[29] T.A. Fligge, J. Kast, K. Bruns, M. Przybylski, J. Am. Soc. Mass Spectrom. 10 (1999) 112.
[30] H.P. Happersberger, M. Przybylski, M.O. Glocker, Anal. Biochem. 264 (1998) 237.
[31] M.O. Glocker, S. Nock, M. Sprinzl, M. Przybylski, Chem.-Eur. J. 4 (1998) 707.
[32] S. Ghaemmaghami, T.G. Oas, Nat. Struct. Biol. 8 (2001) 879.
[33] J.S. Rossier, C. Vollet, A. Carnal, G. Lagger, V. Gobry, H.H. Girault, P. Michel, F. Reymond, Lab. Chip 2 (2002) 145.
[34] J. Rossier, F. Reymond, P. Michel, Electrophoresis 23 (2002) 858.
[35] S. Sechi, B.T. Chait, Anal. Chem. 70 (1998) 5150.
[36] T. Rabilloud, M. Chevallet, in: T. Rabilloud (Ed.), Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods, Springer, Berlin, Heidelberg, 2000, p. 9.
[37] K.R. Babu, A. Moradian, D.J. Douglas, J. Am. Soc. Mass Spectrom. 12 (2001) 317.
[38] K.R. Babu, D.J. Douglas, Biochemistry 39 (2000) 14702.
[39] V. Cunsolo, S. Foti, C. La Rosa, R. Saletti, G.W. Canters, M.P. Verbeet, Rapid Commun. Mass Spectrom. 15 (2001) 1817.
[40] T. Kashiwagi, N. Yamada, K. Hirayama, C. Suzuki, Y. Kashiwagi, F. Tsuchiya, Y. Arata, N. Kunishima, K. Morikawa, J. Am. Soc. Mass Spectrom. 11 (2000) 54.
[41] R. Grandori, I. Matecko, N. Muller, J. Mass Spectrom. 37 (2002) 191.
[42] R. Grandori, I. Matecko, P. Mayr, N. Muller, J. Mass Spectrom. 36 (2001) 918.
[43] R.L. Winston, M.C. Fitzgerald, Mass Spectrom. Rev. 16 (1997) 165.
[44] R. Grandori, J. Mass. Spectrom. 38 (2003) 11.
[45] B. Herbert, M. Galvani, M. Hamdan, E. Olivieri, J. MacCarthy, S. Pedersen, P.G. Righetti, Electrophoresis 22 (2001) 2046.
[46] M. Galvani, M. Hamdan, B. Herbert, P.G. Righetti, Electrophoresis 22 (2001) 2058.
[47] M.B. Smolka, H.L. Zhou, S. Purkayastha, R. Aebersold, Anal. Biochem. 297 (2001) 25.
[48] S.P. Gygi, B. Rist, S.A. Gerber, F. Turecek, M.H. Gelb, R. Aebersold, Nat. Biotechnol. 17 (1999) 994.
[49] K.D. Powell, M.C. Fitzgerald, Anal. Chem. 73 (2001) 3300.