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Integrated Microanalytical Technology Enabling Rapid and Automated Protein Identification

Simon Ekström,[†] Patrik Önnerfjord,[†] Johan Nilsson,[‡] Martin Bengtsson,[‡] Thomas Laurell,[‡] and György Marko-Varga[§,*]

Departments of Cell and Molecular Biology and Electrical Measurements, Lund University, P.O. Box 124, 221 00 Lund, Sweden, and Preclinical Research & Development, Cell & Molecular Biology, AstraZeneca, P.O. Box 34, 221 00 Lund, Sweden

Protein identification through peptide mass mapping by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has become a standard technique, used in many laboratories around the world. The traditional methodology often includes long incubations (6−24 h) and extensive manual steps. In an effort to address this, an integrated microanalytical platform has been developed for automated identification of proteins. The silicon micromachined analytical tools, i.e., the microchip immobilized enzyme reactor (μ-chip IMER), the piezoelectric microdispenser, and the high-density nanovial target plates, are the cornerstones in the system. The μ-chip IMER provides on-line enzymatic digestion of protein samples (1 μL) within 1−3 min, and the microdispenser enables subsequent on-line picoliter sample preparation in a high-density format. Interfaced to automated MALDI-TOF MS, these tools compose a highly efficient platform that can analyze 100 protein samples in 3.5 h. Kinetic studies on the microreactors are reported as well as the operation of this microanalytical platform for protein identification, wherein lysozyme, myoglobin, ribonuclease A, and cytochrome *c* have been identified with a high sequence coverage (50−100%).

As proteomics research evolves, so does the technology used in this field. The past decade's explosive progress in the fields of protein science, bioinformatics, and cell and molecular biology has resulted in an increased demand for development of instrumental technology that allows identification and detailed structural studies of proteins to be automatically performed at high speeds and with a high sensitivity. Presently, it is not possible to use nucleotide sequence data to gain insight into how different basic biological processes such as cell differentiation, receptor activation, signal transduction, and malignant transformation and numerous other processes work in living organisms. Therefore, studies of protein expression, interaction, posttranslational modifications, and resulting repercussions in biological systems must be made at the protein level. Peptide mapping is a frequently used technique for identification of proteins and studies of novel posttranslational modification.[1,2] Briefly, the protein is digested with a proteolytic enzyme, and the resulting digest mixture is analyzed with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), generating a peptide map that is unique for the digested protein. The observed peptide masses are then used to search existing protein and DNA databases. The current bottlenecks of this technique are the long incubation times required for digestion (6−24 h) and the extensive manual sample preparations. Miniaturization of analytical systems is generally considered to be the strategy that will overcome the requirements of process speed for performing efficient evaluation studies, e.g., of drug action and mechanism. This will allow early detection of eventual side effects, which is of great importance for the life science industry. Silicon micromachining presents several interesting and advantageous features for achieving miniaturized systems, such as low manufacturing cost in batch processing, high mechanical strength, and reproducible structures when fabricating well-defined and small flow channels, minute orifices, and thin membranes. Monocrystalline silicon is an excellent material with mechanical properties in many aspects comparable to those of stainless steel. This, combined with its chemical robustness, makes silicon a useful construction material for chemical microsystems. By utilizing the versatility of silicon micromachining to fabricate efficient minute volume microstructures, it is possible to make analysis systems that are extremely small.[3−5] The benefits of miniaturization stem from the increased reaction kinetics in low volumes and the possibility to perform sample-handling procedures at a high speed in micro-/nanoliter systems. Also, the sample amounts in life science research fields are often very small and precious. It is therefore desirable that every analysis consume as little sample as possible while providing the desired information. In this perspective, mass spectrometry has evolved as a very important and widely used analytical method in the recent years.[6,7] Ideally, the low sample amount needed for MALDI-TOF MS[8] also

* Corresponding author: (e-mail) gyorgy.marko-varga@AstraZeneca.com.
[†] Department of Analytical Chemistry, Lund University.
[‡] Department of Electrical Measurements, Lund University.
[§] AstraZeneca.

(1) Tsarbopoulos, A.; Karas, M.; Strupat, K.; Pramanik, B. N.; Nagabhushan, T. L.; Hillenkamp, F. *Anal. Chem.* **1994**, *66*, 2062−2070.
(2) Billeci, T. M.; Stults, J. T. *Anal. Chem.* **1993**, *65*, 1709−1716.
(3) He, B.; Tait, N.; Regnier, F. *Anal. Chem.* **1998**, *70*, 3790−3797.
(4) Harrison, J. D.; van den Berg, A. Micro Total Analysis Systems '98, Proceedings of the μ-TAS '98 Workshop, Banff, Canada, 1998; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1998.
(5) Fintschenko, Y.; Berg, A. v. d. *J. Chromatogr. A* **1998**, *819*, 3−12.
(6) Siuzdak, G. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11290−11297.
(7) Yates, J. R. *J. Mass Spectrom.* **1998**, *33*, 1−19.

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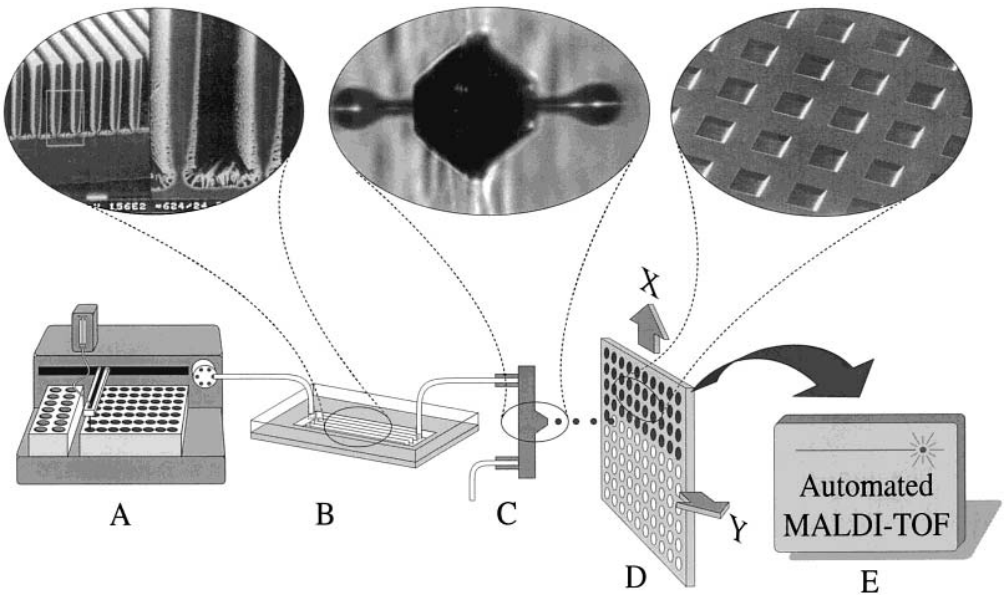

Figure 1. Total analysis system and its different parts;: (A) automated sample pretreatment and injection; (B) μ-chip IMER (the photo inset shows a SEM picture of the lamella structure with the porous layer); (C) the microdispenser used to deposit sample into μ-vials; (D) shallow nanovials (300 $\times$ 300 $\times$ 20 μm) on the MALDI target plate; and (E) automated MALDI-TOF MS analysis.

allows other complementary analyses to be performed on the remaining sample volume, extracting the maximum amount of information that is obtainable from each sample. The integrated microsystem presented herein is designed to be used in situations were it is desirable to quickly screen large amounts of purified protein samples. The goal is to avoid time-consuming manual steps and to make analysis as cost-effective as possible. The system has therefore been designed as a computer-controlled plug-in module, interfacing automated digestion and sample handling to MALDI-TOF MS.

EXPERIMENTAL SECTION

Chemicals. Chymotrypsin, bovine pancreas (Lot No. 85060820-64) was from Boehringer Mannheim (GmbH, Mannheim, Germany). Glutaraldehyde (GA), grade II, 25% (aqueous, Lot No. 56H0107); 3-aminopropyltrieththoxysilane (APTES, Lot No. 127H1073); guanidine·HCl (Lot No. 12H5725); dithiothreitol (DTT, Lot No. 45H007215); iodoacetamide (Lot No. 25H8452); α-*N*-benzoyl-L-arginine ethyl ester (BAEE, Lot No. 17H0861); Trizma base (Lot No. 12H56001); Trizma·HCl (Lot No. 35H5705); trypsin type IX, porcine pancreas (Lot 25H0359); bradykinin (Lot No. 36H5840); ACTH 18-38 (Lot No. 86H4951); angiotensin I (Lot No. 66H5830); cytochrome *c*, bovine heart (Lot No. 84H7145); myoglobin, horse heart (Lot No. 76H7210); ribonuclease A, bovine pancreas (Lot No. 16H7161); albumin, bovine (Lot No. 53H00665); and α-cyanohydroxycinnamic acid (CHCA, Lot No. 51229) were all purchased from Sigma Chemical Co. (St. Louis, MO). Lysozyme (Lot No. 56518) was purchased from ICN Chemicals (Costa Mesa, CA). HCl, acetone, 2-propanol, NH_4HCO_3, KOH, HF, $CaCl_2$, KH_2PO_4, Na_2HPO_4, NaN_3, NaCl, and acetonitrile (ACN) were from Merck (Darmstadt, Germany). Urea (Lot No. 51459), ACTH (Lot No. 91007), trifluoroacetic acid (TFA), and sinapinic acid were obtained from Fluka Chemicals (Buchswald, Switzerland) and used without further purification. Angiotensin I, substance P, ACTH clip 1-17, ACTH clip 18-39, ACTH clip 7-38, and bovine insulin were all part of mass standard kit no. 2-3143-00 (Lot No. 7022801) from Perseptive Biosystems (Framingham, MA). Nitro Cellulose Trans-Blot transfer medium, 0.45 μm, was from Bio-Rad Laboratories (Richmond, CA). The water was purified using a Millipore apparatus (Bedford, MA). All chemicals were used without further purification, and all buffers used were freshly prepared, pH adjusted, and if used in a critical application autoclaved.

System Setup. The integrated microanalytical system is depicted in Figure 1, with photograph insets of the microchip immobilized enzyme reactor (μ-chip IMER), the piezoelectric flow-through dispenser, and the nanovials on the MALDI target plate. The internal volume of the total analysis system was 13 μL, from injection port to dispenser outlet. The pretreatment robot (A in Figure 1) was a Gilson (Villiers-le-Bel, France) ASPEC XL autosampler equipped with a Rheodyne (Berkeley, CA) four-port injection valve with an internal loop of 1-μL volume. The sample rack was adapted for this application, giving a maximum capacity of 576 samples/run. This pretreatment system was controlled with Gilson 719 software from a Pentium PC, and all programs were written in Turbo Pascal. A CMA/100 pump (CMA/microdialysis AB, Solna, Sweden) was used to generate the carrier flow. The micromachined parts included a μ-chip IMER (B in Figure 1), the microdispenser (C in Figure 1), and the high-density nanovial target plate (D in Figure 1). Both the μ-chip IMER and the high-density nanovial MALDI target plate were equipped with temperature-controlled heating elements. All these micromachined parts were manufactured by conventional anisotropic wet etching in KOH. The micromachined parts were connected with $^1/_{200}$-in.-i.d. ($^1/_{16}$-in.-o.d.) PEEK tubing, and an in-house built fixture was used to provide flow connections for the μ-chip IMER. The final system component was an automated MALDI-TOF mass spectrometer (E in Figure 1).

(8) Allmaier, G. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1567−1569.

Pretreatment. The pretreatment was performed by adding a denaturating agent, guanidine·HCl (1 M), and a reducing agent, dithiothreitol (5−10 mM), in digestion buffer to the protein sample. All reagents and samples were dissolved in digestion buffer, 25 mM Tris, pH 8.2. The sample was then heated to 75 °C for 20 min. The protein was subsequently alkylated with iodoacetamide (30 mM); the carboxyamidomethylation reaction took 30 min at room temperature. Prior to injection, the samples were diluted with digestion buffer to a guanidine·HCl concentration of 0.25 M. Finally, 1 μL of sample (containing 1−5 pmol of protein/μL) was injected into a flow of digestion buffer.

The μ-Chip IMER. The μ-chip IMER was fabricated in ⟨110⟩-oriented p-type silicon, which allows vertical anisotropic etching, to generate a surface-enlarging, high-aspect-ratio parallel channel structure.[9] To further enhance the surface area in the μ-chip IMER, it was treated by anodization in an HF/ethanol solution, yielding a thin, porous surface layer on the channel walls. The optimal porosity and the manufacturing procedure for this porous layer were determined for a glucose monitoring systems in an earlier work by Drott et al.[10,11] In that application, porous silicon was shown to give a 170-fold increase in enzyme activity compared to nonporous reactors. The μ-chip IMER used for enzymatic digestion in this work had 32 parallel channels, 11 mm long, 50 μm wide, and 250 μm deep, giving an internal volume of 6 μL.

The immobilization of proteolytic enzymes onto the μ-chip IMER was made according to standard procedures for enzyme coupling to silica matrixes.[12] This was done in three steps, first by silanization in 10% (v/v) aqueous (3-aminopropyl)triethoxysilane, pH-adjusted with HCl to 3.5 at 75 °C for 3 h, followed by extensive washing with water for 1 h. Glutaraldehyde activation was subsequently performed in 0.1 M sodium phosphate buffer, pH 7.0, containing 2.5% glutaraldehyde for 4 h at room temperature, followed by 2 h of washing in 0.1 M sodium phosphate buffer, pH 7.0. Finally, the enzyme was coupled, 2−4 mg/mL enzyme in 0.1 M sodium phosphate buffer, pH 7.0, for 24 h at room temperature. To suppress the reversibility of the formed Shiff's base and stabilize the bound enzyme, the enzyme coupling was performed in the presence of a weak reducing agent, $NaCNBH_3$. All reactions were performed in small beakers, which were gently rocked on a thermostated water bath. In this work, trypsin and chymotrypsin were immobilized onto μ-chip IMERs.

The catalytic efficiency of the trypsin μ-chip IMER was measured using a spectrophotometric assay[13] adapted for this application. The reagent solution containing α-*N*-benzoylarginine ethyl ester (BAEE), dissolved in 50mM Tris, 10 mM $CaCl_2$, pH 8.0, bypassed the μ-chip IMER, directly entering a Waters 486 tunable absorbance detector (Millipore Corp., Milford, MA) to obtain the baseline. Subsequently, a valve was switched, and the reagent passed through the μ-chip IMER before entering the absorbance detector.

On-Line Sample Preparation. In this work, a piezo-actuated flow-through microdispenser developed within our group was used to deposit sample volumes ranging from 65 pL to over 300 nL. The microdispenser was fabricated by anisotropic wet etching of monocrystalline silicon, utilizing the pn-etch stop technique as described by Laurell et al.[14] The dispensing principle is based on a piezoceramic element, which elongates when a voltage pulse is applied, creating a pressure pulse that results in droplet ejection at the orifice. The unique dispensor construction features flow-through capability and a pyramid-shaped nozzle that ensures stable droplet directivity. The internal volume, from inlet to orifice of the microdispenser, was 1.3 μL. Typical operation conditions were a droplet frequency of 50−100 Hz, with a single droplet volume of approximately 65 pL. This particular microdispenser has previously been described in work by Önnerfjord et al.[15] Herein, the dispenser was used for enrichment of samples at low concentration by making multiple sample depositions on the same spot before MALDI-TOF MS analysis. Sample deposition on high-density MALDI target plates was accomplished by stepwise movement of the target plate using an in-house-built computer-controlled *x*−*y* stage, with 5-μm spatial resolution. Deposition of the sample was observed via a microscope (Leitz, Wetzlar, Germany) equipped with a video camera (CCD-72EX, DAGE-MTI Inc., Michigan City, IN).

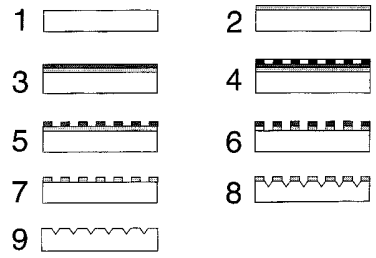

Figure 2. Illustration of the processing steps for the manufacturing of high-density nanovial targets. (1) Starting material, a monocrystalline silicon wafer. (2) The wafer was coated with a protecting layer of silicon dioxide. (3) The silicon dioxide layer was coated with photoresist. (4) A mask with the desired pattern was placed over the wafer and illuminated with UV light. (5) The exposed wafer was developed, and the illuminated photoresist was removed. (6) The silicon dioxide was removed in HF. (7) Remaining photoresist was removed by acetone wash. (8) The silicon wafer was anisotropic wet-etched with KOH. (9) Finally, the remaining silicon dioxide was removed with HF.

Vials ranging in size from 100 × 100 to 400 × 400 μm (intervial spacing of 100−400 μm, respectively) were used for collection of deposited samples. Both pyramidal and shallow (20 μm deep) vials were manufactured in ⟨100⟩-oriented p-type silicon. Figure 2 depicts the process steps in the manufacturing of nanovials. The nanovial chips, each with 100 sample positions in a 10 × 10 format, were mounted onto the MALDI target plate by conductive epoxy or adhesive tape.

For peptide analysis, the sample was deposited directly onto a predeposited thin-layer matrix surface[16] in the shallow nanovials (300 × 300 × 20 μm) using the microdispenser. The predeposited

(9) Laurell, T.; Rosengren, L.; Drott, J. *Biosens. Bioelectron.* **1995**, *10*, 289−299.
(10) Drott, J.; Rosengren, L.; Lindström, K.; Laurell, T. *Thin Solid Films* **1998**, *330*, 161−166.
(11) Drott, J.; Rosengren, L.; Lindström, K.; Laurell, T. *Microchim. Acta* **1999**, *131*, 115−120.
(12) Weetall, H. H. *Methods Enzymol.* **1976**, *44*, 134−148.
(13) Shewert, G. W.; Takenaka, Y. *Biochim. Biophys. Acta* **1955**, 570.
(14) Laurell, T.; Wallman, L.; Nilsson, J. Design and development of a silicon microfabricated flow-through cell for on-line picolitre sample handling. *J. Micromech. Microeng.*, in press.
(15) Önnerfjord, P.; Nilsson, J.; Wallman, L.; Laurell, T.; Marko-Varga, G. *Anal. Chem.* **1998**, *70*, 4755−4760.
(16) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. *Anal. Chem.* **1996**, *68*, 850−858.

matrix was made from CHCA, 20 mg/mL in 4:1 acetone/2-propanol, mixed 1:1 with nitrocellulose, 10 mg/mL in 4:1 acetone/2-propanol, to a final concentration CHCA/NC of 10/5 mg/mL. Alternatively, the matrix CHCA, 8 mg/mL in 1:1 acetonitrile/0.5% TFA (aqueous), was deposited after the sample. The MALDI target plate was heated to 50 °C. Sample cleanup was performed by washing the sample spot surface prior to MALDI-TOF MS analysis by depositing cooled (10 °C) acidic water (1−5% TFA) over the entire target plate. After a few seconds, an air stream was used to remove the water.

Mass Spectrometry Analysis. The MALDI-TOF instrument used was a Voyager DE-PRO (Perseptive Biosystems Inc., Framingham, MA). The instrument was equipped with a linear flight tube of 1.1 m and a delayed extraction ion source and used a nitrogen laser ($\lambda = 337$ nm) with a laser focal spot diameter of approximately 100 μm. The instrument software allowed programming of various sample plate formats for automated analysis. The instrument could be operated in linear (L) or reflector (R) mode. For high-accuracy peptide mapping, the reflector mode was used. The high degree of mass accuracy, 5−100 ppm, obtained with instruments using the delayed extraction principle[17−20] and reflectron detectors makes sequencing and identification amenable.[21] In the automated mode, a 100-position vial target plate could be analyzed in 25−30 min. To ensure accurate mass assignment, an internal two-point calibration or external calibration was used.

RESULTS AND DISCUSSION

Operation of the System. The protein sample was first subjected to pretreatment in the autosampler (A in Figure 1), where it was made amenable for digestion and then injected into the flow of digestion buffer. The sample was subsequently digested on the μ-chip IMER (B in Figure 1) and further transported to the piezoelectric flow-through microdispenser (C in Figure 1). From the microdispenser, the desired amount of sample was ejected onto a high-density nanovial MALDI target plate (D in Figure 1). Finally, the deposited sample was analyzed by MALDI-TOF MS (E in Figure 1), and the resulting peptide map was used for a database search.

Influence of Pretreatment. The variations in structure and size make proteins differ in their susceptibility to enzymatic digestion. To aid enzymatic digestion,[22] a sample pretreatment step was necessary. Several different pretreatment conditions for the protein samples were investigated by varying the concentration of the denaturing agents, urea (1−8 M) or guanidine HCl (1−6 M). It was found that numerous different protocols worked satisfactorily. Because too high concentrations of pretreatment reagents can have a detrimental effect on the MS analysis, it is desirable to use the lowest concentrations possible (<2 M urea or < 0.5 M guanidine·HCl in the injected sample), especially when working with samples containing low concentrations of protein.

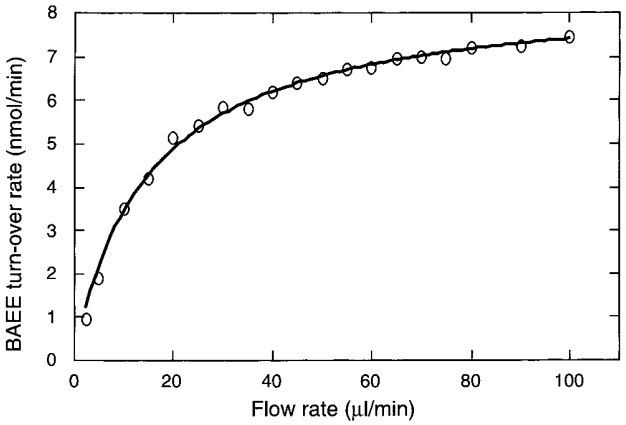

Figure 3. BAEE turnover rate versus flow rate plot for a BAEE concentration of 0.5 mM. The maximum enzyme activity was estimated at the highest deployed flow rate.

The use of different denaturants in the pretreatment step can have a significant impact on the digestion results. For example, when lysozyme was digested on a trypsin μ-chip IMER, the number of matched peaks was 3 when 10 mM DTT was used as denaturant, 17 for 10 mM DTT in 2 M urea, and 23 for 10 mM DTT in 1 M guanidine·HCl. Furthermore, the alkylation of the denatured and reduced protein leads to an increase in the number of observed peptides, although sufficient digestion for successful identification was also obtained without alkylation. The system setup rapidly generates new results, even when alterations are made in the pretreatment protocol, an advantageous feature when optimizing the digestion conditions for a specific protein.

Miniaturized Enzymatic Digestion. Enzyme activity measurements were made to obtain absolute values of the reactor performance for theoretical calculations and comparison with future μ-chip IMER designs. The enzyme activity for trypsin μ-chip IMERs was estimated by applying the Lambert−Beer law to the observed absorbance readings, from which the actual BAEE turnover rate, V_e, could be calculated.[9] The turnover rates obtained were plotted against flow for BAEE concentrations of 0.075−0.5 mM. Figure 3 shows the plot for the BAEE concentration of 0.5 mM. To ensure that first-order enzyme kinetics prevailed, i.e., that the turnover rate was independent of the flow, a value for the maximum enzyme activity at each BAEE concentration was estimated at a flow rate where the turnover had leveled off. By plotting these maximum values against the substrate concentration, estimations of apparent V_{max} ($V_{max\ app}$) and apparent K_m ($K_{m\ app}$) were obtained by fitting the theoretical equation of a first-order enzyme kinetic system[23] to a plot of the data. Additional approximations of $V_{max\ app}$ and $K_{m\ app}$ were obtained by Eadie−Hofstee and Lineweaver−Burk estimations. The estimated $V_{max\ app}$ and $K_{m\ app}$ values are presented in Table 1. As can be seen, there is very little variation between the different methods, which indicates that the estimation is accurate. The kinetic measurements were made at ambient temperature (25 °C). To get an estimate of the gain in reaction kinetics that may be obtained by increasing the temperature in the μ-chip IMER, measurements were also performed at 50 °C, giving an increase in BAEE turnover

(17) Vestal, M. L.; Juhasz, P.; Martin, S. A. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1044−1050.
(18) Whittal, R. M.; Li, L. *Anal. Chem.* **1995**, *67*, 1950−1954.
(19) King, T. B.; Colby, S. M.; Reilly, J. P. *Int. J. Mass Spectrom. Ion Processes* **1995**, *145*, L1−L7.
(20) Brown, R. S.; Lennon, J. J. *Anal. Chem.* **1995**, *67*, 1998−2003.
(21) Jensen, O. N.; Podtelejnikov, A.; Mann, M. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1371−1378.
(22) Riviere, R. L.; Fleming, M.; Elicone, C.; Tempst, P. *Tech. Protein Chem. II* **1991**, 171−179.
(23) Cantor, C. R.; Schimmel, P. R. *Biophysical chemistry, part III*; W. H. Freeman & Co.: San Francisco, 1980; pp 896−988.

Table 1. Estimated Values of $V_{max\ app}$ and Apparent $K_{m\ app}$ for a Trypsin μ-Chip IMER Obtained from the Different Plot Methods

plot method	$V_{max\ app}$ (nmol/min)	$K_{m\ app}$ (mM)
V_e versus S_0	8.9	0.11
Lineweaver−Burke	8.8	0.10
Eadie−Hofstee	8.9	0.11

rate of approximately 25%. This is attributed to a higher enzyme activity and the faster diffusion of analyte molecules. By injection of BAEE solutions in 50 mM Tris, 10 mM $CaCl_2$, pH ranging between 7 and 9.5, the pH optimum for the trypsin μ-chip IMER was determined to be pH 8.2.

Several reports describe different ways to miniaturize proteolytic cleavage procedures, showing the advantages of miniaturized enzymatic digestion.[24−27] The new approach presented in this paper, utilizing the micromachined μ-chip IMER, makes full automation easy, and time-consuming manual analysis steps are circumvented. The use of miniaturized micromachined structures in this application has many advantages. The reduced and automated sample handling leads to minimized sample loss, and reduced sample volumes inherently yield rapid digestion of proteins. The μ-chip IMER digestion was a factor of 200−1000 faster than in-solution digestion. Immobilization of the protease also has the advantage of avoiding autolytic interferences from the proteolytic enzyme in the mass spectra. By using porous silicon as a carrier for the immobilized enzyme in the μ-chip IMER, a large surface area and thus a high catalytic turnover are obtained. While the micromachined reactor might not offer quite the same surface area as a microcolumn with packed material, it has a lower hydrodynamic pressure drop, is less sensitive to carryover effects, and is much simpler to batch manufacture in the desired small sizes. The effect of heating the μ-chip IMER was shown to increase the catalytic turnover of BAEE. When performing protein digestions, the elevated temperature becomes even more important as it also serves to unfold the protein, thereby aiding the enzymatic digestion. In an investigation in which only the temperature was varied, the number of identified peptide peaks increased from 8 when digesting cytochrome *c* on a trypsin μ-chip IMER at room temperature to 15 at 40 °C and 17 at 60 °C.

The ability to alter the digestion time by varying the flow rate provides a powerful means to change experimental conditions, making it possible to achieve the desired degree of digestion or to compensate for enzyme activity loss by simply varying the flow rate. The long-term operational stability of the μ-chip IMER was found to be high. This was proven by using one of the reactors for more than 400 successful digestions of samples containing high concentrations of pretreatment reagents (4 M urea, 2 M guanidine·HCl, 40% ACN) during a time period spanning over 2 months. However, during long-time usage, reactors are bound to eventually lose activity. A simple process, which proved to be useful, allowed recycling of old reactors by stripping off the old immobilized enzyme through acid treatment (6 M HCl, 75 °C overnight), followed by re-immobilization with fresh enzyme.

On-Line Sample Preparation. Sample amounts in the nanoliter to picoliter range are very difficult to handle by conventional pipetting, and therefore special tools have to be used to avoid sample losses and inaccurate positioning of sample.

The microdispenser described easily allows several thousand samples to be deposited on each MALDI plate, and by making micromachined high-density MALDI target plates, it is possible to achieve several tens of thousands of sample positions per plate. Deposition of replicate sample spots in 100 positions on a nanovial target can be achieved in less than 1 min. The use of the flow-through microdispenser enables sample enrichment in each vial at the same time as part of the sample volume proceeds to downstream fractionation at the flow outlet. Unidentified samples or proteins of special interest can then be further analyzed by other methods, such as immunoassays or MS/MS sequencing using nanospray technology. By heating the MALDI target plate to a temperature of 50 °C, the evaporation time was shortened, thus increasing the amount of sample that could be deposited per time unit. The enrichment thereby allows the collection of a high analyte density in the sample spots, which leads to increased sensitivity. This makes it possible to obtain mass spectra from sample solutions of such low concentration that ordinary dried droplet sample preparations would not give usable mass spectra. Typically, microdispenser deposits of 100−300 nL of sample per vial were sufficient to ensure the generation of good mass spectra. The predeposited thin-layer matrix provided a high sensitivity and had the advantage that only the samples had to be microdispensed. The deposition of matrix after the sample provided a somewhat lower sensitivity. An advantage of applying matrix after the sample is that different matrixes can be used for analysis of the same sample (deposited in neighboring nanovials), giving additional sequence information.[28]

Collecting the sample in nanovials is one approach that can be used in order to increase the sensitivity in MALDI-TOF MS analysis. The use of nanovials has been reported by Jespersen et al.,[29] who presented picoliter volume vials etched in silicon wafers. The vials were inverse pyramids of 100 × 100 μm with a volume of 250 pL, and low attomole levels of peptides and proteins were detected with MALDI-TOF MS. Nanovials have also been used for DNA analysis.[30]

The microvial size and geometry were found to influence the MALDI-TOF MS resolution. To investigate this, inverted pyramidal and shallow nanovials, ranging in size from 100 × 100 to 400 × 400 μm, were tested. The experiments revealed that the isotope resolution when analyzing peptides was much lower (up to a factor of 10) for samples inside the deeper nanovials (>100 μm) than for samples that were deposited in shallow nanovials or on top of the silicon surface between the nanovials. It was concluded that this phenomenon originated from the spatial distribution of matrix−sample crystals along the vertical axis within the deep

(24) Gobom, J.; Nordhoff, E.; Ekman, R.; Roepstorff, P. *Int. J. Mass Spectrom. Ion Processes* **1997**, *169/170*, 153−163.
(25) Bergquist, J.; Karlsson, G.; Ekman, R. *Adv. Mass Spectrom.* **1998**, *14*, 1−16.
(26) Dogruel, D.; Williams, P.; Nelson, R. W. *Anal. Chem.* **1995**, *67*, 4343−4348.
(27) Whittal, R. M.; Keller, B. O.; Li, L. *Anal. Chem.* **1998**, *70*, 5344−5347.
(28) Kussmann, M.; Nordhoff, E.; Rahbek-Nielsen, H.; Haebel, S.; Rossel-Larsen, M.; Jakobsen, L.; Gobom, J.; Mirgorodskaya, E.; Kroll-Kristensen, A.; Palm, L.; Roepstorff, P. *J. Mass Spectrom.* **1997**, *32*, 593−601.
(29) Jespersen, S.; Niessen, W. M. A.; Tjaden, U. R.; Greef, J. v. d.; Litborn, E.; Lindberg, U.; Roeraade, J. *Rapid Commun. Mass Spectrom.* **1994**, *8*, 581−584.
(30) Little, D. P.; Cornish, T. J.; O'Donnell, M. J. *Anal. Chem.* **1997**, *69*, 4540−4546.

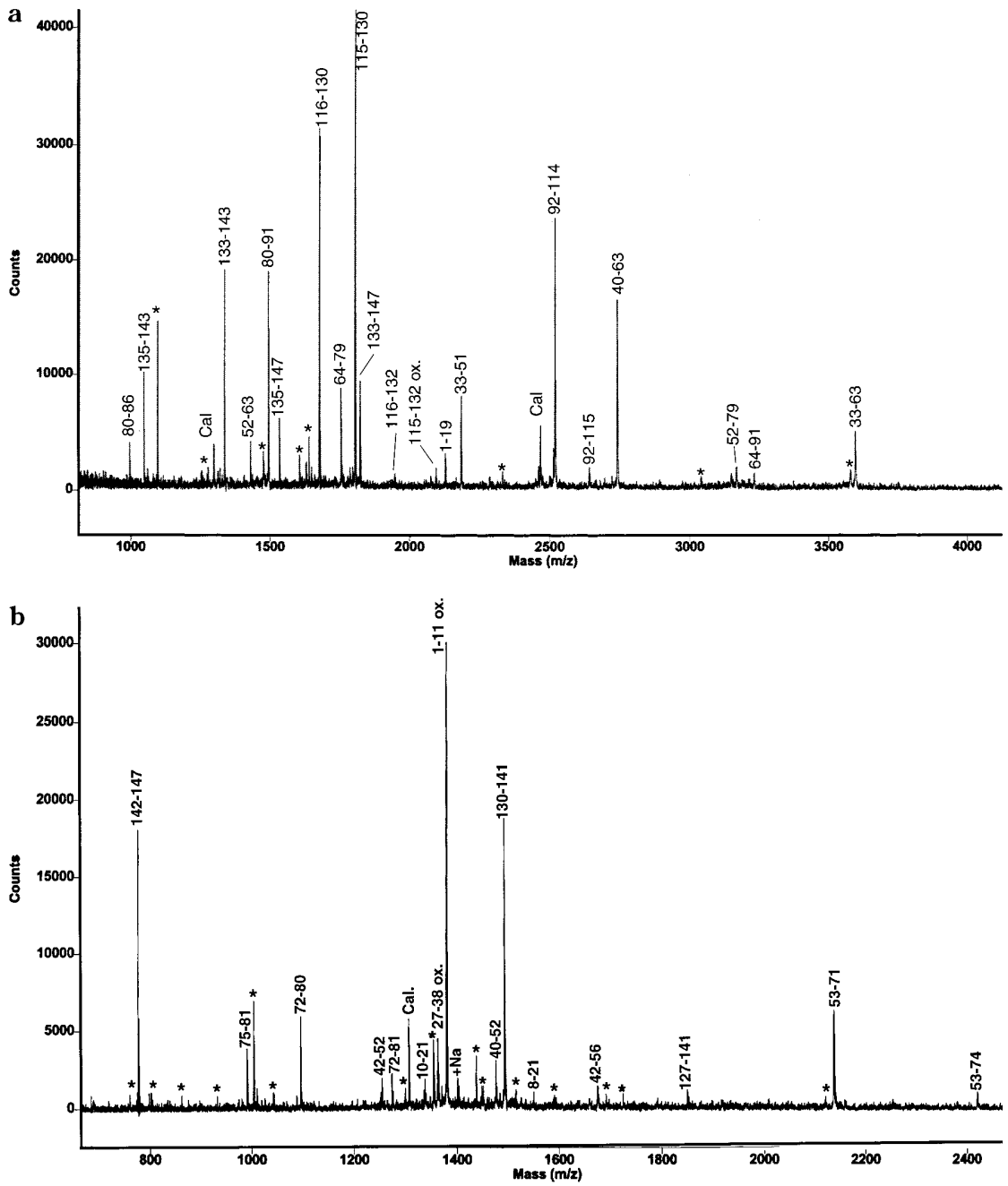

Figure 4. MALDI-TOF MS spectra resulting from digestion of 1 pmol/μL lysozyme. (a) On a trypsin μ-chip IMER, positive identification with MS-Fit as lysozyme (Swissprot accession no. P00698), 20/28 peptides matched providing a sequence coverage of 91%. (b) On a chymotrypsin μ-chip IMER, identified as lysozyme (Swissprot accession no. P00698), 15/30 peptides matched providing a sequence coverage of 64%. Cal, internal calibrants; *, unidentified peaks; +Na, sodium adduct; ox, methionine residue oxidized.

vials. Low resolution leads to decreased mass accuracy, and to avoid this, shallow nanovials with a vial depth of 20 μm were found to be suitable. The use of nanovials for sample collection ensured exact positioning of the sample spots and eliminated the need for "hot spot" searching, and thus providing appropriate conditions for rapid automated MALDI-TOF MS analysis.

MALDI-TOF MS Analysis. The high-density target plates were analyzed, and the generated peptide maps were used for a database search. The database search was done manually, but on-line database search[31] solutions are currently being developed in our laboratory. The protein identifications done in this work were performed with the aid of Internet-accessible programs, MS-Fit, ProFound, and PeptideSearch.[32] All of these programs have different features that aid in assigning the correct match. The seed-layer method used in an instrumental setup described in earlier work by Önnerfjord et al.[33] can be utilized to obtain the mass of intact protein, thereby limiting the database search window. In a

(31) Jensen, O. N.; Mortensen, P.; Vorm, O.; Mann, M. *Anal. Chem.* **1997**, *69*, 1706−1714.

(32) MS-Fit is available at http://prospector.ucsf.edu/cgi-bin/msfit.exe. ProFound is available at http://prowl.rockefeller.edu/cgi-bin/ProFound. PeptideSearch is available at http://193.175.249.95:80/CGI.

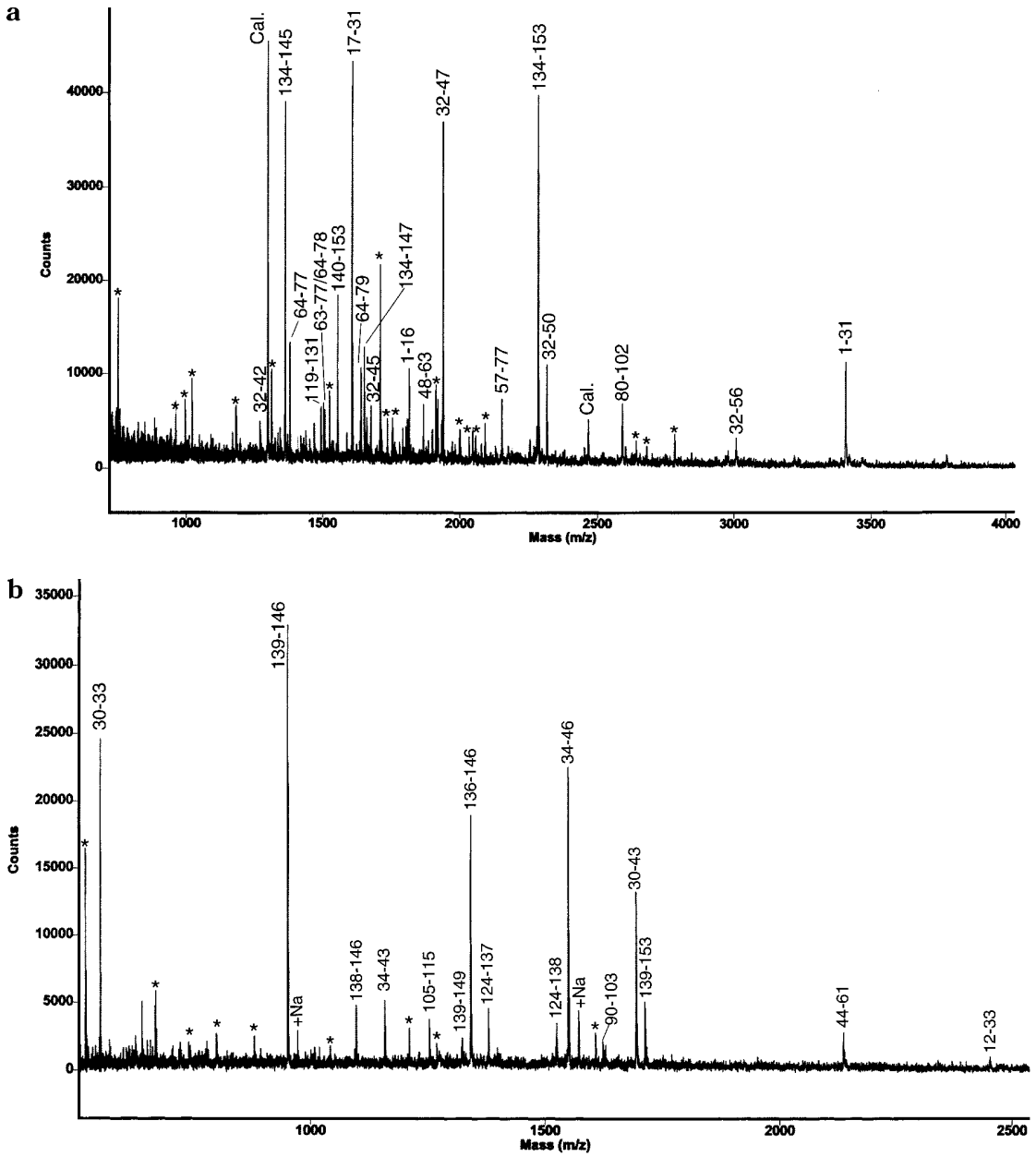

Figure 5. MALDI-TOF MS spectra resulting from digestion of 3 pmol/μL myoglobin. (a) On a trypsin μ-chip IMER, positive identification with MS-Fit as myoglobin (Swissprot accession no. P02188), 20/37 peptides matched providing a sequence coverage of 79%. (b) On a chymotrypsin μ-chip IMER, identified as myoglobin (Swissprot accession no. P02188), 15/24 peptides matched providing a sequence coverage of 68%. Cal, internal calibrants; +Na, sodium adduct; *, unidentified peaks.

report by Jensen et al.,[34] a minimum of five peptide matches with a maximum allowed mass deviation of 50 ppm and a sequence coverage of at least 15% was suggested for an unambiguous match.

Protein samples, containing 1−5 pmol/μL (1−5 μM) lysozyme, myoglobin, ribonuclease A, or cytochrome *c* digested on a trypsin μ-chip IMER, were positively identified in accordance with the above criteria by a database search. In all cases, 8−20 peptides could be assigned to the parent protein, resulting in a sequence coverage of 50−100%. Also, a sample of 5 pmol/μL albumin was digested and positively identified; 10 peptide matches provided sequence coverage of 23%. If the information from a peptide map does not result in an unambiguous identification, a combination of peptide maps from enzymes with different specificity can be used to make the identification.[35,36] To obtain alternative peptide maps, an immobilized chymotrypsin μ-chip IMER was used to digest proteins. Identification of cytochrome *c*, myoglobin, and lysozyme was achieved; 5−15 peptides matched provided sequence coverage of 25−68%. Figures 4a and 5a show spectra

(33) Önnerfjord, P.; Ekström, S.; Bergquist, J.; Nilsson, J.; Laurell, T.; Marko-Varga, G. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 315−322.
(34) Jensen, O. N.; Podtelejnikov, A. V.; Mann, M. *Anal. Chem.* **1997**, *69*, 4741−4750.
(35) Cohen, S. L.; Ferré-D'amare, A. R.; Burley, S. K.; Chait, B. T. *Protein Sci.* **1995**, *4*, 1088−1099.
(36) James, P.; Quadroni, M.; Carafoli, E.; Gonnet, G. *Protein Sci.* **1994**, *3*, 1347−1350.

obtained by digestion of lysozyme and myoglobin performed on a trypsin μ-chip IMER digestion. In Figures 4b and 5b, the spectra obtained from lysozyme and myoglobin digested on a chymotrypsin μ-chip IMER are shown. The somewhat lower value of sequence coverage for digestion on the chymotrypsin μ-chip IMER can be explained by the lower cleavage specificity for this enzyme.

System Performance. Normally, a digestion time of 1 min was sufficient to achieve the desired enzymatic cleavage of the proteins studied. At a flow rate of 6 μL/min, the buffer carried a sample through the system and completed the process of digestion and sample preparation in 2 min. The time between two consecutive injections of sample into the μ-chip IMER was set to 2 min. This was determined by the need to avoid carryover effects, and resulted in an analysis time of less than 3.5 h for 100 samples, not including sample pretreatment and data analysis. It should be noted that, by running several μ-chip IMERs of the same kind in parallel, it is possible to further increase the number of samples that can be processed per time unit.

Since all proteins behave quite differently, the lowest possible concentration of protein in the injected sample that will generate sufficient identification is difficult to estimate. For the proteins studied, 1−5 pmol/μL provided positive identification. By increasing the size of the injected sample plug or injecting the sample continuously, it was possible to analyze samples containing somewhat lower concentrations of protein ($\sim$0.5 pmol/μL). The enrichment of sample by microdispensing helps counteract the negative effects caused by dispersion in the system, but there is also a limit to the enrichment method, as contaminants (buffer salts, pretreatment reagents) are simultaneously enriched. The dispersion is currently the limiting factor, and there are two sources for this: the μ-chip IMER and the flow-through dispenser. By further miniaturization and careful construction of inlets to the micromachined parts, the dispersion can be reduced and sensitivity gained.

Comparison of traditional in-solution digestion and μ-chip IMER digestion without microdispenser sample enrichment using manual sample preparation revealed that, under equal conditions, the μ-chip IMER gave identification results better than or equal to those of in-solution digestion. Furthermore, the microanalytical system, combining the μ-chip IMER, the microdispenser, and the nanovial target plates, provided superior identification results in less time.

CONCLUSIONS

This report presents a new step in the development of tomorrow's totally integrated and miniaturized protein analysis platforms. It is demonstrated that micromachined parts can provide the means necessary for effective unattended automated protein identification. The setup of the system easily allows adaptation for other applications; e.g., the microdispenser can be used alone for sample enrichment of in-gel digested protein samples from 2-DE, and the μ-chip IMER can be used as a precolumn reactor in combination with μ-RP HPLC. The next generation of the microsystem, already under construction, will be even smaller with a higher degree of integration. Smaller μ-chip IMERs in combination with lower internal volume microdispensers will be used to further increase sensitivity and process speed. As biosciences progress, new challenges will jointly be overcome with the technology development, and it is predicted that micromachining technology allowing integrated chemical microsystems will have a major impact on the future development of life science technology.

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