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Silicon microstructures for high-speed and high-sensitivity protein identifications

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Abstract

Silicon microtechnology has been used to develop a microstructure toolbox in order to enable high accuracy protein identification. During the last 2 years we developed and applied monocrystalline silicon structures and established new automated protein analysis platforms. The development of a high throughput protein platform is presented where fully automated protein identifications are performed. It includes the reduction and alkylation of the protein sample in a standard 96- or 384-well plate format prior to injection of 1 μl samples into the continuous flow based microtechnology platform. The processed sample is transferred to a microchip nanovial array target using piezoelectric microdispensing. Identification is made by MALDI-TOF MS and a database search. After the initial sample reduction and alkylation period of 50 min the platform can digest and process protein samples at a speed of 100 samples in 210 min. An optional configuration of the platform, operating the dispenser in the 'static mode', enables on-target enrichment of low abundant proteins and peptides e.g. from 2DE samples. This makes detection at the low attomole level possible. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Silicon microstructures; Proteins

1. Introduction

As the human genome will be mapped within 2 years, we face the post-genomic era with the major challenge being to read the patterns of protein synthesis. The new science of proteomics — protein expression and profiling — promises exciting insights into the function and dynamics of the cell. In this quest some major technical hurdles remain to be overcome.

In pharmaceutical drug development much effort is focused on trying to understand the evolving states of a disease. During most of the drug development phases it is vital to have the appropriate analytical tools to qualitatively and quantitatively characterise and monitor key regulating targets. This will provide new information to enable the screening and testing of potential drug candidates for biological activity with the target. A detailed knowledge of the mechanistic action of these drugs in the complex matrices of the biological model systems can thus be gained.

Protein profiling and proteomics is of key importance for the deeper understanding of many major diseases. The biological material, be that rodent or human cell- and tissue models, forms the basis of biochemical understanding in these models which

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have a significant impact on drug development today. The last few years have shown an explosive progress in the fields of protein science, bioinformatics, cell and molecular biology. These developments have resulted in increased demands for new instrumental technology that allows identification and detailed structural studies of proteins to be automatically performed at high speed and with a high sensitivity. At present it is not possible to solely use nucleotide sequence data to gain insight into the dynamics of different basic biological processes like cell differentiation, receptor activation, signal transduction, malignant transformation and numerous other processes in living organisms. Studies of protein expression, interaction, post-translational modifications and resulting repercussions in biological systems must therefore be carried out at protein level.

The bottleneck in current proteomics technology is characterised by a relatively low throughput due to manual procedures and slow assay protocols. Also, the assays used are typically based on sample volumes at best in the range of 10–100 μl which makes large-scale screening protocols very expensive. Unfortunately, large volumes of biomaterials are today also a prerequisite to enable profiling at low abundant levels, often after time consuming enrichment procedures. The ever increasing demands for lowered detection levels cannot be stressed enough.

Miniaturization of analytical systems is generally considered to be the strategy that will overcome several of these shortcomings and thus generate improved process speed for performing efficient evaluation studies e.g. of drug mechanism of action [1]. This will allow early detection of eventual side effects, which are of great importance for the life science industry. Microstructure technology 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, orifices, and thin membranes [2]. By utilising the versatility of silicon micromachining to fabricate efficient minute volume microstructures it is possible to make biochemical analysis systems that are extremely small. The actual need for miniaturisation arises from the fact that reaction kinetics as well as sample handling procedures can be performed at a high speed and high sensitivities 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 consumes minimal sample volumes 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 [3–8]. Ideally, the small sample consumption of MALDI-TOF MS also allows other complementary analyses to be performed on the remaining sample, extracting the maximum amount of information that is obtainable from each sample [9–11].

This paper summarises the most recent developments of a miniaturised proteomics platform utilising a set of microstructured tools that efficiently interfaces automated sample handling robots in 96 or 384 plate formats at one end and MALDI-TOF MS at the other. The microstructure toolbox performs important sample processing and sample handling steps such as proteolytic digest and on-target sample enrichment. The benefits of flow-through microdispensing and critical issues on the design of nanovial MALDI — target plates are discussed.

2. Experimental

2.1. Chemicals

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: 7022801, Perseptive Biosystems, Framingham, MA, USA). α-Cyano-hydroxycinnamic acid were purchased from Sigma (St. Louis, MO, USA). Lysozyme (Lot: 56518) was purchased from ICN (Costa Mesa, CA, USA); acetonitrile from Merck (Darmstadt, Germany); sinapinic acid were obtained from Fluka (Buchs, Switzerland), and used without any further purification. Stock solutions of the proteins were made by dissolution in phosphate buffer (10 m*M*), and working solutions were made by diluting the stock solution in 0.1% TFA. A mass standard kit was used on a daily basis for external mass calibration. The water was purified using a

Millipore apparatus (Bedford, MA, USA). KOH and HF from (Merck) were used for the microstructuring of silicon.

2.2. Sample preparation

The seed-layer method [5] was used to prepare the homogeneous sample surfaces, where the typical procedure was as follows: (i) a diluted matrix solution (1–2 mg/ml in acetonitrile) was applied on the MALDI target (ii) the sample was mixed 1:1 with matrix solution (15 mg/ml in 50% acetonitrile, 0.3% TFA) and applied onto the same spot (iii) the sample was allowed to dry at room temperature for 5 min before the target was inserted into the mass spectrometer. In the high density applications, the sample solution (ii) was filled into the dispensing unit and dispensed onto the sample plate.

2.3. Instrumentation

The MALDI-TOF instrument was a Voyager DE-RP PRO (Perseptive Biosystems, Framingham, MA, USA) mass spectrometer. The instrument, equipped with a delayed extraction ion source, uses a nitrogen laser at 337 nm and can be operated in both linear (L) and reflector (R) mode at accelerating voltages of 20–25 kV. The sample targets were either made of polished stainless steel or gold. The in-house developed flowthrough piezoelectric microdispenser was fabricated using silicon micromachining and has been described elsewhere [12,13]. The dispenser used had a 40×40 μm nozzle and 65 pl droplets were generated by applying a voltage pulse to the piezoelectric element. The microdispenser was used in order to enable high precision nl–pl sample depositions onto the target surface. Sample deposition on high density MALDI targets was accomplished by controlled stepping of the target plate using an in-house constructed computer controlled x–y stage. The translation in each direction was controlled by stepper motors covering a maximum area of 180×180 mm at a resolution of 10×10 μm. The translation sequence of the x–y stage and the pulsing of the dispenser was controlled from software running under LABVIEW (National Instruments, Austin, TX, USA) on a Pentium PC. Visualisation of the sample spotting was obtained by using a microscope (Leitz, Wetzlar, Germany) equipped with a video camera (CCD-72EX, DAGE-MTI Michigan City, IN, USA). Typical pulse conditions, obtained using a pulse generator (Model 8111A, Hewlett-Packard, Palo Alto, CA, USA) were 50–100 Hz (pulse length 10 μs), using an applied voltage of 10–20 V given by a DC power supply (Model E3612A, Hewlett-Packard).

3. Results and discussions

3.1. Microstructure design and manufacturing

3.1.1. Microstructure fabrication

All microfabricated components were structured using anisotropic etching in KOH. The microdispenser parts and the MALDI-target nanovials (100–300 μm in width) were fabricated in ⟨100⟩-silicon, p-type (1–10 Ωcm) and the micro enzyme reactor (μIMER) was etched in ⟨110⟩-silicon, p-type (10–20 Ωcm) and was composed of 30 parallel vertical trenches, 50 μm wide, 250 μm deep and 1200 μm long, internal volume 6 μl, Fig. 1a. To obtain an increased surface area in the μIMER a porous silicon layer was generated in the walls of the channel array by anodising the IMER chip in an HF–ethanol solution. Fig. 1b shows a cross-section of the μIMER. Porous silicon typically offers surface areas of 300 m^2/g, which makes it an excellent intrinsic carrier matrix in chip integrated analysis systems. Standard procedures for immobilising enzyme onto conventional glass matrices are applicable to silicon. Trypsin and chymotrypsin microreactors were prepared for the on-line protein digest system. A more detailed description of the microreactor fabrication and enzyme activation of porous silicon is described in [14,15].

The small protruding nozzle of the microdispenser was defined by pn-etch stop processing, an electrochemical stop etch technique [16]. A principal scheme of the nozzle etching is displayed in Fig. 2a and the obtained result is seen in the SEM picture in Fig. 2b. The thin membrane (7 μm) in the pressure generating side of the microdispenser channel, Fig. 3a, was also defined by this process. A detailed description of the microdispenser design and principal function has been given elsewhere [13].

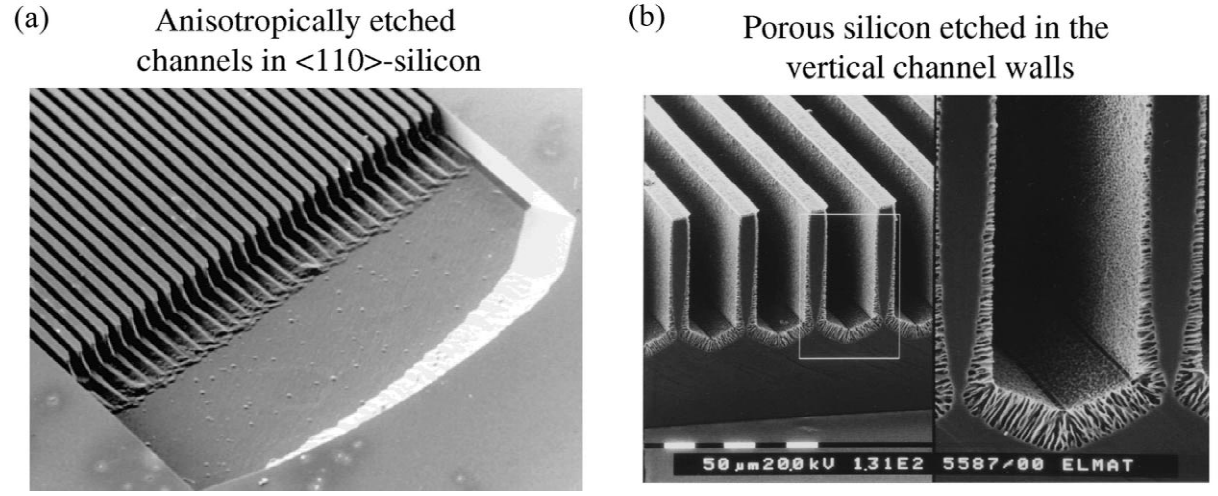

Fig. 1. (a) Overview of the anisotropically etched channel array in the microreactor. (b) Cross-section of the trench type channel arrays in the µIMER with the porous silicon surface layer.

3.2. Microfluidic dispensing

When adapting inkjet technology to chemical applications a fundamental difference is that fluid properties such as viscosity, hydrophobic interaction and evaporation/crystallisation must be allowed to vary considerably. This puts completely new demands on the inkjetting/microdispensing device and the conditions for stable and reproducible droplet formation are far from ensured when, for example, employing microdispensing on-line to a gradient elution in a chromatographic separation. Fig. 4 shows the influence of viscosity on droplet formation, for viscosities ranging between 1 and 65 mPas. As the viscosity increases (higher glycerol concentrations) a longer 'tail' is formed after the main

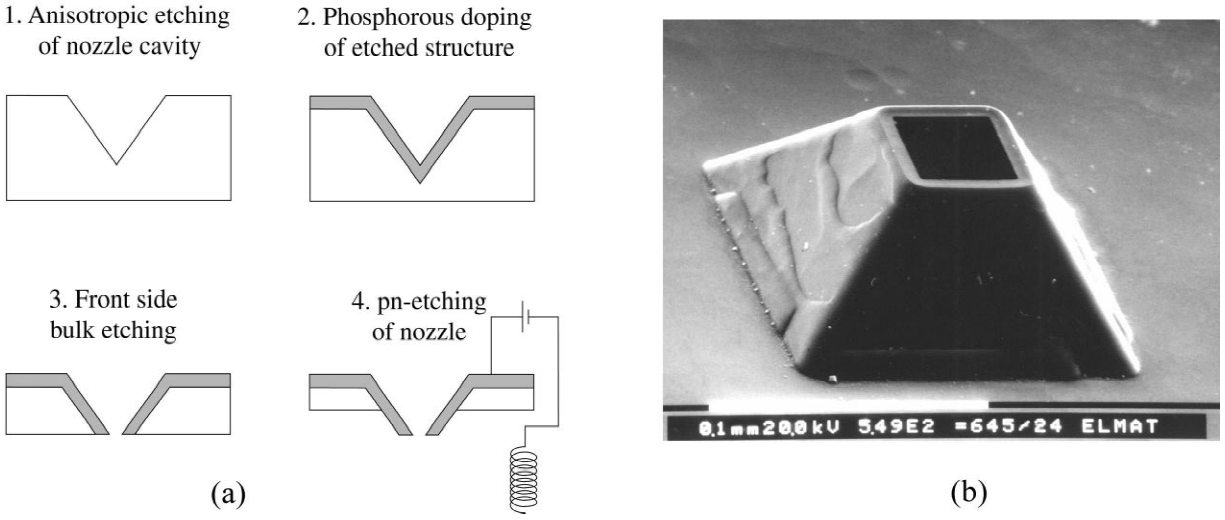

Fig. 2. (a) Principal process scheme for the nozzle fabrication. (b) SEM of the silicon microfabricated dispenser nozzle.

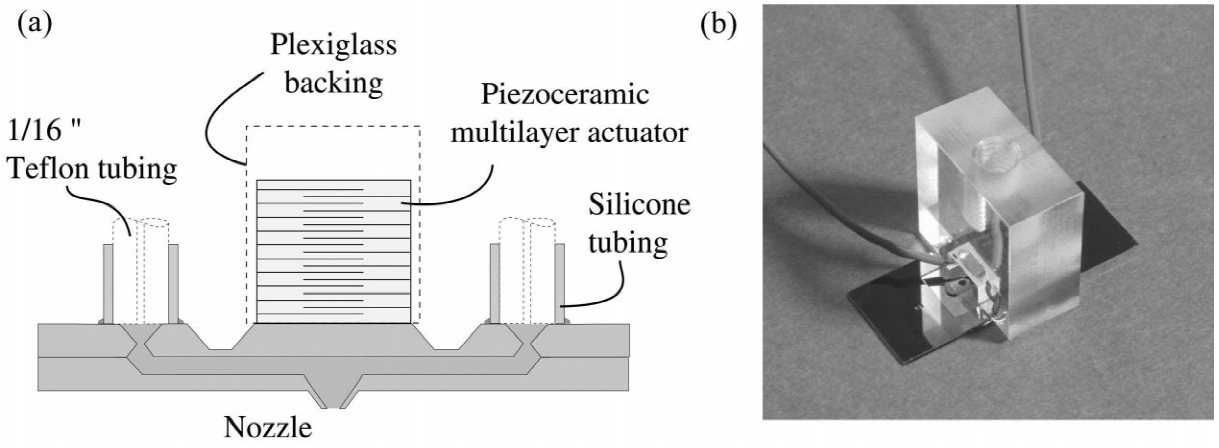

Fig. 3. (a) Schematic drawing of the microdispenser design. (b) Assembled microdevice.

droplet, risking the tail fraction breaking up into sub droplets in an uncontrolled manner. The target plate may thereby be contaminated with fractions in false positions. To overcome these problems our group has investigated these situations and adapted the original flowthrough microdispenser [17] to the fluid parameter situation commonly encountered in proteomics assays. The introduction of a pyramid-shaped nozzle in the microdispenser improves the droplet formation stability and droplet directivity, as well as minimising wetting problems at the orifice vicinity. Earlier studies involve characterisation and optimisation of the dispenser, including successful liquid handling of various mobile phase compositions as well as the use of several MALDI matrices [5,6,8]. The dispenser has also proven to be useful for preconcentrating samples of low concentration levels by using multiple sample depositions on the same spot, see Section 3.6, spot-on-a-chip technology.

3.3. Sample preparation

3.3.1. Automated MALDI analysis

In drug discovery and development there is currently a trend towards high throughput screening (HTS) applications, where the speed of data generation and cost per analysis are key factors to consider.

In order to execute high throughput protein analysis, the analytical technique must be fast, reliable and automated. MALDI MS fulfils the speed criteria since a mass spectra can be obtained within hundreds of microseconds. The automation criteria is also covered since several manufacturers supply automation capabilities with the instruments currently on the market. However, the large quantitative deviations generally found in MALDI-MS sample preparations have had a profound effect upon the progress of HTS systems using MALDI MS. On the other hand, improved sample preparation methods and data collection strategies [18] have opened up new possibilities and an increased use of MALDI MS in HTS applications can be expected in the near future. A recent report by Little et al. [3] presents high-throughput analysis of oligonucleotide samples using piezoelectric pipetting into sample wells (prefilled with matrix) on a silicon chip. An array of 100 positions was filled with a sample solution within 4 min and analysed within 43 min.

Our development of the microanalytical toolbox has enabled us to address several of the demanding analytical protein chemistry issues. The use of small sample spots, matching the size of the laser focal point, is one of the major advantages in the automation process, since searching for high intensity locations (hot-spots) can thereby be avoided. However, this advantage can only be fully utilised when homogeneous sample preparations are obtained using the seed-layer methodology [5]. This method is based on applying the sample–matrix mixture onto a predeposited highly diluted matrix spot. The pre-

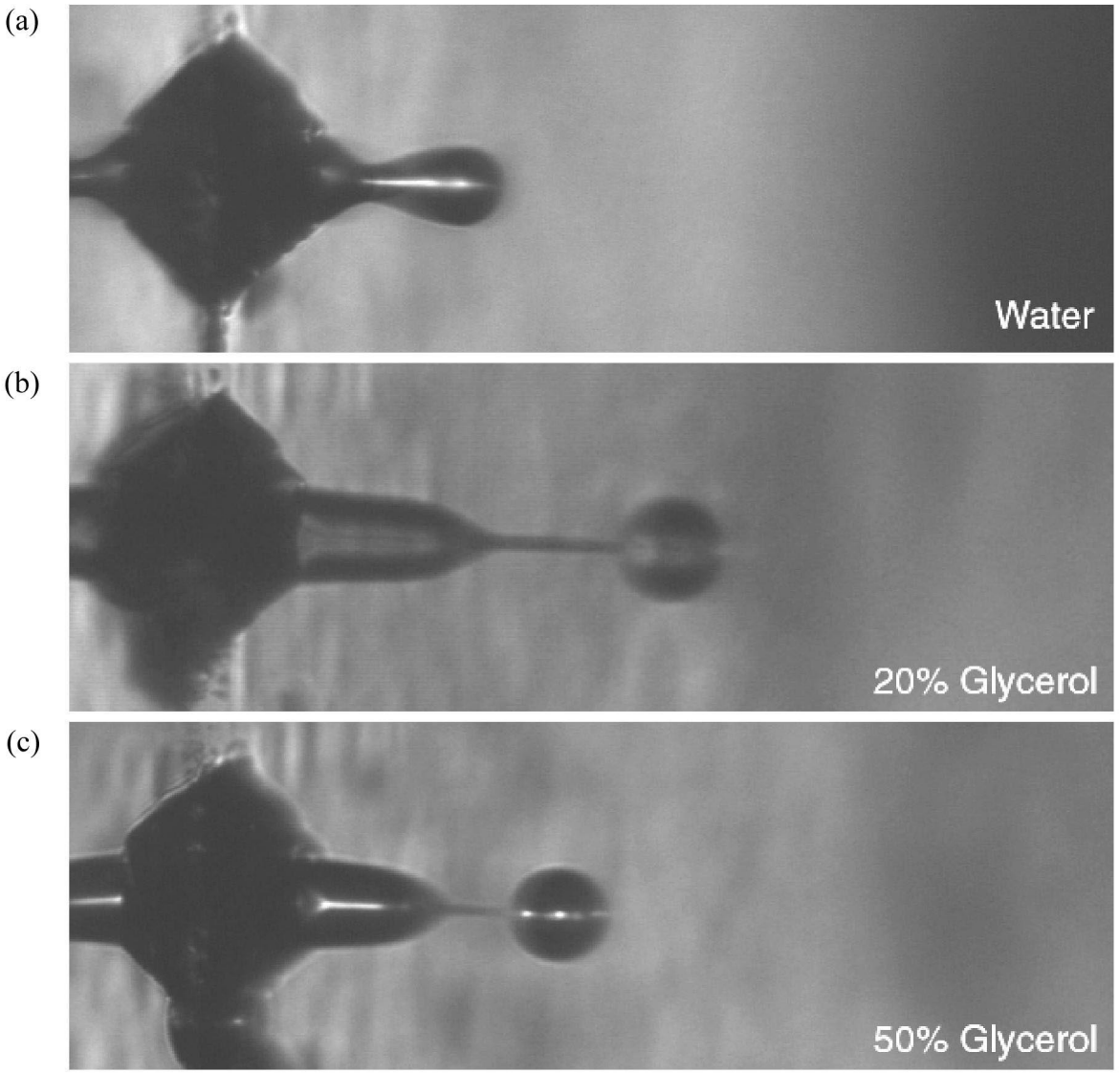

Fig. 4. Droplet formation versus increasing viscosity (a) water (b) 20% glycerol in water, (c) 50% glycerol in water.

deposited crystals act as seeds for the new sample which provides crystals much smaller in size and more evenly distributed than what is obtained with conventional methods. This 'seed-layer' method was developed, optimised and compared to the dried-droplet method using peptides and proteins in the 1000–20 000 Da range. It was found that the seed-layer method increases the surface homogeneity, spot-to-spot reproducibility and sample wash ability as compared to the commonly used dried-droplet method. This methodology is applicable to α-cyanohydroxycinnamic acid, sinapinic acid and ferulic acid, which all form homogeneous crystal surfaces. Fig. 5 shows the differences in homogeneity obtained by applying a dried droplet as compared with the seed-layer deposition technique. Not only do we obtain an improved homogeneous protein distribution but data also show an improved sensitivity on seed-layer prepared surfaces. This is probably due to the enhanced surface area in the

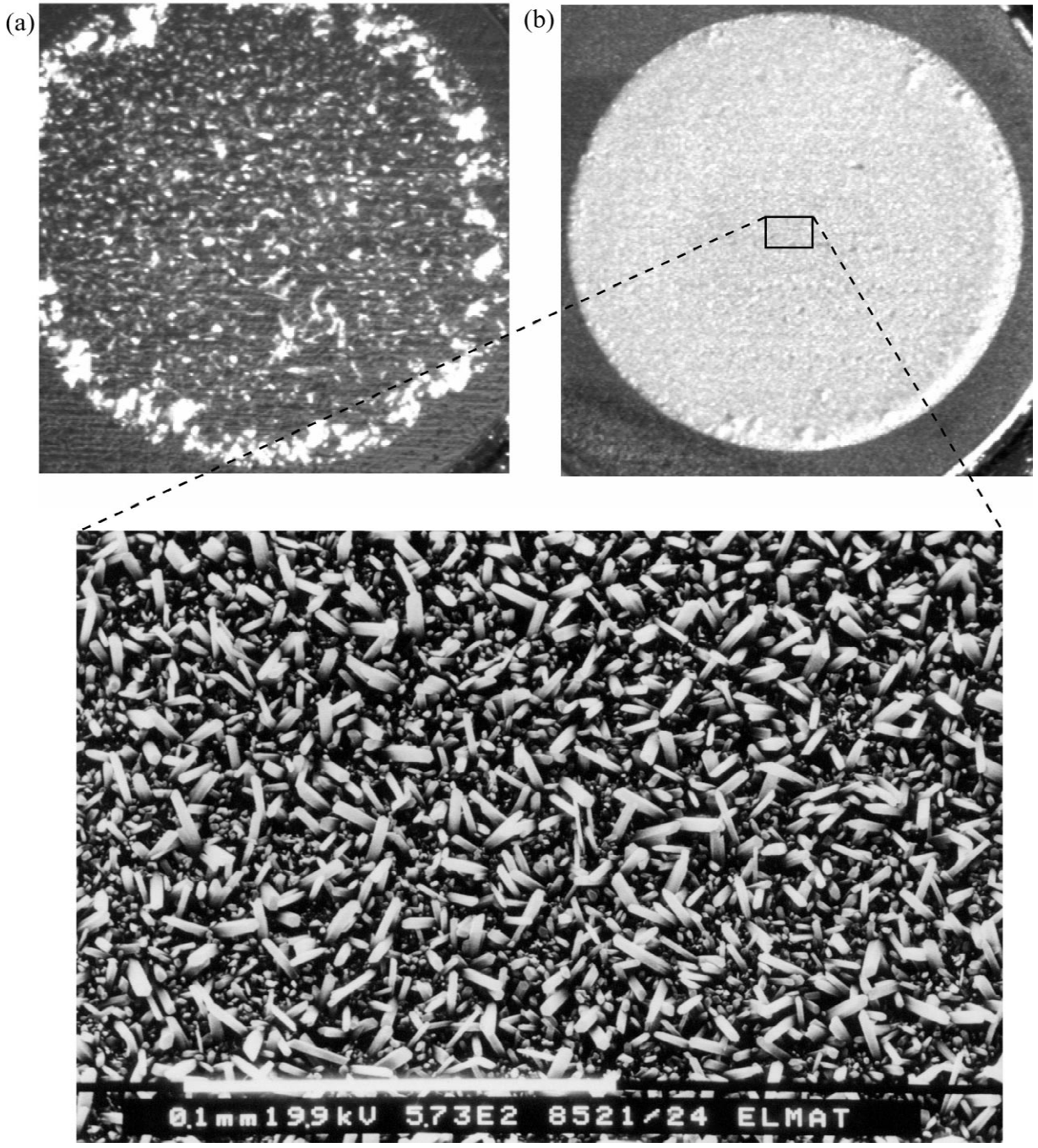

Fig. 5. Crystal images of sinapinic acid (a) dried droplet (b) seed-layer.

finer crystal matrix. Within- and between-spot variations were investigated using statistics at a 95% confidence level ($n=36$). The statistical values were generated from more than 5000 data points collected from 500 spectra. More than 90% of the sample locations results in high intensity spectra with relatively low standard deviations. The time needed to fully process and average 16 laser scans is approximately 15 s, corresponding to a sample throughput of 240 spots/h. In fact, our current limitation to reach improved throughput is the time it takes to move the target in the MALDI instrument.

3.4. Integrated microanalytical toolbox for automated protein identification

The microanalytical system is composed of three components that enable the efficient handling of sub nanolitre sample volumes in a fully robotised and unattended operational mode. The developed microstructures are; the porous silicon protease microreactor (μIMER), the flow-through microdispenser and the nanovial target arrays.

3.4.1. Porous silicon microreactor

The developed porous silicon microreactors were activated with trypsin and the reactor kinetics was characterized with respect to fluid dynamics and temperature, using a colourimetric assay. The obtained spectrophotometric data was analyzed using the Lambert–Beer Law and assuming first order enzyme kinetics.

Briefly, a solution containing α-N-benzoylarginine ethyl ester (BAEE) was continuously injected directly into an absorbance detector to obtain the baseline level. Subsequently a valve was switched and the reagent was passed through the μ-chip IMER, where the enzymatic reaction yielded a colored compound as product, before entering the absorbance detector. The observed shift in absorbance readings was the result of enzymatic cleavage of the substrate (BAEE). By applying the Lambert–Beer Law, the actual BAEE turnover rate, V_e, was calculated. The obtained turnover rates were plotted against flow for BAEE concentrations of 0.075–0.5 m*M*. By plotting the maximum turnover rates against the substrate concentration, apparent V_{max} and K_m were obtained by fitting the theoretical equation of a first order enzyme kinetic system to the plot. From the corresponding Lineweaver–Burk and Eadie–Hofstee plots estimates of apparent V_{max} and K_m were derived [19], see Table 1.

3.4.2. Microdispenser and nanovial arrays

Since deposition of sample amounts in the nl–pl range are very difficult to handle by conventional pipetting, special tools need to be developed to avoid sample loss and inaccurate positioning of sample. In this work the in-house developed piezoactuated flowthrough microdispenser was used to deposit sample volumes ranging from 50 pl (a single droplet) to over 100 nl. The microdispenser holds an internal volume of 250 nl between inlet and the nozzle. Droplet frequencies up to 5000 Hz have been demonstrated, allowing a dispense volume flow up to 15 μl/min.

Nanovials shaped as inverted pyramids, defined by the anisotropic properties of silicon, were etched in array formats, 10×10, of vial sizes ranging from 100 to 400 μm [20]. The vial arrays facilitated automated sample handling by the ordered collection of volumes composed of single droplets up to several thousand droplets. The depth of the vial geometry was found to influence the peptide mass resolution and the boundary condition for this limitation was explored.

Developing MALDI-target plates with microvials in silicon wafers holding several tens of thousands of sample positions per plate can easily be manufactured. These sets of MALDI target chips are normally operated and made in smaller units of 100 vials/

Table 1
Estimates of apparent V_{max} and K_m for trypsin calculated by different kinetic models

	V_e versus S_0	Lineweaver–Burke	Eadie–Hofstee
Apparent V_{max} (nmol/min)	8.9	8.8	8.9
Apparent K_m (m*M*)	0.11	0.10	0.11

target for practical reasons and fundamental R&D work.

The first results using the microdispenser in combination with nanovial arrays showed a dramatic increase in sensitivity when interfaced to MALDI-TOF MS. Fig. 6 demonstrates the difference in sensitivity when a conventional dried droplet preparation is compared to a microdispensed nanovial preparation. In the upper mass spectrum (a), using the dried droplet technique, peptide peaks can barely be identified while in the lower spectrum, using microdispensing in nanovials, a clear improvement in signal to noise is obtained. Also, new peptide peaks clearly emerge in the spectrum.

Experiments revealed that the isotope resolution, when analyzing peptides, was much (up to a factor 10) lower for samples analysed inside the vials of inverted pyramid shape, than for samples that were deposited on top of the silicon surface between the vials, Fig. 7c. It was concluded that this phenomenon originated from the spatial distribution of matrix-sample crystals along the vertical axis within the vial. The low resolution consequently leads to decreased mass accuracy and to avoid this shallow vials, i.e. trunkated inverted pyramids, with a vial depth of 50 μm were manufactured. Fig. 7a and b shows an SEM images of the two types of vial arrays that were investigated and Fig. 7c shows their corresponding influence on mass resolution.

3.5. Protein platform

Protein samples were injected into the protease activated microreactor and were digested on-line before proceeding to the flow-through microdispenser, which was interfaced to MALDI-TOF MS via sample dispensing onto the nanovial array targets. From the target plates peptide map fingerprints were mass identified followed by subsequent data base search and protein identification.

In the system set-up, Fig. 8, the protein sample was first subjected to pretreatment in an autosampler where it is made amenable for digestion, a step that took 50 min. The pretreatment robot was a Gilson ASPEC XL autosampler equipped with a six-port injection valve with internal loop 0.5–5 μl volume. The sample rack was adapted for this application, giving a maximum capacity of 576 samples per run. This pretreatment system was controlled with Gilson 719 software from a Pentium PC, and all programmes were written in TURBO PASCAL.

The variations in structure make proteins differ in their susceptibility to enzymatic digestion and to aid enzymatic cleavage the sample pretreatment step is necessary. This was performed by adding a denaturing agent, urea (1–8 *M*) or guanidine–HCl (0.5–6 *M*), and a reducing agent, dithiotretinol (4–8 m*M*), to the protein sample. All reagents and samples were dissolved in digestion buffer, 25 m*M* NH_4HCO_3, pH 7.8 or 25 m*M* Tris, pH 8.2. The sample was then heated at 75°C for 20 min. In cases where refolding of the protein was to be avoided an alkylating agent, iodoacetamide (10–30 m*M*) was added after the denaturation/reduction step. The carboxyamidomethylation reaction took 30 min at room temperature. If high concentrations of pretreatment reagents were used the sample was diluted until the concentration of urea was below 2 *M* or guanidine–HCl was below 1 *M* before injection into the μIMER.

The samples were then consecutively injected into a flow of digestion buffer (25 m*M* NH_4HCO_3, pH 7.8). The flow carried the sample to the microchip IMER wherein the enzymatic digestion of the protein took place. Sample residence time in the microchip IMER was 1 min, which was sufficient to achieve the desired degree of digestion for the studied proteins. To increase efficiency of the digestion process the microchip IMER was heated to a temperature of 50°C. The sample plug subsequently proceeded to the microdispenser where an adjustable fraction of the sample was deposited onto a MALDI-target plate.

The 32-channeled μIMER made out of porous silicon has been shown to give a 170-fold increase in enzyme activity compared to non-porous reactors [14]. While the micromachined reactor does not quite offer the same surface area as a microcolumn with packed material, it has better flow characteristics, lower hydrodynamic pressure drop, is less sensitive to carryover effects and is much simpler to manufacture in the desired dimensions. The longterm operational stability of the microchip IMER was found to be very high. This was proven by using one of the reactors for performing more than 400 protein digestions of samples containing high concentrations of

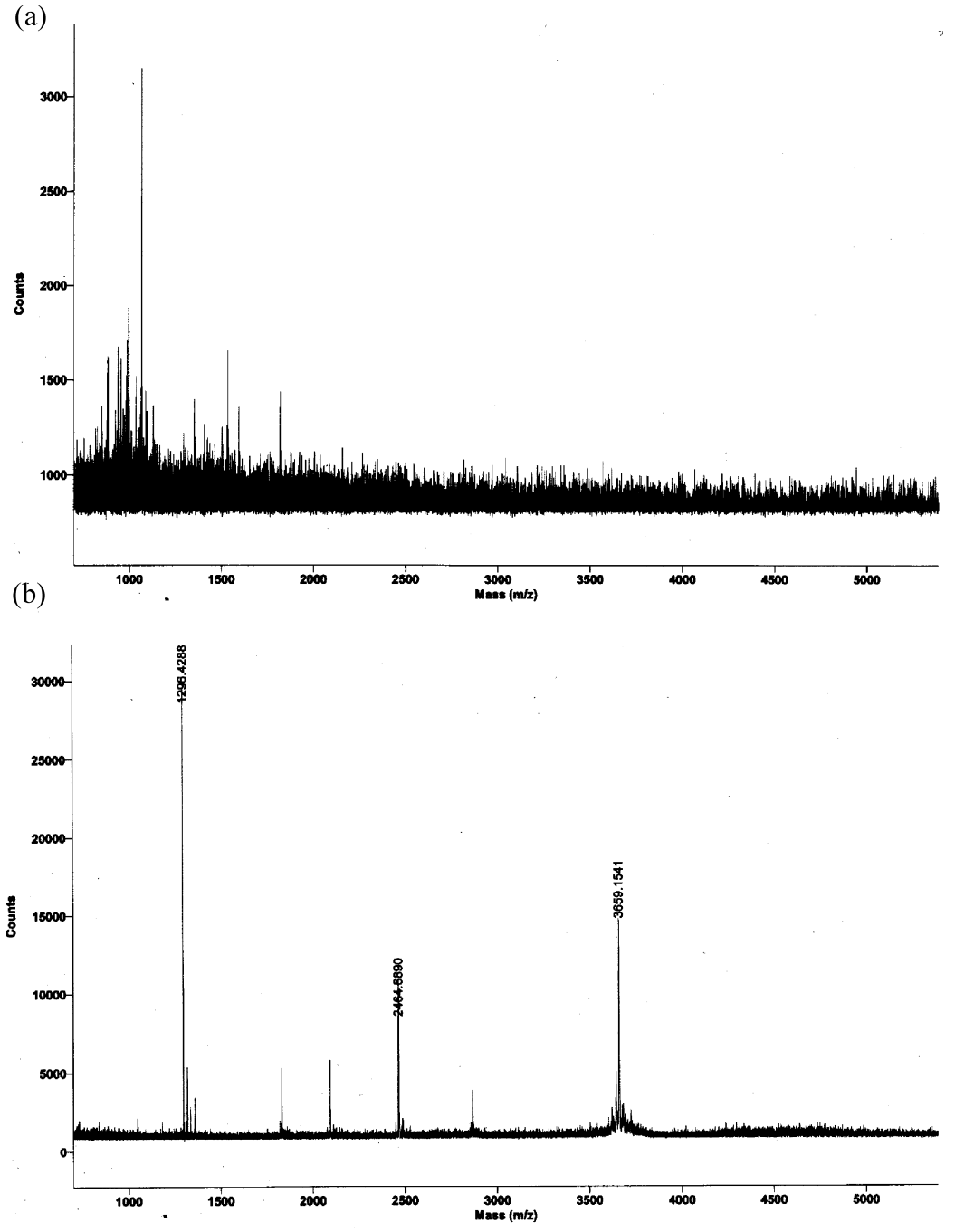

Fig. 6. (a) Mass spectra obtained from ordinary (dried droplet, 50 fmol) sample preparation of a peptide mixture (50 fmol/μl), (b) Mass spectra obtained from 1000 droplets (0.5 fmol) of calibration mixture (50 fmol/μl) deposited in a nanovial.

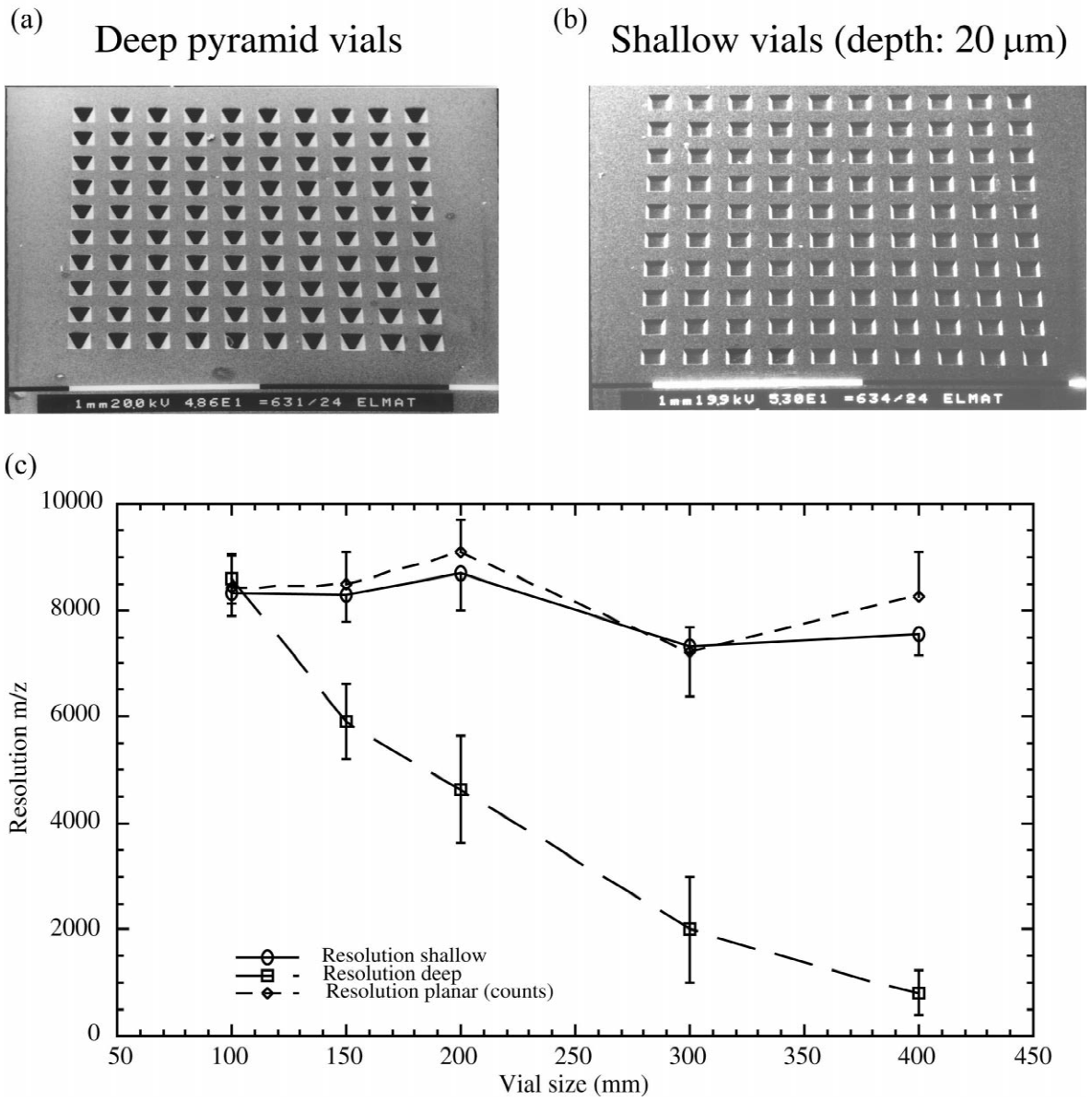

Fig. 7. (a) Inverted pyramid. (b) Trunkated inverted pyramid. (c) Mass resolution vs. vial geometry.

pretreatment reagents, during a time period spanning over 2 months, with no observed loss in catalytic efficiency. Resulting peptide map fingerprints of lysozyme at 1 pmol/μl is shown in Fig. 9 using either chymotrypsin or trypsin immobilized in the microreactor. The use of multiple parallel reactors with different catalytic selectivity can add further dimensions to the protein identification.

The on-line digest system required a 1-min regeneration to avoid carry-over from the preceeding sample. Together with 1 min of protein digest in the μIMER this resulted in a system throughput of 100 samples in 3.5 h. Microdispensing of the samples easily allows several 1000 samples to be deposited on each MALDI-plate.

The minute sample volume consumption when

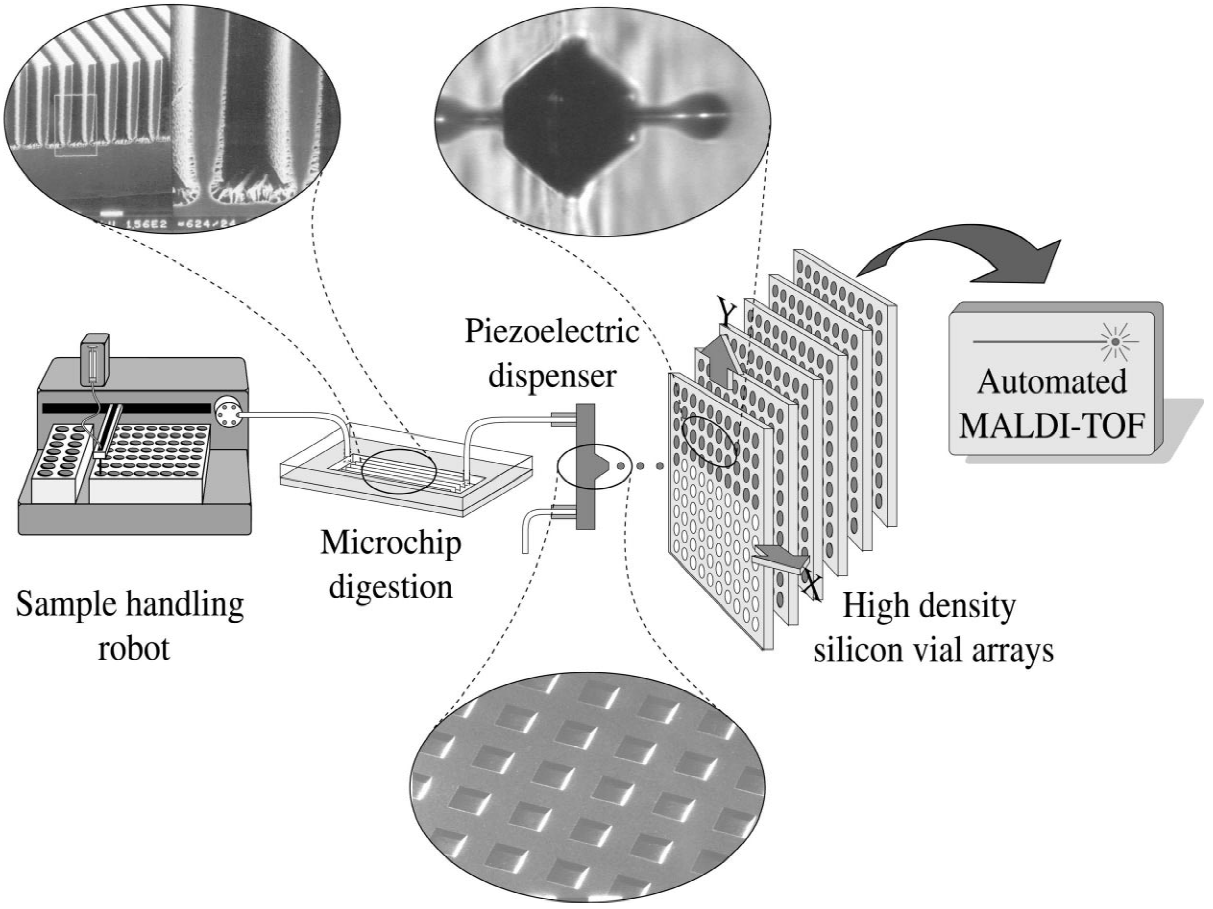

Fig. 8. Schematic drawing of the integrated microanalytical workstation. Inserts show the micromachined parts of the platform.

microdispensing and the flowthrough feature of the device allows on-plate sample enrichment at the same time as not the entire sample volume is deposited on the target. This enables collection of the remaining sample with a fraction collector at the flow outlet [21]. Unidentified samples or proteins of special interest can then be further analyzed by other methods, such as immunoassays, or in the case of key regulating proteins MS–MS sequencing by use of nanospray technology.

3.6. Spot-on a-chip technology

When analysing proteomic samples from e.g. two-dimensional gel separations, high sensitivity techniques are required. For these situations the spot-on-a-chip platform was developed and optimized allowing detection at levels below the 500 fmol level to which the on-line protein microplatform described in Section 3.5 is limited.

As the microdispensing for picolitre sample processing in MALDI-TOF MS was developed it was found that the dispensing technique provided a more quantitative and reproducible mass spectra, when compared to mass spectra obtained from samples prepared by conventional dried droplet methodology. Also, a considerably improved signal-to-noise ratio was obtained [5]. Furthermore, an almost linear increase in MS-signal vs. the number of droplets dispensed on the microchip surface was observed here. To further improve the throughput of the on-target deposition, heating of the MALDI target plate

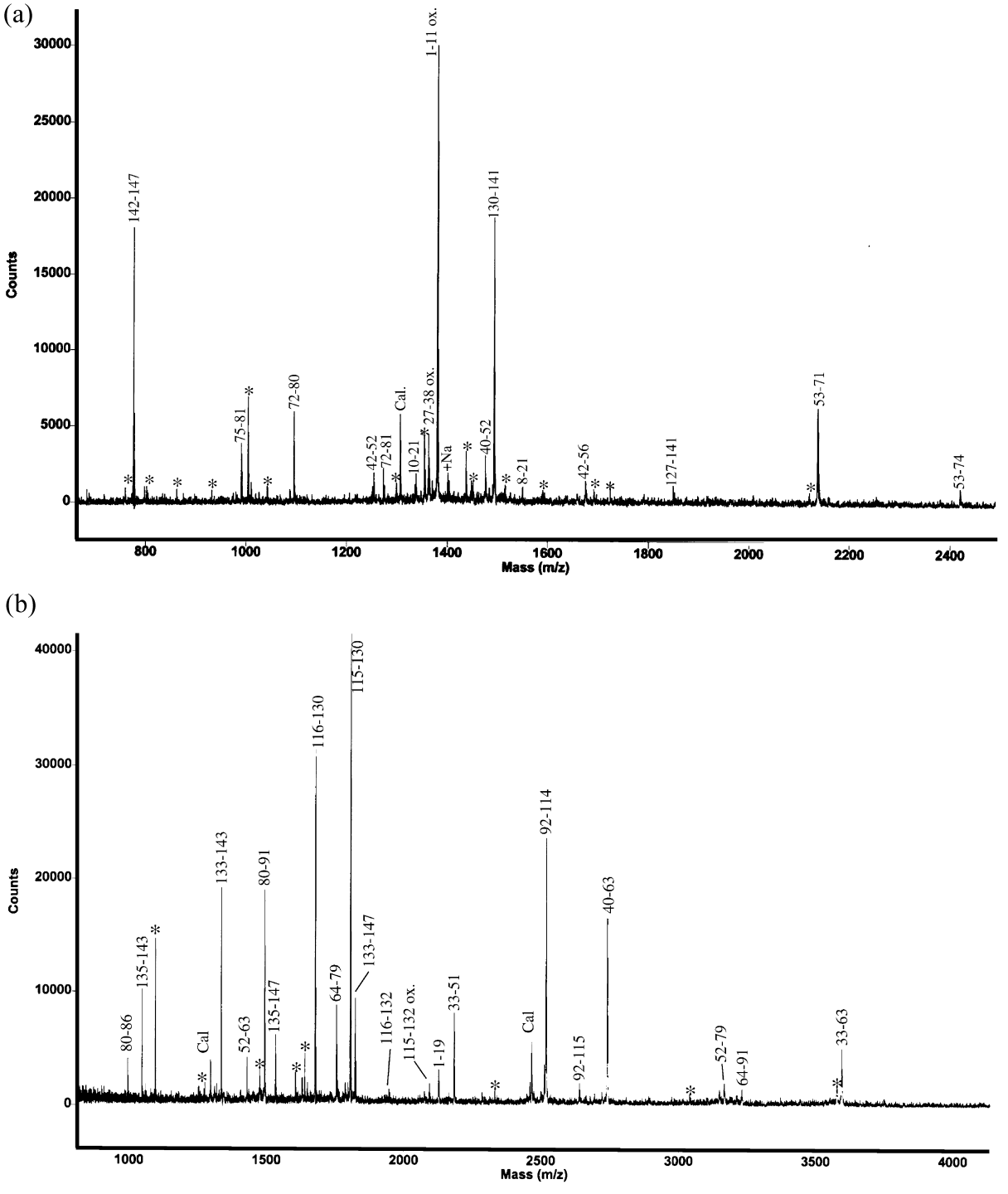

Fig. 9. Digest pattern of lysozyme obtained from (a) upper a trypsin microchip IMER, (b) a chymotrypsin microchip IMER.

(onto which the microchip was fixed) to a temperature of 50°C was performed to shorten the evaporation time. These findings triggered further work on elucidating the effective range of the enrichment strategy.

This enrichment technology was coined spot-on-a-chip technology and allows enrichment and detection of samples in the low attomole range, using MALDI-TOF MS analysis. Typically we can generate mass spectra from two-dimensional gel spots where traditional dried droplet sample preparation fails. Fig. 10 shows the amplification platform developed for high sensitivity protein identifications. The use of nanovials is an important step since high degrees of enrichment are more easily obtained. The sharp edges of the vials function as capillary brakes for the sample volume dispensed into a vial which therefore does not bleed out on the surrounding chip surface. The dispensed volume is maintained on the same chip area, inside the vial, independent of the total volume dispensed, whereas in the case of planar target surfaces the sample spot grows with the dispensed volume. The surface density of sample molecules is thus easier to enrich using nanovials.

An example of low abundant protein detection is given in Fig. 11 where calumenin (I) was identified

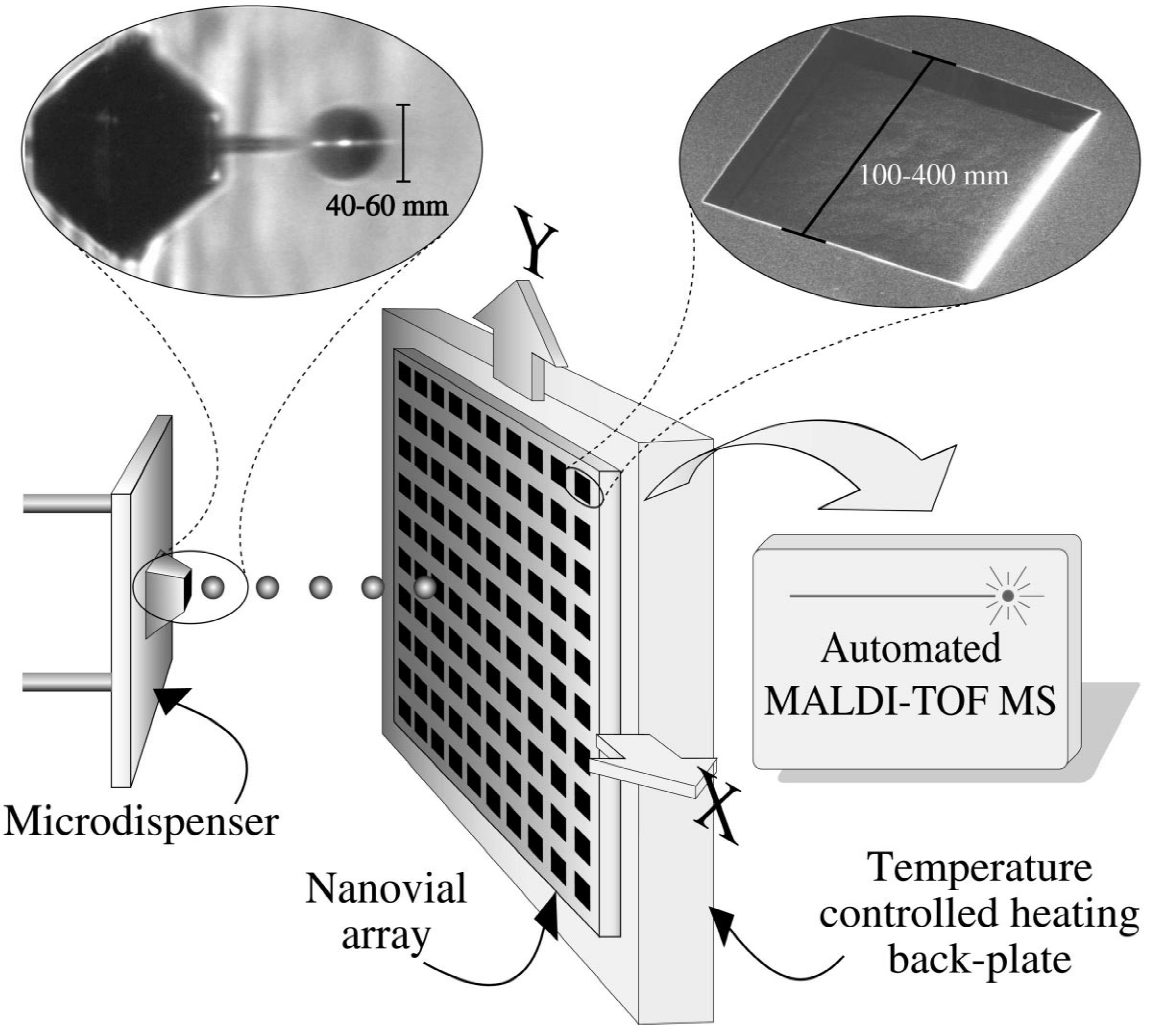

Fig. 10. Schematic drawing of the spot-on-a-chip amplification platform.

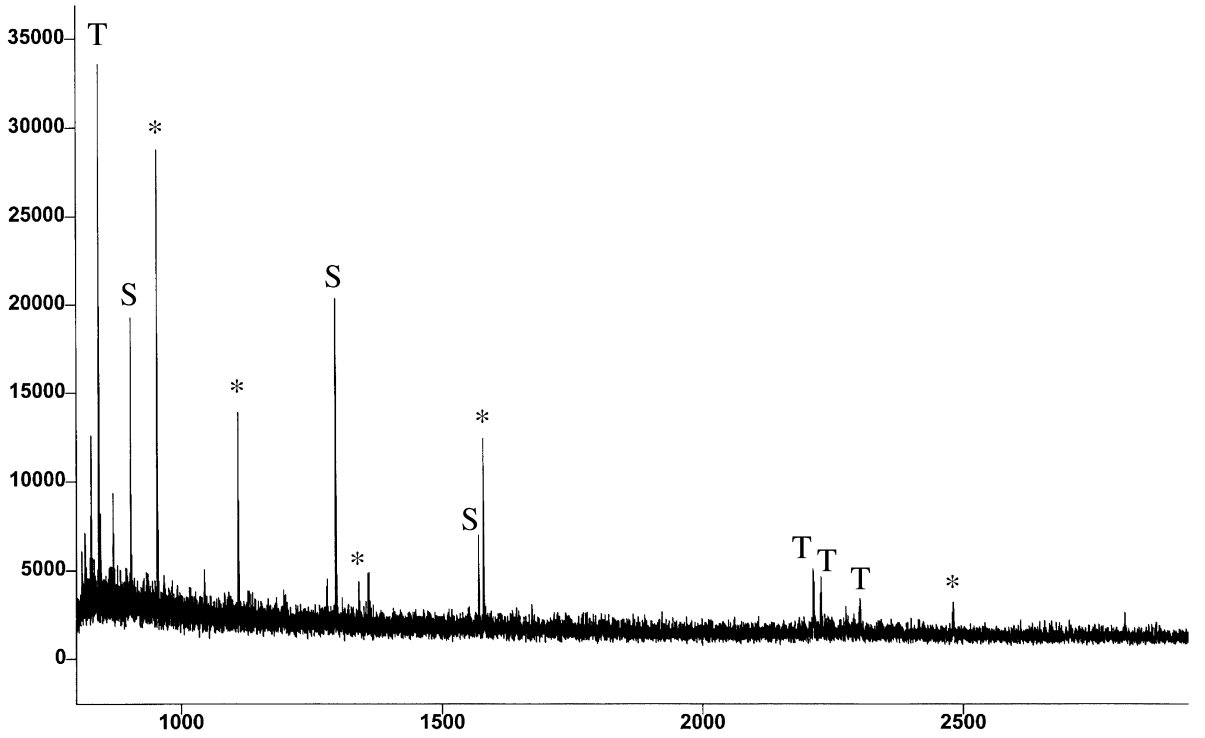

Fig. 11. Mass spectrum resulting from deposition of 500 droplets of reversed-phase nanocolumn purified solution from in-gel digestion of an excised 2-DE spot correctly identified as human calumenin with MS-fit [22]. At a mass accuracy of 10 ppm a sequence coverage of 15% was obtained. T, trypsin autolysis peaks; I, internal standard; *, unidentified peak. Matrix; α-cyanohydroxycinnamic acid (CHCA).

in an excised gel spot from a 2D-PAGE run. As can be seen in the mass spectrum, relevant autotryptic trypsin peaks were obtained (labelled T), which enables a proper mass calibration to be made. By working under restricted environmental conditions, we were able to circumvent keratin contamination. This also allowed an improved sensitivity to be obtained since ion suppression effects caused by other high abundant proteins commonly present (e.g. keratins) were avoided.

The target arrays used had vials sized 300×300 μm as they were found to be ideal, providing the best compromise between a small spot size and the need to have a sufficient amount of analyte to acquire several spectra from each vial. A further advantage of the vial structure is the fact that the sample is guided into the vial by surface tension forces even though the droplets may be slightly off target. This factor is important since it improves the reliability of the spot-on-a-chip technology when operated in automated mode. Furthermore, no 'hot-spot' searching is needed since the desorption laser covers the whole vial upon excitation.

The spot-on-a-chip technology can typically provide a sensitivity amplification currently at the level of 10–50 times compared to an ordinary sample preparation. More important, peaks that are buried by the noise level can become clearly detectable which may be the deciding factor that actually is the difference between a successful identification and a failure. Recently developments have been initiated to map small immunoregulating proteins such as cytokines and chemokines using the spot-on-a-chip platform.

4. Conclusions

This paper presents a new step in the development of tomorrow's totally integrated and miniaturised protein analysis platforms. It is demonstrated that silicon microstructures provide the means necessary

for effective unattended automated protein identifications.

Two integrated protein identification systems are described. The first one includes an upstream protein digest station and performs the total analysis on-line in an automated and unattended mode. The second protein identification system is dedicated to a lower abundance area where identifications can be made in the lower attomole region. The microdispenser is here operated in the static mode. Static mode means non flowthrough operation, i.e. the sample volume is injected directly into the dispenser and subsequently dispensed onto the target, whereby sample enrichment and signal amplification is achieved of samples generated by batch-digestion, or in-gel digested protein samples from 2D gel electrophoresis samples [21].

The next generation of the microsystem, already under construction, will be even smaller with a higher degree of integration. Smaller microchip 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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