Anal. Chem. **2005,** *77,* 827−832

Chip with Twin Anchors for Reduced Ion Suppression and Improved Mass Accuracy in MALDI-TOF Mass Spectrometry

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A new sample target for matrix-assisted laser desorption/ionization mass spectrometry is described. The target consists of pairs of elevated hydrophilic anchor surfaces, positioned in proximity onto a microchip. The anchors are used to obtain separate preparations of sample and external standard, while both anchor surfaces are irradiated simultaneously by the laser pulse. Using a standard, based on six peptides, a 2-fold improvement in mass accuracy is observed. Also, ion suppression is significantly reduced. With a one peptide calibration standard, 22 tryptic fragments from a BSA digest are detected using the twin-anchor concept, whereas only 14 fragments are detected when the sample and standard are laser-ablated as a mixture from a conventional anchor target. A volume of ~30 pL of sample solution of angiotensin I is transferred to the anchor surface, under a thin layer of a perfluorocarbon, to prevent a concentration bias due to evaporation. With this arrangement, a detection limit of 1.5 amol was achieved with a signal-to-noise ratio of 22:1.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), has rapidly become an indispensable technique in proteomics for identification of proteins by peptide mass fingerprinting. In such work, the reliability of identification depends not only on the accuracy of the mass measurements but also on the number of peptides, which can be identified from digests. Proteins of low abundance are of central importance in proteomics, and therefore, the sensitivity of the analytical system is another crucial issue.

It became clear many years ago that refined sample preparation technology, including the use of new types of sample targets, could lead to improved sensitivity. A decade ago, we demonstrated low-attomole sensitivity, by using picoliter-sized vials on a silicon microchip.[1] The idea was based on two fundamental strategies: (1) to confine the minute amount of sample in a very small volume; (2) to consume the entire sample during the analysis. Another advantage of the reduced size target approach is that the ionizing laser irradiates the entire sample, which eliminates the need to search for sweet spots. More recently, microchip-based targets have been utilized by several workers, often combined with piezoelectric dispensers.[2,3] Other new developments include sample preconcentration with nanoscale pipet tips containing reversed-phase chromatography media[4] and the use of new sample support materials.[5−11] Also, reducing the size of the matrix crystals leads to an improved sensitivity.[12] An important new idea was reported by Schuerenberg et al., who suggested a sample confinement by means of hydrophilic anchors on a fluorocarbon-coated plate.[13,14] The sample/matrix crystallizes on a small, defined area and a sensitivity increase of 5−10 times was reported for peptide and DNA samples.[13] Another benefit of the hydrophilic anchor concept, similar to the case of the chip-based microvials, is that the laser beam covers most of the crystallized matrix/sample, and consequently, it is not necessary to search for sweet spots.

In a preliminary investigation, Bogan et al. recently demonstrated a simultaneous ionization from the edges of two adjacent sample spots.[15] As pointed out by these authors, this opens up new possibilities for improved calibration and enhanced sample throughput. In the present study, we report the use of a microchip with geometrically predefined pairs of miniaturized anchors, allowing desorption of physically separated analyte and calibration samples, irradiated within the envelope of a single laser spot.

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10.1021/ac0400966 CCC: $30.25 © 2005 American Chemical Society
Published on Web 01/05/2005

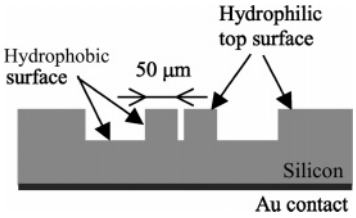

Figure 1. Cross section of the sample target.

Basically, a calibration procedure using an external standard, but mimicking the use of an internal standard was aimed for. The concept results in a better mass accuracy and minimizes the normally observed deterioration of analyte signal caused by ion suppression, when an internal standard is added. Additionally, we describe a new approach for transfer of very small sample volumes to the surface of the miniaturized anchors.

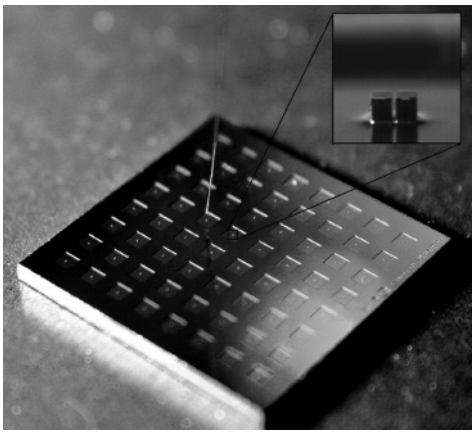

Figure 2. Microchip viewed from an angle. An enlarged picture of an anchor pair is inserted in the top right corner. The dosing capillary can be seen above the chip, adjacent to the enlarged anchor pair.

EXPERIMENTAL SECTION

Chemicals. Acetonitrile, trifluoroacetic acid (TFA), bovine serum albumin (BSA), angiotensin I, angiotensin II, bombesin, adrenocorticotropic hormone (ACTH) clip 1−17, ACTH clip 18−39, and somatostatin 28 were purchased from Sigma-Aldrich (St. Louis, MO). Sequencing-grade trypsin was obtained from Promega (Madison, WI). α-Cyano-4-hydroxycinnamic acid (CHCA) was acquired from Bruker Daltonics (Bremen, Germany). Dimethyldichlorosilane (Repel-silane) was obtained from Amersham Biosciences (Uppsala, Sweden).

Fabrication of the Twin-Anchor Chip. The silicon chips were made from low-resistivity (0.9 Ω-cm, n-type, Phos), thickness 1.3 mm, double-side polished silicon wafers (Virginia Semiconductor Inc., Fredricksburg VA). After cleaning and HF-dipping to remove all native oxide, the chips were immersed in boiling HNO_3 for 10 min, thus forming a thin (∼1.5 nm) very hydrophilic chemical oxide layer.[16] The anchors were formed by anisotropic dry etching in inductively coupled plasma[17] (ICP), using a standard UV-lithography photoresist mask. Directly following the 50-μm-deep etch, a continuous flow of C_4F_8 was employed in the ICP process for 4 min. This resulted in a Teflon-like, highly hydrophobic coating on the surface.[18] Finally, the photoresist was removed. The resulting structure is schematically shown in Figure 1.

After the etching and resist removal, a 250-nm-thick film of Cr/Au was deposited on the backside of the wafer by means of evaporation, to achieve an ohmic contact to the substrate holder. Thereafter, the wafer was cut into 20 × 20 mm sized chips, using a standard dicing saw. A photograph of the chip is shown in Figure 2.

Tryptic Digestion. Denaturation, reduction, and S-alkylation of BSA were performed in accordance with a protocol developed by Norregaard Jensen.[19] The tryptic digestion of 1 pmol/μL BSA was carried out at 37 °C for a period of 18 h with an enzyme-to-substrate ratio of 1/50. The digest was desalted using a Multisep C18 cartridge (ThermoHypersil Chromatography Supplies, Waltham, MA) eluted in acetonitrile/0.1% TFA (50/50).

Instrumentation for Sample Preparation. Sample deposition on the anchors was carried out with a robotic system, constructed in-house. The chip was positioned on an X−Y table, (model TIXY 200, Micro-Controle S.A., Evry Cedex, France) equipped with a motion controller (model MM-4000, Newport, Irvine, CA), providing a resolution of 1 μm. Two CCD cameras (model C2400-75i, Hamamatsu Photonics, Japan and model CS 8630/C, Teli Ltd., Tokyo, Japan), placed at a 90° angle in respect to each other were also included. This arrangement allowed an exact positioning of the chip as well as an alignment of the anchors and the sampling capillary, while the sample deposition process could be monitored by enlarged pictures on video screens. Droplets of the solution of matrix/sample (or matrix/standard) were dispensed onto the individual anchors of the chip from a fused-silica capillary (length 15 cm), the outlet of which was attached to a motor-driven *Z*-axis (model MM-3M-EX-2 and MC-3B-II control unit, Fine Science Tools, Heidelberg, Germany), which made it possible to adjust the height of the capillary outlet with a resolution of <1 μm. The fused-silica capillary had an i.d./o.d. of 20/90 μm (part no. TSP020090, Polymicro Technologies, Phoenix, AZ). The inlet of the capillary was connected to a polyethylene vial, containing the solution to be dispensed. For rinsing the capillary with sample solution, a pressure of ∼1 bar was applied to the vial. For sample deposition, short pressure pulses were applied to the sample container via an electrically controlled valve. The capillary was rinsed with acetonitrile for ∼1 min between changes of sample solution. To increase the reproducibility of sample application, the capillary outlet was carefully cut at a 90° angle, and the last 2 cm of the capillary was coated with Repel-silane after removal of the polyimide layer.

Procedure for Sample Deposition. Matrix, consisting of 4 mg/mL CHCA in a mixed solution of equal proportions of acetonitrile and aqueous 0.1% TFA, was premixed with the BSA digest (1 pmol/μL) in the polyethylene vial. The proportions between the digest and matrix solutions were 50/50 on the basis of volume ratio, and the final volume of the sample was 15 μL. Subsequently, the sample/matrix solution was deposited onto the anchors using the instrumentation described above. One droplet

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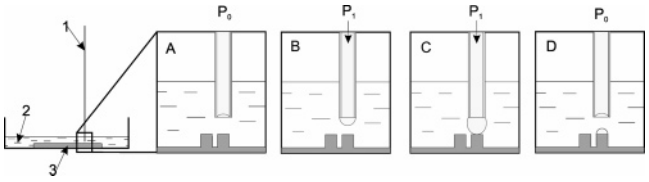

Figure 3. Schematic of the procedure for sample application under liquid fluorocarbon: (1) dosing capillary, (2) liquid fluorocarbon, (3) chip with twin anchors. (A) The dosing capillary is aligned and positioned 50 μm above the appropriate anchor. (B) Pressure (~1 bar) is applied to the dosing capillary, and a small droplet starts to appear at the end of the outlet. (C) The pressure is applied until the droplet touches the hydrophilic surface of the anchor. (D) When the droplet has touched the anchor, the pressure is released and the droplet retracts back into the capillary leaving a small part of the droplet behind on the anchor surface.

at a time was dispensed and left to evaporate. About 5 droplets/anchor was required to cover most of the anchor surface with crystals. The peptide standard was made up in the same way as the digest sample and deposited onto the neighboring anchors. The standard contained 80 fmol/μL angiotensin II, 120 fmol/μL angiotensin I, 160 fmol/μL bombesin, 640 fmol/μL ACTH 1−17, 320 fmol/μL ACTH 18−39, and 640 fmol/μL somatostatin 28. These concentrations were chosen to obtain roughly equal signal strength of the standard and the BSA digest fragments.

Sample Preparation on Standard Targets. For sample preparation on regular stainless steel targets (part no. 25183, Bruker Daltonics), a thin-layer protocol described by Roepstorff et al.[20] was employed. First, 0.5 μL of 20 mg/mL CHCA dissolved in acetone/water (99/1) was applied onto the target and allowed to crystallize. Subsequently, 1 μL of water containing 0.1% TFA was placed on top of the crystals, immediately followed by the application of 0.5 μL of the BSA digest solution (1 pmol/μL). The peptide standard was prepared in the same way onto adjacent target spots.

Sample Preparation under a Layer of Liquid Fluorocarbon. To be able to apply very small volumes of sample onto the twin-anchor chip in a controlled way, these steps were performed under a layer of a volatile liquid fluorocarbon (FC-75 Fluorinert, bp ~100 °C, 3M, St. Paul, MN). During the entire procedure, including rinsing of the capillary and sample deposition, the chip as well as the outlet of the fused-silica capillary were kept under the fluorocarbon surface in a Petri dish (diameter 10 cm). The thickness of the fluorocarbon layer above the chip surface was ~1 mm. In this way, a concentration bias due to premature evaporation of the sample/matrix solution was avoided. The test substance angiotensin I was in an aqueous 0.1% TFA solution. Prior to sample deposition, the sample was mixed in a 1/49 volume ratio with a matrix solution of 1 mg/mL CHCA in a mixture of equal proportions of acetonitrile and aqueous 0.1% TFA. Before the sample was deposited on an anchor, the capillary outlet was positioned in the corner of the Petri dish, away from the chip, and rinsed with ~150 nL of sample/matrix solution. Subsequently, the capillary outlet was positioned ~50 μm above one of the anchors and the sample was transferred as shown in Figure 3. In this way, a series of anchors were loaded with sample and matrix. Finally, the bulk of the fluorocarbon layer was removed with a pipet, and the remaining part of the fluorocarbon was left to evaporate, followed by an instantaneous evaporation of the acetonitrile/0.1% TFA solution, and a cocrystallization of the sample and matrix.

Mass Spectrometry. A Reflex III MALDI system equipped with a SCOUT 384 ion source from Bruker Daltonics was employed for the mass analysis. The twin-anchor chip was mounted on a customized stainless steel target plate (Mikroverktyg AB, Södertälje, Sweden) of the same dimensions as the regular Bruker plate. The midsection of this plate had a recession, to fit the chip, while keeping its surface at the same height as the surrounding surface of the plate. Also, the plate was equipped with small recessed Teflon clamps, to keep the chip in position. In this way, the chip could be conveniently inserted into the mass spectrometer, using the regular inlet of the SCOUT ion source. A 21-in. TV (model CEP2168, Sanyo Industries Ltd.) was added, to obtain an enlarged picture of the chip positioned in the ion source, which facilitated an exact laser illumination of the anchors. The lens and pinhole of the laser optics were adjusted to result in a spot size of 110 × 140 μm. All analyses were performed in positive ion reflector mode.

The analyses for the mass accuracy study were accomplished with a parameter file optimized for ACTH 18−39, 2465 m/z. A matrix deflection setting up until 800 m/z was employed. The mass spectra were based on the signals, accumulated from 100 laser pulses. For the sensitivity evaluation, using angiotensin I, the sample was irradiated with ~200 laser pulses after which no more signal was obtained. Mass calibration was performed with a quadratic curve fit algorithm in the XTof 5.1.5 software (Bruker Daltonics).

RESULTS AND DISCUSSION

High accuracy in mass determination is of central importance for peptide mass fingerprinting in order to achieve a reliable identification of the original proteins. A narrow-mass window for a peptide match provides a platform for a better certainty of protein identity in a database search. The best accuracy is obtained when the internal standard method is employed, and often peaks due to tryptic autolysis products can be used for calibration. However, this strategy is not applicable when there is a lack of autolysis fragments. In such instances, other peptides can be added as internal standards. Unfortunately, addition of an internal standard is known to cause ion suppression of the analyte,[21] which reduces the number of detectable tryptic digest fragments, which is a serious disadvantage in protein identification by peptide mass fingerprinting.

To improve the mass accuracy for the external standard method, without introducing the ion suppression effects observed with the internal standard method, a microchip with dual-sample anchors was developed. The idea was to position the analyte as well as the standard on separate surface areas, both fitting within the circumference of the laser spot. Therefore, it was necessary to reduce the overall size of the twin-anchor arrangement. The distance between the anchors was 10 μm, and the size of the individual anchors was 50 μm. A narrow-bore fused-silica capillary

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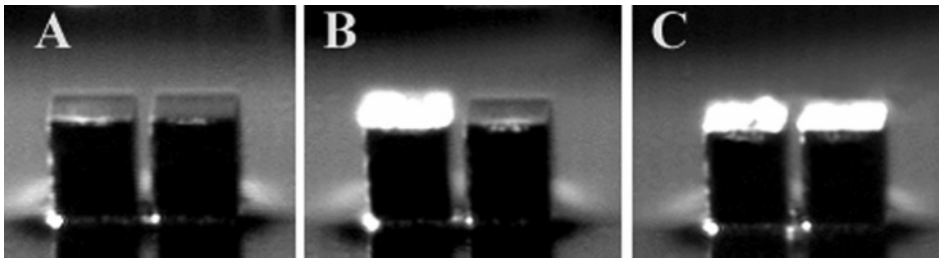

Figure 4. Enlarged view of the twin anchors. The white layer on the anchor surface is due to a light refraction and reveals the presence of crystalline material. (A) No sample has been deposited on either of the anchors. (B) Sample has been deposited onto the left anchor and no sign of cross contamination can be observed. (C) Sample and standard have been deposited on the left and right anchors, respectively.

was utilized to transfer the samples onto the anchors. The computer-controlled motion setup provided the necessary positioning accuracy and also allows an automation of the deposition procedure. Anchor designs on plates with a flat surface were tested during some initial work. With such structures, the sample application process is awkward to control. It was difficult to avoid an overflow of the anchor, despite its hydrophobic boundaries. This occurred particularly when the size of the droplet was larger than the size of the anchor.

This problem was solved with the elevated, pillarlike structure. As soon as the meniscus of the sample droplet makes contact with the hydrophilic surface, the liquid spreads over the entire anchor surface. The sharp surrounding 90° edges of the pillar, combined with the "Teflonized" surface of the pillar walls, and the highly wettable anchor surface creates an extremely large contact angle at the anchor boundaries. As a result, the liquid droplet is strongly confined, which effectively counteracts accidental wetting of the adjacent anchor. Even a fair degree of lateral misalignment between the position of the capillary outlet and the anchor surface can be tolerated. Figure 4 clearly shows that cross contamination is absent, which was also verified with MALDI experiments, where only separate anchors were illuminated by the laser.

The chip was fabricated from phosphorus-doped silicon, which is conductive and thus eliminates the need for metal coatings. The reason for the gold coating on the backside of the chip was to ensure an optimal electrical contact with the stainless steel holder. The silicon dioxide layer on the target is basically an electrical insulator, but no adverse effects were observed from the presence of the oxide. This could be due to its nanometer thickness, which is known to allow electron tunneling.[22] The oxide layer seems to be very stable, since the chip could be reemployed many times after cleaning in an ultrasonic bath between use.

Mass Accuracy. To evaluate the performance of the twin-anchor concept, a series of the BSA digest sample and the standard, containing the six peptides, were prepared separately on the two pillars of the chip-based anchors. For comparison, a series of the BSA digest sample and the peptide mixture were also prepared on conventional stainless steel targets, where the time lag between data acquisitions from the sample and the standard was ~10 s. To maximize the stability of the instrumental conditions, all targets, loaded with samples, were placed in the ion source for 1 h before the analysis, while keeping the high voltage switched on. The comparison of mass accuracy was based on mass determinations of 10 BSA fragments, which were observed in all the mass spectra of both test series. The data obtained are listed in Table 1. These results show that the relative mass error, obtained with the twin-anchor chip procedure, is on average more than 2 times smaller compared to the results, obtained with the conventional external calibration procedure. The results also reveal the presence of a systematic error in the mass determinations for both sets of data. From a more detailed study, we concluded that the cause of these mass shifts is due to surface height differences between the sample and the standard preparations. This can partly be related to a slight tilt of the chip in the ion source with respect to the detector position, but we also observed differences between the crystal morphology of the sample/matrix and the standard/matrix preparations. The importance of this issue is effectively illustrated in the work, described by Moskovets et al.[23] These authors utilized a vacuum deposition process and obtained very planar sample preparations as well as excellent mass accuracy. Another way of improving mass accuracy has been presented by Gobom et al.,[24] which includes a two-step calibration method with a set of externally prepared standards as well as two internal standards for correction of mass deviations resulting from height differences between the sample and the external standard. Such a procedure provides very accurate mass determinations, but at the expense of a more complicated procedure.

Table 1. Results Showing the Relative Mass Errors Obtained with the Twin Anchors Compared to the Results Obtained with a Standard Target[a]

BSA digest peptides		twin anchor		standard target	
AA res	m/z	rel error, ppm	conf int, 95%	rel error, ppm	conf int, 95%
35−44	1249.6211	21	8.1	95	15
360−371	1439.8117	13	5.3	85	9.7
421−433	1479.7954	12	4.8	76	10
347−359	1567.7427	17	6.2	68	9.5
469−482	1724.8346	28	7.7	62	7.9
508−523	1880.9211	40	5.7	57	7.2
529−544	1907.9207	39	8.3	62	7.5
168−183	2045.0279	47	6.0	51	7.1
264−280	2113.8848	48	8.1	57	7.4
118−138	2541.1674	49	7.2	63	6.2

[a] The 10 peptides in the table were identified in all mass spectra. The results are based on 35 analyses on each of the targets.

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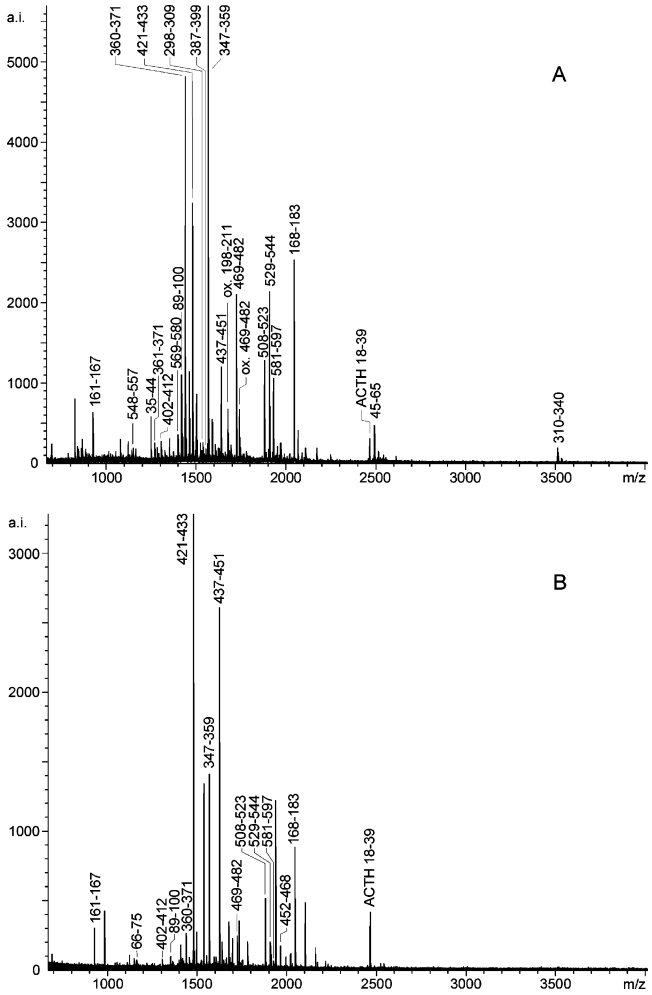

Figure 5. (a) Mass spectrum of the BSA digest (1pmol/μL) and 0.16 pmol/μL ACTH 18−39 prepared on separate anchors of a twin-anchor pair. (b) Mass spectrum of the BSA digest (1pmol/μL) and 0.16 pmol/μL ACTH 18−39 prepared as a mixture on a 400-μm anchor of a regular anchor target. (The BSA-digest and ACTH 18−39 solution (1:1) was mixed (1:5) with the matrix solution consisting of 0.2 mg/mL CHCA in acetonitrile.)

Reduced Ion Suppression. To evaluate the benefits of the twin-anchor concept in terms of reduced ion suppression, the BSA digest and ACTH 18−39 were deposited separately on twin anchors and as a mixture on a regular anchor plate. As can be seen from figure 5, the twin-anchor concept provides a lower ion suppression even when a low concentration of ACTH 18−39 is utilized. The spectrum collected from the twin anchor reveals 22 tryptic fragments, wheras only 14 fragments are observed when the conventional anchor target is used. Also, for different concentrations of the ACTH 18−39, the best results were obtained with separate preparations on the twin-anchor chip. The results are summarized in Table 2. However, some suppression of the BSA fragment ions is still observed, which indicates the presence of gas-phase interactions between analytes desorbed from the physically separated areas. In light of work carried out by Bogan and Agnes,[15] it could be interesting to investigate the influence of the distance between the twin anchors on the magnitude of ion suppression. As pointed out by these authors, a radial mixing of the two separate plumes of volatilized material will occur. This is likely to induce quenching, which can also be concluded from

Table 2. Results Showing the Number of Observed BSA Digest Peaks in Mass Spectra Obtained from Separate Preparations on Twin Anchors as Well as Mixed Preparations of the BSA Digest and ACTH 18−39

	no. of observed BSA digest peaks		
concn of ACTH 18-39, pmol/μL	twin anchor, separated BSA digest and ACTH 18−39	twin anchor, mixed BSA digest and ACTH 18−39	Bruker 400-μm anchor, mixed BSA digest and ACTH 18−39
0	35	35	24
0.16	22	15	14
1.6	10	8	6
8	11	7	7
16	10	6	4
32	7	7	0
160	6	0	0

the results, shown in Table 2. However, the mechanisms of MALDI ionization are not yet fully understood, and an important part of ion suppression could also occur at a more initial stage of the plume formation.

Sensitivity. Although the objective of this study was not directly focused on improving sensitivity, the reduced size of the chip-based anchors prompted us to assess the limit of detection using these sample targets. To carry out such measurements, an exactly defined sample volume has to be deposited onto the anchor. Several advanced techniques have been utilized to transfer small volumes of liquid, including piezoelectric dispensers, dedicated microsyringes, etc.[1−3,25] In our evaluation, we used the fused-silica capillary and the described positioning setup, which proved to be technically straightforward. However, a transfer of defined, very small volumes of liquid is not trivial. Evaporation is a major issue, which has to be taken into account.[26] The rate of evaporation can be derived from Fick's law, according to the following equation:

$$Q = DA(\partial C/\partial r) \qquad \text{(mol/s)} \qquad (1)$$

where Q is the evaporation rate, D is the diffusivity (m^2/s), A is the surface area (m^2), C is the concentration (mol/m^3), and r is the distance from the surface (m).

In our system, we can consider having a hemispherical droplet with a 25-μm radius, consisting of equal amounts of acetonitrile and water. The calculated evaporation rate of such a droplet at room temperature is roughly 200 pL/s. The droplet is therefore rapidly concentrated during sample deposition, which means that it is difficult to know exactly how much of the analyte has been transferred onto the target. Therefore, the sample was transferred under a cover of an immiscible liquid. Such a strategy adequately eliminates the concentration bias caused by evaporation.[26] A volatile perfluorocarbon was utilized as the covering fluid, since water is practically insoluble in perfluorinated solvents.

A solution, containing matrix and 50 fmol/μL angiotensin I was deposited on one of the anchors on the chip, as described in the Experimental Section and depicted in Figure 3. The estimated size of the deposited droplet volume was ~30 pL, which corre-

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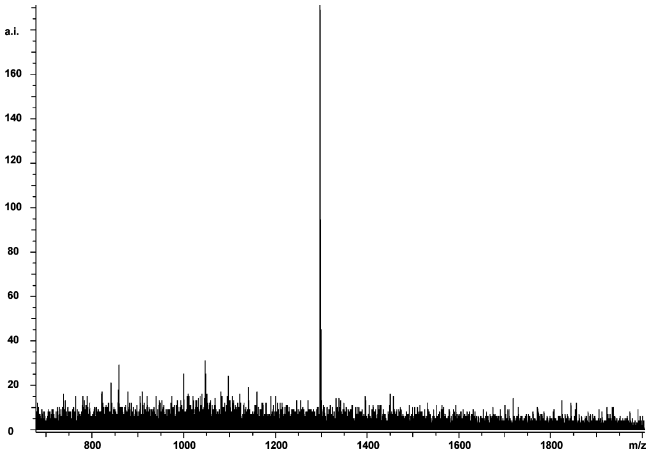

Figure 6. Mass spectrum of 1.5 amol of angiotensin I prepared on one of the twin anchors. The signal-to-noise ratio of the angiotensin I peak is 21:1.

sponds to an absolute amount of ~1.5 amol of angiotensin I. No changes in droplet size could be observed, as long as the layer of the perfluorocarbon was present.

Analyses were performed on a series of eight anchors. Each of the samples was ablated with ~200 laser pulses, after which no more signal was obtained. A representative example of the results obtained is shown in Figure 6, which demonstrates the excellent sensitivity obtained with the chip-based anchors. The average signal-to-noise ratio (for a 95% confidence level) was 22 (±12): 1, which corresponds to a theoretical limit of detection of ~200 zmol. The lowest detecting limit, which has been reported by Keller and Li, is 42 zmol, which is equivalent to ~25.000 molecules.[25] However, these results were obtained when an optimized preparation of a fine layer of matrix crystals by a multiple evaporation process was utilized and where only the strongest spectra from the set of the laser pulses were combined. Our results were obtained without any optimization of the matrix layer. Only one single crystal of ~5 μm was observed on the target. Also, the spectrum, shown in Figure 6, is the result of combining all signals from the 200 laser pulses without any rejection of poor spectra.

Further experiments with diluted solutions of the angiotensin I sample did not lead to useful results. The signal was very weak or disappeared, which we anticipate, is due to adsorption of the analyte onto system components. Similar effects have been reported by others.[13] Thus, if the adsorption effects can be reduced, and a more optimized layer of matrix crystals can be prepared on the targets, the miniaturized chip-based anchor concept should offer a considerable potential for further improvements of the sensitivity in MALDI-MS.

CONCLUSIONS

The described miniaturized twin-anchor concept is very suitable for decreasing ion suppression effects in MALDI MS, induced by the presence of internal standards. For further improvement of mass accuracy, it is important to optimize the interplanarity of the samples on the anchors. The new anchor chip offers many new possibilities. With some modifications of the fabrication layout, a density of 10.000 anchors/chip can easily be obtained, which would provide a platform for a new level of sample capacity. Keller and Li[25] found that the optimal analyte density is 5 molecules/μm^2 and, considering ion detection from a statistical perspective, reaching sensitivities in the yoctomole range would not be unrealistic. A crucial issue is to avoid analyte adsorption and to suppress chemical noise. Therefore, a further reduction of the chip anchor surface area for an additional confinement of the sample volume as well as a reduction of the amount of matrix would be of great interest. We are currently investigating this approach.

ACKNOWLEDGMENT

We thank the Nanochemistry program, funded by the Swedish Foundation for Strategic Research, The Swedish Science Council, The Swedish Agency for Innovation Systems, IRECO Holding AB, and the European Community (Contract NMP4-CT-2003-505311) for financial support.

Received for review May 18, 2004. Accepted October 18, 2004.

AC0400966