Stop and Go Extraction Tips for Matrix-Assisted Laser Desorption/Ionization, Nanoelectrospray, and LC/MS Sample Pretreatment in Proteomics

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Proteomics is critically dependent on optimal sample preparation. Particularly, the interface between protein digestion and mass spectrometric analysis has a large influence on the overall quality and sensitivity of the analysis. We here describe a novel procedure in which a very small disk of beads embedded in a Teflon meshwork is placed as a microcolumn into pipet tips. Termed Stage, for STop And Go Extraction, the procedure has been implemented with commercially available material (C18 Empore Disks (3M, Minneapolis, MN)) as frit and separation material. The disk is introduced in a simple and fast process yielding a convenient and completely reliable procedure for the production of self-packed microcolumns in pipet tips. It is held in place free of obstacles solely by the narrowing tip, ensuring optimized loading and elution of analytes. Five disks are conveniently placed in 1 min, adding <0.1 cent in material costs to the price of each tip. The system allows fast loading with low backpressure (>300 μL/min for the packed column using manual force) while eliminating the possibility of blocking. The loading capacity of C_{18}-StageTips (column bed: 0.4 mm diameter, 0.5 mm length) is 2−4 μg of protein digest, which can be increased by using larger diameter or stacked disks. Five femtomole of tryptic BSA digest could be recovered quantitatively. We have found that the Stage system is well-suited as a universal sample preparation system for proteomics.

The mass spectrometric analysis of proteolytically derived peptides has aided the discovery and characterization of numerous key proteins in biological research. Proteomics, a new field of research centered on the identification, quantitation, and characterization of proteins and their interplay, is based to a large extent on the robustness, sensitivity, speed, and throughput of mass spectrometric procedures.[1−3] The peptide samples obtained by proteolysis are, however, usually not directly analyzed, because they are in buffers and concentrations that are not compatible with high-sensitivity mass spectrometric analysis. Concentration and purification is routinely achieved by binding of the peptides to reversed-phase material in microcolumns or microtips, as microcolumns are called when the material is placed in a pipet tip. The peptides are then eluted off-line in one or several steps for subsequent analysis by mass spectrometry. Even when peptides are analyzed online in LC/MS, peptide purification is usually required to avoid long loading times or column blocking.

The design of microtips for the off-line sample preparation has gone through a number of changes. The key problems are the retention of bead material in the device, the ease and reproducibility of the packing procedure, the robustness of the packed column, and compromised sensitivity for small amounts of protein when exposed to relatively large volume of the microcolumn.

Originally, Wilm and Mann packed Poros R2 beads (Perseptive) in glass capillaries, loaded the sample by centrifugation, and eluted in a similar way using a high percentage of organic solvent directly into the spray capillary used for nanoelectrospray.[4] This was later expanded to the use of two columns in series, the second of which was packed with Oligo R3 material, which retains more hydrophilic peptides including phosphopeptides.[5] The opening at the tapered end of these capillaries needs to be partially widened by controlled crushing of the tip to allow packing. Too much force results in an opening that does not retain beads and renders the capillary useless. These problems are not encountered with high-pressure-packed capillary columns in a nondisposable format.[6,7] Such systems were used for fraction collection for MALDI and enabled the coupling of HPLC to MALDI-MS. However, they are less suited for high-throughput analysis of individual samples because of elaborate handling and the risk of carryover. The delicate procedure using smashed glass capillaries, mentioned above, was in some aspects improved and adapted for MALDI samples using disposable GELoader tips (Eppendorff) with a small piece of glass fiber disk as frit.[8,9] The higher pressure required

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in sample loading and eluting with these tips is delivered by argon from a gas bottle. The laborious "stamping out" or corking the glass fiber disk and positioning the frit in a horizontal position were avoided in a parallel approach where the lower end of GELoader tips was squeezed manually using forceps.[10,11] However, users develop different levels of mastership in this procedure and rarely exceed a success rate of 80−90%. Problematic is especially the sporadic blocking of these latter microtips, presumably by rearrangement of beads closing the opening. When this occurs after the sample is loaded, a very time-consuming rescue operation has to be initiated, and often, the sample is lost. Circumventing the problems of microtip production, chromatographic beads were also added directly to peptide solutions.[12,13] However, this sacrifices the well-defined location of the beads and risks transfer losses. Alternatively, beads with large diameters are retained in GELoader tips, obviating the need for frits or squeezing.[14]

In our laboratory, we have placed a small piece of fused-silica capillary into GELoader tips to serve as the frit. The inner diameter of the fused-silica capillary was chosen to be smaller than the bead diameter, a precaution others and we believed necessary to retain beads. However, a single bead positioned at the entrance of the capillary results in blocking. This can easily occur during packing or later due to rearranged beads. Very recently we reported that the "keystone effect" results in reliable retention of chromatographic material. This effect describes the process in which beads of a diameter larger than one-fifth of an opening shape an arch over the opening and thereby form a self-assembled particle frit (SAP frit).[15] This frit type, along with the glass fiber disks mentioned above, results in devices that cannot block. Nevertheless, for both microtip types, the preparation and packing procedure is somewhat complicated, and the resulting columns are not reproducible in volume and often have a high backpressure. Commercial tips containing chromatographic material polymerized into pipet tips (ZipTips, Millipore) have been introduced and are widely used. However, because of some shortcomings, they did not result in the replacement of self-packed microtips. The binding properties of these tips and the required elution volumes have been found unsatisfactory by some researchers.[16,17] Besides their high material costs, ZipTips are also not readily coupled to nanoelectrospray needles.

Although solid-phase extraction (SPE) traditionally involves loose beads, some vendors have incorporated beads into a matrix of supportive material. 3M (Minneapolis, MN) uses a mesh of poly(tetrafluoroethylene) (PTFE, Teflon) at a mass ratio of ∼1:10 to fix beads in their position and keep them at a distance from each other (Empore Disks). The fixed position of beads enables solid-phase extraction devices with a large diameter and of short column length. The mechanical construction prevents the formation of primary flow paths and facilitates the equal flow of analyte solution throughout the entire SPE disk. As a consequence, the flow rate allowed by SPE cartridges increased over those using lose beads at identical linear velocity. Furthermore, the loading capacity of equal column volumes is improved, and the risk of blocking is reduced. This type of disk has previously been investigated for preconcentration of tryptic peptide mixtures prior to HPLC[18] and CE.[19]

Here, we present novel microtips that overcome the above-discussed limitations while additionally offering other beneficial properties and high versatility. Corked out PTFE membrane disks containing embedded reversed-phase beads (Empore Disks) simultaneously serve as column material and frit. They are placed in a robust, highly reproducible, and fast procedure into pipet tips. The resulting microtips are characterized by minimal material costs, a small bed volume, large loading capacity, high loading and elution flow rates, and excellent recovery.

EXPERIMENTAL SECTION

Reagents. Buffers used were buffer A (0.5% acetic acid, 0.02% heptafluorobutyric acid (HFBA)), buffer B (80% acetonitrile, 0.5% acetic acid, 0.02% HFBA), and matrix solution (α-cyano-4-hydroxycinnamic acid (HCCA)[20] saturated in 70% acetonitrile and 0.1% trifluoroacetic acid (TFA)).

Empore Disk C18 (product number 2215) was purchased from 3M, MN. All other reagents were from Sigma-Aldrich, St. Louis, unless noted otherwise.

BSA was reduced/alkylated and digested by trypsin in a buffer containing either $H_2{}^{16}O$ or 95% $H_2{}^{18}O$ (Icon, Mt. Marion, NY). A stock solution of 1000 pmol BSA/μL was diluted to 100 pmol BSA/μL in 20 mM ammonium bicarbonate, reduced in 1 mM DTT for 30 min at room temperature, and alkylated by addition of 5 mM iodoacetamide for 30 min at room temperature in the dark. An equal aliquot was diluted in 95% $H_2{}^{18}O$ or $H_2{}^{16}O$, respectively, with roughly 50 mM ammonium bicarbonate to a final concentration of 1 pmol BSA/μL after the addition of trypsin. Trypsin (modified, Promega) was added at 1:20. After 48 h at room temperature, the samples were stored at −20 °C. All dilutions were obtained from the 1 pmol BSA/μL stock directly or in series using buffer A.

Unlabeled and $^{13}C_6$-arginine-labeled soluble fraction of cell lysate from *Escherichia coli* were a kind donation from Tobias Maier, Dr. Dean J. Naylor, and Dr. Ulrich Hartl group at the Max-Planck-Institute for Biochemistry, Martinsried, Germany. A 50-μL portion of a 4-mg protein/mL solution was concentrated in a speed vac to ∼10 μL, and 20 μL of 8 M urea in 100 mM Tris−HCl (pH = 8) was added. After reduction (2 μL 1.5 mg/mL DTT, 20 min, 90 °C) and alkylation (2 μL 10 mg/mL iodacetamide, 30 min, room temperature, in the dark), 1 μg of Lys-C (Wako, Osaka, Japan) was added, and the sample was left at 37 °C for 15 h. The sample was diluted by addition of 60 μL of a 50 mM ammonium

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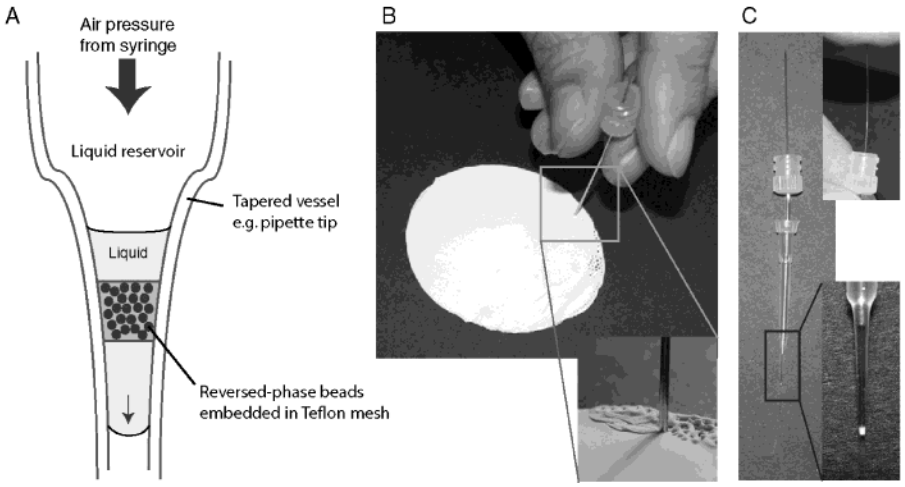

Figure 1. Schematic representation and production of StageTips: (A) The reversed-phase beads for micropurification are embedded in a Teflon mesh and are held in place solely by the tapering of the vessel. Liquid is added to the reservoir and passes the column under pressure from an air-filled plastic syringe. The analyte is retained and can be washed and eluted in an analogous manner using appropriate solvent conditions. (B) A blunt-tipped hypodermic needle is driven manually, in cookie cutter fashion, through an Empore Disk C18 (inset, closeup). (C) Insertion of the cut-out disk from the needle into its position in a pipet tip (inset top, capillary pushed gently by hand; inset bottom, closeup of the StageTip with the needle still inserted at the top, the membrane already positioned at the bottom, and the capillary going through the needle as it fixes the membrane).

bicarbonate solution prior to the addition of 1 μg of trypsin and incubation at 37 °C for 20 h. After filtration through a C_{18}-StageTip (column bed: 0.9 mm diameter, 0.5 mm length), the sample was combined with 5 μL of acetonitrile that eluted bound material from the StageTip and diluted to the final concentration of 0.4 μg/μL using 0.1% TFA in 2% acetonitrile.

Use of StageTips. All solutions were loaded from the top of the tip using a pipet. The liquid was pressed through the tip using an air-filled 1-mL plastic syringe fitted with a pipet tip that was trimmed on both ends to serve as adapter between syringe and StageTip (following in principle previous work[10]). The pressure was roughly adjusted by advancing the plunger to a predefined position.

StageTips were conditioned by 5 μL of methanol and equilibrated using 10 μL of buffer A. Samples were loaded in buffer A or at least a 1:10 dilution therein and passed a single time through a StageTip. The tips were then washed using 10 μL of buffer A. For MALDI-MS, samples were eluted from C_{18}-StageTips (0.4 × 0.5) in 1 μL of matrix solution. For nanoelectrospray MS, 1 μL of 50% methanol/5% formic acid was used. From the larger C_{18}-StageTips (0.9 × 0.5 and 0.9 × 1.0), peptides were eluted in 5 μL of buffer B.

Mass Spectrometric Analysis. For MALDI-MS analysis, samples were eluted from StageTips using matrix solution[6] and deposited directly onto the target to give a dried droplet preparation[21] unless noted otherwise in the text. MALDI-TOF experiments were performed on a Voyager-DE STR (Applied Biosystems, Foster City, CA). Electrospray experiments were conducted using a QSTAR Pulsar (MDS-Sciex, Toronto, Canada). Nanoelectrospray tips were obtained from Protana Engineering A/S (Odense, Denmark). For LC/MS, Vydac 218MSB3 bulk material (3-μm prototype reversed-phase material, a generous gift from Grace Vydac, Hesperia, CA) was packed at 10-cm length into pulled fused-silica capillaries (PicoTip, New Objective, Woburn, MA) with 100-μm i.d. and 8-μm tip opening. Particles formed a self-assembled particle frit (SAP-frit) at the tapered end according to the principle of the stone arch bridge.[22] Peptides were loaded using a sample loop. A gradient of buffer C (0.5% acetic acid) to buffer D (80% acetonitrile, 0.5% acetic acid) eluted the analytes at 200 nL/min flow having the following profile: D: 2.5 → 10% (0 → 5 min), 10 → 25% (5 → 30 min), 25 → 60% (30 → 40 min), 2.5% (40 → 50 min).

Data Analysis. MALDI spectra were displayed using the program "M over Z" (Proteometrics, New York, NY). LC/MS spectra were analyzed in Analyst (Applied Biosystems, Foster City, CA). Scripts in Analyst created peak lists on the basis of the recorded fragmentation spectra. These lists were searched against an *E. coli* database using Mascot (Matrix Science, London) on our in-house server. Thirty high-scoring, arginine-containing peptides covering a broad elution time window were then used to yield quantitative information by opening the corresponding spectra and measuring the peak ratios of the unlabeled and labeled peptides using in-house software.

RESULTS AND DISCUSSION

Principle of StageTips. Figure 1 A shows the principle of the StageTips system for sample preparation in proteomics. Reversed-phase beads are embedded in a Teflon matrix forming a very small disk having a typical volume of ~60 nL for MALDI and nanoelectrospray sample preparation. This disk is positioned in a tip, serving as a frit and as packing material at the same time. Use of Teflon together with the small volume of the disk minimizes irreversible peptide adsorption, a frequent problem with other kinds of frits or if larger packing volumes are involved. Depending on the application, different thickness, diameter, and packing material can be chosen. Analyte solution is loaded either

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by sucking it into the StageTip or pipetting it on top of the disk and pressing the liquid through using an air syringe (see Experimental Section). After washing, elution buffer is placed on top of the disk and pressed through using the air syringe.

Production and Handling of StageTips. The C_{18} chromatographic beads are embedded in a soft PTFE mesh by the manufacturer (3M, Minneapolis, MN) from which disks can reproducibly be corked out using a hollow tool, such as a blunt-tipped hypodermic needle in exactly the same way that cookies are cut (Figure 1B). One mesh is sufficient for producing several thousands of such membrane disks. The disk sticks to the inside of the needle and is pushed out into a tapered vessel, such as a pipet tip, using a piece of fused silica or similar material (Figure 1C). This follows the procedure described by Yang and his colleagues for a device to concentrate samples prior to capillary electrophoresis;[19] however, these researchers fixed the membrane between two pieces of fused silica in a PTFE tube. In contrast, in StageTips, the membrane is held in place solely by the constriction of a vessel such as a pipet tip. This allows much easier assembly and makes the full diameter of the disk available to solvent flow, minimizing flow irregularities. Air can be pressed through the tip without any problem as long as the material does not dry. Liquid displaces air from the wetted column. However, air bubbles will be observed at the exit of the column throughout its use. They form as a result of the pressure drop between the column and the postcolumn region.

An occasional user can easily manufacture ~5 C_{18}-StageTips/min. The costs of C_{18}-StageTips are largely governed by the cost of work time and by the expense of the pipet tips. Material costs per disk are <0.1 cent.

Although in this investigation, we routinely loaded all solutions from the top of the tip, the friction between tip wall and membrane was sufficiently high to also allow reversed flow. This was useful when small gel pieces from in-gel digests blocked a C_{18}-StageTip. Multiple passages of analyte solution or eluent through a C_{18}-StageTip are possible; however, this was not found necessary, because in all of our work, a single passage allowed efficient binding or elution, respectively, as is shown below.

Reagents and Reproducibility Control. To evaluate C_{18}-StageTips, stable-isotope-labeled protein digests were produced. BSA was digested with trypsin either in $H_2{}^{16}O$ (^{16}O-digest) or 95% $H_2{}^{18}O$ (^{18}O-digest)[16,23,24] and used as an example for the analysis of single proteins. A more complex peptide mixture was obtained using the soluble fraction of lysate from *E. coli* cells that were grown in minimal medium containing either unlabeled arginine or $^{13}C_6$-arginine (Ong, S.-E.; Kratchmarowa, I.; Mann, M. J. *J. Proteome Res.*, ASAP on Web 12/13/2002.).

The $H_2{}^{16}O$ content of 95% $H_2{}^{18}O$ results in mass spectrometric peak-splitting as, during cleavage, one ^{16}O and one ^{18}O or two ^{18}O atoms are incorporated at the C terminus of the tryptic peptides. The rate of ^{18}O incorporation is peptide-dependent (as discussed, for example, in ref 16). However, under the conditions chosen here, we have observed that all measured peptides incorporated ^{18}O atoms equally with an ~0.2 ratio between single and double incorporation.

To evaluate the reproducibility of our MALDI-based assay for quantitation and to establish significance thresholds for quantitative differences, we mixed 10 μL of each 20 fmol/μL tryptic BSA ^{16}O and ^{18}O digest on a StageTip, eluted in matrix solution directly onto the target, and recorded the sum of 300 spectra five times. This experiment was repeated five times, yielding 25 spectra in total. The peak ratio between the ^{16}O and $^{18}O_2$ signal in all spectra was determined for six BSA peptides and defined their 100% value. The standard deviations within the five spectra recorded for one preparation were between 1 and 12% for the different peptides. Among the five preparations, deviations ranged from 4 to 11%. All ratios fell within 82−119% (shown as gray area in Figures 2 and 3).

Flow Rate during Loading. The flow rate during sample loading onto a microcolumn is potentially a crucial factor, because the analyte requires sufficient time to distribute and adhere efficiently to the solid phase. We investigated the influence of loading speed on peptide retention by loading 10 μL of a 20 fmol/μL tryptic BSA ^{16}O digest at various speeds ranging from 8 to 300 μL/min on C_{18}-StageTips (one membrane, column bed: 0.4-mm diameter, 0.5-mm length (0.4 × 0.5)). For quantitation, the same C_{18}-StageTip was additionally loaded with 10 μL of a 20 fmol/μL tryptic BSA ^{18}O digest at 30 μL/min as internal standard. This allowed compensating for any loss as a result of incomplete or variable recovery. The peptides were eluted in matrix solution (as first described in ref 6) and analyzed by MALDI-MS as reported in MALDI sample pretreatment, below. Six measurements were done for low flow rates (8 to 20 μL/min) and three each for medium (30 to 50 μL/min) and high (85 to 300 μL/min) flow rates (Figure 2). Surprisingly, the $^{16}O/^{18}O$ peak ratios for the peptides are largely identical in the investigated flow range, showing that the flow rate did not exhibit a detectable influence on the binding. This suggests that the loading capacity of the C_{18}-StageTip is large compared to the 200 fmol tryptic BSA digest loaded and that a large proportion of the surface area of the C_{18}-StageTip is accessible. When much larger sample amounts close to the capacity of the disk are used, the loading speed will exert a noticeable influence (see below). We conclude that in normal operation, StageTips allow very fast and simple loading.

Flow Rate during Elution. To elucidate whether the flow rate of the eluent has any influence on the recovery of bound analyte from C_{18}-StageTips, we again used the $^{16}O/^{18}O$ peak ratios of BSA peptides. As before, 200 fmol of tryptic BSA ^{16}O digest was loaded onto StageTips, but at equal speed, and eluted in 1 μL of matrix solution at various speeds ranging from 12 to 300 μL/min. For quantitative comparison, C_{18}-StageTips were loaded with 200 fmol of tryptic BSA ^{18}O digest and eluted at 30 μL/min. Sample and standard were eluted onto a MALDI target in parallel and mixed. The resulting $^{16}O/^{18}O$ peak ratios are shown in Figure 3. It can be seen that the elution speed did not exert a detectable influence within the range investigated. Even a flow of 300 μL/min (which corresponds to an elution in 0.2 s) results in complete recovery of the bound material. High-speed elution allows for nearly quantitative recovery of the bound peptides, as is shown by the low amounts of peptides eluted in a second elution step. We do not observe memory effects that were reported for Empore disks placed between fused-silica tubings prior to CE.[25] In that arrange-

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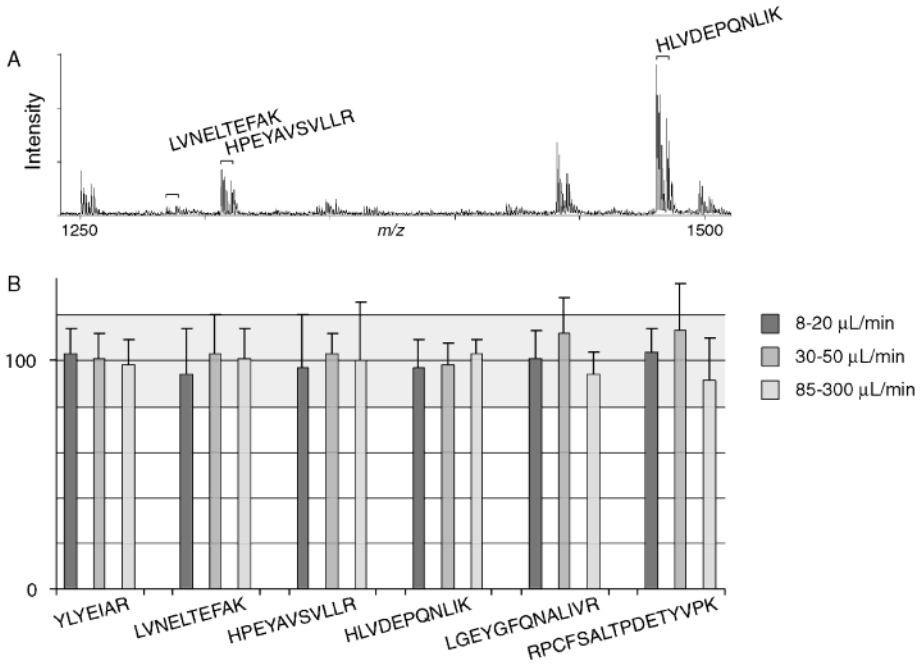

Figure 2. Influence of the loading flow rate on analyte binding to C_{18}-StageTips: (A) Example data of a MALDI-TOF spectrum of 200 fmol of tryptic BSA ^{16}O digest loaded at 300 μL/min on a C_{18}-StageTip (0.4 $\times$ 0.5) with 200 fmol of ^{18}O digest added at 20 μL/min to the same microtip as internal standard. The $^{16}O/^{18}O$ peak pairs, spaced by 4 Da and each split further by natural isotopes, are marked for three peptides. (B) Ratios of $^{16}O/^{18}O$ peaks from several repetitions in the indicated loading flow ranges were extracted from MALDI mass spectra. The standard deviation of this measurement series or, if it was larger, of the control experiment described in the text is shown as an error bar. The gray bar marks the area covering all measured peak ratios of the control experiment. The data show that loading flow rate has no influence on the recovery of peptides in the parameter range investigated.

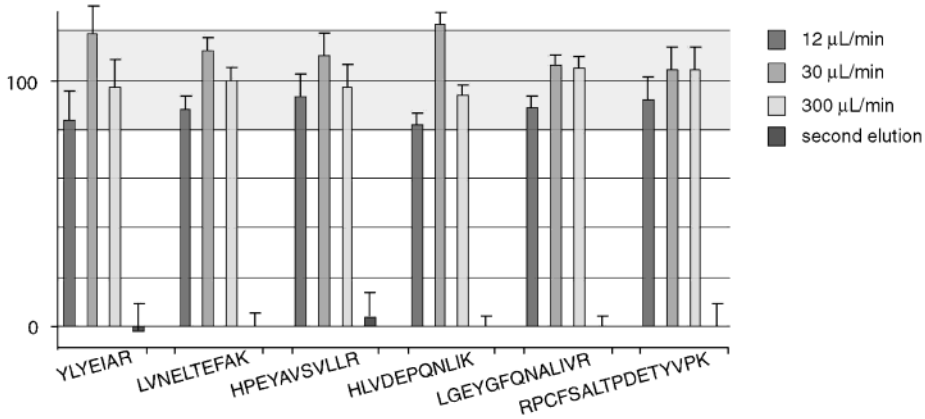

Figure 3. Influence of the elution flow rate on analyte binding to C_{18}-StageTips. Ratios of $^{16}O/^{18}O$ peaks from several repetitions in the indicated elution flow ranges were extracted from MALDI mass spectra. The gray area indicates the distribution of control measurements. The standard deviation of this control measurement is added here as error bars. The data show that elution flow rate has no influence on recovery of peptides in the parameter range investigated.

ment, the support given by the fused-silica capillary introduces a large low-mixing area at the inlet and outlet of the microcolumn. In StageTips, the disk is held in place obstacle-free solely by the taper of the tip. This ensures that the top and bottom surfaces of the column material are open for unhindered passage of liquid and allows uniform loading and elution of analytes.

In our routine work, we now employ a loading and elution speed of ~40−60 μL/min for MALDI-MS analysis and the same for loading for nanoelectrospray. The elution speed for nanoelectrospray is 15−20 μL/min for practical reasons described below. With typical sample volumes, loading takes 10−20 s, and elution, <2 s.

Loading Capacity. A 20-pmol (1.3 μg) portion of tryptic BSA digest was loaded onto a C_{18}-StageTip (0.4 $\times$ 0.5) at 20 μL/min and 60 μL/min and eluted. The eluate was diluted to 1 mL, and a 10-μL aliquot was mixed 1:1 with 20 fmol/μL tryptic BSA ^{18}O digest so that a 1:1 peak ratio would be expected upon mass spectrometric analysis in case of full recovery. After concentration of this mixed sample on a second StageTip, MALDI-MS revealed an influence of loading speed on recovery. The 60 μL/min loading speed allowed the retention of 70−80% of the peptide material by the microtip, while 10−20% was found in the flow-through, which was analyzed in the same way. Reducing the loading speed to 20 μL/min increased the retention to 80−90% (data not shown).

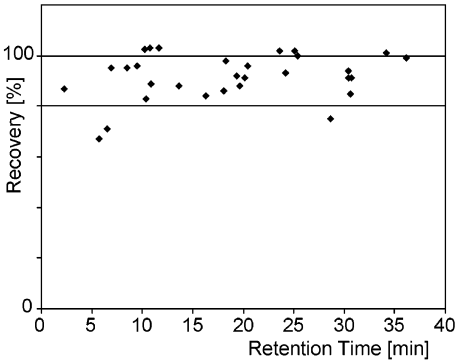

Figure 4. Recovery of tryptic peptides of the soluble fraction of *E. coli* cell lysate from a C_{18}-StageTip (0.9 × 1.0) plotted over their retention time on a reversed-phase column used for LC/MS analysis of the mixture.

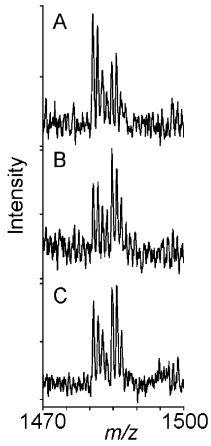

Figure 5. Recovery of analyte bound to C_{18}-StageTips at low femtomole amounts. (A) MALDI-TOF spectrum of 5 μL of 0.5 fmol/μL tryptic BSA ^{16}O digest micropurified on a C_{18}-StageTip (0.4 × 0.5) with 5 fmol of ^{18}O digest added on the target as standard. (B) Repeated experiment of A showing variation in $^{16}O/^{18}O$ ratios at these sample levels. (C) 5 fmol of each, tryptic BSA ^{16}O and ^{18}O digest, mixed as control on the target.

Binding as well as elution of peptides from the microtip will vary among peptides, depending on their properties, such as their hydrophobicity. To further investigate StageTip capacity, we therefore chose the soluble fraction of $^{13}C_6$-arginine-labeled *E. coli* cell lysate as a complex sample containing a wide variety of peptides. The peptide equivalent of 50 μg of soluble fraction of *E. coli* cell lysate was subjected to Lys C and trypsin digestion and loaded at 10 μL/min onto a larger C_{18}-StageTip (0.9 × 1.0, two membranes stacked on top of each other; column bed, 0.9 mm diameter, 1.0 mm length). An identical sample of unlabeled proteins served as standard, added just prior to LC/MS analysis. Using LC/MS for subsequent analysis allows not only dealing with the sample complexity but also selecting peptides that reflect different hydrophobicity as reflected by their retention time. A random set of arginine-containing peptides covering a broad retention time range was chosen, and their peak ratios were measured (Figure 4). We observed an average recovery of 92% of peptide material. No strong bias toward the retention of peptides with certain hydrophobicity values was apparent. On a single disk of the same diameter, only an average of 75% of the peptide material was retained (data not shown). These experiments show that the limiting factor was retention and not elution. We calculated an estimated loading capacity at 95% recovery of 25 μg for a 0.9 × 0.5 C_{18}-StageTip (320-nL bead volume) and 4.5 μg for a 0.4 × 0.5 C_{18}-StageTip (60-nL bead volume). Taking into account possible uncertainties of total protein determination with the Bradford test and completeness of digest, as well as the results of the BSA quantification, a conservative capacity value is 2−4 μg for the C_{18}-StageTip (0.4 × 0.5). This corresponds to ∼50 pmol of a single protein and is large compared to the protein amount used in MALDI and nanoelectrospray MS analysis. For sample pretreatment of complex peptide mixtures in LC/MS, amounts of >20 μg can be loaded onto C_{18}-StageTips (0.9 × 0.5), which is more than the typical loading capacity for 75-μm diameter columns, and even larger capacity StageTips can easily be produced. Since irreversible binding at low sample amounts is not a concern (see below), a StageTip with a loading capacity well above the expected protein amount can be chosen without detrimental effects for the experiment.

Recovery at Low Sample Amounts. The recovery of minute amounts of peptides on microtips can be problematic. This may be caused either by the peptides' not binding or by their irreversible adsorption to high-affinity sites. We addressed recovery to characterize the usefulness of C_{18}-StageTips for work with low amounts of peptides. Dilution series of tryptic BSA ^{16}O and ^{18}O digests in buffer A were prepared in parallel down to 5 fmol/μL and analyzed immediately as follows: To avoid sample loss to the walls of Eppendorf tubes and errors due to liquid delivery, 1 μL from each dilution of ^{16}O digest was diluted into 9 μL of buffer A, which was placed directly in the reservoir of a C_{18}-StageTip (0.4 × 0.5). The lowest concentration of tryptic BSA digest investigated was, therefore, 500 amol/μL with 5 fmol loaded. The peptides were eluted with 1 μL of matrix solution into 1 μL of ^{18}O digest of the appropriate dilution freshly placed on the target. Figure 5 shows the $^{16}O/^{18}O$ peak ratio of the BSA peptide LGEYGFQNALIVR (m/z 1480.71). The peak ratio is as expected from a complete recovery of the loaded material. Because of the low volumes and amounts used, the precision of this measurement is low, with a variation of 40% for five repetitions (two examples are shown in Figure 5A and B). However, their mean value is at the peak ratio expected for complete recovery. On the basis of these measurements, irreversible adsorption is not a concern, at least down to peptide concentrations of 0.5 fmol/μL. It is reassuring to note that the Teflon mesh holding the beads in C_{18}-StageTips does not adversely affect the recovery of bound peptides.

The recovery of peptides from C_{18}-StageTips is superior to commercial ZipTips, for which only 70% of 1 pmol and below 10% of 50 fmol tryptic BSA digest are recovered.[16] Although small sample losses arguably are not of supreme importance at high analyte amounts, they still introduce uncertainties into the interpretation of results, for example, if peptide losses are peptide dependent. At minute sample amounts, recovery becomes a precondition for success of the experiments, and ZipTips appear to be impractical, but C_{18}-StageTips do provide the needed performance.

MALDI Sample Pretreatment. We employed C_{18}-StageTips for efficient concentration and clean up of small analyte quantities prior to MALDI-MS[21] analysis. StageTips (0.4 × 0.5) were prepared

using either GELoader tips or standard 10 μL pipet tips, which are more economical. The microtip was then preconditioned using 5 μL of methanol and equilibrated using 10 μL of buffer A. The sample was loaded in buffer A, washed using 10 μL of buffer A, and eluted in 1 μL of matrix solution. The eluate was placed directly on the target. As shown above, neither loading speed nor elution speed up to at least 300 μL/min had a significant influence on the recovery of subpicomole amounts of analyte. A 1-μL portion of matrix solution was found fully sufficient for elution, and this value can even be reduced to 0.5 μL when needed. In our hands, some of the practical advantages of StageTips over ZipTips include simpler sample handling (since the sample has to be passed multiple times through ZipTips), as well as their larger loading capacity, recovery, and low cost.

Nanoelectrospray Sample Pretreatment. The concentration and cleanup of samples for nanoelectrospray MS analysis[26,27] is very similar to the aforementioned protocol for MALDI-MS. In this case, StageTips were prepared using GELoader tips exclusively, because they allow the elution of the bound analyte directly into the spray capillary. The fine end of the tip was inserted into the spray capillary, and flow was initiated by applying very low pressure using a 1 mL air-filled syringe. Elution was achieved using 50% methanol/5% formic acid.

Two stop points for the flow can be observed, both involving the bottom end of the disk. The first time the flow stops is when the front of the solution reaches the end of the microcolumn. If the pressure is then increased carefully, a second stop can be observed when the solution is about to leave the microcolumn. This may be used in the future for flow control in a lab-on-chip kind of approach. Currently, the second stop indicates that the operator should either switch to centrifugation for further solution transport or carefully retract the StageTip to prevent the air following the solution to press the eluate out of the spray capillary. In any case, a final spin is advisable to collect the sample into the tip of the spray capillary.

LC/MS Sample Pretreatment. As mentioned above, proteomic samples for LC/MS applications need to be pretreated before application to very small columns. Particulate matter needs to be removed, the sample should be prepurified, the concentration of organic solvent should be reduced, and the sample volume should be adjusted to a practical loading volume for the LC/MS system.

Considering the high flow rate and small mesh size of StageTips, we realized that one of their applications in the context of LC/MS could be their use simply as filters to remove material that may block cap columns from samples. The StageTips were preconditioned following the standard procedure. Up to 10 mL of sample was passed through a single disk. The sample volume that can be filtered by one single StageTip depended on the amount of particulate matter, and a new StageTip was used when the flow rate was reduced significantly. The fraction of analyte bound to the StageTip was eluted with 2 μL of 70% acetonitrile and combined with the flow-through. Even though capacity is irrelevant in this filtering application, larger C_{18}-StageTips (0.9 $\times$ 0.5) are preferable over C_{18}-StageTips (0.4 $\times$ 0.5) because of their larger diameter. As an alternative to GELoader tips, we have used 200-μL and 1000-μL pipet tips in the production of C_{18}-StageTips, because they offer a larger reservoir for sample loading.

In addition to their use as a filter unit, the high loading capacity of C_{18}-StageTips and their convenient usage suggests their use for purification and concentration of analytes prior to LC/MS. A C_{18}-StageTip (0.9 $\times$ 1.0) can retain 95% of a tryptic digest of 50 μg of soluble *E. coli* cell lysate, discussed in more detail above. The modular aspect of StageTips allows increasing the column length as required by stacking additional layers of membrane. Initially, we placed additional Poros R2 material on top of the C_{18}-StageTip but we have discontinued this practice after discovering that the loading capacity of C_{18}-StageTips alone is sufficient.

Since very large sample volumes can be accommodated by repeatedly pipetting into the reservoir of C_{18}-StageTips, they can be employed for the same applications as the widely used cartridges from Michrom (Auburn, CA). These cartridges require the sample to be loaded in a less convenient manner from a syringe that additionally introduces the risk of carryover. Another advantage is the low elution volume of C_{18}-StageTips, resulting in more concentrated samples. In practice, 10 column volumes is a safe choice for elution of MicroTrap (20-μg capacity, column volume 5 μL) or MacroTrap (200-μg capacity, column volume 50 μL). Less than 3 μL is sufficient for elution from C_{18}-StageTips (0.9 $\times$ 1.0). Furthermore, the StageTip size can be modularly increased, which optimizes the extraction with respect to elution volumes required and shortens the subsequent loading time of the sample onto the capillary LC column.

The content of organic solvent in the eluate is usually reduced by vacuum centrifugation prior to LC/MS. The low elution volume of StageTips may allow replacing this step by a simple dilution, reducing the risk of sample loss. Although some further work would be required to judge practical advantages of StageTips over commercial cartridges, our laboratory has already adapted them exclusively for this purpose with good success.

Conclusion and Prospects. We have presented a novel type of microtip, StageTips, suitable for sample preparation prior to MALDI-MS, nanoelectrospray MS, and LC/MS analysis. The production of these StageTips is simple, reproducible and economical. Their handling is easy, and one-time usage eliminates the risk of carryover. StageTips have a high loading capacity and allow for excellent recovery of even minute amounts of analyte because of the obstacle-free fitting of the microcolumn in the tapered tip. These tips are extremely resistant toward blocking and sample loss while allowing flow rates of 300 μL/min. These properties make StageTips a very useful tool in proteomics.

Their low backpressure makes the new StageTips highly amenable for automated sample preparation prior to mass spectrometric analysis. Furthermore, the embedded beads have a well-defined position and therefore allow the modular stacking of materials with orthogonal binding properties, an area which we are currently investigating. Autosamplers can be envisaged utilizing StageTips analogous to those presented here but for direct loading of peptides on columns for LC/MS, allowing sample storage on solid-phase rather than in solution.

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