J. Am. Soc. Mass Spectrom. (2016) 27:686–698
DOI: 10.1007/s13361-015-1332-6

RESEARCH ARTICLE

Proteolytic Digestion and TiO_2 Phosphopeptide Enrichment Microreactor for Fast MS Identification of Proteins

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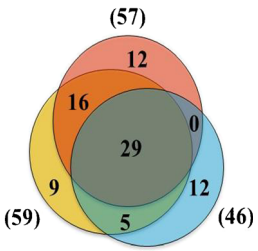

Abstract. The characterization of phosphorylation state(s) of a protein is best accomplished by using isolated or enriched phosphoprotein samples or their corresponding phosphopeptides. The process is typically time-consuming as, often, a combination of analytical approaches must be used. To facilitate throughput in the study of phosphoproteins, a microreactor that enables a novel strategy for performing fast proteolytic digestion and selective phosphopeptide enrichment was developed. The microreactor was fabricated using 100 μm i.d. fused-silica capillaries packed with 1–2 mm beds of C18 and/or TiO_2 particles. Proteolytic digestion-only, phosphopeptide enrichment-only, and sequential proteolytic digestion/phosphopeptide enrichment microreactors were developed and tested with standard protein mixtures. The protein samples were adsorbed on the C18 particles, quickly digested with a proteolytic enzyme infused over the adsorbed proteins, and further eluted onto the TiO_2 microreactor for enrichment in phosphopeptides. A number of parameters were optimized to speed up the digestion and enrichments processes, including microreactor dimensions, sample concentrations, digestion time, flow rates, buffer compositions, and pH. The effective time for the steps of proteolytic digestion and enrichment was less than 5 min. For simple samples, such as standard protein mixtures, this approach provided equivalent or better results than conventional bench-top methods, in terms of both enzymatic digestion and selectivity. Analysis times and reagent costs were reduced ~10- to 15-fold. Preliminary analysis of cell extracts and recombinant proteins indicated the feasibility of integration of these microreactors in more advanced workflows amenable for handling real-world biological samples.

Keywords: Microreactor, Proteolytic digestion, Phosphopeptide enrichment, Mass spectrometry

Received: 27 October 2015/Revised: 20 December 2015/Accepted: 27 December 2015/Published Online: 16 February 2016

Introduction

The analysis of phosphoproteins represents a topic of major interest to many biological researchers because of the key roles that phosphorylation plays in the regulation of various cellular activities, as well as the implications that aberrant phosphorylation has in the development of a number of diseases such as cancer and neurodegenerative disorders. A typical bottom-up mass spectrometry (MS)-based phosphoproteomic study involves a number of steps that include cell processing/lysis, protein separation/purification, proteolytic digestion, phosphopeptide enrichment, microanalytical separations, and MS detection. Alternatively, conventional biological experiments rely on the isolation/purification of a protein of interest, followed by characterization by using a variety of physicochemical methods. Often, a combination of different analysis strategies must be used to enable a comprehensive characterization of individual phosphoproteins, or of the phosphoproteome, as a whole. The steps of proteolytic digestion and phosphopeptide enrichment, as commonly practiced, are time-consuming and represent the main bottlenecks in achieving throughput. For example, in-solution proteolytic digestion is usually performed overnight at substrate:enzyme ratios of 50–100:1 (w/w), being often accompanied by the generation of undesired enzyme autolysis products. Some of the most common techniques for phosphopeptide enrichment involve the use of immobilized metal affinity chromatography (IMAC) and/or of TiO_2 particles. In the case of complex samples, prefractionation steps, using strong anion/cation exchange or hydrophilic interaction chromatography, precede the phosphopeptide enrichment process [1–3]. In comparison to IMAC, the use of TiO_2 particles has gained particular momentum in the bioanalytical

Electronic supplementary material The online version of this article (doi:10.1007/s13361-015-1332-6) contains supplementary material, which is available to authorized users.

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community because of the much improved selectivities that can be achieved for enrichment in phosphopeptides. The sample enrichment process on such particles, although very effective, tends to be also time-consuming as it involves multiple conditioning, rinsing, and centrifugations steps (1–2 h).

To accelerate the in-solution proteolytic digestion process, a number of approaches have been developed [4, 5]. For example, microwave-assisted [6] digestion, proposed by Pramanik et al. in 2002 [7], successfully reduced the digestion time from hours to 10 min. Turapov et al. reported a fast digestion method using a PCR-type thermocycler [8], with the caveat that many proteases cannot withstand the elevated temperatures. Ultrasound-assisted digestion, first introduced in 2005 [9], was carried out in less than 60 s. Alternative techniques, making use of pressure [10], alternating electric fields [11], and infrared radiation [12] have also become powerful tools for accelerating the in-solution proteolytic digestion process. To eliminate the need for additional instrumentation, reduce sample and reagent consumption, and enable process automation, a variety of enzymatic microreactors have been developed as stand-alone devices or as part of micro-total analysis systems (μ-TAS). The majority of these microreactors fall under the category of immobilized enzymatic reactors (IMERs). By immobilizing the enzyme, high enzyme-to-substrate ratios can be achieved, and undesired autolysis products are eliminated. Various methods have been introduced for the enzyme-immobilization process [13–27]. Landers and coworkers introduced a digestion approach that uses electroosmotic flow (EOF) to pump proteins through a proteolytic digestion system packed with immobilized trypsin gel beads [17]. Liu and coworkers immobilized trypsin on different substrates, including titania and alumina sol-gel [22], gold nanoparticles [23], and nanozeolites [24]. Wu et al. developed a microdevice consisting of an immobilized proteolytic enzyme on the surface of acrylic acid-grafted PDMS channels [25]. To improve sensitivity and detection limits, Hustoft et al. coupled for the first time an immobilized trypsin reactor on-line with open-tubular LC-MS, to detect attomole amounts of isolated cancer proteins [26]. Regnier and coworkers proposed another innovative on-line system that integrates affinity selection, buffer exchange, and a continuous flow (cf)-IMER, which was capable of converting native proteins to peptides in a few minutes at elevated temperature with high recovery and great reproducibility [27]. Krenkova et al. introduced an enzyme reactor containing trypsin and LysC immobilized on a porous polymer monolith, which was demonstrated for the fast digestion (6 min) of high molecular weight human immunoglobulin G [16]. In regard to phosphopeptide enrichment using IMAC, TiO_2, immunoprecipitation, or a combination of any two strategies, only a limited number of microdevices have been reported. Yates and coworkers developed a TiO_2-based automated online multidimensional phosphopeptide enrichment system coupled to ESI-MS. The study successfully demonstrated the superior performance of TiO_2 as a phosphopeptide enrichment resin, allowing high-throughput analyses for assessing the phosphorylation states [28]. Heck's group, using the Agilent microfabricated platform, described a TiO_2-based chip coupled to Q-TOF/MS, which identified 1012 unique phosphopeptides corresponding to 960 different phosphorylation sites in human leukocytes [29, 30]. Ficarro et al reported an online nanoflow multidimensional fractionation protocol for high efficiency phosphopeptide analysis [31].

Although most immobilized microfluidic reactors are very effective in achieving their purpose, several key issues must be addressed before enabling trouble-free implementation for routine lab operations. The fabrication of an enzyme-immobilized reactor is time-consuming, complex, and expensive. For example, the fabrication of a monolithic structure inside a microchannel may require up to 10 h, or even more, if surface modifications are necessary. Commercial enzymatic cartridges are high-priced. Covalent attachment or immobilization through noncovalent interactions or encapsulation of enzymes has the potential to affect the enzyme activity. Sample preparation and cleanup alone, such as for phosphopeptide enrichment with highly selective TiO_2 spin tips, requires several hours for completion. The undesired adsorption of proteins on polymer-based microdevices fabricated, for example, from PDMS or PMMA, poses additional concerns to achieving good detection limits. To circumvent some of the above-described concerns, in this work we propose a novel and very simple strategy for the fabrication of a microreactor that enables both proteolytic digestion and phosphopeptide enrichment. Commercially available C18 and TiO_2 particles were packed into fused silica capillaries to act as enzymatic digestion and enrichment microreactors. Unlike in conventional immobilized enzyme systems, in this strategy the protein samples are the ones that are adsorbed on the C18 beads, and the proteolytic enzyme is delivered through infusion. The newly generated peptides are then eluted onto the TiO_2 microreactor for enrichment in phosphopeptides. This analysis approach enabled protein digestion in 1–3 min, digestion/phosphopeptide enrichment in ~5 min, and complete sample analysis in ~30–90 min. In comparison to conventional protocols, this represents ~10- to 15-fold speed-up of the analysis process.

Experimental

Reagents

Hemoglobin α/β, bovine serum albumin, carbonic anhydrase, α-lactalbumin, fetuin, α-casein, β-casein, cytochrome *c*, urea, dithiothreitol (DTT), acetic acid, trifluoroacetic acid (TFA), and ammonium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequencing-grade trypsin was acquired from Promega Corporation (Madison, WI, USA). Zorbax SB-C18/5 μm particles and SPEC-PTC18/SPEC-PTSCX pipette tips were from Agilent Technologies (Santa Clara, CA, USA), and Titansphere Phos-TiO/10 μm particles, styrene divinylbenzene (SDB)/graphitic carbon (GC) cartridges, and lactic acid were products of GL Sciences (Torrance, CA, USA). HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA). DI water was obtained from a MilliQ Ultrapure water system

(Millipore, Bedford, MA, USA). SK-BR-3 breast cancer cells were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA), fetal bovine serum (FBS) from Gemini Bio Products (West Sacramento, CA, USA), and McCoy's 5A cell culture medium from Life Technologies (Carlsbad, CA, USA).

Sample Preparation and Analysis Using Conventional Proteolytic Digestion and Phosphopeptide Enrichment Protocols

Standard bovine proteins, or mixtures of proteins, 1 μM each in NH_4HCO_3 (50 mM), were denatured with urea (8 M) in the presence of DTT (5 mM) at 60 °C for 1 h, diluted 10-fold with NH_4HCO_3 (50 mM), digested overnight with trypsin (50:1 substrate:enzyme ratio) at 37 °C, quenched with glacial CH_3COOH (1% v/v final concentration), and then subjected to C18/SCX cleanup. In preparation for nano-HPLC-MS/MS experiments, or for loading on the microreactor, the samples were brought to dryness and dissolved in a solution of H_2O/CH_3CN/TFA 98:2:0.01 v/v to a final concentration of 0.5–1 μM in each protein. TiO_2 phosphopeptide enrichment was performed in the presence of lactic acid using the Titansphere Phos-TiO Kit following the manufacturer's protocol (adsorption at pH <3 in the presence of TFA and lactic acid, and elution at pH >10 in a solution containing NH_4OH). The phosphopeptide-enriched samples were subjected to SDB/GC cleanup, brought to dryness in a vacuum centrifuge, and dissolved in H_2O/CH_3CN/TFA 98:2:0.01 v/v to reach a final concentration corresponding to the initial concentration of 1 μM in each protein. The phosphopeptide samples were further analyzed by nano-HPLC-MS. For simple infusion-MS analysis, all peptide samples were dissolved in a solution of CH_3CN/H_2O/TFA 50:50:0.01 v/v, phosphopeptide samples in CH_3CN/H_2O/28% NH_4OH 50:40:10 v/v, and infused through a 50 μm i.d. × 360 μm o.d. × 1 m long fused silica capillary at 300 nL/min.

SK-BR-3 Cell Processing

SK-BR-3 cells were cultured in McCoy's 5A supplemented with 10% FBS in an incubator (5% CO_2 and 37 °C), harvested when the cells reached full confluence and stored at –80 °C. For processing, the frozen cells were thawed at room temperature and lysed through sonication for 5 min at 4 °C. The protein content was measured with the Bradford assay (SmartSpec Plus spectrophotometer; Bio-Rad, Hercules, CA, USA). Conventional tryptic digestion of the cell extracts was performed overnight at 37 °C, at substrate:enzyme ratio of ~50:1 (w/w). The digest was further cleaned-up with C18/SCX pipette tips and prepared for MS analysis (1–2 μg/μL) in a solution of H_2O/CH_3CN/TFA 98:2:0.01 v/v [32, 33].

Sample Analysis

MS analysis was performed with an LTQ or LTQ-XL mass spectrometer (Thermo Electron, San Jose, CA, USA), in positive-ion mode, with the capillary voltage set at 2.0–2.2 kV. The samples were either infused with a syringe pump 22 (Harvard Apparatus, Holliston, MA, USA) at 300 nL/min through the microreactor or an empty fused silica capillary, or analyzed with an Agilent 1100 micro-LC separation system, using a nano-HPLC column operated at ~180 nL/min (100 μm i.d. × 360 μm o.d., 10 cm long, packed in-house with Zorbax C18/5 μm particles) [33], with a 10 min concentration gradient of H_2O/CH_3CN/TFA from 96:4:0.01 v/v (solvent A) to 10:90:0.01 v/v (solvent B). Fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA), and syringes (250 μL) from Hamilton (Reno, NV, USA). The samples were analyzed via a data-dependent method by performing Zoom/MS^2 scans on the top 3 (fast nano-HPLC-MS/MS) to top 10 (infusion MS/MS) most intense peaks in each MS scan (5 microscans averaged) [32]. Data were acquired over a mass range of 500–2000 m/z. Collision induced dissociation (CID) was performed with isolation width 3 m/z, normalized collision energy 35%, activation Q 0.25, and activation time 30 ms. The raw data files were searched against UniProt protein databases using the Proteome Discoverer 1.4 software package (Thermo Electron). Phosphorylation was enabled as a dynamic modification on Ser, Thr, and Tyr. Maximum missed cleavage allowance was set to 2, precursor ion mass tolerance at 2 Da, fragment ion tolerance at 1 Da, and the peptide-level FDR at <3%. Each combination of treatments was replicated three times.

Results and Discussion

The development of the proteolytic digestion and phosphopeptide enrichment protocols included the fabrication and testing of microreactors of various dimensions, operated at different flow rates under various sample loading and elution conditions, as well as validation by performing comparisons with conventional sample preparation and analysis protocols.

Microreactor Fabrication

The microreactors were fabricated from fused-silica capillaries (100 μm i.d.) that were packed with a slurry of C18/silica (5 μm) or TiO_2 (10 μm) particles delivered by a 250 μL gastight syringe. The particles were retained in the microreactor by a 1 cm long capillary (20 μm i.d. × 90 μm o.d.) inserted in one end of the microreactor, which also had the role of the electrospray ionization (ESI) emitter. The choice of the solvent for the slurry preparation was critical to preventing particle deposition prior to finalizing the microreactor fabrication process. Isopropanol proved to be an adequate solvent for packing the C18/silica particles. For the heavier TiO_2 particles, a more viscous solution, prepared by adding lactic acid to isopropanol, had to be made. Lactic acid is often used for improving the selectivity of the phosphopeptide enrichment process, and is recommended in the phosphopeptide sample loading solvent in concentrations as high as 25% for the commercial TiO_2 spin tips. Mixed with isopropanol, it enabled trouble-free particle loading. Particle beds of 1–2 mm in length, which contained enough particles for the

retention of a sufficient amount of peptides to achieve similar detection levels to conventional nano-LC separations interfaced to MS, were prepared. As accurate control of a 1 mm long particle bed length was difficult to achieve, most proteolytic digestion studies were performed with C18 microreactors of 2 mm in length. For phosphopeptide enrichment, the microreactor was first loaded with a ~1 mm long section of a C18 bed that acted as a filter for preventing the TiO_2 particles from blocking the ESI emitter, and then with a 2 mm long TiO_2 bed. For performing combined proteolytic digestion and phosphopeptide enrichment, microreactors comprising three sections were prepared (i.e., ~1 mm C18 filter, 1–2 mm TiO_2 for phosphopeptide enrichment. and 2 mm C18 particles for proteolytic digestion). All particle loading operations were performed manually, under a microscope, to enable control of the particle section lengths. The microreactor system was then washed at 2 μL/min with isopropanol, CH_3CN/H_2O 80:20 v/v, and preconditioned with the same solution as used for sample preparation (e.g., H_2O/CH_3CN 98:2 v/v at acidic or basic pH). Overall, the preparation of the microreactors could be completed in 30–40 min.

Microreactor for Proteolytic Digestion

Microreactors that perform fast proteolytic digestion protocols rely on the use of immobilized enzymes for ensuring high enzyme:substrate ratios and preventing the generation of undesired autolysis products. In this work, an alternative, easy-to-implement and cost-effective approach was developed that relies on the adsorption of the protein (i.e., the substrate) on hydrophobic C18/silica particles, and the delivery of the enzyme through continuous flow to the microreactor. The completion of the analysis involves the following steps: (1) adsorption of the protein or protein mix on C18 particles through hydrophobic interactions, (2) infusion of the enzyme over the adsorbed proteins in a solvent that preserves the retention of the newly generated tryptic peptides, (3) removal of undesired buffer/contaminant components, and (4) elution of the peptides from the microreactor in a solvent system compatible with ESI-MS detection. The performance of the microreactor was evaluated for various sample (0.1–1 μM) and trypsin (0.1–5 μM) concentrations, incubation times (1–10 min), and buffer compositions, and the results were compared with those obtained from experiments in which the proteins were digested with a conventional overnight protocol. Optimized microreactor operational conditions are provided in Table 1. A mixture of 10 proteins at concentrations typical for infusion experiments (e.g., 1 μM each), in a solvent system that enables protein adsorption on C18 particles (H_2O/CH_3CN 98:2 v/v, pH ~4 or pH ~7.8), was infused for 5 min over the microreactor. Once the proteins were adsorbed on the hydrophobic C18 particles, a trypsin solution at 5 times higher concentration than the protein

Table 1. Analytical Processing Steps for the Proteolytic Digestion, Phosphopeptide Enrichment, and Combined Proteolytic Digestion/Phosphopeptide Enrichment Microreactors

Proteolytic digestion microreactor	Solution composition	Concentration	Flow rate	Infusion time
Microreactor preconditioning	Acidic rinse solution: H_2O/CH_3CN/TFA	98:2:0.01 v/v	2 μL/min	5 min
	Basic rinse solution: $H_2O/CH_3CN/NH_4HCO3$	98:2 v/v, 50 mM NH4HCO3	2 μL/min	5 min
Sample loading	Sample in acidic solution, or	1 μM	2 μL/min	5 min
	Sample in basic solution	1 μM	2 μL/min	5 min
Enzymatic reaction	Trypsin in basic solution	1.0–5.0 μM	2 μL/min	1–3 min
Tryptic reaction quenching	Acidic rinse solution: H_2O/CH_3CN/TFA	98:2:0.01 v/v	2 μL/min	5 min
Sample elution for analysis	Acidic elution solution: H_2O/CH_3CN/TFA	50:50:0.01 v/v	300 nL/min	25 min

Phosphopeptide enrichment microreactor	Solution composition	Concentration	Flow rate	Infusion time
Microreactor preconditioning	Acidic rinse solution A: H_2O/CH_3CN/TFA	20:80:0.4 v/v	2 μL/min	5 min
Equilibration with lactic acid	Acidic equilibration solution B: solution A/lactic acid	97.5:2.5 v/v	2 μL/min	5 min
Sample loading	Sample in acidic solution B	1 μM	1 μL/min	10 min
Lactic acid removal	Acidic rinse solution A: H_2O/CH_3CN/TFA	20:80:0.4 v/v	1–2 μL/min	5 min
pH change	Rinse solution C: H_2O/CH_3CN	50:50 v/v	1–2 μL/min	5 min
Sample elution for analysis	Basic elution solution: H_2O/CH_3CN/28% NH_4OH	40:50:10 v/v	300 nL/min	50 min

Microdigestion/phosphopeptide enrichment microreactor	Solution composition	Concentration	Flow rate	Infusion time
Microreactor preconditioning	Acidic rinse solution: H_2O/CH_3CN/TFA	98:2:0.01 v/v	2 μL/min	5 min
Sample loading	Sample in acidic rinse solution	1 μM	2 μL/min	5 min
Enzymatic reaction	Trypsin in basic solution	5 μM	2 μL/min	90 sec
Quenching tryptic reaction	Acidic rinse solution: H_2O/CH_3CN/TFA	98:2:0.4 v/v	1–2 μL/min	5 min
Sample elution from C18/adsorption on TiO2	Rinse solution B: Solution A/lactic acid	97.5:2.5 v/v	1 μL/min	10–15 min
LA removal	Rinse solution A: H_2O/CH_3CN/TFA	20:80:0.4 v/v	1–2 μL/min	5 min
pH change	Rinse solution C: H_2O/CH_3CN	50:50 v/v	1–2 μL/min	5 min
Sample elution for analysis	Elution solution: H_2O/CH_3CN/28% NH_4OH	40:50:10 v/v	300 nL/min	25–50 min

(e.g., 5 μM), in a basic buffer solution optimal for enabling the proteolytic digestion process (H_2O/CH_3CN 98:2 v/v, 50 mM NH_4HCO_3, pH ~7.8), was pumped over the microreactor at 2 μL/min for a time ranging from 1 to 10 min (not including the time necessary for rinsing the dead-volume associated with the transfer capillary from the micropump to the microreactor (i.e., ~2 μL for a capillary of 50 μm i.d. × 1 m length). To quench the enzymatic digestion process, a rinse step with acidic solution was performed (H_2O/CH_3CN/TFA 98:2:0.01 v/v, 2 μL/min for 5 min). Ultimately, the peptides were eluted in a high organic content solvent (H_2O/CH_3CN/TFA 50:50:0.01 v/v) at a flow rate of 300 nL/min for 25 min. Although higher elution flow rates would have resulted in overall shorter analysis times, the choice of 300 nL/min was dictated by the maximum tolerable flow by the ESI emitter (20 μm i.d. × 90 μm o.d.) that did not result in a loss of ESI efficiency.

Figure 1a displays the results obtained for different trypsin infusion times over the microreactor (i.e., 1, 1.5, 3, 5, and 10 min, respectively). A total of 8–9 proteins were identifiable by 28–38 unique peptides, a maximum being observed at 1.5 min incubation time, and a decrease in numbers after 3–5 min. A similar decrease in protein identifications, albeit for experiments conducted on longer time-scales, was reported by Klammer et al [34]. A possible explanation for this observation takes into account the forces that control the adsorption/desorption of proteins and peptides on hydrophobic surfaces. Peptides and proteins are adsorbed on C18 particles as a result of hydrophobic interactions. The overall outcome is controlled by the balancing of various forces that arise as a result of intramolecular, solvation, and surface effects. Initially, the sample proteins are adsorbed on the C18 particles, and if the surface is saturated with sample proteins, the adsorption/retention of trypsin is minimized. An increase in the duration of trypsin infusion will increase the length of the enzymatic reaction and lead to a more complete digestion process, a decreased number of missed K/R cleavage sites, and eventually to an increased number of identifiable proteins. Nevertheless, at prolonged reaction times, the larger molecular weight (MW) hydrophobic trypsin, and the buffer solution itself, can also displace the smaller MW peptides, or even some proteins, deteriorating the experimental outcome. This explanation was confirmed by experimental evidence. While the large majority of peptides eluted from the microreactor with the high organic content eluent, a few tryptic peptides were already observable at the start of the elution process, at a stage when the organic solvent could not have reached yet the microreactor, indicating that there were physical processes at work that prevented complete retention of all peptides. For lower sample concentrations (e.g., 0.1 μM) the concentration of trypsin had to be adjusted accordingly, best results being obtained for infusions with 1 μM trypsin solutions. Owing to low intensity peptide signals at these concentrations, the results were somewhat less reproducible in terms of number of unique peptide and protein identifications. The best conditions with the present experimental setup were achieved for a proteolytic digestion process conducted for 1.5–3 min. A substantial improvement in the average number of identified peptides (from 38 to 54) was observed if the microreactor preconditioning and

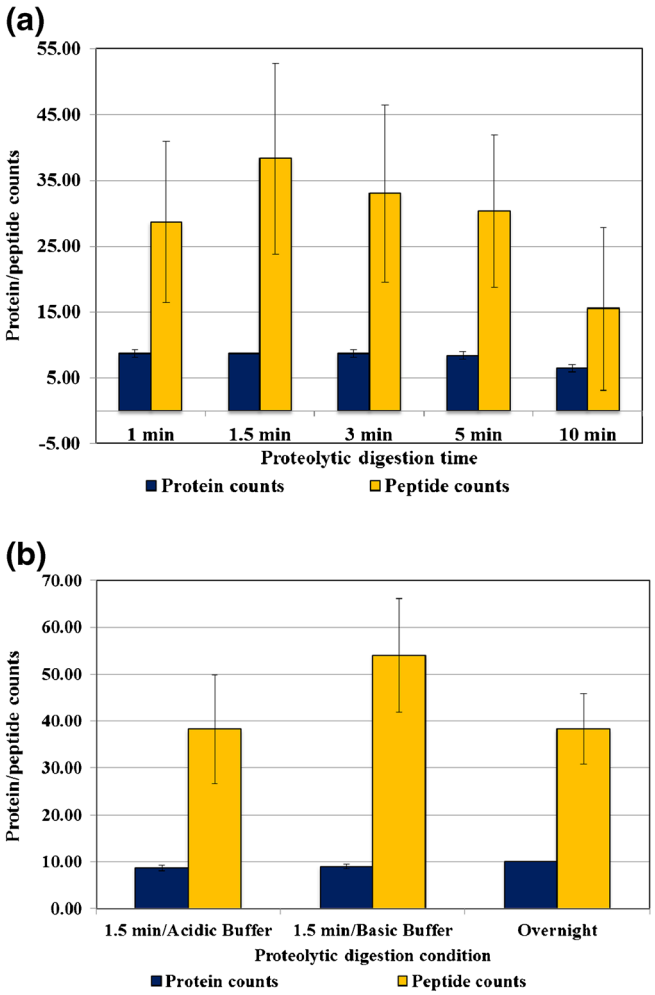

Figure 1. Protein and peptide identifications using various conditions for enzymatic protein digestion. (**a**) Proteolytic digestion performed with the microreactor at various incubation times with trypsin. Conditions: protein concentrations 1 μM, trypsin concentration 5 μM, sample loading/peptide elution in acidic buffer (see Table 1). (**b**) Comparison of the microreactor performance with a conventional overnight digestion protocol. The samples were either digested on the microreactor or digested overnight in a vial and loaded on the microreactor for further processing. Microreactor digestion conditions: protein concentrations 1 μM, trypsin concentration 5 μM, sample loading in acidic or basic buffer, peptide elution in acidic buffer (see Table 1). Overnight digestion conditions: protein denaturation in urea/DTT, proteolytic digestion at substrate:enzyme ratio 50:1, buffer NH_4HCO_3 50 mM, pH ~7.8, C18/SCX cleanup of the tryptic peptide sample, peptide sample (1 μM) loading in acidic solution on the microreactor, and rinsing/elution using the same conditions as for microcolumn digestions but without trypsin infusion. See Table 1 and "Experimental" section for details. Note: three replicates were conducted for each experiment

sample loading solution was changed from an acidic (H_2O/CH_3CN/TFA 98:2:0.01 v/v, pH~4) to a basic buffer system such as the one used for the digestion process (H_2O/CH_3CN 98:2 v/v, 50 mM NH_4HCO_3, pH ~7.8) (Figure 1b). This improvement resulted from the elimination of the equilibration time needed

for achieving the change from acidic to basic environmental conditions that are favorable to the digestion process.

To evaluate the completeness of the digestion process, the proteolytic digestion results obtained with the microreactor were compared with those of an overnight digestion protocol. For this purpose, a sample containing a mixture of 10 proteins was denatured and digested overnight with trypsin (substrate:enzyme ratio 50:1), cleaned-up with C18/SCX cartridges, reconstituted to a final concentration of 1 μM, loaded on the microreactor, and processed using the same experimental conditions as for microreactor digestion, but without the trypsin infusion step (Figure 1b). The microreactor proteolytic digestion process performed within 1.5 min with basic buffer loading conditions resulted in the identification of nine proteins by an average of 54 unique peptides per run and 83 unique peptides in combined three replicate experiments. The overnight process returned all 10 protein IDs, matched by an average of 38 unique peptides per run, and 50 unique peptides in three replicate experiments. A larger number of missed tryptic sites in the peptides generated with the microreactor accounted for the larger number of unique peptides, the percent peptides with missed K/R cleavage sites being 53%–59% and 19%–21% for microreactor and overnight proteolysis protocols, respectively. The typical range for missed K/R sites with the overnight digestion protocols was 20%–30%. While this is an outcome of incomplete enzymatic digestion, the presence of such peptides results in a more complete amino acid sequence coverage, and a more reliable identification by MS. α-Lactalbumin could not be identified in any of the experiments conducted using the microreactor. Although it has a relatively small molecular weight (~16 kDa), its globular structure and the lack of a protein denaturing step led most likely to resisting proteolytic digestion with trypsin [35, 36]. The performance of the microreactor compared with overnight digestion in terms of unique peptides and % amino acid sequence coverage, is provided in Figure 2, and shown as a range for three replicate

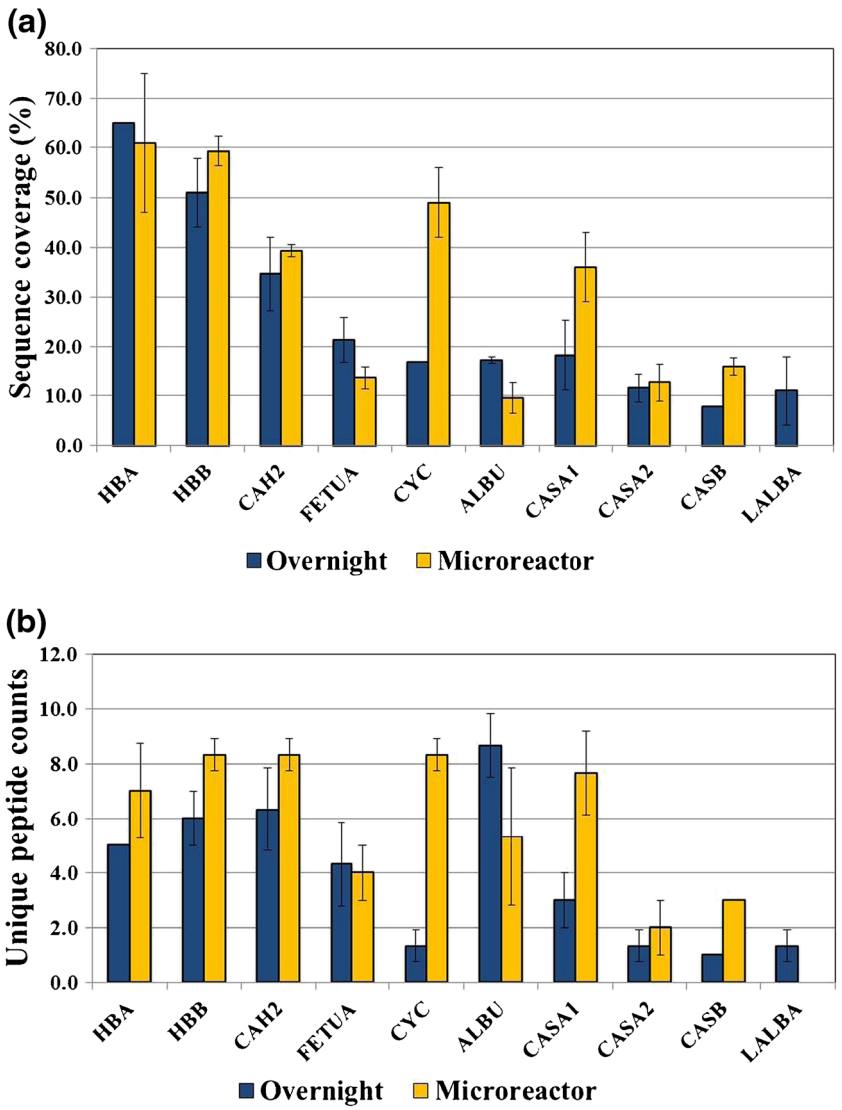

Figure 2. Bar graph comparison of results generated with the overnight and microreactor digestion protocols. (**a**) Amino acid sequence coverage; (**b**) Unique peptides. Experimental conditions were the same as in Figure 1

experiments. Microreactor digestion resulted in each protein being identified by 3–9 unique peptides accounting for 7%–75% sequence coverage (~30% average), except for α-casein S2, which was not added to the mix but was present as a minor component of the α-casein standard. Overnight digestion resulted in each protein being identified by 1–10 unique peptides accounting for 7%–65% sequence coverage (~26% average). Supplementary Table 1 provides an alignment of peptide identifications in all experiments. The data also confirmed the results of earlier studies that evaluated the effectiveness of enzymatic digestion of proteins adsorbed on various surfaces [37]. These studies showed that the enzymatic products for proteins adsorbed on surfaces differ from the ones proteolyzed in solution, most likely because of the different protein sites that are exposed to the enzyme. The same study showed that the attainable sequence coverage for cytochrome *c* adsorbed on C18 beads can reach values as high as 89%, but only after 2 h incubation time with C18 beads or after multiple protein passages through a C18-bead packed micro gel loader tip, followed by 2 h or 30 min proteolytic digestion, respectively [37]. In the case of the microreactor, the attainable sequence coverage for cytochrome *c* was 43%–57% after 90 s of proteolysis.

The reproducibility of peptide identifications in three replicate experiments was assessed by comparing the microcolumn to overnight digestion by using two strategies: (1) direct MS detection of peptides eluting from the microcolumn, with peptides being generated either through digestion on the microcolumn or overnight and loaded on the microcolumn (as discussed above), and (2) nano-HPLC-MS/MS analysis of peptides generated and collected from the microcolumn or directly from overnight digestion. The Venn diagrams in Figure 3a and b show that while a larger number of unique peptides were identifiable from the microcolumn than the overnight enzymatic digestion protocols (i.e., 83 versus 50), the reproducibility of unique peptide identifications in three replicate runs dropped from 50% to 35%, when changing from the overnight to the microreactor digestion conditions. The larger number of peptides with missed cleavages that contributed to a larger sequence coverage also contributed to a somewhat lower reproducibility in peptide identifications. For building the Venn diagrams for Figure 3c and d, the solution of tryptic peptides that eluted from the microcolumn had to be diluted to provide sufficient volume for an HPLC injection. The concentration of all samples analyzed by HPLC was 0.5 μM and the sample consumed for analysis was 4 pmol (assuming 100% recovery from the digestion process). BSA was not part of this protein mixture. To reduce the impact of peptides with missed K/R cleavages, proteolytic digestion on the microcolumn was performed for 3 min. In addition, to avoid possible bias induced by the data-dependent acquisition process, all samples were analyzed twice by HPLC. The Venn diagrams from Figure 3c and d represent the overlap of three replicates of two averages each, and confirmed a similar trend, 55% versus 39% overlap of peptide IDs with overnight versus microreactor digestion. While these

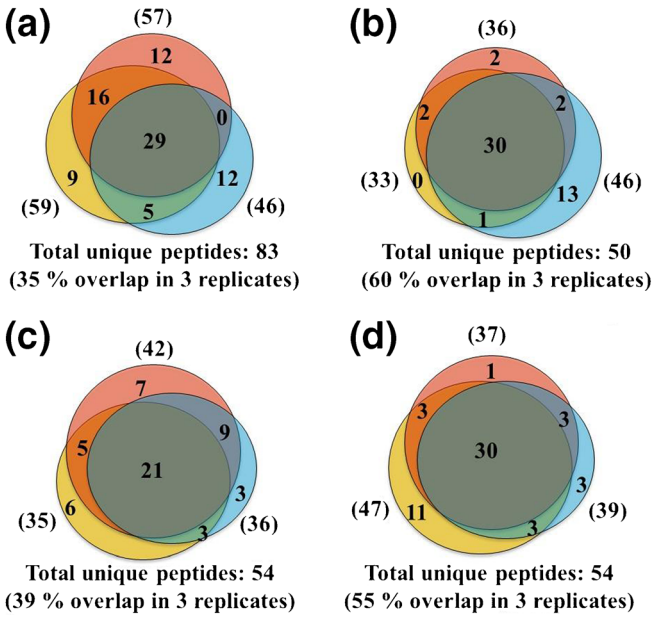

Figure 3. Venn diagram comparisons for unique peptide identifications in proteolytic digestion reactions performed with the microreactor and overnight protocols. (**a**) Microreactor digestion (90 s) with direct MS/MS analysis (1 μM sample, 10 μL loading); (**b**) Overnight digestion with tryptic peptide loading on the microreactor and direct MS/MS analysis (1 μM sample, 10 μL loading); (**c**) Microreactor digestion (3 min) with sample collection for nano-HPLC-MS/MS analysis (0.5 μM sample, 8 μL injection); (**d**) Overnight digestion followed by nano-HPLC-MS/MS analysis (0.5 μM sample, 8 μL injection). See Table 1 and "Experimental" section for details. Note: for **c** and **d**, the count of unique peptides for each experiment is the result of two combined nano-HPLC-MS/MS runs

experimental conditions resulted in a lower number of total peptide IDs (54 versus 83), a better reproducibility, approaching that of the conventional protocol, was evident. The percent of peptides with missed cleavages dropped as well, from 53%–59% to 39%–53%.

Additional parameters that were evaluated for the microreactor included detection limit and breakthrough volume (for conditions of sample loading at 2 μL/min, basic buffer, 1.5 min incubation with trypsin, and sample elution at 300 nL/min). Experiments performed with a mixture of nine proteins (no BSA) revealed that all proteins (except α-lactalbumin) were detectable from 1 μM, seven from 0.1 μM, and four from 0.01 μM solutions, respectively. Overall, this corresponded to a total sample consumption of 10, 1, and 0.1 pmol in an infusion experiment that eluted the great majority of peptides in a time-window of ~10–15 min. Supplementary Table 2 enlists the identifiable peptides for each protein. Sample breakthrough volumes were determined with 1 μM protein mix solutions infused at 2 μL/min over microreactors containing C18 packing of 5–12 mm in length by connecting the outlet of the microreactor to an Agilent HPLC-UV detector equipped with a microflow cell and operated at 254 nm. The particle beds in this study were longer than the ones used for proteolytic

digestions (~2 mm) to minimize measurement errors that could be induced by the variability in packing length and uniformity. After accounting for the dead volumes associated with the transfer capillaries, sample breakthrough started to occur after ~ 7, 11, and 13 min from microreactors containing 5, 10, and 12 mm C18 packing, respectively. Breakthrough was considered to occur when the absorbance of the solution emerging from the microreactor reached ~1% of the absorption of the 1 μM protein mix solution when infused directly through the UV detector without an inline mounted proteolytic reactor. Therefore, we estimated that for short, 2 mm microreactors, the optimal loading time of 1 μM solutions (total sample amount ~2 μg) should not exceed 3–5 min (i.e., the time that maximizes the number of detectable peptides while minimizing the protein losses due to breakthrough).

Microreactor for Phosphopeptide Enrichment

The performance of the TiO_2 microreactor was evaluated with peptides generated from the mixture of 10 proteins (1 μM each) through an overnight digestion protocol. Among the 10 bovine proteins, only α-casein, including subunits 1 and 2, β-casein, and fetuin contained phosphorylated peptides. Selective enrichment in phosphopeptides relies on their retention on TiO_2 particles at pH <3 (when the phosphate groups are still partially ionized while the acidic aspartic and glutamic acid amino acids are not), and elution at pH >10 when buffer anions compete for the Lewis acidic Ti^{4+} sites of TiO_2 and liberate the phosphopeptides. The parameters that were optimized for the phosphopeptide enrichment microreactor included the parameters that affect selectivity, i.e., the composition and flow rate (1–2 μL/min) of the solutions involved in sample loading and rinsing. The commercial TiO_2 spin tips, which contain the same type of particles as the microreactor, deliver selective enrichment in phosphopeptides when conditioned properly in the presence of lactic acid (25% in a solution of CH_3CN/H_2O/TFA, 80:20:0.4 v/v). To minimize the interference of such a high concentration of lactic acid with MS detection, the performance of the enrichment process was evaluated at lower, 0.25% and 2.5%, lactic acid concentrations. While at 0.25% the selectivity of commercial tips was deteriorated, at 2.5% lactic acid concentration the selectivity was the same as at 25%. The spin tip enrichment in phosphopeptides from a mixture of peptides generated through the overnight digestion of a 10 protein mix, followed by nano-HPLC analysis with a 10 min gradient on a 10 cm × 100 μm i.d. capillary packed with C18 particles (5 μm), enabled the identification of all four phosphoproteins (α-casein S1, α-casein S2, β-casein, and α-2HS-glycoprotein), typically by 10–13 phosphopeptide variants corresponding to 6–8 unique peptide sequences (Table 2). The selectivity in phosphopeptide enrichment was 100%, with no nonphosphorylated peptides detected. The same lactic acid concentration of 2.5% was further used for performing phosphopeptide enrichment with the microreactor. Two rinse steps, one for removing the excess lactic acid and one to facilitate the pH change to basic conditions in preparation for phosphopeptide elution, were necessary for completing the enrichment process. Long rinse times and high flow rates increased the selectivity in phosphopeptide enrichment but decreased the recovery rates, indicating that a proper balance must be achieved to obtain both the desired selectivity and recovery rate. Lack of adequate rinsing resulted in loss of selectivity. Two examples of optimized rinse-step combinations that resulted in selective enrichment are provided in Table 2 (only phosphopeptides, or 5–6 phosphopeptides out of a total of 6–7 peptides corresponding to 5–6 unique amino acid sequences, were detected). With few exceptions, the TiO_2 microreactor enabled the identification of all four phosphoproteins with a selectivity of 95%–100% in phosphopeptide enrichment.

Ultimately, the optimal conditions for the operation of the microreactor included the use of lactic acid at concentration of 2.5% and two rinse steps performed at 1–2 μL/min for 5 min. Before sample loading, the TiO_2 microreactor was preconditioned with an acidic/high organic content solution (CH_3CN/H_2O/TFA 80:20:0.4 v/v), and then with the same solution containing also lactic acid (acidic solution/lactic acid 97.5/2.5 v/v) at 2 μL/min for 5 min for each solution (Table 1). The protein mix digest was pumped through the microreactor at a flow rate of 1 μL/min for 10 min (a low flow rate was chosen to facilitate the retention of phosphopeptides). The amount of protein loaded through infusion on a microreactor with a 2 mm TiO_2 bed was ~2 μg (i.e., at the same level that was determined for the C18 microreactors), and at a sample/TiO_2 packing load recommended by the manufacturer for the spin tips. Following sample loading, the microreactor was rinsed again with the acidic/organic solution and then just with an organic solution (CH_3CN/H_2O 50:50 v/v) at 1–2 μL/min, for 5 min, to remove the lactic acid and neutralize the environment, respectively. The enriched phosphopeptides were eventually eluted using a basic/high organic content solution ($CH_3CN/H_2O/28\%NH_4OH$ 50:40:10 v/v/v) at a flow rate of 300 nL/min for 50 min. As

Table 2. Selectivity of the Phosphopeptide Enrichment Process for the Spin Tip and Microreactor. Conditions: 10 protein mix overnight digest (1 μM), phosphopeptide enrichment in the presence of 2.5% lactic acid for both TiO_2 spin tip and TiO_2 microreactor

	Lactic acid removal H_2O/CH_3CN/TFA 20:80:0.4 v/v	pH-adjustment H_2O/CH_3CN 50:50 v/v	Total proteins identified	P-proteins identified	Peptide spectral counts	P-peptide spectral counts	Total unique peptides	Unique P-peptides
TiO_2 Microreactor	2 uL/min, 5 min	1 uL/min, 5 min	3–4	3–4	16–17	16–17	4–5	4–5
	1 uL/min, 5 min	1 uL/min, 5 min	4–5	3–4	22–23	21–22	6–7	5–6
TiO_2 spin tip/ HPLC/MS	Centrifuging, 300 *g*	N/A	4	4	18–19	18–19	10–13	10–13

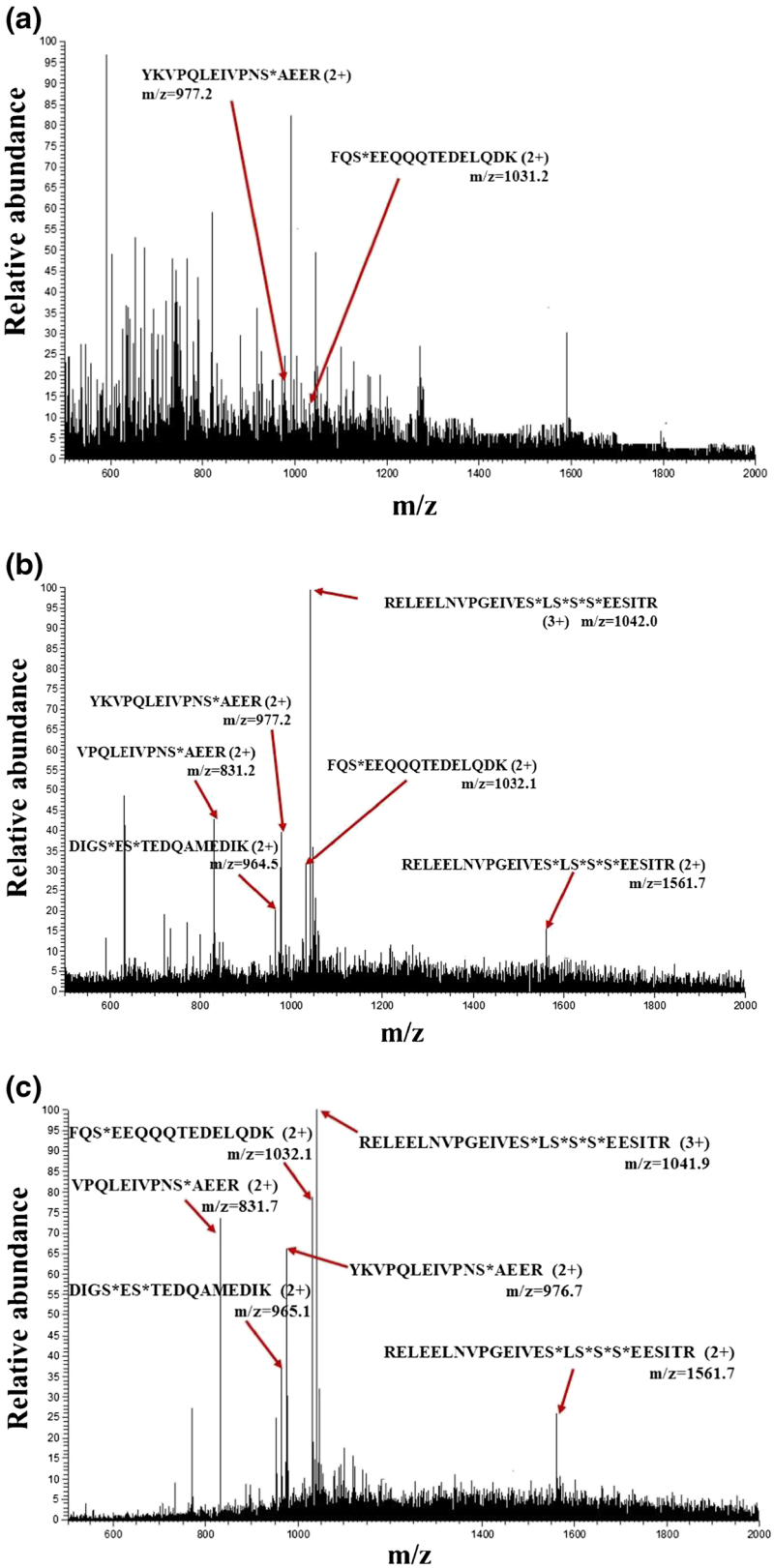

Figure 4. Full mass scans acquired during the analysis of standard protein mixture digests. (**a**) Capillary infusion of the whole protein mixture digest without phosphopeptide enrichment; (**b**) Capillary infusion of the whole protein mixture digest after TiO_2 spin tip phosphopeptide enrichment; (**c**) Infusion of phosphopeptides enriched on the microreactor. Conditions: sample concentration 1 μM, infusion at 300 nL/min, MS data acquisition performed using data-dependent analysis with all peptide identifications being confirmed by tandem MS. See "Experimental" section for details. Note: not all identifiable phosphopeptides are shown in the mass spectra

observed in Figure 4, both conventional and microreactor enrichment protocols were highly selective. A full MS scan for a sample without any enrichment identified only two phosphopeptides, YKVPQLEIVPNsAEER and FQsEEQQQTEDELQDK, with low abundance in the mass spectrum (Figure 4a). Figure 4b and c show the MS scans for the same protein mix digest enriched in phosphopeptides by using a commercial TiO_2 spin tip and the microreactor, respectively. The majority of the intense ions in both MS scans could be assigned to phosphopeptides. Peptide sequences and IDs were confirmed by tandem MS.

The back-pressure of both proteolytic and TiO_2 enrichment microreactors was negligible. When mounted on the outlet of an Agilent 1100 micro-HPLC pump, operated with solvent A, the measured change in backpressure was only 3–4 bar for the C18 and 5–6 bar for C18/TiO_2/C18 microreactors, indicating that the use of simple, low-cost infusion pumps and low-pressure connectors is adequate for the implementation of such experiments in any bioanalytical laboratory. In terms of reusability, after thorough flushing with high organic content solvents, the C18 microreactors were reusable when the protein concentrations were ~1 μM. Carryover that would affect the measurements of low protein concentration (<1 μM) was, however, observed. The TiO_2 particles were not reusable. Given the ease of preparation, the use of new microreactors for each experiment is, therefore, recommended. As both types of particles are broadly used in chromatographic separations and enrichment in phosphopeptides, respectively, and as chemical modifications to the particles were not performed, problems related to stability were not observed. Unspecific binding to the TiO_2 particles was minimized with the addition of the lactic acid prior and during sample loading, as described above.

Microreactor for Proteolytic Digestion and Phosphopeptide Enrichment

The rationale for designing a microreactor for performing both proteolytic digestion and phosphopeptide enrichment relies on the ability to elute peptides from hydrophobic media in acidic/high-organic content solvents and selectively retain only phosphopeptides in the same solution on TiO_2 particles. Using conditions that were optimized in the previous experiments, a microreactor containing a C18 filter (1 mm), TiO_2 bed for phosphopeptide enrichment (1–2 mm), and C18/silica particles for proteolytic digestion (2 mm) was prepared and tested with the same 10 standard protein mix (1 μM each). The sample was loaded on the microreactor at 2 μL/min for 5 min, retained on the 2 mm C18 bed, and subjected to proteolysis with a trypsin solution (5 μM) infused at 2 μL/min for 1.5 min. Following a rinse-step (H_2O/CH_3CN/TFA 98:2:0.4 v/v, 2 μL/min, 5 min) for quenching the enzymatic reaction, the peptides were eluted from the C18 onto the TiO_2 microreactor with a solution of (H_2O/CH_3CN/TFA 20:80:0.4)/lactic acid, 97.5:2.5 v/v (see Table 1). After additional rinse steps to remove the lactic acid and facilitate the change to high pH, the sample was eluted with a solution of CH_3CN/H_2O/28%NH_4OH 50:40:10 v/v, at 300 nL/min, during a time-window of 25–50 min. Higher flow rates for the rinse steps enabled a faster switch to high pH eluting conditions and faster recovery of peptides from the microreactor. MS analysis was performed under high pH conditions, which enabled efficient (+) ESI-MS detection of peptides because roughly half the peptides that result from a tryptic digestion process have a pI-value >8. The interference of background ions is minimized at high pH, the overall outcome resulting in improved signal/noise ratios [38, 39].

A comparison of the performance of the microdigestion/phosphopeptide enrichment microreactor with that of a control experiment that used a conventional protocol involving overnight protein digestion and phosphopeptide enrichment with TiO_2 spin tips, followed by nano-HPLC-MS/MS (10 min gradient), is provided in Figure 5. In both experiments, only phosphopeptides were detected, indicating that a high selectivity, approaching 100%, is achievable with the proposed microreactor. Nevertheless, the time required for performing the analysis with the conventional protocol was roughly 15 h (12 h overnight digestion, 2 h phosphoenrichment, 1 h HPLC analysis, including column equilibration and rinse-steps), while with the microreactor was only ~1.5 h. Table 3 enlists the phosphopeptide sequences identified by the two approaches (i.e., on-column digestion/enrichment/infusion-MS and overnight digestion/spin tip enrichment/nano-HPLC/MS analysis). Microreactor processing returned 7–10 phosphorylated variants of nine unique peptide amino acid sequences per analysis, while conventional processing returned 10–13 phosphorylated variants of eight sequences. Supplementary Table 3 provides an alignment of the peptide identifications. Combined from three replicate analyses, 14 and 16 phosphorylated peptides were identifiable with the microreactor and conventional protocols, respectively (Table 3), each method displaying a few unique sequences not identified by the other. The peptide IDs were compared with what was previously reported by Larsen

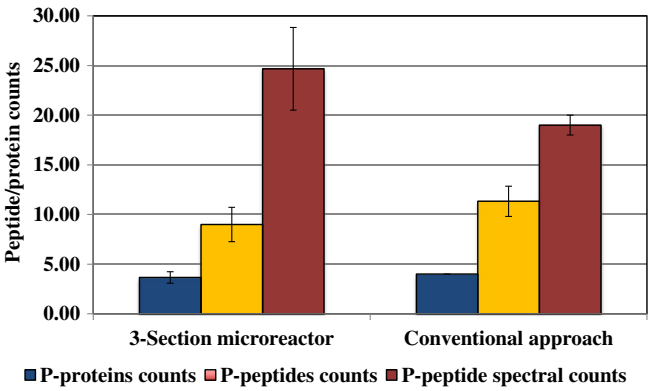

Figure 5. Comparison of the proteolytic digestion/phosphopeptide enrichment microreactor performance with conventional overnight digestion/TiO_2 spin tip enrichment/nano-HPLC-MS/MS protocols. Note: three replicates were conducted for each experiment. See Table 1 and "Experimental" section for details

Table 3. Phosphopeptide sequences identified in a mixture of bovine proteins by using overnight conventional enzymatic digestion/TiO_2 spin tips followed by nano-HPLC-MS/MS, and a 3-section microreactor with direct infusion MS analysis. Note: (a) combined results of 3 replicate analyses are shown; (b) phosphorylated amino acid residues are shown in lower case letters

Protein	Overnight digestion/TiO_2 spin tip/nano-HPLC-MS 8 unique peptide sequences 10-13 phosphopeptides per analysis 16 phosphopeptides per 3 replicates	Proteolytic digestion/TiO_2 microreactor 9 unique peptide sequences 7-10 phosphopeptides per analysis 14 phosphopeptides per 3 replicates
Alpha-S1-casein precursor	DIGsESTEDQAMEDIK	
Alpha-S1-casein precursor	DIGSEsTEDQAMEDIK	
Alpha-S1-casein precursor	DIGsEsTEDQAMEDIK	DIGsEsTEDQAMEDIK
Alpha-S1-casein precursor		*K*Y*K*VPQLEIVPNsAEER
Alpha-S1-casein precursor	VNELs*K*DIGsEsTEDQAMEDIK	
Alpha-S1-casein precursor	VPQLEIVPNsAEER	VPQLEIVPNsAEER
Alpha-S1-casein precursor	Y*K*VPQLEIVPNsAEER	Y*K*VPQLEIVPNsAEER
Alpha-S2-casein precursor	TVDMEsTEVFTK	TVDMEsTEVFTK
Alpha-S2-casein precursor		TVDMEsTEVFT*K*K
Alpha-S2-casein precursor		TVDMEStEVFTK
Alpha-S2-casein precursor		KTVDMEsTEVFTK
Beta-casein precursor	FQsEEQQQTEDELQDK	FQsEEQQQTEDELQDK
Beta-casein precursor	FQsEEQQQtEDELQDK	
Beta-casein precursor	IE*K*FQsEEQQQTEDELQDK	
Alpha-2-HS-glycoprotein	HTFSGVAsVESSSGEAFHVGK	HTFSGVAsVESSSGEAFHVGK
Alpha-2-HS-glycoprotein	HTFSGVASVEsSSGEAFHVGK	
Alpha-2-HS-glycoprotein	HTFSGVAsVEsSSGEAFHVGK	HTFSGVAsVEsSSGEAFHVGK
Alpha-2-HS-glycoprotein	HTFSGVASVESsSGEAFHVGK	HTFSGVASVESsSGEAFHVGK
Alpha-2-HS-glycoprotein	HTFSGVASVEssSGEAFHVGK	
Alpha-2-HS-glycoprotein	HTFSGVAsVEssSGEAFHVGK	HTFSGVAsVEssSGEAFHVGK
Alpha-2-HS-glycoprotein		HTFSGVASVESssGEAFHVGK

et al [40]. All peptides but one from α-S2, corresponding to six unique sequences of α-S1, α-S2, and β-casein peptides, carrying one or two phosphorylation groups were detectable with the microreactor setup, but none carrying 3–4 phosphorylation groups. The use of a different ionization method (ESI versus MALDI) and a mass spectrometer with a more limited mass range (ion trap versus TOF) were the reason for this outcome. Important also to note is that in the microreactor experiments, ESI-MS was performed directly from the TiO_2 elution buffer, at pH conditions that do not favor the detection of multiple phosphorylated peptides (pH >10.5). The use of a different enrichment protocol (lactic acid versus 2,5-dihydroxybenzoic acid for improving selectivity) could have been an additional contributor. Nonetheless, one β-casein (3+) peptide carrying four phosphorylation sites, which was not identified by the Discoverer software package, produced a tandem mass spectrum that enabled manual verification of three phosphate group losses and some additional fragment ions (Figure 4b and c).

Analysis of Biological Samples

Without sample prefractionation and/or HPLC separation steps, the proposed microreactor functions best for the analysis of simple mixtures of proteins, as shown above, or of isolated proteins from cell extracts. To test its capacity for handling samples of biological origin, a cell extract obtained from SK-BR-3 breast cancer cells was analyzed. Typically, such extracts prepared by denaturation/overnight digestion and analyzed by a 4-h long nano-HPLC-MS/MS gradient yielded 700–1000 protein IDs. The length of the gradient was essential, a short 10 min gradient reducing the numbers to only 65–70 protein IDs matched by 140–150 unique peptides. Without denaturation, the number of identified proteins did not change, but the matching peptides dropped by ~15%. The proteolytic digestion of such a cell extract with the microreactor followed by direct MS analysis yielded results that were commensurate with the fast nano-HPLC gradients (i.e., 38–44 protein IDs), but a lower number of matching peptides (i.e., 47–55). The microreactor contained a 5-mm long bed of C18 particles for enabling the loading of larger sample amounts (10–20 μg), and peptide elution occurred in three steps with progressively larger percentages of CH_3CN in the eluent (10%, 25%, and 50%). Supplementary Table 4 enlists the proteins identified in three microreactor digestion/direct infusion MS and three overnight digestion/with and without denaturation/nano-HPLC-MS/MS (10 min gradient) analyses. Mainly abundant proteins could be identified (keratins, actins, GAPDH, fatty acid synthase, tubulin, etc.), but also a few proteins of interest such as nucleophosmin and histone proteins. For a cytoplasmic SK-BR-3 cell extract spiked with bovine α/β-casein, the proteolytic digestion/phosphoenrichment microreactor was able to return 11 proteins matched by 12 peptide IDs, of which nine were phosphopeptides, preponderantly from the bovine casein proteins (75% selectivity). The effectiveness of the microreactor became most evident in experiments aimed at the characterization of recombinant proteins. Typically, after expression in an adequate system, such proteins are purified by column chromatography and analyzed by SDS-PAGE for assessing MW and purity. The microreactor was tested for the characterization of the PH domain-containing family F member 2 protein (PKHF2_Human/Q9H8W4) expressed in *E. coli*, with a purity

of ~95%, as determined by SDS-PAGE. The protein has roles in TNF-induced apoptosis. Overnight proteolytic digestion followed by a 4-h long nano-HPLC-MS/MS gradient returned 29 unique peptides accounting for ~77% protein sequence coverage, while fast, 10-min long HPLC gradients yielded 11–15 unique peptides accounting for ~59%–64% coverage. Proteolytic digestion with the microreactor (3 min with 5 μM trypsin) followed by infusion-MS yielded essentially identical results to the overnight digestion/fast nano-HPLC protocol, returning 9–11 unique peptides and 45%–66% sequence coverage in three replicate experiments, respectively (Supplementary Table 5). The protein concentration was 1 μM. At a level of 10 μM protein concentration, the sequence coverage was ~69%. As contaminants, only abundant *E. coli* proteins such as ribosomal and glutathione S-transferase GstA proteins could be identified sporadically by 1–2 peptides. The results corroborated the protein sequence and the SDS-PAGE purity data. Although additional work will be necessary for optimizing the microreactor for a specific biological application, altogether, the preliminary results confirm the ability of the microreactor to handle semi-complex samples and utility for protein characterization.

Conclusions

A microreactor that enables fast proteolytic digestion and selective enrichment in phosphopeptides, followed by on-line ESI-MS detection, was described in this work. Compared with conventional sample preparation protocols, the microreactor has multiple advantages, including the following: (1) enables a novel, inversed strategy for performing enzymatic digestion of proteins (i.e., with the sample adsorbed on the surface of C18 particles and with a high concentration trypsin solution flowing over the adsorbed substrate); (2) obviates the need for high-cost immobilized enzyme particles, while simultaneously eliminating problems associated with altered activity of the enzyme due to covalent attachment; (3) warrants fast, straightforward, and cost-effective fabrication requiring only common reagents, commercial C18 and TiO_2 particles, and a simple particle loading process in a fused silica capillary; overall preparation of the microreactor can be accomplished in a few min and can be implemented in any bioanalytical laboratory; (4) facilitates high performance enzymatic digestion of proteins in <3 min, and selective enrichment and identification of phosphopeptides; the overall sample analysis process can be completed in 30–90 min (i.e., 10–15 times faster than commonly used bench-top protocols that require 1–2 days for completion; (5) minimizes concerns related to the generation of autolytic enzyme fragments because of the short analysis times; (6) minimizes concerns related to the presence of high concentration enzymes in solution as a result of continuous removal of the enzyme from the system; and (7) ratifies broad applicability for the analysis of simple protein mixtures and potential for integration in more sophisticated, multiplexed workflows. To the best of our knowledge, such a combined proteolytic digestion/phosphopeptide enrichment microreactor is reported for the first time. It is envisioned that the integration of the microreactor with high-performance separations [41], and/or within microfluidic devices with high-throughput and multifaceted functionality, will facilitate the fast analysis of complex samples of biological origin.

Acknowledgments

This work was supported by grant NSF/DBI-1255991 to IML. The authors thank Professor Daniel Capelluto from Virginia Tech for providing the PKHF2 recombinant protein used for testing the effectiveness of the microreactor.

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