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Protein digestion and phosphopeptide enrichment on a glass microchip

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Abstract

This work describes an integrated glass microdevice for proteomics, which directly couples proteolysis with affinity selection. Initial results with standard phosphopeptide fragments from β -casein in peptide mixtures showed selective capture of the phosphorylated fragments using immobilized metal affinity chromatography (IMAC) beads packed into a microchannel. Complete selectivity was seen with angiotensin, with capture of only the phosphorylated form. On-chip proteolysis, using immobilized trypsin beads packed into a separate channel, was directly coupled to the phosphopeptide capture and the integrated devices evaluated using β -casein. Captured and eluted fragments were analyzed using both capillary electrophoresis (CE) and capillary liquid chromatography/mass spectrometry (cLC/MS). The results show selective capture of only phosphopeptide fragments, but incomplete digestion of the protein was apparent from multiple peaks in the CE separations. The MS analysis indicated a capture bias on the IMAC column for the tetraphosphorylated peptide fragment over the monophosphorylated fragment. Application to digestion and capture of a serum fraction showed capture of material; however, non-specific binding was evident. Additional work will be required to fully optimize this system, but this work represents a novel sample preparation method, incorporating protein digestion on-line with affinity capture for proteomic applications.

Keywords

Microchip; Phosphopeptide; Mass spectrometry; Protein digestion; IMAC

1. Introduction

The development of lab-on-a-chip or micro-total analysis systems (μ -TAS) has gained a significant amount of attention over the past decade. Microchip technology, originally shown to expedite electrophoresis separations, offers the additional promise of allowing

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integrated sample preparation in micron-sized chambers or channels fabricated in glass or polymer analytical devices [1,2]. In addition to the potential for automating sample preparation and analysis, scaling down the analytical processes also offers faster total speed and potential savings on reagent costs. With respect to application of these devices for proteomics, the method of detection determines what sample preparation steps are required in the analysis [1]. For proteins, mass spectrometry (MS) is most often utilized for detection because it can provide accurate information for protein identification in a sample as well as provide information about post-translational modifications present. Toward this end, a large number of papers have illustrated integration of mass spectrometry detection directly from the end of the microdevices [3–7]. To exploit the potential capabilities of sample preparation microchips for protein analysis using MS, efforts have been directed toward demonstrating on-chip digestion, and separations that could be integrated with MS detection on a single device [8–14].

With the global proteomic effort gaining momentum, methods that facilitate understanding the post-translational modification (PTM) of proteins become increasingly important. Protein phosphorylation, for example, is known to play an important role in a number of processes including signal transduction [15,16]. Knowledge of the particular residue that is phosphorylated can provide insight into a signaling pathway via an understanding of how the protein's activity is regulated and which enzymes are responsible for the regulation. A number of methods have successfully been developed to enrich phosphopeptides for analysis [17–19], the most amenable of which to microdevices is immobilized metal ion affinity chromatography μ MAC). In IMAC, an immobilized metal (Fe^{3+} or Ga^{3+}) on a solid support (such as Sepharose) can selectively retain and pre-concentrate phosphorylated proteins and peptides; this method has been successfully applied for phosphopeptide enrichment by a number of groups [20–22]. A typical procedure for phosphopeptide enrichment with IMAC incorporates digestion of the protein with trypsin to generate peptide fragments, normally performed overnight.

The present work describes a new application of microdevices for combined digestion of proteins followed by on-line phosphopeptide capture with IMAC on a single glass microdevice. Agarose beads containing immobilized trypsin were packed into one channel, with agarose beads containing ferric ion packed into a subsequent channel. The frit for solid phase (beads) packing was made simply by reducing the diameter of the channel in the microchip. In a similar manner, this microdevice was also used for enrichment of glycopeptides through lectin affinity capture. Both of these methods could be directly integrated with MS detection from the same microdevice through the proper microchip interface.

2. Experimental

2.1. Materials

Trypsin, β -casein, ovalbumin and cytochrome *c*, purchased from Sigma–Aldrich (St. Louis, MO), were each dissolved in 0.1 M NH_4HCO_3 (pH 8.0) at a concentration of 1 mg/mL. Immobilized trypsin beads, agarose beads with immobilized iminodiacetic acid, and agarose beads with immobilized Concanavalin A, were all purchased from Pierce (Rockford, IL).

Acetic acid, FeCl_3 , and sodium phosphate were purchased from Fisher. β -casein phosphopeptide standards, phosphoangiotensin standard, and ammonium bicarbonate were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Microchip fabrication

Microdevices for on-chip protein digestion were fabricated in borofloat glass coated with chrome and photoresist (Nanofilms, Westlake Village, CA) using standard photolithographic techniques and wet chemical etching. Both top and bottom plates were etched, with the top plates etched 30 μm deep while the bottoms were etched 200 μm deep. Reservoirs were fabricated in the top plates using diamond tipped drill bits (Crystalite, Westford, OH), then the plates were bonded together using thermal bonding at a temperature of 675 $^{\circ}\text{C}$. Channels in the top plate extended the entire length from inlet to outlet reservoirs; bottom channels intersected the inlet reservoir, but ended before the outlet reservoir, thus forming a weir to hold the 50–145 μm beads in the channel. Channels were 300 μm wide at the center; channel length varied from 1 to 2.5 cm with 2 cm selected as optimal for these experiments. For integrated devices a center reservoir was included offset from the main channel by 3 mm to allow packing of the second channel and simultaneous flow from both the inlet and center reservoirs.

2.3. Trypsin digestion

Standard trypsin digestions were performed in 0.1 M NH_4HCO_3 (pH 8.0) using a 1:50 enzyme:protein ratio at 37 $^{\circ}\text{C}$ overnight in a water bath. This was used for comparison with microchip digestions, as well as for peptide generation for microchip IMAC studies. Microchip digestions were performed by first washing the trypsin immobilized agarose beads with 0.1 M NH_4HCO_3 three times then packing the beads into the device by filling the inlet reservoir with a suspension of beads and applying vacuum at the outlet reservoir. Beads were packed until they filled the channel, and any remaining beads were removed from the reservoir. Protein solution was then passed through the packed channel using a syringe pump (WPI Sarasota, FL) at a rate between 0.5 and 1 $\mu\text{L}/\text{min}$ until approximately 15 μL was collected from the outlet reservoir. Connection of the syringe pump to the microdevice was through a non-commercial interface utilizing traditional HPLC fittings (Upchurch Scientific, Oak Harbour, WA). Trypsin digestion during electroosmotic driven flow was also performed on these packed devices. A potential of 1000 V was applied between the inlet and outlet reservoirs to induce electroosmotic flow of the protein solution through the packed channel. Solution was collected at the outlet between 20 and 40 min after initiation of the voltage and analyzed by CE.

2.4. Microchip immobilized metal affinity chromatography

Microdevices for IMAC were prepared by washing the agarose beads with immobilized iminodiacetic acid three times with 2% acetic acid. The beads were further washed with 0.1 M FeCl_3 to immobilize Fe^{3+} then rinsed with 2% acetic acid to remove any unbound Fe^{3+} ions. Beads were then packed into the column in the same manner as the trypsin immobilized beads and equilibrated with 0.1% acetic acid. Peptides from standard digests were mixed with acetic acid to decrease the pH to around pH 4–5 for efficient capture before being flowed through the packed channel at 1.0 $\mu\text{L}/\text{min}$ using the syringe pump. Peptides

from single protein digestions as well as mixed peptide samples were evaluated. After sample loading, the microchip packed bed was washed with 0.1% acetic acid to remove non-specifically bound peptides and 50 mM NH_4HCO_3 (pH 8.7) was used to elute the phosphopeptides from the IMAC beads. About 10 μL of elution buffer was collected from the outlet reservoir for analysis by CE separation.

2.5. Integrated microchip digestion/capture

Microdevices for integrated functionality were fabricated and filled in the same manner as the single process devices. These devices contained two adjoining channels separated by a weir, with an offset center reservoir for filling the second channel. Each type of bead was first washed then packed using vacuum at the outlet reservoir, with the trypsin immobilized beads being packed first. Once packing was completed for both channels, β -casein solution, 1 mg/mL in 0.1 M NH_4HCO_3 buffer pH 8.0, was placed into a 19 μL sample loop and injected into the chip at a flow rate of 1.0 $\mu\text{L}/\text{min}$ using the syringe pump. A second syringe pump, attached at the center reservoir, was used to flow 4% acetic acid into the system also at 1.0 $\mu\text{L}/\text{min}$. The acetic acid was required to lower the pH of the effluent from the trypsin digestion column before entering the IMAC column to allow binding of phosphopeptides to the column. After the 19 μL sample was loaded, the IMAC packed channel was washed with 0.1% acetic acid to remove non-specifically bound peptides. Phosphopeptides were then eluted from the IMAC beads in ~ 10 μL of 50 mM NH_4HCO_3 buffer (pH 8.7) and analyzed by CE.

2.6. Peptide analysis

Capillary electrophoretic analyses of the standard protein digestions, and of material collected from the outlet reservoirs of the microchips were performed in an HP 3D CE (Agilent, Walbraun, Germany) Separations were carried out in a bare silica 50 μm I.D. 33 cm capillary (27 cm effective length) using 50 mM phosphate (pH 2.5) as the separation buffer. Sample injections were performed using pressure (0.05 bar) for 3 s followed by separation at 10 or -10 kV. Proteins and peptides were detected using UV absorbance at 214 nm. β -casein phosphopeptide standards and phospho-angiotensin standard were used to spike the phosphopeptides collected from the IMAC for CE analysis. Phosphopeptides eluted from the IMAC microchip were also analyzed by capillary LC/MS. A 1 μL aliquot of the material collected at the outlet reservoir was diluted to 10 μL and ~ 2 –4 μL of diluted sample was loaded on a C18 capillary column. The peptides were eluted into the MS using a gradient elution profile (A: 0.1% acetic acid, B: acetonitrile with 0.1% acetic acid; 100% A decreased to 20% A in 40 min). MS analysis was performed on a ThermoFinnigan LCQ classic.

2.7. Serum analysis

Human serum was diluted 100-fold with 0.1% trifluoroacetic acid (TFA) in water and filtered through a 0.22 μm syringe filter. Five hundred microliters of this solution was injected onto a reverse phase column ($\mu\text{BONDPACK}$ C18, Waters Corp., Milford, MA) and a gradient elution (1 mL/min) of 0 to 100% acetonitrile with 0.8% TFA was performed over the course of 90 min. Analytes were detected by UV absorbance at 214 nm and fractions were collected every 1 min. Using a slot blot method, fractions were analyzed using an

antibody specific for phosphotyrosine, phosphoserine, and phosphothreonine residues. One fraction (23 min into the HPLC run) showed significant binding of the antibody, so this fraction was analyzed on the integrated device to determine the phosphopeptides present. In total, 20 μ L of this serum fraction was flowed through the integrated trypsin/IMAC microdevice as previously described and the eluent from the IMAC support was analyzed by CE with UV detection at 214 nm.

3. Results

3.1. Phosphopeptide enrichment using the IMAC microchip

To test the specificity of IMAC enrichment on a microchip, standard phosphopeptides were mixed with non-phosphopeptides and loaded on IMAC microchips. In the first experiments, 20 μ g of peptides from cytochrome *c* digested with trypsin was mixed with 16 μ g of phospho-angiotensin and loaded on an IMAC microchip. The captured peptides were eluted from the device and the eluent was separated by CE. Electropherogram a in Fig. 1 shows the electrophoretic separation of the cytochrome *c* digest mixed with phospho-angiotensin; CE analysis of the standard phospho-angiotensin (data not shown) indicate that the peak at a migration time of about 10.5 min is the phospho-angiotensin. Electropherogram b of Fig. 1 shows the CE analysis of the eluent run under the same conditions, verifying that only phospho-angiotensin was enriched on the IMAC column from the peptide mixture. In a second set of experiments standard monophosphopeptide (5 μ g) and standard tetraphosphopeptide (5 μ g) from β -casein were mixed with 24 μ g of the trypsin digested cytochrome *c* peptides and loaded on an IMAC microdevice. The captured peptides were eluted and analyzed using CE separation as shown in electropherogram a of Fig. 2. Through the standard addition method, the monophosphopeptide was identified (electropherogram b), but the tetraphosphopeptide was not detected in this separation. Since the tetraphosphopeptide is expected to have a large negative charge, the polarity of the separation was reversed and the peptides eluted from the IMAC column reanalyzed. The reverse polarity CE separation shows a single peak in the eluted sample (electropherogram c), which was identified as the tetraphosphopeptide using the same standard addition method (electropherogram d).

Extraction of phosphopeptides from β -casein using an IMAC microchip was further tested using β -casein digested with trypsin using the standard procedure. The resulting peptide mixture was loaded on an IMAC microchip, and analyzed using CE (Fig. 3). A normal polarity separation showed the presence of two phosphopeptides enriched using the IMAC microchip (electropherogram a); spiking with standard monophosphopeptide (electropherogram b) showed a significant increase in the area of the second peak (~28 min) relative to the first peak. The second peak was therefore identified as the monophosphopeptide peak. The eluted phosphopeptides were also analyzed using a reverse polarity CE separation (data not shown) showing the presence of the tetraphosphopeptide, but also containing an additional peptide peak.

3.2. Integrated protein digestion and IMAC enrichment on a microchip

To obtain the best digestion with syringe pump driven flow through the immobilized trypsin column, the digestion of protein at different flow rates is compared in Fig. 4. These results were used to select a flow rate of 1 $\mu\text{L}/\text{min}$ for use with the integrated device. The problem with the integrated protein digestion and IMAC microchip is the different pH buffer solution required for trypsin digestion and IMAC enrichment. Thus, when protein was flowed through the trypsin beads to the IMAC beads, 4% acetic acid was pumped in the side inlet (Fig. 5) to the IMAC microchannel at the same time to adjust the pH of the digested protein solution from pH 8.0 to pH 4–5. During elution, 50mM NH_4HCO_3 (pH 8.8–9.0) was flowed through the side inlet, but since the IMAC enrichment microchannel was shorter than the trypsin digestion channel and the syringe was not removed from the inlet of the trypsin channel, the 50 mM NH_4HCO_3 only flowed through the IMAC channel due to the lower back pressure in this direction. Fig. 6 is the CE separation for the elution from the integrated device, with trace a showing the normal polarity separation. Three major peaks are evident, however, the monophosphopeptide peak was actually identified as the minor peak near the end of the trace by spiking with the monophosphopeptide standard. This separation is shown in trace b. Trace c shows the reverse polarity separation with the large area peak identified as the tetraphosphopeptide by standard addition (trace d), but an additional peak is also present in these separations. To further confirm the presence of the phosphopeptides, the elution from the integrated chip was analyzed with capillary LC/MS, which gave the total ion chromatogram and selected ion chromatograms shown in Fig. 7. The largest peak was identified as a 25 amino acid tetraphosphopeptide, but interestingly enough, not the fully digested product which contains only 19 amino acids. The additional peaks in the chromatogram represent the fully digested tetraphosphopeptide along with the monophosphopeptide fragments as indicated by the database search results listed in Table 1.

To determine the utility of this method for a more complicated sample, serum was fractionated on a reverse phase column and the fractions probed using a generic phosphorylation antibody. A fraction with phosphoproteins was identified and an aliquot passed through the integrated microdevice. A fraction was collected after the trypsin digestion as well as an elution fraction. Fig. 8 shows the CE separations of these two fractions.

4. Discussion

Mass spectrometry is the preferred detection method in proteomics because of the sensitivity and the ability to identify specific proteins present in a sample. Normal sample processing for MS detection requires trypsin digestion of sample proteins, with affinity purifications sometimes utilized to identify specific peptide fragments, such as those with post-translational modifications. Microdevices have been utilized for both interfacing with the MS detection, and for the trypsin digestion part of sample preparation, but incorporation of an affinity capture step with the digestion had not been reported.

4.1. Phosphopeptide and glycopeptide enrichment on an IMAC microchip

The results with standards, both phospho-angiotensin and β -casein fragments, clearly show the utility of this method to selectively enrich phosphorylated peptides from tryptic digests. Figs. 1 and 2 illustrate the selectivity of the process, with very little non-specific adsorption of the cytochrome *c* peptides under the conditions used. Use of the β -casein fragment standards provided some concern on first analysis, as the CE separation showed elution of only a single fragment, which was identified as the monophosphopeptide. Standard addition of the tetraphosphopeptide to the elution fraction showed no additional peaks, however, indicating that the large negative charge due to the four phosphate groups was actually reversing the electrophoretic migration of this fragment. The subsequent reverse polarity separation shown in Fig. 2 illustrates that the tetraphosphopeptide was also retained then eluted from the IMAC microdevice. β -casein has five serine phosphorylation sites with complete trypsin digestion producing the monophosphopeptide (amino acids 48–63) and tetraphosphopeptide (amino acids 16–40) utilized as standards [20]. As shown in Fig. 3, two peaks were seen in the normal polarity separation of the microchip IMAC eluent of trypsin digested β -casein. The other peak could be the result of incomplete digestion, or indicate that another type of casein was present in the sample; there are α -s1, α -s2, β , and γ caseins, all of which have different phosphorylation sites [20]. The efficiency of the IMAC chip was investigated but not determined in this study due to analysis of the eluent by CE separations. The UV absorbance detection method did not provide sufficient sensitivity for efficiency measurements.

This microdevice can also be used for glycoprotein or glycopeptide enrichment by replacing the IMAC beads with beads containing immobilized concanavalin A (Con A) or other lectins. In this work, Con A beads were packed into the microchannel and loaded with a mixture of ovalbumin, a glycoprotein, with lysozyme, bovine serum albumin, cytochrome *c* and β -casein. Elution from the column using methyl α -D-mannopyranoside was followed by CE separation that showed only a single peak identified as ovalbumin (data not shown). Testing of multiple devices showed equivalent results, illustrating the ability to easily create a general affinity capture technique for proteomic analysis on a microdevice.

4.2. Integrated protein digestion and IMAC enrichment microchip

To integrate protein digestion on-line with phosphopeptide enrichment on one microchip, two methods for sample movement through the protein digestion channel were evaluated: pressure-induced flow and EOF [23]. Sample loading by EOF is easy to integrate with other microchip processes; however, a shortcoming of the EOF method is buffer limitation, as a high concentration of salt decreases the flow rate. In addition, integration of this microchip directly with MS detection is an eventual goal, and decoupling of the electrospray voltage from the EOF voltage can be complicated. Syringe pump flow was therefore selected for this device, which proved to allow better control of flow through the two inlets during integrated processing. For syringe pump flow, because it is easily controlled, the effect of flow rate on digestion was evaluated. Fig. 4 shows the effect of changing the flow rate through the immobilized trypsin bed. The protein digestion efficiency is based on the reaction time between protein and enzyme, thus lower flow rates provide higher efficiency as seen in Fig. 4 by the disappearance of the intact protein peak. The flow rate chosen for the integrated

studies, 1 $\mu\text{L}/\text{min}$, was not optimal, but three other factors play a role. The amount of protein loaded for CE detection is much greater than will be needed for direct MS detection from the chip, thus the digestion should be faster. The analysis time will depend on sample size and flow rate, thus increasing the flow rate provides a faster analysis. At the same time, however, because flow through the IMAC column during loading is twice as fast due to the need to reduce the pH of the solution, too fast of a rate will not provide time for the phosphopeptide capture step. For all cases, the incomplete digestion is also due to the limited amount of immobilized trypsin in the channel due to the large diameter (50–145 μm) of the Sepharose bead packing. Use of smaller diameter particles or immobilization of the trypsin onto a sol-gel matrix formed within the channel would both provide more efficient digestion in the same channel length.

The results of the integrated digestion/phosphopeptide capture of β -casein (Fig. 6) show four separated phosphopeptides in the normal polarity CE separation. Through standard addition, the smallest peak at the end of the electropherogram was identified as the monophosphopeptide and the peak in the reverse polarity CE analysis is tetraphosphopeptide. The number of other peaks and their size suggests that digestion was not complete on the microdevice. In comparison with Fig. 3, which showed significant digestion to the monophosphopeptide when the digestion was performed off-line, the on-line digestion showed very little fully digested monophosphopeptide product. To further confirm the assumption of incomplete digestion, cLC/MS was used to analyze the elution buffer from the integrated trypsin and IMAC microchip (Fig. 7).

The TIC and the selected ion chromatograms of the elution buffer shown in Fig. 7, indicate that the highest intensity peak represents tetraphosphopeptide intensity. This is not unexpected as the tetraphosphopeptides have four phosphate groups, which can interact with the IMAC column, providing a higher binding affinity than that for the monophosphopeptide, which contains only a single phosphate group. The tetraphosphopeptide fragments also contain additional carbonyl groups, which are known to interact with the immobilized Fe^{3+} groups. One issue is that the MS results in Fig. 7 indicate that the most significant ion peaks arise from a fragment that is not fully digested by the trypsin, but the reverse polarity separation in Fig. 6 indicates that the largest peak co-migrates with the tetraphosphate standard. A number of explanations are possible for this discrepancy. The tetraphosphate standard could contain the longer 25 amino acid fragments, the electrophoretic mobilities of the fully digested and the longer fragments could be the same since they will be highly influenced by the charge on the phosphate group. It is also known that the MS sensitivity of peptides is poorer if phosphate groups are present, thus the longer fragment may be more easily ionized resulting in the higher MS signal. As for the significantly lower ion intensity of the monophosphopeptide seen in Fig. 7, in addition to the higher affinity of the IMAC for the tetraphosphopeptides, Table 1 shows at least five different size monophosphopeptides present. This corroborates the multiple peaks seen in the normal polarity separation in Fig. 6, which shows at least four peaks in the electropherogram.

The reproducibility of this method with standard samples was evaluated using multiple microdevices and repeat digestion/capture on single devices. The trypsin bed was washed

with water between repeat experiments and the IMAC bed was cleaned using the manufacturer's instructions or by treatment with a high salt buffer before being reloaded with Fe^{3+} for a subsequent extraction. Blank control experiments showed no captured peptides, and digested standards showed the expected peaks in over 50 experiments. This method was therefore tested with a more complex sample to determine the issues to be addressed in subsequent generations of the device. Application of this method to a serum fraction, indicated that additional work will be required to make this a viable method for proteomics. As seen in Fig. 8, there was a definite change in the electropherograms before and after the IMAC purification. Both separations show mostly unresolved peptides except for the single peak, which is significantly reduced following IMAC. This is not completely unexpected. The serum sample, though a single fraction from a reverse phase separation, still is expected to have a large number of proteins, with trypsin digestion giving an even larger number of peptides. In addition, it was already shown that digestion is not complete in the trypsin column, and the amount of protein in the fraction may have further overloaded the digestion column. This would also explain the lack of specific peaks in the eluent from the IMAC column.

Analysis of the eluent using cLC/MS indicated the presence of a non-phosphopeptide component, suggesting non-specific capture of additional material by the IMAC. It has been shown that carboxylic acids can also bind to the Fe^{3+} ions on the IMAC column, and two methods have been reported to decrease this non-specific binding. Ficarro et al. [22] have reported an improvement for sample pre-treatment, along with modified loading and elution buffers to decrease the non-specificity of the IMAC enrichment. Replacement of the Fe^{3+} ion with a Ga^{3+} ion has also been shown to be more selective for the phosphate moiety in IMAC experiments [21]. Further work has shown that TiO_2 might also provide a more selective capture agent for phosphopeptides that might be utilized in these devices [24].

Compared with the traditional methods for off-line trypsin digestion and IMAC phosphopeptide purification, the integrated microdevice method is significantly faster, and should be able to achieve better sensitivity if MS detection is employed on-line. To utilize this method with real samples, a number of improvements will be required including increasing the capacity of both the trypsin digestion and the IMAC capture beds by replacing the large diameter beads with smaller beads or monolithic matrices such as sol-gels. The specificity of the IMAC will have to be improved, and the flow rates through both beds will have to be optimized using protein concentrations suited for MS detection rather than UV detection. In the end, direct interfacing of these microchip sample preparation steps with MS detection, possibly through an on-chip desalting column, should provide rapid throughput with good sensitivity and selectivity.

5. Conclusion

Phosphopeptide capture from standard solutions and protein digests has been achieved on a simple microdevice using IMAC beads packed into a microfluidic channel. Replacement of the IMAC beads with immobilized lectin beads allowed capture of a glycoprotein in this same device. The phosphopeptide capture method has been integrated with on-chip trypsin digestion, requiring additional flow through a side inlet to decrease the pH of the digestion

solution for effective binding to the IMAC beads. Using β -casein, the integrated device showed selective enrichment of the two expected phosphopeptide fragments along with four additional fragments, all of which were shown to be phosphorylated using MS analysis. The additional fragments indicate incomplete digestion, indicating the digestion capacity of the device was insufficient as it is currently utilized. Flow rate investigations were performed, but additional work will be required once this microdevice is fully developed for direct MS detection. Evaluation of the method using a complex sample showed non-specific binding which can be affected by changes to the IMAC column or additional sample treatment.

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Fig. 1.

CE separation of (a) 20 μ g cytochrome *c* trypsin digestion peptides mixed with 16 μ g phospho-angiotensin; (b) the elution buffer after loading the mixture on an IMAC microchip. CE conditions: 33 cm 50 μ m I.D. bare silica capillary, 50 mM phosphate buffer pH 2.5, 3 s pressure (0.05 bar) injection, 10 kV separation voltage, 214 nm absorbance detection.

Fig. 2.

Mono (5 μ g) and tetraphosphopeptide (5 μ g) standards were mixed with cytochrome *c* peptides (20 μ g) and loaded on an IMAC microchip; the eluent was analyzed by CE using both normal polarity (top; +10kV) (a) IMAC eluent, (b) IMAC eluent with added monophosphopeptide; and reverse polarity (bottom; -10kV) separations, (c) IMAC eluent, (d) IMAC eluent with added tetraphosphopeptide. Other CE conditions as in Fig. 1.

Fig. 3.
 β -casein was digested with trypsin and loaded on an IMAC microchip. The eluent was analyzed using normal polarity CE separations: (a) eluent, (b) eluent spiked with monophosphopeptide. CE conditions as in Fig. 1.

Fig. 4. CE separation of β -casein (1 mg/mL) digested in the microchip using different flow rates through the device: (a) 0.5 $\mu\text{L}/\text{min}$, (b) 1 $\mu\text{L}/\text{min}$, (c) 5 $\mu\text{L}/\text{min}$, (d) 10 $\mu\text{L}/\text{min}$. CE conditions as in Fig. 1; arrow indicates undigested protein peak.

Fig. 5. Diagram of the integrated trypsin digestion and affinity capture process along with a picture of the actual microdevice.

Fig. 6.

CE separations of eluent from the integrated digestion and capture of β -casein (1 mg/mL) on the microdevice. Top panel shows the normal polarity (+10 kV) separation of (a) eluent, (b) eluent spiked with monophosphopeptide; bottom panel shows the reverse polarity (-10kV) separations of (c) eluent, (d) eluent spiked with tetraphosphopeptide. CE conditions as in Fig. 1.

Fig. 7. cLC/MS results for the collected eluent from the integrated microchip: (a) total ion chromatogram for the eluent, (b) selected ion chromatogram for one tetraphosphopeptide fragment, (c) selected ion chromatogram for one of the monophosphopeptide fragments.

Fig. 8. CE separations of the serum fraction before digestion, after digestion on the microchip, and the eluent after digestion and IMAC capture and elution. CE conditions same as in Fig. 1.

Table 1Database search results for the identified phosphopeptides of β -casein

Sequences	M_w
RELEELNVPGEIVES*LS*S*S*EESITR	3124
IEKFQS*EEQQQTEDELQDK	2433
FQS*EEQQQTEDELQDK	2063
KIEKFQS*EEQQQTEDELQDK	2561
NVPGEIVES*LS*S*S*EESITR	2354
FQS*EEQQQTEDELQDKIHHPFAQTQSL	3186
FQS*EEQQQTEDELQDKIHHPFAQTQ	2986