

The Proteomic Reactor: A Microfluidic Device for Processing Minute Amounts of Protein Prior to Mass Spectrometry Analysis

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Received June 26, 2006

Gel-free proteomics has emerged as a complement to conventional gel-based proteomics. Gel-free approaches focus on peptide or protein fractionation, but they do not address the efficiency of protein processing. We report the development of a microfluidic proteomic reactor that greatly simplifies the processing of complex proteomic samples by combining multiple proteomic steps. Rapid extraction and enrichment of proteins from complex proteomic samples or directly from cells are readily performed on the reactor. Furthermore, chemical and enzymatic treatments of proteins are performed in 50 nL effective volume, which results in an increased number of generated peptides. The products are compatible with mass spectrometry. We demonstrated that the proteomic reactor is at least 10 times more sensitive than current gel-free methodologies with one protein identified per 440 pg of protein lysate injected on the reactor. Furthermore, as little as 300 cells can be directly introduced on the proteomic reactor and analyzed by mass spectrometry.

Keywords: strong cation exchange • reduction • alkylation • proteolytic digestion • online • cell lysis • integrated approach

Introduction

Different proteomic approaches have been developed over the years that commonly require the processing of proteins into peptides before they are analyzed by mass spectrometry. In gel-based experiments, such as the well established 2D gel electrophoresis approach,¹ the separated proteins are difficult to extract from the polyacrylamide gel.² Instead, proteins are enzymatically cleaved into peptides in the gel³ before they can be extracted and analyzed. Unfortunately, protein/peptide loss and contamination are hard to avoid with 2D gel electrophoresis,⁴ and the proteins are digested in a large volume of solution (typically 25 μ L).

Gel-free proteomics has been proposed as a complementary approach to 2D gel electrophoresis.⁵ These gel-free approaches rely on either solution based protein separations such as size exclusion,⁶ reverse-phase liquid chromatography,⁷ free-flow electrophoresis (FFE)^{8,9} and 2D chromatography,¹⁰ or peptide separations.^{11–14} In these approaches, the proteins are digested in a large volume of solution either before or after separation. Even though gel-free approaches bypass the need for gels, they do not address the fundamental issue of efficiency of protein processing.

Various alternatives to in-solution digestion have been proposed, but they have notable limitations. For example, two groups have reported approaches for processing proteins using immobilized trypsin in monolithic columns followed by peptide fractionation,^{15,16} Unfortunately, no protein preconcentration is associated with these approaches. This means that the samples must have low amounts of contaminants and high levels of proteins to perform well. Another group reported protein separation coupled to immobilized trypsin^{17,18} In these studies, protein separation is performed at the same time as the digestion followed by mass spectrometry. However, peptide fractionation is not performed in this approach, which drastically limits the number of proteins that can be handled by the systems. Craft et al.^{19,20} digested proteins by absorbing them on a hydrophobic support, introducing trypsin, gradually eluting them, and then analyzing the resulting peptides by mass spectrometry. Unfortunately, this technique required a high amount of sample (200 ng per protein) and was only successful for standard protein solutions that were contaminant free. In general, these approaches require further development before being readily amenable to real biological samples.

Our current study presents the development of a single microfluidic device, termed the *proteomic reactor*. The proteomic reactor not only rapidly extracts proteins directly from cells, but it can also preconcentrate, clean up, derivatize, and digest proteins. Furthermore, by combining the proteomic reactor with simple HPLC–ESI–MS/MS, proteomic samples can be rapidly analyzed (Figure 1a). Briefly, the proteomic reactor contains a small bed of packed strong cation exchange

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a)

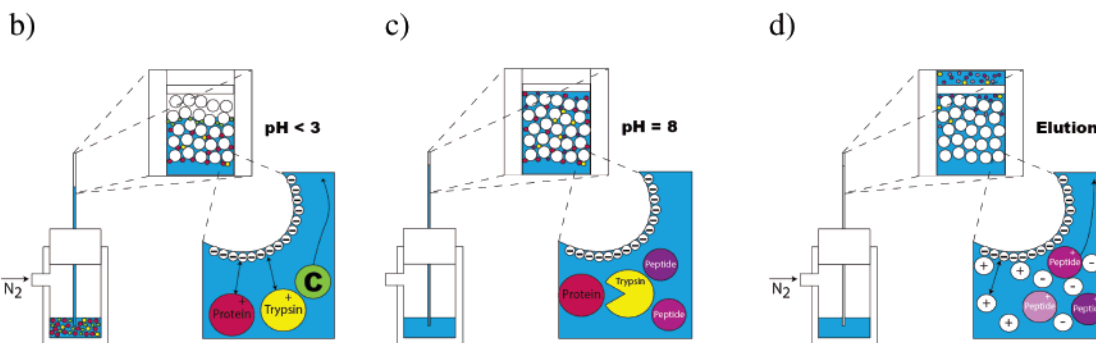
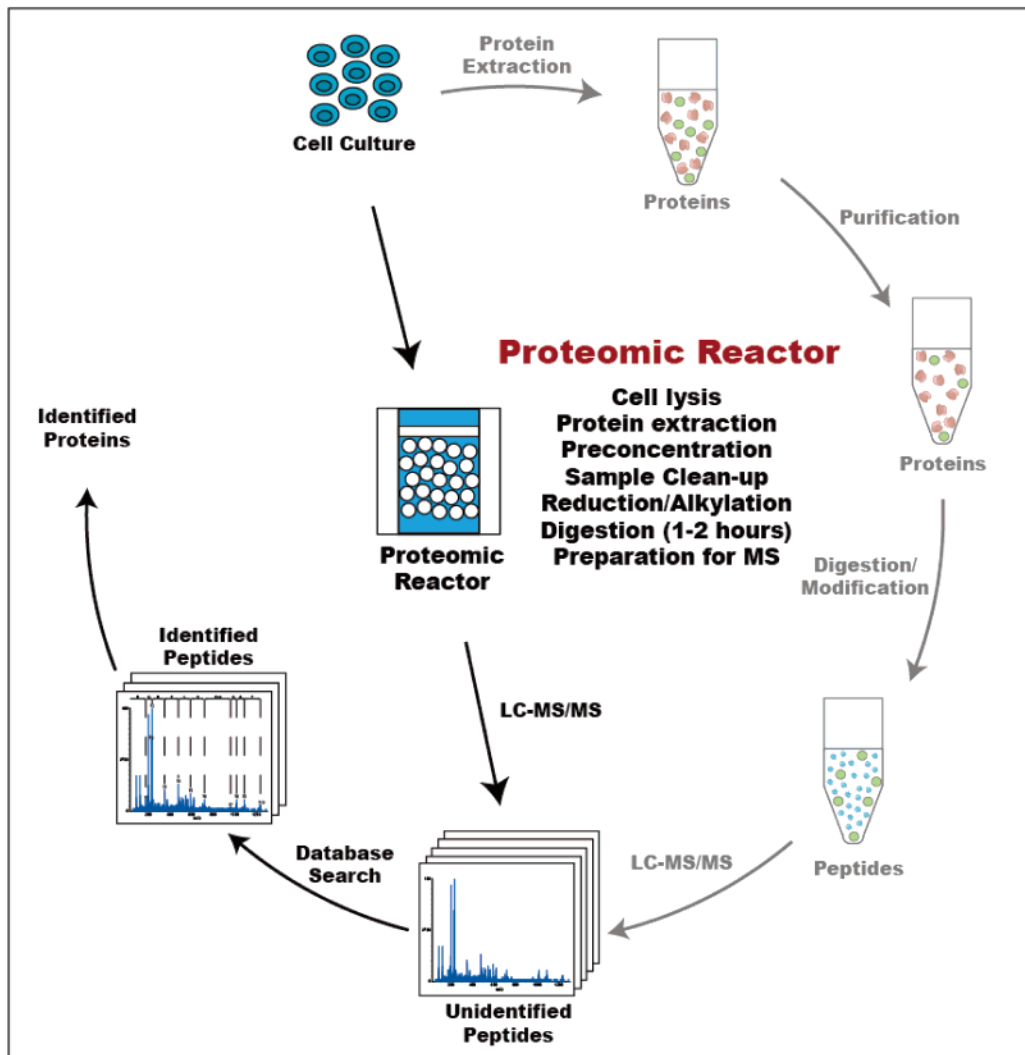


Figure 1. (a) Sample treatment steps done using the proteomic reactor. (b) Schematic representation of the proteomic reactor. Pressurized nitrogen is used to push the liquid into the reactor. The protein and trypsin are bound to the SCX. (c) Trypsin is activated by adjusting the pH at 8. The flow is stopped to let the digestion proceed without losing peptides. (d) Peptides are eluted by using an ammonium bicarbonate solution.

material (SCX). The proteomic samples (either proteins or cells) are lysed and/or loaded onto the reactor at a low pH (Figure 1b). Because the vast majority of proteins have a pI higher than the pH condition in the reactor, they will be positively charged and absorbed onto the reactor material. The nonionic deter-

gents are readily washed away while the proteins bind to the SCX material. Proteins can then be derivatized by adding the reagents to the reactors. After this, proteins are digested by increasing the pH, which in turn, will activate the trypsin loaded onto the reactor with the protein sample (Figure 1c). The

products of the reaction, typically peptides, are then eluted using a buffer readily compatible with HPLC–ESI–MS/MS (Figure 1d).

The first advantage of the proteomic reactor is that it extracts and concentrates proteins from complex mixtures using approximately 1 μL of packed SCX material. The strong affinity of proteins toward the SCX material at low pH enables minute amounts of proteins to be easily extracted from mid to high μL solution volumes. Furthermore, the reactor's affinity for proteins is enhanced when the pH and salt levels are modulated. The change in pH and salt levels cause proteins to undergo a transition from a solid state on the SCX material to a free solution state. This transition from a solid to a solution state is advantageous for capturing and processing proteins on the reactor. During sample loading, the pH is low and the proteins bind to the SCX material on the reactor. During digestion/alkylation, the pH and salt contents are higher, therefore, the proteins translate to a solution phase. The interstitial volume is limited to approximately 50 nL, which indicates that the overall preconcentration effect ranges from 20 to 2000-folds if 1–100 μL of starting material is used.

Experimental Section

Cell Culture. Mouse P19 testicular cancer cells were cultured at 37 °C in 5% CO₂ in DMEM medium (GIBCO–BRL, Burlington, ON) supplemented with 10% fetal calf serum. Cells were harvested and washed twice with phosphate buffered saline (PBS). For online cell lysis with the reactor, cells were counted and suspended in an appropriate amount of PBS to obtain the desired amount of cells. Offline cell lysis was performed using a modified RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na deoxycholate, 1 mM EDTA). Collected cells were placed on ice with RIPA buffer for 10 min, and cell lysates were centrifuged for 10 min at 14 000 rpm to pellet cell debris. Supernatants were collected, and the protein concentration was assessed using a Bradford protein assay kit (Bio-Rad, Hercules, CA). Cell culture density was evaluated prior to harvesting.

On the basis of the cell density of the samples and their corresponding Bradford assay results, we evaluated how many cells were needed to provide a given amount of protein. The independent results from the cell density and Bradford assay analysis of three 100-mm plates gave an average of 1 μg for 3100 \pm 400 of cells.

In-Solution Digestion. The in-solution digestion of P19 cell lysate was performed as follows. The RIPA buffer was exchanged using a centricon YM-3 filter (Millipore, Billerica, MA) and washed three times with 20 mM TRIS, 150 mM NaCl pH 7.4 buffer. The protein concentration was then determined by Bradford assay. Three aliquots of P19 lysate corresponding to 10 μg of total protein were reduced, alkylated, and digested by the addition of trypsin. BSA was used as a positive control for the digestion. Digestion was performed in a total of 100 μL of solution. The solvent was then evaporated and the sample was reconstituted in a final volume of 40 μL of 200 mM ammonium bicarbonate and 4 μL of 50% formic acid, which is the same solvent used for the reactor sample. This solution (5 μL) was injected in the mass spectrometer.

Reactor Assembly. The reactor was assembled by connecting a 200 μm inner diameter capillary tubing into an inline micro-filter (UpChurch Scientific, Oak Harbor, WA) which was used as a frit. All tubing was washed with 500 μL of 10 mM potassium phosphate buffer (pH 3) prior to digestion. A slurry of SCX

material (12 μm beads; The Nest Group, Southboro, MA) was then introduced from the other end of the capillary tubing by applying 200 PSI of nitrogen. Four centimeters of capillary tubing was packed creating a protein binding capacity of 12 μg . Each reactor was then equilibrated by passing 50 μL of 10 mM potassium phosphate buffer (pH 3).

Reactor Digestion. To ensure that no cross-contamination occurs, a fresh proteomic reactor was used for each analysis. Different aliquots of P19 samples (0.1–10 μg of total protein) were acidified prior to the proteomic reactor digestion by diluting them in a ratio of 1:9 in 50mM H₃PO₄. Trypsin (Promega, Madison, WI) (0.5 $\mu\text{g}/\mu\text{L}$ in 10 mM potassium phosphate buffer (pH 3)) was added to the sample in a ratio of 1:5. The samples were loaded on the reactor using a pressure of 200 PSI producing an approximate flow rate of 10 $\mu\text{L}/\text{min}$. Next, the reactor was washed three times with 20 μL of wash buffer (8mM potassium phosphate buffer, 20%(v/v) acetonitrile) and then washed further with 25 μL of deionized water to remove the wash buffer. Once washed, the reactor was dried using nitrogen gas for 30–60 s. The proteins that were attached to the reactor were reduced using a solution of 100 mM DTT (dithiothreitol) and 10 mM ammonium bicarbonate for 30 min. The reactor was filled with enough solution to only hydrate the beads, at which point the pressure was dropped to atmospheric pressure and the reaction was allowed to proceed for 30 min. The pressure is dropped when a droplet form at the end of the reactor which indicates that the reactor is filled. The reactor was then dried and washed with 4 μL of 10 mM potassium phosphate buffer to decrease the pH to 3 and quench the reaction. The alkylation and the activation of trypsin were done concomitantly by introducing a digestion/alkylation solution (100 mM Tris-HCl pH 8, 10mM Iodoacetamide) into the reactor. The reactions proceeded at atmospheric pressure and at room temperature for 2 h. Peptides were then eluted using 40 μL of 200 mM ammonium bicarbonate.

Online Cell Lysis. A fritted fused silica tube of 75 μm ID was connected upstream on each reactor. A known amount of cells was placed in suspension using 20 μL of PBS and 5 μL of 1 M H₃PO₄ and introduced onto the frit. Two μg of trypsin was added to 40 μL of modified RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, and 1 mM EDTA), which was acidified using 4 μL of 1 M H₃PO₄ and then introduced on the reactor. Phosphoric acid was also added to ensure that the proteins bound to the reactor following the online cell lysis. The cells and frit were then removed, and the rest of the digestion was performed as mentioned above.

Chromatography–Mass Spectrometry. Samples from the reactor were acidified using 4 μL of 50% formic acid. For the LCQ DecaXP instrument (Thermo-Finnigan, San Jose, CA), 5 μL of each sample was injected on a 4 cm \times 75 μm ID precolumn packed with 5 μm C18 beads (Waters, Milford, MA) using a micro flow HPLC 1100 system (Agilent, Palo Alto, CA). The peptides were then separated using a 2-h linear gradient (Solvent A: Water with 0.1% formic acid, Solvent B: Acetonitrile with 0.1% formic acid (J. T. Baker, Phillipsburg, NJ)) on a 5 cm \times 75 μm ID column packed with 5 μm C18 beads in a 10 μm picotip emitter (New Objective, Woburn, MA). The flow rate through the column was approximately 200 nL/min. One MS was acquired, and the top three peaks were further analyzed by MS/MS. A peak could be sequenced a maximum of three times before being excluded for 3 min. The online cell lysis analyses were performed on an LTQ (Thermo-Finnigan, San

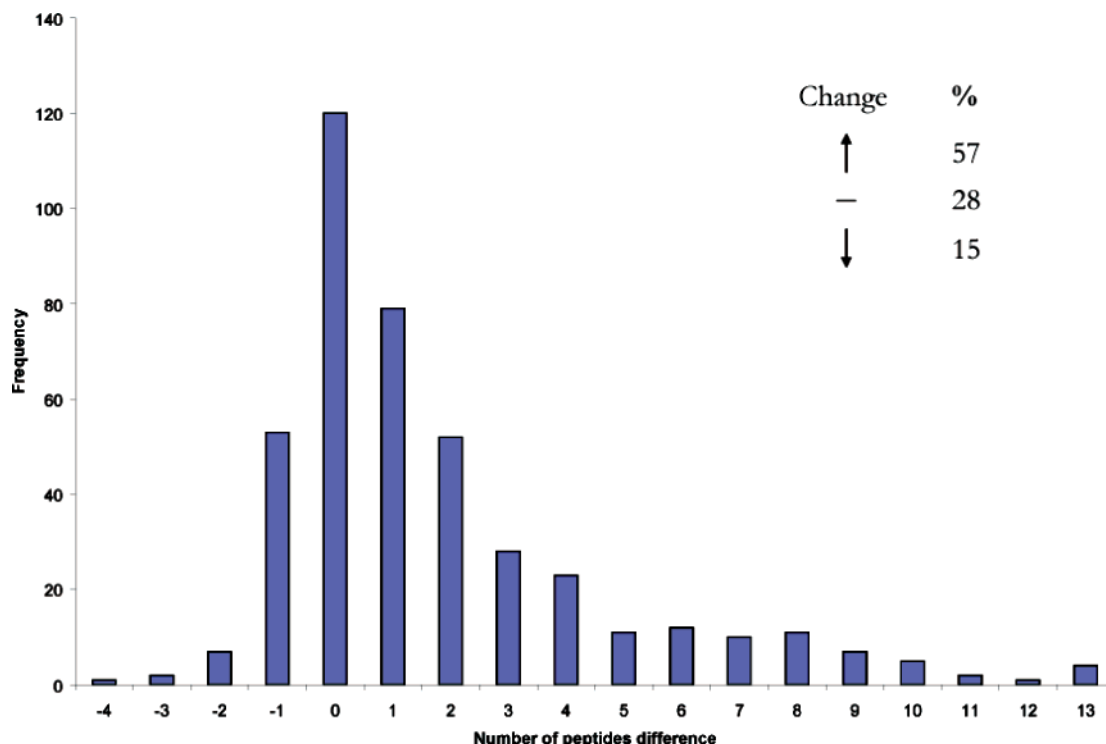


Figure 2. Chemical reaction performed using the proteomic reactor. Effect of DDT reduction and iodoacetamide alkylation on the identification of proteins from mouse P19 total cell lysate. The change in number of identified peptides per protein was computed. A positive difference indicates an increase in the number of peptides identified when both reduction and alkylation are performed. The percentages represent the proportion of proteins having, respectively, an increase, the same, or a decrease in number of peptides identified. Analyses were done in duplicate.

Jose, CA) in a similar fashion, except that the total amount of the sample was injected on a 4 cm × 200 μm ID precolumn using a linear gradient over 1 h.

Data Analysis. The MS/MS spectra were analyzed using Mascot (Matrix Science, Boston, MA) using the following parameters: oxidation(M) as an optional modification, a maximum of 2 tryptic miscleavages, a peptide tolerance of 2 Da and a MS/MS tolerance of 0.8 Da, *mus* taxonomy and 2+ and 3+ precursor ions only. Only the proteins with at least one new peptide were kept (Require Bold Red option in Mascot). A Mascot peptide score threshold of 15 was used for the peptide scores on the LCQ, and a score of 30 for the LTQ based on the manual observation of results.

Results and Discussion

We first established the maximum protein binding capacity of the proteomic reactor. Aliquots (5 μg) of bovine serum albumin (BSA) were successively loaded on the proteomic reactor. The flow-through solutions were collected after each injection, and the presence of protein was assessed by SDS-PAGE followed by coomassie-blue staining. The presence of protein in the flow-through was only observed from the third aliquot onward. Thus, the binding capacity of the reactor in the present format is between 10 and 15 μg of protein. Furthermore, multiple washes of the reactor were performed and no noticeable proteins were observed in the flow-through. This observation indicates that protein interaction with the SCX material is not affected by the wash conditions when proper pH and salt contents are maintained. Two elutions were performed to verify the elution of protein from the reactor.

Most of the proteins were eluted in the first elution, but some were present in the second elution. A final stringent elution indicated that some proteins remained attached to the beads. The incomplete elution of protein is acceptable to retain the compatibility of the elution buffer with mass spectrometry. For this reason and the fact that SCX resin is highly cost-effective (~\$0.02/reactor), a new reactor was used for each analysis.

The second advantage of the reactor is that chemical reactions can be performed directly on the proteins concentrated on the reactor. For example, we performed reduction and alkylation of cysteins for a complex protein mixture (P19 cell lysate) loaded on the reactor. Briefly, protein processing was tested under two conditions: first, one protein aliquot underwent a reduction/alkylation reaction on the reactor prior to its digestion; and second, another protein aliquot was directly digested on the reactor. The resulting peptides were analyzed using the LCQ mass spectrometer as described in the Experimental Section. The experiments were repeated twice. The resulting data were compared for sequence coverage of the identified proteins. Furthermore, an in-house software was used to track individual peptides and to verify their protein annotation from sample to sample. Ninety-four proteins were observed in all of the sample with different sequence coverage. The difference in the number of peptides identified in both conditions for each aligned protein was calculated (Figure 2). Overall, 57% of the identified proteins had an increased sequence coverage when reduction/alkylation was performed on the proteomic reactor (i.e., there was an increase in the number of identified peptides per protein). This clearly indicates that chemical reactions can be performed on minute

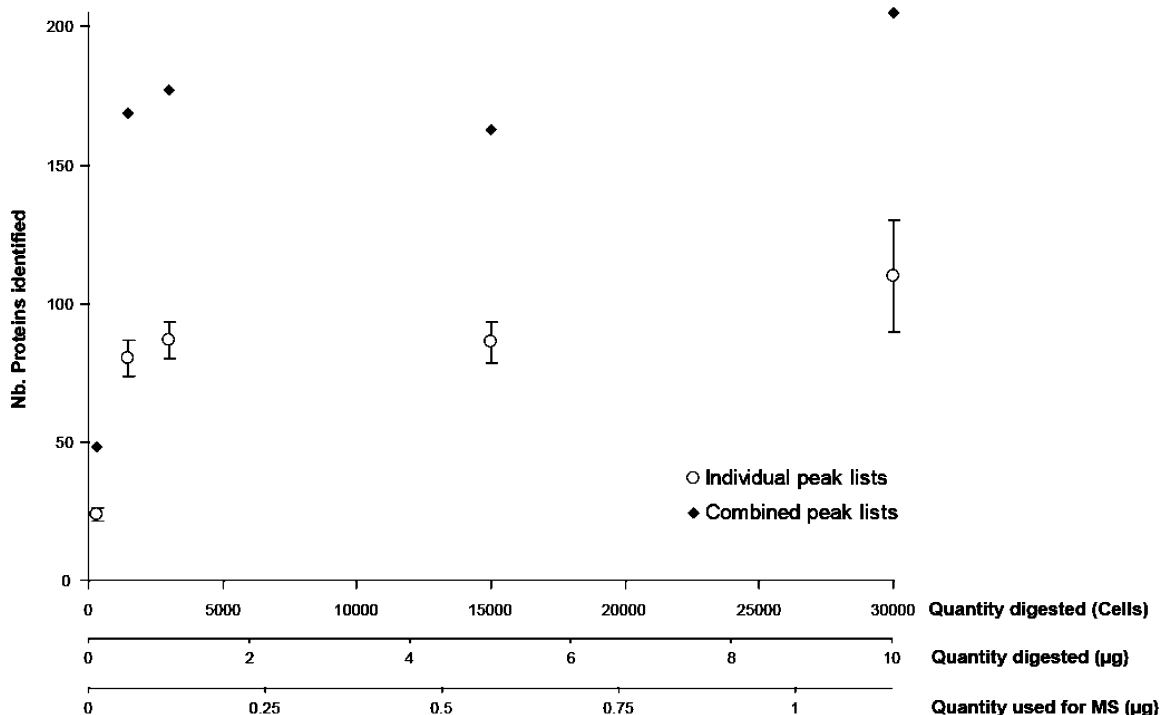


Figure 3. Evaluation of the performance of the proteomic reactors for the processing of proteomic samples. The sample used is total cell lysate from mouse P19 cells. The circles represent the average of triplicate digestion for each protein quantities on reactor of 40 mm × 200 µm packed with 12 µm SCX beads. Error bars correspond to one standard deviation. The different x axes represent respectively the number of cells corresponding to the protein content used in the digestion, (determined by Bradford assay) and the quantity used for mass spectrometry. ♦ represents the results when the peak lists from the three replicates were merged prior to the Mascot search.

amounts of proteins on the reactor at a low pH in a solid-state like reaction or at a higher pH in a solution state in a nanoliter volume.

The third advantage of the proteomic reactor is that enzymatic catalysis can be performed on the reactor and benefit from an enhanced concentration effect. The benefits of doing protein digestion on the reactor was demonstrated using different levels of protein lysate loaded on the reactor and analyzing the peptides derived from the digestion by HPLC-ESI-MS/MS. Briefly, five different amounts of protein from 0.1 to 10 µg of total protein lysate from P19 were processed in triplicate on the reactor and analyzed by HPLC-ESI-MS/MS. This effectively corresponds to the amount of proteins that would be obtained from 300 to 30 000 cells as evaluated from a Bradford assay and cell counts. Figure 3 shows the average number of proteins identified and the total number of unique proteins found according to the amount of protein digested. Proteins were detected in all the samples, but no significant gain was observed when more than 0.5 µg of protein was loaded. In practice, only 11% of the total volume of digest from each sample was injected on the HPLC-ESI-MS/MS. This means that of the 0.1 µg of protein loaded and processed on the reactor, only 11 ng were analyzed on the HPLC-ESI-MS/MS. An average of 25 proteins were identified when 11 ng of protein digest from the reactor was injected on the HPLC-ESI-MS/MS. This suggests that an average of 440 pg of protein lysate is required for every identification obtained. Figure 3 shows that the reactor works better when the saturation point of the binding capacity is attained (10 µg); however, even at 0.5 µg, 75% of the identifiable proteins can still be detected. Although we did not change the size of the proteomic reactor according to protein content, it is possible to make shorter or longer proteomic reactors to accommodate different levels of proteins.

Table 1. Number of Unique Proteins Identified When Performing Online Lysis of Cells on the Proteomic Reactor^a

| number of cells digested | number of unique proteins detected |
|--------------------------|------------------------------------|
| 300 | 17 |
| 3000 | 57 |
| 30 000 | 251 |

^a The analyses were performed two times, and peak lists were combined before their submission to Mascot.

The reactor's performance for digesting proteins was compared to an in-solution digestion using the same amount of protein (10 µg). Briefly, 10 µg of P19 cell lysate was digested in-solution and on the reactor to obtain, in both cases, 44 µL of peptide solution. The resulting peptides for each method were then analyzed by HPLC-ESI-MS/MS as described in the Experimental Section. The in-solution digestions were performed three times and 22 unique proteins were identified. In comparison, the reactor digestions identified 205 unique proteins when performed three times. Of the 22 proteins identified in the in-solution digestion, 21 were also identified in the proteomic reactor digestion. The improved performance of the reactor is due to (1) the efficient concentration of the samples and the buffer exchange prior to digestion, and (2) the efficient digestion of the sample in nanoliter volumes.

The integrative aspects of the proteomic reactor also allow cells to be directly lysed online. For example, we introduced different number of mouse P19 cells (300, 3000, and 30 000 cells) directly into the proteomic reactor. The cells were then rinsed and lysed directly onto the reactor where the proteins were absorbed and processed as described above. Table 1 shows the results obtained using an LTQ mass spectrometer. More proteins were identified as the amount of cells introduced

on the reactor increased, but as much as 17 proteins were identified from only 300 cells.

Conclusion

The microfluidic proteomic reactor provides an integrated platform to efficiently and reliably extract, purify, and concentrate down to nanoliter volumes of complex proteomic samples. Furthermore, we have demonstrated that chemical reactions, such as cysteine reduction and alkylation, can be performed on the reactor. The reactor digestion also led to approximately 10 times more protein identifications than in-solution digestions. As well, cells can be directly introduced and processed on the reactor, thus greatly simplifying the processing of proteomic samples. We successfully demonstrated that as little as 300 cells can be processed and analyzed on the reactor. We expect that the performance of the reactor will be further enhanced by coupling it to protein fractionation approaches and to more efficient mass spectrometers.

Acknowledgment. We thank Nathalie Major for a critical review of the manuscript and Jean-Philippe Lambert for providing the cells. We also thank the Canada Research Chair, the Canadian Foundation for Innovation, NSERC, and MDS Inc. for funding.

References

- (1) Hamdan, M.; Righetti, P. G. *Mass Spectrom. Rev.* **2003**, *22*, 272–284.
- (2) Aebersold, R. H.; Leavitt, J.; Saavedra, R. A.; Hood, L. E.; Kent, S. B. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 6970–6974.
- (3) Wilm, M.; Shevchenko, A.; Houthaevae, T.; Breit, S.; Schweigerer, L.; Fotsis, T.; Mann, M. *Nature* **1996**, *379*, 466–469.
- (4) Havlis, J.; Shevchenko, A. *Anal. Chem.* **2004**, *76*, 3029–3036.
- (5) Lambert, J. P.; Ethier, M.; Smith, J. C.; Figeys, D. *Anal. Chem.* **2005**, *15*, 3771–3787.
- (6) Ihling, C.; Sinz, A. *Proteomics* **2005**, *5*, 2029–2042.
- (7) Martosella, J.; Zolotarjova, N.; Liu, H.; Nicol, G.; Boyes, B. E. *J. Proteome Res.* **2005**, *4*, 1522–1537.
- (8) Moritz, R. L.; Clippingdale, A. B.; Kapp, E. A.; Eddes, J. S.; Ji, H.; Gilbert, S.; Connolly, L. M.; Simpson, R. J. *Proteomics* **2005**, *5*, 3402–3413.
- (9) Burggraf, D.; Weber, G.; Lottspeich, F. *Electrophoresis* **1995**, *16*, 1010–1015.
- (10) Linke, T.; Ross, A. C.; Harrison, E. H. *J. Chromatogr. A* **2006**, *1123*, 160–169.
- (11) Dai, J.; Shieh, C. H.; Sheng, Q. H.; Zhou, H.; Zeng, R. *Anal. Chem.* **2005**, *77*, 5793–5799.
- (12) Jessani, N.; Niessen, S.; Wei, B. Q.; Nicolau, M.; Humphrey, M.; Ji, Y.; Han, W.; Noh, D. Y.; Yates, J. R., 3rd; Jeffrey, S. S.; Cravatt, B. F. *Nat. Methods* **2005**, *2*, 691–697.
- (13) Washburn, M. P.; Wolters, D.; Yates, J. R., 3rd. *Nat. Biotechnol.* **2001**, *19*, 242–247.
- (14) Wu, C. C.; MacCoss, M. J.; Howell, K. E.; Yates, J. R., 3rd. *Nat. Biotechnol.* **2003**, *21*, 532–538.
- (15) Duan, J.; Sun, L.; Liang, Z.; Zhang, J.; Wang, H.; Zhang, L.; Zhang, W.; Zhang, Y. *J. Chromatogr. A* **2006**, *1106*, 165–174.
- (16) Lim, L. W.; Tomatsu, M.; Takeuchi, T. *Anal. Bioanal. Chem.* **2006**, DOI 10.1007/s00216-006-0458-6.
- (17) Slys, G. W.; Schriemer, D. C. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1044–1050.
- (18) Slys, G. W.; Schriemer, D. C. *Anal. Chem.* **2005**, *77*, 1572–1579.
- (19) Craft, D.; Doucette, A.; Li, L. *J. Proteome Res.* **2002**, *1*, 537–547.
- (20) Craft, D.; Li, L. *Anal. Chem.* **2005**, *77*, 2649–2655.

PR060312M