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Technical Note

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Biphasic Microreactor for Efficient Membrane Protein Pretreatment with a Combination of Formic Acid Assisted Solubilization, On-Column pH Adjustment, Reduction, Alkylation, and Tryptic Digestion

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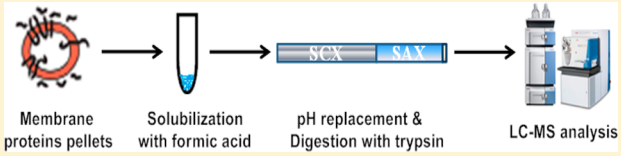
Supporting Information

ABSTRACT: Combining good dissolving ability of formic acid (FA) for membrane proteins and excellent complementary retention behavior of proteins on strong cation exchange (SCX) and strong anion exchange (SAX) materials, a biphasic microreactor was established to pretreat membrane proteins at microgram and even nanogram levels. With membrane proteins solubilized by FA, all of the proteomics sample processing procedures, including protein preconcentration, pH adjustment, reduction, and alkylation, as well as tryptic digestion, were integrated into an "SCX-SAX" biphasic capillary column. To evaluate the performance of the developed microreactor, a mixture of bovine serum albumin, myoglobin, and cytochrome c was pretreated. Compared with the results obtained by the traditional in-solution process, the peptide recovery (93% vs 83%) and analysis throughput (3.5 vs 14 h) were obviously improved. The microreactor was further applied for the pretreatment of 14 μg of membrane proteins extracted from rat cerebellums, and 416 integral membrane proteins (IMPs) (43% of total protein groups) and 103 transmembrane peptides were identified by two-dimensional nanoliquid chromatography-electrospray ionization tandem mass spectrometry (2D nano-LC-ESI-MS/MS) in triplicate analysis. With the starting sample preparation amount decreased to as low as 50 ng, 64 IMPs and 17 transmembrane peptides were identified confidently, while those obtained by the traditional in-solution method were 10 and 1, respectively. All these results demonstrated that such an "SCX-SAX" based biphasic microreactor could offer a promising tool for the pretreatment of trace membrane proteins with high efficiency and throughput.



Integral membrane proteins (IMPs) play a critical role in mediating a host of biological and physiological processes, including receptor function, transduction, ion transport, and propagation of signaling cascades, and therefore, they are often regarded as molecular targets for drug discovery.[1−5] Nevertheless, IMPs analysis remains challenging due to the highly hydrophobic nature, resulting in poor solubility in aqueous buffer and tendency of aggregation. Recently, a variety of additives, such as chaotropes,[6,7] detergents,[8−11] organic solvents,[12,13] and organic acids[14−16] have been used to solubilize membrane proteins. However, to avoid the adverse effect on enzyme activity, separation efficiency, and mass spectrometry (MS) signals, the removal or dilution of additives is indispensable before digestion and MS analysis, which not only is labor-intensive but also might result in poor recovery of IMPs.

Besides, highly concentrated formic acid (FA) is an efficient solubilizing agent, easily removed, and compatible with downstream liquid chromatography (LC)-MS analysis.[3,14] However, for the proteolytic digestion of proteins solubilized with FA, acid-compatible chemical reagents or enzymes, such as cyanogen bromide (CNBr)[7,18] or pepsin,[14,19,20] are commonly adopted. The fragments of CNBr cleavage are often too large for electrospray ionization (ESI)-MS analysis. Although pepsin is effective for the cleavage of transmembrane domains (TMDs) of IMPs,[19] the low cleavage specificities result in a dramatic increase in the theoretical peptide list, which not only is time-consuming for data searching but also causes high false discovery rate (FDR), ultimately leading to poor protein identification.[21]

As the prevalent enzyme choice in current shot-gun based proteomic analysis, trypsin exhibits excellent specific cleavage behavior for protein digestion and generates peptides with suitable mass for ESI-MS analysis.[22−25] However, trypsin should work under alkaline conditions that are incompatible with FA based solubilization. To solve this problem, Martinou et al.[17] demonstrated a strategy by adding ammonium bicarbonate to adjust sample buffer to pH 8.5, compatible with subsequent reduction, alkylation, and trypsin digestion. However, IMPs with more TMDs might be precipitated during solvent replacement, which is especially unfavorable for a trace amount of membrane protein analysis. In addition, this process

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was performed in solution in an Eppendorf tube, which is difficult to couple with LC-MS/MS analysis.

In recent years, microreactors have been developed to improve the recovery and throughput for proteomic samples treatment and showed advantages in trace sample analysis.[22,26−28] However, studies on membrane proteins using microreactors are relatively limited.[29,30] Herein, on the basis of the excellent complementary retention behavior of proteins on strong cation exchange (SCX) and strong anion exchange (SAX) materials, we developed an "SCX-SAX" based biphasic microreactor for the preparation of membrane proteins at the microgram and even the nanogram level. With the combination of membrane protein solubilization by FA and pH adjustment on an SCX-SAX capillary column, as well as on-column reduction, alkylation and tryptic digestion, the microreactor was successfully applied for the analysis of trace membrane protein extracted from rat cerebellums. Compared to traditional approaches, the microreactor demonstrated the advantages of high efficiency, high throughput and high recovery, indicating that such a microreactor might provide an attractive technology for trace membrane proteome profiling.

EXPERIMENTAL SECTION

Reagents and Materials. Bovine serum albumin (BSA), myoglobin (Myo), cytochrome c (Cyt C), protease inhibitor cocktail, dithiothreitol (DTT), iodoacetamide (IAA), trifluoroacetic acid (TFA), 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS), polyethylene glycol diacrylate (PEGDA), and azodiisobutyronitrile (AIBN) were bought from Sigma (St. Louis, MO). Trypsin was ordered from Promega (Madison, WI). FA and urea were obtained from Fluka (Buches, Germany). Acetonitrile (ACN, HPLC grade) was ordered from Merck (Darmstadt, Germany). Deionized water was purified by a Milli-Q system (Millipore, Milford, MA). Other chemicals were of analytical grade.

SCX particles (10 μm, 1 000 Å pore) and SAX particles (10 μm, 1 000 Å pore) were obtained from TOSOH (Tokyo, Japan). Luna C18 particles (5 μm, 100 Å pore) were obtained from Phenomenex (Torrance, CA). Fused-silica capillaries (150 or 200 μm i.d./365 μm o.d.) were brought from Sino Sumtech (Handan, China). The rat cerebellums were ordered from the Experimental Animal Center of Dalian Medical University (Dalian, China).

Preparation of Membrane Protein Samples. Five male Sprague-Dawley rats (180−200 g) were killed by decapitation, and the cerebellums were dissected quickly and freshly frozen in liquid nitrogen followed by storage at −80 °C. For IMP extraction, the procedure followed the previously described method with minor modifications.[31] The details of the extraction method are shown in the Supporting Information.

Preparation of Biphasic Microreactor. As shown in Figure 1A, the biphasic microreactor was prepared by packing SCX and SAX particles in sequence in a capillary with an on-column monolithic frit, prepared according to our previous work but with minor modifications.[32] The details are shown in the Supporting Information. With such a monolithic frit prepared (2 cm long), SAX and SCX particles suspended in 500 mM NaCl were packed into the capillary in sequence with well-controlled length (3.5 cm for each) using a manual pump.

Preparation of Samples with Microreactor. The mixture of BSA, Myo, and Cyt C and membrane proteins extracted from rat cerebellums were selected to evaluate the performance of the microreactor, following the flowchart

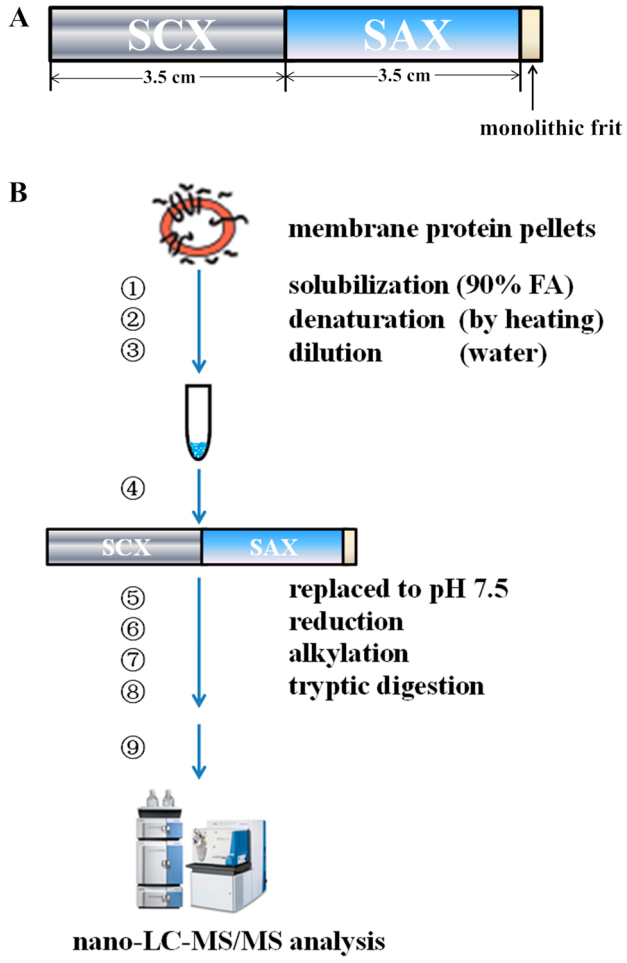

Figure 1. Structure of biphasic microreactor (A) and flowchart for sample analysis (B).

shown in Figure 1B. Briefly, sample was solubilized with 90% FA (v/v) to a final concentration of 9 mg/mL and sonicated for 40 min (step 1), followed by heating at 90 °C for 20 min for denaturation (step 2). After the microreactor was flushed with 0.1% FA (v/v), the sample was diluted to 1% FA (v/v) (step 3) and loaded directly onto the SCX segment of biphasic microreactor at 10 μL/min with a micropump (Dionex, Thermo Fisher, Waltham, MA) (step 4). Then, the microreactor was washed with 5 mM NH_4HCO_3 buffer (pH 7.5) to adjust the pH microenvironment, and the unretained proteins on SCX particles were recaptured onto the SAX segment (step 5). For protein reduction, 100 mM DTT dissolved in 5 mM NH_4HCO_3 was loaded onto the microreactor and incubated for 30 min at room temperature for reduction (step 6), followed by washing with 5 mM NH_4HCO_3 to remove unreacted DTT. Subsequently, 10 mM IAA dissolved in 5 mM NH_4HCO_3 was loaded for alkylation at room temperature for 30 min in the dark (step 7), followed by washing with 5 mM NH_4HCO_3 to remove unreacted IAA. Finally, 2 mg/mL trypsin dissolved in 5 mM NH_4HCO_3 (pH 7.5) was quickly loaded, making most of the trypsin freely moving in the reactor, and then, the microreactor was incubated at 37 °C for 2 h for protein digestion (step 8). After protein pretreatment, the microreactor was directly connected with a C18 capillary separation column for 1D/2D nano-LC-ESI-MS/MS analysis (step 9). For comparison, the mixture of BSA, Myo, and Cyt C was treated

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with an SCX trap column based microreactor, according to the published protocol[27] with the same procedure described above.

In-Solution Sample Preparation. For the control experiment, the in-solution digestion was performed for the mixture of BSA, Myo, and Cyt C and 50 ng of membrane proteins (1 μg/mL, 50 μL) extracted from rat cerebellums, respectively. The details are shown in the Supporting Information.

SDS-PAGE Analysis. SDS-PAGE analysis was performed to compare the protein recovery in the steps of loading and pH replacement with biphasic and SCX trap column based microreactors, respectively, using mini Protein 3 cell (Bio-Rad, Hercules, CA) with 12% separating gel and 4% stacking gel. Silver staining was used for staining the SDS-PAGE gel with a fast silver stain kit (Beyotime, Jiangsu, China) according to the manufacturer's instruction.

Nano-LC-ESI-MS/MS Analysis. 1D nano-RPLC-ESI-MS/MS analysis was carried out to analyze the mixture of BSA, Myo, and Cyt C and trace membrane proteins from rat cerebellum, respectively. For analysis of microgram amounts of membrane proteins from rat cerebellum, 2D nano-SCX-RPLC-ESI-MS/MS analysis was performed on an LTQ XL with 6-step salt elution, respectively. The experimental details are shown in the Supporting Information.

Database Searching. All nano-LC-MS/MS Raw files were converted to *.mgf file by pXtract v1.0[33] and searched with MASCOT search engine v2.3.2 against database Swiss-Prot 1006 Mammalia for the mixture of BSA, Myo, and Cyt C and IPI. RAT. v3.75 (39 713 protein entries) for membrane proteins extracted from rat cerebellums. Peptides were searched using fully tryptic cleavage constraints, and up to two internal cleavage sites were allowed for tryptic digestion. Cysteine carbamidomethylation was set as static modification; Met oxidation was set as variable modification. For LTQ XL analysis, the mass tolerances were 2 Da for parent ions and 1 Da for fragment ions, and for LTQ-Orbitrap Velos analysis, the mass tolerances were 10 ppm for parent ions and 0.5 Da for fragment ions. After database searching, the results were filtered by pBuild[33] to control FDR[34,35] at the peptide level less than 1% and reduce the apparent redundancy in protein identification. For the mixture of BSA, Myo, and Cyt C, the following settings were applied to filter the results: significant threshold, $p < 0.05$; expect cut off, 0.05; required bold red.

Bioinformatics Analysis. The grand average of hydropathicity (GRAVY) values of identified peptides was calculated using ProtParam program (http://tw.expasy.org/tools/protparam.html). Peptides with positive and negative GRAVY values are named as hydrophobic and hydrophilic peptides, respectively.[36] The TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) algorithm was used to predict TMDs of identified proteins. Proteins with at least one predicted TMD were regarded as IMPs.[37]

RESULTS AND DISCUSSION

Recovery and Efficiency of Biphasic Microreactor. For the biphasic microreactor, the maximum loading amount of proteins in acidic buffer was 16 μg, measured by frontal analysis with BSA as a sample, according to ref 38. In view of different retention behaviors of acidic, neutral, and basic proteins on ion-exchange particles during pH replacement and the difficulty in obtaining standard membrane proteins with different pIs, a mixture of BSA, Myo, and Cyt C, with pIs ranging from 4 to 9, was chosen as the sample to evaluate the protein recovery of biphasic microreactor. The passing-through effluents during sample loading and unretained fractions during pH adjustment were collected and analyzed by SDS-PAGE. As shown in Figure 2, no significant protein loss was observed in both steps (Figure

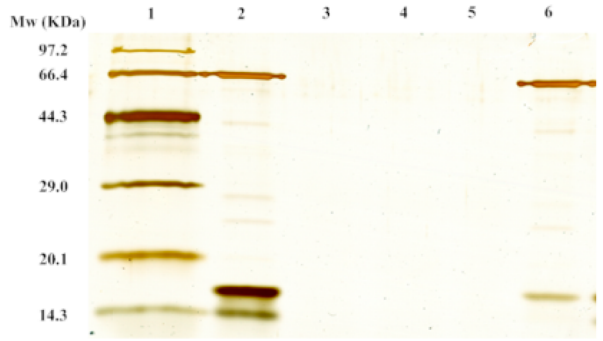

Figure 2. Colloidal silver-stained gel image of markers, effluents in sample loading, and pH replacement steps with biphasic and SCX microreactors. Lane 1: marker; Lane 2: a mixture of BSA, Myo, and Cyt C; Lane 3: flow-through fraction in sample loading step with biphasic microreactor; Lane 4: flow-through fraction in sample loading step with SCX microreactor; Lane 5: unretained fraction in pH replacement step with biphasic microreactor; Lane 6: unretained fraction in pH replacement step with SCX microreactor.

2, lanes 3 and 5). For comparison, the recovery of an SCX based microreactor[27] was also evaluated. Almost all the proteins were retained for sample loading (Figure 2, lanes 4). However, during pH adjustment, BSA and Myo were severely leaked (Figure 2, lanes 6). These results demonstrated the superiority of SCX-SAX over SCX based microreactor on improving the protein recovery during pH adjustment.

After pH replacement, proteins retained on both microreactors were on-column reduced, alkylated and digested by trypsin, followed by LC-MS analysis. For comparison, the mixture of three proteins was also pretreated by a conventional in-solution process. The identified sequence coverages of proteins pretreated with three methods were listed in Table 1. Equivalent results could be obtained with a biphasic

Table 1. Sequence Coverages of Proteins Obtained with Samples Treated by SCX-SAX Microreactor, SCX Microreactor, and In-Solution Methods

	SCX-SAX, %	SCX, %	in solution, %
BSA	81	54	80
Myo	94	77	94
Cyt C	66	62	65

microreactor and in-solution method but were superior to the SCX based microreactor, which further demonstrated the low sample loss of the biphasic microreactor. Furthermore, with such a biphasic microreactor, the total sample preparation time was shortened to 1/4 of that required by the traditional in-solution method (3.5 vs 14 h), which is beneficial for achieving high throughput membrane proteome analysis.

In addition, the recovery of the whole sample preparation with biphasic microreactor was evaluated using 4 μg of the mixture of BSA, Myo, and Cyt C as the sample and compared with the traditional in-solution process. The produced tryptic digests were quantified by UV detection (214 nm) according to previous work.[31,39] As a result, for biphasic microreactor based sample preparation, 3.73 ± 0.15 μg of tryptic digests was

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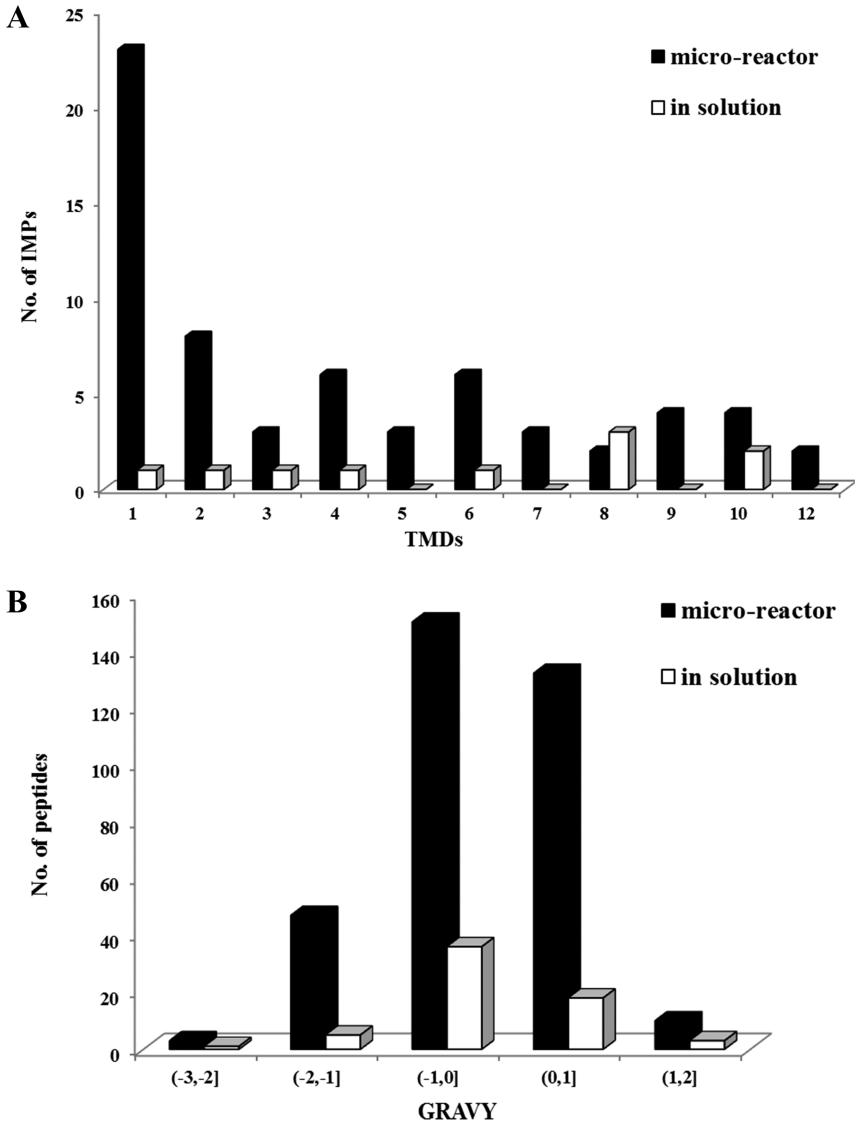

Figure 3. Comparison of (A) TMDs of IMPs and (B) GRAVY of peptides distribution identified from 50 ng (1 μg/mL, 50 μL) of membrane proteins from rat cerebellum by biphasic microreactor and in-solution methods coupled with 1D nano-LC-MS/MS.

obtained, with the recovery as 93 ± 3% (n = 3), while those obtained by the in-solution method were 3.31 ± 0.29 μg, with the recovery as 83 ± 7% (n = 3). Such a high recovery of the microreactor should be attributed to the low protein loss on the SCX-SAX column during pH replacement and in situ operation of all steps, without extra sample cleanup, transfer, and lyophilization, which might lead to sample loss.

Analysis of Microgram Amounts of Membrane Proteins. Fourteen micrograms of membrane proteins from rat cerebellum was analyzed to evaluate the performance of biphasic microreactor for real sample preparation. The sample was in parallel pretreated with three biphasic microreactors and identified by nano-2D LC-ESI-MS/MS. By database searching, 682 ± 74 protein groups were identified with FDR less than 1%, corresponding to 2055 ± 255 (n = 3) peptides. With the combination of identified proteins from triplicate analysis, a total of 975 protein groups were identified with 3268 peptides matching, among which 416 (43%) proteins were predicted by TMHMM[40] to be IMPs. The details of protein and peptide identification were listed in the Supporting Information, Excel file.

We further compared the results obtained by our protocol with those previously reported[14,41−43] for rat brain membrane proteome analysis. Chen et al.[42] developed a digestion scheme using three kinds of detergents compatible with MS to improve membrane protein identification, and 22.6% of proteins were predicted to contain at least one TMDs. Using ionic liquid and urea solubilization coupled to MudPIT, Tao et al.[43] identified 27.66% and 17.03% of total proteins were IMPs. Ma et al.[14] developed a membrane proteome profiling system, in which proteins were solubilized by FA, online digested by a pepsin based immobilized enzyme reactor, and analyzed by SCX-μRPLC-ESI-MS/MS, and 39% of the identified proteins were predicted as IMPs. Therefore, in comparison, a higher percentage of IMPs was identified with our protocol (43%). Although compared to Lu's work,[41] in which two sequential solubilization steps with CHAPS and SDS as additives were used, the identified IMP percentage was a little higher (52−58%, 43%); it took 52 h for sample preparation, much longer than that spent by us (3.5 h). All these results indicated the superiority of our developed protocol for IMPs analysis.

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For the in-depth analysis of 416 IMPs, 58% (240 protein groups) contained 2−16 TMDs (Supporting Information, Figure S-1A), higher than the percentage (43%) reported from Ma et al.,[14] which further demonstrated the good performance of our developed microreactor for multitransmembrane proteins analysis. Furthermore, the distribution of GRAVY values of peptides identified was analyzed (Supporting Information, Figure S-1B), and 1081 peptides (33% of the total 3268 peptides) were hydrophobic (GRAVY > 0). In addition, with our protocol, 103 peptides containing partial or whole TMDs were identified and recognized as transmembrane peptides (Supporting Information, Table S-1), which was important and challenging for IMPs analysis.

Analysis of Nanogram Amounts of Membrane Proteins. With high recovery and high pretreatment efficiency, the biphasic microreactor was further applied for the pretreatment of 50 ng (1 μg/mL, 50 μL) of membrane proteins from rat cerebellum, followed by nano-RPLC-ESI MS/MS analysis, and the results were also compared with those obtained by the in-solution protocol. After data searching, in triplicate runs, in total, 141 proteins were identified with the sample treated by biphasic microreactor, 4-fold of that identified by in-solution protocol (34 proteins), among which 45% (64 proteins) were IMPs with at least one TMD, higher than that obtained by in-solution treatment (29%, 10 proteins). As shown in Figure 3A, more IMPs were identified by our protocol than that obtained with the in-solution method in most TMDs. In addition, IMPs with 5, 7, 9, and 12 TMDs were exclusively identified with our protocol. These results further showed the superiority of our protocol for the analysis of trace membrane proteins with more TMDs.

Furthermore, the distributions of GRAVY values of identified peptides in both methods were compared (Figure 3B). The hydrophilic peptides (GRAVY $\leq$ 0) and hydrophobic peptides (GRAVY > 0) were improved to 3.8-fold (200 vs 42) and 5.8-fold (142 vs 21) with our protocol. It is worth noting that, with the biphasic microreactor and in-solution method, the difference on the identified hydrophobic peptides was more notable than the identified hydrophilic peptides. Furthermore, 17 transmembrane peptides were identified with our protocol, while only 1 was identified with the in-solution method. These results further demonstrated great promise of our approach for hydrophobic peptides analysis with high sensitivity.

The good performance of the biphasic microreactor for IMPs and hydrophobic peptides analysis with a trace amount was presumably attributed to three reasons. First, the high recovery and good efficiency of the "SCX-SAX" based biphasic microreactor were achieved for sample preparation. Second, the direct connection of the microreactor with a packed C18 capillary column for LC-ESI-MS/MS analysis, without additional lyophilization and transfer steps, further reduced sample loss. Third, higher enzyme-to-substrate ratio was more efficient than free trypsin for the digestion of low concentration IMPs, in accordance with the recently published work.[25]

CONCLUSION

An "SCX-SAX" based biphasic microreactor for membrane proteins pretreatment was developed. Compared with previously reported strategies, our protocol could offer improved compatibility for FA based IMPs solubilization and tryptic digestion. The whole sample preparation procedure, including preconcentration, pH replacement, reduction, alkylation and tryptic digestion, was in situ performed in the microreactor with high sample recovery, high efficiency and high throughput. Furthermore, all operation steps were in situ performed without an extra-column process and could further be directly coupled with LC-MS/MS. Through the applications in trace membrane proteome sample analysis, such a biphasic microreactor showed great advantages of decreased sample loss, high sensitivity and high throughput, which are significant for trace membrane proteomics analysis. Besides, the biphasic microreactor is applicable not only for membrane proteome but also for nonmembrane proteins.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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