

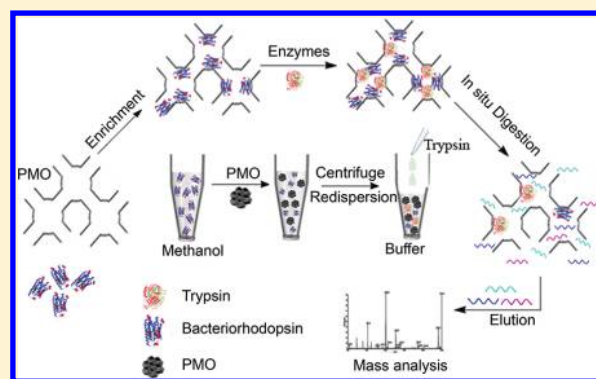
# Periodic Mesoporous Organosilica as a Multifunctional Nanodevice for Large-Scale Characterization of Membrane Proteins

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## Supporting Information

**ABSTRACT:** A versatile protocol has been developed for large-scale characterization of hydrophobic membrane proteins based on the periodic mesoporous organosilica (PMO) acting as both an extractor for hydrophobic substrate capture and a nanoreactor for efficient *in situ* digestion. With introduction of organic groups in the pore frameworks and the presence of hydrophilic silanol groups on the surface, PMO can be well-dispersed into not only an organic solution to concentrate the dissolved membrane proteins but also an aqueous solution containing enzymes for sequential rapid proteolysis in the nanopores. The unique amphiphilic property of PMO ensures a facile switch in different solutions to realize the processes of substrate dissolution, enrichment, and digestion effectively. Furthermore, this novel PMO-assisted protocol has been successfully applied for identification of complex membrane proteins extracted from mouse liver as proof of general applicability.



Membrane proteins are the main undertaker of many essential biological membrane functions, which play vital roles in the biological processes such as molecule transport and signal transduction.<sup>1,2</sup> Hence, deep insights of membrane proteins will be of significant values for biological function study. However, low abundance and the hydrophobicity caused by the existence of the nonpolar amino acids in the bilayer of membrane proteins make their identification still a great challenge.<sup>3</sup> Various efforts have been developed to facilitate the characterization of membrane proteins to address the limitations suffering from conventional methods. Mass spectrometry compatible detergents<sup>4–10</sup> and organic solvents<sup>11–15</sup> have been widely used to dissolve the hydrophobic membrane proteins, although such reagents could cause the decrease of the protease activity for digestion. Considering the typical lack of tryptic cleavage sites of membrane proteins, less and nonspecific proteases<sup>16–18</sup> or even chemical reagents such as CNBr<sup>19–24</sup> have also been proposed to digest membrane proteins in lieu of trypsin, increasing the total number of peptides produced. However, lacking degradation specificity reduces the confidence of the score reported and the use of CNBr is followed with a security problem and undesirable modification. Therefore, developing new methods to profile membrane proteins is desired for wide practical applications. Recently, a few examples have been developed to promote the analysis of membrane proteins<sup>25–28</sup> such as solvent-free matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis of membrane protein via a mini ball mill approach,<sup>25</sup> two-phase, on-membrane digestion method,<sup>26</sup> or centrifugal proteomic reactor.<sup>28</sup> Although these achieve-

ments did improve the identification of membrane proteins, they still suffered from time-consuming, high-cost, and labor-intensive procedures.

In order to enhance the proteolytic efficiency, various enzymatic reactors based on microchips and mesoporous materials have been developed.<sup>29–32</sup> With theoretical predictions and experimental validations, it was demonstrated that the porous materials could efficiently accelerate the biochemical reactions by enriching reactants into its inner pores and confining the reactions at the nanoscale.<sup>30</sup> Nanomaterials with hydrophobic properties have been used to enrich peptides/proteins effectively through hydrophobic–hydrophobic interaction. Periodic mesoporous organosilica (PMO) materials possess an organobridged hybrid composition as an intrinsic part of the pore walls, leading to superior performance over the classical mesoporous silica with similar structured parameters and morphology for the applications such as peptide/protein adsorption.<sup>33</sup> These specific features make them promising candidates for the analysis of hydrophobic membrane proteins. However, to our best knowledge, this kind of property has rarely been applied to the analysis of hydrophobic membrane proteins.

Herein, we propose a new approach for large-scale characterization of hydrophobic membrane proteins based on a large-pore PMO which acts as both an extractor for

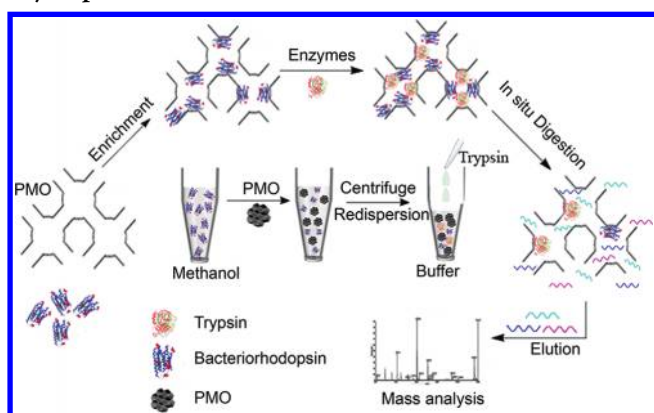
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hydrophobic substrate enrichment and a nanoreactor for efficient *in situ* digestion. Compared to previous work, by introducing the homogeneously distributed organic groups in the pore frameworks, PMO can be highly dispersed into an organic solution in which the uniform dissolved hydrophobic membrane proteins are quickly captured into the nanodevices through the hydrophobic–hydrophobic interaction. Due to the presence of hydrophilic silanol groups on the surfaces, the protein-loaded PMO is also well-dispersed into an aqueous solution containing enzymes where the tryptic digestion can proceed rapidly in the nanopores. The unique amphiphilic property of PMO ensures a facile switch in different phase systems to realize the multiprocess of substrate dissolution, concentration, digestion, and MS measurement avoiding cross influence (Scheme 1). Furthermore, conventional ordered

**Scheme 1. Schematic Illustration of the PMO-Assisted Strategy Employed for Efficient Identification of Hydrophobic Membrane Proteins**



mesoporous silica (FDU-12) with similar pore sizes and pore volumes whose surface is more hydrophilic has also been used for comparison. As a proof of broad applicability, we show the successful identification of the complex membrane proteins extracted from mouse liver by this PMO-assisted protocol.

## EXPERIMENTAL SECTION

**Chemicals.** A triblock copolymer,  $\text{EO}_{106}\text{PO}_{70}\text{EO}_{106}$  (F127), bis(trimethoxysilyl)ethane (BTME), 1,3,5-trimethylbenzene (TMB), tetraethyl orthosilicate (TEOS), potassium chloride (KCl, 99.5%, AnalaR, Australia), ethanol (99.5%, Asia Pacific Specialty, Australia), fuming hydrochloric acid (37%, Lab-Scan, Analytical Science, Thailand), and ammonium bicarbonate were purchased from Aldrich. Methanol (HPLC), trypsin (from bovine pancreas), dithiothreitol (DTT), iodoacetamide (IAA), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), and wild-type bacteriorhodopsin (BR) isolated from *Halobacterium salinarum* strain S9 as purple membranes were obtained from Sigma.  $\alpha$ -Cyano-4-hydroxycinnamic acid (CHCA, 99%), acetonitrile (ACN, 99.9%), and trifluoroacetic acid (TFA, 99.8%) were purchased from Merck.

**Synthesis and Characterization of Materials.** PMO materials were synthesized according to the reported method.<sup>34</sup> Typically, 0.5 g of F127, 2.5 g of KCl, and 0.5 g of TMB were dissolved in 30 g of HCl (0.1 M). The mixture was stirred for 6 h at a fixed temperature (0 °C) before the addition of 2.0 g of BTME. After stirring for 24 h, the resultant mixture was hydrothermally treated at 100 °C for 24 h. The precipitate was

filtered and washed twice with a mixture of 60 mL of ethanol and 5 mL of HCl (2 M) at 60 °C to remove the template. The final products were filtered and dried in air at room temperature.

In a synthesis of FDU-12,<sup>29</sup> 0.5 g of triblock copolymer F127, 0.6 g of TMB, and 2.5 g of KCl were dissolved in 30 mL of 2 M HCl and stirred at 15 °C for 24 h. To this solution, 2.08 g of TEOS was added under stirring. After stirring for 24 h at 15 °C, the mixture was autoclaved at 100 °C for 24 h and for another 72 h at 140 °C. The solid product was collected by filtration and dried at room temperature in air. The organic templates were completely removed by microwave-assisted digestion.

Transmission electron microscopy (TEM) images were directly taken with a JEOL 2011 microscope operated at 200 kV by dispersing the samples on a Cu grid with carbon films. Nitrogen sorption isotherms of samples were obtained by a Quantachrome's Quadrasorb SI analyzer at 77 K. Before the measurements, the samples were degassed at 383 K for at least 8 h in vacuum. The infrared spectra were obtained by using an FT-IR360 manufactured by Nicolet. Small-angle X-ray scattering (SAXS) measurements were performed on a Bruker NanoStar U system using Cu K $\alpha$  radiation at 40 kV and 35 mA. CD spectra were obtained by a JASCO J-715 circular dichroism (CD) spectropolarimeter.

**Membrane Protein Digestion with Materials.** Solubilization of BR using methanol was carried out using sonication in water bath for 15 min. Briefly, 10, 20, 50, and 100 ng/ $\mu\text{L}$  of BR was dissolved in 50  $\mu\text{L}$  of methanol (HPLC grade), respectively. After sonication in ice bath for 15 min, PMO or FDU-12 was added into the solubilized protein with a final concentration of 1 mg/mL. The mixture was then agitated at 298 K for 15 min for complete protein adsorption in PMO/FDU-12. Then, PMO/FDU-12 were pelleted by centrifugation and resuspended in 15  $\mu\text{L}$  of ammonium bicarbonate buffer (25 mM, pH  $\sim$  8.0). For the proteolysis in PMO or FDU-12, 5  $\mu\text{L}$  of trypsin in ammonium bicarbonate buffer (25 mM, pH  $\sim$  8.0) was added into the above-mentioned system with a final enzyme/substrate ratio of 1:10 (w/w), and then the mixture was incubated at 37 °C for 10 min. After the digestion, the mixture was centrifuged. Then the deposits were resuspended in 10  $\mu\text{L}$  of 0.1% TFA/50% ACN aqueous solution (v/v) and shaken for 30 min to elute the digested peptides. Then the eluate was directly detected by MS.

**In-Solution Digestion.** In-solution digestion was performed according to a published method.<sup>11,13</sup> An amount of 5  $\mu\text{g}$  of BR was dissolved in 50  $\mu\text{L}$  of ammonium bicarbonate buffer (25 mM, pH  $\sim$  8.0) containing 60% (v/v) methanol using sonication in a water bath for 15 min and incubated at 37 °C with trypsin at an enzyme/substrate ratio of 1:10 (w/w). The digestion products were analyzed by MS.

**MALDI-TOF-MS Analysis.** The digestion products were analyzed on an Applied Biosystems 5800 proteomics analyzer (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, MALDI-TOF-MS), using CHCA (99%) as matrix. The MALDI plate was externally calibrated by the tryptic digests of myoglobin in six spots. All mass spectra were obtained in the positive ion reflector mode via an accumulation of 2000 laser shots under a laser intensity of 3500 instrument units. Mass spectrometric data analysis was performed with the GPS Explorer software from Applied Biosystems with Mascot as a search engine and NCBIInr as a database with one missed cleavage site accepted.

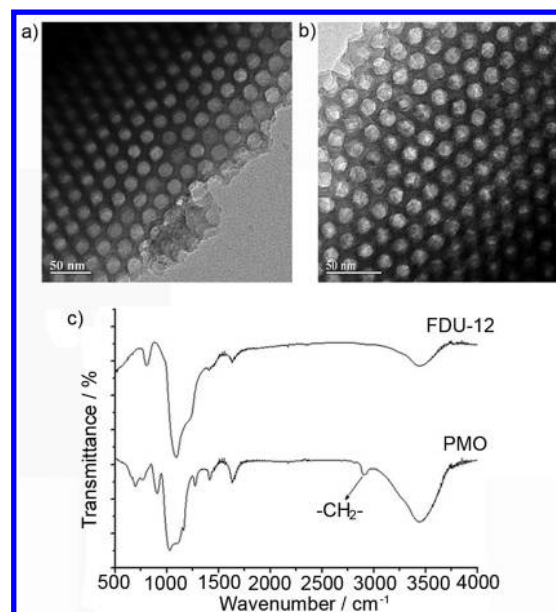
**Preparation of Mouse Liver Membrane.** The treatment was according to published literature.<sup>26</sup> Mice were killed after being starved for 24 h, and their liver was excised. After a wash with PBS and removal of the gall bladder and blood vessels, the livers were minced into pieces and homogenized in an ice-cold solution containing 50 mM HEPES (pH 7.4), 1 mM  $\text{CaCl}_2$ , and 1 mM phenylmethylsulfonyl fluoride (PMSF). Unbroken cells and debris were removed by centrifugation at 3200g for 15 min at 4 °C. The resulting supernatant was centrifuged for 30 min at 4 °C, and the pellet was collected and washed with the above buffer three times. Protein concentration of the membrane fraction was determined by Bradford assay.

**Analysis of Liver Membrane Proteins.** Before digestion, the rat liver membrane fraction was reduced with 10 mM DTT in 25 mM ammonium bicarbonate buffer at 37 °C for 1 h and alkylated by 55 mM IAA in the same buffer in the dark at room temperature for 45 min. For in-solution digestion, 20  $\mu\text{g}$  of membrane protein was dissolved by ammonium bicarbonate buffer (25 mM, pH  $\sim$  8.0) containing 60% (v/v) methanol and incubated overnight (12 h) with trypsin at an enzyme/substrate ratio of 1:10 (w/w). The porous materials catalyzed digestion was performed by adding an additional 200  $\mu\text{g}$  of PMO, and the digestion time was shortened to 2 h.

The digested peptides were analyzed by reversed-phase liquid chromatography–electrospray ionization–tandem mass spectrometry (RPLC–ESI-MS/MS) using a Thermo Scientific LTQ Orbitrap XL mass spectrometer. Liquid chromatography was performed on a nano Acquity UPLC system (Waters Corporation, Milford, U.S.A.) connected to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with an online nanoelectrospray ion source (Michrom Bioresources, Auburn, U.S.A.). The mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. The mass spectra were searched using the Bioworks software (version 3.3.1, Thermo Scientific) based on the SEQUEST algorithm. The database used to search was the mouse UniProtKB/Swiss-Prot database (release 2011\_10\_19, with 29 996 entries). To reduce false positive identification results, a decoy database containing the reverse sequences was appended to the database. The searching parameters were set up as follows: partial trypsin (KR) cleavage with two missed cleavages was considered; fixed carbamidomethyl modification of 57.02 Da for cysteine; the variable modification was oxidation (M); the peptide mass tolerance was 50 ppm and the fragment ion tolerance was 1.0 Da. The peptide identification criteria were set up based on  $\Delta\text{CN}$  ( $\geq 0.1$ ) and Xcorr (double charges,  $\geq 2.31$ ; triple charges,  $\geq 2.87$ ) with false discovery rate (FDR) less than 1.0%.

## RESULTS AND DISCUSSION

To evaluate the PMO as a promising host for analysis of membrane proteins, PMO and FDU-12 materials were prepared and characterized. Well-resolved scattering peaks can be observed in the SAXS patterns of PMO and FDU-12, which are both typically assigned to face-centered cubic (fcc) structures with a space group of  $Fm3m$  (Figure S1, Supporting Information). The fcc mesostructures of PMO and FDU-12 were further confirmed by TEM. Parts a and b of Figure 1 are TEM images of PMO and FDU-12 along [110] directions, and the pore sizes are about 20 nm, respectively. Compared with FDU-12, PMO could work as a good candidate for rapid analysis of hydrophobic proteins/peptides due to the existence of  $-\text{CH}_2$  groups in the framework revealed from the FT-IR



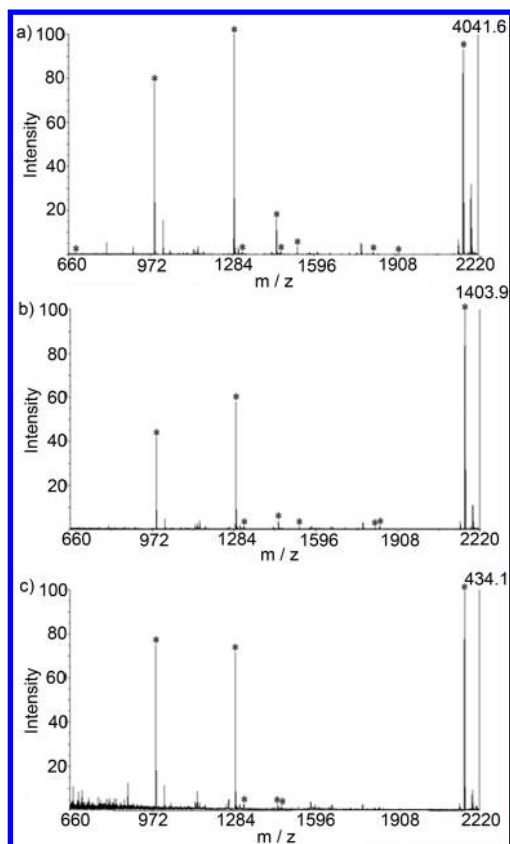
**Figure 1.** TEM images of (a) PMO and (b) FDU-12; (c) FT-IR spectra of PMO and FDU-12.

spectra (Figure 1c). Protein substrates dissolved in methanol were first captured and concentrated by PMO, followed by redispersion in an ammonium bicarbonate buffer solution containing trypsin for efficient digestion, whereas the reaction time was significantly reduced to minutes due to the nanoscale confinement.<sup>28</sup> In this way, the pure methanol or any other organic solvents can be used to dissolve hydrophobic membrane proteins effectively and be readily removed before tryptic digestion to avoid the negative effects of reagents.

To prove the high efficiency of the proposed method, the performance of the PMO nanoreactor has been evaluated by using MALDI-TOF-MS and LC–ESI-MS/MS to analyze BR, a integral membrane protein, which has been frequently used as a model membrane protein substrate. The mass spectra of tryptic digests of BR with and without the assistance of PMO are shown in Figure 2. It is noteworthy that the same enzyme/substrate ratio of 1:10 (w/w) was used in the digestion both in-solution and in-nanoreactor for convenient comparison. Only two matched peptides are observed in Figure 2a after 10 min of in-solution digestion of BR, whereas the number of matched peptides and the sequence coverage increase from 2 to 14 and 14% to 26% in 10 min after being assisted by PMO (Figure 2c), which is even much better than 12 h in-solution reaction with only eight peptides detected (Figure 2b). The digested products were also measured by LC–ESI-MS/MS (Figure S2, Table S1, Supporting Information), and 20 peptides were obtained by this PMO-assisted protocol. Figure 4a shows that FDU-12 can also improve the digestion of BR with eight matched peptides, which is less than those of the PMO-assisted protocol. These results may be attributed to the amphiphilic surfaces of the mesoporous silica which can provide a suitable accommodation for both hydrophilic and hydrophobic molecules. Considering the existence of both  $-\text{CH}_2$  groups and  $\text{Si}-\text{O}-\text{Si}$  groups in the framework, PMO is more suitable for analysis of membrane proteins compared with FDU-12. Among the detected peptides (Table 1), two peaks at  $m/z = 987.65$  and  $m/z = 1230.68$  are hydrophobic peptides located in the transmembrane region of helix no. 7 of BR, indicating that some of the hydrophobic regions can be attacked by trypsin





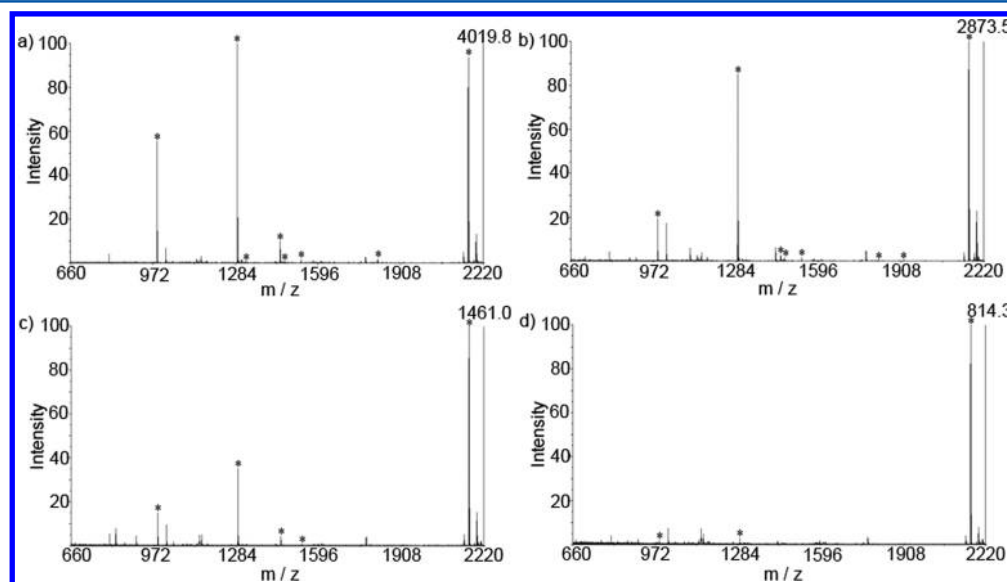


**Figure 3.** MALDI-MS spectra of digests of bacteriorhodopsin with different concentrations at (a) 50, (b) 20, and (c) 10 ng/ $\mu$ L obtained by PMO-assisted digestion in 10 min. Identified peptide peaks are labeled with asterisks.

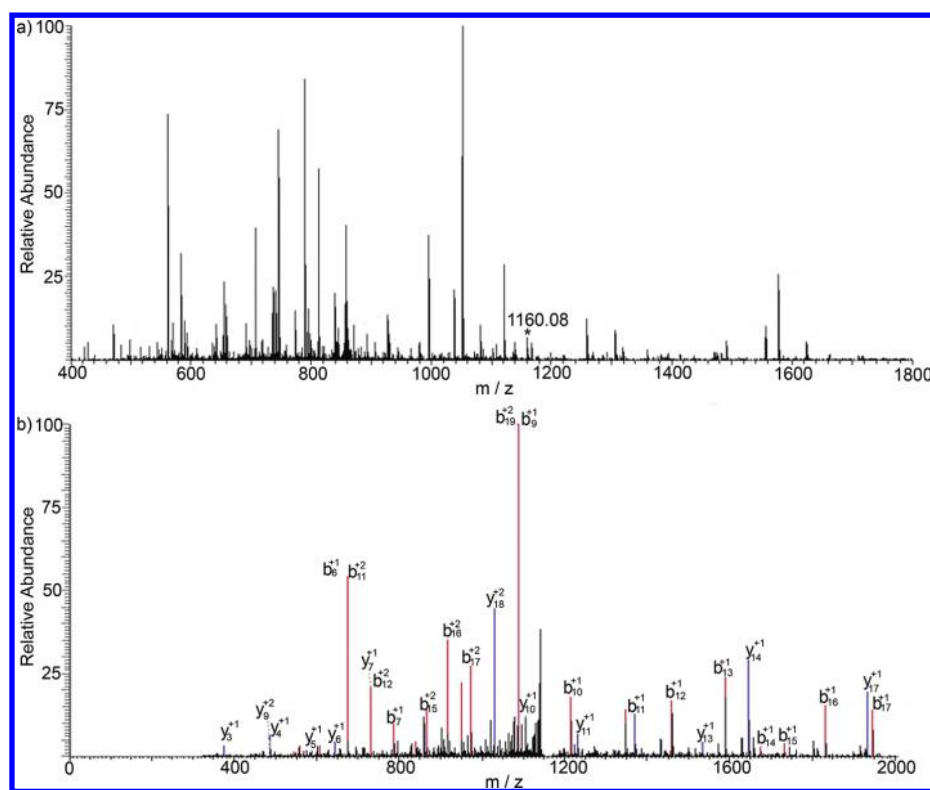
surfactant interference.<sup>35,36</sup> In this protocol, up to 1% SDS was used in methanol–BR solution to investigate the effects of SDS on BR analysis in the PMO-assisted protocol. For comparison, 1% SDS was also added to the methanol-based in-solution digestion system. The results (Figure S5, Supporting

Information) show that nine peptides can be observed in MS spectra with the presence of PMO, whereas no peptide is detected in solution digestion.

To further investigate the application of such a PMO-assisted protocol for large-scale characterization of a real-case biological sample, membrane proteins extracted from mouse liver by methanol was carried out for proteolytic profile using this PMO nanodevice and the commonly used methanol-based in-solution method, respectively. After MS/MS analysis by the Thermo LTQ Orbitrap XL mass spectrometer based on the SEQUEST algorithm (Figures S6 and S7, Supporting Information), a total of 1543 unique peptides were identified using the PMO protocol yielding 420 proteins, whereas the in-solution-based approach just allowed identification of 350 tryptic peptides resulting in a total of 177 proteins. For example, Figure 5a is the mass spectrum scan at retention time (RT) 38.55 min in LC–ESI-MS for mouse liver digests with the PMO-assisted digestion strategy and Figure 5b is the corresponding MS/MS spectrum of the  $m/z$  1160.08 which is a peptide from ATP synthase subunit  $\beta$  protein, both of which show good signal-to-noise ratio (S/N). Figure 6a shows the distribution and overlap of the identified membrane proteins by two methods. Almost 90% of proteins identified by methanol-based in-solution digestion are also observed by the PMO-assisted protocol. The physicochemical characteristic of all the identified proteins are analyzed in Tables S2 and S3 (Supporting Information). The transmembrane hidden Markov model (TMHMM) analysis shows that such a multifunctional PMO protocol yields 149 integral membrane proteins possessing 1–16 transmembrane domains (TMDs), where 77 transmembrane proteins have one TMD, 24 proteins have two TMDs, and 48 proteins have 3–16 TMDs, whereas the conventional approach yields a total of 57 integral membrane proteins possessing 1–12 TMDs with 30 proteins having only one TMD. The hydrophobic property of proteins is usually expressed as the grand average of hydrophobicity (GRAVY). Figure 6b shows the GRAVY and isoelectric point (PI) distribution of all the proteins identified by the PMO-assisted digestion. Their GRAVY values vary in the range of –1.5 to 0.8,



**Figure 4.** MALDI mass spectra of digests of bacteriorhodopsin with different concentrations of (a) 100, (b) 50, (c) 20, and (d) 10 ng/ $\mu$ L obtained by FDU-12-catalyzed digestion. All matched peptides are labeled with asterisks.



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