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Improved Mass Spectrometric Analysis of Membrane Proteins Based on Rapid and Versatile Sample Preparation on Nanodiamond Particles

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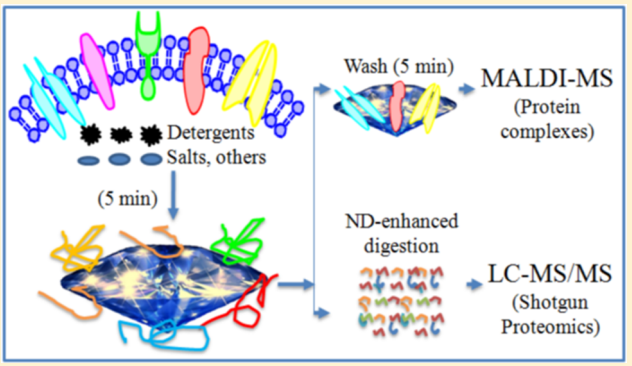
Supporting Information

ABSTRACT: We have developed a novel streamlined sample preparation procedure for mass spectrometric (MS) analysis of membrane proteins using surface-oxidized nanodiamond particles. The platform consists of solid-phase extraction and elution of the membrane proteins on nanodiamonds, concentrating the membrane proteins on the nanodiamonds and separating out detergents, chaotropic agents, and salts, and other impurities that are often present at high concentrations in solubilized membrane preparations. In this manner, membrane-protein extracts are transformed into MS-ready samples in minutes. The protocol is not only fast, but also widely adaptable and highly effective for preparing generic membrane protein samples for both MALDI-MS studies of membrane-protein complexes and shotgun membrane proteomics studies. As proof of concept, we have demonstrated substantial improvements in the MALDI-MS analysis of the particulate methane monooxygenase (pMMO) complex, a three-subunit transmembrane protein solubilized in various detergent buffers. Enzymatic digestions of membrane proteins are also greatly facilitated since the proteins extracted on to the nanodiamonds are exposed on the surface of the nanoparticles rather than in SDS gels or in detergent solutions. We illustrate the effectiveness of nanodiamonds for SDS removal in the preparation of membrane proteins for MS analysis on the proteome level by examining the quality of the tryptic peptides prepared by on-surface nanodiamond digestion of an *E. coli* membrane fraction for shotgun proteomics.



Membrane proteins play unique and prominent functions in cells because of their specialized location and cellular activities. They serve as conduits transmitting molecular and electrical signals from outside to inside the cell and *vice versa*. More than 50% of known drugs act on membrane proteins, and these proteins remain popular targets for drug development.[1] However the characterization of membrane proteins is slow due to their intrinsic hydrophobicity and low abundance.

Mass spectrometry (MS) has become a core tool in membrane proteomics research. Because of their intrinsic hydrophobicity, solubilizing and denaturing reagents are the indispensable chemicals in sample preparation of membrane proteins for MS. Unfortunately, the presence of these compounds subsequently interferes with downstream analysis of the proteins and peptides due to ion suppression, incomplete digestion, and inaccuracies in mass assignments.[2,3] The removal or reduction of these interfering reagents is commonly time-consuming and labor-intensive, and contributes to significant sample loss.

Various approaches have been developed to improve and facilitate the characterization of membrane proteins by MS. A discussion of these advances can be found in several recent reviews.[3−6] Typically, detergents or detergent-like reagents with or without organic solvents, and sometimes denaturants, are used in the analysis of membrane proteins by MALDI-MS. However, even in studies when "MS-compatible detergents" are employed, deleterious effects are often reported, such as the suppression of signals and undesired loss/gain of protein modifications. Thus, the reduction or removal of detergents and interfering contaminants prior to MS analysis certainly is of vital importance. In the "bottom-up" analysis of membrane proteins, there are two major strategies for converting proteins into peptides: "in-gel" and "in-solution" digestion.[7−12] The advantage of in-gel digestion is its robustness against contaminants, but the gel prevents the access of proteolytic

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enzymes, with additional difficulties related to the extraction of hydrophobic peptides. This technique is also time-consuming and labor intensive. In-solution digestion, including filter-based in-solution digestion, is more readily automatable and minimizes sample handling, but studies have reported sample loss, incomplete digestion, and poor reproducibility.[9−11] We report here a new and general approach that is inexpensive, easy to deploy, and effective in overcoming these inherent problems to facilitate the characterization of membrane proteins by MS.

Solid-phase extraction is a popular method to clean up samples of proteins or peptides by extracting these molecules from suspensions containing interfering contaminants or soluble impurities. Indeed, a number of efforts have been reported utilizing surface-modified adsorbents for such purposes.[13−15] This platform has proved to be a promising alternative to the gel- and filter-based approaches mentioned earlier.[15,16] Zhou and colleagues[17] have developed a proteomic reactor using ion exchange particles that not only improves membrane protein identification both in terms of sample requirement and quantitation, but also integrates peptide fractionation. However, certain problems related to peptide extraction, and salt or ionic detergent tolerances, remain.[15,16] Furthermore, the capability of the proteomic reactor in harvesting membrane-protein complexes for MALDI-MS analysis has yet to be demonstrated.

Han and Chang et al.[18,19] have previously demonstrated the SPEED (solid-phase extraction and elution on diamond) platform, where surface-oxidized nanodiamond particles (NDs) have been demonstrated to be highly effective for MS sample preparation of soluble proteins and proteins in human fluids. Oxidized NDs should interact with membrane proteins and hydrophobic/hydrophilic peptides in a similar fashion as in the proteomic reactor of Zhou et al.[20,21] Aside from the ionic interactions, membrane proteins can adhere to the surface of NDs by hydrophobic forces.

As proof of concept, we have used detergent-solubilized micelles of the particulate methane monooxygenase (pMMO), a three-subunit transmembrane protein isolated and purified from the methanotroph *Methylococcus capsulatus* (Bath), to illustrate the advantages of the nanodiamond technology for both MALDI-MS and protein subunit identification. This membrane-bound metalloenzyme has been receiving considerable attention in recent years because of its unique capability to mediate efficient oxidation of methane to methanol under ambient temperatures and pressures.[22−24] In fact, a MALDI-TOF-MS analysis of the protein complex reconstituted in *n*-dodecyl-*β*-maltoside (DDM) has appeared as part of the original characterization of the protein. This system thus offers a benchmark to assess the nanodiamond technology developed here against the earlier approach.[25,26]

To demonstrate the utility of NDs for analysis of a complicated mixture of membrane proteins as well as effective sodium dodecyl sulfate (SDS) removal, we have examined the membrane-enriched fraction of *E. coli* (TB1 strain) for shotgun proteomics. We find that only a single 2-h 1D-LC MS/MS run of tryptic peptides prepared by on-surface nanodiamond digestion of a membrane fraction of *E. coli* (solubilized in 2% SDS) will lead to the identification of more than 400 proteins, 70% of which can be classified as membrane proteins.

EXPERIMENTAL SECTION

Preparation of Oxidized Diamond Nanoparticles. Diamond powders were surface-functionalized with carboxyl and carbonyl groups in strong oxidative acids under microwave heating. The details of the procedure can be found in ref 27. The microwave reactor (100 W, Model Discover, CEM) is placed in a chemical fume-hood to protect the operator from NO_2 produced in the operation. At the end of the microwave treatment, we have also taken precautions to ensure that the residual strong acids are diluted prior to collection of the NDs.

Membrane-Protein Preparation. The growth of *Methylococcus capsulatus* (Bath) was adapted from our previous publication[25] with some modifications. When the cells were ready to be harvested, three-quarters of the culture in the fermenter (about 2.5 L) was collected directly without the assistance of a hollow-fiber filter. New NMS medium was then added to the remaining volume of bacteria for the next batch of cell culture. Bacteria were cultured in the medium containing 30 μM Cu^{2+} ions, the condition required for the high production of pMMO. The transmembrane protein was extracted and solubilized in 2% DDM without purification by size exclusion chromatography as described in the earlier procedure.[25] With this procedure, the membrane protein can reach up to 95% in purity.

The preparation of the membrane-enriched fraction of *E. coli* TB1 cells followed the published procedure of Masuda et al.[28] Membrane proteins were extracted and solubilized in a buffer consisting of 1% dithiothreitol (DTT), 2% CHAPS, 2% DDM, and 4 M urea. Insoluble materials were removed by centrifugation at 12 500 rpm for 15 min at 4 °C.

Facile, Speedy Extraction and Enrichment of Membrane Proteins. To test the protein absorbability of NDs in the presence of various single-component detergents or chaotropic reagents: [DDM (2%), Triton X-100 (2%), Tween-20 (2%), Digitonin (2%), SDS (0.01%, 0.05%, 0.2%, 1%, 2%), CHAPS (2%), urea (8 M)], as well as a mixture of (4 M urea, 2% DDM, 2% CHAPS, and 1% DTT), the solubilized pMMO was captured by direct addition of the nanoparticles. Enrichments were also attempted using other conditions such as in salts (1.5 M Tris HCl, 1.5 M NaCl), or organic solvents (MeOH, ethanol, acetonitrile (ACN)). After extensive trials, we find that at a protein/NDs mass ratio of 1:30 is usually more than sufficient to ensure complete capture of all the hydrophobic proteins from the solutions. The experimental details can be found in the Supporting Information.

Nanodiamond-Assisted MALDI-TOF-MS of the pMMO Complex. For each experiment, 50 μL of solution containing 0.5 μg pMMO was mixed well with 15 μg NDs for 5 min to selectively extract the proteins out of the detergents and contaminants. Pellets of the protein-NDs collected by centrifugation at 12 500 rpm for 3 min were then washed two times by 200 μL of washing buffer (50% ACN). To analyze the protein complex by MALDI-MS, we used a matrix solution consisting of 30 mg dihydroxybenzoic acid (DHB) in 70% ACN/0.1% formic acid (FA). Typically, 10 μL of the matrix solution was added and mixed thoroughly with the 15 μg washed protein-ND sample as described above. Only 1 μL of the mixture containing 1.5 μg of NDs (with ~0.05 μg pMMO) was deposited onto the stainless steel plate for MALDI-TOF-MS analysis. For comparison, an aliquot (0.5 μL, with about 0.01 μg pMMO) of the protein sample without nanoparticle

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treatment was also deposited on the probe, to which 0.5 μL DHB solution was admixed and then vacuum-dried.

Nanodiamond Surface-Enhanced Digestion of Membrane Proteins. Digestion of pMMO on the surface of the NDs was carried out according to the procedures of an earlier publication with some modifications.[18] Details of the digestion protocol can be found in Supporting Information.

For digestion of the complicated membrane proteome from *E. coli*, we have developed a new procedure that takes advantage of the unique tolerance of the NDs for SDS. First, 20 μg of the proteins in the pellet was completely solubilized in 50 μL solution containing 2% SDS, 4 M urea, and 25 mM NH_4HCO_3. To speed up the solubilization, the sample was subjected to ultrasonication for 15 min in a water-bath sonicator. In-solution reduction and alkylation were performed by adding 5 μL of 250 mM DTT for 1 h at room temperature before adding 6 μL of 550 mM iodoacetamide (IAA), and then the mixture was kept in the dark for 45 min. To extract the proteins by the SPEED protocol, 50 μL of MeOH and 200 μg of nanoparticles were added and incubated for 5 min. A 500 μL portion of 4 M urea was then added into the solution in order to dilute impurities before collecting the protein-nanodiamond complexes by 13 000 rpm/3 min/RT centrifugation. The protein-nanoparticle pellet was washed twice with 500 μL of 4 M urea to clean up any residual impurities (especially SDS) and finally once with deionized H_2O. Enzymatic digestion of the proteins adhering to the surface of the NDs was performed at 37 °C overnight by trypsin (enzyme:protein = 1:20) in 50 mM NH_4HCO_3 buffer. Digested peptides were automatically released from the nanoparticles into the supernatant and were collected by a simple centrifugation (13 000 rpm/3 min/RT). Collection of any remaining peptides was done by washing the nanoparticles with 100 μL of 25 mM NH_4HCO_3. Zip-tip C18 procedure was also conducted for desalting digested peptides in compliance with guidelines set by the MS core facility. The final collected peptides were dried in a Savant Speedvac concentrator (Thermo Scientific) and stored at −20 °C until use.

Nano-LC-MS/MS and Bioinformatic Analysis. Details on the mass spectrometric identification of digested peptides from pMMO samples and *E. coli*'s membrane proteins are supplied in the Supporting Information.

For protein identification, at least two unique peptides with minimum of 7 amino acid residues were taken to be required for confident assignments. The false discovery rate was evaluated by searching against a randomized decoy database created by MASCOT using identical search parameters and validation criteria. Protein localization was assigned by GO Slim annotation using Proteome Discovery version 1.3 (Thermo Scientific). The number of transmembrane helices in each of the annotated proteins was predicted by TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Protein GRAVY scores were determined using the GRAVY Calculator (http://www.gravy-calculator.de/). Proteins annotated in GO as membrane, or with TM domains according to the TMHMM algorithm, or positive GRAVY scores, were classified as membrane proteins.

RESULTS AND DISCUSSION

Rapid and Efficient Extraction, Enrichment of Membrane Proteins. Detergents and salts are indispensable in the isolation and purification of membrane proteins. Before MS or even SDS-PAGE analysis, however, one needs to reduce or completely remove salts, detergents, or other impurities from membrane protein samples to minimize or eliminate their interferences. The frequently applied methods to accomplish this include dialysis or membrane-based centrifugal filtering, but these procedures are tedious and time-consuming. Alternatively, we can resort to a combination of ion exchange beads with reversed-phase adsorptive materials, although here there might be concerns related to sample loss and low impurity tolerance. In this work, we demonstrate the ease and versatility of working with the SPEED platform in the preparation of membrane proteins for SDS-PAGE, as well as for MS analysis especially. To test the adsorption of NDs toward membrane proteins, we have selected the multisubunit integral membrane protein pMMO as a test protein. We have also extended the study to include the membrane proteome of *E. coli* to evaluate the efficacy of the SPEED approach for shotgun proteomics of a model organism.

pMMO Protein Complex. Figure 1 shows SDS-PAGE analysis of the pMMO associated with the ND-protein complexes derived from 6 μg of pMMO enriched by SPEED from various commonly used preparation media: DDM (2%), Triton X-100 (2%), Tween (2%), Digitonin (2%), CHAPS (2%), urea (8 M), and a mixture of detergents and denaturants composed of 2% DDM, 2% CHAPS, and 4 M urea in 25 mM NH_4HCO_3. Since it takes less than 5 min to harvest the solubilized membrane proteins from the large sample volume (500 μL of working solutions) at low protein concentration (~50 nM), it is evident that the SPEED platform works very satisfactorily.

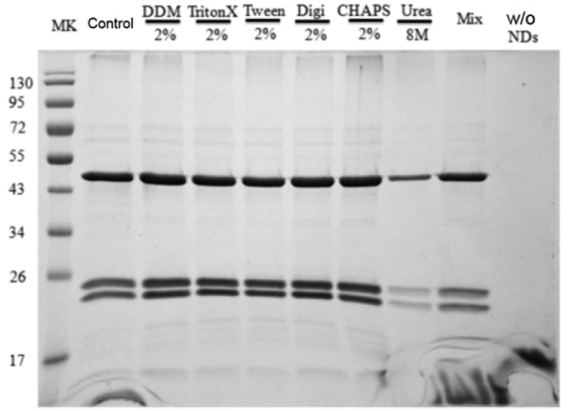

Figure 1. SDS-PAGE analysis of pMMO extracted from solutions containing different detergents or a mixture of detergents (mix) using NDs. In this experiment, 200 μg of NDs are used to extract 6 μg of the pMMO proteins solubilized in 200 μL of the detergent solutions. Control experiment (control, the first well): direct loading of 6 μg proteins into the corresponding PAGE sample well. Digi- stands for digitonin. The last well (w/o NDs) represents sample without the addition of NDs to the pMMO solutions containing the mixture of detergents.

To assess the effectiveness of NDs in extracting pMMO from solutions containing varying concentration of SDS, 0.5 μL of pMMO in 2% DDM is mixed with 200 μL of solutions containing varying amounts of SDS, and NDs are added to capture the pMMO from these solutions. Judging from the intensity of the three protein bands on the SDS-PAGE gels, the ND-protein complexes are stable in solutions containing up to 1% SDS. The tolerance of the pMMO-NDs for SDS can be readily doubled to 2% simply by adding 50% MeOH to the sample solution. A previous study has shown that NDs lose their affinity for cytochrome *c* in solutions containing greater

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than 0.05% SDS.[18] Thus, the membrane proteins are more strongly absorbed by the NDs compared with cytosolic proteins, as expected. Interestingly, when the pMMO is solubilized in a solution composed of 4 M urea, 2% DDM, and 2% CHAPS, the NDs can still maintain absorbability toward the proteins in the presence of 1% SDS without the aid of MeOH (see Figure 2). The disruption of the interaction between SDS and proteins by other nonionic surfactants observed here suggests a novel approach to remove SDS from protein samples using the nanodiamond technology.

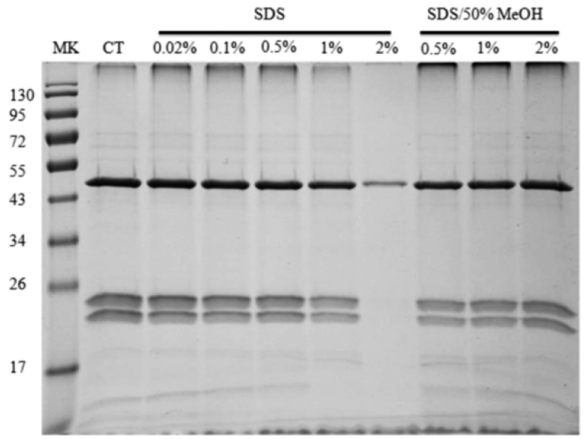

Figure 2. SDS-PAGE analysis of pMMO extracted from solutions containing varying concentrations of SDS in the presence of trace amount of urea, CHAPS, and DDM originating from the starting pMMO extraction buffer. The first well is the control experiment, which is the direct loading of about 6 μg of the pMMO to the well for comparison. The addition of MeOH enhances or restores the ND capturing ability up to 100%. The experimental conditions are the same as those used in Figure 1.

Apart from detergents, we have tried to use the SPEED platform to capture pMMO from solutions containing various salts, as demonstrated in Figure S1 in Supporting Information. Under these salt conditions we do not detect decrease in protein extraction efficiency. These buffers are commonly used in the fractionation of membrane proteins by column chromatography, so the SPEED platform may find a special niche in downstream sample cleanup in such procedures. Moreover, water and many common organic solutions do not adversely affect the binding of membrane proteins to the NDs so they can be used to wash off contaminants from the protein-nanodiamond complexes. To our best knowledge, the SPEED platform that we introduce here is the first and only technique that works so well toward membrane proteins in such a broad spectrum of purposefully admixed interfering agents.

E. coli Membrane Proteome. The ND technology works equally satisfactorily well even for a complex mixture of membrane proteins. It is generally considered a challenging task to prepare samples of the membrane proteome for proteomic analysis. The capability of SPEED to handle a membrane proteome is illustrated in Figure S2, where we show the SDS-PAGE analysis after extraction of the membrane proteins from *E. coli* by SPEED. It is apparent from comparison of the gels that different detergents do not affect the ability of the NDs to capture the membrane proteins. Even the presence of up to 2% SDS can be tolerated by SPEED on the proteome scale with molecular weights as low as 10 kDa. Moreover, different patterns of proteins are observed in the gels when the SPEED is carried out at different pHs. Figure 3 demonstrates the ability of NDs to simplify the membrane proteome of *E. coli* solubilized in a detergent solution consisting of 4 M urea, 2% DDM, and 2% CHAPS at three different pH values. Smaller proteins are extracted at pH 7, while the pattern at pH 2 is identical to that found for the original solution (control sample, CT). There are also many differences (compared to the control solution) in the region of mild-to-low molecular-weight proteins extracted into an alkali solution (pH 12). Although the separation observed here is not sharp, the pH dependence of the affinity for the proteins by the NDs can indeed be exploited for protein analysis.[20] This method offers an economic and fast alternative way to simplify a complicated membrane proteome. This approach has previously been exploited to selectively capture proteins in the human urine at three pH values of 3, 7, and 11 with very distinct protein patterns observed in the SDS gels.[18]

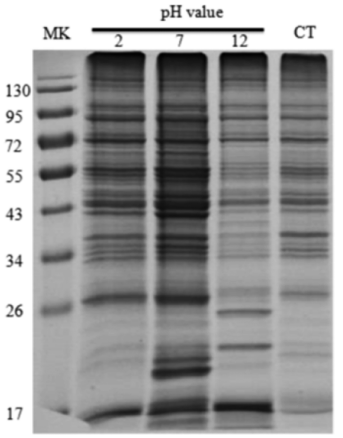

Figure 3. pH-dependent enrichments of *E. coli* membrane proteins by NDs at three different pH values (2, 7, and 12). The control experiment: CT, direct loading of ~20 μg of proteins.

It has been proposed that NDs interact with proteins by different forces including ionic and hydrophobic interactions. The nanodiamond/protein ratio used in our experiments is typically less than 20, which is much lower than that used earlier with soluble proteins (nanodiamond/protein ~50).[18] The intrinsic higher hydrophobicity of membrane proteins ensures stronger interactions between the proteins and the nanoparticles so that a lower nanodiamond/protein ratio suffices for solid-phase extraction and enrichment of membrane proteins.

The present study constitutes the first study that extends SPEED's streamlined application on cytosolic proteins[20] to include the extraction and enrichment of membrane proteins. To augment the initial systematic demonstration of SPEED on cytosolic proteins, we have conducted a parallel study summarizing the tolerance of surface-oxidized NDs toward commonly encountered contaminants in routine membrane-protein preparation protocols. These results are summarized in Table 1.

Nanodiamond-Assisted MALDI-MS Analysis of the pMMO Complex. Many approaches have been utilized to solubilize membrane proteins for improving MALDI-MS analysis such as the use of organic solvents, strong acids, and detergents. The performance of the MALDI technique, however, deteriorates markedly for samples containing excessive amounts of salts or detergents.[29−31] Kong et al.

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Table 1. Compability of Buffer Components for Extraction and Enrichment of Membrane Proteins

detergents	5% DDM; 5% Triton X-100; 5% Tween-20; 5% Digitonin; 5% CHAPS; 1% SDS in a detergent mixture (2% DDM, 2% CHAP, 4 M urea); 2% SDS in 40% MeOH.
salts	1.5 M NaCl; 1.5 M Tris HCl (pH 8); 1 M NH_4HCO_3
organic solvents	ACN; MeOH; ethanol
acidic solutions	5% FA; 5% trifluoro acetic acid (TFA)
others	6 M urea; 100 mM DTT; 200 mM IAA

have previously shown that the SPEED platform is superior for improved MALDI-MS analysis of soluble proteins.[20] Following up on this seminal work, we have developed SPEED further to analyze membrane protein complexes by MALDI-MS. We illustrate the power of the SPEED platform with the subunit analysis of pMMO, a membrane-bound protein complex comprising three distinct subunits 45 kDa (α or PmoB), 30 kDa (γ or PmoC), and 28 kDa (β or PmoA), respectively. The heterosubunit arrangement of the enzyme makes this system a good model to study the behavior of a transmembrane complex by MALDI-MS. Indeed, this membrane protein has also been used as a model protein for demonstrating PVDF-aided MALDI mass spectrometric analysis of membrane proteins.[32] This group presented improvements of intact pMMO subunit analysis by MALDI-MS of the protein solubilized in DDM micelles. We demonstrate here similar MS improvements; moreover, our approach can be applied to a broad range of detergents and salts. The advantage of the SPEED platform is that the NDs not only concentrate membrane proteins but also effectively remove impurities within minutes from the protein samples to optimize the application of modern mass spectrometry to the problem at hand.

The pMMO complexes solubilized by various detergents are extracted by NDs and MALDI-MS analysis is conducted on centrifugation-recovered protein-nanoparticle complexes. The left part of Figure 4 compares the results obtained with and without the use of NDs prior to MALDI-MS analysis for pMMO complex solubilized in the mild detergent 2% Triton X-100. The observed masses of all three subunits of pMMO (42 646 Da for PmoB, 29 687 Da for PmoC, and 28 287 Da for PmoA) have been clearly resolved in spectrum with the use of ND, and are in excellent agreement with their predicted masses (42 649 Da, 29 689 Da, and 28 294 Da, respectively, based on the gene sequences).[33] Equally high quality MALDI-MS spectra can be routinely acquired when the Triton X-100 is replaced with DDM and other commonly used detergents (Figure S3). The beneficial effect of SPEED is obvious. With the assistance of the NDs, there is clearly a big improvement in peak intensities with concomitant enhancements in the signal-to-noise (S/N). Since the patterns of the observed signals are the same as what we have obtained from MALDI-MS of this membrane-protein complex in detergent micelles,[25] the results suggest that the interactions of NDs with pMMO under the conditions of the SPEED experiment do not change the stoichiometry. However, unlike the interactions of lysozyme and other water-soluble proteins studied with NDs,[34,35] the native conformation of protein might not be totally preserved in the case of the membrane-protein complex here. X-ray absorption experiments on the copper ions of this multicopper protein clearly indicate that the native structure of the pMMO has been somewhat disrupted, although some of the copper ions are still associated with the protein scaffold and have retained the Cu^I oxidation state.

MALDI-MS analysis of pMMO has also been performed on protein samples directly suspended in other buffers such as 4 M urea, 1.5 M NaCl, and 1.5 M Tris-HCl pH 8.8 (see the right side of Figure 4 and Figure S4). Because salts and urea are destructive reagents at high concentrations, the obtained MS spectra here are totally different from the MALDI-MS of the pMMO complexes solubilized in the four mild detergents mentioned earlier (see left part of Figure 4). The most notable differences are in the peak intensities of the subunits, especially the absence of PmoC peak at m/z 29 700 Da in 1.5 M NaCl. With the NDs, the proteins are extracted onto the surface of the nanoparticles from the highly contaminated solutions, followed

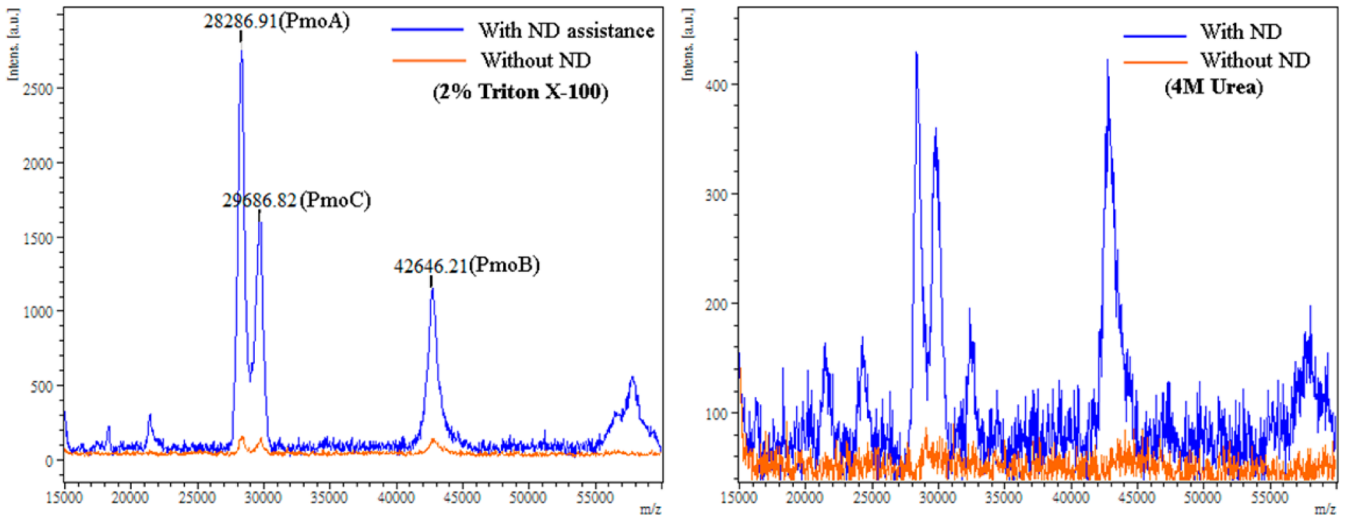

Figure 4. Demonstration of nanodiamond-assisted MALDI-MS for the analysis of the pMMO complex solubilized in the mild detergent 2% Triton X-100 (left) and in 4 M urea (right). For experiments conducted without the aid of the nanoparticle (labeled "without ND"), 0.5 μL protein-detergent micelles at a concentration of 50 nM and 0.5 μL matrix are directly deposited on the MALDI plate for analysis. The blue signals (labeled as "with ND") are obtained by MALDI-TOF analysis of the pMMO complex extracted onto the surface of diamond nanoparticles (for details see section on Nanodiamond Surface-Enhanced Digestion of Membrane Proteins in the Experimental Section).

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by a quick cleansing step, and MALDI-MS signals with good *S*/*N* are observed (see the right part of Figure 4, blue signals). It is not surprising that the presence of high concentration of salts or urea precludes the direct analysis of pMMO by MALDI-TOF-MS (see the right part of Figure 4 and Figure S4, bronze signals).

The SDS-PAGE analysis of pMMO-ND complex captured under various denaturing conditions (see Figures 1 and S1) shows no loss in any of the three subunits. However, the band intensities observed are somewhat different from the MALDI-MS intensities of the pMMO in mild detergent micelles (see left part of Figure 4 and Figure S3). These results suggest that the ionization efficiencies of the three subunits are not the same, as might be expected.

Taken all together, the observed MALDI-MS signals of (i) pMMO eluted from ND-pMMO complexes with mild, nondestructive detergents and (ii) pMMO suspended in destructive reagents reveal that MALDI-MS analysis with the aid of NDs may be used to probe the effects of a strong detergent on the assembly and conformation of a membrane-protein complex.

ND Surface-Enhanced Digestion of Membrane Proteins. As demonstrated earlier, nanodiamonds not only preconcentrate proteins on their surface but also attract certain analytes, e.g., proteases, from the solution. Thus, the NDs serve as ideal substratum for the proteolytic digestion of the membrane proteins adhering to the surface of the nanoparticles, a method which we will henceforth refer to as "ND surface-enhanced" protein digestion. As demonstrated in Figure S5, all the pMMO protein bands disappear just after 5 min of digestion. In the literature, accelerated enzymatic digestions are typically accomplished with the assistance of additional physical forces such as high intensity focused ultrasound,[36] microwave,[37] and high pressure.[38] With our protocol, no specialized facility is required. Figure S5 (lane 1 vs lane 2) also presents the additional advantage of the digestion protocol since there is no noticeable sample loss accompanying the reduction and alkylation of ND-adsorbed pMMO proteins.

To check the effectiveness of our ND surface-enhanced digestion on pMMO, we have varied the duration of incubation with the chymotrypsin enzyme and analyzed the digested peptides by nano-LC-MS/MS. The results are shown in Table S1. It is interesting that the sequence coverage does not change much when we increase the time of the digestion, and the results are the same for all three of the protein subunits. Although the best sequence coverage is obtained with 1 h of incubation, 5 min digestion suffices for the proteomic identification of the membrane protein with high confidence (Figure 5). This surprising result has not been noted in any other earlier reports.

Perhaps the efficiency of the proteolytic digestion of the pMMO is understandable, given that the native structure of the pMMO has been disrupted upon extraction of the protein complex onto the surface of the nanodiamond particles. Although the extent of unfolding of the protein scaffold remains to be clarified, outside of the detergent micelles or the bilayer membrane, the transmembrane helices of membrane proteins are expected to be preferentially associated with the hydrophobic surface of the nanodiamonds. With the backbone of the polypeptides more exposed to the solution, the protein should become more susceptible to proteolyic digestion, which might account for why only 5 min is required for the digestion to be nearly complete.

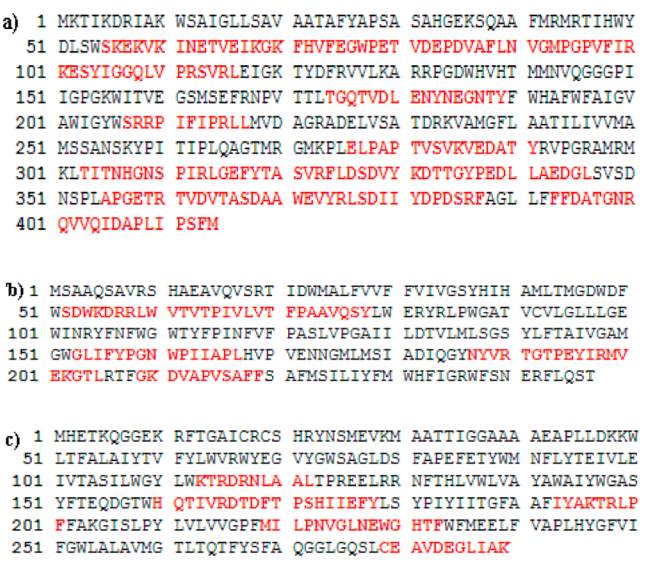

Figure 5. Sequence coverage of pMMO (red amino acids) digested within 5 min by on-surface nanoparticle digestion: 50% for PmoB (a), 30% for PmoA (b), and 23% for PmoC (c).

The power of SPEED as a new SDS removal and digestion platform has also been demonstrated with the analysis of whole membrane proteome from *E. coli*. As SDS is the most effective detergent in disrupting membranes and solubilizing proteins, it is preferably utilized in gel-based and filter-based membrane proteomics workflows.[7,9,11,12] However, the presence of this detergent in the peptide samples not only hinders the reversed-phase LC resolving power but also leads to some serious problems with analysis by mass spectrometry.[39]

We have discussed in the previous sections that NDs extract membrane proteins from 2% SDS solutions with ease. The residual SDS associated with the protein-ND complexes can be washed out with urea. SDS, therefore, has been used in our protocol to solubilize and facilitate the reduction and alkylation of membrane proteins in the solution phase before these proteins are extracted for ND surface-enhanced digestion. It is interesting that we do not observe any significant effects of SDS on the LC-MS/MS analysis of digested peptides generated by the SPEED procedure (data not shown), indicating the effectiveness of SDS removal by the ND technology. More than 400 proteins have been identified from a single 2-h 1D LC-MS/MS run of less than 1 μg of digested peptides prepared by ND surface-enhanced digestion of the *E. coli* membrane proteome, with a false discovery rate of 3.5%. Of these detected proteins, 70% are classified as membrane proteins (Figure S6 and list of identified proteins in Supporting Information) by using TMHHM, GO terms, and GRAVY scores.

Strong cation exchange chromatography (SCX) has recently emerged as an effective technique for SDS cleanup,[40,41] albeit with nontrivial sample loss. Using this method, Li and co-workers[40] have reported the identification of more than 300 proteins in a 250-min 1D LC-MS/MS analysis of the membrane proteome of *E. coli* prepared by SDS-assisted digestion. The best protein coverage obtained by the same 1D LC-MS/MS analysis came from the RapiGest-assisted digestion with 400 proteins identified, a number similar to what we have obtained in the present study. However, aside from sample loss, the removal of SDS by the SCX method requires additional sample handling to remove salts. When the shotgun proteomics is undertaken by the multidimensional protein identification

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technology (the MudPIT approach), the removal of SDS by SCX can be integrated into the first separation stage of a 2D LC-MS/MS system to reduce labor and sample loss.[41] However, in our work, the removal of SDS with the ND-based SPEED technology happens at the protein level by a simple washing step with urea. Nevertheless, the MudPIT approaches have come up with an impressive number of identified proteins from analyses of the membrane proteome of *E. coli*.[40−42]

The identification of proteins in membrane proteomes has not been the intent of our study. Rather, the goal of our work is to develop a general platform for membrane proteome cleanup, and toward this end, we have surveyed the membrane proteins solubilized in 2% SDS harvested with NDs by a 1D LC-MS/MS analysis to substantiate the protein bands observed by SDS-PAGE, as illustrated in Figure S2. As demonstrated by the fast shotgun proteomics of the *E. coli* membrane proteome, it is clear that the ND technology presented here constitutes a simple method of SDS removal and an effective substratum for membrane protein digestion. So, our SPEED platform brings to the proteomics community an attractive alternative to the two traditional "solution based" and "gel based" digestion approaches of membrane proteins. We are mindful that the "in-gel" digestion remains the only practical method for the assignment of individual protein bands on SDS-PAGE to subunits or specific proteins.[22]

CONCLUSIONS

We have developed a rapid streamlined sample-preparation procedure for mass spectrometric analysis of membrane proteins using surface-oxidized nanodiamond particles. This method extends the SPEED (solid-phase extraction and elution on diamond) platform, which has previously been found to be highly effective for MS sample preparation of soluble cytosolic proteins, to the extraction and enrichment of membrane proteins on nanodiamond particles. The technology is shown to yield stable protein-nanodiamond complexes, facilitating SDS-PAGE analysis of membrane proteins as well as improved MALDI-MS analysis and large scale shotgun proteomic studies.

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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