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A New Mass Spectrometry-compatible Degradable Surfactant for Tissue Proteomics

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SUPPLEMENTAL MATERIAL

Supplemental information includes the supplemental methods, results, and supplemental scheme 1 as well as 11 supplemental Figures, and 6 supplemental Tables.

Supplemental Scheme 1. Comparison of the degradation mechanisms of RapiGest (RG), ProteaseMax (PM) and MaSDeS under acidic conditions.

Supplemental Figure S1. High-resolution Fourier transform ion cyclotron resonance (FT-ICR) MS analysis of MaSDeS degradation in 10% formic acid (FA).

Supplemental Figure S2. Assessment of MaSDeS thermo-stability.

Supplemental Figure S3. MS evaluation of MaSDeS stability at 65 °C.

Supplemental Figure S4. Evaluation of the influence of MaSDeS on enzymatic digestion and MS analysis.

Supplemental Figure S5. The sequential tissue extraction method reduces blood protein contamination in the second heart tissue extract.

Supplemental Figure S6. SDS-polyacrylamide gel electrophoresis (PAGE) and protein assay analyses of sequential extracts from liver and lung.

Supplemental Figure S7. The effect of MaSDeS on protein identification.

Supplemental Figure S8. MS analysis of equal protein amounts (1 µg) from pig heart tissue extracts (Pre-extract, Control, and 0.2% MaSDeS).

Supplemental Figure S9. Quantitative MS results showing that the use of MaSDeS significantly improved the detection of membrane proteins in heart tissue extracts.

Supplemental Figure S10. MaSDeS degrades slowly under acidic conditions at 37 °C, in contrast to the rapid degradation observed for the two commercially available acid-labile surfactants, RapiGest (RG) and ProteaseMAX (PM).

Supplemental Figure S11. Buffer containing 0.2% of PM, RG, or MaSDeS was used to homogenize pig heart, liver, and lung tissue.

Supplemental table S1. Protein/peptide list: equal volume loading test in heart tissue

Supplemental table S2. Protein/peptide list: equal volume loading test in liver tissue

Supplemental table S3. Protein/peptide list: equal volume loading test in lung tissue

Supplemental table S4. Protein/peptide list: equal amount loading test in heart tissue

Supplemental table S5. Enriched function and human disease list

Supplemental table S6. Protein information of interactome

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Abstract

Tissue proteomics is increasingly recognized for its role in biomarker discovery and disease mechanism investigation. However, protein solubility remains a significant challenge in mass spectrometry (MS)-based tissue proteomics. Conventional surfactants such as sodium dodecyl sulfate (SDS), the preferred surfactant for protein solubilization, are not compatible with MS. Herein, we have screened a library of surfactant-like compounds and discovered an MS-compatible degradable surfactant (MaSDeS) for tissue proteomics that solubilizes all categories of proteins with performance comparable to SDS. The use of MaSDeS in the tissue extraction significantly improves the total number of protein identifications from commonly used tissues, including tissue from the heart, liver, and lung. Notably, MaSDeS significantly enriches membrane proteins, which are often under-represented in proteomics studies. The acid degradable nature of MaSDeS makes it amenable for high-throughput mass spectrometry-based proteomics. In addition, the thermostability of MaSDeS allows for its use in experiments requiring high temperature to facilitate protein extraction and solubilization. Furthermore, we have shown that MaSDeS outperforms the other MS-compatible surfactants in terms of overall protein solubility and the total number of identified proteins in tissue proteomics. Thus, the use of MaSDeS will greatly advance tissue proteomics and realize its potential in basic biomedical and clinical research. MaSDeS could be utilized in a variety of proteomics studies as well as general biochemical and biological experiments that employ surfactants for protein solubilization.

Keywords

Surfactant; Proteomics; Membrane Proteins; Mass Spectrometry; Tissue

INTRODUCTION

Mass spectrometry (MS)-based tissue proteomics is increasingly recognized as a highly valuable tool for biomarker discovery and mechanistic investigation since damaged tissues near the disease source contain the highest concentration of potential disease markers.^{1–11} Nonetheless, there are still significant challenges in tissue proteomics such as protein solubility issues, particularly for membrane proteins.^{12–15} Membrane proteins comprise approximately one-third of the proteome,^{12–14, 16–18} playing an important role in many essential cellular functions and accounting for a significant proportion of drug targets.^{19, 20}

To effectively extract proteins from tissues, surfactants (surface-acting agents, also known as detergents) are commonly used in biochemical research.²¹ Sodium dodecyl sulfate (SDS), owing to its outstanding performance in solubilizing proteins, is the most widely used surfactant.^{13, 21–24} Unfortunately, even very low concentrations (<0.01%) of SDS can severely suppress the ionization of proteins/peptides.^{21, 23, 25} Consequently, numerous methods have been developed to remove SDS prior to MS analyses.^{21, 22, 26–30} Although, gel-based methods can effectively remove surfactants, the protein coverage, sensitivity, and reproducibility of protein identification/quantification using these methods are relatively low in comparison to methods using direct in-solution digestion liquid chromatography (LC)/MS-based shotgun method.¹³ Unfortunately, in-solution removal of SDS has been known to be difficult and require multiple clean-up steps that often result in protein loss,

degradation, and are also time-consuming.^{21, 22, 27, 28} Some non-ionic detergents such as the non-ionic saccharides are MS-compatible at relatively low concentrations (<0.1%), but these surfactants disrupt lipid-lipid and lipid-protein interactions instead of protein-protein interactions and, therefore, are considered relatively mild surfactants.²³

To address these problems, acid-labile MS-compatible ionic surfactants have been developed.^{31–33} These surfactants contain an acid-labile functional group between the hydrophilic head and hydrophobic tail of the surfactant molecule. Upon addition of acid to the surfactant-containing sample, the surfactant quickly degrades into innocuous non-surfactant byproducts, thus eliminating the need to remove the detergent prior to MS analysis.^{31, 33, 34} However, commonly used acid-labile surfactants appear to be only mild denaturants and, consequently, are not as effective as SDS at solubilizing proteins.^{33, 34} Consequently, SDS still remains the surfactant of choice for the total solubilization of tissues and cells.^{21, 22, 28}

Herein, we aimed to develop a strong MS-compatible degradable ionic surfactant that can effectively solubilize proteins during sample preparation with similar performance to SDS. We have screened a library of forty-three surfactant-like compounds and discovered a mass spectrometry-compatible degradable surfactant (MaSDeS) that can solubilize all categories of proteins with comparable performance to SDS at the concentrations used in this study. Importantly, MaSDeS significantly enriched membrane proteins, which are often under-represented in proteomics studies. In addition, MaSDeS is slowly degradable in acidic conditions, which obviates the need to remove the surfactant prior to MS analysis and enable the high-throughput proteomics analysis. Furthermore, it is thermostable and, thus, can be used in experiments that require relatively high temperature to facilitate protein extraction and solubilization. These favorable characteristics make MaSDeS an ideal MS-compatible surfactant for proteomics studies. Here, we have shown that MaSDeS outperforms other MS-compatible surfactants, including ProteaseMAX (PM), RapiGest (RG), PPS Silent Surfactant (PPS), octyl β -D-glucopyranoside (OG), n-dodecyl β -D-maltoside (DDM), and digitonin (DGT), by both improving protein solubilization and increasing the number of identified proteins in tissue proteomics.

EXPERIMENTAL METHODS

Sequential tissue extraction

All experiments involving animals were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and using protocols approved by the University of Wisconsin Institutional Animal Care and Use Committee. Swine heart, liver, and lung tissue samples were excised from healthy Yorkshire domestic pigs, snap frozen in liquid N₂, and stored under –80 °C before use. All steps of the sequential tissue extraction procedure were performed in a cold room (4 °C). First, the frozen pig tissue samples (approximately 200–300 mg) were cut into small pieces (approximate 2 mm³) and immediately washed twice with cold PBS buffer containing a protease inhibitor cocktail (Roche, Switzerland). The washed tissue pieces were transferred to HEPES buffer (0.25 M sucrose, 25 mM HEPES at pH 7.4, 50 mM NaF, 0.25 mM Na₃VO₄, 0.25 mM PMSF, 2.5 mM EDTA, and 1 mg/mL protease inhibitor cocktail) and homogenized 5–6 times using a Polytron electric

homogenizer (Model PRO200, PRO Scientific Inc., Oxford, CT, USA) for 5–7 s on ice. The homogenate was centrifuged at 120,000 g for 30 min at 4 °C and the supernatant was removed and saved as the “Pre-extract”. The remaining pellet was re-homogenized in HEPES buffer with or without (Control) surfactant. After homogenization, the samples were incubated at 4 °C and placed in a vortex at 900 rpm for 30 min followed by 30 min of centrifugation at 16,000 g at 4 °C. All supernatants were collected and store at –80 °C for later study.

Protein solubility evaluation

After the first extraction described above, the homogenized tissue samples were evenly divided into smaller aliquots. In one aliquot, 25 mM ammonium bicarbonate (ABC) buffer was used as a control. Different surfactants were individually added to the other aliquots. The final concentrations of the surfactants in the aliquots were 0.1%, 0.2%, and 0.5%, respectively. The 25 mM ABC buffer was used to equalize the volume in all of the aliquots. An equal volume of each aliquot was subsequently resolved using 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) with a voltage of 55 V for 30 min and 120 V for an additional 1.5 h. Visuals of separated protein bands were created using Coomassie Brilliant Blue R-250. The protein concentration of each sample was determined using the Bradford assay (Bio-Rad, Hercules, CA) in accordance with the manufacturer’s protocol.

Measurement of critical micelle concentration (CMC)

The critical micelle concentration was determined using the Optimizer-BlueBALLS kit (G-Biosciences, MO, USA). In brief, 0.25 mL of different concentrations (mg/mL, %) of MaSDeS and SDS, from 0.0003 to 5%, were added to 1 Optimizer-blueBALL and then incubated for 4 h at room temperature. During the 4 h incubation, all samples were periodically vortexed. At the end of the incubation period, each sample was centrifuged at 6,000 g for 15 min. The supernatants were then transferred to a 96-well plate and the optical density was read at 650 nm.

Comparison of degradation rates

Surfactants were dissolved in 25 mM ABC buffer to a final concentration of 0.2% and incubated in acid (10%) or at high temperature (65 °C and 80 °C) to investigate their degradation rates. Each sample was diluted 50 times with isopropyl alcohol (IPA) buffer (IPA: acetonitrile: acetic acid: water = 20:40:1:39) and methanol with 1% ammonium hydroxide (1:2 v/v). All samples were analyzed using a 7T linear ion trap/Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (LTQ/FT Ultra, Thermo Scientific Inc., Bremen, Germany) equipped with an automated chip-based nano ESI source (Triversa NanoMate; Advion Bioscience, Ithaca, NY, USA). Each sample was introduced into the mass spectrometer using a spray voltage of –1.5 kV and a gas pressure of 0.5, resulting in a flow rate of 50–200 nL/min. Survey MS spectra in the mass range of m/z 120–450 were acquired in the FT-ICR with a resolution of 10,000 at 400 m/z and the maximum injection time was set at 3000 ms. The degradation rate (%) was the signal intensity of the hydrophilic head (degradpartition product) divided by the signal intensity of the hydrophilic head plus the signal of the intact surfactant.

Protein digestion

Each protein sample (20 µg for equal amount loading and 20 µL for equal volume loading experiments) was reduced with 20 mM dithiothreitol (DTT) at 65 °C and alkylated with 25 mM iodoacetamide (IAA) at room temperature, with each step requiring 1 h. The samples were all adjusted to a pH of 8. Modified trypsin [1:50 (w/w, trypsin/protein)] was added to each sample and the samples were incubated at 37 °C for 20 h to allow for complete digestion. Subsequently, 5% of acetonitrile with 1% of formic acid was added to each sample to stop the reaction. The samples were then centrifuged at 16,000 g at 4 °C for 1 h before the supernatants were collected for MS analysis.

MS analysis of tissue samples (Q Exactive orbitrap)

A Q Exactive mass spectrometer (Thermo Scientific, Bremen, Germany) with a nanoelectrospray ion source coupled to a nano flow UPLC (Waters LC nanoACQUITY, MA, USA) was used to perform MS analyses. A 180 µm × 20 mm trap column (Symmetry C18) and a 75 µm × 100 mm Waters C18 analytical column (1.7 µm particle BEH) with mobile phases A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile) were also used. The pump flow rate was set to 0.35 µL/min, and peptides (1 µg) were contained in the trap column for 10 min before elution was achieved. A linear gradient of 5–35% B was used for the first 130 min followed by a rapid increase to 95% B over the following 20 min. The column temperature was kept at a constant 28 °C. The electrospray voltage was set at 1.9 kV. The Orbitrap mass analyzer performed full MS scans over the range m/z 300–2000 with a mass resolution of 70,000 (at m/z 200). The automatic gain control target value was 1.00E+06 with a maximum injection time set at 100 ms. The 15 most intense ions with charge states ≥ 2 were fragmented in the HCD collision cell using normalized collision energy of 30%. Tandem mass spectra were acquired in the Orbitrap mass analyzer set to a mass resolution of 17,500 at m/z 200 (automatic gain control target 1.00E+05, 30 ms maximum injection time). The ion selection threshold was 3,300 for MS/MS. Dynamic exclusion of the sequenced peptides for 20 s was used to minimize the repeat sequencing of peptides.

Protein identification and quantification

The SEQUEST-based Proteome Discoverer (Thermo Scientific; version 1.4) was used to analyze all MS/MS samples and was set to search *Sus scrofa* database (24,476 entries), which was downloaded from NCBI (ftp://ftp.ncbi.nih.gov/genomes/Sus_scrofa/protein/)³⁵ with trypsin as the enzyme. The search settings that were used allowed for data containing two missed cleavages and the mass tolerances for the precursor and fragment ion masses were 10 ppm and 0.02 Da, respectively. The carbamidomethyl of cysteine was specified as a fixed modification, whereas deamidated asparagine and glutamine as well as oxidated methionine were set as variable modifications. The data was further searched against a decoy database and filtered using a 1% false discovery rate. Peptides with high confidence, rank 1, and $\Delta C_n < 0.1$ were accepted. The proteins identified as common trypsin autolysis peaks as well as keratin contamination were excluded. The function that included distinct proteins in the search was enabled. The Proteome Discoverer plugin, InforSense, was used for collecting Gene Ontology data. Further information regarding other relevant

biological processes for each protein was retrieved from the AmiGo database. For quantification, normalized peptide intensity was used to adjust for median signal variances from run-to-run. All peptides corresponding to their respective proteins in each run were calculated for their geometric mean, which provided the protein's final abundance. The lowest protein intensity divided by 10 was assigned to unidentified proteins.

Bioinformatics and statistical analyses

The protein information, such as subcellular locations and biological processes, were classified during the database search by Proteome Discoverer. If the proteins could not be classified by Proteome Discoverer, the Gene Ontology and Uniprot databases were manually checked for those proteins. TMHMM was performed to predict transmembrane helices in proteins.³⁶ STRING database/software was used to perform function enrichment and interactome prediction.³⁷ This study used SPSS software (SPSS 18.0, Chicago, IL) and two-tailed tests, with a statistically significant p-value of less than 0.05, which was applied to all tests. The Mann-Whitney U and Kruskal-Wallis tests were used to determine the differences between groups.

Western blot

Equal amounts of protein (50 µg) from different samples were loaded and resolved on 12.5% SDS-PAGE gels. When transferring the proteins to a PVDF membrane, a Fast Semi-Dry Blotter (Fisher Scientific, Waltham, MA, USA) was used in accordance with the manufacturer's protocol. The membrane was placed in a protein-free blocking buffer (Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature and incubated with primary antibodies overnight (4 °C). The membranes were then washed with TBST five times before incubation with horseradish peroxidase-conjugated secondary antibodies for 50 min at room temperature. Before the membranes were developed using enhanced chemiluminescence detection, they were washed with TBST five more times.

RESULTS

Identification of a mass spectrometry-compatible degradable surfactant (MaSDeS)

To identify an optimized surfactant for tissue proteomics, we screened a library of forty-three surfactant-like compounds (synthesized by Promega Corporation; structural and other information regarding these surfactants can be found in the patent)³⁸ based on their effectiveness in solubilizing proteins from tissue extracts and discovered a compound, sodium 3-(((1-(thiophen-3-yl)undecyl)oxy)carbonyl)amino)propane-1-sulfonate (Figure 1A), as the best candidate. This surfactant has high structural similarity to SDS including a sulfonate anion as the hydrophilic head and a long alkyl chain as the hydrophobic tail. In addition to the traditional features of surfactant molecules, it has an acid-labile functional group (thiophenyl carbamate) which separates the hydrophilic head and hydrophobic tail, and plays an essential role in the acid degradability of the surfactant. Under acidic conditions, the surfactant will undergo acid hydrolysis resulting in a neutral compound and a small zwitterionic species (Figure 1A) (Supplemental Scheme 1).

Next, we assessed the degradation rate of this surfactant in acidic conditions (pH 2–3) and found that it is progressively degraded over the course of 24 h (and thus referred to as Mass Spectrometry-compatible Degradable Surfactant, MaSDeS, hereafter) (Figure 1B and Supplemental Figure S1). Subsequently, we determined the CMC (the minimal surfactant concentration at which micelles are observed) of this surfactant to be 0.007% in comparison with 0.03% for SDS at room temperature, confirming that very low concentrations of MaSDeS are sufficient to form micelles in solution (Figure 1C). Furthermore, we found that MaSDeS is thermostable. After incubation at either 65 °C or 80 °C for 24 h, only 4% and 6% of degradation products were detected by FT-ICR MS, respectively (Supplemental Figure S2–3). It is important to note that MaSDeS, at concentrations up to 0.5% surfactant, had minimal effects on digestion efficiency and MS analysis, proving the MS compatibility of this surfactant (Supplemental Methods and Supplemental Figure S4).

The effectiveness of MaSDeS for solubilizing proteins from commonly used tissues

Given the favorable characteristics of MaSDeS, we next investigated the effectiveness of this surfactant for solubilizing proteins from commonly used tissues (heart, liver, and lung) with a fast and simple sequential extraction method to minimize contamination from highly abundant blood proteins (Figure 1D, Supplemental Results). After the first homogenization, the Pre-extract contained highly abundant soluble proteins such as albumin and other blood proteins (Supplemental Figure S5). The pellets from the pre-extraction were then homogenized again with and without surfactant (Figure 1D). We found that the inclusion of MaSDeS in the extraction buffer greatly improved the solubilization of proteins in all three types of tissue (heart, liver, and lung) extracts and performed at a level comparable to SDS, as evidenced by SDS-PAGE (Figure 1E and Supplemental Figure S6A–B) and Bradford protein assay (Figure 1F and Supplemental Figure S6C–D) at 0.1%, 0.2%, and 0.5% of the surfactant concentration. Subsequent LC-MS/MS analysis of equal volumes of swine tissue extracts, from all three tissues, confirmed the increase in the total amount of protein in samples containing MaSDeS. Each type of tissue was homogenized, equally divided, and then either MaSDeS or 25 mM of ABC buffer (for Control) was added. The equal volume experiment allowed us to assess the effectiveness of MaSDeS for solubilizing and identifying proteins during MS analyses. Each sample was run in triplicate (Supplemental Tables S1–3). Over 90% of proteins could be identified in at least 2 runs showcasing the high reproducibility of this extraction method. The number of proteins identified in swine heart, lung, and liver extracts, generated with extraction buffer containing MaSDeS, were significantly improved compared to the Control extracts (Supplemental Figure S7). In the MaSDeS-containing heart tissue extracts, the results represented a greater than twofold increase in the number of proteins identified (Supplemental Figure S7A). The protein intensity distribution and the sum of the protein intensities in each extract revealed that tissue extracts containing MaSDeS have much higher protein intensity profiles than their respective controls (Supplemental Figure S7B–C), suggesting an increase in the total amount of proteins solubilized in MaSDeS-containing extracts. These results further underscore the effectiveness of MaSDeS for solubilizing proteins, which greatly improves protein identification.

Improvement of protein identification using MaSDeS

The swine heart tissue served as a model to investigate the improvement of protein identification using MaSDeS. An equal amount of protein with or without 0.2% MaSDeS (MaSDeS and Control, respectively) was analyzed by LC-MS/MS, we observed an increase in the total number of proteins identified in MaSDeS-containing tissue extracts. A total of 1,113 proteins were identified in samples containing MaSDeS versus 778 proteins identified in Control in triplicate analyses (note that the reason the number of identified proteins is lower than other tissue proteomics studies is due to the fact that the swine protein database remains incomplete) (Figure 2A, Supplemental Figures S5A and S8, Supplemental Table S4). High reproducibility was observed across MS technical replicates with $80.5 \pm 12.3\%$ and $79.6 \pm 2.0\%$ of the proteins identified in Control and MaSDeS extracts, respectively, identified in all three replicates. Additionally, the coefficient of variation (CV) for the protein intensities in different MS technical replicates was less than 30% for approximately 80% of the proteins identified in the Control and MaSDeS-containing extracts, allowing for increased confidence in protein quantification (Supplemental Figure S8C).

Next, to determine what categories of proteins were solubilized by MaSDeS, we performed Gene Ontology analysis to investigate the subcellular locations of the proteins identified in heart tissue extracts with and without MaSDeS (Figure 2B). A total of 481 membrane proteins (43% of the total identified proteins) were identified in swine heart tissue samples extracted with buffer containing MaSDeS, which is nearly a two-fold increase compared with Control. In addition, the summed intensities of the membrane proteins identified in swine heart tissue extracts containing MaSDeS were approximately two times higher than in Control (Figure 2C). Furthermore, proteins identified in MaSDeS-containing extracts displayed the highest frequency of transmembrane helices (Figure 2D).

To further validate these findings, the presence of well-known membrane proteins, such as the $\text{Na}^+\text{-K}^+$ ATPase, voltage-dependent anion channel 1 (VDAC1), and phospholamban (PLN), in the extracts were confirmed by Western blot (Figure 2E) and label-free quantitative MS (Figure 2F-J and Supplemental Figure S9). Notably, the enrichment of these proteins in MaSDeS-containing extracts was comparable to that observed in samples with SDS, as shown in the Western blot data (Figure 2E).

Characterization of proteins uniquely identified in MaSDeS-containing extracts

Interestingly, of all of the proteins uniquely identified in the heart tissue extracts containing MaSDeS, membrane and endoplasmic reticulum (ER)/Golgi proteins were enriched in comparison to proteins from other subcellular locations (Figure 3A). Among them, 226 proteins were characterized as membrane proteins. The main biological processes that the 226 membrane proteins uniquely identified in extracts containing MaSDeS are most likely involved in are metabolic processes, transport, and cell communication, based on the protein annotations from the Gene Ontology database (Figure 3B). Additionally, the TopFun software was used to identify the potential functions and disease associations of the 226 membrane proteins (Supplemental Table S5). The proteins were implicated in the regulation of significant cardiac functions and diseases, such as cardiovascular system development, heart contraction, the respiratory electron transport chain, and sarcoplasmic reticulum

calcium ion transport, as well as cardiomyopathy and cardiac arrhythmia (Supplemental Table S5).

Furthermore, the STRING database predicted that 130 out of the 226 membrane proteins could form five interactomes, with several significant networks that were involved in membrane trafficking, mitochondrial electron transport chain, cell adhesion, and cytoskeletal organization (Figure 4 and Supplemental Table S6). For instance, there are 14 proteins involved in the assembly of the mitochondrial NADH-ubiquinone oxidoreductase complex (NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13 (NDUFA13), NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 4 (NDUFS4), etc.).³⁹ Other membrane proteins that were identified, such as VDAC3, TIMM50, TOMM22, and metaxin-1 (MTX1), are involved in the formation of channels in the inner or outer mitochondrial membranes that allow for the diffusion and transport of proteins, small molecules, and chemicals in and out of the mitochondria.⁴⁰ The proteins PLN, ankyrin-1 (ANK1), and ankyrin-2 (ANK2) indirectly regulate the movement of ions (e.g. calcium and sodium) and energy metabolism in cardiomyocytes.^{41, 42} Furthermore, the membrane proteins integrin beta-1 (ITGB1), integrin beta-2 (ITGB2), and integrin alpha-5 isoform 1 (ITGA5) form a network involved in the regulation of cell adhesion and cytoskeletal organization.⁴³ The functions of each protein shown in the interactome network are listed in Supplemental Table S6.

Comparison of MaSDeS with other commonly used MS-compatible surfactants

We compared MaSDeS and other commonly used MS-compatible surfactants, including ionic (ProteaseMAX (PM) and RapiGest (RG)), zwitterionic (PPS Silent Surfactant (PPS)), and non-ionic (octyl β -D-glucopyranoside (OG), n-dodecyl β -D-maltoside, also known as n-dodecyl- β -D-maltopyranoside (DDM), and digitonin (DGT)) surfactants^{23, 44} based on their ability to solubilize proteins from heart, liver, and lung tissues (Figure 5). At low concentrations (<0.1%), non-ionic saccharide surfactants, such as DDM and OG, are known to be MS-compatible.^{23, 25, 44} The existing acid-labile MS-compatible surfactants, PM, RG and PPS, have been developed to improve in-gel proteolytic performance for bottom-up MS analysis.^{20, 31, 33, 34} Recently, PM has also been utilized in other applications, including cell lysis.⁴⁵ However, these surfactants have not yet been tested for their performance in tissue proteomics and, thus, we compared these commonly used MS-compatible surfactant with MaSDeS side-by-side for their abilities to extract proteins from heart, liver and lung tissue. The extraction was performed as follows: each type of tissue was homogenized, equally divided, and then either surfactant (to a final concentration of 0.2% surfactant) or 25 mM of ABC buffer (for Control) was added. The SDS-PAGE and protein assay results showed that MaSDeS performs significantly better in solubilizing proteins than these commonly used surfactants, RG, PM, PPS, DDM, DG and DGT, in all three types of tissues (Figure 5). PM and RG demonstrated much better protein solubilization ability than PPS, OG, DDM, and DGT.

We next compared the degradation rate of MaSDeS with the two leading commercially available acid-labile surfactants, RG and PM, in acidic conditions (pH 2–3) and found that MaSDeS degrades much slower than PM and RG (Supplemental Figure S10). Subsequently,

LC-MS/MS analyses of equal volume experiments were performed to assess the effectiveness of MaSDeS, PM, and RG for identifying proteins during MS analyses. Each sample was run in triplicate (Supplemental Table S1–3). Over 90% of proteins could be identified in at least 2 runs showcasing the high reproducibility of this extraction method. The number and intensity of proteins identified in swine heart, lung, and liver tissue extracts generated with extraction buffer containing MaSDeS were significantly increased (Figure 6, Supplemental Figure S11) compared to the Control, PM, and RG extracts.

DISCUSSION

MaSDeS is a strong MS-compatible surfactant for tissue proteomics

Protein solubility is a major challenge in tissue proteomics, particularly for membrane proteins.^{12–14} In this study, we have identified an MS-compatible degradable surfactant (MaSDeS), which can effectively solubilize all categories of proteins, including membrane proteins, from tissue sources commonly used in proteomic analyses. As demonstrated, the performance of this surfactant was comparable to the strongest surfactant, SDS, in solubilizing proteins from tissue extracts. It is MS-compatible and greatly outperforms the existing MS-compatible surfactants.

In general, surfactants (also known as detergents) can be categorized into three major groups: ionic, non-ionic, and zwitterionic.²³ Ionic surfactants, such as the classical anionic surfactant SDS, are strong denaturants and highly efficient at solubilizing proteins.²⁴ This stems from two features of SDS: a long hydrocarbon tail that interacts with polypeptides and breaks intra-protein interactions and an anionic head group that can interact with positively charged amino acids in the polypeptide chain. The interaction between the anionic head group and the polypeptide chain disrupts inter- and intra-protein electrostatic interactions, thereby promoting the disruption of protein tertiary structure and preventing protein aggregation.²³ Ionic surfactants such as SDS, despite being the strongest surfactants, are unfortunately not compatible with MS analyses since they can inhibit enzymatic digestion and severely suppress the ionization of proteins/peptides.^{21, 23, 25} Non-ionic surfactants, such as OG, DDM, DGT, Triton X-100, and NP-40, also contain a hydrocarbon tail but, unlike ionic detergents, lack a charged head group. Consequently, these surfactants cannot effectively disrupt protein-protein interactions and, therefore, are considered relatively mild solubilizing agents.²³ Although some of the non-ionic saccharide surfactants such as DDM and DG are MS-compatible at low concentrations (<0.1%),^{44, 46, 47} other polymer-based non-ionic surfactants such as Triton X-100 and NP-40 can cause significant signal suppression and high polymer background in addition to the formation of adducts with the proteins,²⁵ and thus are not selected in this study. Zwitterionic surfactants, such as CHAPS, are also MS-compatible at low concentrations (<0.1%) but, unfortunately, they also lead to adduct formation and signal suppression.²⁵ Zwitterionic surfactants have intermediate protein solubilizing abilities; being stronger than non-ionic detergents but not as strong as ionic surfactants.²³

Subsequently, MS-compatible surfactants have been developed such as RG and PM, which are ionic surfactants,^{31–33} in addition to PPS, which is zwitterionic. These surfactants differ from conventional surfactants in that they contain an acid-labile functional group. Thus,

upon addition of acid to the surfactant-containing sample, the surfactant quickly degrades into innocuous non-surfactant byproducts—eliminating the need to remove the detergent prior to MS analysis.^{31–33} It should be noted that, while these surfactants are supposed to enhance trypsin digestion efficiency, they are frequently used in combination with gel-based methods.^{33, 48, 49} This may be partly explained by the fact that higher concentrations of RG (>0.5%) have been shown to influence the digestion efficiency.³¹ The data presented here showed that PM and RG perform at a similar level in solubilizing proteins, but are inferior to MaSDeS and SDS (Figure 5). The zwitterionic surfactant PPS was not as effective as RG or PM for protein solubilization, which is consistent with previous studies comparing RG and PPS.⁵⁰ In addition, non-ionic surfactants, such as DDM, have also been utilized to solubilize integral membrane proteins for the MS analysis of protein complexes.⁴⁴ Nevertheless, the commonly used MS-compatible non-ionic surfactants, OG, DDM, and DGT, poorly solubilized proteins in aqueous solution compared with ionic surfactants (Figure 5), which is consistent with previous studies.²³ Thus, our data demonstrates that MaSDeS's protein solubilizing capability is greater than those of currently available MS-compatible surfactants and is on par with those of SDS, the gold standard surfactant for protein solubilization (Figure 1D and 1E, Figure 5). Furthermore, the CMC of this surfactant is very low compared to SDS at room temperature (Figure 1C). Low CMC provides an important advantage of MaSDeS as less surfactant is needed for protein solubilization.

The unique characteristics of MaSDeS: slow degradation and thermostability

Unlike PM and RG, which degrade rapidly under acidic conditions (both PM and RG were completely degraded within 30 min under acidic conditions), MaSDeS started degrading after an hour of incubation at pH 2–3 (Supplemental Figure S10). The observed differences in the degradation times for these surfactants under acidic conditions are likely due to the differences in their chemical properties. RG contains a ketal functional group, which is highly susceptible to hydrolysis under acidic conditions (Supplemental Scheme 1A). Consequently, at pH <5, this functional group is rapidly cleaved, generating a hydrophilic anionic molecule and a hydrophobic molecule ($\text{CH}_3(\text{CH}_2)_{10}\text{COCH}_3$).³¹ On the other hand, MaSDeS has a similar molecular structure to PM with the exception that it contains a thiophene ring in place of the furan ring in PM (Supplemental Scheme 1B). Under acidic conditions (pH <2), the carbonyl oxygen atom would be protonated and the C-O σ -bond would be cleaved, forming a carbocation that is stabilized by a furan ring (or thiophene ring) as an intermediate. Compared to the thiophene group (in MaSDeS), the furanyl group (in PM) is a better electron-donating group and, therefore, promotes rapid cleavage of the C-O σ -bond (see mechanistic details in Supplementary Scheme 1B). Subsequently, the resulting carbamate species will undergo decarboxylation [loss of carbon dioxide (CO_2)]. This provides a strong driving force for degradation of the surfactant into CO_2 and a zwitterionic species, similarly as proposed for PM.³³ Therefore, it is expected that MaSDeS, with its thiophene ring, is slowly degraded into an alcohol, CO_2 , and a zwitterionic species (Figure 1A), compared to PM, which will degrade more quickly due to the furan ring (Supplemental Scheme 1B). This slow degradation under acidic conditions will allow membrane proteins to remain in solution for a longer time and, thus, may have contributed to the better solubilization and increase in membrane protein identifications in MaSDeS-containing samples (Supplemental Figure S10).

In addition to relatively slow degradation under acid conditions, MaSDeS was also found to be thermostable, with minimal degradation observed up to 12 h after incubation at 80 °C (Supplementary Figure S2). This is likely related to the fact that the furan ring is a better electron-donating group than the thiophene ring as discussed above. The thermostability allows MaSDeS to be used in experiments that require relatively high temperature to facilitate protein extraction and solubilization. For example, some membrane proteins are difficult to be solubilized at room temperature even in the presence of high concentrations of SDS.⁵¹ These unique characteristics of MaSDeS, thermostability and relatively slow degradation under acidic conditions (Supplemental Figure S2–3 and Supplemental Figure S10), may make MaSDeS useful in a wide range of tissue proteomics applications. For instance, histones and myofilament proteins are extracted using acidic conditions^{7, 52–54} and, thus, the addition of MaSDeS could help maintain and/or increase protein solubility during the extraction process. It has also been demonstrated that high temperature (60 °C) chromatographic separation dramatically improved the recovery of hydrophobic peptides derived from the transmembrane domains of integral membrane proteins.^{12, 23}

MaSDeS improves membrane protein identification in tissue proteomics

Membrane proteins play an important role in many essential cellular functions and account for a significant proportion of drug targets.^{20, 55–59} However, membrane proteins are notoriously difficult to study due to their hydrophobic nature, which makes them difficult to solubilize. Consequently, membrane proteins are under-represented in proteomics studies.^{12, 13, 16, 23, 60} As demonstrated in this study, the use of MaSDeS during tissue extraction leads to a significant enrichment of membrane proteins. We have shown that the number of membrane proteins identified in extracts generated using buffer containing MaSDeS was greater than for control extracts (no surfactant) (Figure 2B and Supplemental Table S4). Notably, 43% of the total identified proteins in the swine heart tissue extract are membrane proteins. Additionally, based on the quantitative MS results, the total amount of membrane protein identified in MaSDeS-containing extracts was significantly greater than in the Pre-extract and Control extracts (Figure 2C and Supplemental Figure S9). Furthermore, Western blot analysis of membrane protein revealed that MaSDeS significantly enriched membrane proteins comparable to SDS (Figure 2E). The levels of well-known membrane proteins were significantly enriched in heart tissue extracts containing MaSDeS in comparison to Control extracts. Such membrane proteins included the Na⁺-K⁺ ATPase, VDAC1, and PLN, which are essential membrane proteins involved in excitation-contraction coupling in the heart,⁶¹ as well as cadherin, a transmembrane protein involved in cell-cell adhesion.⁶²

The 226 membrane proteins uniquely identified in the swine heart tissue extracts containing MaSDeS formed interactomes that are involved in the regulation of cardiac function. Our data suggests that the inclusion of MaSDeS in the extraction buffer allowed for the identification of these important membrane proteins, which would have otherwise gone undetected without this surfactant. Thus, MaSDeS has the potential to be highly useful for membrane proteome analysis.

CONCLUSIONS

To recapitulate, we have identified an MS-compatible surfactant, MaSDeS, which can effectively solubilize all categories of proteins, including membrane proteins, with performance that is comparable to the strongest surfactant, SDS, at the concentrations employed in this study. The use of MaSDeS greatly simplifies the sample preparation process for tissue proteomic analyses since it is acid-labile and therefore does not need to be removed prior to MS analysis. This makes MaSDeS amenable for high-throughput proteomic analyses. Moreover, the incorporation of MaSDeS in the sequential tissue extraction leads to a significant enrichment of membrane proteins, which are often under-represented in proteomic studies. Furthermore, MaSDeS is thermostable and, consequently, can be used in cases where high temperature is needed to facilitate protein extraction and solubilization. These favorable characteristics of MaSDeS will make it a powerful tool for the analysis of tissues and other challenging biological samples. We expect it will expedite the use of tissue proteomics in clinical diagnosis and can be applicable to a wide range of biological applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ABC	Ammonium bicarbonate
BSA	Bovine serum albumin
CMC	Critical micelle concentration
CV	Coefficient of variation
DDM	n-Dodecyl β -D-maltoside
DGT	Digitonin
DTT	Dithiothreitol
ER	Endoplasmic reticulum
FA	Formic acid
FT-ICR	Fourier transform ion cyclotron resonance
IAA	Iodoacetamide
IPA	Isopropyl alcohol

MaSDeS	Mass spectrometry-compatible degradable surfactant
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
OG	Octyl β -D-glucopyranoside
PLN	phospholamban
PM	ProteaseMAX
PPS	PPS Silent Surfactant
RG	RapiGest
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Figure 1. The identification and characterization of an MS-compatible degradable surfactant (MaSDeS) with comparable performance to SDS

(A) A comparison of the structures of SDS and MaSDeS. M_r , most abundant molecular weight. (B) Determination of the CMC of MaSDeS and SDS at room temperature. (C) MaSDeS degrades under acidic conditions (10 % formic acid in 25 mM ABC buffer) at 37 °C. (D) Schematic depicting the sequential tissue extraction procedure. Pre-extract, first HEPES extraction; Control, second HEPES extraction without the addition of surfactant; MaSDeS and SDS, second HEPES extraction with buffer containing MaSDeS or SDS, respectively. (E) SDS-PAGE analysis and (F) Bradford protein assay results of extracts from heart tissue using 0.1%, 0.2%, or 0.5% of MaSDeS or SDS, respectively. An equal volume of each extract was evaluated by SDS-PAGE and Bradford protein assay. Both SDS-PAGE and Bradford protein assay results show that protein solubilization with MaSDeS is comparable to that obtained with SDS.

Figure 2. The use of MaSDeS significantly improved the detection of membrane proteins (A) A Venn diagram showing the number of proteins identified in heart tissue extracts without (Control) and with 0.2% MaSDeS as well as the overlap between these extractions. (B) The Gene Ontology annotations for the subcellular localizations of the identified proteins in each extract. (C) The summed intensities of the identified membrane proteins demonstrate that extracts generated with buffer containing MaSDeS have a greater abundance of membrane proteins than the Control. (D) The TMHMM software, which uses the Hidden Markov Model algorithm, was utilized to predict the number of transmembrane helices in each identified protein based on their protein sequence. The data shows that the proteins identified in extracts containing MaSDeS have a higher frequency of transmembrane helices than the proteins identified in the Control. (E) Western blot and (F–J) quantitative MS results demonstrate that membrane proteins were present in the greatest amount in extracts generated with buffer containing MaSDeS. The abundance of membrane proteins in heart tissue extracts containing 0.2% MaSDeS was similar to that in extracts containing 0.2% SDS. *, $p < 0.05$; N.S., not significant.

Figure 3. Gene Ontology annotation of the cellular location and biological processes for proteins uniquely identified in samples containing MaSDeS

(A) Among the 416 proteins uniquely identified in heart tissue extracts with 0.2% MaSDeS, membrane and ER/Golgi proteins were enriched in comparison to the proportion of total proteins. Of the 1,113 proteins identified in extracts containing MaSDeS and the 416 unique proteins only identified in MaSDeS-containing samples, 43.2% and 55.3%, respectively, were categorized as membrane proteins. Similarly, 5.7% and 7.9% of the total and uniquely identified proteins, respectively, were categorized as proteins localizing to the ER/Golgi. (B) Analysis of the biological processes of the membrane proteins uniquely identified in heart tissue extracts with added MaSDeS according to the gene ontology database. The results showed that the top 3 significant processes of the 226 unique membrane proteins are metabolic processes, transport, and cell communication.

Figure 4. The interactome analysis of the 226 membrane proteins uniquely identified in heart tissue extracts containing MaSDeS

The proteins in the pink circles serve as channels in the inner or outer mitochondrial membranes, allowing for the diffusion and transport of proteins, small molecules, and chemicals in and out of the mitochondria. The proteins in the blue circles are involved in the regulation of cell adhesion or cytoskeleton organization/assembly. The proteins in the orange circles are well-known proteins involved in excitation-contraction coupling in the heart.

Figure 5. Comparison of MaSDeS with other leading MS-compatible surfactants in protein solubilization

The commonly used MS-compatible surfactants, including ionic (ProteaseMAX (PM), RapiGest (RG), PPS Silent Surfactant (PPS)) and non-ionic (Octyl β -D-glucopyranoside (OG), n-Dodecyl β -D-maltoside (DDM), and Digitonin (DGT)) surfactants, were investigated for their ability to solubilize proteins when compared with MaSDeS and SDS. Protein solubilization from three commonly used tissue sources were used for testing. An equal volume of each sample was evaluated by the SDS-PAGE results, (A) heart tissue, (C) liver tissue, and (E) lung tissue, as well as protein assay with (B) heart tissue, (D) liver tissue, and (F) lung tissue. Both Western blot and protein assay results showed that MaSDeS possessed the highest capability for solubilizing proteins. All of samples have significant differences with Control. *indicates the differences with MaSDeS. * $p < 0.05$, ** $p < 0.01$, N.S. not significant.

Figure 6. Comparison of PM, RG, and MaSDeS on protein identification

Heart (A, B), liver (C, D), and lung (E, F) tissues were homogenized with and without 0.2% PM, RG or MaSDeS to evaluate protein identification under equal volume loading. (A, C, E) The identified protein number. (B, D, F) Sum of protein intensity. Each sample was run in triplicate. All of the given protein intensities were presented in Log10 form. MaSDeS containing samples showed higher number of protein identifications and higher sum of intensities compared to Control, PM- and RG-containing samples in all three types of tissues. All of surfactant-containing samples have significant differences with Control. *means the sample has a statistically significant difference from MaSDeS sample. *, $p < 0.05$.

0.05. **, $p < 0.01$. # means the sample has a statistically significant difference from Control sample. #, $p < 0.05$. ##, $p < 0.01$.

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