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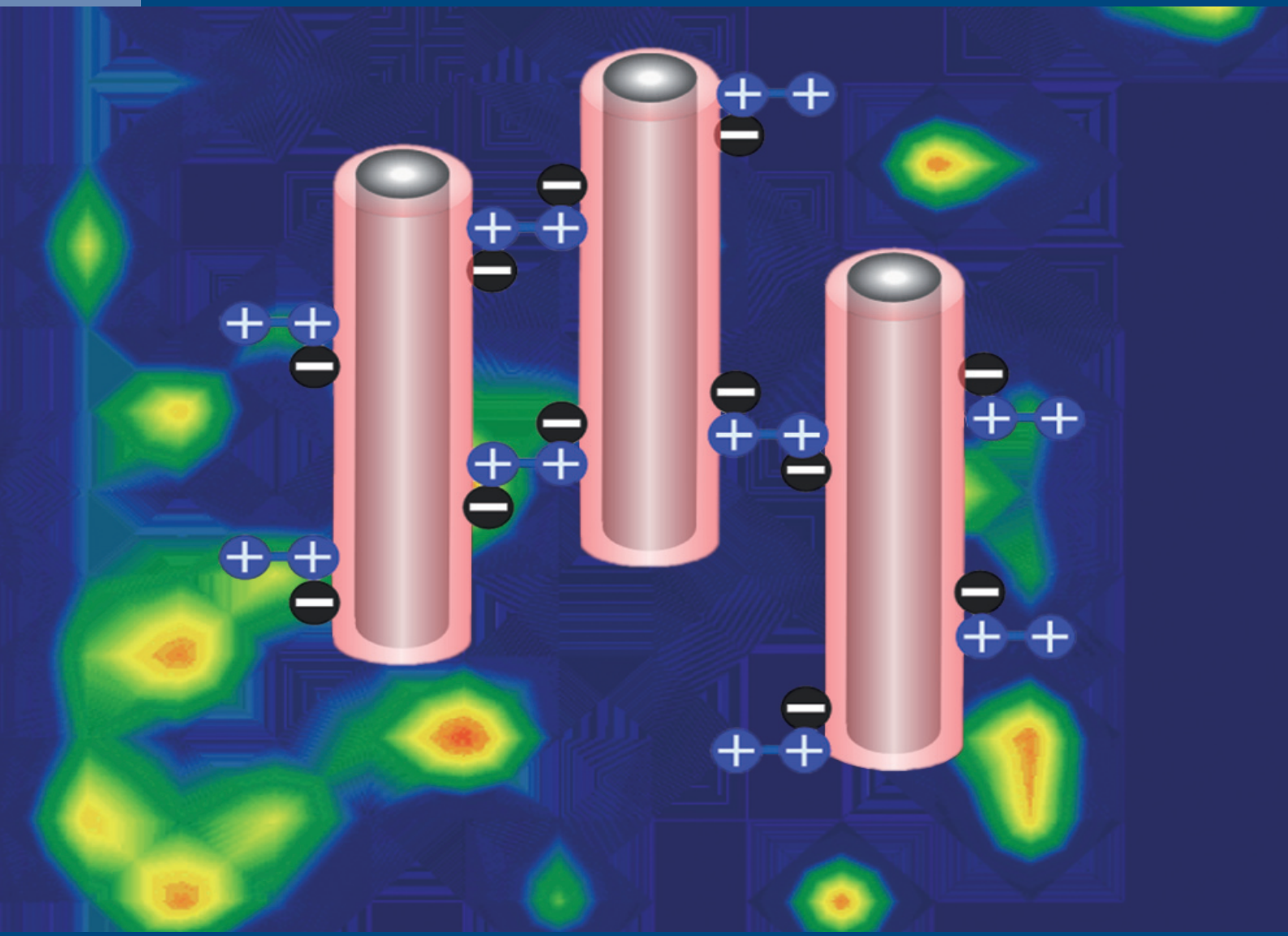

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RESEARCH ARTICLE

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Improved in-solution trypsin digestion method for methanol–chloroform precipitated cellular proteomics sample

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Methanol–chloroform based protein precipitation is an essential step in many liquid chromatography–tandem mass spectrometry-based cellular proteomics applications. However, re-solubilization of the total protein precipitate is difficult using regular in-solution digestion protocol. Sodium deoxycholate is reported as an efficient surfactant for re-solubilization of membrane fractions. In this study, we demonstrated an application combining methanol–chloroform based protein precipitations and deoxycholic acid assisted re-solubilization of pellets to evaluate the improvement of protein identifications in mass spectrometry-based bottom-up proteomics. We evaluated the modified method using an equal amount of Raw 264.7 mouse macrophage cell lysate. Detailed in-solution trypsin digestion studies were presented on methanol–chloroform precipitated samples with or without deoxycholic acid treatments and compared with popular sample digestion methods. A mass spectrometric analysis confirmed an 82% increase in protein identification in deoxycholic acid-treated samples compared to other established methods. Furthermore, liquid chromatography–tandem mass spectrometry analysis of an equal amount of proteins from methanol–chloroform precipitated, and methanol–chloroform/deoxycholic acid-treated macrophage cell lysate showed a 14% increase and 27% unique protein identifications. We believe this improved digestion method could be a complementary or alternative method for mammalian cell sample preparations where sodium dodecyl sulfate based lysis buffer is frequently used.

KEYWORDS
deoxycholic acid, methanol–chloroform precipitation, proteomics sample preparation, raw macrophages, trypsin digestion

1 | INTRODUCTION

The shotgun proteomics method is widely used for identifying and quantifying proteins in subcellular compartments. Besides, biochemical methods are often used to purify organelles or membranes to study a target protein [1]. However, many membrane and organelle associated proteins are hydrophobic. SDS is a strong detergent used in the lysis buffer for protein extraction and solubilization. However, there is

Article Related Abbreviations: ABC, ammonium bicarbonate; DCA, deoxycholic acid; FA, formic acid; IAA, iodoacetamide; MeOH-Chl, methanol–chloroform; MeOH-Chl-DCA, methanol–chloroform-deoxicholic acid; MeOH-Chl-NaDCO, methanol–chloroform- Na-deoxicholate; SDC/or NaDCO, sodium deoxycholate.

some problem associated with the downstream mass spectrometric analysis of samples that are prepared with SDS. The presence of highly surface-active anionic surfactants can also interfere with ESI in positive ion mode [2]. Moreover, it has been observed that the presence of SDS in samples reduces the S/N ratio [3,4]. Besides, for bottom-up proteomics, the presence of SDS reduces trypsin activity [5]. Thus, SDS needs to be removed before trypsin digestions in MS-based bottom-up proteomics experiments.

There are many ways SDS can be removed before mass spectrometric analysis. These include electrophoretic separation, hydrophobic interaction chromatography, organic solvent precipitation, ion-pairing reagents, potassium dodecyl sulfate precipitation, oligosaccharide based spin column, filter assisted sample preparation (FASP), and enhanced filter assisted sample preparation (eFASP) [6-10]. These methods have different advantages and disadvantages. The different organic solvents in combination with salts or acids are reported to be used in organic solvent-based precipitation of proteins and thus removes SDS. Among these, the two most popular methods are acetone purification and methanol–chloroform precipitation [7,11–13]. However, bottom-up proteomics experiments following organic-solvent precipitation of proteins suffer from under-representation of precipitated proteins in the MS detection platform. The MS-compatible solvent system is not capable to solubilize all of the hydrophobic components of the precipitate [14].

It has been shown in previous work by Takeshi Masuda et al. that a phase transfer surfactant, sodium deoxycholate (SDC) can improve protein identification in enriched membrane fractions of *E. coli* and HeLa cells [15]. The shotgun proteomics method on standard filtration device has been developed with improved identification using exchange buffer 8 M urea or SDC [8,16–19]. Sprenger and co-authors did a remarkable study on the in-solution digestion efficiency of several denaturants, such as urea, RapiGest, and SDC with or without spin-filter aided SDS removal and concluded that SDC is superior among them [20]. A significant amount of protein loss happens when proteins are recovered from the spin filter membranes. Several studies have been done utilizing SDC during gel or in-solution digestion sample preparations [21–26]. Besides, the application of SDC to plasma proteomics sample preparations was done successfully [27,28].

Motivated by all these works, here in this study, we evaluated deoxycholic acid (DCA) assisted solubilization of methanol–chloroform precipitated cellular proteome from RAW 264.7 mouse macrophage. We solubilize the methanol–chloroform precipitated proteome in DCA or SDC and the samples were further digested with trypsin to evaluate the protein identification numbers compared to the conventional methods, such as DRSC, urea, eFASP, and acetone precipitation. Detailed experiments were conducted by taking an equal amount of macrophage cell lysate and performing methanol–chloroform precipitation and solubilizing the precipitates with or without DCA. Besides, we performed LC–MS/MS analysis on equal amount of proteins normalized after methanol–chloroform purification and methanol–chloroform/DCA treatments to compare the protein classes increased due to DCA treatments. We believe this modified method could be an alternative method for in-solution digestion-based bottom-up proteomics application of cellular proteomics.

2 | MATERIALS AND METHODS

2.1 | Chemicals

SDC, DCA, iodoacetamide (IAA), formic acid (FA), and ammonium bicarbonate (NH_4HCO_3) were purchased from Sigma–Aldrich (St. Louis, MO, USA). DMEM was purchased from Corning Cell gro (Tewksbury, MA, USA). Methanol and acetonitrile were purchased from Alfa Aesar (Tewksbury, MA, USA). Sequencing grade modified trypsin was purchased from the Promega Corporation (Madison, WI, USA). Pierce BCA Protein Assay kit was purchased from the Thermo Fisher Scientific (Waltham, MA, USA).

2.2 | Protein purification and digestions

Cell Culture, protein extraction, and quantification procedures are provided in the Supporting Information.

2.2.1 | Methanol–chloroform purification and digestion in ammonium bicarbonate

In the first phase, 150 μg of Raw 264.7 macrophage cells, in three replicates (Tag: MeOH-Chl), were purified using the methanol–chloroform method described before [29]. Briefly, proteins were diluted to 1 μg/μL. One volume of protein is mixed with four volumes of methanol and vortexed for 30 s. Then, mixed with one volume of chloroform and vortexed for another 30 s. Afterward, three volumes of water were added and vortexed for 30 s and centrifuged at 20 000 × *g* for 10 min at room temperature. The upper phase was discarded while keeping the white precipitate. Then, another three volumes of methanol were added, and gently mixed without breaking the pellets. Finally, centrifugation at 20 000 × *g* precipitated the proteins as pellets. The supernatant was discarded and proteins in the pellets were taken for bottom-up proteomics. The proteins were then reduced and alkylated, digested with trypsin (MS Grade) at a 1:100 enzyme/protein concentration for 16 h at 37°C. Formic acid was added afterward to drop the pH and to stop trypsin activity. The samples were then desalted using a C18 desalting column (Thermo Scientific, IL, USA). After completely drying by speed vacuum, peptides

were dissolved in 0.1% formic acid and stored at −20°C before LC–MS/MS analysis.

In the second phase, 250 µg of Raw 264.7 macrophage cells in nine aliquots were taken and then purified using MeOH-Chl method described previously [29]. They were then suspended in 300 µL, 50 mM NH_4HCO_3. From these nine replicates, we prepared three replicates for MeOH-ChI purified samples by combining three replicates in one microcentrifuge tube. Proteins were normalized in each replicates, then reduced, alkylated, digested, desalted, centrifuged, and the supernatants were stored at −20°C before LC–MS/MS analysis.

2.2.2 | DCA-assisted tryptic digestion of methanol–chloroform purified proteins

Proteins (150 and 250 µg) were extracted from three biological replicates using the MeOH-Chl method, as described above [29]. This is followed by our modified method (Tag: MeOH-Chl-DCA). The samples were first air-dried for 5 min. All Solutions (10 mM DTT, 10 mM IAA), including trypsin stock solution (20 µg /200 µL), were prepared in 50 mM NH_4HCO_3, and pH 7.5. DCA solution was prepared in 7 N NH_4OH in water. After that, NH_4HCO_3 was added to make the final solution in 2% DCA in 50 mM NH_4HCO_3. Then, the pH was adjusted to ≈7.0–8.0 using conc. NH_4OH. One hundred microliters of 2% DCA in 50 mM NH_4HCO_3 was added to the sample and then the sample was rested at 25°C for 30 min. A 300 µL of 50 mM NH_4HCO_3 solution was added to the sample and rinsed with a 200 µL pipette tip; the samples were dissolved. For 250 µg of cell lysates samples, protein amount in three biological replicates was quantified and normalized. Thus, the same amount of proteins could be used for digestion in regular and DCA treated solutions. After reduction and alkylation, the final volume was increased to 1 mL with 50 mM NH_4HCO_3. The proteins were then digested with trypsin (MS Grade) at a 1:100 enzyme/protein concentration for 16 h at 37°C. Formic acid was added afterward to drop the pH, stopping trypsin activity and precipitate the DCA. Next, centrifugation at 20 000 rpm for 30 min at 4°C removed the surfactant DCA. The samples were then desalted using a C18 desalting column (Thermo Scientific, IL, USA). After drying by speed vacuum completely, peptides were dissolved in 0.1% formic acid and stored at −20°C before LC–MS/MS. A detailed protocol of each step is included in Supporting Information S2–S4. Sodium deoxycholate (NaDCO) assisted tryptic digestion of methanol–chloroform purified proteins is detailed in Supplementary Information S4.

DCA assisted digestion of discarded protein pellets after MeOH-Chl purification (Tag: Digested-Pellet-DCA) was provided in the supplementary data [29]. Detailed methods for acetone purification and digestion, detergent removal spin column filtration (DRSC), urea, and eFASP protocols are also provided in the Supporting Information [8].

2.3 | LC–MS/MS experiments and data analysis

Detailed MS methods and data analysis are provided in the Supporting Information.

3 | RESULTS AND DISCUSSION

In our regular experiments with cell lysates, we always observed recalcitrant behavior of the MeOH-Chl precipitated sample in dissolving into the tryptic digestion buffer of 50 mM NH_4HCO_3. SDC (NaDCO) was used to dissolve isolated membranes and DCA is used to equilibrate micron filters of a detergent removal method, named Enhanced Filter Assisted Sample Preparation (eFASP). We came up with a modification in the method where precipitated sample pellets were dissolved in 0.5% DCA in 50 mM NH_4HCO_3 and subsequently digested in a total concentration of 0.2% DCA in 50 mM NH_4HCO_3. Similarly, pellets were dissolved with 0.5% NaDCO in 50 mM NH_4HCO_3 and digested in 0.2% NaDCO in 50 mM NH_4HCO_3. We anticipated that the use of surfactants, i.e. DCA or NaDCO could take MeOH-Chl precipitates fully into the solution (Figure 1A and B). Hence this would lead to improved protein solubilization, efficient digestion, and increased protein identification. An extra advantage of DCA is that it can be precipitated from the solution after digestions.

To test this, we first took 150 µg of RAW 264.7 cell lysates in three replicates for control and the modified experiments. We first did MeOH-Chl precipitation of proteins and further dissolved and digested the pellets in regular tryptic lysis buffer, 50 mM NH_4HCO_3 (Figure 1A). Similarly, we dissolved the MeOH-Chl precipitated proteins in 0.5% DCA in 50 mM NH_4HCO_3 or 0.5% NaDCO in 50 mM NH_4HCO_3 (Figure 1B). Finally, the dissolved proteins were diluted and tryptic digested in 0.2% respective surfactant solution in 50 mM NH_4HCO_3. Acidification, followed by centrifugation, removed the surfactants. After desalting, samples were analyzed in LC–ESI–Thermo Velos Pro mass spectrometer. For comparison, we also conducted other protein precipitations/purification methods on 150 µg of cell lysate. We compared the DCA sample preparation with acetone purification, detergent removal spin column purification (DRSC), urea, and eFASP followed by tryptic digestion in Ammonium Bicarbonate buffer (see Supporting Information S4–S7).

In an alternative experiment, samples from three biological replicates were prepared using 250 µg of cell lysate. As mentioned previously, significant sample loss occurs after MeOH-Chl purification, thus nine samples were prepared for the regular method. After MeOH-Chl purification, these samples were combined to make three replicates for regular NH_4HCO_3 method whereas another three 250 µg of cell lysate replicates

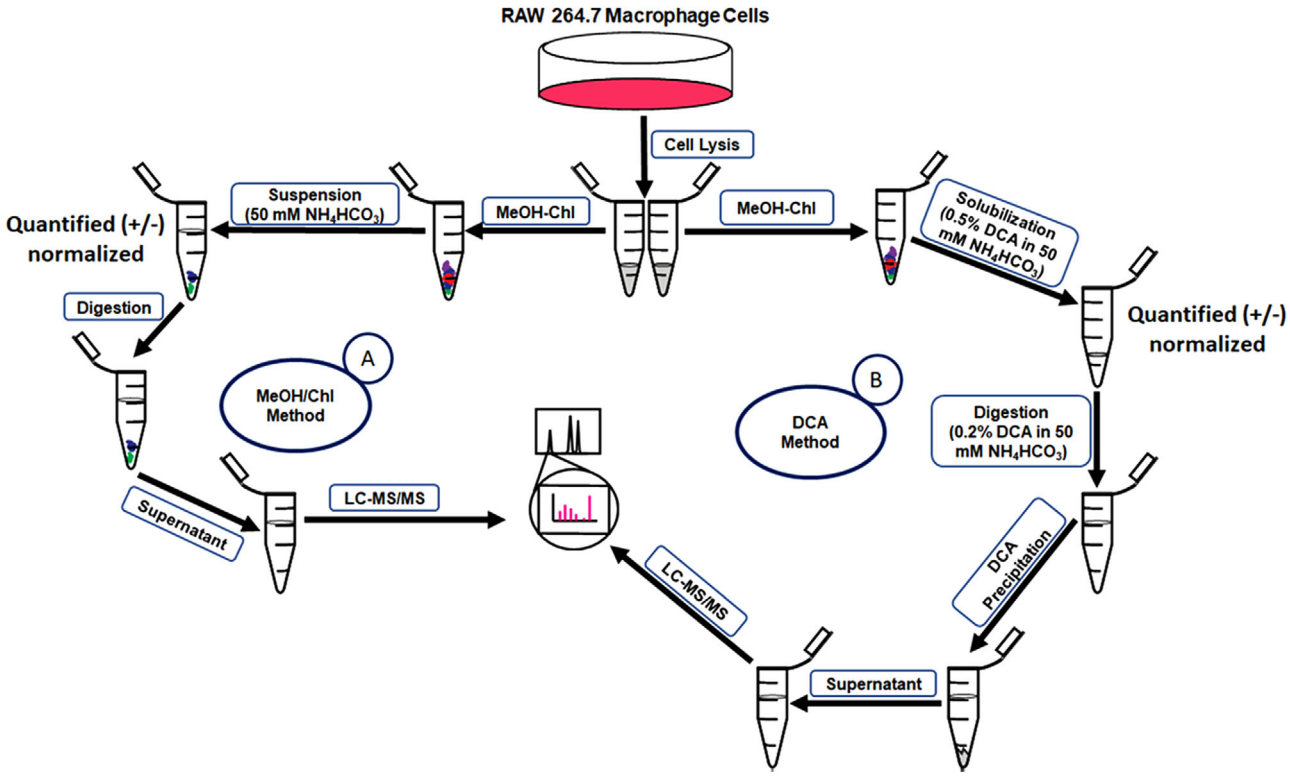

FIGURE 1 Sample preparations steps are shown with two experimental schemes. Panel (A) shows MeOH-Chl purification followed by digestion in 50 mM ammonium bicarbonate. Panel (B) shows MeOH-Chl-DCA method where MeOH-Chl precipitated pellet was dissolved in DCA and digested with trypsin

were used for DCA treated solution. This way it is easier to normalize the amount of proteins in the regular and modified methods for each biological replicates with minimum sample variations (please see Supporting Information Table S1, 250 µg cell lysate gave 68 µg of MeOH-Chl precipitated proteins and 162.5 µg when treated with DCA). Protein amounts were normalized to the equal amount before digestion. LC–ESI–Thermo Orbitrap Fusion Lumos Tribrid Mass Spectrometer was used for high-resolution data and panther gene ontology functional classifications were utilized. We also prepared a third set of sample in this phase of the experiments. We took the left-over pellets after regular tryptic digestion and used DCA to dissolve this leftover pellet and re-digested in the surfactant solution (Supporting Information S4 and S5 and Figure S1C).

In the first stage of our experiment, we took 150 µg of RAW 264.7 macrophage cell lysate, performed MeOH-Chl purification and then reconstituted the pellets in regular Tryptic digest buffer 50 mM ABC and our modified buffer 0.5% DCA or 0.5% NaDCO in 50 mM ABC buffer, at pH ≈ 7.5. The visual assessment showed (Figure 2) both DCA and NaDCO took all the pellets into the solution. This was further validated through a BCA assay of the dissolved proteins to quantify roughly the amount of proteins solubilized. A similar analysis was performed using 250 µg of proteins. From both the analysis we confirmed approximately three times more proteins were dissolved when the surfactant was added (Supporting Information Table S1).

In Figure 3, we compared four regular sample preparation methods (e.g., Acetone purification, DRSC filtration, urea, and eFASP) in regular tryptic digestion in 50 mM ABC versus our two modified sample preparation methods using DCA and NaDCO. RAW 264.7 macrophage cell lysates were taken and different sample purification methods, e.g. MeOH-Chl purification, acetone purification, DRSC, and eFASP were used to remove detergents from samples followed by in-solution digestion in 50 mM ABC. At the same time, we took pellets from MeOH-Chl precipitation and used 0.5% DCA or 0.5 % NaDCO in 50 mM ABC buffer, at pH ∼7.5 for dissolving the precipitate. The sample solutions then diluted to 0.2% respective surfactants concentration with 50 mM ABC for tryptic digestion. We also compared this method with the urea method with sonication to solubilize the precipitates.

Finally, the data of the three biological replicates were merged. We show the data sets where at least one peptide or two peptides were identified for each identified protein from all the three replicates. This rule was followed for all sample sets. Protein IDs that were not represented in all the three replicates were excluded from analysis (Figure 3A; Supporting Information Tables S2 and S3). Scatter plots and

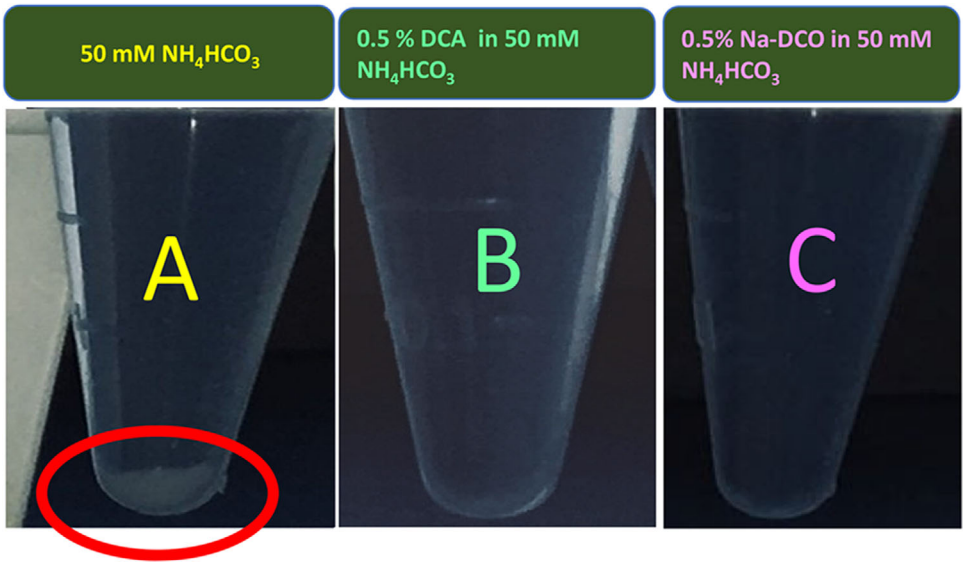

FIGURE 2 Visual assessment of MeOH-Chl precipitated proteins dissolved in (A) 50 mM NH_4HCO_3, (B) DCA in 50 mM NH_4HCO_3, or (C) NaDCO in 50 mM NH_4HCO_3

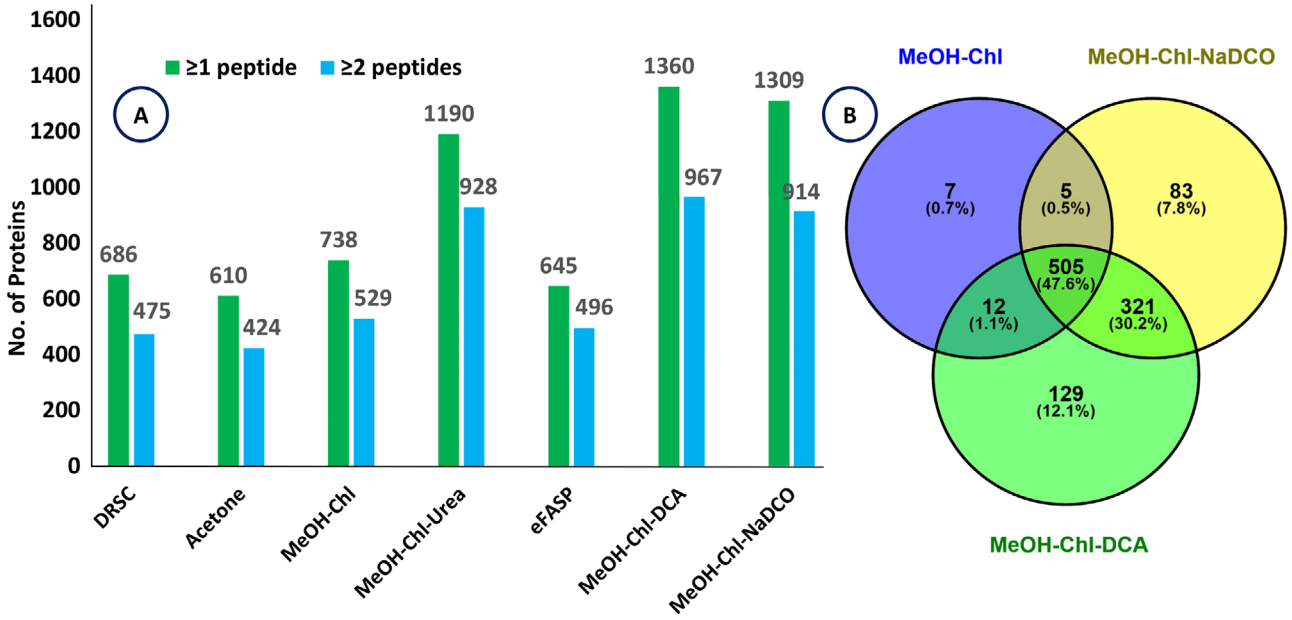

FIGURE 3 Number of proteins identified in different sample preparation methods from an equal amount of RAW 264.7 macrophage cell lysates (un-normalized) (A) and Venn diagram comparison for MeOH-ChI and MeOH-ChI-DCA/NaDCO methods (B)

pairwise correlations showed a significant correlation among all the biological replicates (Supporting Information Figure S2A).

In these sets of data, we observed samples prepared through MeOH-Chl purification gave higher protein identification (738 and 529, at least one or two peptides respectively from each protein identified) compared to Acetone or DRSC method. Urea methods with sonication gave comparable results with the DCA method but DCA method performed better. The protein identification from MeOH-Chl methods increased when DCA or NaDCO was used for digestion. We observed the highest number of identification from DCA (1360 and 967, for at least one and two peptides, respectively, per identified proteins) and NaDCO (1309 and 914, for at least one and two peptides, respectively, per identified proteins) treated tryptic digested samples. This is an 84% (number of identified peptides per protein ≥ 1) or 82% (number of identified peptides per protein ≥ 2) increase in protein identification for DCA compared to regular MeOH-Chl with NH_4HCO_3 buffer method. For NaDCO, the identification is 77% (number of identified peptides per protein ≥ 1) or 72% (number of identified peptides per protein ≥ 2) higher

compared to the regular MeOH-Chl with NH_4HCO_3 buffer method.

We also generated a Venn diagram with identified proteins from MeOH-Chl, MeOH-Chl-DCA, and MeOH-Chl-NaDCO methods where the number of peptides identified per protein ≥ 2 (Figure 3B). From the Venn diagram, 450 proteins (321 +129) were uniquely identified with the MeOH-Chl-DCA method compared to the control (MeOH-Chl). On the contrary, only 12 proteins (7+5) were identified uniquely to the MeOH-Chl method that was not identified in the MeOH-Chl-DCA method. Thus, this is 37.5 (450/12) times more identification of unique proteins in the modified method with DCA, compared to the control with the NH_4HCO_3 (ABC) buffer. For the MeOH-Chl-NaDCO method, 404 proteins (321 + 83) were uniquely identified compared to the control (MeOH-Chl). On the contrary, only 19 proteins (12 +7) were uniquely identified in the MeOH-Chl method that were not identified in the MeOH-Chl-NaDCO method. Therefore, this is 21.2 (404/19) times more identification of unique proteins in our modified method with NaDCO, compared to that of control with ABC buffer. This suggested that the modified method was highly efficient in solubilizing more proteins. MeOH-Chl purification technique is a widely used protein precipitation technique but we observed a significant amount of protein loss after precipitation. Since NaDCO is the sodium salt of DCA and we got similar results from both, we decided to proceed further with DCA for the next stage of the experiments.

To further evaluate proteins identified by the method, we quantified and normalized the same amount of proteins in MeOH-Chl and MeOH-Chl-DCA treated samples. Sample preparations details were described before. Three replicates samples were dissolved in 0.5% DCA in 50 mM NH_4HCO_3. This was further diluted to 0.2% DCA solution with a 50 mM NH_4HCO_3 and tryptic digested in this condition. We have found previously that resuspension in DCA dissolved three times more proteins into the solution compared to the ABC buffer alone. Thus, in this stage, we ensured we had an equal amount of cell lysate and after MeOH-Chl precipitation, we normalized protein amount in control and DCA treated samples. This way we can evaluate the protein classes that were solubilized due to the DCA treatment. After digestion, these three replicates were sent for LC–MS/MS analysis with a high-resolution mass spectrometer. One replicate was an outlier, so we excluded that from the data analysis. The left-over pellets of these three biological replicates, after overnight digestions, were solubilized with DCA and digested with trypsin. All samples were sent to Purdue Proteomics Facility for the analysis in an LC–ESI–Thermo Orbitrap Fusion Lumos Tribrid mass spectrometer. Scatter plots and pairwise correlations showed excellent reproducibility of each LC–MS/MS dataset (Supporting Information Figure S2B).

The results of all three biological replicates were combined and the protein IDs that were commonly identified in all replicates are shown in Figure 4A and Supporting Information Table S4. MeOH-Chl-DCA method ended up identifying 3217 proteins (peptides ≥ one per protein) or 2560 proteins (peptides ≥ two per protein). On the contrary, MeOH-Chl purification in combination with ABC identified 2872 proteins (peptides ≥ one per protein) or 2242 proteins (peptides ≥ two per protein). This is 12–14% less than our modified DCA method. The left-over pellet after digestion of the MeOH-Chl method identified 2439 proteins (peptides ≥ one per protein) or 1782 proteins (peptides ≥ two per protein). This confirmed that a large number of proteins were not taken for in-solution digestion unless DCA was used for dissolving the precipitates.

To compare these data sets, we generated a Venn diagram (Figure 4B). From the Venn diagram, with at least two peptides identified per proteins, we can project, at least 594 proteins (428+166) were identified uniquely with the DCA method compared to the control, MeOH-Chl method. On the contrary, only 276 proteins were identified uniquely in the MeOH-Chl method, which were not identified in the DCA method. This is 2.15 times (594/276) more identification of unique proteins in our modified method with DCA compared to the control. Also, in the left-over pellets, digested with the DCA method, 241 (166+75) proteins were uniquely identified. From this data, we confirmed twice as much as higher unique protein identification with the MeOH-Chl-DCA method.

In a simple Supporting Information Table S5, we summarized the identification of proteins among the MeOH-Chl method and our modified MeOH-Chl-DCA method. When we started with an equal amount of starting material (150 μg), we observed our modified method yields 82% (peptides ≥ two per protein) increase in protein identification compared to the regular method. Since, we confirmed through BCA assay that the addition of surfactants took in three times more proteins into the solution and MS analysis has shown an 82% increase in protein ID, we decided to normalize protein amount in both samples after MeOH-Chl precipitation. This way we can see different functional categories of proteins uniquely identified in the samples due to the modified method. We found, there is an increase of 14% (peptides ≥ two per protein) protein identification in our modified method and among these proteins, 27% was unique protein ID. The proteins were then classified according to the panther gene-ontology functional classifications to organize the overall category of protein identified due to the surfactant treatments.

Proteins present in all biological replicates (at least two peptides identified per protein) were filtered. This filtered data sets from control (MeOH-Chl) and modified method with DCA (MeOH-Chl-DCA) were used for generating gene-ontology classification based on the panther classification system into the molecular function, biological process,

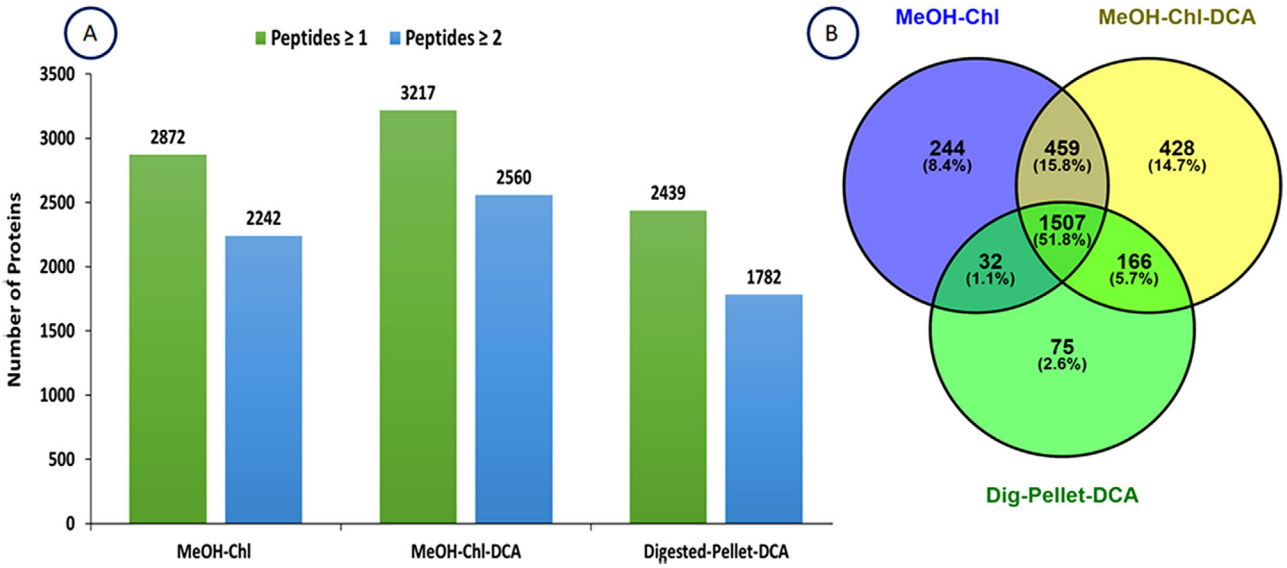

FIGURE 4 Number of proteins identified in normalized samples of MeOH-ChI and MeOH-ChI-DCA and leftover digested pellet with DCA treatment (A) and Venn diagram of comparison of identified proteins (B)

cellular component, and protein class. Gene ontology classification based on protein classes are shown in Supporting Information Figure S3A and B. In Supporting Information Figure S3A, we showed with DCA we have higher identification in protein type in all subcategories, whereas in Supporting Information Figure S3B in the normalized sample, we see higher identification in most of the subcategories. Other "functional classifications" comparative diagram is shown in the supporting Supporting Information Figure S4A and B. Data for all functional classes and their subcategories are shown in Supporting Information Table S6.

4 | CONCLUDING REMARKS

Methanol–chloroform based protein precipitation is a widely used protein sample preparation methods. A significant amount of sample loss occurs during this purification process. In this paper, we showed that the addition of surfactant DCA increased protein solubility and more protein ID in LC–MS/MS-based protein identification methods. Our comparison of different methods with cell lysates showed 82% more protein identification in the presence of DCA compared to the MeOH-Chl precipitate digested in Ammonium bicarbonate buffer. Normalization of protein amount after precipitations gave us a 14% increase in protein ID as well as 27% unique protein ID. The left-over pellets after digestion, showed a good number of proteins identified from there; further validating the fact that there is sample loss involved in the traditional method. From all results being summarized, we conclude that the modified method will increase protein identification in MeOH-Chl purification based cellular proteomics.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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