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Online removal of sodium dodecyl sulfate via weak cation-exchange in liquid chromatography-mass spectrometry-based proteomics

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Abstract

Biological research often requires the use of sodium dodecyl sulfate (SDS) to solubilize protein samples; however this detergent is not compatible with direct mass spectrometry (MS) analysis. Here we report an online high-throughput proteomics method that permits standard in-solution digestion of SDS-containing samples followed by direct liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis using weak cation exchange chromatography (WCX). This approach, named as *online removal of sodium dodecyl sulfate* (Online reSDS), exploits the properties of WCX, in a high organic and mildly acidic medium, to retain positively charged peptides by both hydrophilic interaction and electrostatic attraction whilst simultaneously repelling negative SDS molecules. This method was optimized to successfully analyze complex samples that contain up to 1% of SDS. Furthermore, online reSDS improves the identification of peptides with posttranslational modifications (PTMs), such as deamidation and phosphorylation, without preliminary enrichment. In conclusion, we show that reSDS can facilitate research in proteomics by allowing the use of SDS in a wide range of LC-MS/MS applications with simplified sample processing procedures.

Keywords

Sodium dodecyl sulfate, weak cation exchange, post-translational modifications, deamidation, phosphorylation, membrane proteins.

Introduction

Sodium dodecyl sulfate (SDS) is an anionic detergent widely used for solubilizing challenging biochemical samples including amphipathic membrane proteins¹⁻⁶ and aggregated/amyloid macromolecules.^{7, 8} SDS is also a common component of commercially available sample preparation kits designed for extraction of specific molecules or proteomes. However, although SDS is commonly used in biochemical analyses it is not compatible with liquid chromatography coupled to mass spectrometry (MS), including tandem MS (LC-MS/MS) due to ion suppression effects, spray shield contamination and unmanageable background noise.⁹⁻¹¹ Efficient removal of SDS, then, is a critical step prior to the successful MS analysis.

In-gel electrophoresis is a common strategy to process SDS-containing samples in proteomic sample preparation for MS analysis.¹²⁻¹⁴ However, as previously discussed by Wiśniewski and colleagues, in-gel digestion is highly dependent on manual operations, which are not required during in-solution digestion.¹⁵ Thus, accommodating the use of SDS for in-solution strategies would be beneficial and is a priority for improved proteomics sample preparation. Various methods have been employed for removal of SDS from in-solution preparations, which have been recently reviewed by Kachuk et al.¹⁶ Off-line SDS removal strategies have been developed including precipitation of SDS by addition of salts,¹² addition of organic solvents,¹⁷⁻¹⁹ molecular weight exclusion¹⁸ or affinity separation.²⁰ Electrostatic interaction technologies such as strong-cation exchange²¹ and anion-exchange²² have also been applied successfully as off-line LC-MS/MS strategies to remove SDS. In addition, filter-aided sample preparation,²³ such as the FASP strategy proposed by Mann's team²⁴ is a widely used off-line method.

Vissers et al.^{25, 26} reported a LC-MS/MS method that is able to trap online up to 0.1% SDS from single protein digests by the use of anion-exchange trapping columns. The accumulation of SDS

in the trapping column limited its application to samples containing low concentrations of SDS and the assiduous replacing of overloaded trapping columns is the most significant constraint of this method. Other online strategies have been developed recently to facilitate analysis by capillary electrophoresis²⁷⁻²⁹ although none of them is applicable to LC-MS/MS.

Here we have built upon our experience on hydrophilic interaction chromatography and electrostatic effects³⁰⁻³² to develop a novel approach for SDS removal from protein samples. Our approach uses a WCX stationary phase to selectively retain positively charged peptides whilst allowing negatively charged SDS to be washed out from the capillary during the sample loading step. The method presented here (as *online removal of sodium dodecyl sulfate* or Online reSDS), demonstrates an improved direct analysis of SDS containing samples by LC-MS/MS without the requirement of any specific pre-processing step. Additionally, low-concentration samples that have been extracted or solubilized using commercial kits containing SDS would finally be susceptible to high-throughput LC-MS/MS analysis with the significant advantage that this fact represents.

Experimental procedures

Reagents and chemicals

Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. SDS was purchased from Bio-Rad (Hercules, CA, USA). HPLC grade water and acetonitrile (ACN, HPLC grade) were purchased from Thermo Scientific Inc. (Bremen, Germany). Protease inhibitor cocktail tablets were obtained from Roche (Basel, Switzerland), and sequencing-grade modified trypsin was purchased from Promega (Madison, WI, USA).

Tryptic digestion of bovine serum albumin (BSA) and ovalbumin

A tryptic digested protein mixture of BSA and chicken ovalbumin was used for method optimization. Trypsin digestion was performed essentially as previously described³³ with minor modifications. Briefly, 1 mg of each protein was dissolved in 8 M urea in 25 mM ammonium bicarbonate (ABB). Proteins were chemically reduced using 20 mM dithiothreitol (DTT) at 37 °C for 3 h and subsequently alkylated using 80 mM iodoacetamide (IAA) for 45 min in the dark at room temperature. During digestion, to maintain trypsin activity, samples were diluted to <1 M urea using 25 mM ABB. Proteins were digested at an enzyme-to-substrate ratio of 1:50 (w/w) overnight at 37 °C. Enzymatic digestion was quenched by adjusting to pH 3 using formic acid (FA). Tryptic peptides were desalted using a 50 mg Sep-pack C18 cartridge (Waters, Milford, MA, USA). Peptides were eluted from the cartridge with 1 mL of 75 % ACN, 0.1 % FA. Eluates were dried in a vacuum concentrator (Eppendorf, Hamburg, Germany).

Analysis of the BSA/ovalbumin protein digest by LC-MS/MS

Online reSDS LC. Dried peptides were initially reconstituted in 0.1% FA and then further diluted in ACN with 0.1% FA to give a peptide concentration of 100 ng/μL in 75 % ACN, 0.1 % FA. Reconstituted peptides (100 ng) were loaded into a Dionex UltiMate 3000 UHPLC by injection into one of two 200 μm x 10 cm capillary columns (sample traps) containing PolyCAT A (3 μm, 300 Å) (Columbia, MD, USA) as the stationary phase. PolyCAT A consists of silica coated with polyaspartic acid and is a WCX material with 60-70% of the residues being isoaspartyl- (iso-Asp) groups. For the details of the column packing see Gallart-Palau *et al.*³⁴ The trapping columns were connected using a ten-port valve (a diagram of the instrument configuration is available in **Fig. S-1**). Connecting the trapping columns in parallel allowed for processing of two independent samples simultaneously with no waiting time. After peptide loading the trapping column was flushed for 90 min at 4 μL/min with 90 % ACN, 0.1 % FA in order to wash out SDS, salts and other impurities

(the “online reSDS” step). After flushing, the position of the 10-port valve was switched to connect the peptide-loaded trapping column to a third identical PolyCAT A capillary column to perform peptide separation. At the same time the next sample was loaded onto the unused PolyCAT A trapping capillary for online reSDS treatment. Separation of peptides was performed using a typical HILIC (hydrophilic interaction liquid chromatography) gradient with 0.1% FA in water as mobile phase A and 0.1 % FA in ACN as mobile phase B at a flow rate of 1 μ L/min. A 90-min gradient was applied as follows: 75% B for 2 min, 75 – 40 % B for 58 min, and 40 – 2 % B for 10 min, followed by 4 min at 2 % B, then 2 – 75 % B for 1 min and then maintained at the initial conditions isocratically for 15 min. Subsequently, peptides were detected using a mass spectrometer (see section “Mass spectrometry analysis of the BSA/ovalbumin protein digest”).

Reverse Phase (RP) LC separation of the BSA/ovalbumin protein digest

Dried peptides were reconstituted in 0.1% FA and 100 ng of the reconstituted peptides were loaded into a Dionex UltiMate 3000 UHPLC by injection into a reverse phase Acclaim PepMap RSL column (75 μ m ID $\times$ 15 cm, 2 μ m particle size, Thermo Scientific Inc.). Separation of peptides was performed at a flow rate of 300 nL/min using a 60-min gradient with 0.1% FA as mobile phase A and 100 % ACN, 0.1 % FA as mobile phase B. The 60-min gradient was applied as follows: 3 – 7% B for 1.7 min, 7 – 30 % B for 43.8 min, 30 – 54.2 % B for 4 min, 54.2 – 72 % B for 1 min followed by 2 min at 72 % B, then 72 – 3 % B for 1 min and then maintained at the initial conditions isocratically for 7 min. Subsequently, peptides were detected by the mass spectrometer (see section “Mass spectrometry analysis of the BSA /ovalbumin protein digest”).

Mass spectrometry analysis of the BSA/ovalbumin protein digest

Analysis of the BSA/ovalbumin protein digest was performed with a linear quadrupole ion trap-Fourier transform (LTQ-FT) Ultra mass spectrometer (Thermo Scientific Inc, Bremen, Germany). Online ionization was performed with a Michrom CaptiveSpray ionization source (Bruker-Michrom Inc., Auburn, USA) using an electrospray potential of 1.5kV and an ion transfer tube temperature of 180 °C. Centroid data in positive mode was acquired using Xcalibur version 2.0 SR2 (Thermo Scientific Inc., Bremen, Germany). An FT-ICR cell, at a resolution of 100,000 and maximum injection time of 1,000 msec, was used to acquire the full-MS scans (350-1600 m/z range) with an automatic gain control (AGC) target for FT of 1.0e+06. MS/MS data was acquired with a maximum injection time of 200 msec in the linear ion trap with AGC target for full-MS scans of 3.0e+04 and MSⁿ of 1.0e+04. Positive, singly charged peptides were excluded for fragmentation. Precursor ion charge state screening was enabled. The ten most intense ions with a 500-count threshold were fragmented by collision-induced dissociation (MS²) with normalized collision energy of 35 %, activation Q of 0.25, isolation width for precursor ion of 2 Da and activation time of 30 ms.

Culture of HEK 293F cells

HEK 293F cells were cultured as previously described.³⁵ Briefly, cells were obtained from Invitrogen (MA, USA) and cultured in 125 mL sterile vented PETG flasks (NALGENE, MA, USA) using Free-Style 293 expression medium (GIBCO Invitrogen). Cells were seeded at 3×10^5 /mL and grown at 37 °C in a shaking incubator with 5% CO₂ and 120 rpm as stable conditions. Prior to the experiments, cells were pelleted by centrifugation at 1300 rpm for 3 min and subsequently washed three times in 1X PBS to remove any remaining of culture medium.

Analysis of HEK 293F cells by online reSDS

SDS-assisted in-solution digestion of HEK 293F cells. The SDS-assisted in-solution digestion protocol was essentially as previously described for in-solution digestion of protein samples^{36, 37} with the main exception that urea was substituted in this study by SDS. Briefly, combined cell pellets from three cultured flasks (Roche, Basel, Switzerland) were mechanically disrupted by sonication in 300 μ L of 25 mM ABB containing 1 % SDS and a complete protease inhibitor cocktail (Sonics Vibra Cell, Sonics & Materials Inc., Newtown, CT, USA). The cell-homogenates were centrifuged at $10\,000 \times g$ for 10 min and the supernatants collected. Protein content was quantified by bicinchoninic acid (BCA) protein assay. Subsequently, 0.5 mg of proteins were chemically reduced with 35 mM DTT for 1 h at 60 °C and subsequently alkylated in 85 mM IAA for 1 h at room temperature in the dark. To ensure trypsin endopeptidase activity, the samples were diluted 100 times with 25 mM ABB, thereby decreasing the concentration of SDS to <0.01%. Samples were digested overnight at 37 °C using 1:20 enzyme-to-substrate ratio using sequencing-grade modified trypsin. Subsequently, a second trypsin digest was performed (1:125 enzyme-to-substrate ratio) for two hours. Enzymatic reactions were quenched by the addition of FA to a final concentration of 0.5%. The peptide samples were then desalted using a C-18 Sep-pack 1 g cartridge (Waters, Milford, MA) and elution was carried out with 5 mL of 75% ACN, 0.1% FA. Eluates were dried using the vacuum concentrator and reconstituted for the HPLC fractionation in 200 μ L of 0.1% FA. Experiments were performed in duplicate.

RP-LC fractionation of HEK 293F cells extract processed by SDS-assisted in-solution digestion. Fractionation of samples by HPLC using a reverse-phase BioBasic-18 column (4.6 $\times$ 250 mm, 5 μ m, Thermo Fisher Scientific Inc) was performed in a Shimadzu Prominence UFLC system (Shimadzu, Kyoto, Japan), monitoring absorbance at 280 nm as previously described.³⁸⁻⁴⁰ Briefly, peptides (from 0.5 mg of proteins) were separated at 1 mL/min using a 75-min gradient

as follows: 5 % mobile phase B (90 % ACN, 0.1 % FA) for 4 min, 5–12 % mobile phase B for 2 min, 12–50 % mobile phase B for 44 min, 50–80 % mobile phase B for 5 min, and 90–100 % mobile phase B for 5 min, followed by 10 min at 100 % mobile phase B, then returning to the initial conditions for 1 min and then maintained isocratically for 4 min. Fractions were collected every minute and neighboring fractions combined to give 23 fractions according to the chromatographic profile. The method of combining fractions was performed in accordance to the multidimensional chromatography strategy previously developed by our group.³² The combined fractions were dried in a vacuum concentrator and reconstituted in 75 % ACN, 0.1 % FA prior to online reSDS analysis.

Online reSDS LC analysis of HEK 293F cells extract. Online reSDS analysis of the HEK 293F cells extract was performed using a Dionex UltiMate 3000 UHPLC (Thermo Fisher Scientific Inc., Bremen, Germany). Digested peptides (from 293F cells: 1–2 µg per fraction, 1 µL total volume) were injected into a PolyCAT A trapping capillary column as described above for the analysis of the BSA/ovalbumin protein digest, with the exception that due to a high salt content of the samples, the flow rate used in the online reSDS step to flush the PolyCAT A trapping column was increased to 5 µL/min for the last 4 minutes of the gradient. Separation of peptides was performed as described for the separation of BSA /ovalbumin protein digest using the 90 min-HILIC gradient at 0.4 µL/min.

Sample preparation of HEK 293F cells by Filter Aided Sample Preparation (FASP)

The FASP of HEK 293F cells was performed following the protocol II described by Wiśniewski *et al.*²⁴, with minor modifications. Briefly, combined cell pellets from three cultured flasks were mechanically disrupted by sonication in 4% (w/v) SDS, 100 mM Tris/HCl pH 7.6, 0.1M DTT in

a ratio 1:10 (v/v) sample-to-buffer for 5 min at 95°C. Cell lysate was then clarified by centrifugation at $16,000 \times g$, 4°C for 5 min. A total of ~ 0.5 mg of proteins per sample were processed by FASP in multiple filter units (maximum 250 µg of protein per filter unit; Microcon Ultracel YM-3, Sigma-Aldrich). In every filter unit 30 µL of cell lysates (proteins concentration ~ 8 µg/µL) were diluted with 200 µL of 8M urea in 100 mM Tris/HCl pH 8.5 (UA buffer). Filter units were then centrifuged at $14,000 \times g$, 4°C for 15 min. The flow-through was discarded and 100 µL of 5 mM IAA in UA buffer were added to the filter units. Alkylation of Cys was performed during 20 min. Subsequently, 100 µL of UA were added to the filter units and they were centrifuged at $14,000 \times g$, 4°C for 15 min. This step was repeated twice. After alkylation, 100 µL of 50 mM ammonium bicarbonate were added to the filter units and they were centrifuged at $14,000 \times g$, 4°C for 15 min. This step was repeated twice. Tryptic digestion was performed adding 40 µL of 50 mM ammonium bicarbonate with sequencing grade trypsin at 1:100 enzyme-to-substrate ratio, overnight at 37°C. After digestion the filter units were transferred to new collection tubes and centrifuged at $14,000 \times g$, 4°C for 10 min. Subsequently, 50 µL of 500 mM NaCl were added. Finally, the filter units were centrifuged at $14,000 \times g$, 4°C for 10 min. The filtrates were acidified to a final concentration of 0.5% FA and finally combined. Experiments were performed in duplicate.

Digestion of HEK 293F cells extract by urea-assisted in-solution digestion for characterization of post-translational modifications

In-solution digestion of HEK 293F cells lysate under urea denaturing conditions was performed as previously described^{36, 37} with minor modifications. Briefly, pellets from three culture flasks were combined and resuspended in 8 M urea in 25 mM ABB and mechanically disrupted by sonication in the presence of protease inhibitors (inhibitor cocktail). Homogenates were

centrifuged at $10\,000 \times g$ for 10 min and the supernatants collected and the protein content quantified using a Bradford protein assay. Next, 0.5 mg of protein was chemically reduced using 10 mM DTT for 1 h at 37 °C and alkylated using 20 mM IAA for 1 h at room temperature in the dark. The samples were then diluted to <1 M urea and incubated overnight at 37 °C in 1:20 enzyme-to-substrate ratio of sequencing-grade modified trypsin. Subsequently, a second trypsin digest was performed (1:125 enzyme-to-substrate ratio) for two hours. Tryptic digestion was quenched by acidification to a final concentration of 0.5% FA. Desalting of samples was performed using C-18 Sep-pack cartridge and elution was carried out with 75% ACN, 0.1% FA. Eluates were then dried in the vacuum concentrator and reconstituted for HPLC fractionation in 200 μ L of 0.02% ammonium hydroxide in water. Experiments were performed in duplicate.

RP-LC fractionation of HEK 293F cells extracts processed by urea-assisted in-solution digestion and FASP

Fractionation of the HEK 293F cells extract (0.5 mg of proteins) that were digested under urea conditions or FASP was performed by high pH RP chromatography. A Shimadzu Prominence UFLC system equipped with a XBridge C18 column (4.6×250 mm, 5 μ m, 130 Å; Waters, Milford, MA) was used as previously described³¹ with minor modifications. Briefly, peptides were separated at 1 mL/min with 0.02% ammonium hydroxide in water as mobile phase A and 0.02% ammonium hydroxide in 80% ACN as mobile phase B. Separation was carried over a 75-min gradient as follows: 5 % mobile phase B for 4 min, 5–12 % mobile phase B for 2 min, 12–50 % mobile phase B for 44 min, 50–80 % mobile phase B for 5 min, and 90–100 % mobile phase B for 5 min, followed by 10 min at 100 % mobile phase B, then returning to the initial conditions for 1 min and then maintained isocratically for 4 min. Absorbance at 280 nm was monitored and fractions collected every minute were partially dried and pooled as above into a total of 23

fractions. The combined fractions were dried in a vacuum concentrator and reconstituted in 0.1% FA.

LC separation of HEK 293F cells extracts processed by by urea-assisted in-solution digestion and FASP

RP-LC separation prior MS analysis of peptides was performed by injecting 1-2 μg of peptides from each fraction (1 μL total volume) into a 200 μm x 10 cm capillary column packed with Aeris PEPTIDE stationary phase (3.6 μm , XB-C18) (Phenomenex Inc., Torrance, CA, USA). Separation of peptides was carried with 0.1% FA in water as mobile phase A and 0.1 % FA in ACN as mobile phase B using a flow rate of 1 $\mu\text{L}/\text{min}$. A 60-min gradient was applied as follows: 6% B for 1 min, 6 – 32 % B for 44 min, 32 – 50 % B for 4 min, 50– 80 % B for 0.1 min followed by 0.9 min at 80 % B, then 80 – 5 % B for 1 min and then maintained at 5% B for 9 min.

Mass spectrometry analysis of HEK 293F cells extract

Peptides from online reSDS, FASP and urea-assisted in solution digestion were sprayed using a Michrom Thermo CaptiveSpray nanoelectrospray ion source (Bruker-Michrom Inc., Auburn, CA) using 1.5 kV spray voltage as previously described.³⁰ Data acquisition by the Orbitrap Elite mass spectrometer was performed in positive mode using Xcalibur 2.2 SP1.48 software (Thermo Fisher Scientific Inc., Bremen, Germany). Fragmentation using high-energy collisional dissociation (HCD) at 32% of normalized collision energy was performed for the 10 most intense precursors with charge higher than 2+. Precursors were isolated with a 2 Da isolation width. Precursor ion scan (MS) (350-1600 m/z range) and ion fragmentation (MS/MS) were both recorded in Orbitrap during data acquisition. For full FT-MS and FT-MS/MS, the automatic gain control values were set at 1×10^6 .

Data analysis

Raw MS/MS data was converted into Mascot Generic Format (mgf) files using Thermo Proteome Discoverer (version 1.4.1.14, Thermo Fisher Scientific Inc., Bremen, Germany). Proteins were identified by Mascot (version 2.3.02, Matrix Science, Boston, MA, USA) using an in-house server. Characterization of PTMs was performed by Mascot using Proteome Discoverer 1.4.1.14. Database searches were performed with a MS tolerance of 10 ppm and a MS/MS tolerance of 0.8 Da or 0.05 Da for the analysis of data obtained using the LTQ-FT Ultra mass spectrometer or the Orbitrap Elite mass spectrometer respectively. Carbamidomethylation at cysteine (Cys) residues was set as a fixed modification, oxidation at methionine (Met), deamidation at asparagine (Asn) and glutamic acid (Glu) and phosphorylation at serine (S), threonine (T) and tyrosine (Y) were considered as variable modifications. A false discovery rate (FDR) of 0.01 was used at peptide level in Proteome Discoverer. For the analysis of the data obtained from the BSA/ovalbumin protein digest a tailored database was constructed. The UniProt human database (downloaded on July 25th 2016, consisting of 70849 sequences and 23964784 residues) was used for the protein database search of the HEK 293F cells extract. The grand average of hydropathy (GRAVY) and isoelectric point (pI) of identified peptides were calculated using an in-house script. The charge of peptides at pH 2 was calculated based on the number of lysine (+), histidine (+) and arginine (+) and the N-terminal amino group (+). Venn diagram analysis was performed using Venny 2.0.2. (BioinfoGP CNB-CSIC, Spain). Alignment of base-peak chromatograms was done using Sieve software (version 1.4.1.14, Thermo Fisher Scientific Inc). Extracted ion chromatograms of identified peptides were generated with Igor Pro 4.0.7.0 (WaveMetrics Inc., Lake Oswego, OR, USA). Statistical significance was established by one-way ANOVA with Bonferroni correction (p -value <0.05), unless otherwise specified. ANOVA analyses and graphics were performed using

GraphPad Prism 6 (GraphPad Software, Palo Alto, USA). Chi-square analyses were performed with XLSTAT Free version 2018.1 (Addinsoft, New York, USA). The open source software Funrich⁴¹ was used for functional analyses. Specific analysis of the membrane proteome content was performed by search comparison of the HEK 293F cells extract to the membrane proteins predicted and curated in the latest version (12/12/2016) of the Human Protein Atlas.⁴² Extracted ion chromatograms (XIC) at 5 ppm were generated using the MZmine 2 software⁴³ as previously indicated.³⁴ Results were reported as mean $\pm$ standard error of the mean (SEM), if not otherwise specified.

Data availability

Data generated in this study have been made publicly available through the ProteomeXchange consortium⁴⁴ via the partner repository PRIDE under the following identifier PXD006035. Reviewer account details: Username: reviewer19100@ebi.ac.uk; Password: ECZa2Dr6

Results and discussion

Chemical principle and development of the online reSDS method

The use of PolyCAT A HPLC in HILIC mode has been applied mainly to the LC-MS/MS analysis of histones⁴⁵ as it allows separation of peptides by charge state and hydrophilicity.⁴⁶ At a pH $\sim$ 2.8 (achieved by the use of 0.1% FA), most peptides display a net positive charge due to neutralization of negative charges. While the n-Asp residues of PolyCAT A (the stationary phase) are also neutralized under these conditions, the more acidic iso-Asp residues (pKa $\sim$ 3.1) remain substantially unprotonated. This neutralization of negative charge in peptides at this pH leads to their retention by both hydrophilic interaction and electrostatic attraction due to positive charges

within peptides. However, under these conditions SDS maintains its negative charge due to a lower pKa (pKa ~ 1.9), and consequently it is not retained by PolyCAT A.

In our online reSDS strategy, PolyCAT A capillaries are used simultaneously for peptide trapping and separation. The trapping stage is carried out under isocratic conditions of low pH and high concentrations of organic solvent, which not only allows for retention of positively charged peptides and the removal of SDS, but also results in salts and impurities being flushed out of the system. Subsequently, by the application of a HILIC gradient, desalted peptides are transferred to a second PolyCAT A capillary column for chromatographic separation and MS analysis as illustrated in **Fig. 1a**. The use of two PolyCAT A columns, connected in parallel through a ten-port valve as shown in **Fig. 1b**, represents one of the main strengths and novelties of our approach as it allows for the serial analysis of SDS-containing samples and reduces waiting time between sample loading.

Online reSDS performance in the analysis of the BSA/ovalbumin protein digest

To maximize the retention of peptides by the PolyCAT A stationary phase and to obtain a uniform elution of the peptides along the chromatographic timeline, we initially optimized the conditions of the gradient by performing a SDS-free experiment. Samples prepared for online reSDS have to be solubilized in organic solvents (**Fig. S-2, Tables S-1 and S-2**) and a minimum concentration of 70% should be used to solubilize particularly challenging samples. However, although low solubility of acidic and hydrophilic peptides is a common concern with salt-free ion exchange systems under HILIC conditions,^{31, 47} in our approach the additional retention by electrostatic attraction overcomes this issue by providing greater flexibility in the range of organic solvent that can be used. A critical requirement of the online reSDS strategy is the efficient trapping of peptides

during the online desalting and removal of SDS molecules. To ensure effective peptide retention during this step, a higher concentration (90%) was used to strengthen hydrophilic interaction during SDS desalting under isocratic conditions.

Following optimization of the chromatographic parameters, the effectiveness of online reSDS for SDS-containing samples was validated by spiking BSA/ovalbumin protein digests with increasing concentrations of SDS (up to 1%). The online reSDS strategy proved to be successful for all tested SDS concentrations with no significant SDS effects detectable on the chromatographic profiles obtained (**Fig. 2**). As shown in Fig. 2, alignment of the chromatographic profiles of samples processed in different SDS concentrations gave alignment scores that ranged between 82.6 and 88.9%, indicating the reliability of this approach to analyze SDS-containing samples. The observed increase in the total number of peptides identified with increasing SDS concentration was not statistically significant, and presumably reflects the expected efficiency of SDS at solubilizing peptides (**Table S-3**). Furthermore, it has been reported that pH ~ 3 significantly enhances the solubilizing ability of SDS,⁴⁸ which could account for the lack of solubility problems seen here when the peptides were solubilized with 0.1% FA and higher concentration of organic solvent.

Analysis of the physicochemical properties of the peptides identified by LC-MS/MS (data shown in **Fig. S-3a-c**) revealed enhanced identification of more highly charged peptides (peptide net charge > +2) using online reSDS compared to RP chromatography. This result demonstrates that the electrostatic attraction of PolyCAT A (under HILIC conditions) results in more efficient trapping and enrichment of more highly charge peptides in the online reSDS strategy. A comparison of the number of unique peptides identified by RP urea-assisted in-solution analysis and by our online reSDS approach showed albeit with considerable overlap, there were two subsets of unique peptides that were exclusively identified by each methodology. These differences in

overlapping can be attributed to the different physicochemical properties of the chromatographic analyses (HILIC vs. RP) (**Fig. S-3d**). These observations indicated that online reSDS should be considered as an approach complementary to RP-LC-MS/MS but is better suited for direct LC-MS/MS analysis of SDS-containing samples that cannot be analyzed by conventional RP-LC-MS/MS-based proteomics.

Impact of SDS concentration on electrospray ionization during online reSDS

The use of SDS is a limiting factor for MS applications even at very low concentrations as it causes severe analyte signal suppression in electrospray ionization (ESI).^{9, 49} In this study we evaluated the occurrence of adverse SDS effects during online reSDS by comparing the extracted ion chromatogram (XIC) of peptides identified in increasing concentrations of SDS. Contrary to previous reports^{49, 50} the ion intensity of peptides identified by online reSDS was constant and unaffected by the presence of SDS (**Fig. 3**). Importantly, the peptides used for this analysis were selected randomly, covering the full range of observed signal intensities.

LC-MS/MS analysis of HEK 293F cells extract digests

To further test the performance of our method we applied it to the analysis of more complex samples. Two independent aliquots of HEK 293F cells were lysed in the presence of 1% SDS and the proteins in the lysates subjected to SDS-assisted in-solution digestion. Prior to MS analysis, sample complexity was reduced by HPLC fractionation. The resulting neighboring fractions were combined based on our previously reported multidimensional chromatography strategy³² and then analyzed individually by online reSDS. As expected and previously observed with urea, we observed here that the use of 1% of SDS directly reduced the endoprotease activity of trypsin. Therefore, successful digestion of the samples was in our experiments achieved after performing

a 100-fold dilution of the initial 1% SDS concentration before overnight trypsin digestion. This dilution step can be adapted according to the complexity of the sample and the trypsin endoprotease capacity. In our experiments, we calculate that the final amount of SDS present in every fraction of the HEK 293F cells extract prior to online reSDS was higher than 0.6% in every fraction of the sample (assuming an equal distribution of SDS across fractions during HPLC fractionation). According to our previous experiments the initial concentration of SDS used to solubilize the sample could thus be further increased up to a theoretical concentration of 1% of SDS/fraction prior to online reSDS if required.

Using the online reSDS strategy, we identified an average of 2940 proteins in the HEK 293F cells extract digests (**Dataset S-1**) based on the identification of an average of 46079 unique peptides (**Dataset S-2**). Functional analysis of the identified proteins determined that online reSDS is not biased and does not enrich proteins from a specific cellular compartment (**Fig. 4a**) as the cellular compartment distribution of proteins identified was similar to that previously reported at the protein level using FASP¹⁵ and at the gene level from microarray approaches.^{15, 51}

Although state-of-art of proteomics technologies currently allows characterization of a large number of proteins from a single sample, the representation of organelle-derived membrane proteins in the raw datasets is limited due to technical challenges associated with their physicochemical properties.¹³ Membrane proteins often display an amphipathic property that allows them to bind to the lipid bilayer membrane while keeping functional domains in the surrounding aqueous milieu.¹³ This particular characteristic, together with their low abundance in vivo, makes their study using proteomics challenging. However, in the present study ~40% of the total proteins identified in the HEK 293F cells extract were membrane proteins (**Fig. 4b**), which is also consistent with previous reports that analyzed the proteome composition.^(15,47)

Performance comparison of online reSDS with FASP on the analysis of complex extracts

To compare the performance of online reSDS with another classic in-solution digestion strategy that involves the use of SDS, we performed a comparison between online reSDS, FASP and urea-assisted in solution digestion. No significant differences were observed in the number of unique peptides between online reSDS and urea as shown in **Figure 4c (Dataset S-2-4)**. On the contrary, FASP obtained a significantly lower number of unique peptides that might be attributed to a most intense sample processing. Besides, significant differences in the number of identified proteins were also observed between all the three tested methodologies (**Figure 4d ; Datasets S-1 and S-5**), where the SDS-free strategy, urea-assisted in solution digestion, provided the highest number of identified proteins (**Dataset S-6**). These comparison demonstrates the efficiency and performance of reSDS, therefore online reSDS represents an important in-solution alternative strategy when the use of SDS is required in the sample processing.

Direct analysis of protein posttranslational modifications from complex extracts by online reSDS

We then analyzed the performance of online reSDS characterizing different types of PTMs from complex sample extracts (i.e. Gln and Asn deamidation and Ser, phosphorylation) (**Fig. 5**). In this experiment the PTMs data obtained by online reSDS were compared to those obtained from FASP and urea-RP, common in-solution digestion strategies for the characterization of PTMs by LC-MS/MS. We found that online reSDS significantly improved the identification of charge-related modified peptides (**Figure 5a**), specially identifying phosphorylated peptides (Ser, Thr and Tyr) compared to the other two tested in-solution methodologies, as shown in **Figure 5b**. Besides,

identification of Gln and Asn deamidation was significantly improved by online reSDS compared to FASP (**Figure 5c and d**). These results suggest that on-line reSDS represents a promising strategy for the analysis of the PTMs from complex samples, especially Ser, Thr and Tyr phosphorylation without a priori enrichment, a step often required in standard shotgun proteomics.

Online reSDS takes full advantage of the application of multidimensional chromatography for the separation and quantification of PTMs.³² This is especially useful for quantification of Asn and Gln deamidation since the first dimension separation by RP allows co-elution of the modified and un-modified peptides,³² which can then be collected in the same fraction for subsequent separation by PolyCAT A chromatography using online reSDS as shown in **Fig. S-4a-d**. In the future, we anticipate that the use of commercially packed WCX capillaries could significantly improve the analysis of complex extracts when used as part of online reSDS. Based on the proof-of-concept provided by this study we consider that online reSDS represents an efficient LC-MS/MS method that will complement the common use of FASP and RP-urea methodologies in shotgun proteomics.

Conclusions

The strategy (online reSDS) presented in this study permits straightforward LC-MS/MS analysis of SDS-containing samples. SDS can be completely removed online by the electrostatic repulsion generated between the negatively charged sulfate group of SDS and the negatively charged WCX stationary phase in the acidic condition typically used on LC-MS/MS. The combination of multidimensional RP-WCX chromatography in online reSDS represents an alternative method when SDS is used in sample processing for LC-MS/MS-based proteomic analysis and especially improves the characterization of peptides modified by PTMs.

Author contributions

A.S. and X.G.-P. are co-first authors and contributed equally to this work. A.S. designed the experiments, packed capillary columns, performed experiments, analyzed data and wrote the manuscript. X.G.-P. designed the experiments, prepared samples, analyzed data and wrote the manuscript. B.D. cultured cell lines and performed proteomics. S.K.S. conceived the project, designed the experiments, supervised the project and wrote the manuscript. All the authors have read and approved the final version of the manuscript.

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Supporting Information

The following is the supporting data related to this article:

Figure S-1: Diagram of the configuration for the online reSDS.

Figure S-2: Optimization of the gradient used for the separation of peptides.

Figure S-3: Physicochemical properties of the peptides identified from the BSA/ovalbumin protein digest by online reSDS at different SDS concentrations.

Figure S-4. Separation of deamidated peptides and their un-modified pairs from the HEK F293 cells extract digests using online reSDS.

Table S-1: Total and unique peptides obtained by on-line reSDS during the analysis of a BSA/ovalbumin protein digest.

Table S-2: Multiple comparison test for the total and unique peptides obtained by online reSDS from the BSA/ovalbumin protein digest.

Table S-3: Total and unique peptides identified by on-line reSDS from the BSA/ovalbumin protein digest spiked with increasing concentrations of SDS.

Dataset S-1: List of proteins identified by online reSDS from the HEK 293F cells extract digest.

Dataset S-2: List of unique peptides identified by online reSDS from the HEK293F cells extract digest.

Dataset S-3: List of unique peptides identified by FASP from the HEK293F cells extract digest.

Dataset S-4: List of unique peptides identified from the HEK293F cells extract digested by urea-assisted in solution digestion.

Dataset S-5: List of proteins identified by FASP from the HEK 293F cells extract digest.

Dataset S-6: List of proteins identified from the HEK 293F cells extract digested by urea-assisted in solution digestion.

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Figures

a

b

Figure 1. Diagram illustrating the principle of the online reSDS. **a.** Peptide trapping uses a weak cation exchange (WCX) stationary phase and is carried out at pH ~ 2.8 which allows binding of positively charged peptides by hydrophilic and electrostatic interaction. At the same time, the stationary phase repels negatively charged SDS by electrostatic repulsion (green arrows). Subsequently, peptides are separated on a second WCX capillary column. Mobile phase flow is depicted as a pink line. **b.** Diagram of the two possible configurations of the 10-port valve used to connect in parallel the two PolyCAT A trapping columns.

Figure 2. Validation of the online reSDS approach using the BSA/ovalbumin protein digest spiked with increasing concentrations of SDS. **a.** LC-MS chromatograms with different percentages of SDS (0-1% SDS). **b.** Peak alignment and alignment scores from base-peak chromatograms of all tested conditions.

Figure 3. Evaluation of the signal suppression by the analysis of extracted ion chromatograms (XICs) corresponding to four randomly selected peptides. **a-d.** XICs of peptides for the different SDS percentages (0 %, 0.1 %, 0.2 %, 0.5 % and 1 % SDS). Horizontal lines represent minimum and maximum integrated areas for each condition. XIC peak heights have been adjusted to fit the mean of the integrated area for each condition. No significant differences were observed for the peak intensity of the four selected peptides between the different concentrations of SDS tested.

Figure 4. Functional enrichment study and method comparison of the HEK F293 cells extract digests by online reSDS. **a.** Distribution of the identified proteins by cellular compartment categories (considering the categories with more than 5% of gene enrichment). **b.** Diagram illustrating the percentage of proteins categorized as membrane proteins identified by online reSDS. Categorization of membrane proteins was performed by comparison between the reSDS protein list and the list of membrane proteins curated in The Human Protein Atlas. Further functional enrichment analysis describes the identified subclasses of membrane proteins. **c.** Comparison of the number of unique peptides obtained by online reSDS, FASP and urea-assisted

in solution digestion and **d.** Number of identified proteins by online reSDS, FASP and urea-assisted in solution digestion.. *Statistical significance was assessed by one-way ANOVA with Bonferroni correction (p -value < 0.05).

Figure 5. Comparison of the number of post-translationally modified unique peptides identified by FASP, reSDS and urea-assisted in-solution digestion from HEK F293 cells extract digests. **a.** Ratio modified/unmodified unique peptides calculated considering modified peptides those that contain a charge-related modification (including phosphorylation and Q/N deamidation). **b.** Number of phosphorylated unique peptides. **c.** Number of deamidated (Gln (Q)) unique peptides and **d.** Number of deamidated (Asn (N)) unique peptides. *Indicates significant differences assessed by ANOVA with Bonferroni correction (p -value < 0.05). **Indicates significant differences assessed by Chi-square test (p -value < 0.05).

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