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Cleavable Crosslinkers as Tissue Fixation Reagents for Proteomic Analysis

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Formaldehyde fixation is widely used for long-term maintenance of tissue. However, due to formaldehyde-induced crosslinks, fixed tissue proteins are difficult to extract, which hampers mass spectrometry (MS) proteomic analyses. Recent years have seen the use of different combinations of high temperature and solubilizing agents (usually derived from antigen retrieval techniques) to unravel formaldehyde-fixed paraffin-embedded tissue proteomes. However, to achieve protein extraction yields similar to those of fresh-frozen tissue, high-temperature heating is necessary. Such harsh extraction conditions can affect sensitive amino acids and post-translational modifications, resulting in the loss of important information, while still not resulting in protein yields comparable to those of fresh-frozen tissue. Herein, the objective is to evaluate cleavable protein crosslinkers as fixatives that allow tissue preservation and efficient protein extraction from fixed tissue for MS proteomics under mild conditions. With this goal in mind, disuccinimidyl tartrate (DST) and dithiobis(succinimidylpropionate) (DSP) are investigated as cleavable fixating reagents. These compounds crosslink proteins by reacting with amino groups, leading to amide bond formation, and can be cleaved with sodium metaperiodate (*cis*-diols, DST) or reducing agents (disulfide bonds, DSP), respectively. Results show that cleavable protein crosslinking with DST and DSP allows tissue fixation with morphology preservation comparable to that of formaldehyde. In addition, cleavage of DSP improves protein recovery from fixed tissue by a factor of 18 and increases the number of identified proteins by approximately 20% under mild extraction conditions compared with those of formaldehyde-fixed paraffin-embedded tissue. A major advantage of DSP is the introduction of well-defined protein modifications that can be taken into account during database searching. In contrast to DSP fixation, DST fixation followed by cleavage with sodium metaperiodate, although effective, results in side reactions that prevent effective protein extraction and interfere with protein identification. Protein crosslinkers that can be cleaved under mild conditions and result in defined modifications, such as DSP, are thus viable alternatives to formaldehyde as tissue fixatives to facilitate protein analysis from paraffin-embedded, fixed tissue.

Introduction

Formaldehyde-fixation-paraffin-embedding (FFPE) or ultralow temperature freezing (at −80 to −190 °C) are the two most commonly employed strategies in tissue preservation for diagnostic and research purposes.[1] Although controlled freezing is considered the best way to preserve biomolecules and live cells, it requires specialized and costly facilities for tissue preservation. In addition, unless freezing is performed under high-pressure or vacuum conditions, tissue fine structure can be distorted, which hampers histological tissue evaluation.[2] On the other hand, FFPE tissue can be stored at room temperature, which reduces storage costs, while maintaining tissue architecture.[2b] For these reasons, formaldehyde fixation followed by paraffin embedding has become the most common procedure for the long-term preservation of clinical samples, leading to the generation of large FFPE tissue repositories worldwide.[2b,3] Routinely, FFPE tissue from diseased and paired control areas is collected in hospitals for analysis and stored in sample archives. Because stored FFPE tissue is linked to pathological, clinical, and outcome information, it represents a tremendous potential for biomarker discovery and mechanistic studies.[3b,4] In this regard, there has been growing interest in developing strategies for mass spectrometry (MS)–proteomic studies on FFPE tissue in recent years.[3b,5] This is not only due to the possibility of linking MS–proteomic results to relevant clinical data, but also because proteins that might be closely linked to a disease mechanism are present in higher amounts in the tissue of interest than in blood, which is the most common sample employed for diagnostic and prognostic clinical assays.[3b,6] However, MS–proteomic analyses on FFPE tissue are hampered by

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difficulties related to protein crosslinks generated after formaldehyde fixation. Formaldehyde is a reagent that fixes tissue mainly through the formation of methylene bridges, preferentially between protein primary amines.[2b,7] The main problem derives from the reduced solubility of crosslinked proteins, which results in limited and biased protein extraction from FFPE tissue.[3b,4] In recent years, this problem has been addressed, particularly through adaptation of "antigen retrieval methods" used in immunohistochemistry to allow MS–proteomic studies on FFPE tissue.[5b,d,8] Heating FFPE tissue to fairly high temperatures (60–115 °C for 20 min–2 h) greatly improves protein extraction from fixed tissue and is one of the most important factors for protein recovery.[3b] However, heating proteins to high temperatures can lead to chemical modifications, aggregation, and loss of informative post-translational modifications. The extent of modification of proteins in fixed tissue is currently unclear and it is uncertain if all proteins are solubilized under these conditions. Additional information is also required on how different fixation times or storage periods might affect crosslink reversal.[3b,6,8c,9] Furthermore, it is known that formaldehyde-derived crosslinks are more heterogeneous and complex than originally assumed, which leads to undefined and unspecified modifications on peptides, resulting in ambiguous or negative results upon matching experimental data to in silico databases.[2b,3b] This, together with the fact that formaldehyde fixation does not prevent nucleic acid degradation completely, has led to the investigation of alternative fixation strategies,[10] including the use of alcohol-based (i.e., Methacarn, UMFIX, FineFIX), zinc-based (Z7), and commercial non-crosslinking fixatives (i.e., RCL2, PAXgene, HOPE). Although these reagents have shown promising results with respect to tissue morphology and nucleic acid preservation,[10–11] none of them have replaced formaldehyde. A few MS–proteomic studies have been performed with alternative, non-crosslinking fixatives to show that protein extraction yields and identification rates approach those of fresh-frozen tissue,[11b,12] but there is currently no information on the long-term stability of the morphology of tissue sections fixed in this manner.

Cleavable crosslinkers combine the advantage of creating covalent bonds between proteins for tissue fixation with the possibility of retrieving proteins under defined chemical conditions for MS-based proteomics analysis. It is, however, currently not clear whether they can fix tissue with similar success to that of formaldehyde and the extent to which they can facilitate subsequent proteomics analyses. We therefore evaluated two cleavable crosslinkers, disuccinimidyl tartrate (DST) and dithiobis(succinimidylpropionate) (DSP), as tissue fixatives with respect to preservation of tissue morphology, protein extraction yield, and LC-MS/MS-based protein identification rate. These reagents crosslink proteins through primary amino groups with two potential advantages over formaldehyde: 1) the crosslinking reaction is more specific, leading to the generation of fewer side products; and 2) the protein crosslinks can be cleaved under mild, chemically defined conditions. Because protein crosslinks are cleaved prior to protein extraction, we expect that protein recovery will be higher than that of FFPE tissue. In addition, the introduction of defined protein modifications is expected to facilitate peptide identification by database searching of MS/MS spectra. To assess these characteristics, we evaluated FFPE, DST, and DSP fixation on rat liver tissue and compared them with fresh-frozen tissue.

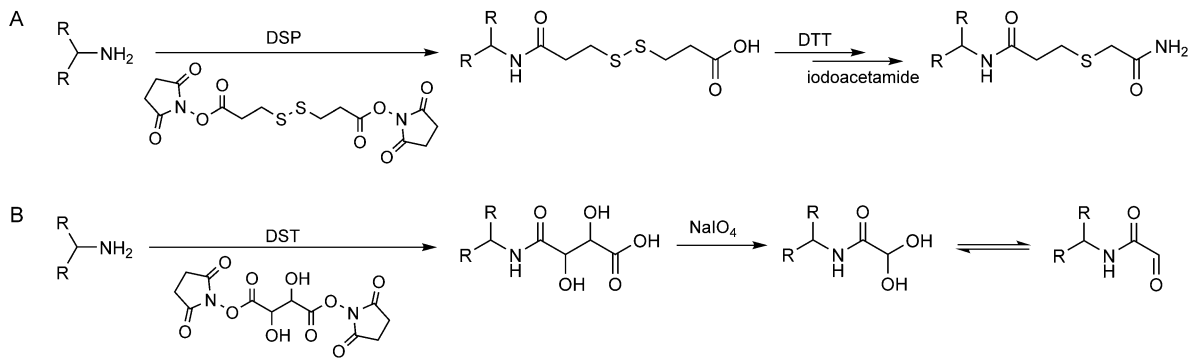
Results and Discussion

Peptide modification

A synthetic peptide (*N*-acetyl-Asn-Leu-Glu-Phe-Lys-NH_2-amide), with a single primary amino group, was used as a model to identify modifications that might be introduced by DST and DSP. The peptide was reacted with DSP or DST, followed by reaction of the resulting products with DTT/iodoacetamide or sodium metaperiodate, respectively.

In both cases, modification of the primary amino group on lysine was observed. If DSP is used, the main product corresponds to the addition of hydrolyzed DSP to the peptide ($C_5H_7O_2S_2$; Scheme 1 A). Reaction with DTT and iodoacetamide led to cleavage and alkylation of the thiol, which resulted in the addition of $C_5H_7N_1O_2S_1$ to the peptide, as expected. This indicates that the main modification after tissue fixation with DSP and subsequent cleavage/alkylation of the crosslinks with DTT/iodoacetamide should be a mass increment of 145.120 Da.

Reacting the peptide with DST resulted in the addition of hydrolyzed DST ($C_4H_4O_5$, +132.006 Da), which led to the addition of $C_2H_2O_3$ and C_2O_2 (+74.000 and +55.990 Da) after cleav-



Scheme 1. Reaction products after A) DSP or B) DST modification and crosslinker cleavage.

age with sodium periodate (Scheme 1 B). In addition to this expected peptide modification, another product corresponding to the addition of 160.000 Da was observed. We were unable to assign this mass increase to any theoretically expected DST modification. Treatment with sodium periodate had no effect on this product.

Tissue surrogate generation and SDS-PAGE analysis

Tissue surrogates (TSs) are formed if proteins are crosslinked. They are an easy way to evaluate whether a crosslinker can be employed as a tissue fixative.[13] Figure 1 A shows that both crosslinkers result in lysozyme TSs; this indicates that both reagents lead to protein crosslinking under the employed conditions and could serve as fixatives. This was confirmed by non-reducing SDS-PAGE on TSs formed after 30 min and 24 h. The addition of extraction buffer (2% SDS, 8 M urea in 50 mM Tris buffer pH 7.6) to the TSs formed after 30 min led to complete (formaldehyde-TS, form-TS) or partial (DST- and DSP-TSs) solubilization. There was still some monomeric lysozyme in all cases in the solubilized portion next to dimers and higher order multimers (Figure 1 B, lanes 3–5). On the other hand, the addition of extraction buffer to 24 h TSs did not allow the solubilization of lysozyme from the formaldehyde TS, whereas small amounts of solubilized monomer and dimers were observed for the DST- and DSP-TSs (Figure 1 B, lanes 6–8). This indicates that the initial kinetics of formaldehyde crosslinking are slower than those for DST and DSP, due to the higher reactivity of *N*-hydroxysuccinimide (NHS) esters, but that crosslinking is complete with formaldehyde after 24 h, whereas some soluble protein is left for DST and DSP. This indicates that hydrolysis of NHS esters in DSP and DST, under aqueous conditions at pH 7.5, brings the crosslinking reaction to a halt prior to completion, whereas formaldehyde continues to react. Because tissue fixation requires 24 h of incubation to allow diffusion of the crosslinker throughout the tissue, anhydrous conditions were employed in subsequent experiments to avoid hydrolysis of the NHS esters before crosslinking was complete.

Incubating the 24 h DSP-TSs with cleavage solution (15 mM DTT/30 mM iodoacetamide) resulted in solubilization of lysozyme with very little or no dimers and multimers remaining (Figure 1 B, lane 10). Similar results were observed after incubation of 24 h DST-TSs with 15 mM sodium metaperiodate (Figure 1 B, lane 9). These initial results confirm that DSP and DST crosslinks can be cleaved and that MS–proteomic analysis after tissue fixation should be favored for these crosslinkers.

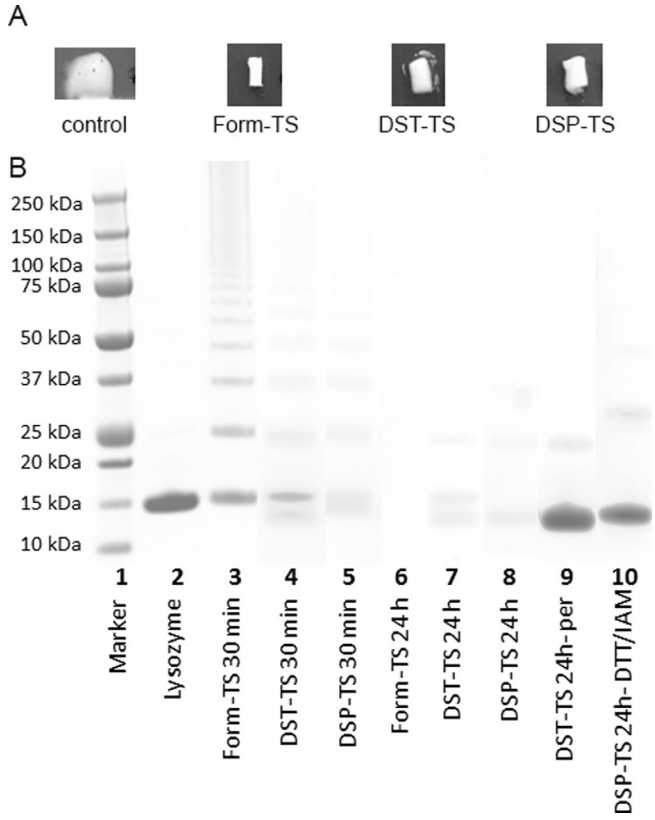


Figure 1. A) Surrogate tissue formation from lysozyme. Left to right: control (lysozyme), formaldehyde tissue surrogate (Form-TS), DST tissue surrogate (DST-TS), DSP tissue surrogate (DSP-TS). B) SDS-PAGE analysis of TS formation and solubilization due to crosslinker cleavage. Lane 1: marker, lane 2: control, lane 3: Form-TS after 30 min of reaction, lane 4: DST-TS after 30 min of reaction, lane 5: DSP-TS after 30 min of reaction, lane 6: Form-TS after 24 h of reaction, lane 7: DST-TS after 24 h of reaction, lane 8: DSP-TS after 24 h of reaction, lane 9: DST-TS treated for 24 h with 15 mM sodium metaperiodate, lane 10: DSP-TS treated for 24 h with 15 mM DTT/30 mM iodoacetamide.

Tissue fixation and protein extraction/identification from fixed tissue

Rat liver tissue slices were fixed with DST or DSP under anhydrous conditions (DMSO) to avoid hydrolysis of the NHS ester moieties and to maintain the reactivity of the crosslinkers for 24 h. Fixed tissue was embedded in paraffin, hematoxylin and eosin (H&E) stained, and compared with FFPE tissue. Both DST and DSP allow preservation of the tissue architecture in a way that is comparable to that of formaldehyde (Figure 2). The only difference between formaldehyde and DST/DSP fixation is the less intense eosin staining in DST/DSP-fixed tissue, which can be explained by the fact that these reagents convert primary/secondary amino groups into amides that are no longer protonated (positively charged), leading to reduced binding of the anionic dye eosin.[14]

We subsequently investigated the effect of crosslinking and cleavage on protein extraction and identification for different fixation strategies under mild extraction conditions. To do so, we compared the total amount of protein extracted from fresh-frozen tissue to that of nonfixed paraffin embedded (PE), FFPE, DST-FPE, and DSP-FPE tissue (three biological replicates in all cases). After protein extraction, proteins were precipitated, digested, and the peptides were analyzed by LC-MS/MS. Table 1 shows that the different steps needed for paraffin removal and tissue rehydration lead to protein loss (ca. 30%) in comparison with PE and fresh-frozen tissue in the absence of any fixation. On the other hand, extraction of PE tissue and DSP-fixed tissue gave similar amounts of extracted protein; this indicates that DSP fixation followed by cleavage does not affect protein extraction and that losses relative to fresh-frozen

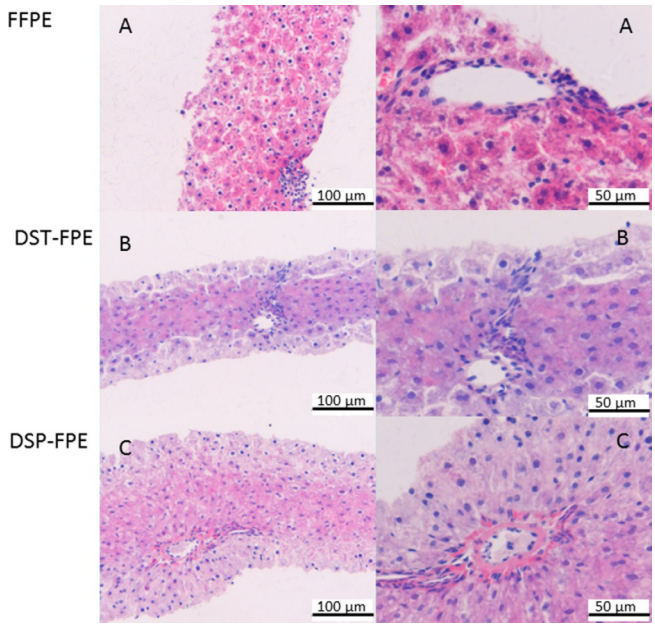


Figure 2. H&E staining of rat liver tissue slices fixed with 4% (*w/v*) formaldehyde (A), 1% (*w/v*) DST in dry DMSO (B), or 1% (*w/v*) DSP in dry DMSO (C).

Table 1. Amount of protein extracted from the same amount of fresh-frozen liver tissue and from PE, DSP-FPE, DST-FPE, and FFPE fixed tissue.

	Total protein concentration Mean ($n=3$, µg per slice)	RSD [%]	Relative to fresh-frozen [%]	Relative to PE [%]
fresh-frozen	143	5	100	
PE	99	12	69	100
DSP-FPE	91	18	64	93
DST-FPE	15	41	10.5	22
FFPE	5	38	3.5	7

[a] Measurements were performed by Micro BCA assay (see the Experimental Section). Mean and relative standard deviation (RSD) values from three biological replicates are given.

tissue are due to the paraffin removal and tissue rehydration steps.

DST fixation and sodium metaperiodate cleavage led to significantly reduced protein extraction compared with that of DSP, but was still slightly higher than that for formaldehyde fixation (3 times more protein extracted).

To investigate whether *cis*-diol cleavage did occur in DST-fixed tissue, we performed periodic acid–Schiff (PAS) staining. During PAS staining, the tissue is incubated with periodic acid to generate reactive aldehyde groups that further react with pararosaniline to form a Schiff base; this gives the tissue a characteristic pink color. In the case of liver tissue, it is necessary to remove glycogen by pretreatment with α-amylase to avoid interference.[15] There is strong PAS staining for FFPE and DST-PE tissue prior to treatment with α-amylase (Figure 3, left), whereas only DST-PE tissue gave strong PAS staining after glycogen removal (Figure 3, right). This confirmed that metaperiodate-mediated cleavage of the DST crosslinker was successful, leading to reactive aldehyde groups.

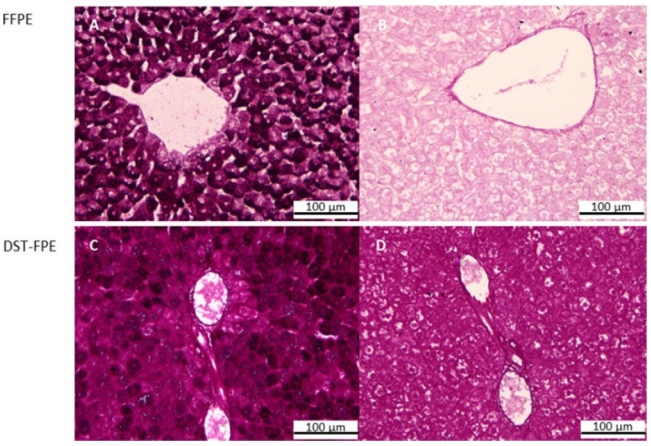


Figure 3. PAS staining of rat liver tissue slices fixed with 4% (*w/v*) formaldehyde (FFPE) A) prior to and B) after glycogen removal, and fixed with 1% (*w/v*) DST in dry DMSO (DST-FPE) C) prior to and D) after glycogen removal.

We hypothesize that low protein extraction after DST fixation and cleavage may be due to further reaction of the generated reactive aldehyde groups with amino groups, for example, in proteins, possibly leading to renewed crosslinking, which hampers protein extraction.

To assess the extent to which protein identification rates could be improved, equal amounts of extracted protein were digested by trypsin and subjected to LC-MS/MS analysis followed by database searching. This resulted in comparable numbers of identified proteins for fresh-frozen, PE, and DSP-fixed tissue (Table 2).

Table 2. Number of proteins and number of modified proteins identified by LC-MS/MS from fresh-frozen liver, PE, DSP-FPE, DSP-FPE and FFPE liver tissue using equal amounts of protein.

	No. of proteins identified			No. of modified proteins identified	
	Mean	RSD [%]	Av. sequence coverage [%]	Mean	RSD [%]
fresh-frozen	623	2	28	–	–
PE	597	0.4	27	–	–
DSP-FPE	611	1	14	433	3
DST-FPE	337	17	8	4	83
FFPE	511	2	24	–	–

Mean and RSD values from three biological replicates are given.

FFPE tissue gave a slightly lower number of identified proteins (ca. 85%), whereas DST-fixed tissue led to a clearly reduced identification rate (ca. 55% that of fresh-frozen tissue). Average sequence coverage per protein was low for DST-fixed tissue (8%) and was clearly higher for DSP-fixed tissue (14%). Sequence coverage was highest for fresh-frozen and PE tissue (27–28%) and only slightly lower for FFPE tissue (24%) (Table 2). The low sequence coverage for DST might be due to

ill-defined protein modifications, as observed at the peptide level, and to renewed crosslinks that cannot be taken into account during database searching. LC-MS/MS analysis and database searching resulted, on average, in only 4 proteins with the expected DST modifications out of a total of 337 identified proteins (Table 2), whereas DSP gave 433 modified proteins out of 611 identified proteins. The reason for the reduced sequence coverage for DSP is currently unexplained, but might also be due to modifications that were not taken into account, such as oxidation of sulfur or the recently described cross-reactivity of iodoacetamide with methionine.[16]

Conclusion

Cleavable crosslinkers allow reversible tissue fixation with preservation of microscopic morphology, while, at the same time, facilitating MS–proteomic analysis. Evaluation of these aspects was based on 1) a model peptide to identify possible modifications that might be introduced into fixed proteins, 2) a "surrogate tissue" model to investigate DST/DSP crosslinking capabilities and the effect of cleavage on protein solubility, and 3) rat liver tissue to assess the preservation of tissue morphology and the effect on protein extraction and LC-MS/MS-based protein identification.

One of the main challenges in MS–proteomic analysis of FFPE tissue is defining the exact modifications that affect proteins during tissue fixation. Formaldehyde fixation leads to the formation of crosslinked intra- and interprotein networks. Furthermore, formaldehyde fixation can lead to the formation of adducts and reaction products different from those of the expected methylene bridges.[2b,13a] Although some of these reactions have been described, the identification of the precise protein modifications that occur in FFPE tissue requires further investigation. It is possible that other (not yet identified) protein modifications are formed during the process, which leads to negative results upon matching experimental MS/MS spectra to those generated in silico from peptides in a given sequence database. Thus, the successful analysis of fixed tissue relies not only on proper extraction, denaturation, and protein digestion, but also on the precise knowledge of possible peptide modifications. Taking all of this into consideration, our first objective was to investigate which modifications DSP and DST introduced into proteins during tissue fixation. To achieve this objective, we employed a synthetic peptide (*N*-acetyl-Asn-Leu-Glu-Phe-Lys-NH_2-amide) as a model because it allowed easy monitoring of different reactions and interpretation of the results. Our results show that lysine-containing peptides are modified by DST or DSP through the addition of $C_2H_2O_3$ or C_2O_2 (+74.000, +55.990 Da) and $C_5H_7N_1O_2S_1$ (145.120 Da), respectively. In addition, DST leads to an unspecific modification of +160.000 Da, which we could not assign to expected DST modification products. The DSP crosslinking reaction is more specific, which results in well-defined reaction products. This indicates that DSP crosslinking is advantageous compared with DST for tissue fixation in view of MS–proteomics.

To investigate the capability of DST and DSP to crosslink proteins in tissue and the effect of crosslinker cleavage on protein extraction from fixed tissue, we employed a surrogate tissue model based on lysozyme. The surrogate tissue model was introduced by Fowler et al.[13] to study protein recovery from archival FFPE tissue. In this model, a protein solution is crosslinked through the addition of formaldehyde to form a gel plug that can undergo dehydration and paraffin embedding. This model has been employed to investigate different protocols for protein extraction from FFPE tissue, protein LC-MS/MS analysis, and protein identification.[13] Our results show that DSP and DST generate a TS that can be solubilized under the employed cleavage conditions. These results indicated that the investigated cleavable crosslinkers were suitable as reversible fixatives for MS proteomics. However, we also observed that crosslinking was not complete after 24 h, in comparison to formaldehyde under aqueous conditions at pH 7.5. This is likely due to hydrolysis of the NHS ester moieties during crosslinking. Subsequent crosslinking reactions were thus performed in anhydrous DMSO. Although tissue architecture was preserved under these conditions, this is a disadvantage of the cleavable crosslinkers. Future generations of crosslinkers should thus combine a water-insensitive activated group for protein crosslinking with a linker that can be cleaved under defined and mild chemical conditions, resulting in well-defined chemical modifications.

Finally, we evaluated the capability of DST and DSP to fix tissue and preserve its morphology. Our results demonstrate that both DST and DSP allow preservation of the tissue architecture in a way that is comparable to that of formaldehyde. However, the selection of adequate protein crosslink reversal conditions is crucial. Protein crosslinking reversal should result in protein extraction from fixed tissue that is comparable to that of nonfixed tissue and, moreover, should allow comprehensive MS–proteomics studies. Our results show that protein extraction is severely hampered if protein crosslinking is not fully reversible (DST-FPE and FFPE), whereas extraction yields similar to those of nonfixed tissue (PE) are obtained if protein crosslinking is fully reversible (DSP-FPE). In addition, if protein modifications due to crosslinking are not well defined, protein identification is affected, even if the same amount of extracted protein is used (337 proteins in DST-FPE vs. 611 proteins in DSP-FPE tissue from the same initial extracted protein amount).

In summary, DSP is a suitable, cleavable fixative that allows preservation of tissue morphology and MS–proteomic analysis after paraffin embedding with comparable yields to those of nonfixed tissue. Under mild extraction conditions, DSP-FPE tissue compares well to fresh-frozen tissue with respect to protein extraction yield (64%), which is equivalent to nonfixed PE tissue (69%), whereas protein recovery from FFPE tissue is only 5%. DSP fixation allows tissue preservation and excellent protein extraction/identification without the need for harsh extraction conditions, such as elevated temperature, which might affect post-translational modifications or certain amino acids and lead to protein aggregation. Another cleavable crosslinker DST, which we expected to perform equally well, proved disappointing when it came to the final MS–proteomic analysis because it introduced ill-defined modifications and did not allow

the extraction of nearly as much protein after sodium metaperiodate mediated cleavage.

Experimental Section

Chemicals: DST and DSP were purchased from Pierce Biotechnology (Rockford, USA). Formaldehyde (4% solution) was from Klinipath (Duiven, The Netherlands). University of Wisconsin organ preservation solution was from DuPont Critical Care (Waukegab, USA). Angiotensin II, ammonium bicarbonate, α-amylase, β-mercaptoethanol, D,L-dithiothreitol (DTT), bioreagent-grade DMSO, Dulbecco's phosphate-buffered saline (PBS), formic acid, hematoxylin, iodoacetamide, lysozyme, myoglobin, periodic acid, 50 mM phosphate buffer, Schiff reagent, SDS, sodium metaperiodate, trizma base, trifluoroacetic acid (TFA), and urea were from Sigma–Aldrich. Precision Plus Protein Dual Color Standard and Coomassie Brilliant Blue R-250 staining solution were from BioRad (USA). NuPAGE MES SDS running buffer was from Novex (Carlsbad, USA). Acetonitrile (HPLC supragradient grade) and xylol (reagent grade) were from Biosolve (Valkenswaard, The Netherlands). Trypsin was from Promega (sequencing-grade modified trypsin), eosin was from Merck, absolute ethanol (AnalR NORMAPUR) was from VWR International (Fontenay-sous-bois, France), and water was purified by means of an Arium Ultrapure water system (conductivity 18.2 MΩ cm; Sartorius Stedim Biotech, Göttingen, Germany).

Peptide modification: A synthetic peptide (*N*-acetyl-Asn-Leu-Glu-Phe-Lys-NH_2-amide) was reacted with DST and DSP followed by incubation with cleavage solutions (15 mM sodium metaperiodate or 15 mM DTT/30 mM iodoacetamide, respectively). For DST or DSP modification, peptide solution (1 μL; 5 mM in 0.1% formic acid) was added to DST or DSP (50 μL, 0.4 mg mL^{-1}) in DMSO. The mixture was left to react for 30 min (600 rpm in a thermomixer, room temperature). Afterwards, DST-derivatized peptides were diluted to 0.5 mL with sodium acetate buffer (50 mM; pH 5). DST crosslinks were cleaved by the addition of sodium metaperiodate (1 μL, 750 mM) to the DST-derivatized peptide solution (0.1 mL; 1 h, 600 rpm in a thermomixer, room temperature). DSP-derivatized peptides were diluted with 50 mM ammonium bicarbonate buffer to 0.5 mL. DSP crosslinks were cleaved by the addition of DTT (1 μL, 750 mM; 30 min, 600 rpm, room temperature) to the DSP-derivatized peptide solution (0.1 mL) followed by the addition of 1.5 M iodoacetamide (1 μL; 30 min, 600 rpm in a thermomixer, room temperature, in the dark). The different reaction steps were monitored by LC-MS/MS on an Agilent series 1100 capillary LC system (Waldbronn, Germany) comprised of a degasser, a binary pump with stream splitter and flow controller, a thermostated autosampler (4 °C), and a thermostated column compartment (40 °C). The derivatized peptides were analyzed with an Atlantis dC18 column (Waters; Etten-Leur, The Netherlands; 1.0 mm × 150 mm, particle size 3 μm). Mobile phase A consisted of 0.1% (*v/v*) formic acid in ultrapure water. Mobile phase B was 0.1% (*v/v*) formic acid in acetonitrile (HPLC-S gradient grade; Biosolve; Valkenswaard, The Netherlands). The injection volume was 1 μL. Separation was performed with an increasing gradient of B (3–90% at 4% min^{-1}) at a flow rate of 50 μL min^{-1}. The mass spectrometer was a Bruker HCT ultra ETD II ion trap (Bruker Daltonics, Bremen, Germany) with an ESI source operated in positive mode. Data were acquired over a scan range of *m/z* 100–2000. The original MS spectra were analyzed with the Data Analysis software (Bruker Daltonics, v. 3.4).

Surrogate tissue generation and SDS-PAGE analysis: TSs were generated as described by Fowler et al.[13b] Briefly, a solution of lysozyme (150 mg mL^{-1}) in phosphate buffer (50 mM, pH 7.5) was mixed (equal volumes) with a solution (10 mg mL^{-1}) of DST or DSP in dry DMSO, or a 4% (*w/v*) solution of formaldehyde, and left to react overnight. As a control, lysozyme (0.1 mL, 150 mg mL^{-1}) in phosphate buffer (50 mM, pH 7.5) was mixed with DMSO (0.1 mL). The protein crosslinking and cleavage processes were monitored by SDS-PAGE. To do so, 2% SDS (92 μL) and urea (8 M) in Tris buffer (50 mM, pH 7.6) were added to the TSs after 30 min or 24 h reaction times. As a control, a solution of protein (2 μL, 150 mg mL^{-1} lysozyme in 50 mM phosphate buffer) was mixed with DMSO (2 μL) before 2% SDS (92 μL) and 8 M urea in Tris buffer (50 mM, pH 7.6) were added. To monitor the cleavage of the crosslinkers, cleavage solution (92 μL) was added to the TSs. DST-TSs were cleaved with sodium metaperiodate (15 mM in 50 mM acetate buffer pH 5) for 1 h (600 rpm in a thermomixer, room temperature, in the dark). DSP-TSs were cleaved with DTT (15 mM, 46 μL) for 30 min at 60 °C followed by the addition of iodoacetamide (30 mM, 46 μL; 30 min, room temperature, in the dark). After completion of the reaction, each sample (8 μL) was mixed with PBS (8 μL) and fivefold-concentrated loading buffer (4 μL; 10% SDS, 10 mM DTT, 20% glycerol, 0.2 M Tris·HCl pH 6.8, 0.05% Bromophenol Blue). SDS-PAGE was performed in a Mini-Protein III cell (Bio-Rad) with 4–12% Bis·Tris gels (NuPAGE, Novex) by loading sample (10 μL). Precision Plus Protein Dual Color Standard was used as a molecular-weight marker and 20 times diluted NuPAGE MES SDS solution as running buffer. Proteins were stained with Coomassie Brilliant Blue R-250.

Tissue fixation, dehydration, paraffin embedding, and staining: All experiments were approved by the committee for care and use of laboratory animals of the University of Groningen and were performed according to strict governmental and international guidelines. Rat liver tissue slices (diameter 5 mm, about 200 μm thickness, and 5 mg wet weight) were obtained as described previously,[17] and stored in ice-cold University of Wisconsin organ preservation solution until fixation. Fixation was performed with 1) 4% (*w/v*) solution of formaldehyde, 2) 1% (*w/v*) solution of DST in dry DMSO, or 3) 1% (*w/v*) solution of DSP in dry DMSO, for 24 h at room temperature. As a control, rat liver tissue slices were preserved in University of Wisconsin organ preservation solution for 24 h. The fixed tissue was dehydrated (60% ethanol overnight, 80% ethanol 45 min, 96% ethanol 45 min, 100% ethanol 45 min, 100% ethanol 45 min, 100% ethanol 45 min, xylene 45 min, xylene 45 min, xylene 45 min) and embedded in paraffin (24 h). H&E staining was performed on 4 μm sections, as described previously.[17] PAS staining was performed by paraffin removal from 4 μm tissue sections (Ultraclear 15 min, two times), tissue rehydration (100% ethanol 2 min, 100% ethanol 2 min, 96% ethanol 2 min, 96% ethanol 2 min, 70% ethanol 2 min, water 2 min), treatment with 1% (*w/v*) periodic acid in water for 20 min followed by reaction with the Schiff reagent for 20 min. The PAS staining protocol was also performed after removal of glycogen by incubation at 37 °C for 30 min with 1000 U mL^{-1} α-amylase.[15]

Protein extraction from fixed tissue, quantification, and digestion: Fixed PE rat liver tissue slices were deparaffinized (paraffin melting at 60 °C and removal, washing with xylene (1 mL, 4×), shaking at 300 rpm, 20 min) and rehydrated (100% ethanol 5 min, 100% ethanol 15 min, 80% ethanol 10 min, 60% ethanol 10 min, 60% ethanol 20 min, water 5 min, water 5 min with shaking at 300 rpm). To cleave the crosslinks, DST-fixed tissue was incubated with 2% periodic acid (3 h, room temperature, in the dark, 300 rpm) and DSP-fixed tissue was incubated with DTT (250 mM; 2 h, room temperature, 300 rpm) followed by incubation with iodoacetamide (500 mM; 1 h, room temperature, in the dark, 300 rpm). After crosslinker cleavage, the tissue was washed with

water to remove excess cleavage reagents. Subsequently, tissue slices were snap frozen (liquid nitrogen) and homogenized with a mortar and pestle. After tissue grinding, lysis buffer (0.2 mL; 2% SDS, 50 mM Tris buffer pH 7.6, 8 M urea, 0.1 M β-mercaptoethanol) was added, the sample was vortexed for 1 min, and sonicated in a water bath for 2 min. The sample was centrifuged (14000 rpm for 10 min, Eppendorf Centrifuge 5417R) and the supernatant transferred to a new Eppendorf tube (protein extract). For total protein analysis, proteins were precipitated (acetone precipitation, see below for details) from protein extract (10 μL) and redissolved in SDS (20 μL, 4%, *w/v*). This solution (10 μL) was diluted to 0.5 mL with PBS, and the total protein concentration was determined by means of the Micro BCA assay (Pierce Protein Research Product, Thermo Scientific) following the manufacturer's protocol by measuring the absorbance at $\lambda = 550$ nm on a plate reader (Molecular Devices, THERMOmax). For proteomics analysis, acetone protein precipitation was performed on a volume of protein extract corresponding to 10 μg protein. After precipitation, proteins were digested with trypsin. Briefly, trypsin (25 μL; 4 μg mL^{-1} in 50 mM ammonium bicarbonate buffer) was added to the precipitated proteins and the mixture was shaken at 600 rpm for 4 h. DTT was added to the sample to a final concentration of 10 mM and the sample was incubated for 30 min (60 °C, 600 rpm). After cooling of the sample, iodoacetamide was added to the sample to a final concentration of 20 mM and left to react for 30 min (room temperature, in the dark, 600 rpm). After completion of the reaction, trypsin was again added at a trypsin/protein ratio of 1:20 (*w/w*), and allowed to react for 16 h at 37 °C. To stop trypsin digestion, TFA was added to a final concentration of 1% (*v/v*).

LC-MS/MS analysis and protein identification: MS measurements were performed on an Ultimate 3000 nano LC system (Dionex, Germering, Germany) online coupled to a hybrid linear ion trap/Orbitrap mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific, Germany). A digest (1 μL) of each sample was loaded on a C_{18} trap column (C18 PepMap, 300 μm i.d. ×5 mm, 5 μm particle size, 100 Å pore size; Dionex, The Netherlands) and desalted for 10 min at a flow rate of 20 μL min^{-1} with 0.1% TFA in water. Then the trap column was switched online with the analytical column (PepMap C18, 75 μm i.d. ×150 mm, 3 μm particle and 100 Å pore size; Dionex, The Netherlands) and peptides were eluted with the following binary gradient: 0–25% solvent B in 120 min followed by 25–50% solvent B for 60 min; solvent A consists of 2% acetonitrile and 0.1% formic acid in water and solvent B consists of 80% acetonitrile and 0.08% formic acid in water. The column flow rate was set to 300 nL min^{-1}. For MS detection, a data-dependent acquisition method was used: a high-resolution survey scan from 400 to 1800 Th was performed in the Orbitrap instrument (target value of automatic gain control (AGC) 10^6, resolution 30000 at *m/z* 400; lock mass set to 445.120025 Th (protonated $(Si(CH_3)_2O)_6$)). Based on this survey scan, the 5 most intense ions were consecutively isolated (AGC target set to 10^4 ions) and fragmented by collision-activated dissociation (CAD) by applying 35% normalized collision energy in the linear ion trap. After precursors were selected for MS/MS, they were excluded for further MS/MS analysis for 3 min.

Bioworks (v. 3.3) was used as peak picking software and data files were submitted to Mascot (v. 2, Matrix Science, London, UK) to interrogate the nonredundant UniProt database (release 2014_03; taxonomy: *Rattus norvegicus*, 7914 entries) for protein identification. Peptides with a Mascot ion score >25 (i.e., a peptide probability cutoff value of 0.01) were accepted as true identifications. Modifications: carbamidomethylation of cysteine was set as fixed and oxidation of methionine as variable modification. Additional variable modifications were set for DSP modification of lysine (87.998 and 145.120 Da) and DST modification of lysine (55.990 and 74.000 Da). A maximum of two missed cleavages were allowed. Mass tolerance for precursor ions was set to 10 ppm and for fragment ions to 0.5 Da. The false discovery rate, as determined by performing a search against a decoy database, was below 1%. The MS proteomics data have been deposited with the ProteomeXchange Consortium[18] through the PRIDE partner repository with the dataset identifier PXD002169.

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