

'Tissue surrogates' as a model for archival formalin-fixed paraffin-embedded tissues

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High-throughput proteomic studies of archival formalin-fixed paraffin-embedded (FFPE) tissues have the potential to be a powerful tool for examining the clinical course of disease. However, advances in FFPE tissue-based proteomics have been hampered by inefficient methods to extract proteins from archival tissue and by an incomplete knowledge of formaldehyde-induced modifications in proteins. To help address these problems, we have developed a procedure for the formation of 'tissue surrogates' to model FFPE tissues. Cytoplasmic proteins, such as lysozyme or ribonuclease A, at concentrations approaching the protein content in whole cells, are fixed with 10% formalin to form gelatin-like plugs. These plugs have sufficient physical integrity to be processed through graded alcohols, xylene, and embedded in paraffin according to standard histological procedures. In this study, we used tissue surrogates formed from one or two proteins to evaluate extraction protocols for their ability to quantitatively extract proteins from the surrogates. Optimal protein extraction was obtained using a combination of heat, a detergent, and a protein denaturant. The addition of a reducing agent did not improve protein recovery; however, recovery varied significantly with pH. Protein extraction of >80% was observed for pH 4 buffers containing 2% (w/v) sodium dodecyl sulfate (SDS) when heated at 100°C for 20 min, followed by incubation at 60°C for 2 h. SDS-polyacrylamide gel electrophoresis of the extracted proteins revealed that the surrogate extracts contained a mixture of monomeric and multimeric proteins, regardless of the extraction protocol employed. Additionally, protein extracts from surrogates containing carbonic anhydrase:lysozyme (1:2 mol/mol) had disproportionate percentages of lysozyme, indicating that selective protein extraction in complex multiprotein systems may be a concern in proteomic studies of FFPE tissues.

Laboratory Investigation (2007) **87**, 836–846; doi:10.1038/labinvest.3700596; published online 28 May 2007

KEYWORDS: antigen retrieval; formalin-fixed; paraffin-embedded tissue; lysozyme; protein extraction; proteomics

Many diseases are characterized by the expression of specific proteins;¹ in some cases, malignant cells yield unique 'protein profiles' when total cellular protein extracts are analyzed by two-dimensional gel electrophoresis or matrix-assisted laser desorption ionization mass spectrometry.² High-throughput proteomic studies may be useful to differentiate normal cells from cancer cells, to identify and define the use of biomarkers for specific cancers, and to characterize the clinical course of diseases. Proteomics can also be used to isolate and characterize potential drug targets and to evaluate the efficacy of treatments. When fresh or frozen tissue is used for proteomic analyses, the results cannot be related directly to the clinical course of diseases in a timely way. Instead, researchers frequently reduce the number of proteins of interest to a manageable number and then attempt to use immunohistochemistry to understand the implications of proteomic

changes in archival formalin-fixed paraffin-embedded (FFPE) tissue for which the clinical course has been established. Unfortunately, immunohistochemistry is at best a semiquantitative proteomic method, and the choice of 'interesting' proteins must occur without advance knowledge of the clinical course of the disease or the response to therapy. In addition, immunohistochemical reagents are only available for a small fraction of the potentially interesting protein targets. If modern proteomic methods could be applied to archival FFPE tissues, then these powerful techniques could be used to both qualitatively and quantitatively analyze large numbers of tissues for which the clinical course has been established.

Several proteomic studies on archival FFPE tissues have been reported in recent years. In 1998, Ikeda *et al*³ reported a heat-induced antigen retrieval procedure for extracting pro-

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Received 11 March 2007; revised 2 May 2007; accepted 3 May 2007

tein from FFPE tissue sections for analysis by two-dimensional gel electrophoresis. Additionally, Prieto *et al*⁴ identified a number of proteins from archival tissues by mass spectrometry through the use of a commercial kit for extracting proteins from FFPE tissue sections. Crockett *et al*⁵ identified >300 proteins by mass spectrometry in an extract from an archival FFPE cell block using an enzyme digestion method. In a recent comparative study of proteins extracted from fresh and FFPE tissue sections from the same case, Shi *et al*⁶ showed that most identified proteins extracted from the FFPE tissue overlapped with those extracted from the fresh tissue. Although these results are encouraging, there are a number of challenges that must be addressed to develop better and more reproducible techniques for extracting and identifying proteins useful for proteomic analysis from archival FFPE tissue. The study by Crockett *et al*⁵ perhaps best illustrates the current state of our ability to use archival FFPE tissues for proteomic studies. Liquid chromatography-tandem mass spectrometry was used to compare proteins identified in a fresh cell lysate to the same cells processed as an FFPE cell plug. A total of 263 common proteins were identified. However, 278 proteins (54%) identified in the fresh cell lysate were not seen in the FFPE cells, and 61 proteins (23%) identified in the FFPE cells were not seen in the fresh cell lysate. This suggests incomplete, and possibly selective, protein recovery from the FFPE cells and misidentification of proteins, possibly owing to the failure to completely reverse formaldehyde-protein modifications.

Formaldehyde fixes proteins in tissue by reacting with basic amino acids—such as lysine, asparagine, and glutamine⁷—to form methylol adducts. These adducts can then form crosslinks through Schiff base formation. Both intra- and intermolecular crosslinks are formed,⁸ which destroy enzymatic activity and often immunoreactivity. These formaldehyde-induced modifications reduce protein extraction efficiency and may also lead to misidentification of proteins during proteomic analysis. Therefore, to perform high-throughput proteomics on fixed tissues, we must first identify formalin-induced modification of proteins and then develop protocols for reproducible extraction and, ideally, demodification of proteins from FFPE tissue.

Previously, we have modeled the effects of formalin fixation of proteins in solution. In these studies, intermolecular formaldehyde crosslinks of 6.5 mg/ml solutions of ribonuclease A (RNase A) were reversed by mild heating at 65°C for 4 h at pH 4.^{9,10} Published proteomic experiments do not suggest recovery of essentially unmodified proteins following extraction from paraffin blocks, however, suggesting that in fixed, dehydrated, and embedded tissues, protein-formaldehyde adducts undergo further modifications that are not observed in aqueous solution.

In this paper, we describe a procedure for the formation of a 'tissue surrogate' as a model system for studying protein recovery from archival FFPE tissues. Cytoplasmic proteins, such as lysozyme and RNase A, at concentrations

approaching the protein content in whole cells are fixed with 10% neutral-buffered formalin. The resulting opaque gel is then processed through graded alcohols, xylene, and paraffin embedded according to standard histological procedures. Tissue surrogates formed by this method enable us to quickly evaluate tissue extraction protocols and to more easily identify formalin-induced protein modifications and their reversal. In this study, we evaluate tissue extraction protocols for their ability to extract proteins from tissue surrogates composed of one or two proteins, as well as from tissue surrogates consisting of HeLa cell plugs in 1% agarose.

MATERIALS AND METHODS

Bovine pancreatic RNase A (type III-A), chicken egg white lysozyme, and bovine carbonic anhydrase, β -mercaptoethanol (BME), sodium dodecyl sulfate (SDS), citraconic anhydride, guanidine HCl, and Tris HCl buffer were purchased from Sigma (St Louis, MO, USA). Aqueous 37% formaldehyde and xylene were purchased from Fisher Scientific (Pittsburgh, PA, USA). Absolute ethanol was purchased from Pharmco-AAPER (Brookfield, IL, USA), and Paraplast and Paraplast Plus tissue embedding medium were purchased from Oxford Labware (St Louis, MO, USA).

Formation of Tissue Surrogates

The tissue surrogates were formed by mixing a cytoplasmic protein solution at a concentration of 150 mg/ml in deionized water with an equal volume of 20% phosphate-buffered formalin using the following procedure. The end of a 2-ml disposable syringe was removed to create an open-ended tube (Figure 1a). The syringe barrel was drawn back to the 1-ml mark, and 500 μ l of the cytoplasmic protein solution was dispensed into the open end of the syringe. An equal volume of 20% formaldehyde in 20 mM phosphate buffer, pH 7.4, was then added to the syringe and rapidly mixed with the protein solution. An opaque gel formed within 2 min, and the resulting surrogate was allowed to stand at room temperature for at least 24 h to complete the fixation process (Figure 1b).

Dehydration and Embedding

The solid, fixed tissue surrogate was gently ejected from the syringe barrel (Figure 1c). Dehydration and paraffin embedding were then conducted by the following protocol.¹¹ The tissue surrogate was washed with distilled water and then dehydrated through a series of graded alcohols: 70% ethanol for 10 or 30 min; 85% ethanol for 10 or 30 min; 100% ethanol for 10 or 30 min; and a final 100% ethanol dehydration for 10 min, 30 min, or overnight (Figure 1d). The tissue surrogate was then incubated through two changes of xylene, 10 or 30 min each (Figure 1e), and placed in hot liquid paraffin overnight (Figure 1f). The processed surrogates were embedded into a tissue cassette using a TissueTek embedding console (Miles Scientific, Naperville, IL, USA) and cooled until the paraffin hardened (Figure 1g). The tissue surrogate

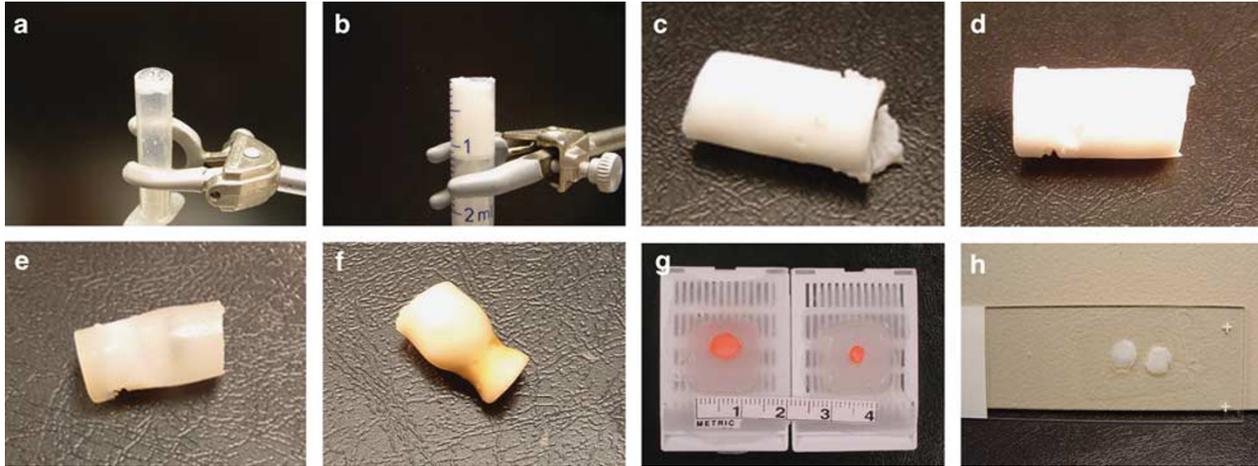


Figure 1 Illustration of the tissue surrogate method. (a) Solution of lysozyme (150 mg/ml) immediately after mixing with an equal volume of 20% formalin. (b) The mixture after 24 h of fixation. (c) The tissue surrogate extruded from the syringe. (d) The tissue surrogate after processing through a series of graded alcohols. (e) The tissue surrogate after processing in xylene. (f) The tissue surrogate after incubation in hot liquid paraffin. (g) Side-by-side comparison of surrogates incubated for 30 min (left) and overnight (right) in 100% ethanol. The tissue surrogates were stained in a 0.001% Eocin Y solution for 30 min before paraffin embedding. (h) Pair of tissue surrogates after 50- μ g sectioning and mounting on a glass slide.

can be sectioned, if desired, as shown in Figure 1h. Samples of 1–2 mg of the surrogate were saved after fixation and after each histological step to determine how each phase of histological processing affected the properties of the surrogate components.

Formation of HeLa Cell Plugs in 1% Agarose

HeLa cells were grown in plastic Corning T-75 flasks (Fisher Scientific) in minimal essential medium (Invitrogen, Carlsbad, CA, USA) at 37°C, supplemented with 5% CO₂. After 48 h, the cells, at > 90% confluency, were detached with 0.05% trypsin-EDTA (Invitrogen) and spun down in a table top centrifuge at 300 \times g. The cell pellet was resuspended in Dulbecco's phosphate-buffered saline (DPBS; Invitrogen), and aliquots were extracted for cell counting. The HeLa cells were then fixed with an equal volume of 20% formalin in DPBS for 30 min. After fixation, 50- μ l aliquots of the fixed cells ($\sim 1 \times 10^6$ cells per aliquot) were dispensed into 1.5-ml centrifuge tubes. An equal volume of 2% agarose in DPBS was added to each cell aliquot and mixed. The cell plugs were allowed to gel at 4°C overnight before proceeding through the dehydration and paraffin-embedding steps as outlined for the whole tissue surrogates.

Deparaffinization and Rehydration of Surrogates

Tissue surrogate samples before embedding in paraffin, 50- μ m sections of paraffin-embedded tissue surrogates, and agarose or HeLa cell plugs were transferred to 1.5-ml polypropylene microcentrifuge tubes and deparaffinized by removing the excess paraffin and incubating the surrogate in two changes of xylene for 10 min each. The surrogates were then rehydrated through a series of graded alcohols for 10 min each: 100% ethanol, 100% ethanol, 85% ethanol, and

70% ethanol. The cleared surrogates and cell plugs were then incubated in distilled water for a minimum of 30 min.

Solubilization and Recovery

The rehydrated 'tissue surrogates' and HeLa cell plugs were resuspended in a panel of recovery buffers consisting of 20 mM Tris HCl at pH 4, 6, or 9—with or without, 2% (w/v) SDS, 0.2 M glycine or BME. Solutions of 6 M guanidine HCl with BME¹² and aqueous 0.05% citraconic anhydride¹³ were also evaluated. The surrogates were then homogenized with a disposable pellet pestle (Kontes Scientific, Vineland, NJ, USA), followed by two 10-s cycles of sonication on ice using a Sonic Dismembrator, model 550, fitted with a 0.125-inch tapered microtip (Fisher Scientific). The homogenized surrogates were heated in a water bath at 60°C for 2 h, 80°C for 2 h, 100°C for 30 min, or were subjected to a thermal program that consisted of heating at 100°C for 20 min, followed by a 2-h incubation at 60°C. Tissue surrogates retrieved at 121°C were processed in a model 2100 steam antigen retrieval unit (PickCell Laboratories, Leiden, the Netherlands). After protein extraction, any remaining insolubilized material was pelleted at 14 000 \times g for 20 min, and the supernatant was saved for further analysis.

Analysis of Protein Composition

The composition of all surrogate preparations was characterized by electrophoresis of dithiothreitol-treated samples in the presence of 0.1% SDS. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on precast NuPAGE Bis-Tris 4–12% gradient polyacrylamide gels (1 \times 80 \times 80 mm) using 2-(*N*-morpholino)ethanesulfonic acid-SDS running buffer at pH 7.3 (Invitrogen, Carlsbad, CA, USA). Molecular mass standards and the Coomassie blue-based

colloidal staining kit were also purchased from Invitrogen. Gel images were documented using a Scanmaker i900 flat-bed scanner (Microtek, Carson, CA, USA) and annotated in Adobe Photoshop, version 7.1. The composition of individual gel lanes was analyzed and percentages were determined using Un-Scan-it Gel 6.1 analysis software (Silk Scientific Corp., Orem, UT, USA).

Determination of Protein Content in Retrieved Surrogate Samples

For the quantitative recovery experiments, 5 μ l volumes of the cytoplasmic protein solutions were aliquoted into 1.5 ml microcentrifuge tubes and rapidly mixed with an equal volume of 20% formaldehyde in 20 mM phosphate buffer, pH 7.4. The resulting surrogate aliquots were then histochemically processed, rehydrated and retrieved as outlined above. The total protein content in the recovered reaction supernatant was assessed colorimetrically using a Pierce BCA protein assay (Rockford, IL, USA) according to a standard microplate protocol. The standard curve was generated using bovine serum albumin standards (25–2000 μ g/ml working concentrations). Samples containing reducing agents were assessed using a non-interfering protein assay kit from EMD Biosciences (San Diego, CA, USA). The absorbance of all samples was read on a Spectramax M5 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Percent recovery was calculated relative to an aliquot of the non-formalin-treated protein solution or HeLa cell aliquots.

RESULTS

Formation of Tissue Surrogates and Histological Processing

We found that 150 mg/ml solutions of lysozyme, RNase A, or a 1:2 mol ratio of carbonic anhydrase:lysozyme formed opaque gels within 2 min when mixed with an equal volume of 20% neutral buffered formalin. After overnight fixation, the surrogates were firm and sliced easily with a razor blade for sampling. To determine the optimal histological processing conditions, lysozyme tissue surrogates were formed, as shown in Figure 1a–c, and processed through a series of graded alcohols (Figure 1d) and xylene (Figure 1e) before paraffin embedding. When we passed lysozyme tissue surrogates through graded alcohols and xylene for 10 min per treatment, they shrank by over 66%, hardened noticeably upon paraffin embedding, and continued to shrink further following paraffin embedding (Figure 1f). The surrogates also took on a waxy, semitransparent appearance after treatment with xylene. Increasing the processing time to a 30-min incubation through each of the graded alcohols and xylene did not prevent the FFPE tissue surrogate from shrinking. However, extending the final 100% ethanol step to an overnight incubation reduced shrinkage considerably, and the surrogate was of a more uniform consistency. Figure 1g illustrates the differences between the surrogates processed through ethanol overnight or just 30 min. The reason for this

behavior is not clear. It is possible that prolonged incubation in ethanol is required to completely dehydrate the protein plug, which, in turn, prevents shrinkage during the remaining histological steps. The protein plugs are not as dense as real tissue and, accordingly, may be more susceptible to shrinkage during histological processing.

Evaluation of the Effects of Histological Processing on Tissue Surrogates

To determine the effects of fixation and histological processing on the components of the tissue surrogate, 1.5 mg samples of the lysozyme surrogate processed through to formalin only, 100% ethanol overnight, xylene, or paraffin embedding were rehydrated and retrieved according to the procedures outlined in the Materials and Methods section. The rehydrated surrogate sections were ground and resuspended in a solution of 20 mM Tris HCl, supplemented with 2% SDS, as described by Shi *et al.*⁶ After heating, the solubilized lysozyme surrogates were analyzed by SDS-PAGE. At pH 4, 84–5% of total protein was successfully solubilized (Table 1). All samples showed intermolecular crosslinks with the formalin-only treated surrogate composed of a mixture of monomer, dimer, trimer, and tetramer species, constituting approximately 15, 19, 23, and 17% of total protein content, respectively (Figure 2, lane 1). Lower molecular weight species, indicating chain scission during processing, accounted for approximately 25% of the recovered protein. Tetrameric and pentameric lysozyme was present in the samples processed through 100% ethanol and xylene, while processing through to the paraffin-embedding stage resulted in highly-crosslinked species, with oligomers in excess of 100 kDa. Control experiments (not shown) demonstrated that the progressive increase in crosslinking is associated with post-fixation tissue processing, and not the length of time that the tissue surrogates are exposed to formalin. As revealed by electrophoresis, the formaldehyde-induced crosslinks in the formalin-only treated surrogate were not reversed after heating, contrary to previous findings in studies of proteins that were fixed at lower concentrations.^{9,10} This finding is interpreted to indicate that the number of intermolecular

Table 1 Recovery of lysozyme from tissue surrogate samples

| Processing stage | % Protein recovery |
|---------------------|--------------------|
| Formalin-fixed only | 92.80 \pm 6.29 |
| 100% ethanol | 90.42 \pm 2.69 |
| Xylene | 95.05 \pm 5.00 |
| Paraffin-embedded | 84.22 \pm 5.40 |

Mean \pm s.d. of three samples for each stage. Surrogate samples (1.5 mg) histologically processed to different stages, from formalin only to paraffin embedding, were rehydrated and resuspended in 20 mM Tris HCl, pH 4.0, with 2% SDS. Total protein in the supernatants was assessed colorimetrically after heating at 100°C for 20 min, followed by 60°C for 2 h.

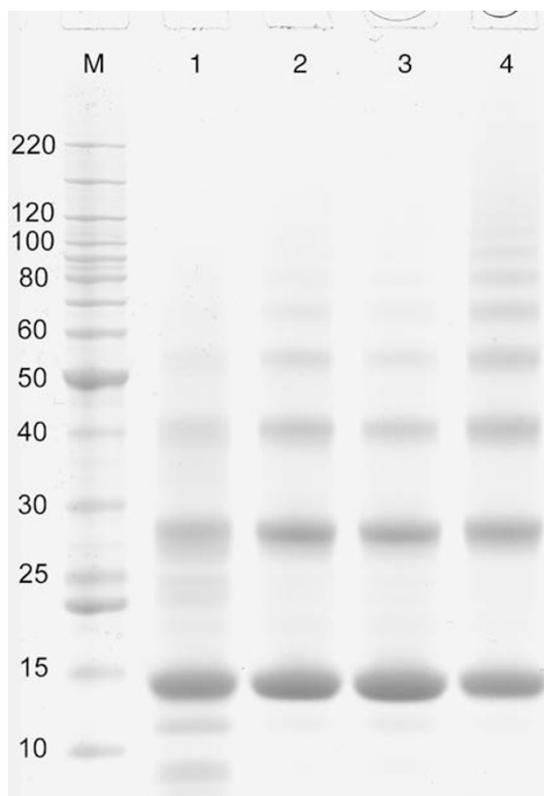


Figure 2 SDS-PAGE of proteins extracted from lysozyme tissue surrogates processed to different points. Lane M: molecular weight marker; lane 1: surrogate after formalin fixation; lane 2: surrogate after processing through a graded alcohol series; lane 3: surrogate after processing in xylene; lane 4: surrogate after paraffin embedding. The surrogates were rehydrated and subjected to a protocol of heating at 100°C for 20 min followed by a cycle of heating at 60°C for 2 h, in 20 mM Tris HCl, pH 4.0, with 2% SDS.

formaldehyde crosslinkages formed by proteins in formalin solution increases with increasing protein concentration.

Effects of Detergent and Temperature on Recovery Efficiency

To optimize protein recovery from the FFPE tissue surrogates, a number of variables were examined, including pH, the use of detergent, the use of protein denaturants and reducing agents, and temperature. We first evaluated a number of heat-induced antigen retrieval techniques that have been applied in immunohistochemistry for FFPE tissues.^{6,10} These results are listed in Table 2. Heating deparaffinized lysozyme tissue surrogates in 20 mM Tris HCl, at pH 4, 6, or 9, at 100°C for 20 min, followed by incubation 60°C for 2 h resulted in the solubilization of only 2–6% of the total protein. The addition of 2% SDS to the above protocol improved protein solubilization by more than 15-fold, with >80% of total lysozyme recovered from the tissue surrogate. At pH 4, a further increase in recovery efficiency of ~10% was observed for surrogates retrieved in 20 mM Tris HCl, pH 4 with 2% SDS supplemented with 0.2 M glycine. These results are consistent with previous surveys of protein retrieval techni-

Table 2 Effects of detergent and pH on recovery of protein from FFPE lysozyme tissue surrogates

| Recovery buffer | % Recovery (n = 3) |
|---|--------------------|
| 20 mM Tris HCl, pH 4 | 5.8 ± 0.50 |
| 20 mM Tris HCl, pH 6 | 3.1 ± 0.50 |
| 20 mM Tris HCl, pH 9 | 2.3 ± 0.40 |
| 20 mM Tris HCl+2% SDS, pH 4 | 83 ± 10 |
| 20 mM Tris HCl+2% SDS, pH 6 | 88 ± 4.1 |
| 20 mM Tris HCl+2% SDS, pH 9 | 84 ± 5.8 |
| 20 mM Tris HCl+2% SDS+0.2 M glycine, pH 4 | 95 ± 6.7 |
| 20 mM Tris HCl+2% SDS+0.2 M glycine, pH 6 | 81 ± 9.3 |
| 20 mM Tris HCl+2% SDS+0.2 M glycine, pH 9 | 79 ± 7.2 |

Lysozyme tissue surrogate samples (1.5 mg) histologically processed to paraffin embedding were rehydrated and resuspended in the indicated recovery buffer. Total protein in the supernatants was assessed colorimetrically after heating at 100°C for 20 min, followed by 60°C for 2 h. The % recovery values are the mean ± s.d.

ques from archival FFPE human tissues.⁶ Significant proteolysis was evident in tissue surrogate sections recovered at pH values less than 3 or greater than 9 (data not shown).

Heating time and temperature also affected protein recovery efficiency as shown in Table 3. Heating lysozyme tissue surrogate samples for 2 h at 60–65°C in 20 mM Tris HCl, pH 4 with 2% SDS solubilized only ~25% of the total protein. Increasing the recovery temperature to 80–100°C improved the extent of protein recovery to >60%. Optimal protein solubilization was achieved using a thermal program that consisted of incubating the tissue surrogates at 100°C for 20 min, followed by a cycle of heating at 60°C for 2 h. This resulted in >80% of total protein recovered from the surrogate samples. Figure 3 illustrates the effect of temperature on the recovery of lysozyme tissue surrogates in 20 mM Tris HCl, pH 4, with 2% SDS. Although the solubilized protein in lane 1 is highly enriched in lysozyme monomer, only 64% of the total protein from the surrogate was recovered. In contrast, the solubilized protein in lane 2 contains a significant quantity of lysozyme crosslinked oligomers, but 83% of the total protein from the surrogate was recovered. This demonstrates that there is not a direct correlation between protein recovery from the tissue surrogate and reversal of the protein-formaldehyde modifications. Surrogates retrieved at temperatures >100°C (ie, at 121°C) underwent significant heat-induced degradation, as seen by the bands running below the lysozyme monomer in lane 3.

Effects of Other Buffer Formulations on Recovery Efficiency

Additional conditions from published antigen retrieval and FFPE proteomic tissue studies were also evaluated for efficacy. These results are shown in Table 4. Namimatsu *et al*¹³ reported improved immunohistochemical staining of

Table 3 Effect of temperature on recovery of protein from FFPE lysozyme tissue surrogates

| Temperature/time | % Recovery (n = 3) |
|-------------------------------|--------------------|
| 60°C for 2 h | 26 ± 2.7 |
| 80°C for 2 h | 64 ± 2.4 |
| 100°C for 30 min | 67 ± 3.7 |
| 100°C for 20 min/60°C for 2 h | 83 ± 10 |

Lysozyme tissue surrogate samples (1.5 mg) histologically processed to paraffin embedding were rehydrated and resuspended in 20 mM Tris HCl+2% SDS, pH 4. Total protein in the supernatants was assessed colorimetrically after heating at the indicated temperatures and times. The % recovery values are the mean ± s.d.

FFPE tissue sections heated in solutions of citraconic anhydride at pH 7.4. Heating lysozyme tissue surrogate samples in freshly prepared 0.05–0.1% (w/v) citraconic anhydride, pH 1–2, resulted in excellent protein recovery, with >90% of the lysozyme being solubilized. Adjusting the pH of the citraconic anhydride solutions to 7.4 decreased the protein recovery by >13-fold. SDS-PAGE of the surrogates treated with citraconic anhydride indicated the presence of ~15% monomeric protein, with ~85% of the protein remaining in the form of higher order oligomers that were not reversed during treatment (data not shown).

Tissue surrogates heated in 6 M guanidine HCl supplemented with 0.5 M BME, a disulfide-reducing agent, resulted in a protein recovery of 58%. Recovery efficiency was increased to >70% in solutions of 20 mM Tris HCl with 2% SDS and 0.5 M BME (Table 4). Addition of protein denaturants such as guanidine or SDS was found to improve tissue surrogate solubility. However, reduction of disulfide bonds did not improve either protein recovery or reversal of formaldehyde crosslinkages. In heat-coagulated lysozyme, reduction of scrambled disulfide linkages is required for regeneration of native protein.¹² Figure 4 compares formalin-fixed lysozyme tissues surrogates heated in the presence of BME with a lysozyme solution that was boiled for 10 min to coagulate the protein before treatment with BME. After treatment with BME (lane 2), monomeric protein, and peptide fragments resulting from protein hydrolysis, was present in the heat-coagulated lysozyme sample. In contrast, oligomeric protein remained in the FFPE tissue surrogate after treatment with the reducing agent (lane 1). Thus, any increased protein flexibility brought about by the elimination of disulfide linkages does not facilitate the reversal of the formaldehyde crosslinkages.

Several methods for extracting soluble protein from archival FFPE tissue for proteomic studies have been reported in recent years. Results obtained applying these methods to lysozyme tissue surrogates are also reported in Table 4. In 1998, Ikeda *et al*³ extracted proteins from FFPE tissue sections for analysis by two-dimensional gel electrophoresis using RIPA buffer supplemented with 2% SDS. Heating

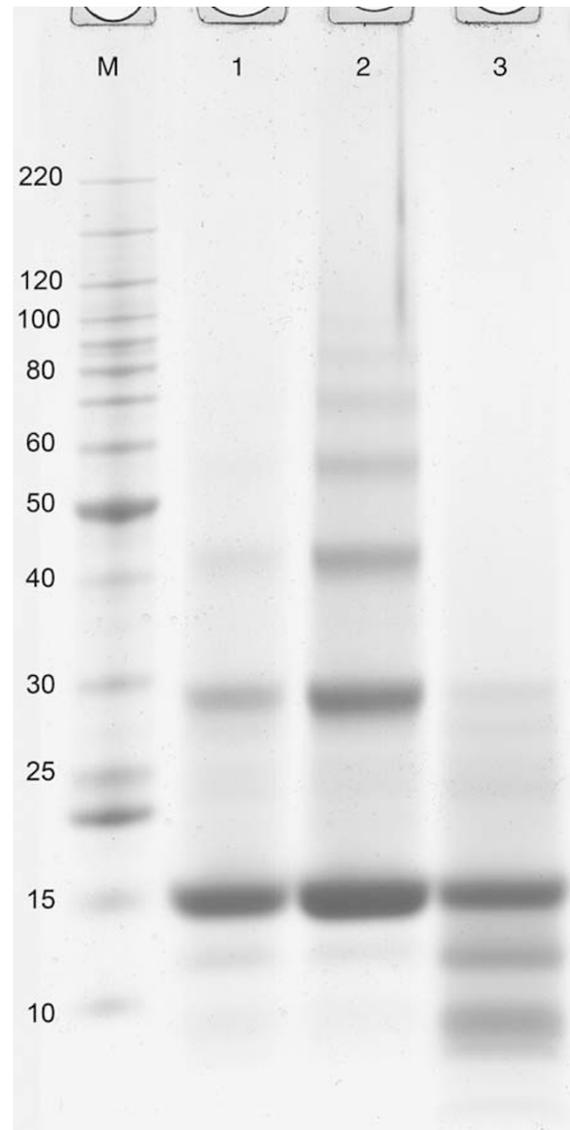


Figure 3 SDS-PAGE of proteins extracted from formalin-fixed paraffin-embedded lysozyme tissue surrogates retrieved at pH 4 in 20 mM Tris HCl, with 2% SDS. Lane M: molecular weight marker; lane 1: surrogate heated at 80°C for 2 h; lane 2: surrogate incubated at 100°C for 20 min followed by a cycle of heating at 60°C for 2 h; lane 3: surrogate processed at 121°C for 20 min in the model 2100 steam antigen retrieval unit.

samples of the lysozyme tissue surrogate in RIPA buffer at 100°C for 20 min, followed by a 2-h incubation at 60°C, recovered only 2% of the surrogate protein. Extraction of the FFPE tissue surrogate using a commercially available FFPE tissue extraction buffer⁴ yielded only about 17% solubilized lysozyme.

Protein Retrieval from Surrogates Formed from Rnase A, Carbonic Anhydrase and Lysozyme, or from Hela-Agarose Cell Plugs

To evaluate the utility of the tissue surrogate as a model for FFPE tissue, surrogates from several proteins were formed

Table 4 Effect of buffer formulation on recovery of protein from FFPE lysozyme tissue surrogates

| Recovery buffer | Temperature/time | % Recovery (n = 3) |
|--|-------------------------------|--------------------|
| 0.05% Citraconic anhydride, pH 2 ¹³ | 100°C for 20 min/60°C for 2 h | 91 ± 5.7 |
| 0.05% Citraconic anhydride, pH 7.4 ¹³ | 100°C for 20 min/60°C for 2 h | 6.6 ± 0.40 |
| 6 M Guanidine+0.5 M BME, pH 4 ¹² | 100°C for 20 min/60°C for 2 h | 58 ± 2.5 |
| 20 mM Tris HCl+2% SDS+0.5 M BME, pH 4 | 100°C for 20 min/60°C for 2 h | 74 ± 2.4 |
| RIPA buffer ³ | 100°C for 20 min/60°C for 2 h | 2.0 ± 0.20 |
| Liquid tissue buffer ⁴ | 95°C for 90 min | 17 ± 6.3 |

Lysozyme tissue surrogate samples (1.5 mg) histologically processed to paraffin embedding were rehydrated and resuspended in the indicated recovery buffer. Total protein in the supernatants was assessed colorimetrically after heating at the indicated temperatures and times. The % recovery values are the mean ± s.d.

and histologically processed to paraffin. Although aqueous lysozyme (pI = 11.0) or RNase A (pI = 9.45) solutions at 75 mg/ml formed solid gels upon formalin fixation, a solution of carbonic anhydrase (pI = 6.0) did not gel after 24 h. This suggested that the isoelectric point of the protein may affect its ability to form tissue surrogates. However, a surrogate consisting of 33 mol% carbonic anhydrase and 66 mol% lysozyme formed a solid gel within 1–2 min. RNase A or lysozyme surrogates were of similar consistency and, after deparaffinization and recovery, exhibited similar banding patterns, as shown in Figure 5. In both surrogates retrieved at pH 4.0, there was ~15% monomeric protein and ~85% higher order oligomers, indicating the presence of intermolecular formaldehyde crosslinks.

In the mixed carbonic anhydrase:lysozyme tissue surrogate, analysis of the surrogate was complicated by the presence of two proteins, indicating that further analysis by two-dimensional gel electrophoresis or mass spectrometry may be necessary to fully identify all of the protein components (Figure 6). In samples extracted at pH 4.0, ~72% of total protein corresponded to monomeric lysozyme, whereas monomeric carbonic anhydrase and a band of the correct size for a lysozyme:carbonic anhydrase heterodimer accounted for 19 and 3.5%, respectively. In the mixed surrogate extracted at pH 6.0, there was a relatively greater concentration of heterodimeric protein, as well as possible minor higher order oligomers.

Comparative extraction studies on tissue surrogates formed from other proteins or from HeLa-agarose cell plugs were performed to further evaluate the utility of tissue surrogates as a model for FFPE tissues. The results of these studies are shown in Table 5. Tissue surrogates produced from 75 mg/ml solutions of RNase A formed oligomeric complexes similar to the fixed lysozyme solutions, and formed solid tissue surrogates after a 1–2 min fixation in buffered formalin. Heating the deparaffinized surrogate sections in 20 mM Tris HCl, pH 4, with 2% SDS for 20 min at 100°C, with a subsequent heating cycle at 60°C for 2 h, recovered the greatest amount of protein (81%).

For the mixed surrogate, 82% of the total protein was recovered in the 20 mM Tris HCl buffer with 2% SDS at pH

4, but the total protein recovery decreased to 68% when the pH was increased to 6 (Table 5). A greater percentage of carbonic anhydrase was recovered at pH 6 than at pH 4, indicating that the recovery of individual proteins may be dependent upon pH. The pH-dependent recovery from a single-protein-agarose plug formed by fixing carbonic anhydrase in a 1% agarose matrix supports this hypothesis. In recovery trials with the carbonic anhydrase:agarose tissue surrogate, 46% of total carbonic anhydrase was recovered from the agarose plug at pH 6, as opposed to only ~30% recovery observed at pH 4.

Similar effects of pH and buffer composition were observed in a more complex whole cell model, as reported in Table 6. HeLa cells were formalin-fixed in 1% agarose and histologically processed through paraffin embedding using the same procedure as was used for the single- and mixed-protein tissue surrogates. The most effective protein extraction buffer studied was 20 mM Tris HCl containing 2% SDS, with 35% total cellular protein solubilized at pH 6. RIPA buffer containing 2% SDS extracted 15% of total cellular protein.³ HeLa-agarose cell plugs extracted with a commercially available FFPE tissue extraction buffer⁴ recovered 5% of the total protein.

DISCUSSION

In this study, we describe a tissue surrogate model system for studying the recovery of proteins from FFPE tissues. Previous studies have shown the potential of the use of high-throughput proteomic techniques on proteins extracted from FFPE tissues. However, a number of challenges must be addressed to develop better and more reproducible protocols for performing molecular analysis on proteins from archival tissues. The simple tissue surrogate described here has identified some of these challenges, which include incomplete recovery of protein, selective recovery of protein, incomplete reversal of formaldehyde modifications, and protein degradation (chain scission). The tissue surrogate model will enable future studies aimed at improving our current tissue handling techniques so as to facilitate recovery of proteins from FFPE tissues that are suitable for proteomic analyses.

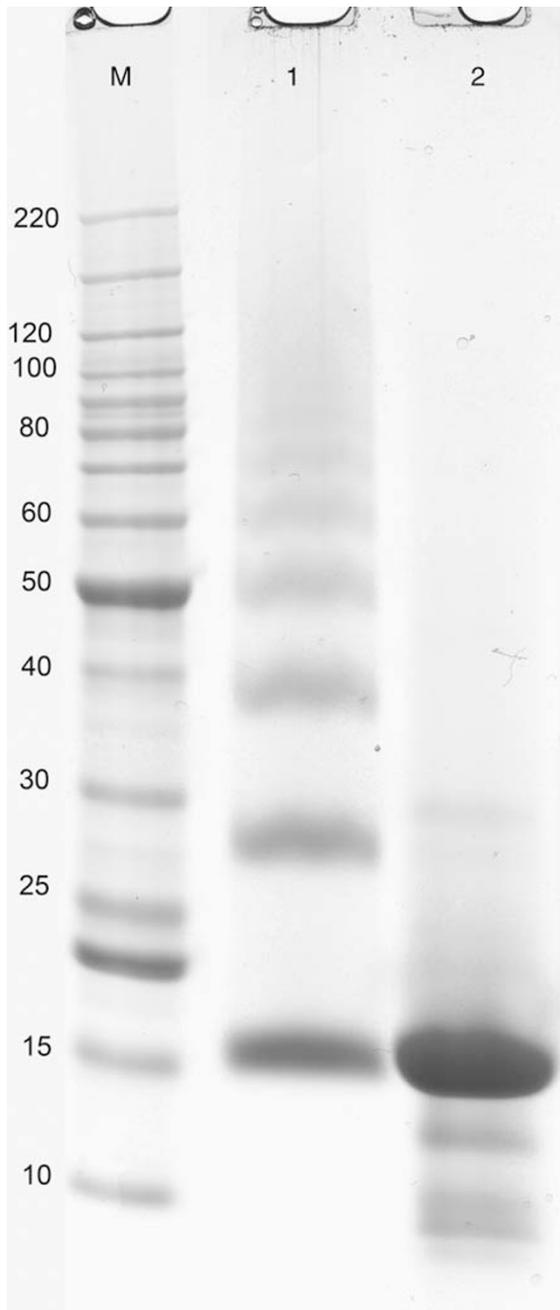


Figure 4 SDS-PAGE of recovery of lysozyme in the presence of BME. Lane M: molecular weight marker; lane 1: FFPE Lysozyme tissue surrogate; lane 2: 75 mg/ml solution of lysozyme heat collagulated for 10 min at 100°C in 10 mM sodium phosphate buffer, pH 7.4. Both preparations were resuspended in 20 mM Tris HCl, pH 4, with 2% SDS and 0.5 M BME and heated at 100°C for 20 min followed by a cycle of heating at 60°C for 2 h.

Our previous studies demonstrated that protein-formaldehyde adducts and crosslinks formed in aqueous solution are easily reversed by mild heating under acidic conditions.^{9,10} However, brief exposure of formaldehyde-treated proteins to ethanol resulted in the formation of additional modifications that were not easily reversed

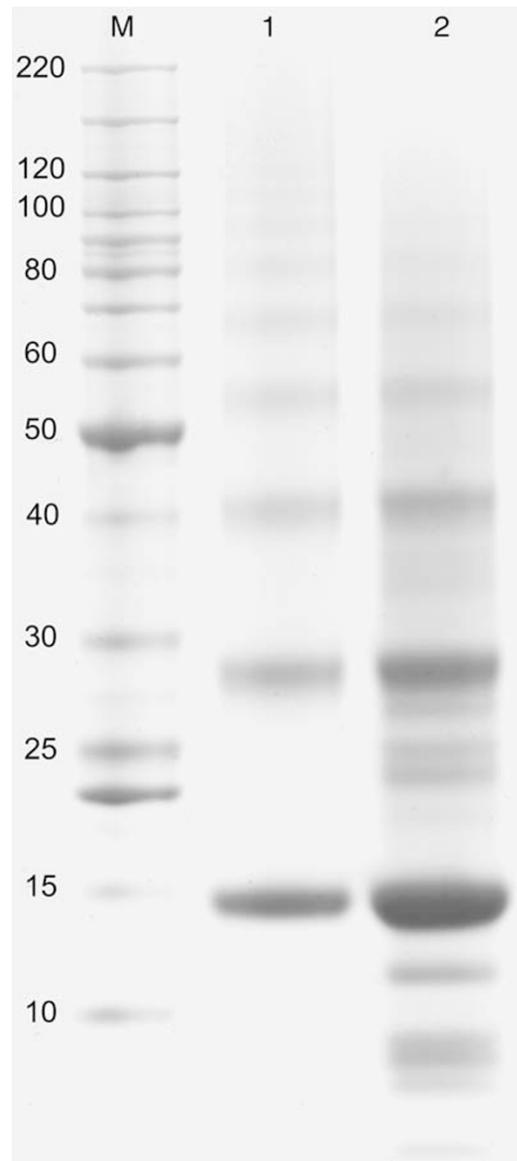


Figure 5 Gel image of proteins extracted from FFPE tissue surrogates, retrieved at pH 4.0 in 20 mM Tris HCl, with 2% SDS. Lane M: molecular weight marker; lane 1: chicken egg white lysozyme tissue surrogate; lane 2: bovine RNase A tissue surrogate.

(unpublished data). This led us to conclude that many protein-formaldehyde adducts undergo further chemical reactions during the subsequent steps used in tissue histology. To investigate this hypothesis, we developed a tissue surrogate model for studying the effects of tissue histology on the properties of formaldehyde-treated proteins. Solid tissue surrogates were successfully formed from several classes of proteins, and in most cases, >80% recovery of protein from the FFPE surrogate was documented. We found that a final forming surrogate concentration of 75 mg/ml was optimal for rapidly forming surrogates that sectioned easily for study. At higher concentrations, the surrogates became brittle after paraffin

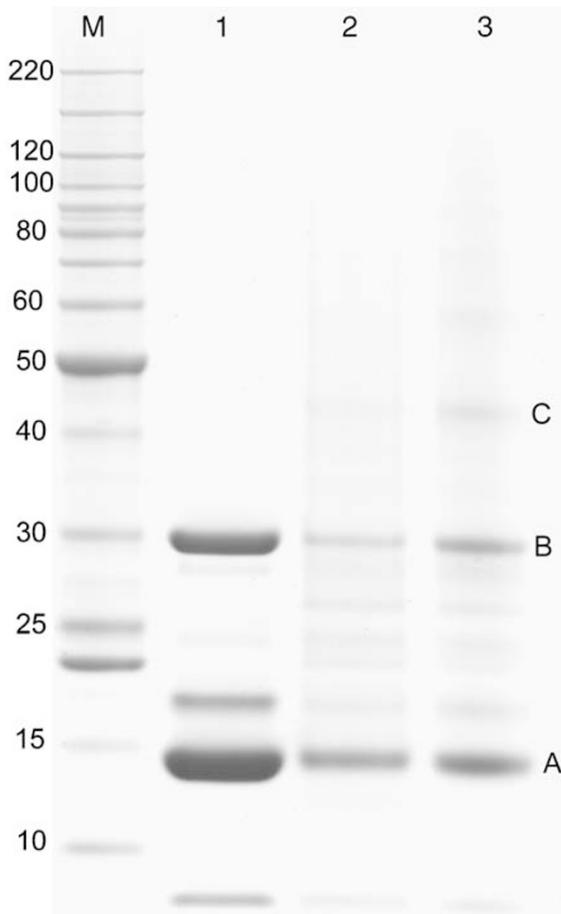


Figure 6 Gel image of proteins extracted from a mixed carbonic anhydrase:lysozyme tissue surrogate. Lane M: molecular weight marker; lane 1: A 1:2 mol ratio mixture of native, non-formalin-treated carbonic anhydrase and lysozyme; lane 2: mixed surrogate with 1:2 mol ratio carbonic anhydrase:lysozyme, solubilized and retrieved in 20 mM Tris HCl, pH 4.0, with 2% SDS; lane 3: mixed surrogate with 1:2 mol ratio carbonic anhydrase:lysozyme, solubilized and retrieved in 20 mM Tris HCl, pH 6.0, with 2% SDS. Protein bands corresponding to lysozyme monomer (A), carbonic anhydrase monomer (B), and the putative lysozyme-carbonic anhydrase heterodimer (C) are indicated.

embedding; and at lower concentrations, they failed to form solid gels following overnight incubation. The data suggest that the protein pI may affect surrogate formation, because proteins with higher pIs formed tissue surrogates more readily than those with lower pIs. Crosslinking, as observed by gel electrophoresis, was documented in all one- and two-protein surrogates studied, suggesting that these surrogates are a reasonable model system for the study of formalin-induced protein modifications in FFPE tissue.

In this study, we have performed a comprehensive evaluation of protein extraction protocols using FFPE tissue surrogates that were formed by fixing lysozyme, RNase A, or carbonic anhydrase:lysozyme [1:2] in 10% buffered formalin; dehydrating the protein gels through graded alcohols; incubating in xylene; and finally embedding the

Table 5 Recovery of protein from tissue surrogates formed from other proteins

| Tissue surrogate | pH of recovery buffer | % Recovery (n = 3) |
|-----------------------------|-----------------------|--------------------|
| RNase A | 4 | 81 ± 12 |
| RNase A | 6 | 79 ± 2.3 |
| RNase A | 9 | 65 ± 12 |
| Carbonic anhydrase:lysozyme | 4 | 81 ± 7.5 |
| Carbonic anhydrase:lysozyme | 6 | 68 ± 3.1 |
| Carbonic anhydrase:agarose | 4 | 30 ± 2.5 |
| Carbonic anhydrase:agarose | 6 | 46 ± 12 |

Tissue surrogate samples (1.5 mg) histologically processed to paraffin embedding were rehydrated and resuspended in recovery buffer (20 mM Tris HCl+2% SDS) at the indicated pH. Total protein in the supernatants was assessed colorimetrically after heating at 100°C for 20 min, followed by 60°C for 2 h. The two-protein tissue surrogates were composed of carbonic anhydrase:lysozyme (1:2 mol/mol). The % recovery values are the mean ± s.d.

protein gels in paraffin. After removal of the paraffin and rehydration of the surrogates through graded alcohols, a number of buffers, detergents, and protein denaturants were screened to identify the most effective conditions for recovering proteins from the surrogates.

We found that efficient protein recovery required heat, a protein denaturant, and a detergent. SDS, which serves the dual role of a protein denaturant and a detergent, was the single most effective ingredient for promoting protein recovery. The most efficient extraction of lysozyme from FFPE tissue surrogates was obtained using buffers containing 2% SDS; there was a more than 13-fold greater protein recovery than in buffers without SDS. These results are consistent with previously reported studies of FFPE tissues,^{6,14} in which SDS was found to be essential for protein extraction and antigen retrieval. Use of Triton X-100, which is an excellent detergent but a poor protein denaturant, resulted in poor recovery (data not shown). Likewise, guanidine HCl, which is an excellent protein denaturant but not a detergent, was only modestly effective. Temperature and processing times also affected recovery efficiency from FFPE tissue surrogates. Heating the surrogates at temperatures at, or below, the denaturation temperature of the component proteins resulted in poor recovery efficiency. Increasing the processing temperature to 80°C for 2 h or 100°C for 30 min improved the extent of protein extraction 10-fold. Heating the surrogate samples in buffer at 100°C for 20 min, followed by a longer incubation at 60°C further improved recovery efficiency without causing the heat-induced proteolysis (chain scission) seen at temperatures above 100°C. The addition of 0.2 M glycine, intended to serve as a formaldehyde scavenger, increased recovery efficiency to 95% at pH 4, when the surrogate samples were heated at 100°C for 20 min, followed by a 2-h incubation at 60°C. A similar effect was observed in surrogates heated at 80°C in the presence of

Table 6 Extraction of proteins from HeLa cells fixed in a 1% agarose plug

| Recovery buffer | Temperature/time | % Recovery (n = 3) |
|-----------------------------------|-------------------------------|--------------------|
| 20 mM Tris HCl+2% SDS, pH 4 | 100°C for 20 min/60°C for 2 h | 15 ± 3.3 |
| 20 mM Tris HCl+2% SDS, pH 6 | 100°C for 20 min/60°C for 2 h | 35 ± 2.9 |
| 20 mM Tris HCl+2% SDS, pH 9 | 100°C for 20 min/60°C for 2 h | 18 ± 1.4 |
| Liquid tissue buffer ⁴ | 95°C for 90 min | 5.3 ± 1.5 |
| RIPA ³ | 100°C for 20 min/60°C for 2 h | 15 ± 2.0 |

HeLa cells (1×10^6) were formalin-fixed in an equal volume of 1% agarose. After histological processing and paraffin embedding, the cell plugs were rehydrated and resuspended in the indicated buffer. Total protein in the supernatants was assessed colorimetrically after heating at the indicated temperatures and times. The % recovery values are the mean \pm s.d.

glycine, with recoveries of \sim 79% observed at pH 4, as compared with \sim 64% total protein recovered in the presence of 2% SDS alone.

As discussed above, buffers containing 6 M guanidine HCl or reducing agents, such as BME, but without SDS, did not improve protein extraction; and solutions containing BME alone did not yield protein recoveries $>$ 60%. Although the use of solutions containing citraconic anhydride, pH 1–2, enabled recovery of 80–90% of lysozyme from tissue surrogates, the toxicity of anhydrides makes them less desirable as protein extraction reagents. Finally, protein recovery from the tissue surrogates was sensitive to the pH of the recovery buffer. With the exception of citraconic anhydride, optimal protein recovery was achieved using buffers in a pH range of 4–6. Most protein extraction protocols evaluated recovered \sim 15% monomeric protein, with \sim 85% multimeric complexes, suggesting the presence of formaldehyde crosslinks. Thus, detergents, protein denaturants, or disulfide reducing agents did not promote reversal of formaldehyde-induced protein modifications. From this observation, we conclude that these agents improve protein recovery from the tissue surrogates through their ability to render the proteins soluble, rather than through promoting reversal of formaldehyde-protein crosslinkages.

Surrogates formed from RNase A behaved similarly to those formed from lysozyme, with the most efficient protein recovery observed at pH 4–6 in the presence of 2% SDS. Protein extracts from surrogates containing carbonic anhydrase:lysozyme (1:2 mol/mol) appeared to contain disproportionate percentages of lysozyme. As shown in Figure 6, 72% of total protein by mass corresponded to monomeric lysozyme, whereas monomeric carbonic anhydrase and a band of the correct size for a lysozyme:carbonic anhydrase hetero-dimer accounted for 19 and 3.5%, respectively. In the mixed surrogate extracted at pH 6.0, there was a relatively greater concentration of hetero-dimeric protein, and trace levels of higher molecular weight oligomers. This differential extraction efficiency was corroborated by parallel extractions of carbonic anhydrase fixed in 1% agarose. In this single-protein system, only 30% extraction was obtained in SDS-containing retrieval buffers at pH 4, whereas protein recovery

increased to 46% at pH 6. These observations suggest that protein extraction is pH dependent, perhaps related to the physical properties of the protein, such as its isoelectric point. Subsequently, total, or at least representative, protein extraction from FFPE tissues may require multiple treatments at different pH values to compensate for this effect.

Using identical extraction protocols on HeLa cells fixed and paraffin embedded in a 1% agarose matrix also validated the observation that 2% SDS is an effective protein extraction reagent, while highlighting the challenges of studying protein extracted from whole cells and tissues. This study further validates the utility of tissue surrogates formed from cytoplasmic proteins as models for archival FFPE tissue. After fixation and paraffin embedding, we were able to rapidly evaluate a battery of tissue extraction and antigen retrieval protocols for efficacy. As the amount of total protein in each surrogate was known, quantification of the amount of protein recovered by colorimetric assay was easily obtained. In whole tissues, analysis of protein extracts is only possible through mass spectrometry or two-dimensional gel electrophoresis, which is time consuming. Misidentification of proteins extracted from formalin-fixed tissue is a potential problem because the high concentration of protein in whole tissues may promote significant intermolecular crosslinks. The protein composition of surrogates containing one or two components is easily visualized by SDS-PAGE or micro-capillary electrophoresis. In addition, tissue surrogates can simplify the identification of formaldehyde-induced adducts and crosslinks by mass spectrometry. We hope that tissue surrogates will facilitate future studies to standardize protein recovery protocols and identification of proteins from archival formalin-fixed tissues.

In summary, these studies highlight some of the problems that must be overcome before proteins extracted from FFPE tissues can be used for proteomic studies. First, our studies demonstrate that reversal of protein-formaldehyde adducts does not assure quantitative extraction of proteins from FFPE tissues. It may ultimately turn out that there is no one 'universal' method that can accomplish both tasks, but that instead each step will need to be optimized separately. It is also evident that failure to quantitatively extract the entire

protein component from FFPE tissues may result in sampling bias owing to the preferential extraction of certain proteins. This behavior may be linked to protein physical properties, such as the isoelectric point. Multiple extractions steps (perhaps using a range of pH values) may be necessary to achieve quantitative, or at least representative, extraction of proteins from FFPE tissues. Based on our results with tissue surrogates and cell plugs, it is clear that reversal of protein-formaldehyde modifications in the systems we have examined requires heating at high temperatures ($\geq 90^{\circ}\text{C}$) in acidic ($< \text{pH } 5$) buffers. Indeed, this approach appears to substantially reverse formaldehyde-induced protein modifications (Figure 3, lane 3). Unfortunately, these conditions also promote deleterious protein modifications. These pH-dependent modifications include β -elimination of cysteine residues, deamidation of asparagine and glutamine residues, and hydrolysis of peptide bonds at aspartic acid residues.^{15,16} The hydrolysis products running below the lysozyme monomer in Figure 3 (lane 3) and Figure 4 (lane 2) may represent lysozyme peptide fragments produced by such aspartic acid hydrolysis. Accordingly, conditions will need to be developed to abrogate these modifications during reversal of the fixation process,¹⁷ or it will be necessary to account for them during proteomic analysis.¹⁸ These modifications may also interfere with antigen retrieval methods used in immunohistochemistry.¹⁹

It appears that more complex tissue surrogates may be created by incorporating additional proteins of interest to the lysozyme solution. Alternately, RNA, DNA, lipids, or carbohydrates can be added at nanomolar to millimolar concentrations to increase the complexity of the model system to better mimic whole tissue. The use of these more complex tissue surrogates should facilitate the development of protein recovery protocols optimal for proteomic investigation of FFPE tissues.

ACKNOWLEDGEMENT

This work was supported by grant 1R01 CA 107844 from the National Cancer Institute.

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