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Liquid Tissue™: proteomic profiling of formalin-fixed tissues

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Identification and quantitation of candidate biomarker proteins in large numbers of individual tissues is required to validate specific proteins, or panels of proteins, for clinical use as diagnostic, prognostic, toxicological, or therapeutic markers. Mass spectrometry (MS) provides an exciting analytical methodology for this purpose. Liquid Tissue™ MS protein preparation allows researchers to utilize the vast, already existing, collections of formalin-fixed paraffin-embedded (FFPE) tissues for the procurement of peptides and the analysis across a variety of MS platforms.

INTRODUCTION

Proteomic profiling of diseased and normal tissue by mass spectrometry (MS) is at an early stage, yet it promises to decipher proteomic complexities of the tissue microenvironment and to deliver biomarker information with appropriate pathologic and histologic relevance. Mass spectrometric profiling of the complex cellular proteome obtained from diseased tissue has previously been demonstrated with frozen cancer tissue (1–5). However, the use of frozen tissue for such analysis is problematic for several reasons. These difficulties include collecting sufficient tissue samples for large studies (especially human tissue), the need to design the collection of tissues in a prospective manner, the cumbersome nature of first processing then analyzing frozen tissue, and the effort/expense of storing such tissue on a long-term basis.

In contrast, formalin fixation of tissue is a routine process that provides an easily stored archive of tissue that is pathologically well-defined. Extensive archives of animal and human fixed tissue have been collected, assembled, and stored at room temperature for decades; the majority of which contain associated clinical and experimental information representing an extremely valuable untapped reservoir of potential biomarkers. However, current methods of proteomic analysis for formalin-fixed tissue are limited to immunohistochemistry (IHC) (6,7). This methodological limitation is due to the protein cross-linking properties of formalin, which preserves the proteins but renders them insoluble. This process makes the proteins within these samples incompatible with many of the discovery tools used in proteomics today, leaving these fixed archival tissue collections inaccessible for further exploration of biological knowledge (8–12).

Expression Pathology has developed a patent pending process, termed Liquid Tissue™, for the extraction of peptides directly from formalin-fixed tissue. This report demonstrates the analysis of peptides obtained from formalin-fixed tissue utilizing the Liquid Tissue process across a variety of MS platforms, including microcapillary reversed-phase liquid chromatography (μRPLC) tandem mass spectrometry (MS/MS), matrix-assisted laser desorption ionization (MALDI) tandem time-of-flight (TOF/TOF), and surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF). Results from SELDI-TOF MS analysis reveal tissue-based patterns of protein expression on a variety of chromatographic chip surfaces, while multiple protein identifications were obtained from μRPLC-MS/MS, all from a single Liquid Tissue extract. These results demonstrate that the Liquid Tissue methodology provides the ability to unlock the proteome of the world's vast reservoir of archived tissue for large-scale discovery and validation of biomarkers to improve disease diagnosis and therapy.

MATERIALS AND METHODS

Chemicals and Reagents

Formic acid (HCOOH) and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO, USA). High-performance liquid chromatography (HPLC)-grade acetonitrile (ACN; CH_3CN) was obtained from EMD Chemicals (Gibbstown, NJ, USA). All buffers and reagents were used as supplied from the manufacturer and prepared in double-distilled water using a NANOPure Diamond® water system (Barnstead International, Dubuque, IA, USA). The Liquid Tissue MS Protein Prep kit was from Expression Pathology (Gaithersburg, MD, USA).

Tissue Processing and Peptide Extraction

A formalin-fixed paraffin-embedded (FFPE) colon cancer tissue with well-defined adenocarcinoma was analyzed by standard histological methods in order to identify a cancerous region consisting of at least 70% cancerous epithelium with little to no observable lymphocytic infiltration. A 5 μm-thick section was cut, placed on a glass slide, and stained with hematoxylin and eosin (H&E) for identification of the cancerous region. A 10 μm-thick section was placed on a second slide for tissue dissection and peptide procurement. Paraffin was removed by treatment in SubX organic solvent (Surgipath Medical Industries, Richmond, IL, USA) twice for 5 min, followed by tissue rehydration through multiple graded ethanol solutions and distilled water. The cancerous region of interest was identified microscopically using the H&E-stained region to locate the cells for macrodissection with a sterile 30-gauge hypodermic needle in the rehydrated tissue section. The collected sample, approximately 120,000 cells, was placed into a 1.5-mL low-binding microcentrifuge tube containing 40 μL Liquid Tissue buffer.

Peptides were extracted utilizing the Liquid Tissue MS Protein Prep kit according to the manufacturer's protocol. Briefly, the cellular material, suspended in the Liquid Tissue buffer, was incubated at 95°C for 90 min, then cooled on ice for 3 min. Trypsin was added (approximately 15–18 U) followed by incubation at 37°C overnight. Dithiothreitol was added to a final concentration of 10 mM, and the samples were heated for 5 min at 95°C. The Liquid Tissue digestate was stored at -20°C until analysis.

Nanoflow RPLC-MS/MS Analysis

μRPLC-MS/MS was performed using an Agilent 1100 capillary LC system (Agilent Technologies, Palo Alto, CA, USA) coupled online to a linear ion trap (LIT) mass spectrometer (LTQ; Thermo Electron, Waltham, MA, USA). Separations were performed using

75 µm inner diameter (i.d.) × 360 µm outer diameter (o.d.) × 10 cm long fused silica capillary columns (Polymicro Technologies, Phoenix, AZ, USA) that were slurry packed in-house with 3 µm, 300 Å pore size C-18 silica-bonded stationary phase (Vydac, Hysperia, CA, USA). An aliquot of the Liquid Tissue extract (corresponding to approximately 6000 cells) was desalted using C-18 ZipTip™ microcolumns (Millipore, Billerica, MA, USA), lyophilized, and resuspended in a minimal amount of 0.1% TFA prior to µRPLC-MS/MS analysis. Following sample injection, the column was washed for 20 min with 98% mobile phase A (0.1% formic acid in water) at a flow rate of 0.5 µL/min. Peptides were eluted using a linear gradient of 2% mobile phase B (0.1% formic acid in ACN) to 42% solvent B in 40 min, then to 98% B in an additional 28 min, all at a constant flow rate of 250 nL/min.

The LIT-MS was operated in data-dependent MS/MS mode, in which each full MS scan was followed by five MS/MS scans, respectively, where the five most abundant peptide molecular ions were selected for collision-induced dissociation (CID) using a normalized collision energy of 35%. Dynamic exclusion was utilized to minimize redundant selection of peptides previously selected for CID. The heated capillary temperature and electrospray voltage were set at 160°C and 1.5 kV, respectively. Data were collected over a broad mass-to-charge (m/z) precursor ion selection scan range of 400–2000.

Tandem mass spectra were searched against the UniProt human proteomic database (01-04-05 release) from the European Bioinformatics Institute (www.ebi.ac.uk/integr8) using SEQUEST® (Thermo Electron). For a fully tryptic peptide to be considered legitimately identified, it had to achieve stringent charge state and proteolytic cleavage-dependent cross correlation (X_{corr}) scores of 1.9 for $[M+H]^{1+}$, 2.2 for $[M+2H]^{2+}$, and 3.1 for $[M+3H]^{3+}$, and a minimum delta correlation (ΔC_n) of 0.08. SEQUEST results were further filtered using software developed in-house to determine unique peptides and proteins.

SELDI-TOF Analysis

In preparation for the SELDI-TOF analysis, 25 µL Liquid Tissue colon cancer extract (corresponding to approximately 75,000 cells) were lyophilized and resuspended in 10 µL binding/wash buffer [phosphate-buffered saline (PBS), pH 7.4, containing 0.1% Triton® X-100]. The sample was then applied sequentially to the different SELDI surfaces in the following manner.

The immobilized metal affinity capture (IMAC3) ProteinChip® Array (Ciphergen Biosystems, Palo Alto, CA, USA) was loaded with copper (100 mM $CuSO_4$), washed with HPLC-grade water, and incubated with 50 mM sodium acetate, pH 4.0. Following a brief HPLC-grade water wash, the array was then pre-equilibrated in binding/wash buffer. Sample was applied to the array surface using a ProteinChip Bioprocessor (Ciphergen Biosystems) and incubated for 2 h at ambient temperature with gentle agitation. Sample was aspirated from the array surface and stored at 4°C until further use. The array was washed twice with binding/wash buffer, followed by two final 5 mM Tris, pH 8.0, washes. The ProteinChip Bioprocessor was subsequently removed, and the array was air-dried prior to analysis.

The weak cation exchange (WCX2) ProteinChip Array was pretreated with 10 mM HCL and washed with HPLC-grade water followed by pre-equilibration in binding/wash buffer (50 mM sodium acetate, pH 4.5, containing 0.1% Triton X-100). Sample obtained from the IMAC3 ProteinChip Array surface was lyophilized and resuspended in 10 µL WCX2 binding/wash buffer. Sample was applied to the WCX2 array surface using a ProteinChip Bioprocessor and incubated for 2 h at ambient temperature with gentle agitation. Sample was aspirated from the array surface and stored at 4°C until further use. The array was washed twice with binding/wash buffer, followed by two final HPLC-grade water washes. The ProteinChip Bioprocessor was subsequently removed, and the array air-dried prior to analysis.

The strong anion exchange (SAX2) ProteinChip Array was pre-equilibrated with SAX2 binding/wash buffer (50 mM Tris, pH 8.0, containing 0.1% Triton X-100). Sample obtained from the WCX2 ProteinChip Array surface was lyophilized and resuspended in 10 µL SAX2 binding/wash buffer. Sample was applied to the SAX2 array surface using a ProteinChip Bioprocessor and incubated for 2 h at ambient temperature with gentle agitation. The array was washed twice with binding/wash buffer, followed by two final 5 mM Tris, pH 8.0, washes. The ProteinChip Bioprocessor was subsequently removed, and the array air-dried prior to analysis.

For the gold ProteinChip Array surface, 5 µL liquid tissue colon cancer extract (approximately 15,000 cells) were lyophilized and resuspended in 3 µL HPLC-grade water. One microliter of a saturated sinapinic acid solution in 50% ACN, 0.5% TFA was mixed with the sample and directly spotted onto the gold array surface.

A total of 1 µL of a saturated sinapinic acid solution in 50% ACN, 0.5% TFA was added to each spot of the IMAC3, WCX2, and SAX2 ProteinChip Arrays. Data acquisition settings were dependent upon the ProteinChip array type used: laser intensities of 215–220, detector sensitivities of 8–10, and a molecular mass range 0–100,000 Da, with 130 averaged shots per spectrum. Data were collected and analyzed using ProteinChip software version 3.2 (Ciphergen Biosystems).

MALDI-TOF/TOF Analysis

MALDI-TOF/TOF analysis was performed on a Voyager 4700 MALDI-TOF/TOF™ mass spectrometer (Applied Biosystems, Foster City, CA, USA). Sample was co-crystallized with an equivalent amount of α-cyano-4-hydroxycinnamic acid in 50% ACN, 0.1% TFA, and spotted directly on a stainless steel MALDI target plate. Data was collected at a laser frequency of 200 Hz, and the collision energy for CID was 1 keV (air was used as collision gas). Post-acquisition baseline correction and smoothing was carried out using software provided with the instrument.

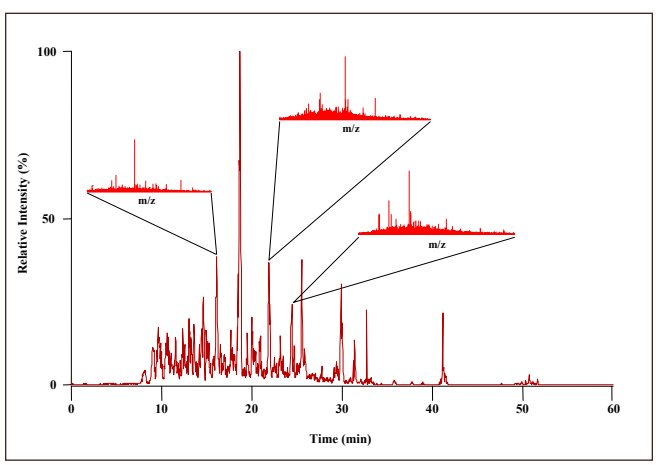

Figure 1. Microcapillary reversed-phase liquid chromatography tandem mass spectrometry (µRPLC-MS/MS) analysis of proteins extracted from formalin-fixed colon cancer tissue. The efficiency of extracting proteins from formalin-fixed colon cancer tissue using the Liquid Tissue method is exemplified by the complexity of the resultant MS base peak chromatogram. Over 600 peptides were identified from this µRPLC-MS/MS analysis of formalin-fixed paraffin-embedded (FFPE) colon cancer tissue. m/z, mass-to-charge ratio.

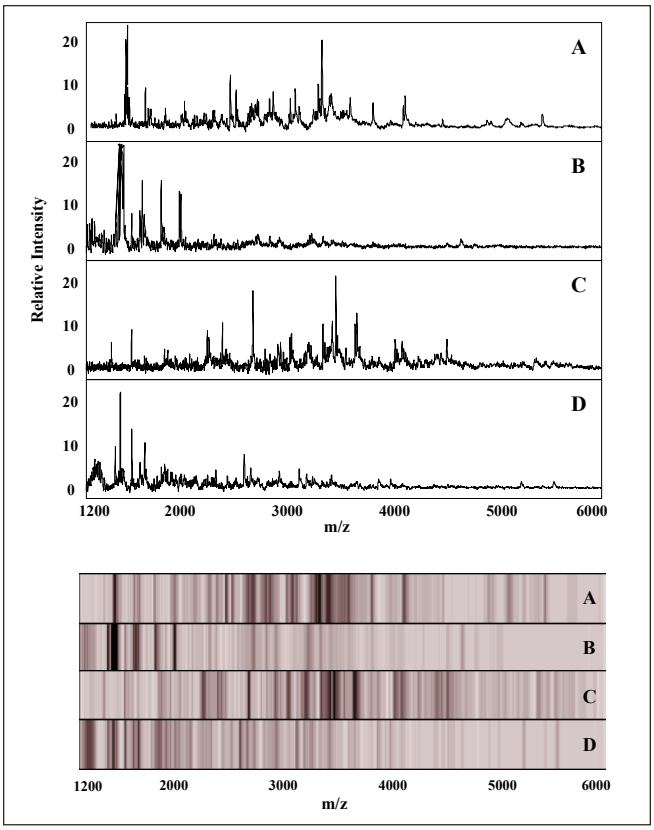

Figure 2. Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) analysis. An analysis of the Liquid Tissue extract was performed using SELDI-TOF MS. Colon cancer tissue extract was processed on the following ProteinChip surfaces (top panel): (A) immobilized metal affinity chromatography (IMAC3), (B) strong anion exchange (SAX2), (C) weak cation exchange (WCX2), (D) Au Chip. In each case, the sample was lyophilized and resuspended in the appropriate binding buffer for each chip surface. The sample was processed on the IMAC3 ProteinChip array first, followed by WCX2, and then SAX2. The bottom panel shows the virtual gel representation of each SELDI-MS spectrum (labeled as in the top panel). The Gold ProteinChip was processed separately using an aliquot of the original sample. m/z, mass-to-charge ratio.

RESULTS AND DISCUSSION

Even though the complexity makes qualitative and quantitative analysis of a cell's proteome difficult at best, observations carried out at the protein level are inherently attractive because of the cause and effect relationship between protein expression and biological function. Informative pathologic context and relevance makes tissue-based studies important for linking proteins directly to biological and disease processes. Fresh frozen tissue is difficult to obtain in large numbers, expensive to store, and difficult to process, whereas large collections of well-defined FFPE tissue with clinical history (and often patient outcome) are readily available. Unfortunately, effective and routine analysis of proteins in formalin-fixed tissue has been limited to IHC. This limitation is due to the chemical cross-linking properties of the common fixative reagent formalin, preventing standard protein preparation methodologies from extracting soluble protein from fixed tissue. Samples processed using the Liquid Tissue methodology allow for the proteomic analysis of formalin-fixed tissue not previously attainable.

One analytical method that can be used in conjunction with Liquid Tissue protein extraction is MS. Multidimensional LC separation methods when combined with MS provide sufficient separation and resolution to resolve, detect, and identify multiple proteins simultaneously, while providing broad proteome coverage of a single Liquid Tissue protein preparation. In addition, MS pattern determination utilizing SELDI technology can be employed to develop proteomic profiles characterizing individual Liquid Tissue protein preparations.

A colon cancer Liquid Tissue extract was prepared from a FFPE tissue and analyzed on numerous MS platforms to demonstrate both the practicality and versatility of this approach. Microcapillary RPLC-MS/MS analysis of the peptides obtained from this colon cancer tissue extract resulted in the detection of thousands of species as shown by the base peak chromatogram provided in Figure 1. A single injection of a diluted extract resulted in the actual identification of 629 unique, fully tryptic peptides representing over 350 unique proteins. Interestingly, a 20-fold lower injection volume yielded almost the same results: 501 unique fully tryptic peptides corresponding to 289 unique proteins. In each case, nearly 40% of the unique proteins were identified by two or more unique peptides. Many of the proteins identified in this study by multiple unique peptides have previously been shown to have differential expression levels in a comparison of normal mucosa and colorectal cancer tissue, including annexin V, α-tropomyosin, and triosephosphate isomerase (13).

While individual protein identification is important for correlating potential expression levels with defined histological states, the ability to evaluate proteomic patterns in various samples also plays a significant role in biomarker discovery. Although still in its infancy, proteomic pattern diagnostics using SELDI-TOF MS holds great promise as a diagnostic tool. This technique uses the principles of retentate chromatography to selectively bind biological samples to the ProteinChip Array surface. Nonspecifically bound peptides, proteins, salts, and other interfering compounds are washed away. The peptides/proteins retained on the array surface are then analyzed by TOF-MS after applying matrix to the sample. This technique has been successfully employed for the early detection of numerous cancers, including ovarian (14), prostate (15), cervical (16), and pancreatic (17) cancers. SELDI-TOF MS of the colon cancer tissue extract revealed complex peptide spectra on a variety of chromatographic surfaces (Figure 2). Spectra obtained on the WCX2 and SAX2 array surfaces are even more interesting, since sample limitation required application of the same tissue extract consecutively on these surfaces. For example, after the sample was applied to the IMAC3 chip, the solution of peptides that did not

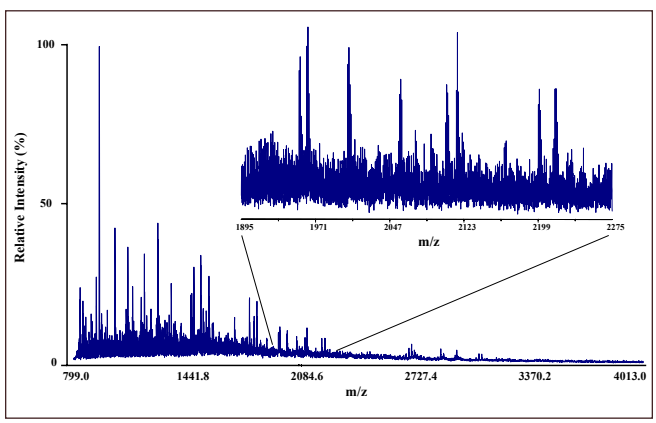

Figure 3. Matrix-assisted laser desorption ionization tandem time-of-flight (MALDI-TOF/TOF) analysis of proteins extracted from formalin-fixed paraffin-embedded (FFPE) colon cancer tissue. Peptides extracted from formalin-fixed colon cancer tissue using the Liquid Tissue method were analyzed using a Voyager 4700 high-resolution MALDI-TOF/TOF mass spectrometer showing a large number of observable peptides, including well-resolved low-level signals (inset). m/z, mass-to-charge ratio.

bind to this surface was collected, relyophilized, and then applied to the WCX2 chip. Likewise, the solution of peptides that did not bind to the WCX2 chip were collected, relyophilized, and applied to the SAX2 chip. Even with the loss of peptides bound to the previous ProteinChip array type, complex spectra from a single sample applied on multiple chromatographic surfaces was still observed. The gold ProteinChip surface was used to evaluate the global colon cancer Liquid Tissue extract. This approach to proteomic pattern analysis offers a convenient alternative when sample is severely limited and is insufficient to apply fresh samples to each ProteinChip array surface.

As expected, the proteomic profiles of the peptides extracted from the colon cancer tissue extract were dependent on the chromatographic surface (i.e., IMAC3, WCX2, and SAX2) used. The complexity of the spectra, however, is quite high, showing that the extraction of peptides from formalin-fixed tissue using Liquid Tissue is quite efficient. As has been done primarily for plasma and serum samples, these results show that proteomic profiles generated from formalin-fixed tissues also have the potential to be coupled with SELDI-TOF MS for the identification of biomarkers of specific diseases. Studying formalin-fixed tissues has many advantages over studying biofluids, in that retrospective samples are easily obtained, and issues related to sample collection are not as critical.

There is an active area of research that is attempting to use higher resolution MS platforms to obtain proteomic patterns. To meet this demand, we investigated the capability of obtaining proteomic profiles of peptides extracted from FFPE tissues using a high-resolution MALDI-TOF spectrometer. Peptides extracted from a FFPE colon cancer tissue using the Liquid Tissue method were spotted onto a target plate, and the spectrum was acquired using a Voyager 4700 high-resolution MALDI-TOF/TOF mass spectrometer. The resulting spectrum revealed a large number of observable peptides as shown in Figure 3. Expansion of a region within this spectrum shows that a large number of highly resolved, low-level signals are readily detectable using this direct MALDI approach (Figure 3, inset). These results show the complexity and flexibility of the Liquid Tissue methodology to allow proteome investigation of peptides extracted from FFPE tissue on a variety of mass spectrometry platforms.

The development of methods to use state-of-the-art proteomic discovery tools to analyze FFPE tissue provides an exciting new opportunity to identify disease-specific biomarkers in pathologically defined samples. There are vast archives of formalin-fixed tissues, human and animal, spanning every conceivable condition (disease, time, treatment, and outcomes), allowing acquisition of the necessary numbers of samples to carry out in-depth analyses on a disease of interest.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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