

APPLICATION NOTE

NanoLC-MS/MS Analysis of Proteins from Microdissected Cells

INTRODUCTION

Despite the analytical advantages demonstrated by the use of laser microdissection for cell-type specific genomic analysis, similarly productive applications of corollary proteomic analysis of microdissected cell populations have been limited. Obtaining high quality proteomic analysis results using nanoLC-MS/MS, a key analytical method of proteomics, has typically required more cells than can readily be collected by laser capture/catapult microdissection (LCM). Additionally, when time or tissue sample sizes are limiting, as is often the case with fresh or frozen clinical specimens, research strategies that rely on pooling cells captured from multiple sections are unfeasible.

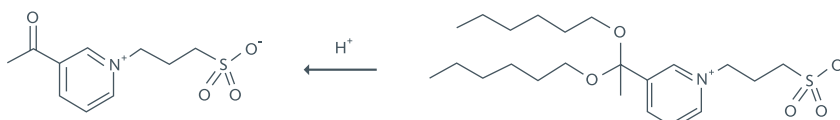
To increase protein yield and mass spectrometer signal strength from small populations of cells, Thomas P. Conrads, PhD, an Associate Professor at the University of Pittsburgh Cancer Institute's Hillman Cancer Center, has developed a single-tube protocol for preparing proteins from cells captured by LCM. The single-tube protocol relies on the special characteristics of PPS Silent® Surfactant to act as an aggressive detergent for cell disruption and protein solubilization, while eliminating buffer changes and sample cleanup steps that otherwise reduce protein yield. The new protocol is now enabling an early stage endometrial cancer biomarker discovery study based on nanoLC-MS/MS data derived from cells acquired by LCM from 150 tissue samples to identify candidate proteins indicative of early stage metastasis.



Early stage endometrial cancer biomarker discovery study team at the University of Pittsburgh Cancer Institute. Sue Abbatiello PhD, Aaron Lucas, Brian Hood PhD, and Thomas Conrads PhD

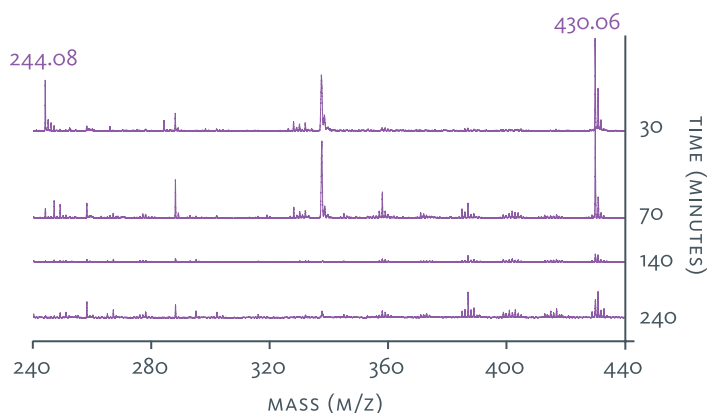
BACKGROUND

PPS Silent Surfactant (3-[3-(1,1-bisalkyloxyethyl)pyridin-1-yl]propane-1-sulfonate) is a cleavable surfactant capable of disrupting cell membranes and solubilizing proteins. At low pH, PPS hydrolyzes rapidly into cleavage products that have no remaining surfactant properties and do not interfere with mass spectrometry detection of proteins.



Acid hydrolysis of PPS Silent Surfactant eliminates detergent interference, enabling mass spectrometry analysis of peptides resulting from trypsin digestion of complex protein mixtures.

Figure adapted from Vanderbilt University doctoral thesis dissertation *Design and Synthesis of Novel Cleavable Detergents from Protein and Peptide Analysis by Mass Spectrometry* (Jeremy Norris, PhD).



OBJECTIVE

Develop a single-tube protocol to prepare cancer biopsy tissue cell populations captured by laser microdissection for proteomic analysis. Final solution in the tube must be directly compatible with nanoLC injection and contain protein at sufficient concentration to enable confident protein identification from mass spec detection data resulting from a preparation of as few as 5,000 cells. This protocol must include all steps necessary for liquid-phase digestion upstream of the nanoLC-MS/MS analysis, and must not require a clean-up step to get rid of salts or detergents prior to nanoLC-MS/MS.

PROTOCOL

Cells from patient biopsy endometrial cancer tissue samples were selected by laser catapult microdissection and received directly in approximately 100 μ L volume of the following buffer: 0.2 mM PPS (surfactant), 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM DTT. After boiling samples for 10 minutes, trypsin was added in a 1:100 (trypsin:total protein) ratio and digestion proceeded 16 hours at 37°C. In order to cleave the PPS surfactant, samples were acidified with 100 μ L formic acid and incubated for 2 hours at 30°C. This volume was sufficient to acidify the solution to a pH of less than 2. The LCM digests were then lyophilized to dryness and resuspended in 15 μ L of 0.1% formic acid for subsequent nanoLC-MS/MS analysis. Cell collection, membrane disruption, protein solubilization, and trypsin digestion were carried out in a single tube.

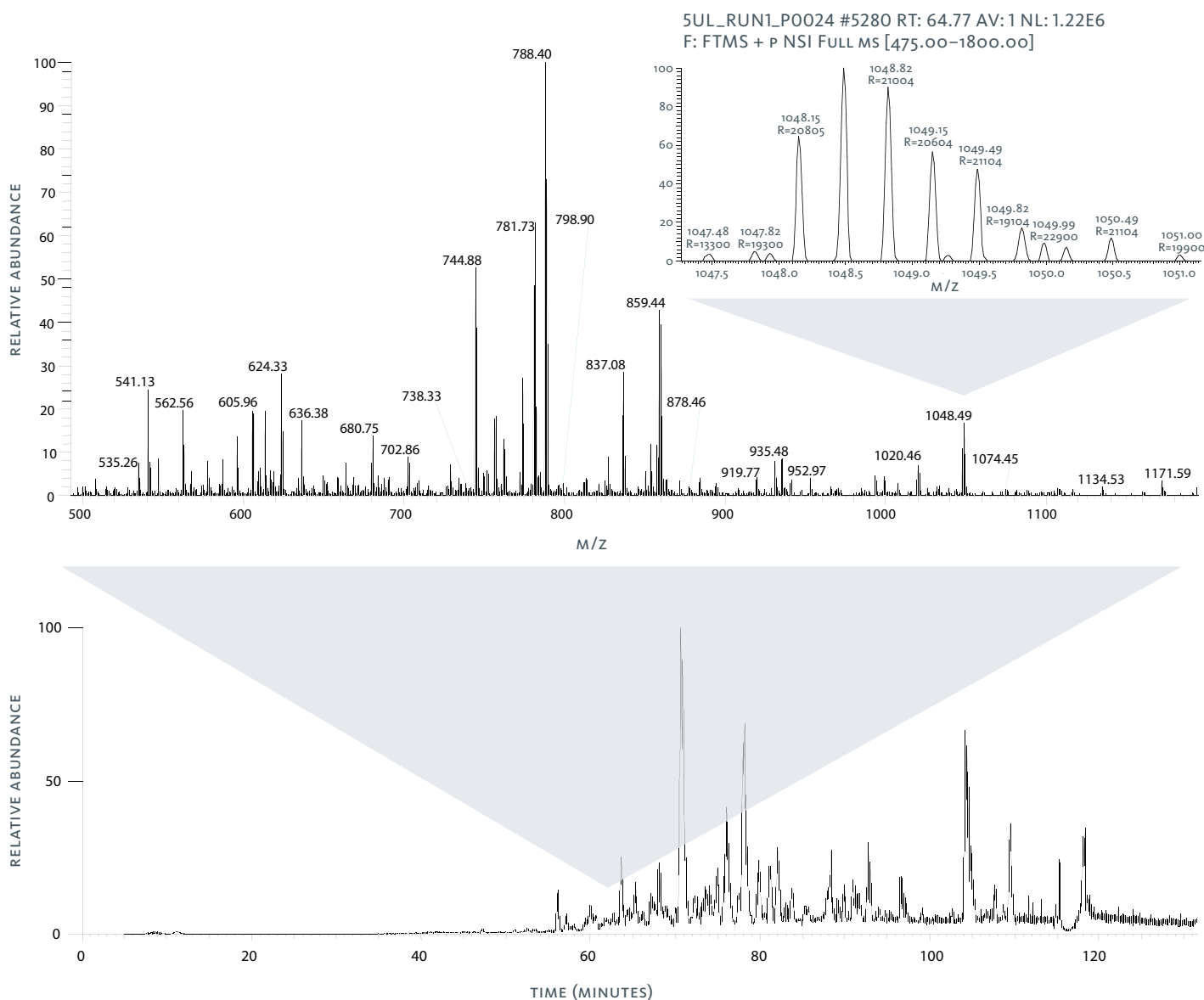
The endometrial tissue LCM protein digests were analyzed in duplicate by nanoflow reversed-phase liquid chromatography (nanoRPLC) coupled online via electrospray ionization to a hybrid linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap, ThermoFisher Scientific). The instrument was configured to collect a high resolution, high mass measurement accuracy broadband mass spectrum from which the seven most-abundant peptide molecular ions were dynamically selected for collision-induced dissociation (CID).

Data files representing approximately 30,000 CID spectra each were all searched using SEQUEST and the UNIPROT derived human proteome database downloaded from the European Bioinformatics Institute (EBI) (<http://www.ebi.ac.uk/integr8/EBI-Integr8-HomePage.do>).

Only tryptic peptides with up to two missed cleavage sites meeting specific SEQUEST scoring criteria [$\Delta C_n \geq 0.1$ and charge state dependent cross correlation ($X_{corr} \geq 2.0$ for $[M+H]^+ \geq 2.2$ for $[M+2H]^{2+}$ and ≥ 3.75 for $[M+3H]^{3+}$] were considered as legitimate identifications. A gene ontology classification of the proteins identified was performed according to cellular compartment, biological process and molecular function.

FINDINGS

REPRESENTATIVE RAW LTQ-ORBITRAP DATA FROM SAMPLE ID "P24"



Representative base peak chromatogram and mass spectra from a protein digest of 10,000 endometrial cancer cells procured by LCM. Each sample was analyzed in duplicate by nanoRPLC coupled online via ESI to an LTQ-Orbitrap MS.

PROTEIN IDENTIFICATION

Table 1. Proteins Identified by nanoLC-MS/MS

SAMPLE ID	PROTEINS IDENTIFIED	PEPTIDES IDENTIFIED
Patient 24	985	1972
Patient 26	844	1736

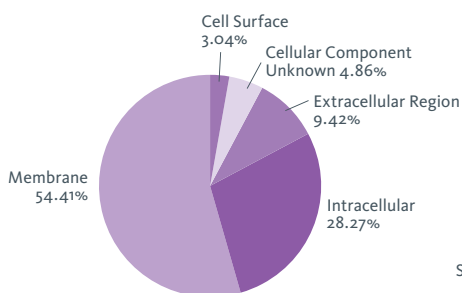
The single tube protocol allows confident identification by nanoLC-MS/MS of approximately 500 proteins per sample collected by microdissection.

Table 2. Proteins Identified ≥ 2 peptides by nanoLC-MS/MS

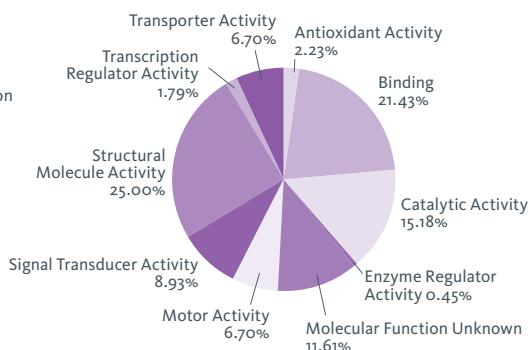
SAMPLE ID	PROTEINS IDENTIFIED
Patient 24	620
Patient 26	468

GENE ONTOLOGY CLASSIFICATION OF IDENTIFIED PROTEINS

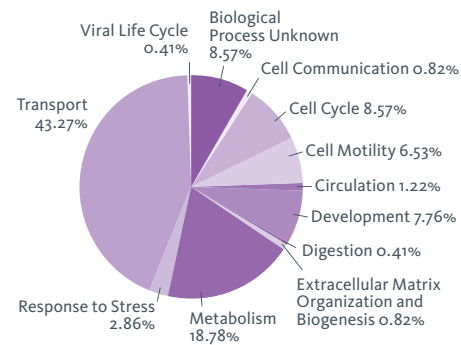
CELLULAR COMPARTMENT (POO24)



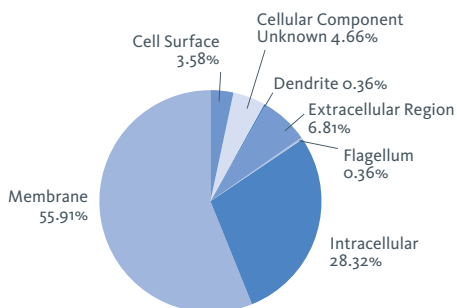
MOLECULAR FUNCTION (POO24)



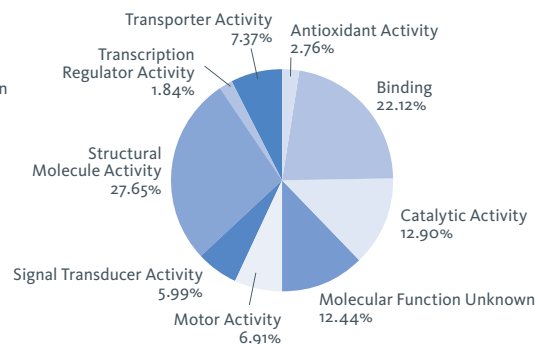
BIOLOGICAL PROCESS (POO24)



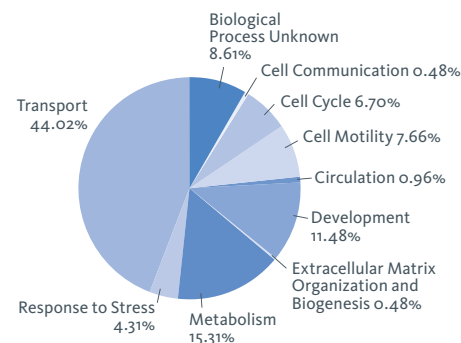
CELLULAR COMPARTMENT (POO26)



MOLECULAR FUNCTION (POO26)



BIOLOGICAL PROCESS (POO26)



Gene ontology association analysis indicates the single tube protocol results in efficient membrane protein extraction and solubilization.

CONCLUSION

The special characteristics of PPS Silent Surfactant include aggressive surfactant action and susceptibility to acid hydrolysis into non-surfactant products that do not interfere with downstream nanoLC-MS/MS analysis. These properties have enabled the design of a single-tube protocol for preparing protein digests for nanoLC-MS/MS proteomic analysis from small cell populations derived by microdissection of endometrial cancer biopsy specimens. In the single tube protocol, PPS Silent Surfactant is used to disrupt cell membranes, extract proteins, and solubilize proteins to aid trypsin digestion, prior to its hydrolytic cleavage into non-surfactant products. This protocol serves to accomplish all steps necessary for a liquid phase digestion upstream of the nanoLC-MS/MS analysis, without introduction of salts or detergents that would otherwise require a clean-up step prior to injection. The single-tube protocol overcomes limitations related to inadequate protein concentration levels that have prohibited conventional nanoLC-MS/MS analysis of small cell type specific populations derived by microdissection. Gene ontology association analysis indicates that a high proportion of membrane proteins are recovered using the protocol.

“The PPS reagent allows us to capture cells, efficiently extract proteins, and digest, all in a single tube that is directly compatible with downstream LC and MS analysis without the need for involved clean-up that would result in substantial losses of clinically valuable material. We were astonished to find that greater than half of the proteins identified are classified as membrane proteins, clearly an attribute of the approach that relies on solubilization and denaturation of the lysate proteins by the PPS surfactant. This sample preparation protocol is central to our clinical proteomic investigation of early endometrial cancer tissue biopsies.”

Thomas P. Conrads, PhD
Associate Professor of Pharmacology
University of Pittsburgh Cancer Institute
Hillman Cancer Center
Clinical Proteomics Facility



ORDERING INFORMATION

PPS Silent Surfactant is available in 1 mg and 10 mg vials. To place an order for PPS Silent Surfactant, please contact Protein Discovery by phone, fax, or e-mail

Tel: (865) 521-7400
Fax: (865) 521-3548
E-mail: sales@proteindiscovery.com

DESCRIPTION	PART NUMBER
10 mg vial, 1 vial per pack	21010
1 mg vial, 5 vials per pack	21011



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