[APPLICATION NOTE]

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IDENTIFICATION, QUANTIFICATION AND ANNOTATION OF HEART MEMBRANE PROTEINS USING LABEL-FREE NANOSCALE LC/MS

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INTRODUCTION

Heart diseases resulting in heart failure are among the leading causes of morbidity and mortality in the Western world and can result from either systemic disease (e.g. hypertensive heart disease, ischaemic heart disease) or specific heart muscle disease (e.g. dilated cardiomyopathy (DCM)). Sub-proteome analysis of such disease sub-sets affords a reduction in sample complexity, potentially revealing biomarkers of cardiac failure that would otherwise remain undiscovered.

Label-free nanoscale LC/MS has been used in this study to validate a Triton X-114 based enrichment method for cardiac membrane proteins. Annotation of the sub-cellular location combined with hydropathicity (GRAVY) score analysis for the identified proteins indicates a clear separation between soluble and membrane bound proteins. LC/MS is shown to identify, quantify, and annotate hydrophobic proteins in this study and to demonstrate the power of this technique for diseased-based analysis.

A novel protein identification algorithm was employed for the identification, quantification, and annotation of heart membrane proteins analyzed by nanoscale LC/MS. The algorithm has been designed and is ideally suited for data-independent acquisitions, whereby multiple precursor ions are fragmented simultaneously.

The search algorithm utilizes an iterative three-step process whereby the culmination of each loop increases the selectivity and sensitivity of the next. In addition, the method utilizes limited database queries whereby each query accesses different sets and subsets of peptides from the proteins present in the database.

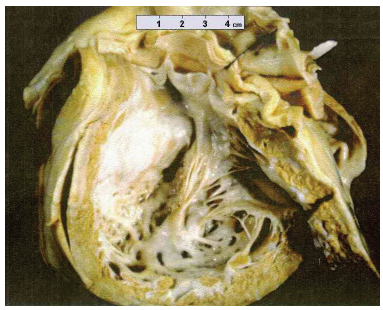

Figure 1a. Dilated cardiomyopathy (DCM).

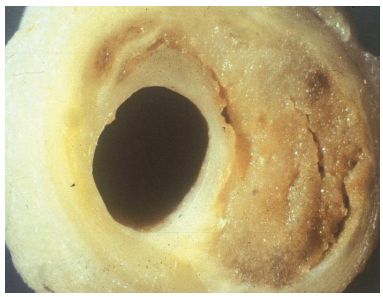

Figure 1b. Ischaemic heart disease.

EXPERIMENTAL

Sample preparation

250 mg of human heart tissue (left ventricle) was ground into a fine powder using N_2. The ground tissue was immediately added to 8 mL of cold PBS and 2 mL of 10% Triton X-114 and left overnight at 4 °C

on a rotary shaker. The suspended sample was then spun at 20,000 g for 30 min at 4 °C to remove debris.

The supernatant was placed at 37 °C for 30 min followed by a 5000 g centrifuge spin for 30 min at 25 °C to separate the detergent and aqueous phase. The aqueous layer was removed to a fresh tube to which 2 mL of 10% Triton X-114 was added. The detergent phase was re-suspended in 8 mL of PBS. Both phases were placed at 37 °C for 15 min followed by a 15 min centrifuge spin at 25 °C. This process was repeated three times in order to wash each phase and remove contaminants.

Proteins were extracted from each phase by acetone precipitation. The precipitated proteins were re-suspended in 0.1% RapiGest™ in 50 mM ammonium bicarbonate buffer. Next, the proteins were reduced (10 mM DTT) and alkylated (10 mM IAA) prior to enzymatic overnight digestion with trypsin – 1:50 (w/w) enzyme/protein ratio – at 37 °C. RapiGest was removed by the addition of 2 µL conc. HCl, followed by centrifugation, and the supernatant collected.

LC/MS conditions

Qualitative and quantitative LC/MS experiments were conducted using a 1.5 h gradient from 5 to 40% acetonitrile (0.1% formic acid) at 250 nL/min on the Waters® Identity^E High Definition Proteomics™ System, using as an inlet the nanoACQUITY UPLC® System. An Atlantis® 3 µm C_{18} 75 µm x 15 cm nanoscale LC column was used. Typical on-column sample loads were 0.1 to 0.5 µg protein digest.

The Identity^E System also included the Q-Tof Premier™ Mass Spectrometer for performing multiplexed, alternate scanning (LC/MS^E) experiments. The Q-Tof Premier was programmed to step between normal (5 eV) and elevated (15 to 40 eV) collision energies applied to the gas cell, using an acquisition time of 1.5 seconds per function over the m/z range 50 to 1990. Samples were run in triplicate.

In addition, accurate mass LC/MS/MS data directed analysis (DDA™) experiments were conducted. The survey scan was 0.48 s with an interscan delay of 0.02 s. Two MS/MS spectra/precursors were acquired for the five most abundant multiply charged ions. The MS/MS scan rate was 0.48 s with 0.02 s interscan delay. The collision energy was automatically set based on the observed precursor m/z and charge state. A dynamic exclusion window was set to 60 s.

Data processing, protein identification, sub-cellular annotation, and GRAVY score calculation

Continuum LC/MS data was processed and searched using ProteinLynx GlobalSERVER™ (PLGS) Software v2.3 for the DDA data, and the Identity^E System's search algorithm for the multiplexed LC/MS^E fragmentation data. The sub-cellular locations – GO component annotation – of the identified proteins were obtained using SRS (European Bioinformatics Institute Hinxton, UK). The general average

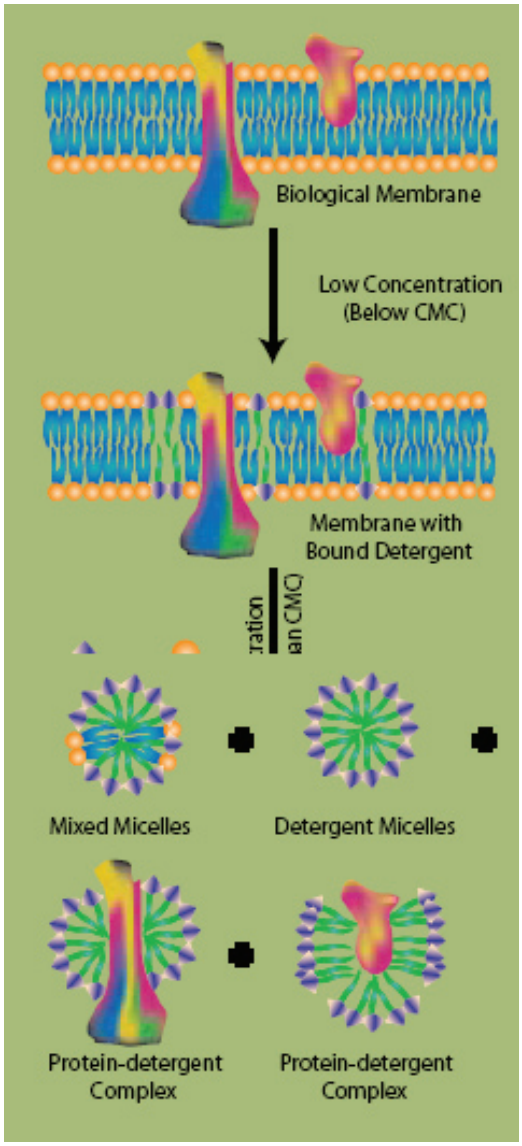

Figure 2. Sample preparation - membrane protein solubilization.

hydro pathicity (GRAVY) score of the proteins was determined with v0.1 of Protalizer (Ernst Moritz Arndt University, Greifswald, Germany).

RESULTS AND DISCUSSION

Identification and annotation

Figures 3 shows typical example LC/MS^E chromatograms from one of the investigated detergent phase samples. An example Identity^E System identification from a membrane associated protein is illustrated in Figure 4.

Quantification

In total, 240 proteins were identified and annotated in the aqueous phase extracts, 113 proteins in the detergent phase extracts, and 60 proteins were commonly identified in both phases. 20 percent of the aqueous phase proteins were membrane-related as were 62 percent of the detergent phase, as shown in Figures 5 and 6.

The absolute concentrations of the identified proteins for the LC/MS^E experiments were determined to assess technical replication for future larger scale studies. The results are summarized in Table 1.

GRAVY score

The GRAVY score of the identified proteins was calculated and expressed as a function of phase, as show in the right-hand pane of Figure 7. The GRAVY score is the average hydropathy score for all the amino acids in the protein. According to Kyte and Doolittle, integral membrane proteins typically have higher GRAVY scores than do globular proteins. Though this score is another helpful piece of information, it cannot reliably predict the protein structure. The left-hand pane of Figure 7 illustrates significant statistical difference in terms of GRAVY score of the proteins identified in both phases, confirming sample preparation method efficacy in terms of enrichment of protein based on their hydrophobicity.

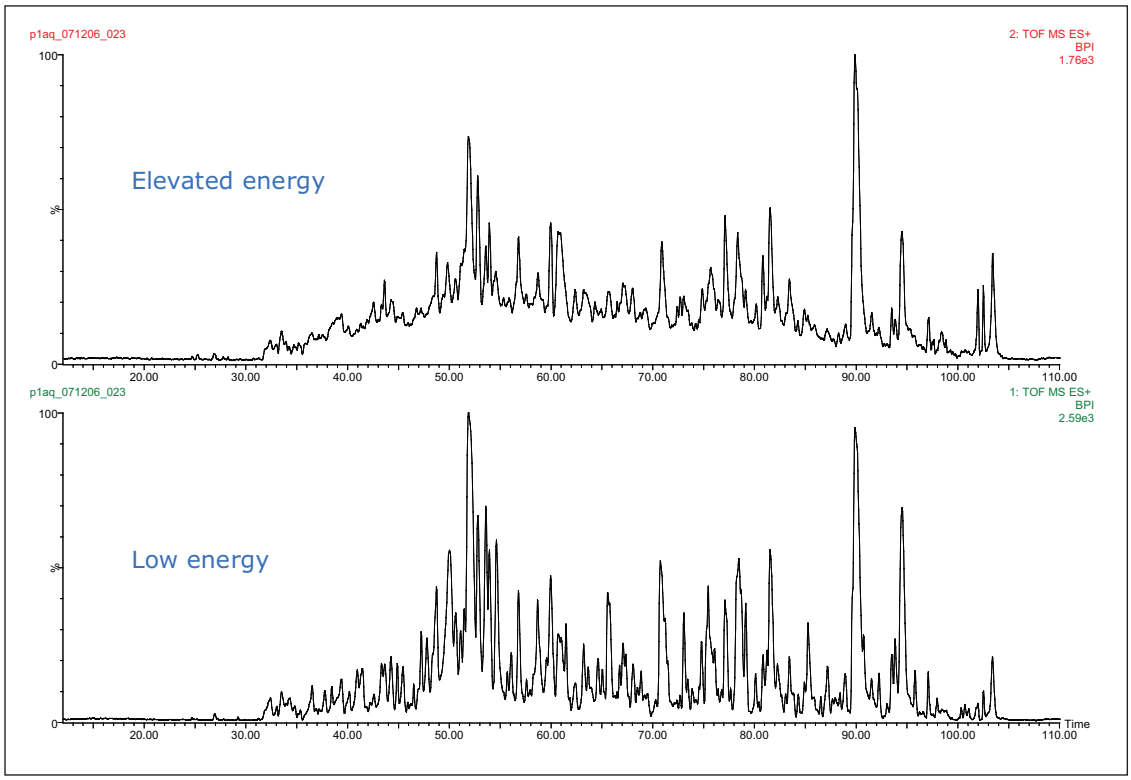

Figure 3. Low- (bottom) and elevated-energy (top) chromatograms of one of the detergent phase samples.

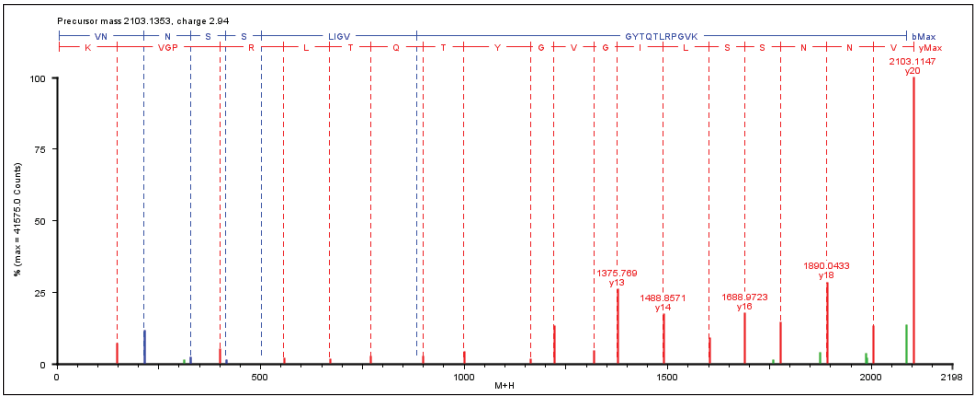

Figure 4. High-energy Identity^E System identification of Outer Mitochondrial Membrane Protein Porin 2 (P45880).

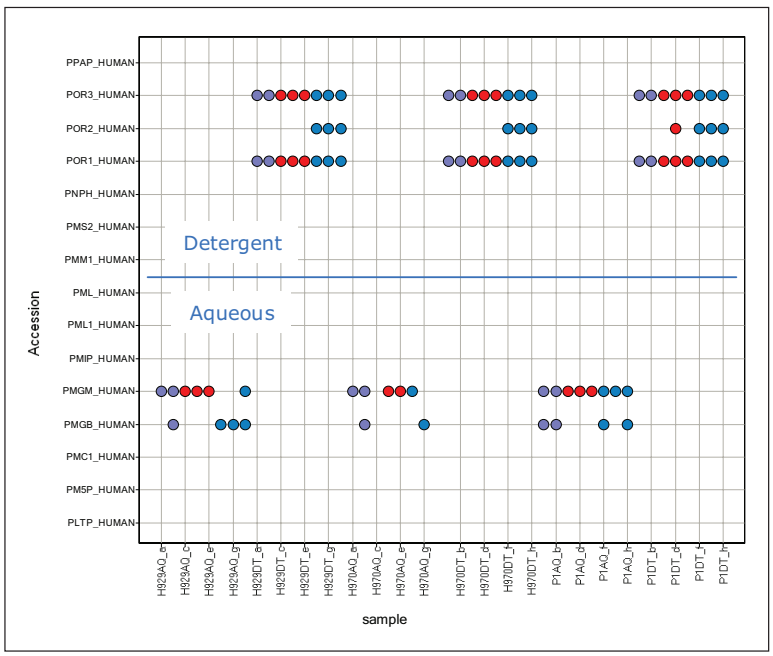

Figure 5. Distribution of a subset of the identified proteins in the aqueous and detergent phase; color annotation by scanning and search algorithm type (purple = DDA; red = PLGS v2.5; blue = Identity^E).

	AQ-1	AQ-2	DET-1	DET-2
	# proteins			
n = 3	84	51	88	52
n = 2	30	16	26	9
	measurement variation			
RSD* (%)	11.0	12.0	11.7	12.4

*Table 1. Technical reproducibility obtained for two aqueous and two detergent phase extracts samples; * statistical outliers removed.*

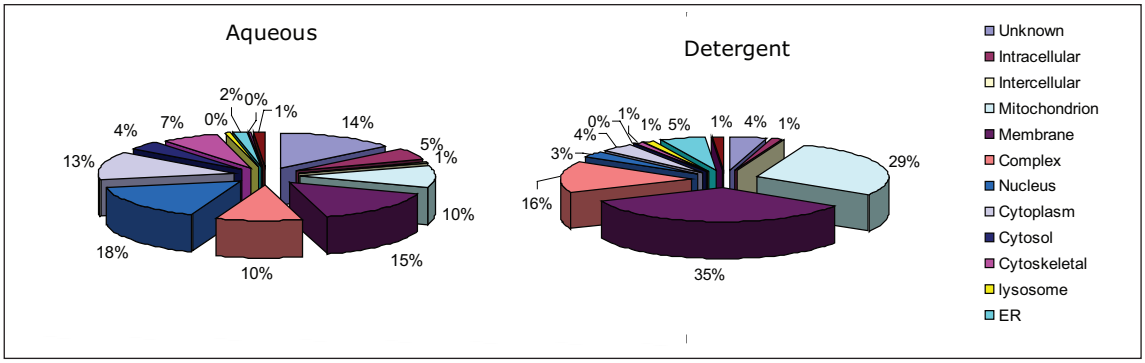

Figure 6. Sub-cellular distribution of the proteins identified in the aqueous (left) and detergent (right) phase extracts.

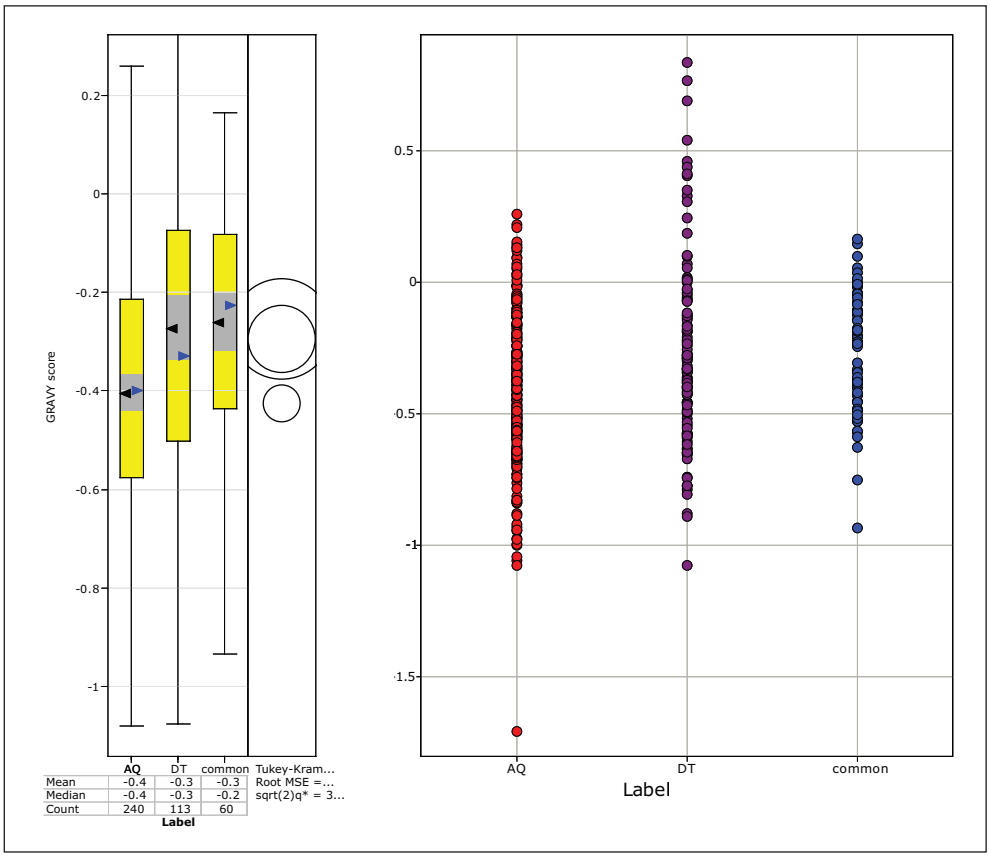

Figure 7. Protein GRAVY score distribution as a function of extraction phase.

CONCLUSION

- Phase separation with Triton X-114 can enrich cardiac membrane proteins
  - Parallel enrichment for cardiac mitochondrial proteins
  - Significant differences between phases
- High degree of technical reproducibility
  - 10 to 15% measurement variation
  - Control tissue pilot study enables sample number determination for disease-based study
  - Relatively small number of samples per disease group of Control, DCM and IHD required for diseased based analysis
- Stringent data analysis
  - ≥2 peptides per protein, ≥95% probability, replication (reproducibility)

References

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