METABOLOMIC PROFILING OF OSTEOBLAST EXTRACELLULAR VESICLES AND MATRIX BOUND VESICLES USING A PROTOTYPE BENCHTOP MULTI REFLECTING TIME-OF-FLIGHT (MRT) MASS SPECTROMETER

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INTRODUCTION

Extracellular vesicles (EVs) are lipid delimited nanoparticles that function in development and intercellular signaling events throughout the body[1]. Within bone there exists a subset of EVs, known as matrix binding vesicles (MBVs), which have been long proposed to associate with the underlying collagenous extracellular matrix to drive early mineralization events during bone development[2]. However, the precise relationship between EVs and MBVs and their differential roles in bone development and metabolism remains a point of contention[3].

This study uses a Xevo™ MRT Mass Spectrometer (MS) coupled to an ACQUITY™ Premier LC system to construct a comparative metabolite profile for EVs and MBVs obtained from MC3T3 pre-osteoblasts under osteogenic culture conditions.

The Xevo MRT MS incorporates multi-reflecting time-of-flight (MRT) technology first commercialized on the SELECT SERIES™ MRT MS. Providing a mass spectral resolution of up-to 100,000 FWHM and sub-ppm mass accuracies at up-to 100Hz scan speeds.

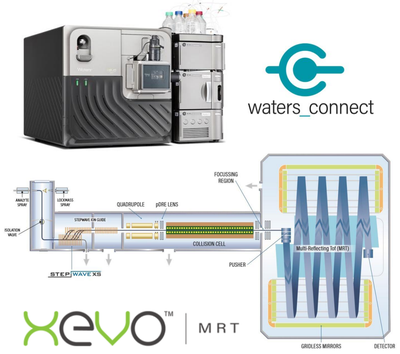

Figure 1. The Xevo MRT MS, a routine benchtop mass spectrometer with a novel ion optics design and multi-reflecting time-of-flight technology.

METHODS

SAMPLE PREPARATION

MC3T3 osteoblast cells were cultured to confluence and differentiated towards a pro-mineralizing phenotype in the presence of 10mm beta-glycerophosphate and 50µg/ml ascorbic acid. EVs were isolated from the media using a differential ultracentrifugation (UC) process and lysed using sonication which incorporated no lysis buffer. MBV's were liberated from the ECM using collagenase digestion and subjected to UC isolation.

Extracts underwent a water:MeOH:MTBE extraction and the aqueous layer analyzed by LC-MS.

LC-MS METHODOLOGY

Chromatographic separation utilized both HILIC (1x50mm BEH™ Amide) and reversed-phase (RP) (1x50 HSS T3) chromatographic methods.

Injection volume: 1 µL, column temp 50°C.

HILIC – flow 0.25 mL/min RP – flow 0.4 mL/min

Time (min)	%B
0.0-0.3	99
2.0	80
2.3-2.5	50
2.5-4.0	99

Time (min)	%A
0.0-0.3	99
2.0	50
3.0	30
3.0-3.3	1
3.3-4.0	99

Data were acquired within the waters_connect™ software platform using a data independent analysis (DIA) strategy, across the mass range 50-1200 Da, with a 10Hz scan speed, and the high energy collision ramp 25-45 V, in both positive and negative electrospray ionization (ESI) modes.

The data were visualized in the waters_connect MSToolkit application and statistical processing was conducted within MARS (MassAnalytica™, Barcelona, Spain), putative compound identifications were through database searches within the MARS software using a human metabolome (HMDB) database.

RESULTS

HILIC DATASET

Data was processed through MARS software to determine differences between the EV and MBV metabolite profile. The PCA plot generated can be seen in *Figure 2,* from this a volcano plot was produced *(Figure 3),* showing differences in the polar metabolite profile between the extracts.

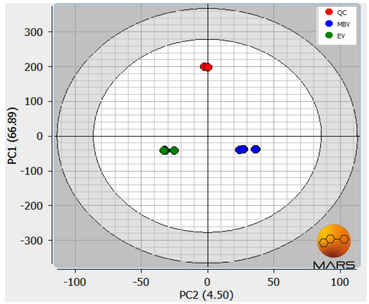

Figure 2. MARS generated unsupervised PCA plot showing separation between EVs (Green) and MBVs (Blue) using HILIC chromatography. The system QC was a NIST urine standard (Red).

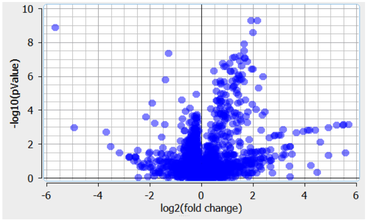

Figure 3. Volcano plot showing the upregulation of polar metabolites in the MBV (left) and EV (right) extracts from HILIC analysis.

From this analysis a number of statistically significant compounds were putatively identified predominantly upregulated in the EVs, these consisted of:

Glycerophospholipids, Glycerolipids, Fatty Acyls and Carboxylic Acids.

RESULTS

REVERSED-PHASE DATASET

Data was processed through MARS software to determine differences between the EV and MBV metabolite profile. The PCA plot generated can be seen in *Figure 4,* from this a volcano plot was produced *(Figure 5),* showing differences in the polar metabolite profile between the extracts.

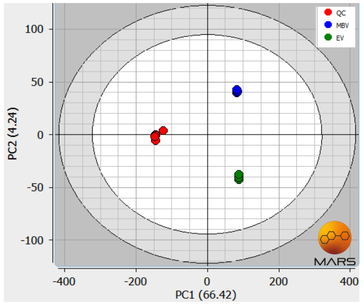

Figure 4. MARS generated unsupervised PCA plot showing separation between EVs (Green) and MBVs (Blue) using RP chromatography. The system QC was a NIST urine standard (Red).

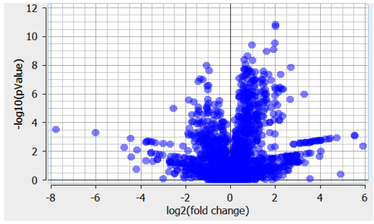

Figure 5. Volcano plot showing the upregulation of polar metabolites in the MBV (left) and EV (right) extracts from RP analysis.

From this analysis a number of statistically significant compounds were putatively identified predominantly upregulated in the EVs, these consisted of:

Glycerophospholipids, Glycerolipids, Fatty Acids, Steroids and Carboxylic Acids.

RESULTS

PUTITIVE IDENTIFICATION

The Xevo MRT MS is a compact benchtop mass spectrometer offering up-to 100,000 FWHM mass resolution and sub-ppm mass accuracy. The mass spectrum of a statistically significant analyte can be seen in *Figure 6,* with a mass resolution of ~70,000 FWHM. The MARS identification of another analyte of interest can be seen in *Figure 7.*

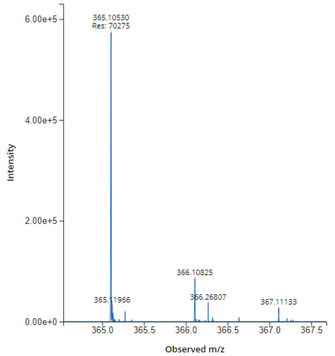

Figure 6. A mass spectrum showing the isotope cluster of 3-b-Galactopyransyl glucose with a ~70,000 FWHM mass resolution. A Fatty Acyl Glycoside found expressed ~50x in the MBVs compared to EVs.

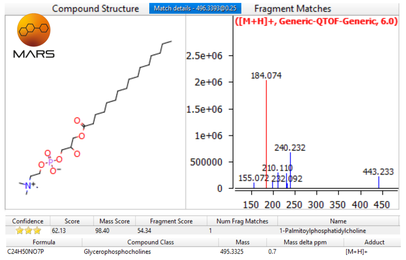

Figure 7. Example identification from MARS showing the match for 1-Palmitoylphosphatidylcholine (LPC 16:0), a phospholipid seen to be ~5x upregulated within the EVs compared to the MBVs.

DISCUSSION

From this limited feasibility dataset we were able to observe a difference in the expression of several compounds between the EV and MBV samples. There are considerably more metabolites which appear upregulated in the EVs compared to the MBVs, with ~80% of the significant differentiating features being more highly expressed by the EVs.

The most significant variation between the two vesicle types were seen within fatty acyls where fold changes of up to 50x were observed between the MBV and EV samples, *e.g.* 3-b-Galactopyransyl glucose which is believed to be a membrane stabilizer.

A second group of analytes with significantly different profiles observed between the EVs and MBVs are phospholipids, these are believed to play a role in mineralization[4] therefore this is of particular interest.

CONCLUSION

- **A comparative metabolic profile of EVs vs MBVs was performed differential compounds putatively identified within this feasibility study were predominantly fatty acyls and phospholipids.**
- **The high mass resolution of the Xevo MRT MS can increase the number of detected compounds by separating species with closely matching *m/z* values.**
- **The high mass accuracy reduces the number of false database hits and provides confidence in putative identifications for OMIC applications.**

References

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