Differential detection of cancer-derived extracellular vesicles using combined antibody functionalized magnetic beads and infrared spectroscopy

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INTRODUCTION

- Extracellular vesicles (EVs) paly a pivotal role in cellular functions including signaling pathways involved in the maintenance of normal physiological processes (1).
- EV surface antigens and molecular cargoes are altered in response to challenges to cellular homoeostasis and may contribute to disease pathogenesis (2).
- Rapid and simple methods for isolating enriched populations of EVs and characterizing their cell-specific biomarkers and molecular cargo are requisite to realizing their diagnostic potential.
- Non-destructive label-free vibrational spectroscopies definitively inform about the relative concentrations of biomolecular classes and, in some cases, specific biomolecules.
- Several advantages over current "omics" technologies, i.e., ability to assess relative concentrations of classes of biomolecules, e.g., lipids, proteins, DNA, RNA, etc., and to analyze the content of individual EVs and identify conformational changes within these biomolecules.
- FTIR has been successfully used to differentiate between case and control clinical samples (3-5) and *in vitro* cell phenotypes (6-8).

STUDY AIMS

- To compare the FTIR spectral signatures of EVs released from different cancer cell lines and phenotypes, and isolated by differential centrifugation or bead-based immunoaffinity capture.
- To model the future detection,in plasma from cancer patients, EV were isolated from the cell-conditioned of two different cancer cell lines: the human epithelial carcinoma A549; and the SV-40 immortalized mesothelial cell line MeT-5A. To model the inflammatory state of tumor micro-environment, the effects of IFN-ɣ stimulation of A549 EVs was determined.

METHODS

- ***Cell Culture:*** Lung cancer cell line, A549, and the SV40-immortalized mesothelial cell line, MeT-5A, were seeded at 1x10^4 cells/mL in tissue culture dishes and cultured for 4 d in RPMI-1640 with 10% fetal bovine serum. Cell-conditioned medium was collected and stored at -80^0C until analyzed. A549 cells were further incubated with IFN-ɣ (100 ng/mL) for 48 h.
- ***EV Isolation by Immunoaffinity Isolation****:* EVs were isolated from cell-conditioned medium (500 µL) using immunoaffinity magnetic bead capture (50 µL EXO-NET®, INOVIQ Ltd, Figure 1).

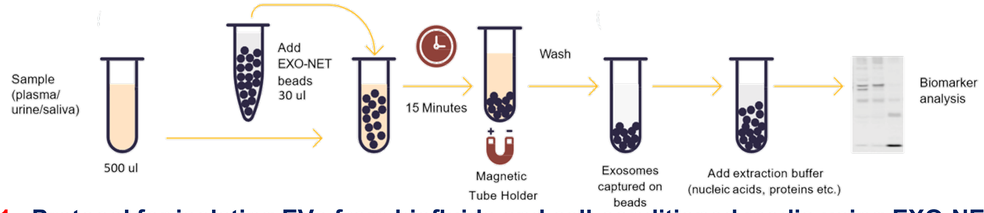

Fig 1. Protocol for isolating EVs from biofluids and cell-conditioned media using EXO-NET®

- ***EV Isolation by Differential Centrifugation of Medium***: 2,000 g for 10 min, 10,000 g for 45 min, 18,000 g for 45 mins and then 100,000 g for 1 h at 4°C in 30% sucrose-deuterium oxide (D_2O).
- ***FTIR Spectroscopy:*** Mapping of air-dried aliquots (5 µL) on a CaF_2 window (0.5 x 0.5 cm) used a Bruker Lumos FTIR microscope (transmission mode; 20 x 4 transverse maps.
- Spectra from whole dried aliquots on CaF_2 used the Bruker Alpha: aliquots (5 µL) with a Platinum ATR module over the region of 4000-400 cm^{-1} (attenuated total reflection mode with the coaddition of either 64 or 256 scans.
- Alternatively, spectra from whole air-dried aliquots (1 µL) deposited on a Si 96-well plate were measured on a Bruker Tensor HTS-XT instrument
- Spectra compensated for atmospheric moisture and CO_2 interference, vector-normalized to the 1800-1050 cm^{-1} region and subjected to smoothing using the Savitsky-Golay algorithm with 13 smoothing points (Bruker OPUS software).
- Further smoothing with nine smoothing points of second derivatives (Savitsky-Golay algorithm, The Unscrambler X software), then Principal Component Analysis (PCA) was applied.

RESULTS

- ***Method of EV Isolation:*** EVs isolated using a differential centrifugation contained a residual concentration of sucrose that absorbed intensely in the IR region and overwhelmed the detector and signal from the extracellular vesicles, which prevented meaningful analysis.
- Samples isolated using EXO-NET were successfully analyzed using IR spectroscopy Lumos with individual points across a dried sample (Fig 2) or averaged spectra for all points across two separate samples (Fig. 3.)
- Subsequent studies, therefore, were performed using EXO-NET isolated EVs.

- ***Cell and Phenotypic Spectral Signatures: S***pectra from whole dried spot were measured using the Tensor instrument, which operates with 96 well-plates, (Fig. 4), or using the ATR mode using an inexpensive Bruker Alpha (Fig. 5), EV from both cell lines and unstimulated vs stimulated A549 cells were clearly differentiated from one another and the beads alone.
- Distinct clustering of the spectra by sample type indicated that the different varieties of cell-derived exosomes were differentiated using several vibrational spectroscopic methods.

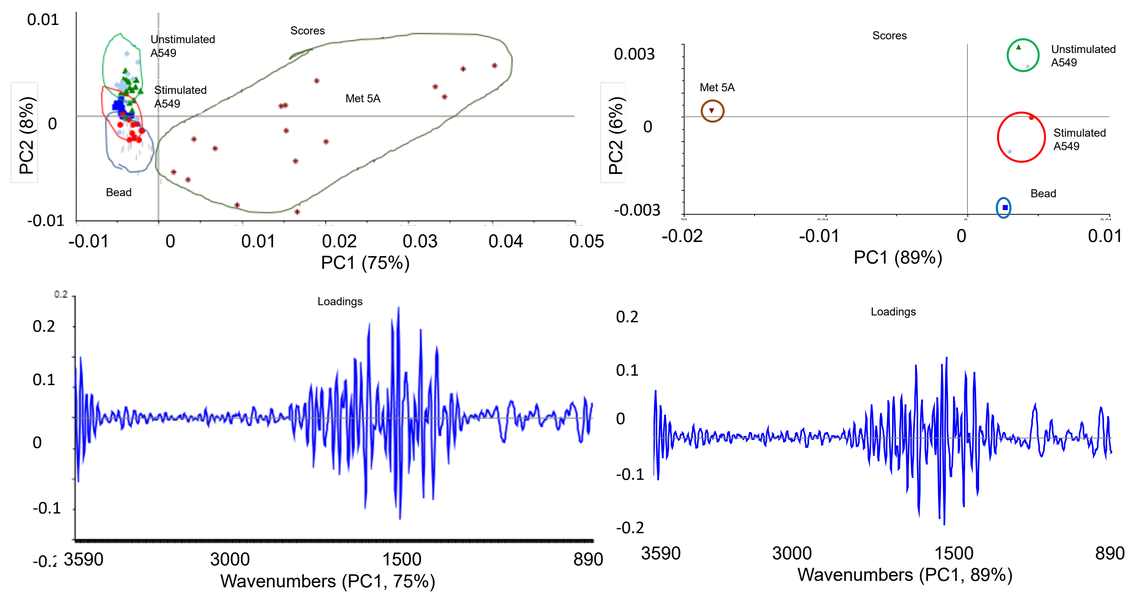

Fig 2. PCA scores and loadings plots of second derivative spectra collected from unloaded beads and beads loaded with exosomes; stimulated A549 cells **(1)**, unstimulated A549 cells **(2)** and MeT-5A cells **(4)**.

Fig 3. PCA scores and loadings plots of the average spectra collected from beads loaded with the three cell varieties and unloaded beads, showing distinct grouping by cell variety.

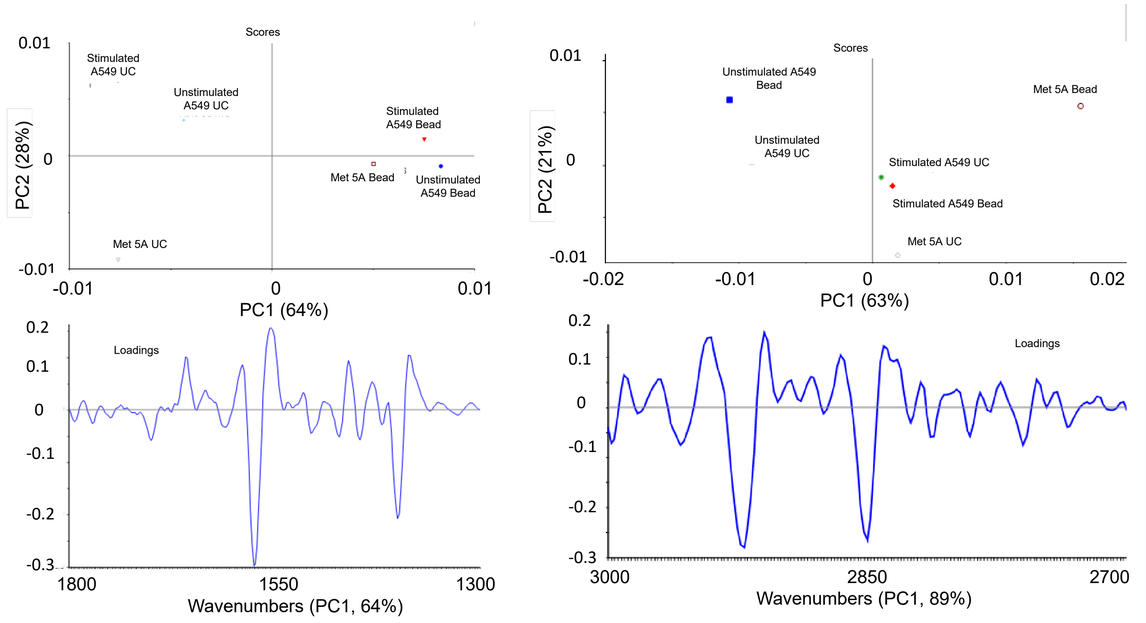

Fig 4. PCA scores (top) and loadings (PC1, bottom) plots for second derivative spectra collected on the Bruker Tensor (1800-1300 cm^{-1}). Spectra are separated by isolation method of the relevant EVs along PC1.

Fig 5. PCA scores (top) and loadings (PC1, bottom) plots for second derivative spectra collected on the Bruker ALPHA, over 3000-2700 cm^{-1} range. The MeT-5A and IFN-γ stimulated A549 cells are separated along PC1 from the regular A549 samples.

CONCLUSIONS

1. Bead-based immunoaffinity capture (EXO-NET) represents a simple and rapid method for preparing enriched subpopulations of EVs for direct, on-bead FTIR spectroscopic analysis.
2. On-bead analysis using vibrational spectroscopy provides a rapid and simple method for determining the relative changes in EV biomolecular contents from different cancer cell types.
3. Distinct clustering of the spectra by sample type occurred, indicating that the different subpopulations of cell-derived exosomes could be differentiated using this vibrational spectroscopic method.
4. Data obtained support the hypothesis that on-bead FTIR analysis of EV differentiates cancer cell type from position in PC1 and the phenotype from position in PC2. Both parameters are determinants of disease classification accuracy and triage to treatment.

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