Exosomes Purification Strategies for New Biomarkers Discovery in Cancer

Federica Anastasi[1,2,*], Marco Cecchini[1], Liam A. McDonnell[2,#]

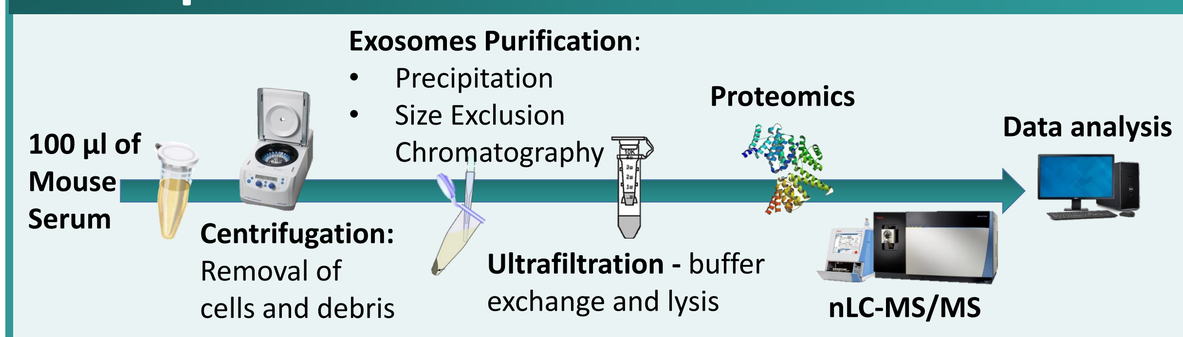
email: * federica.anastasi@sns.it # l.a.mcdonnell@outlook.com

[1] NEST, Scuola Normale Superiore, Pisa, Italy [2] Fondazione Pisana per la Scienza – ONLUS, San Giuliano Terme, Pisa, Italy

1 - Introduction

Exosomes are cell-derived nanovesicles (30-100 nm in diameter) that are secreted from all types of cells and are present in all body fluids. Tumor derived exosomes contain molecules derived from the cancer-cells, and thus represent an opportunity for health monitoring and diagnosis [1]. Two different purification and protein analysis procedures have been developed and compared with the aim to purify exosomes from low sample amount and to study the correlation between exosome cargo and cancer progression.

2 - Experimental Workflow



3 - Materials and Methods

Sample preparation

Exosomes have been isolated and purified from Normal Mouse Serum (Thermo), buffer exchange was performed on 3kDa filters, concentrated exosomes lysed and proteins quantified with microBCA assay. Two different purification procedures were evaluated:

- **Exosomes purification by precipitation** (Total Exosomes Isolation Kit - Invitrogen).
- **Size Exclusion Chromatography** (SEC, qEV columns - Izon).

For the proteomic analysis of SEC-purified exosomes, only the first three exosomes fractions have been processed to ensure the maximum removal of blood proteins from exosomes sample.

Lysis and digestion were performed following two different protocols because of the different amounts of isolated proteins: in solution protein digestion (1%SDC, 10mMTCEP, 100mM TRIS, 40mM CAA) for exosomes isolated by precipitation and protein digestion with SP3 protocol [2,3] for SEC-purified exosomes.

nLC-MS/MS ANALYSIS

Samples were injected into an EASY-nLC 1000 coupled to an Orbitrap Fusion (Thermo Scientific) and analysed with a data-dependent method.

Data analysis

Raw data were analysed with Proteome Discoverer 2.1 (Thermo Scientific). The identified proteins were compared with ExoCarta [4] and EVpedia [5] databases. Panther was used to perform gene ontology analysis comparing the two identified proteins lists with the GO general mus musculus database.

Dynamic Light Scattering (DLS)

DLS analysis was performed on 1 mL PBS suspension of exosomes pellet isolated by precipitation and on 1 mL PBS dilution of the three pooled pure exosomes fraction collected by SEC.

4 - Results

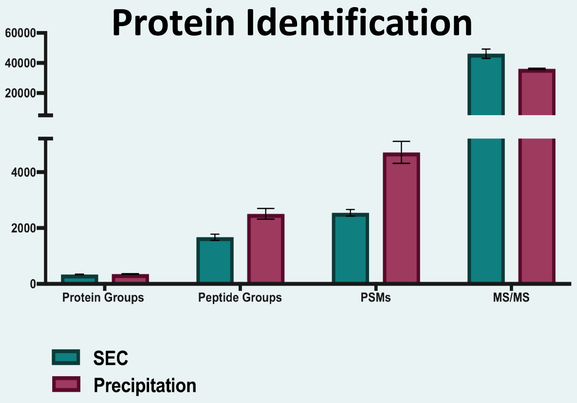
Extracted proteins*	µg
Precipitation	520
Size Exclusion Chromatography	3,5

*total protein amount in 100ul serum = 6mg

N° of identified Protein Groups *	
Precipitation	348
Size Exclusion Chromatography	330

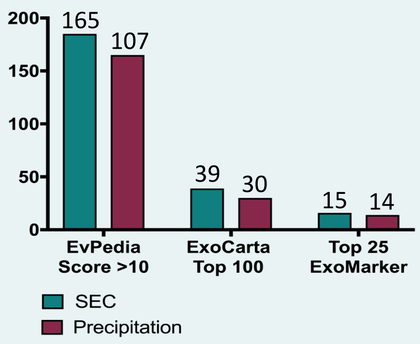
*identified with at least one unique peptide

Protein Identification



Despite the high difference in proteins amount between the two procedures the number of identified proteins are comparable.

Databases Coverage



Data comparison with ExoCarta and EVpedia exosomes databases show that identified proteins from SEC-purified exosomes have higher overlap with the two databases than identified proteins from exosomes purified by precipitation.

Proteins expected to be present in Exosomes [4]		
	Precipitation	**SEC**
Integrins	-	β1, β2, β3, α2, α4, α6, α2β
Tetraspanins	-	CD 81, CD82, CD9, CD97
Growth Factor Receptors	Eps15l1	Eps15l1
Syntenin	-	YES
Annexins	-	ANXA 5, ANXA 7

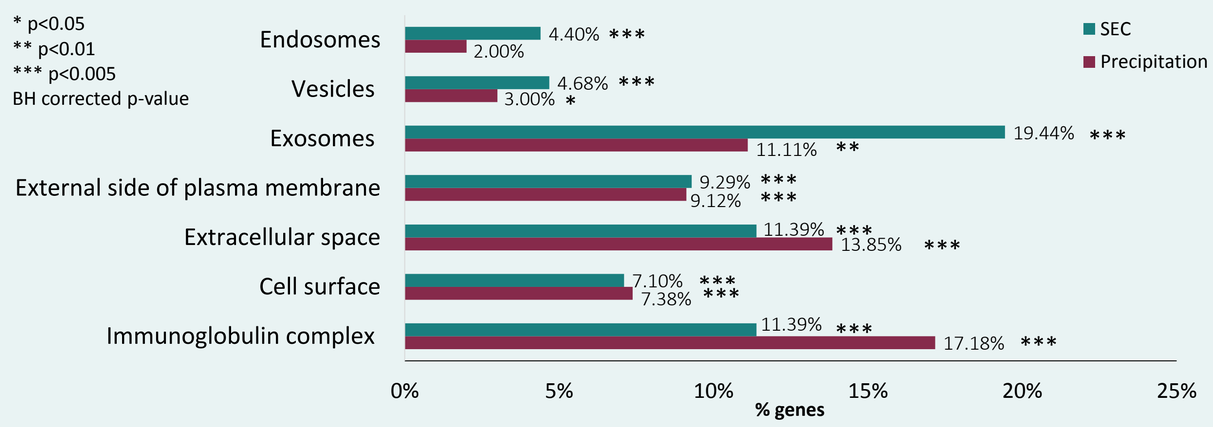
	Precipitation	**SEC**
# of identified blood proteins*	164	123

*complement system, coagulation, immunoglobulins

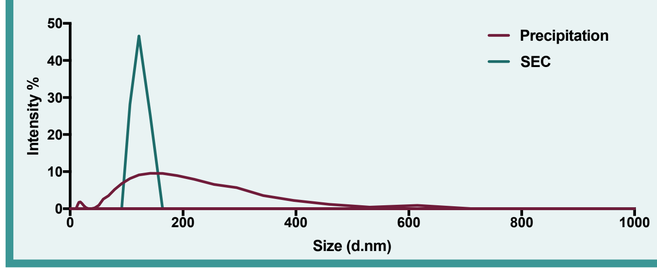
Size exclusion chromatography is able to remove higher amount of blood proteins.

Gene enrichment analysis

% of genes between identified proteins and dataset of singular cellular component in GO mus musculus database



Dynamic Light Scattering Analysis



		Precipitation	SEC
Peak 1	Size (d.nm)	18.2	122.6
	Intensity %	8 %	100%
	St.dev (d.nm)	12.4	13.15
Peak 2	Size (d.nm)	142	-
	Intensity %	91%	-
	St.dev (d.nm)	403.9	-

5 - Conclusions

Proteomics analysis of exosomes by bottom-up-LC-MS/MS have been overwhelmingly performed on exosomes purified by ultracentrifugation, which requires large sample volume and thus cannot be used to analyze the exosomes from the serum of individual mice (~100µl available from a single mouse) for studying cancer mouse models. The exosomes purification methods reported here enables the analysis of exosomes from individual mice. Size exclusion chromatography resulted to perform better in removing higher amount of blood contaminating proteins.

6 - References

[1] Lin J, Li J, Huang B, Liu J, Chen X, Chen X-M *et al*. *Scientific World Journal,* 2015.
[2] de Graaf, E.L.; Pellegrini, D.; McDonnell, L.A., *Journal of Proteome Research*, 2016.
[3] Hughes et al. *Molecular Systems Biology*, 2014.
[4] Keerthikumar, S. et al. ExoCarta, *Journal of Molecular Biology,* 2015.
[5] Kim DK et al. EVpedia, *Bioinformatics*, 2015.
[6] Lötvall J, Hill AF, Hochberg F, et al, *Journal of Extracellular Vesicles,* 2014.