Optimization and characterization of low vacuum filtration procedure - novel method for the isolation of extracellular vesicles

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Introduction

In recent years, many new extracellular vesicles (EVs) isolation methods have been developed. Unfortunately, EVs isolation from high volume sources such as cell culture media or urine still remains challenging. We created and validated a new EVs isolation system dedicated for high volume sources and characterized isolated EVs in terms of their morphology, size, concentration and presence of EVs protein markers.

Aim

The aim of the study was to compare efficiency and quality of EVs isolation using low vacuum filtration (LVF) method with two most commonly used EVs isolation procedures: differential centrifugation and ultracentrifugation.

Methods

Analyzes were performed on EVs isolated from Human Umbilical Vein Endothelial Cells (HUVECs). Three different methods of EVs isolation were applied for the analysis (Fig.2.).

NTA

For NTA analysis, samples were diluted to the volume of 700 µl with filtered PBS. All measurements were performed in triplicates, for 30s and five independent records were collected for each sample. The measurements were analyzed using NTA 3.1. software and normalized to the starting sample and the applied dilution.

FTIR

For FTIR spectroscopy, 5 µl of sample in PBS was evaporated on the diamond crystal to obtain a thin film. The measurements were performed at room temperature, immediately after drying the sample (within approximately 5 min). Scans were performed at a nominal resolution of 4 cm^{-1}. The spectra were composed of 256 scans. Data processing and statistic analyzes were performed by means of OriginPro 2018 software.

Western blot

Proteins extracts (15 µg of proteins) from EVs isolated using different methods were separated by SDS-PAGE electrophoresis using stain-free 4-20% gradient gel and transferred to PVDF membranes. The blots were blocked overnight and incubated for 1h with primary antibodies for VCAM (marker for endothelial cells), Hsp70 (marker for exosomes) and ARF (marker for ectosomes). Membranes were then incubated for 1h with an appropriate HRP-conjugated secondary antibody. Immunopositive bands were visualized using Lumi-light Reagent (Roche) and ChemiDoc™ XRS+System). Individual protein levels were normalized to the total intensity of bands on a given line detected in gel after electrophoresis.

LVF system characteristic

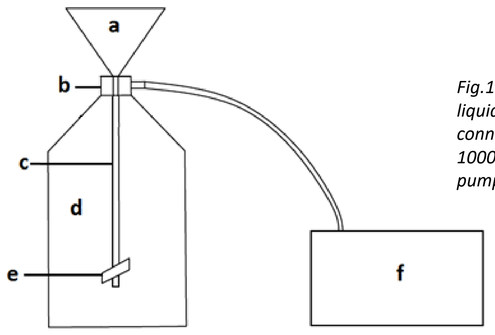


Fig.1. Low vacuum filtration system: a) Closed liquid container, b) Coupler - element connecting the dialysis membrane – MCOW 1000 kDa (c), the vacuum chamber (d), the pump (f) and closed liquid container (a).

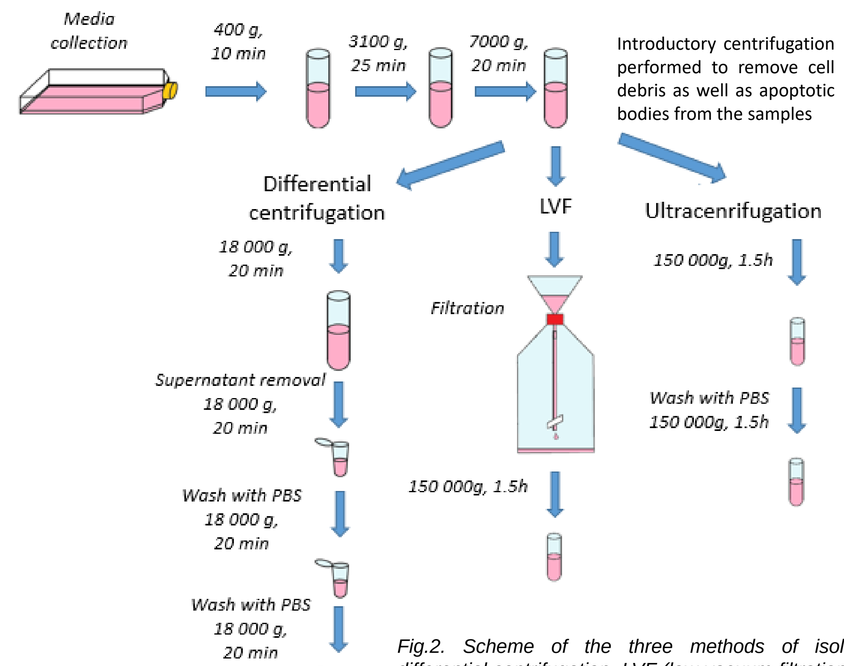


Fig.2. Scheme of the three methods of isolation: differential centrifugation, LVF (low vacuum filtration) and ultrafiltration.

Results

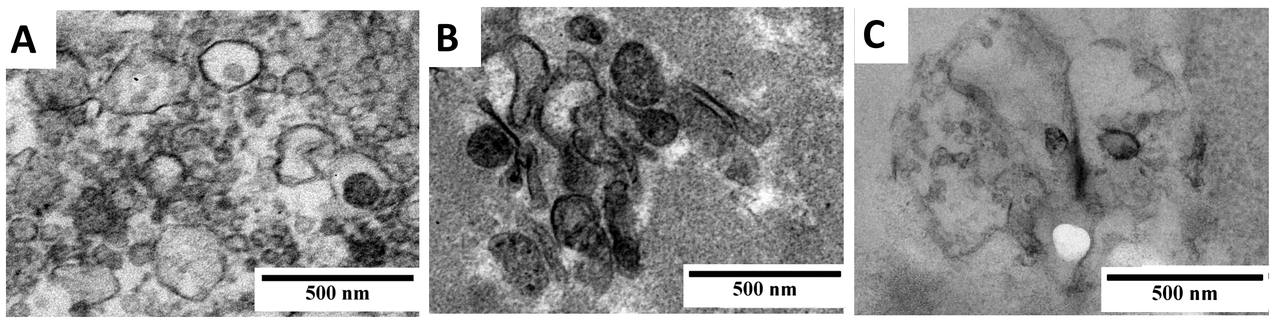
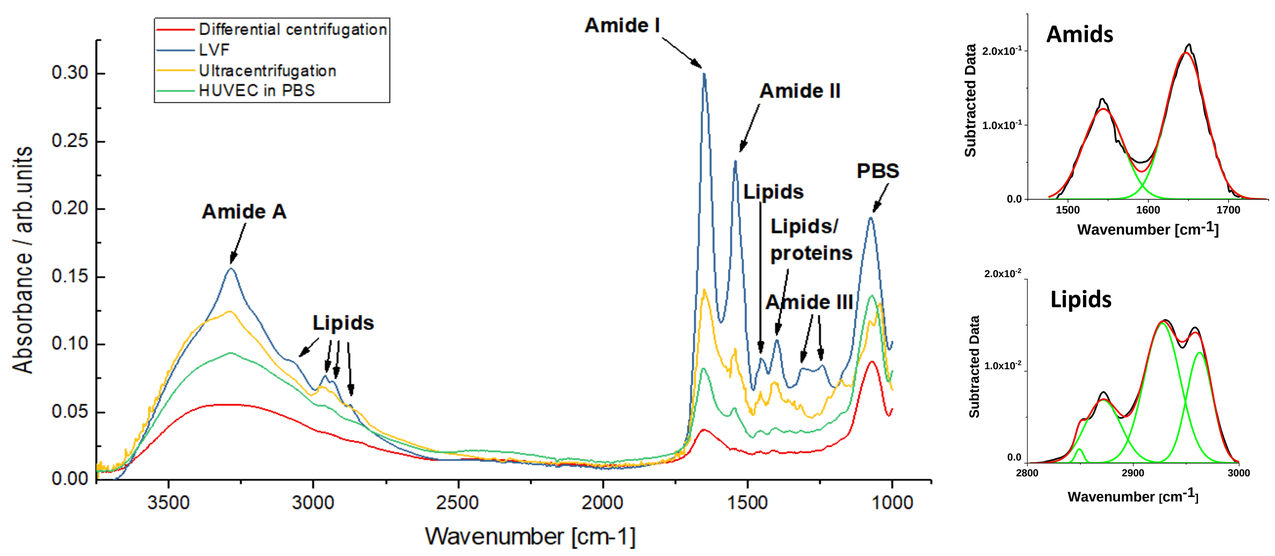


Fig.3. Transmission Electron Microscopy (TEM) images of EVs isolated by: A – differential centrifugation, B- LVF, C- ultracentrifugation.



$$\frac{amid\ I}{lipid} = \frac{area\ under\ the\ peak\ of\ amide\ I}{sume\ of\ area\ under\ lipids\ peaks}$$

	Amide I area	Lipids area	Amide I to lipid
Differential centrifugation	too poor quality to calculate	too poor quality to calculate	too poor quality to calculate
LVF	12.91	0.315	10.22
Ultracentrifugation	5.69	0.22	6.31

Fig.5. FTIR spectra of EVs analyzed using compared methods: ultracentrifugation, differential centrifugation and LVF. Additionally, the spectrum from HUVEC cells is presented on the graph. To assess the quality of samples, amide to lipid ratios were calculated.

Wavenumber [cm^{-1}]	Definition of the spectra assignment
3286	Overlapped –OH stretching vibrations and N-H stretching vibrations from the peptide groups of proteins (amide A)
3076, 2959	CH_3 asymmetric stretch from lipids with low contribution from proteins, carbohydrates, nucleic acids
2930, 2869	CH_2 asymmetric and symmetric stretching vibration from lipids with low contribution from proteins, carbohydrates, nucleic acids
1652	C=O stretching vibrations from the peptide backbone (amide I)
1542	N-H bending vibrations from the peptide groups (amide II)
1450	CH_2 bending (scissoring) vibrations from lipid acyl
1397	CH_3 bending vibrations from lipids and proteins
1309, 1240	C-N stretching mode of proteins, indicating mainly α-helix conformation (amide III)

Tab.3. Interpretation of the peaks in FTIR spectra.

Method	Concentration [EVs/ml]	Sample condensation	Mean size [nm]	Size mode [nm]
Differential centrifugation	$4.74*10^9 \pm 3.91*10^7$	13x	255±142	167
LVF	$7.96*10^9 \pm 5.82*10^7$	22x	260±132	184
Ultracentrifugation	$1.71*10^{10} \pm 1.23*10^8$	35x	224 ±112	170

Tab.1. Results of NTA analysis, normalized to the undiluted samples. Additionally, the sample condensation, in reference to the particles concentration in sample after the initial steps of purification is presented.

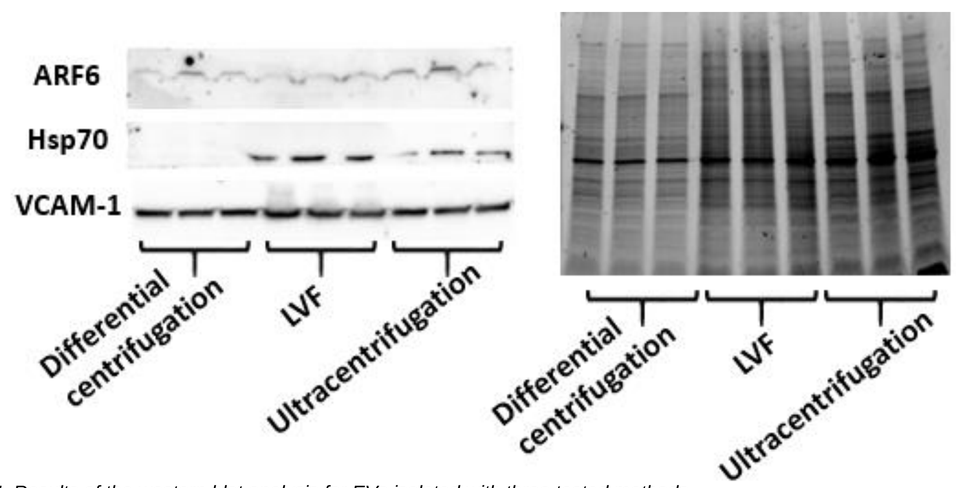


Fig.4. Results of the western blot analysis for EVs isolated with three tested methods.

	Protein concentration [mg/ml]	VCAM-1 band intensity [AU]	Hsp70 band intensity [AU]	Afr-6 band intensity [AU]
Differential centrifugation	2.41	1.50	0.04	0.07
LVF	2.40	1.38	0.48	0.05
Ultracentrifugation	3.73	0.89	0.23	0.07

Tab.3. Results of the western blots analysis. Individual protein levels were normalized to the total intensity of bands on a given line detected in the gel after electrophoresis.

Summary

- The yield for both ultracentrifugation and the LVF method is comparable.
- LVF is the most efficient method for isolation of exosomes and ectosomes fractions which can be separated in additional isolation steps.
- Isolation of EVs by ultracentrifugation method does not allow for isolation of a clear population of exosomes, contamination by ectosomes fractions is high.
- The quality of FTIR spectra for the LVF isolation method is very good
- FTIR analysis could be used to predict the sample content and quality

Acknowledgements

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