MORE THAN MEETS THE EYE: EXTRACELLULAR VESICLE CHARACTERIZATION VIA NANO ES DIFFERENTIAL MOBILITY ANALYSIS, NANOPARTICLE TRACKING ANALYSIS AND MS/MS REVEALS CO-ISOLATED PROTEINS

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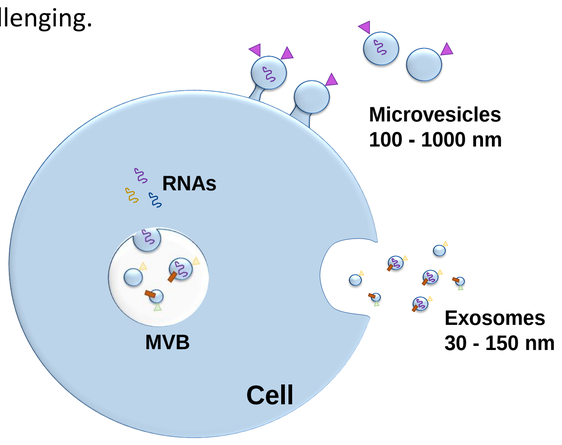
Introduction

Extracellular vesicles (EVs), released by cells and consisting mainly of a lipid bilayer and an aqueous lumen, are in demand due to the presence of biomarkers and as therapeutic platforms. They play a significant role in cellular processes from cargo transport to cell-to-cell communication. Their heterogeneity makes their characterization highly challenging.

We analyzed vesicles via gas-phase electrophoresis on a nano Electrospray Differential Mobility Analyzer (nES-DMA) aka nES Gas-phase Electrophoretic Mobility Molecular Analyzer (nES-GEMMA)[1], determining their surface-dry particle diameter with particle-number based detection. Gas-phase electrophoresis enables the detection of smaller analytes (proteins) next to larger ones (EVs) yielding information on sample constitution and purity. Nanoparticle tracking analysis (NTA) and proteome analysis corroborated our results.



Methods

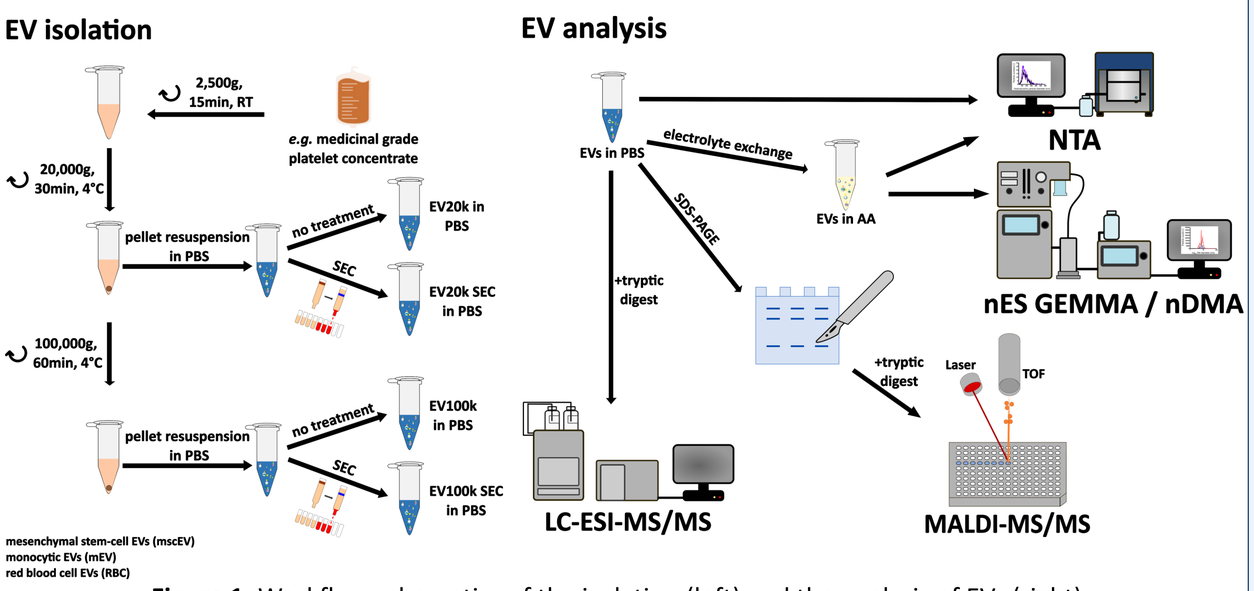


Figure 1: Workflow schematics of the isolation (left) and the analysis of EVs (right).

EV samples

- purified from human blood via ultracentrifugation[2]
- size exclusion chromatography (SEC) → Annexin 5+ fractions pooled
- exchange from phosphate buffered saline (PBS) to 40 mM ammonium acetate (AA), pH 8.4, with centrifugal filters (10 kDa MWCO, polyethersulfone membrane)

NTA

- ZetaView PMX 120 (ParticleMetrix, Meerbusch, Germany)

nES GEMMA

- TSI Inc. Instrument (Shoreview, MN, USA)
  - nES Aerosol generator (Model 3080C) + Corona-discharge, nDMA (Model 3480C), n-Butanol based ultrafine CPC (Model 3776C)
  - Sheath flow rate 8.00 lpm, Macrolon 1.1 lpm (Air 1.0 lpm, CO_2 0.1 lpm), capillary ID 25 µm, 3.0-91.6 nm range

SDS-PAGE

- 4-12% Bis-Tris gel, MS-compatible silver staining according to Shevchenko[2]
- Tryptic in-gel digestion overnight @37°C, ZipTip (C_{18}) purification of digest, elution with matrix on target

MALDI-TOF/RTOF

- UltrafleXtreme (Bruker Daltonics, Bremen, Germany)
- Matrix: 3 mg/mL α-CHCA in Acetonitrile/0.1% TFA (60:40)
- PMF: positive reflectron mode, 40% laser power, m/z 500 – 3500, 8,000 shots, 1 kHz
- Fragmentation: LIFT mode, 12,000 shots

Database search via Mascot search engine (v.2.4.0)

- NCBI protein database: homo sapiens (152,462,470 proteins, 327,411 sequences; Feb 2019)
- Modifications: carbamidomethylation (C), acetylation (protein N-term), oxidation (M), phosphorylation (ST), deamidation (NQ)
- MC 2, mass tolerance: ± 0.3 Da, fragment tolerance: ± 0.5 Da
- Identification criteria: ≥ 2 unique peptides per protein, 5% FDR

LC-ESI-MS/MS

- timsTOF (Bruker Daltonics, Bremen, Germany)
- 100 ms cycle time, scan range of 100-1700 m/z in PASEF mode, source capillary voltage at 1500 V, dry gas flow to 3 L/min at 180°C
- Data analysis via Max Quant Andromeda search
  - Modifications: carbamidomethylation (C), acetylation (protein N-term), oxidation (M)
  - Identification criteria: ≥ 2 unique and razor peptides per protein, 1% PSM, 5% FDR

Conclusion

- ✓ **nES GEMMA suitable for quality assessment of EV-containing samples**
- ✓ **nES GEMMA reveals high amounts of co-purified proteins in EV-containing samples**
- ✓ **Proteinaceous contaminants can be depleted with an additional SEC purification**
- ✓ **SEC has no influence on the proteome of EVs as demonstrated via LC-ESI-MS/MS**

Results

Nanoparticle tracking analysis

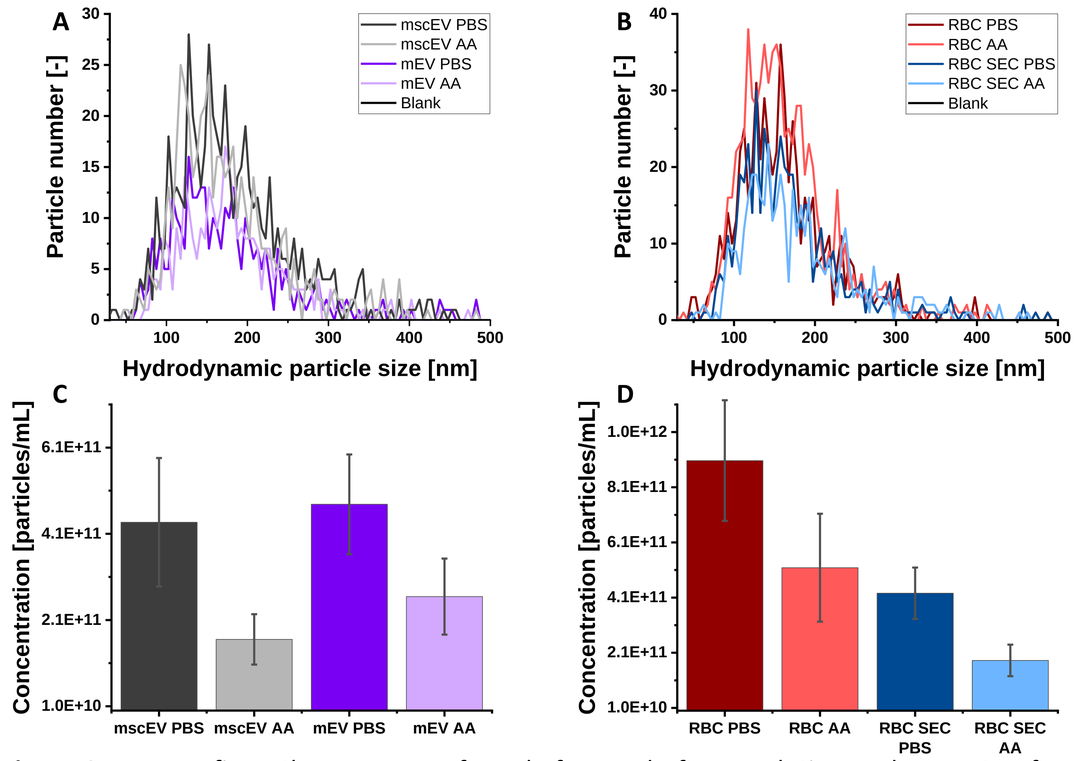


Figure 2: NTA confirms the presence of EVs before and after a solution exchange step from PBS to volatile AA, a necessary prerequisite for nES-GEMMA, (A, B) as well as the SEC purification step (B). Nonetheless, the size distribution is not affected. Even though it does not influence the size distribution of vesicles, solution exchange leads to significant vesicle loss (> 40%) (C,D).

nES GEMMA analysis

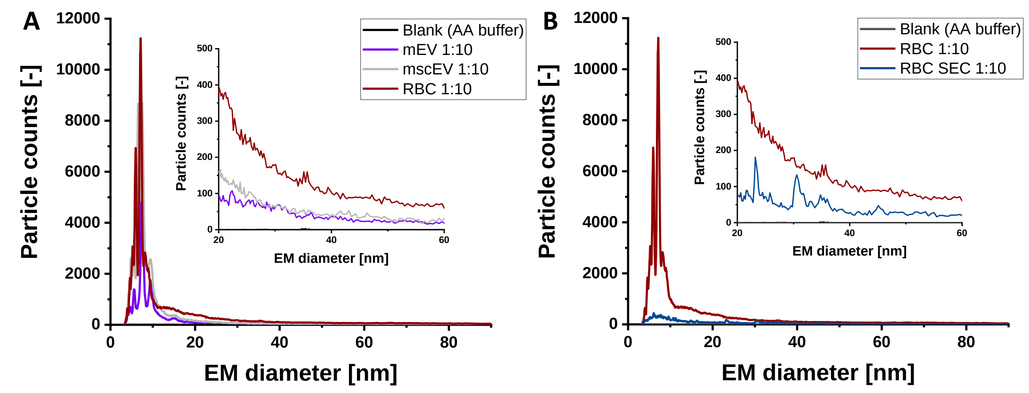


Figure 3: nES GEMMA of EV containing samples reveals vesicle size heterogeneity (from around 20 nm and tailing off to higher EM diameters). Mostly co-isolated proteins are detected in the lower EM diameter range (≤ 20 nm) (A). These exceed vesicle numbers by far. An additional SEC step depletes these contaminants (B).

Identification of co-isolated proteins

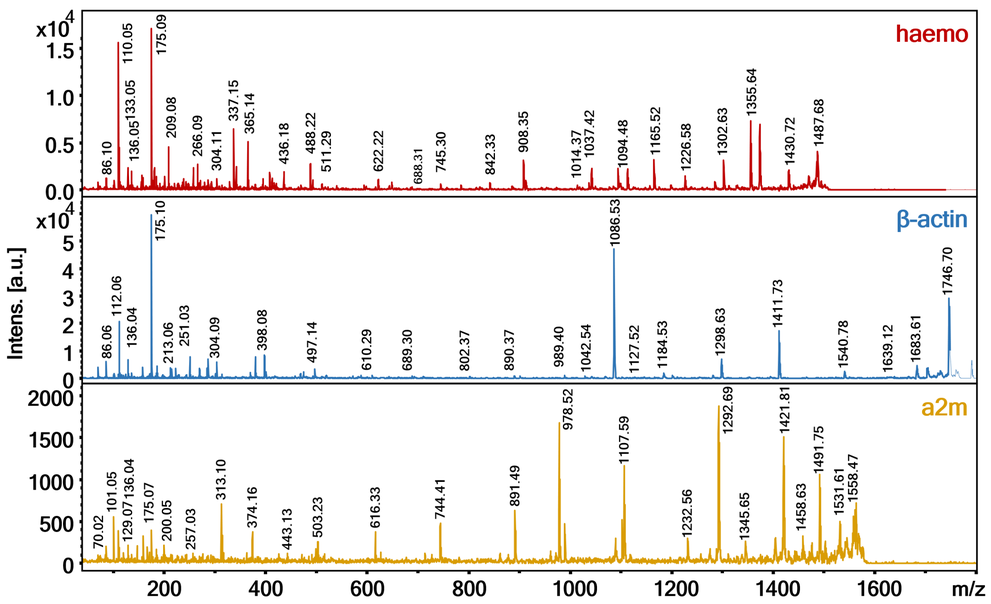


Figure 4: Main co-isolated proteins, haemoglobin (haemo), β-actin(-like-protein) and α-2-macroglobulin (a2m) were identified based on SDS-PAGE, tryptic in gel digest and MALDI MS/MS.[4]

nES GEMMA of identified proteins

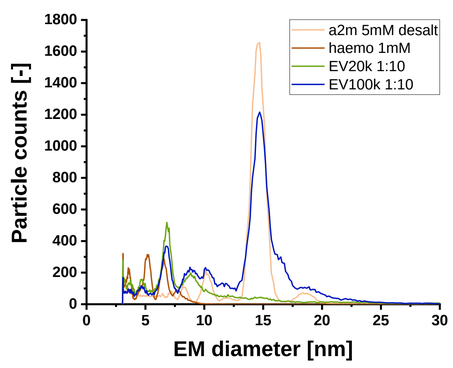


Figure 5: nES GEMMA of EVs and identified proteinaceous contaminants (haemo, a2m) overlap in nm-sizes and pattern.

Proteome analysis of SEC influence

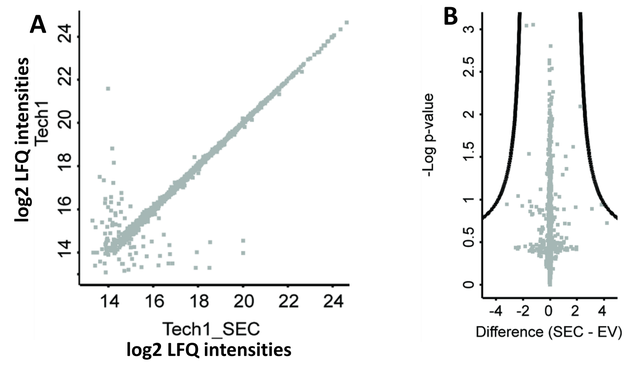


Figure 6: LC-ESI-MS/MS analysis of a technical replicate of samples before and after SEC (A) reveals similar protein expression. No significant difference in expression (B) demonstrates that SEC purification does not influence the vesicle proteome itself, thus applicable for EV isolation.

References
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