Extracellular Vesicles and How to Find Them

A script based, quantitative analysis of Cryo–TEM pictures in comparison to NTA measurements of EVs

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INTRODUCTION

Extracellular vesicles (EVs) are membrane-bound structures that play a crucial role in intercellular communication and molecular transport. Because of these natural functions, EVs hold a great potential as tools in anticancer therapies. Elevated glucose concentrations affect cellular metabolism, thereby modifying composition of the EVs [1] and their lipid bilayer [2]. This in turn can influence EVs behaviour and morphology, which is important in their understanding. **Hypothesis:** Hyperglycemic conditions, by altering the composition and metabolism of EVs, influence their size distribution and physicochemical properties. To investigate this, two complementary methods based on different physical principles were applied: **Nanoparticle Tracking Analysis (NTA)** and **Cryo-Transmission Electron Microscopy (Cryo-TEM)**. In this study, 1.1B4 cells were cultured in normo- (NG; 5 mM glucose) and hyperglycemic (HG; 25 mM glucose) conditions. **Aim:** Visualisation and size distribution comparison between NG and HG conditions.

METHODS

NTA

Nanoparticle tracking analysis was performed using a **NanoSight NS300** instrument equipped with a 532 nm green laser. Samples were diluted 100-fold in phosphate-buffered saline (PBS) to obtain a particle concentration of approximately 20–100 particles per field of view. All solvents were pre-filtered through a 0.45 µm filter. A blank measurement was recorded before sample loading. Each sample was measured in 9 replicates, and the resulting data were averaged for analysis.

Cryo-TEM

Measurements were conducted at NSRC SOLARIS UJ. As a sample carrier, copper meshes with formvar-carbon film (QUANTIFOIL R2/1 grid: Cu 200) were used (**Fig 2. a**). Meshes were cleaned and functionalised in plasma. After that, they were put inside the Vitribot (**Fig 2. b**) and 5 µL of the sample was put onto the hydrophilic side of the mesh and immediately lowered into the liquid ethane. Following the vitrification, meshes were put into plastic carriers, encased in copper rings (**Fig 2. c**) and kept in the liquid nitrogen.

Picture analysis

To obtain size distribution from cryo-TEM images, a custom Python script was developed with the assistance of ChatGPT (https://chatgpt.com/). The script measured vesicle diameters based on hand-marked lines (**Fig 1. a**). A verification function overlaid green perimeters on detected lines, enabling identification of undetected or incorrectly measured features (**Fig 1. b**). Accuracy was validated against a representative image. Out of 1791 measurements in total, 5 lines (0.28%) were not detected and 4 (0.22%) were misidentified, these were manually corrected.

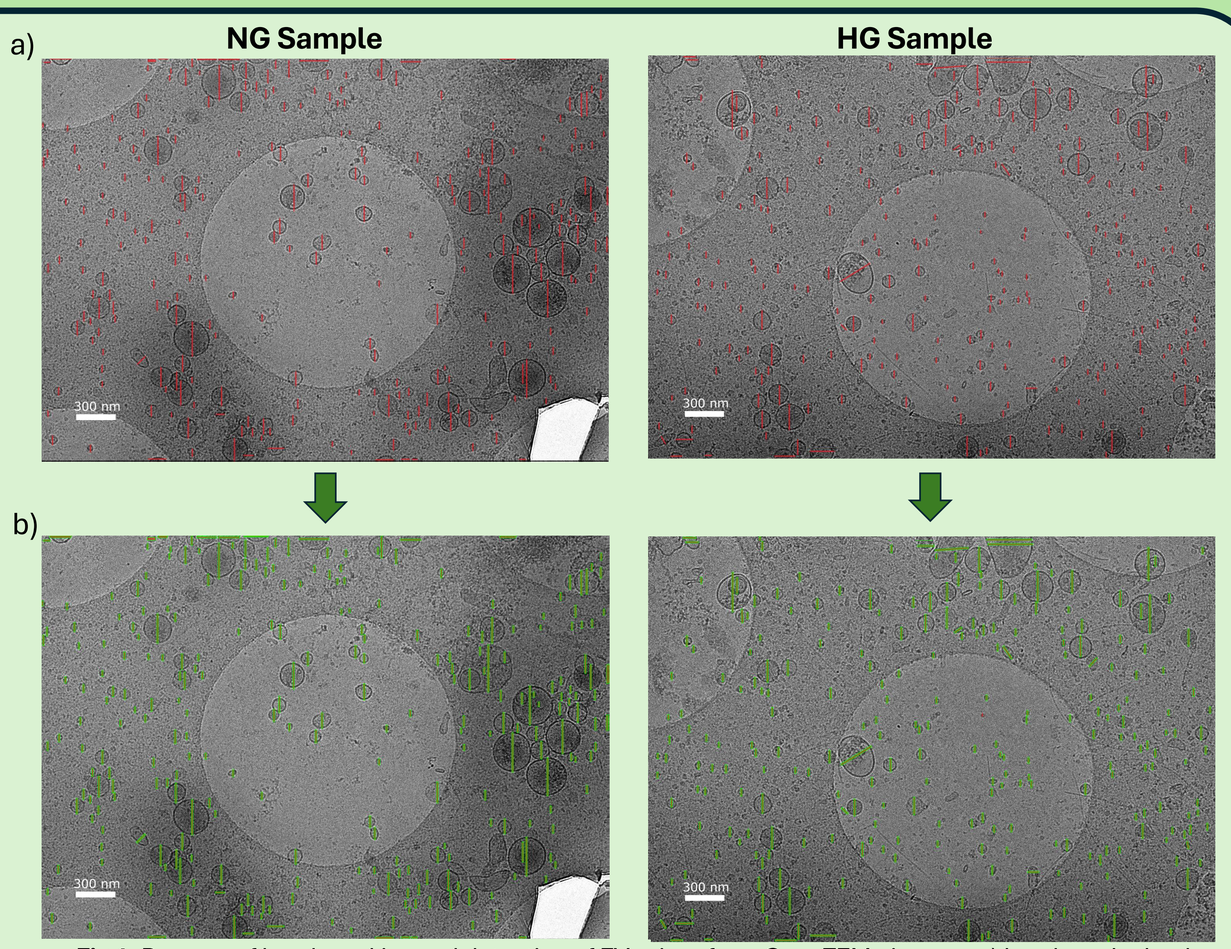

Fig 1. Process of hand-marking and detection of EVs sizes from Cryo-TEM pictures: a) hand-marked red lines in graphic software, b) overlaid green perimeters confirming proper identification

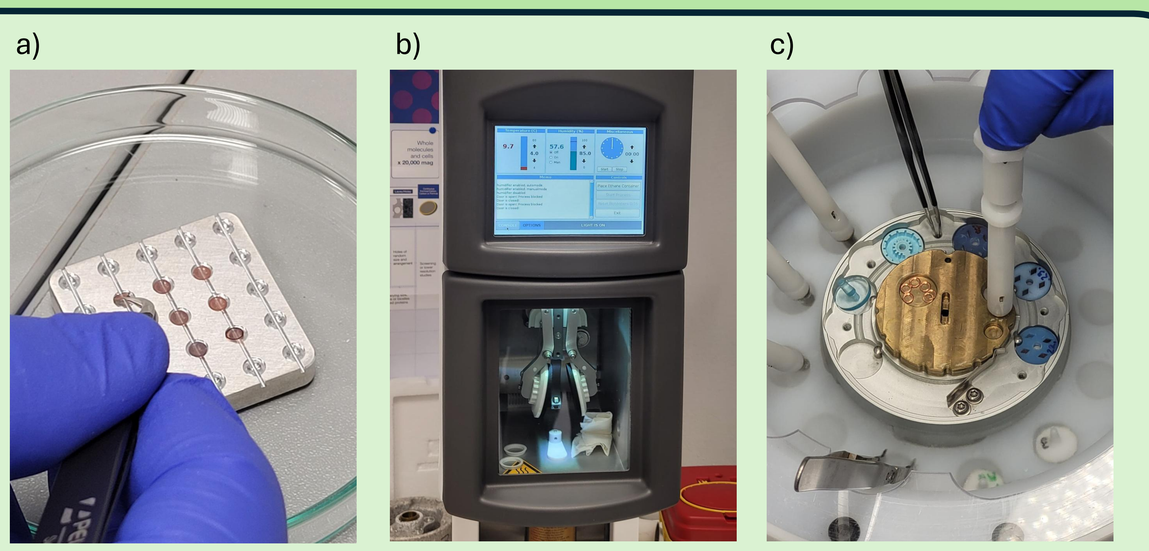

Fig 2. EVs sample preparation for Cryo-TEM measurements: a) copper meshes, b) semi-automated vitrification system Vitribot Thermofisher, c) process of stiffening the meshes.

RESULTS AND DISCUSSION

There is a clear difference between the histograms obtained from Cryo-TEM and NTA (**Fig 3. a,b**), which arises from their distinct measurement principles. Cryo-TEM produces ”images” of EVs by exposing them to an electron beam, whereas NTA illuminates the particles with a laser, detects the scattered light, and tracks their Brownian motion. Because of that, as the NTA result we see a hydrodynamic radius which is far larger than the factual one and depends on the structure of the EV corona. Substracting the two radii can give us insight into the size of the corona.

Another important observation is the similarity in histogram shapes. This suggests that both methods are complementary and with appropriate assumptions, can be considered valid independently. It is also worth noting the small standard deviations (**Fig 3. c**), which indicate that both methods provide precise measurements.

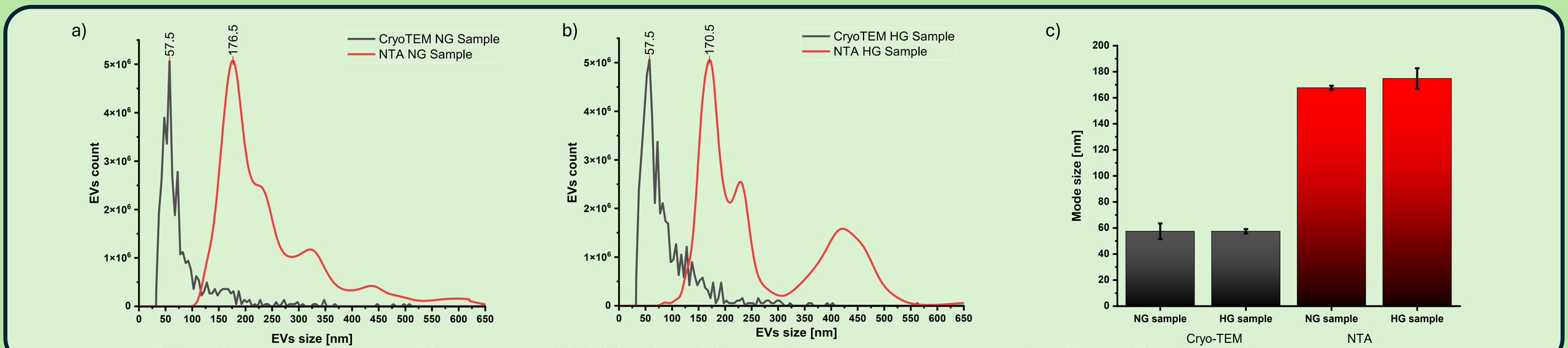

Fig 3. a,b) Superimposed histograms of EVs sizes respectievly for NG Sample and HG sample; c) mode sizes comparision between Cryo-TEM and NTA witd imposed standard deviations

CONCLUSIONS

- The factual size of most EVs was consistent, averaging around 57.5 nm, and was not affected by glucose concentration in the cell medium.
- The hydrodynamic radius of 1.1B4 EVs was estimated to be approximately 113 nm, independent of experimental conditions.
- The script-based approach to size distribution analysis proved highly precise, offering low standard deviations as well as rapid detection and efficient data management.
- Manual hand-marking of the EVs did not compromise the quality of the results.
- Cryo-TEM in comparision with NTA is a more precise technique for size distribution analysis, whereas NTA provides a rapid, cost-effective, and reliable assessment of hydrodynamic radii.

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References

[1] Rząca C, Jankowska U, Stępień E: Proteomic profiling of exosomes derived from pancreatic beta-cells cultured under hyperglycemia. Bio-Algorithms and Med-Systems 2022; 18(1): 151-157. https://doi.org/10.2478/bioal-2022-0085.

[2] Skalska ME, Durak-Kozica M, Stępień EŁ. ToF-SIMS revealing sphingolipids composition in extracellular vesicles and paternal β-cells after persistent hyperglycemia. Talanta. 2025 Jul 15;297(Pt A):128582. doi: 10.1016/j.talanta.2025.128582. Epub ahead of print. PMID: 40684740.