Optimized set-up for purification of extracellular vesicles from human induced pluripotent stem cells

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Introduction to extracellular vesicles

Extracellular vesicles (EVs) are cell-secreted nanoparticles that are primarily involved in intercellular communication processes. In the past few years, EVs have gathered increasing interest by crossing several biological barriers, such as the cell membrane and blood-brain barrier, and they are involved in many pathological processes. For these reasons, EVs have promising applications as drug targets, therapeutic agents, and delivery scaffolds, becoming a multidisciplinary field of research still predominantly in the preclinical and discovery phases. One of the crucial challenges for the use of EVs for scientific and medical applications is isolating them from other fluid components using ultracentrifugation, density gradients, and size exclusion chromatography. However, these methods can be limited by input volume and EVs yield. For this reason, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico and Cytiva combined their respective expertise to develop a robust process for the optimization stage of EVs production from adherent human induced pluripotent stem cells (hiPSCs), which represent a promising cell source for tissue replacement.

Proposed process for EV purification

We identified one approach in the manufacture of EVs that can provide advantages in terms of yield and labor-effectiveness. For this study and following the customer demands, the process sequence reported in Figure 1 would result in no need for an additional chromatography step to enhance the purity of the final EVs product.

Fig 1. Proposed process purification map.

Materials, methods, and analytics

- **Harvest production**: 150 000–300 000 cells/mL (0.35 L) hiPSC from flask culture production
- **Clarification**: Preflow™ UUA 11 cm², 0.2 µm; Supor™ EAV 20 cm², 0.65/0.2 µm, flow rate 100 L/m²/H (LMH), post flush 10 L/m² with PBS
- **Tangential flow filtration (TFF)**: Centramate™ cassettes with Omega™ membrane 100 or 30 kDa (0.02 m²), CFF 2-3 L/min/m², TMP 0.3 bar, VCF 8×, DF 5× with PBS
- **Sterile filtration (0.2 µm)**: Supor EKV filter; Fluorodyne™ EX EDF 20 cm², flow rate 500 LMH, post-flush 10 L/m² with PBS
- **Size and concentration of isolated EVs** assessed by nanoparticle tracking analysis (NTA) technique with Nanosight NS300 (Malvern Panalytical, Westborough, MA, USA). Purity ratio and residual protein content quantified by Webber et al, 2013 equation (1) and BCA protein assay kit:

$$\text{Purity (particles/µg)} = \frac{\text{Total particles (particles/mL)}}{\text{Total protein (µg/mL)}}$$

References and acknowledgments

1. Webber J, Clayton A. How pure are your vesicles? *J. Extracell. Vesicles.* 2013;2:1-6.

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Clarification screening

Feasibility study for EV clarification step

hiPSCs harvest consists of a heterogeneous population of vesicles varying in size from 50 to 300 nm. The aim of this step is to separate the EVs from process impurities such as protein content and cell debris. First screening filters:

- Glass fiber depth filter, nominal pore size 0.2 µm: Preflow UUA membrane
- PES membrane filter, nominal pore size 0.65/0.2 µm: Supor EAV membrane

A single filtration step was sufficient to clarify the harvest from residual contaminants and other high molecular weight cell debris with a turbidity of around 30 NTU. No significant losses were observed during the filtration step. The throughput (L/m²) and yield (%) of the clarification step are shown in Figure 2.

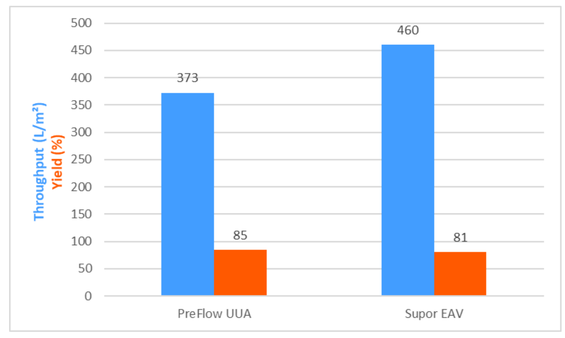

Fig 2. Overview clarification results: throughput and yield.

Tangential flow filtration (UF/DF)

Determination of TFF cassette molecular weight cut-off (MWCO)

Following the clarification, a TFF step was included to efficiently increase the purity of the EVs. The right choice of the MWCO and the type of membrane have a very high impact on this essential purification step for the yield and the final purity of the EVs, The choice for these two parameters is dependent on the final size and the range of the shear stress of the EVs. The objective was to retain EVs in the TFF retentate and to remove protein contaminants in the permeate to increase the target EVs' population size. For this reason, a screening of TFF Omega cassettes (using low crossflow filtration) between 30 and 100 kDa cut off was performed, and results are shown in Figure 3.

VCF: volumetric concentration factor. DF: diafiltration. LMH: liters per square meter per hour.

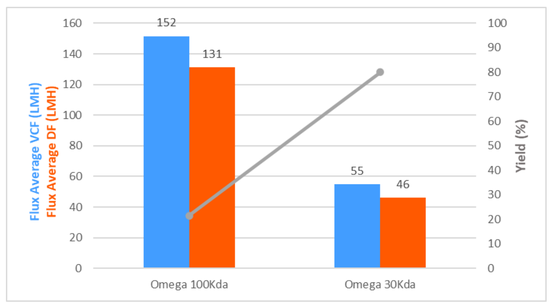

Fig 3. Screening results with different MWCO cassettes: permeate flux average during VCF and DF related to the yield.

Final sterile filtration

Screening of sterile filtration filters

Final sterile filtration is a necessary and challenging step to produce a sterile final product. This step avoids establishing a purification suite in aseptic conditions and reduces the cost of goods of the process purification steps.

Capacity (L/m²) and yield (%) assessed by NTA analysis for two different types of sterile grade filters are shown in Figure 4.

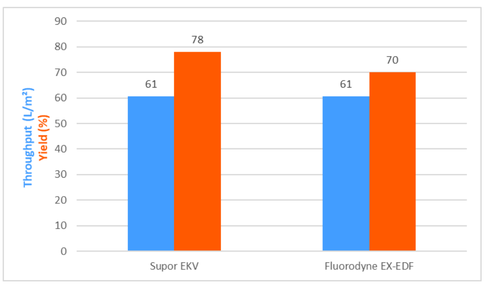

Fig 4. Throughput and yield results on sterile filtration screening.

Final purity assessment

EV purity and enrichment assessment by NTA analysis

In parallel to the filterability trial of each purification step, control analyses for purity and size of the final product were conducted (Fig 5). The EVs purity was assessed using the ratio of the particles and the protein content; the ratios increased during the different purification steps with a total purity ratio of 15×.

Moreover, the size distribution of the EVs after each purification step showed a sharp and monodisperse peak at the end of the purification suite as reported in Figure 6 and Table 1.

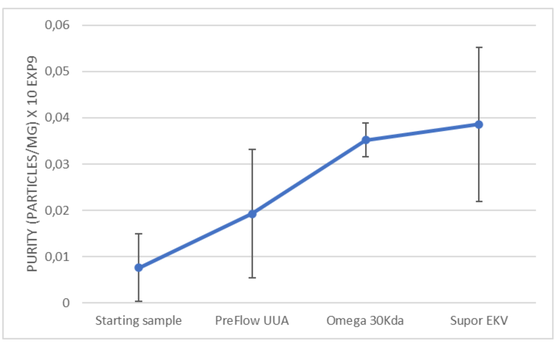

Fig 5. Overview of purity results in the total purification process.

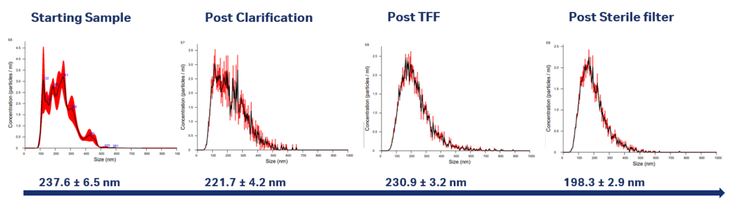

Fig 6. Overview of results on size distribution during purification.

Table 1. Overview of purification results

	Harvest	Clarification	TFF	Sterile
Concentration (particles/mL)	6.2×10^8	1.23×10^9	9×10^9	7×10^9
Mean size (nm)	236.7 ± 6.5	221.7 ± 4.2	230.9 ± 3.2	198.3 ± 2.9

Conclusions and recommendations

Platform proposition for EVs

In this study, a first proof of concept was demonstrated of a reproducible and optimized process to produce EVs from hiPSCs. A clarification step gave a yield of 85%. The TFF step retained 80% of the vesicles with an increase of sharpness confirmed with the NTA profile, and the final sterile filtration had a yield of 78%. A larger cut-off will improve the purity ratio, because more small-sized impurities would be removed. The drawback is a negative impact on the yield of EVs. The total process purity ratio was increased by a factor of 15×. Based on this study, we propose a scalable process shown in Figure 7 for purification of sensitive EVs.

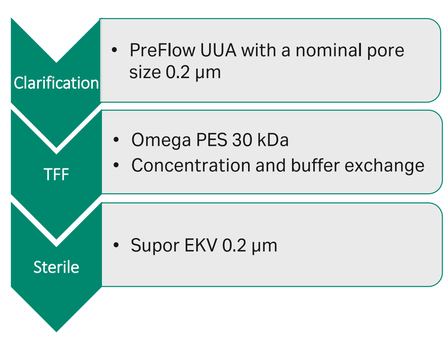

Fig 7. Final process steps for EVs purification.

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