Successful Development of a Scalable and Robust Process for hMSC-EV Production

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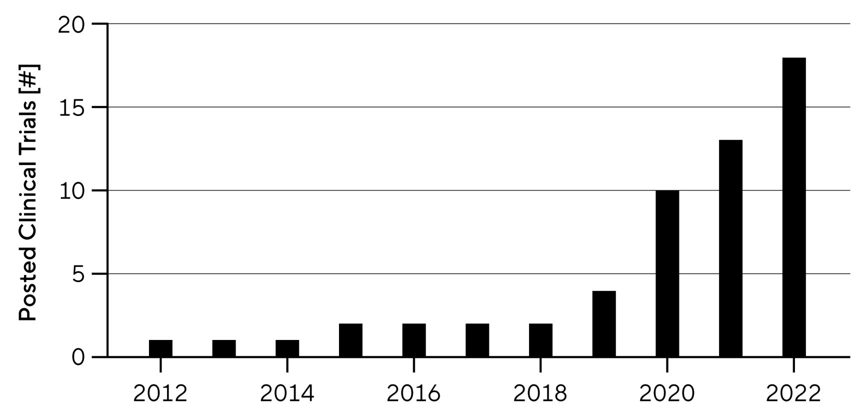
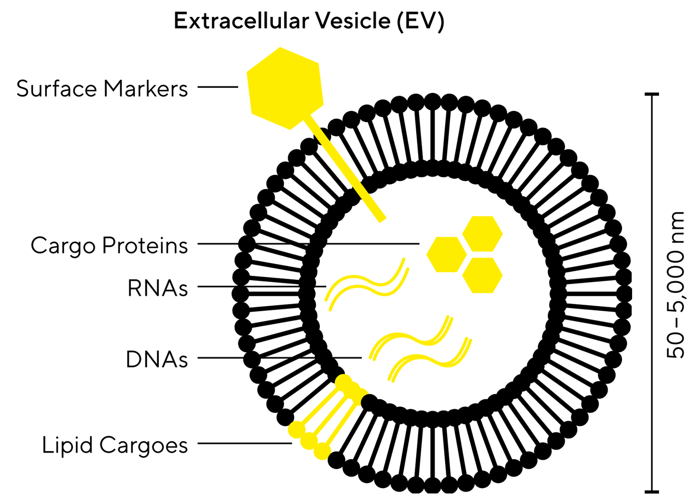
Background & Objective

- Extracellular vesicles (EVs) are nano-sized particles composed of a lipid bilayer.
- EVs are released from many different types of cells, and they contain proteins, nucleic acids (RNA and DNA), and lipid cargoes.
- EVs released from Mesenchymal Stromal | Stem Cells (hMSC-EVs) are rapidly gaining momentum in clinical trials.
- One of the challenges in EV production is scaling up the manufacturing processes using the optimal cell source and collection media.
- RoosterBio® offers reproducible and scalable processes to manufacture high-quality, xeno-free (XF) hMSCs and media for EV production.

Objective: The goal of this study was to develop a robust and scalable upstream process for hMSC-EV production in a stirred tank bioreactor system.

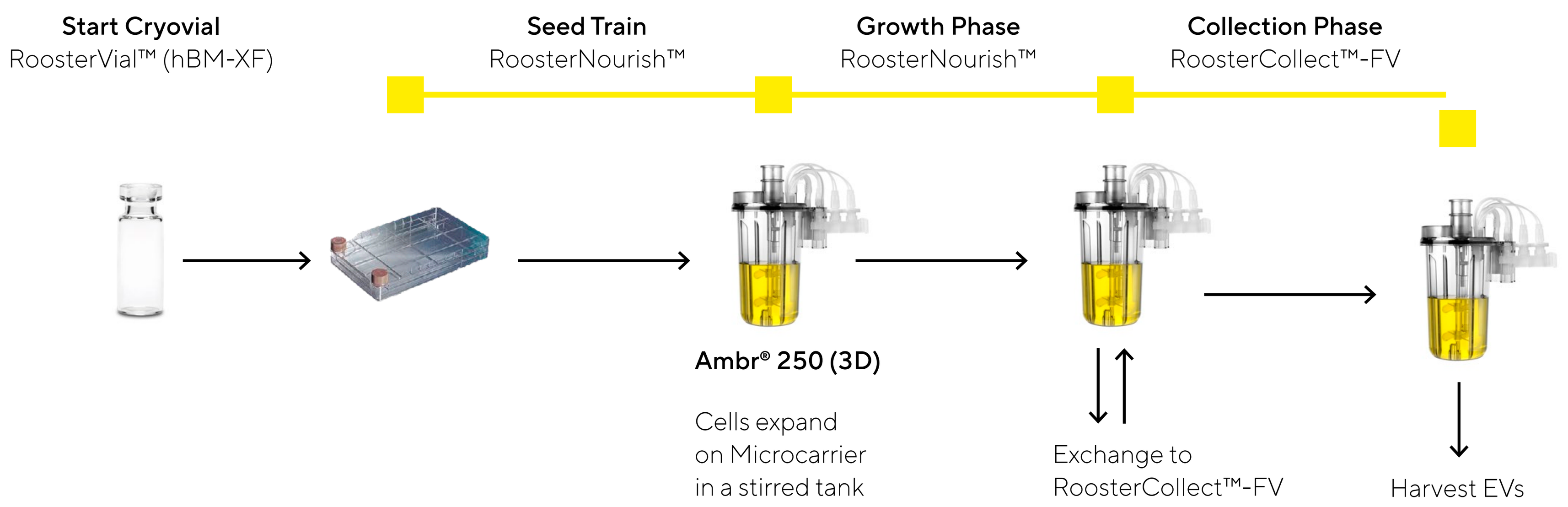


The majority of clinical trials involving the secretome and | or extracellular vesicles use MSCs as producers. (Source: Clinicaltrails.gov)



Process Development Approach

Process Flow Diagram Showing the Adapted Process for EV Manufacturing in Stirred Tank Bioreactors.

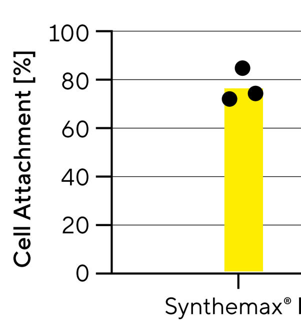


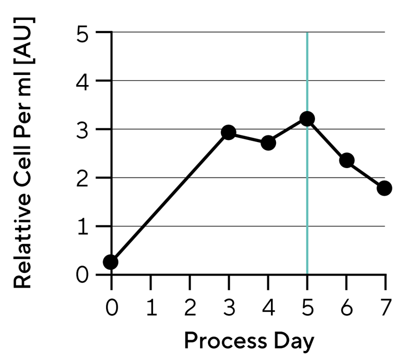
This study was carried out using xeno-free human bone marrow MSCs (RoosterVial™-hBM, RoosterBio®) expanded in RoosterNourish™ (RoosterBio®) in Ambr® 250 (Sartorius). EVs were collected in low-particle media, RoosterCollect™-EV (RoosterBio®).

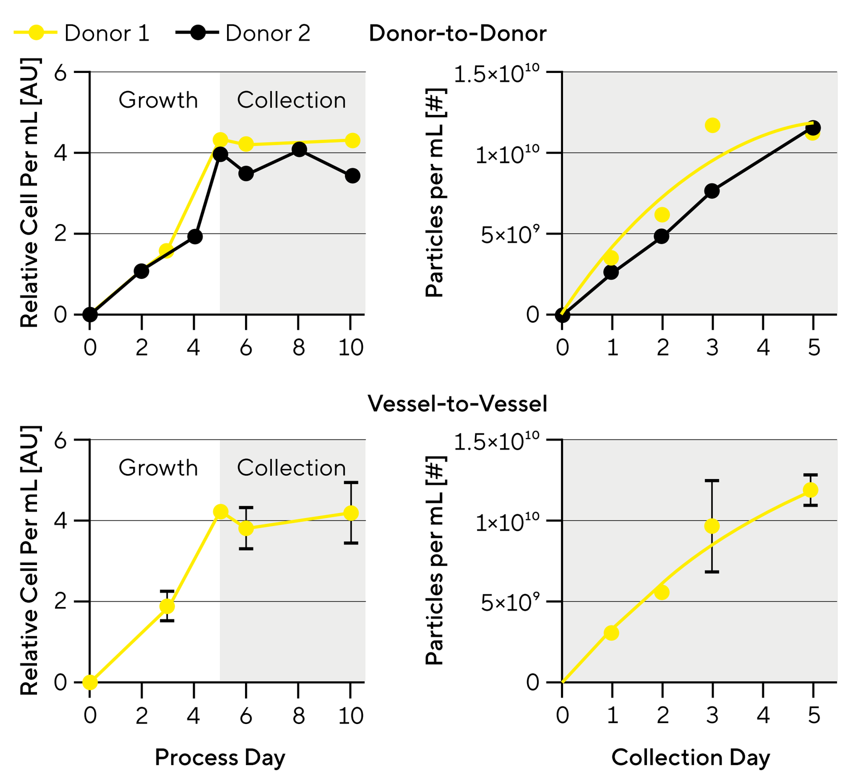
- **Critical process parameters** (CPPs) were evaluated and optimized to increase final cell yield and EV productivity:
  - Microcarrier type
  - Cell growth phase length (pre-collection)
  - Microcarrier density
  - Initial cell seeding density
  - EV collection time
- **Critical quality attributes** (CQAs) were evaluated for particle count and size, identity (tetraspanins by western blot, percent lipidated particles, RNA content) and potency (in vitro wound healing assay).

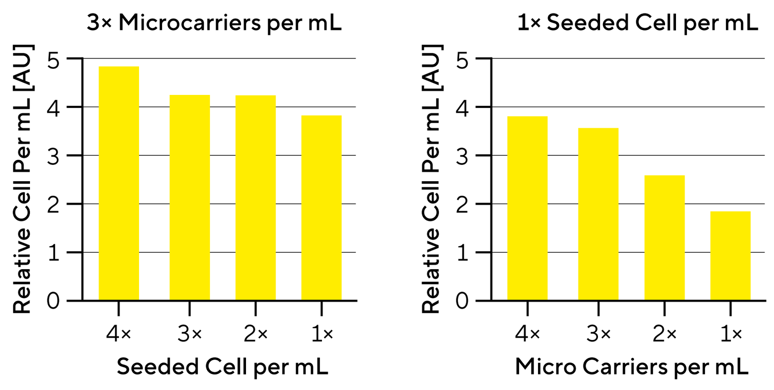
Small Scale (Ambr® 250) Process Development Results

(Right) Synthemax® II (Corning) showed high MSC attachment (~80%) and was selected as the optimal microcarrier. MSC growth reached its peak at day 5 of culture and then plateaus afterwards. Therefore, 5 days was selected as the optimal growth phase length.



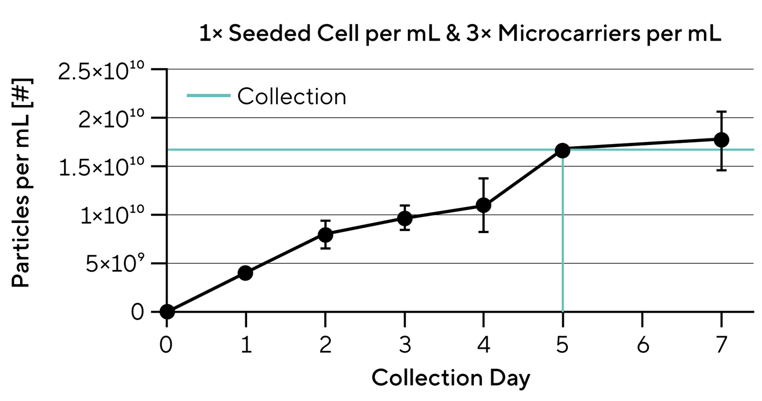






(Above) Increasing microcarrier density up to 3× significantly increases cell yield by day 5, and 1× cell seeding densitiy was sufficient to reach maximal cell yield without cell | microcarrier aggregation.

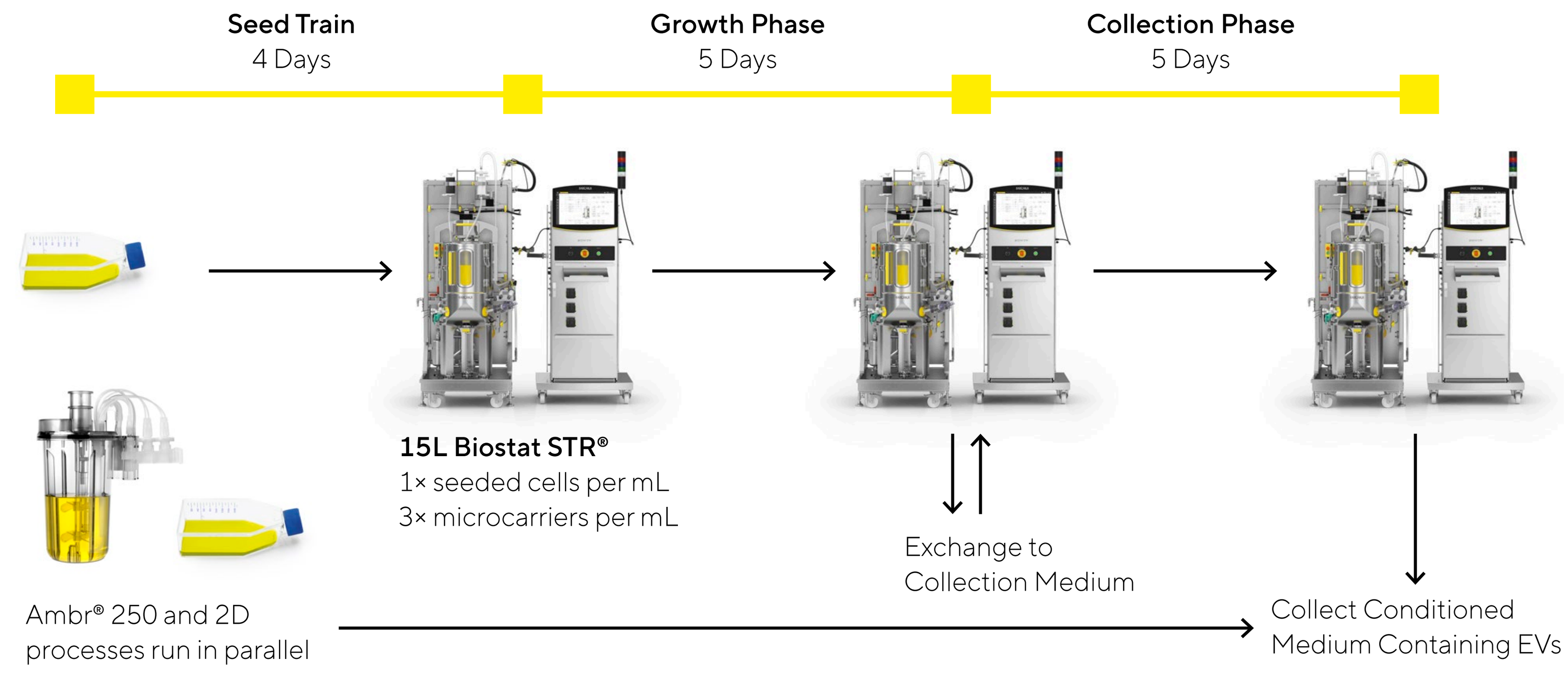
(Below) After exchange into EV collection media, particle yield increased and peaked at day 5 of the collection phase.

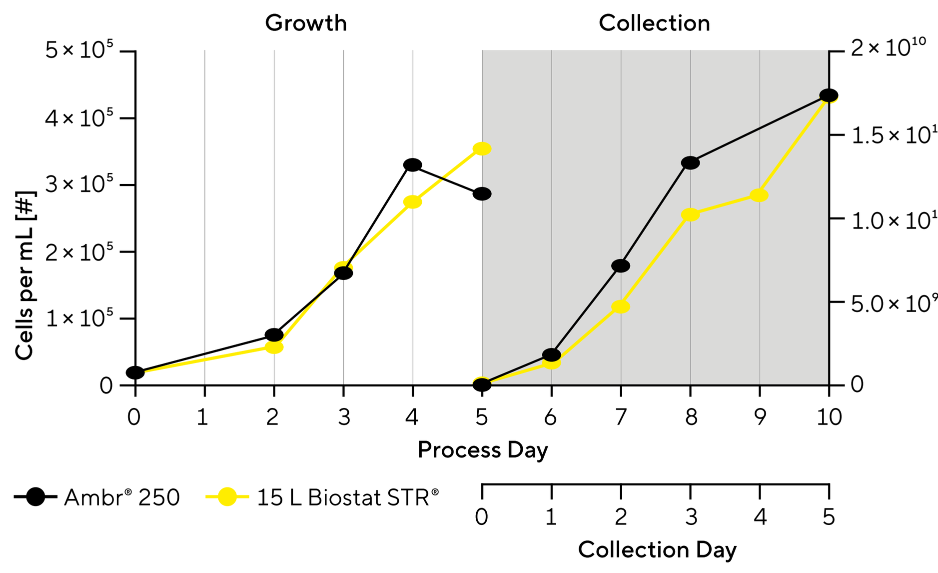


The process using optimally determined CPPs consistently yields comparable cell number and 1–1.5×10[10] particles per mL by harvest day. N=3 vessels for Vessel-to-Vessel Repeatability.

Optimally Determined CPPs From 250 mL Development	
Microcarrier type	Synthemax® II
Microcarrier density	3×
Cell growth time	5 days
Cell seeding density	1×
EV collection media	Rooster Collect™-EV
EV collection duration	5 days

15 L Scale-Up in Biostat STR®



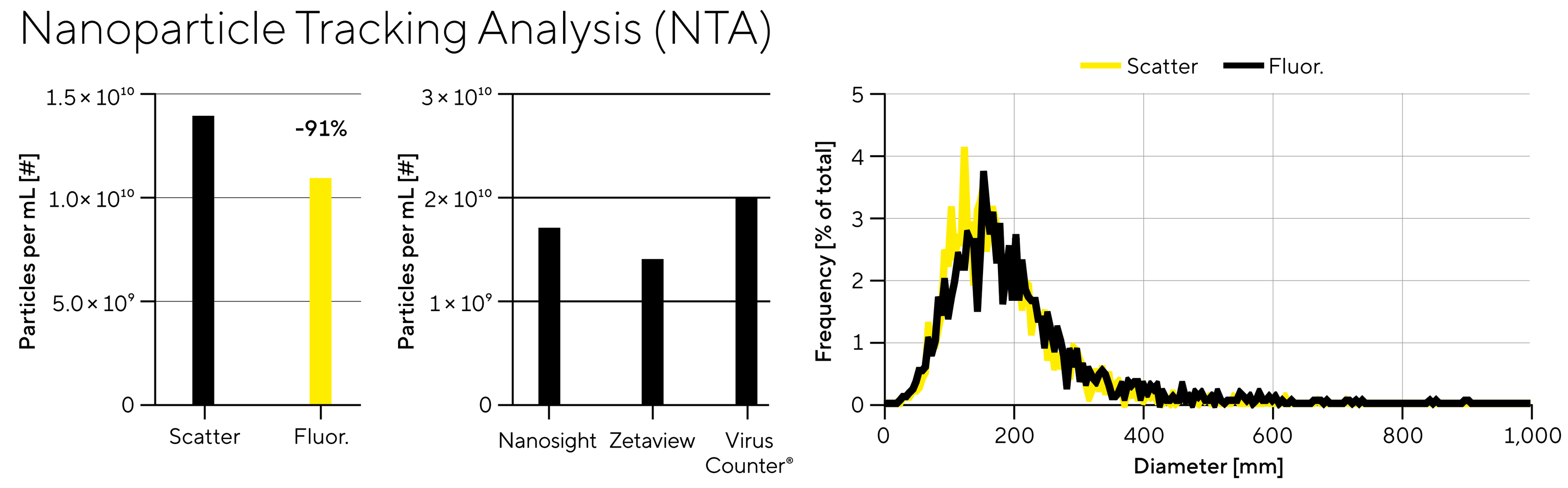


Critical Process Parameters identified at the small scale were verified at the large scale (15 L) in the Sartorius Biostat STR® bioreactor.

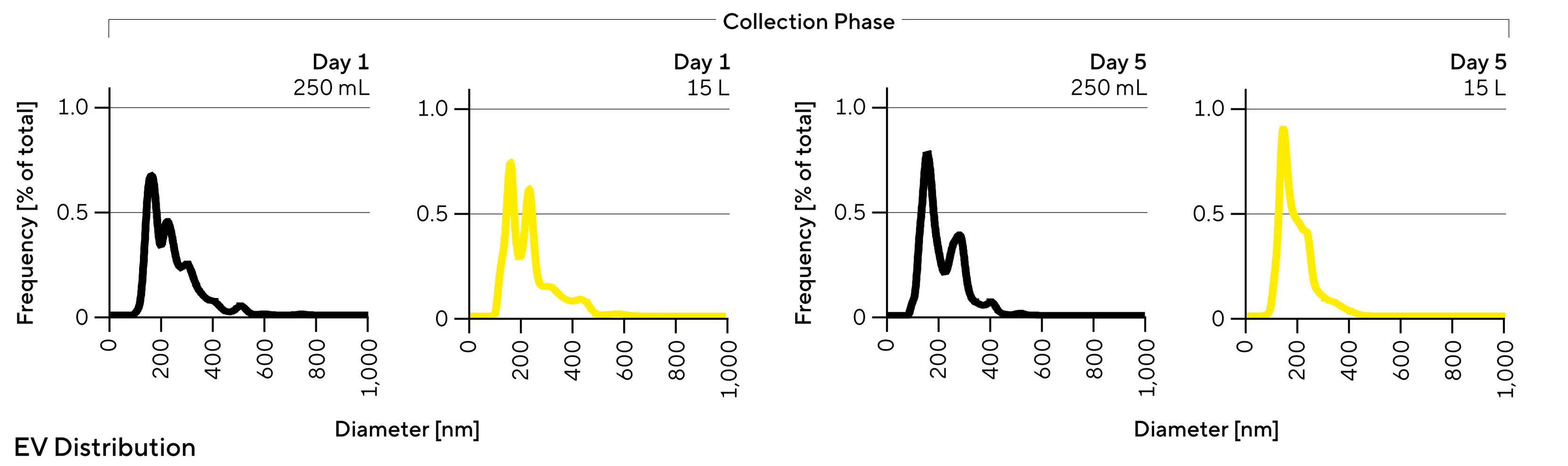
In Biostat STR®, MSCs reached up to ~400,000 cells per mL by the end of the growth phase (day 5), which was comparable to Ambr® 250. Particle production reached ~1.7×10[10] per mL by day 5 of the EV collection phase, which was also consistent with the Ambr® 250 data. Ambr® 250 and 15 L Biostat STR® comparability demonstrate process scalability.

Critical Quality Attribute (CQA) Comparability

Nanoparticle Tracking Analysis (NTA)

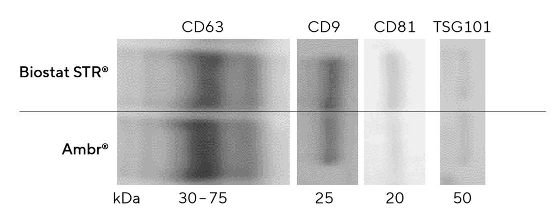


EV Count and Integrity: With NTA, the great majority (~91%) of particles stained positive for the lipid membrane dye Memglow, indicating 91% of the particles were EVs. Similar results are obtained across instruments. Virus Counter® (Sartorius) detects particles positive for lipid membrane dye CellMask Orange.

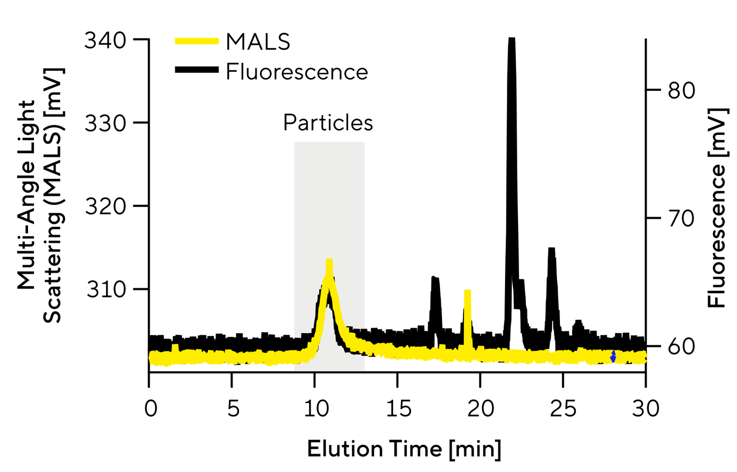


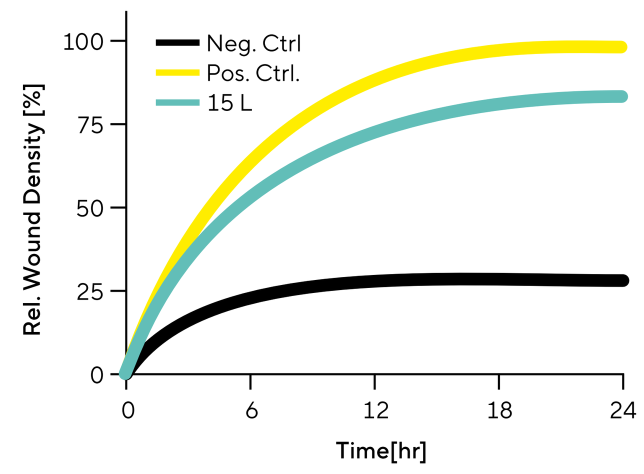
EV Distribution
Particle diameters ranged from 50–450 nm with median ~180 nm. Distributions are similar across platforms and collection phase duration.

EV Identity
Harvested conditioned medium showed presence of conventional EV markers tetraspanins CD63, CD9, and CD81, as well as intraluminal marker TSG101.



PATfix® SEC-MALS Biochromatography and EV Potency Assay





PATfix® SEC-MALS: Earlier elution during size-exclusion chromatography is indicative of larger particles that did not enter the pores of the column and instead eluted in the void volume. Molecules that have entered the pores elute in order of decreasing size. Overlapping MALS and fluorescence signal (~10–12 min) indicates larger particles that are stained positive for lipid membrane dye CellMask Orange and are likely EVs.

EV Potency: EV conditioned medium from 15 L Biostat STR® harvest showed potency effect greater than conditioned medium (Neg. Ctrl) and effect similar to growth medium (Pos. Ctrl) in an *in vitro* HUVEC cell wound healing assay. Cells are grown to a monolayer, a scratch wound is generated, and closure is measured over time using the IncuCyte® (Sartorius) platform. Data fit to a one-phase association model.

Conclusion and Next Steps

- We successfully developed a reproducible and scalable microcarrier-based stirred tank process for EV production using RoosterBio xenofree hMSCs (RoosterVial™-hBM) coupled with RoosterBio® MSC expansion media (RoosterNourish™) and RoosterBio low particle EV collection media (RoosterCollect™-EV).
- The process was developed at the 250 mL scale (Ambr® 250) and its scalability was successfully demonstrated at the 15L scale in Biostat STR®.
- The developed process consistently generates a conditioned medium containing ~1×10[10]–1.5×10[10] particles per mL.
- Particles generated from this process were in the range of 50–450 nm diameter, stained positive with lipid membrane dyes across three independent analytical methods (NTA, Virus Counter, PATfix®), confirming that they are extracellular vesicles, expressed the EV specific tetraspanin markers (CD81, CD63 and CD9) and demonstrated biofunctionality through an in vitro wound healing assay.

Overall, the study demonstrated the successful development of a scalable and robust upstream process for hMSC-EV production.