Investigating Cell–Microcarrier Aggregation to Modulate Hypoxia and EV Biogenesis In Vitro

Juzheng Zhang[1*], Costanza Emanueli[2], Richard M. Day[1]

[1] Division of Medicine, University College London, London, UK

[2] Faculty of Medicine, Imperial College London, London, UK

Background

Mesenchymal stem cell (MSC)-derived extracellular vesicles (EVs) are emerging as promising cell-free therapeutics, carrying pro-angiogenic factors, immunomodulatory cargo, and repair-associated miRNAs. However, clinical translation is constrained by low endogenous EV secretion rates and a lack of scalable manufacturing platforms.

'Borrowed Technology' problem: Current *in vitro* culture systems for EV production are repurposed from cell therapy or recombinant protein manufacturing and are not specifically designed for the unique requirements of EV biogenesis. 2D monolayer cultures are limited by surface-area constraints and contact inhibition, while existing 3D microcarrier-based systems prioritize cell expansion rather than EV yield.

'Failure' of aggregation — a missed opportunity: In conventional bioprocessing, microcarrier aggregation is considered an undesirable phenomenon due to risks of nutrient/oxygen diffusion limitation, metabolic waste accumulation, and reduced harvesting efficiency. Under hypoxic conditions, HIF-1α is stabilized and activates transcription of genes involved in vesicle trafficking, angiogenesis, and cellular stress responses — pathways directly linked to enhanced EV production and bioactivity.

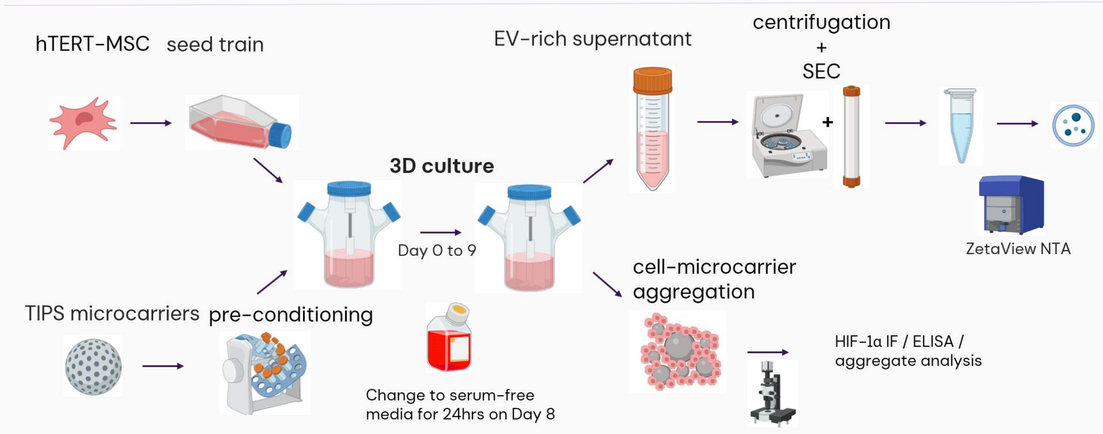
Hypothesis

Induced aggregation of microcarriers can be used as a method to enhance EV biogenesis resulting in increased yield.

Aims

1. Establish conditions for achieving cell-microcarrier aggregation in bioreactors by varying microcarrier properties (size, porosity)
2. Determine whether microcarrier aggregation results in localized hypoxia
3. Correlate the quantity of EVs harvested with microcarrier aggregation and hypoxia

Experimental Workflow



Materials & Methods

Thermally Induced Phase Separation (TIPS) Microcarriers

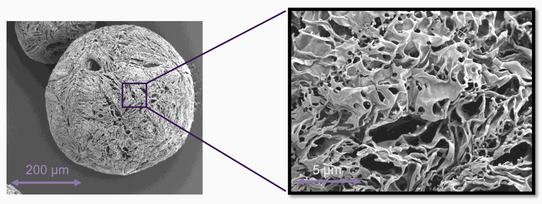


FIGURE 1: SEM images of PLGA TIPS microcarriers

- Poly(DL-lactide-co-glycolide) (PLGA)
- 355–425 μm (small) and 500–600 μm (large)
- Quantity adjusted to provide same surface area (350cm^2)

hTERT-immortalized MSCs

- Immortalized via stable integration of the human telomerase reverse transcriptase (hTERT) gene
- Stable batch-to-batch consistency and predictable growth
- Retain trilineage differentiation potential

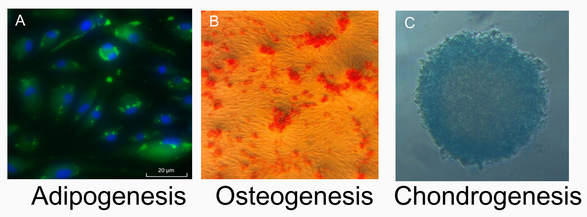
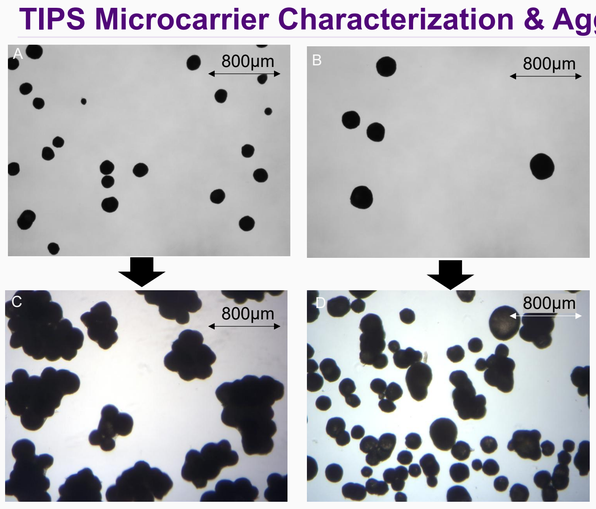


FIGURE 2: Confirmation of tri-lineage differentiation of hTERT MSC. A. Adipogenesis differentiation by DAPI and HCS LiqidTOX Green neutral lipid staining. B. Osteogenesis differentiation by Alizarin Red S staining. C. Chondrogenesis differentiation by Alcian Blue staining

TIPS Microcarrier Characterization & Aggregation



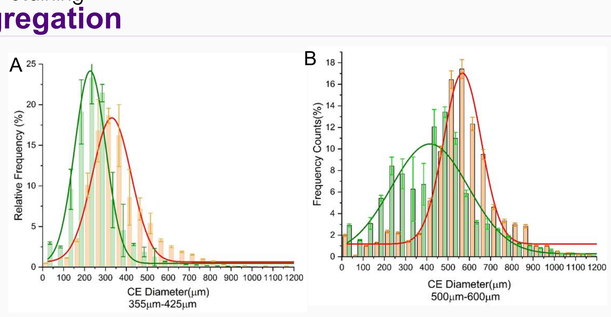


FIGURE 3: TIPS microcarrier images from particle analyzer (Morphologi G3). A. 355–425 μm size range TIPS microcarriers on Day 0. B. 500–600 μm size range TIPS microcarriers on Day 0. C. Aggregation of 355–425 μm size range TIPS microcarriers on harvest (Day 9). D. aggregation of 500–600 μm size range TIPS microcarriers on harvest (Day 9).

FIGURE 4: Size distribution of 355–425 μm (A) & 500–600 μm (B) PLGA TIPS microcarriers under dry (Red) and wet state (Green)

Parameter	355–425 μm	500–600 μm
Mean wet CE diameter (μm)	223.8 ± 21.6	323.3 ± 64.0
Total number of TIPS Microcarrier before seeding	59,306	30,921
% Aggregated (Day 9)	91%	83%
Average number of TIPS microcarrier per aggregate	22 ± 4	8 ± 1

- Size distribution of PLGA TIPS microcarriers shifts towards a smaller range after pre-wetting.
- The size range of PLGA TIPS microcarriers affects the quantity of clumped microcarriers, with smaller microcarriers resulting in more aggregation.

Localized Hypoxia in Aggregated & Single Microcarriers

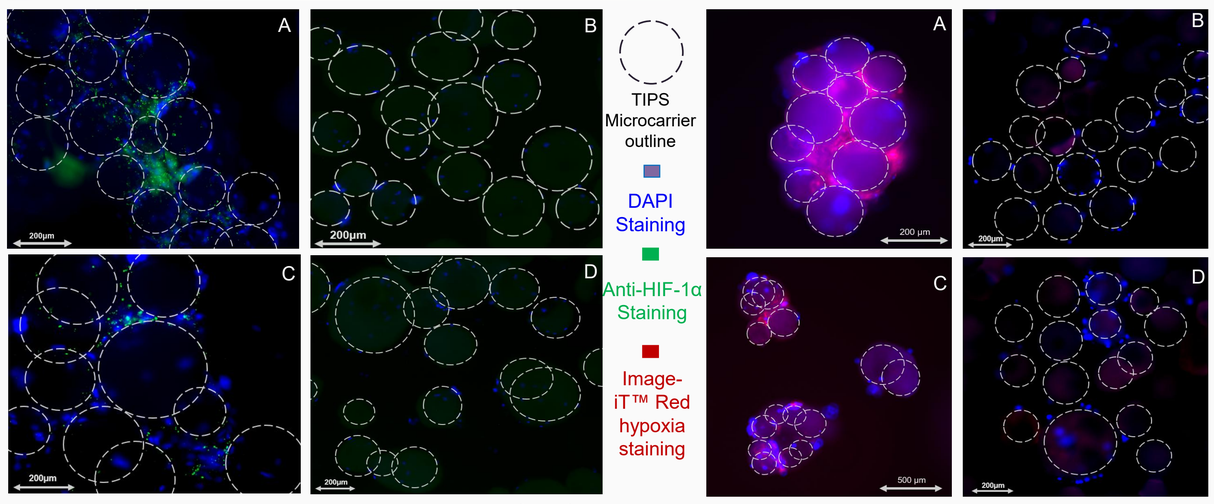


FIGURE 5: Immunofluorescence staining in DAPI (blue) + HIF-1α (green). Top row: 355–425 μm aggregated microcarriers (A) & single microcarriers (B). Bottom row: 500–600 μm aggregated microcarriers (C) & single microcarriers (D).

FIGURE 6: Fluorescence staining with Image-iT™ Red hypoxia staining (red) + DAPI (blue).

- Hypoxic signal localizes to microcarrier aggregate cores; single microcarriers remain normoxic
- HIF-1α expression co-localizes with Image-iT Red signal, confirming hypoxia-driven molecular activation
- Aggregates of smaller microcarriers show stronger signal than larger microcarriers, consistent with bigger, denser clusters associated with the smaller microcarriers

HIF-1α Expression & ZetaNTA Analysis of EV Production

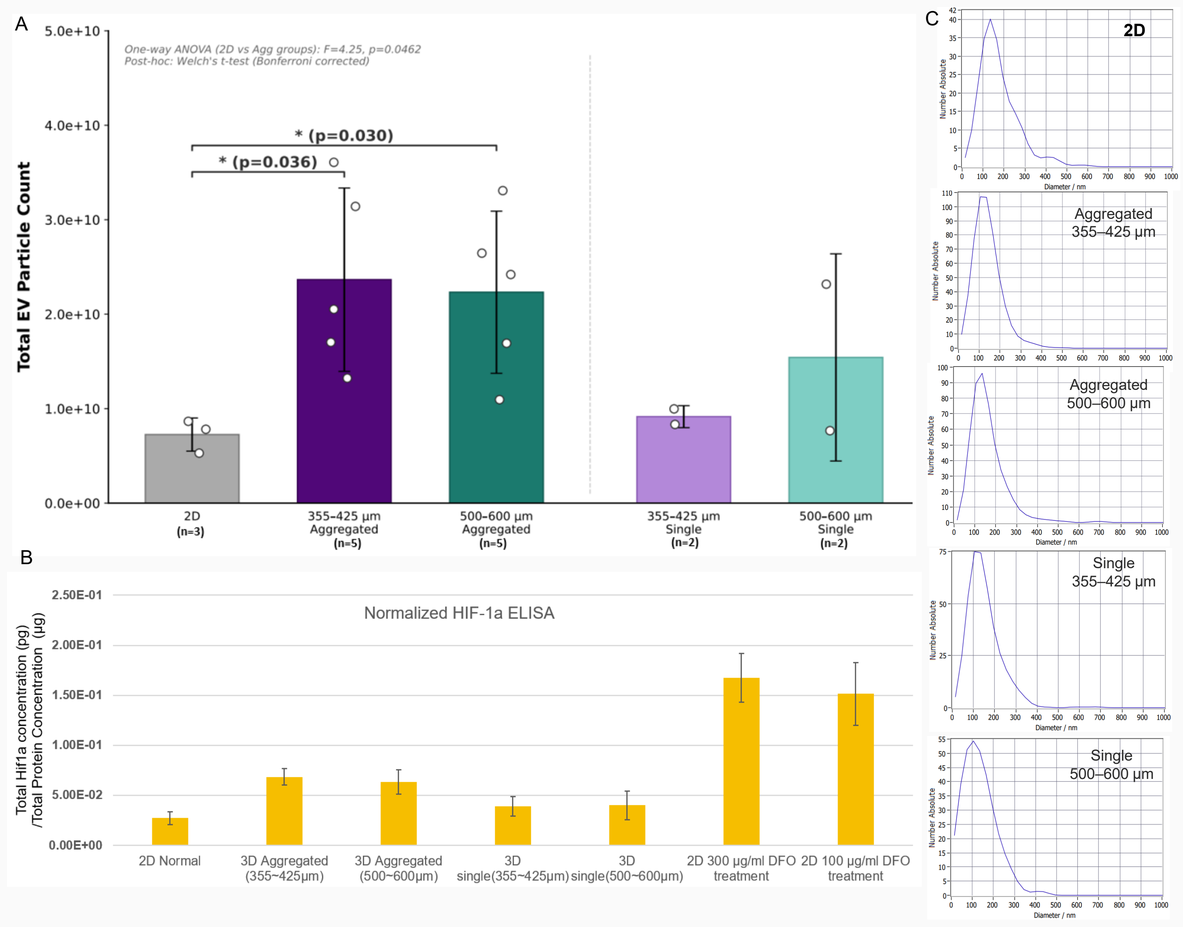


FIGURE 7: A. Total EV Yield in 24 h serum-free collection(ZetaNTA). B. Total HIF-1α expression (normalized, n=3). C. EV size distribution (ZetaNTA)

- Both groups of aggregated microcarriers show higher EV secretion than 2D cultures. Also higher than 3D single microcarriers group.
- EV measurement for 355–425 μm aggregated microcarriers is higher than 500-600 μm aggregated microcarriers corresponding with higher HIF-1α expression
- Hypoxic cells are localized to aggregate cores, corresponding with regions of HIF-1α expression. HIF-1α levels were significantly higher in the aggregated group, and highest in 355–425 μm aggregated group
- Both DFO positive controls (100 & 300 μg/ml) confirmed HIF-1α pathway responsiveness.
- The EV size range for all groups was 50 - 200 nm.

Conclusions

1. Smaller microcarriers exhibit greater aggregation and form larger multi-microcarrier clusters. This may be driven by higher bead density and collision frequency in the bioreactor.
2. Hypoxia localizes to aggregate cores: HIF-1α is enriched in the small-MC group, confirming oxygen diffusion limitation within dense cell–microcarrier clusters.
3. EV production is up to 3.2× higher with small aggregated microcarriers than with 2D monolayers, potentially indicating intrinsic upregulation of vesicle biogenesis under 3D hypoxic conditions.

Collectively, these findings indicate that controlled microcarrier aggregation can be strategically leveraged as a novel approach to enhance EV yield and bioactivity.

Juzheng Zhang, UCL Day Lab, Division of Medicine, UCL
Richard Day, UCL Day Lab, Division of Medicine, UCL