Production of Exosomes in a Hollow Fiber Bioreactor

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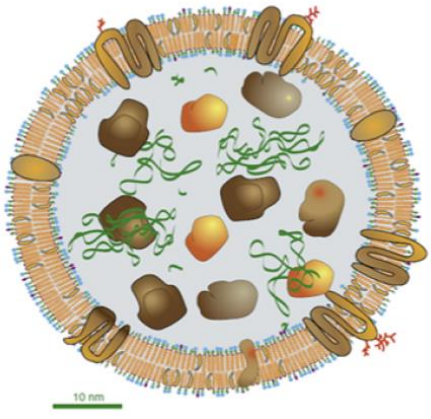
INTRODUCTION

Exosomes are small lipid membrane vesicles (80-120 nm) of endocytic origin generated by fusion of cytoplasmic endosomal multi-vesicular bodies with the cell surface. Protein and miRNA is exported outside cells via exosomes into the circulatory system. Exosomes may serve to shield miRNA contained within them from degradation, allowing them to serve as intercellular communication vectors. It has been demonstrated that the exosomal miRNA can modulate gene expression in other target cells. Cells of all types secrete exosomes and they are indicative of specific cell physiology, and have been targeted as circulating biomarkers of specific diseases. Those secreted by various types of stem cells are of particular interest as they may stimulate tissue repair. Exosomes secreted by mesenchymal stem cells (MSC) have demonstrated wound healing and regenerative effects mediated by stimulating collagen synthesis and through other pathways. Clinical trials are planned for cardiac infarct treatment using exosomes secreted by MSC, based upon positive data from cardiac reperfusion studies in animals. Increasing evidence suggests that tumor cells release excessive amounts of exosomes, which may influence tumor initiation, growth, progression, metastasis, and drug resistance. In addition, exosomes transfer message from tumor cells to immune cells and stromal cells, contributing to the escape from immune surveillance and the formation of tumor niche. As their content is a fingerprint of type and status of the cell when generating them, they could also serve as biomarkers in the prediction of therapeutic outcomes.

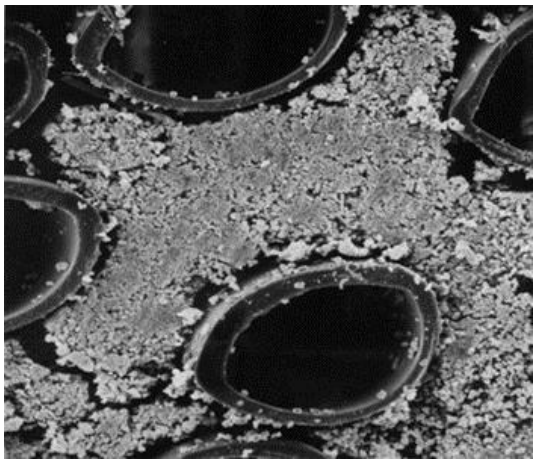


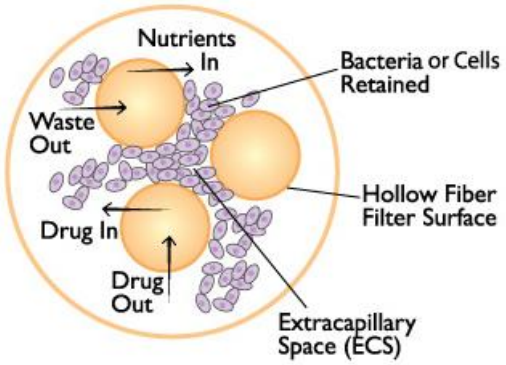
Until recently exosomes were simply considered to contain intracellular garbage. Exosomes are secreted in very low amounts and were an annoyance appearing at the bottom of flow cytometry plots, interfering with “real” data. Exosomes have very high specific activity, but progress in understanding and applying them has been impeded by their low availability and difficulty in producing large quantities for experimentation. In laboratory-scale production, any number of culture, harvest and purification approaches have been used. The most

The use of flask culture for the production of exosomes can generate a large amount of waste. This is an example of the number of flasks used to generate 1X10⁹ cells.

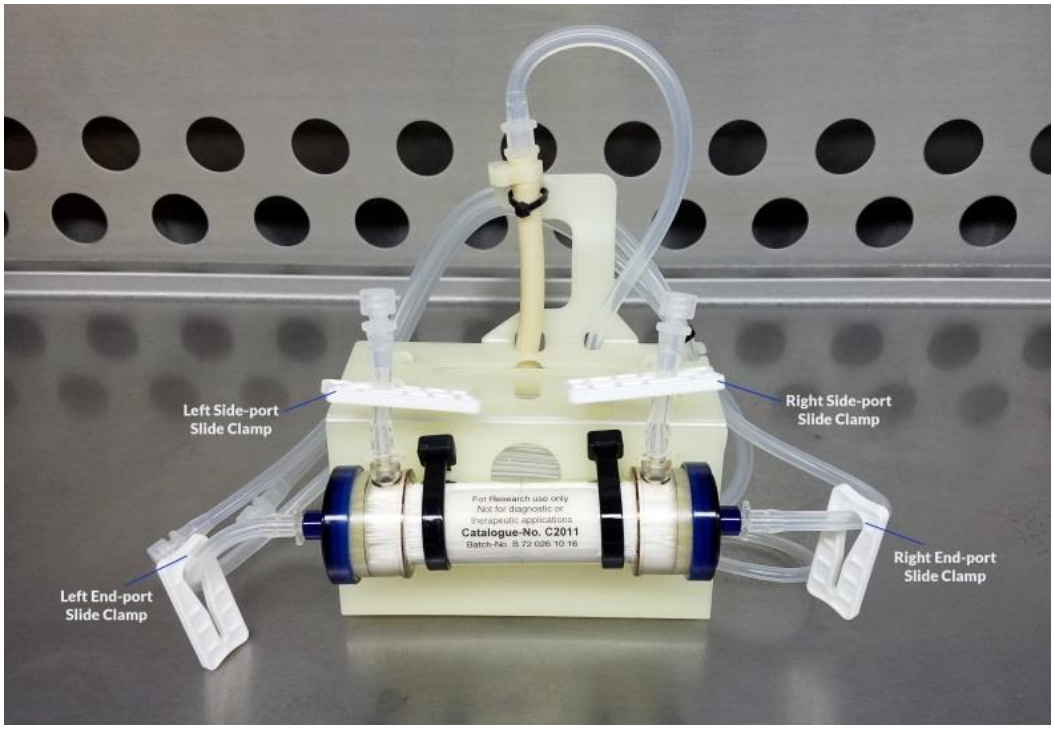
common method uses large numbers of T flasks. Cells are expanded and the final collection performed using only basal medium (without serum), as serum contains significant levels of contaminating endogenous exosomes. This batch-mode protocol can entail a final stage of hundreds of large flasks and many liters of medium to process. Current isolation protocols utilize ultracentrifugation, not practicable for large scale production. The composition and activity of exosomes reflect the physiologic state of the cells when secreting them and this method is far from physiologic or relevant to *in vivo* conditions. In any of these approaches it can take several rounds of splitting and culture expansion to attain this final production cell-mass. This method is wasteful, time consuming, space consuming and not amenable to scale up for clinical applications. They can also present such technical challenges as cells undergoing apoptosis just prior to harvest, contaminating it with difficult to remove membrane fragments and protein aggregates.

Hollow fiber bioreactors (HFBR) offer significant advantages over flask culture, especially for the collection and concentration exosomes under physiologic cell culture conditions. HFBRs are the most efficient way to culture large numbers of cells as they grow *in vivo*. They provide a high amount of surface area so large numbers of cells can be supported at high densities. The molecular weight cut off (MWCO) of the fiber allows nutrients and waste products to pass through the fibers but retain larger secreted products and exosomes in the small volume of the ECS (extra-capillary space) where they accumulate and concentrate. If serum is required for production it can be used in the circulating medium only, while the ECS containing the cells and secreted exosomes can be maintained serum free. Endogenous exosomes in serum cannot cross the fiber, but the factors in serum that support cell growth can cross the fiber. The HFBR can facilitate the use of a protein free medium, such as CDM-HD. The cells are also bound to a porous support so that splitting is not required, cells are free to grow post-confluent



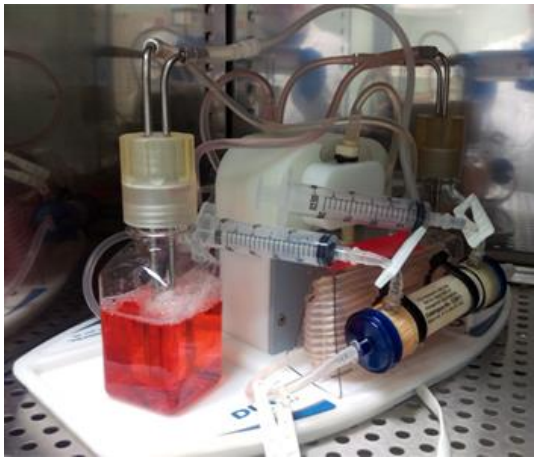


and collection of secreted products can be maintained over several weeks or months of continuous production.



CASE STUDY #1

5 X 10^8 human adipose derived adult MSC were cultured in a FiberCell Systems HFBR for 8 weeks using DMEM/10% FBS in the circulating medium only, with basal DMEM in the ECS. 40 mL of supernatant were harvested weekly from the cartridge. To isolate EV, the collected serum-free conditioned medium was subjected to two steps of centrifugation 1) 3000 g, 20 mins to remove cell debris and 2) 100,000 g to pellet exosomes. Size distribution and concentration of EVs were quantitated by tunable resistive pulse sensing (TRPS; (qNano, IZON Sciences). One interesting feature of this protocol is that the cells did not expand over this period of time as the glucose uptake rate remained constant. MSC that do not proliferate will also not differentiate. At the end of 8 weeks the cartridge was cut open and cells recovered for phenotypic analysis. This demonstrated no change in cell phenotype over the period of

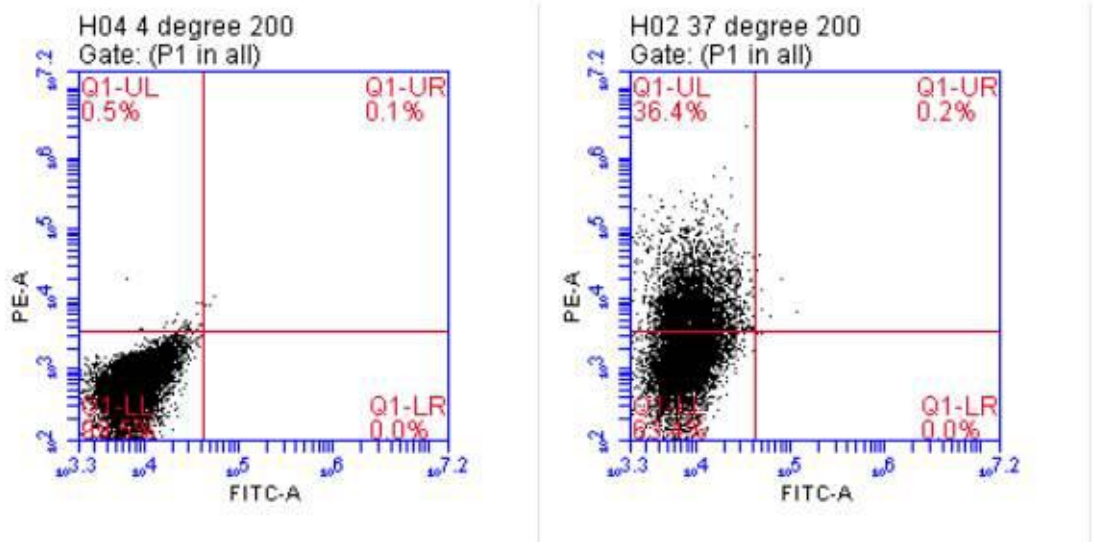


culture.

Comparison of flask culture to a C2011 HFBR for the production of exosomes from mesenchymal stem cells.

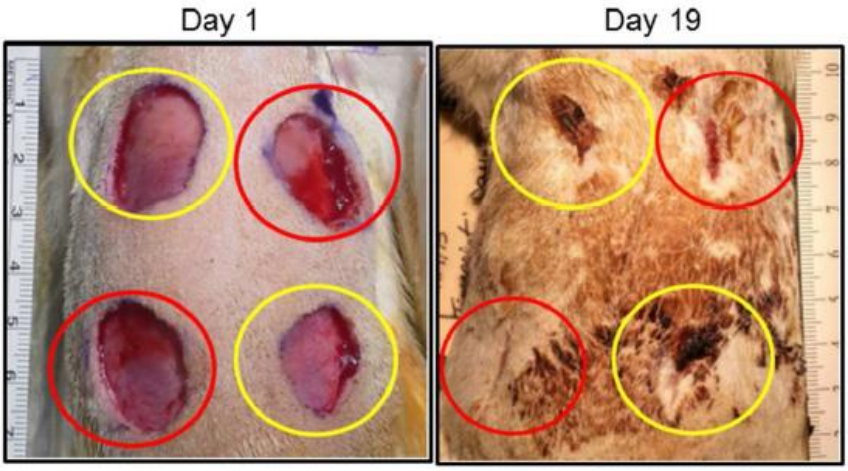
	Collection Volume (mL)	Total Exosome Protein (mg)	Total Exosome Particles (10^{10})
HFBR			
Cartridge #1 (7 weeks, collection every week)	240	11.82	95.78
Cartridge #2 (4 weeks , 6 collections)	120	14.45	326.9
Flasks			
130 T225	4000	0.9	1.6

A flow cytometry dye transfer assay was used to demonstrate the ability of EV to deliver cargo to endothelial cells. A scratch closure test and rat skin wound healing assay was used to measure wound healing activity.



Incorporation of exosomes into human endothelial cells. In order for exosomes to transfer their cargo (nucleic acid, protein), they must somehow be incorporated into the recipient cell.

Lipophilic dye transferred from exosomes and incorporated into cultured cells has been used to support the function of exosomes to deliver cargo into cells. As shown by the shift in the population in the right panel, the cells incorporated DiI from the labeled exosomes, supporting the notion that these exosome preparations from the bioreactor are capable of delivering their cargo (i.e.: nucleic acid, protein) into the cell.



Wound healing in response to topical application of EVs. Left panel shows fresh 2 cm diameter wounds in the back of the rat. Right panel shows degree of wound healing after 19 days. Yellow circles mark vesicle control treatment. Red circles mark EV treated wounds. All treatments were single application. Pictures are illustrative of 9 different animals.

TREATING CELLS WITH HEAT SHOCK

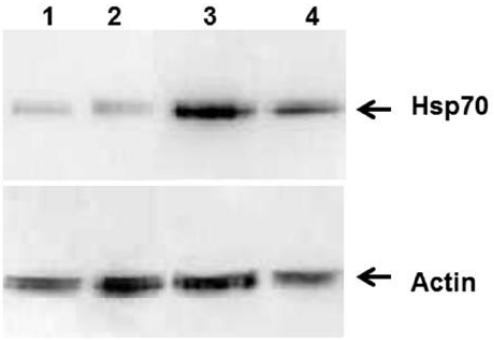


Figure A

Studies with heat shock protein 70 (Hsp70) have revealed a protective effect with regards to oxidative stress, blood vessel development and repair, and wound healing that involves actin cytoskeletal remodeling. As these Hsp70 functions are all positive contributors towards wound healing a heat shock was applied to the cells by warming the central reservoir of medium to 42 C for 30 minutes.

As demonstrated by western blot (Figure A), there is an observable increase in Hsp70 band intensity for EVs isolated from heat-shocked cells compared to those isolated from the non-heat-shocked control cells. Quantitating these bands, using actin as an internal control, revealed an approximate 30 - 40 % increase in EV Hsp70 content from heat-shocked cells (Figure B).

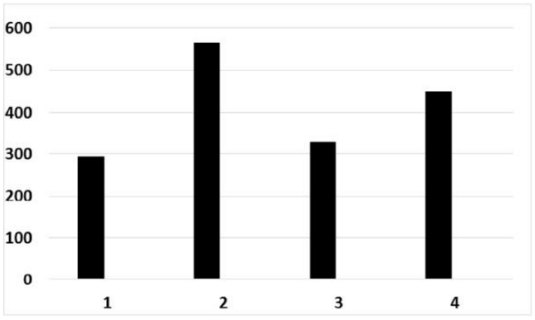


Figure B

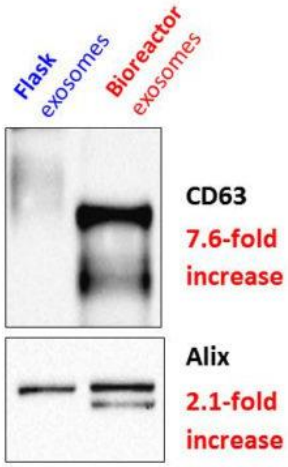
The use of the HFBR allowed a uniform and consistent culture condition manipulation to be applied to a large number of cells, with ease.

CASE STUDY #2 293T CELLS

An HEK 293 culture expressing heterodimeric IL-15 was maintained in a hollow fiber bioreactor for over 4 months of continuous production, with 3 harvests of 20 mL per week.

The HEK293 bioreactor culture yielded an equivalent number of exosomes per harvest as 70 T225 flasks. CD63 and Alix were greatly enriched from the bioreactor compared to flask culture. EV/protein ratio was 10-fold higher in harvests from the bioreactor suggesting a significant reduction in contaminating cell membrane fragments. Purified HEK 293 cells retained their IL-15 biological activity.

Engineered Exosomes Derived From 293 T Cells



Sample	µg yield per ml sup
Conventional culture EVs (comparison protocol)	2.7 ± 0.4
Bioreactor EVs (comparison protocol)	33 ± 3
Bioreactor EVs (optimized protocol)	54 ± 2

>10-fold increase in exosome yield from bioreactor supernatants

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"We developed a methodology for the mass production of highly purified, bioactive EV using a lab-scale hollow fiber bioreactor, which may facilitate further in vivo studies. This method presents many advantages over conventional culture that likely contribute to the higher EV production yield and purity that we observed. First, lab-scale hollow fiber bioreactors enable the sustained maintenance of large numbers of cells (estimated by the manufacturer to be in the order of 109 cells) within a standard

incubator, with minimal maintenance. More importantly, all of the cultured cells secrete EV into the relatively small volume of the culture cartridge extracapillary space (~20 mL) resulting in EV-rich conditioned medium that can be used directly for EV purification. Our findings suggest that this method yields 40-fold more EV particles per volume of conditioned medium vs. conventional cell culture. Thus, to match a single daily harvest of the hollow-fiber bioreactor (20 mL), an estimated 53 large (175 cm^2) flasks producing 800 mL conditioned medium would be required. Moreover, cells grown in hollow-fiber bioreactors readily adapt to sustained growth in protein-free medium, which also facilitates EV purification without serum contaminants, as highlighted by our comparative proteomics study. On the other hand, bioreactor EV preparations may have a more diverse population of EV, as suggested by the increased size range. Thus, further purification of preparations (e.g. by density gradient) may be required for some applications.

*Our findings showing the greatly enhanced yield of EV purified from hollow-fiber cultures may also have significant implications for the development of clinical grade EV therapeutics, given that hollow-fiber methods have already been used to expand primary human cells under cGMP conditions. "**

Hollow fiber bioreactors have the potential to be the method of choice for the production of exosomes up to clinical scale. They have the following advantages:

- Exosomes are concentrated by a factor of 10-100 X
- No interference from endogenous exosomes present in serum
- Reduction in apoptosis reduces cell debris
- No serum starvation, more physiologic cell culture conditions
- Continuous production over several months
- No passaging of cells required
- Closed, single use system
- Manipulations such as heat shock can easily be applied uniformly to large numbers of cells
- Continuous biomanufacturing process
- Clinical scale under cGMP possible
- Production at the gram scale possible using current technology

Exosomes and EVs have garnered tremendous interest over the past few years, showing promise for cancer therapy, disease biomarkers, and regenerative medicine. Their complexity permits them to be a direct indicator of cell health and for the culture conditions during their secretion. There is a demonstrated need for larger scale methods to produce them without serum, in a concentrated state, and under cGMP compliant conditions for human therapy.

Hollow fiber bioreactors from FiberCell Systems represent a more *in vivo* like way to produce exosomes of both the quality and quantity required for laboratory research as well as clinical relevance in a closed, single use system. They are highly concentrated, and with reduced contamination from intracellular proteins and membrane fragments

as a result of reduced apoptosis. They have the capability of producing gram quantities of exosomes using currently available cartridges. A hollow fiber bioreactor is the ideal method for the production of exosomes under cGMP conditions and represent a paradigm shift in advancing both exosome research and clinical applications.

* Watson, DC et al, Biomaterials 105 (2016) 195-206. Efficient production and enhanced tumor delivery of engineered extracellular vesicles.

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