Trends in Molecular Medicine

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Opinion

# Manufacturing Exosomes: A Promising Therapeutic Platform

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**Extracellular vesicles, in particular the subclass exosomes, are rapidly emerging as a novel therapeutic platform. However, currently very few clinical validation studies and no clearly defined manufacturing process exist. As exosomes progress towards the clinic for treatment of a vast array of diseases, it is important to define the engineering basis for their manufacture early in the development cycle to ensure they can be produced cost-effectively at the appropriate scale. We hypothesize that transitioning to defined manufacturing platforms will increase consistency of the exosome product and improve their clinical advancement as a new therapeutic tool. We present manufacturing technologies and strategies that are being implemented and consider their application for the transition from bench-scale to clinical production of exosomes.**

## Highlights

Exosome research has been rejuvenated in recent years, due in part to the evolution in understanding of stem cell mode of action. The paracrine effect of stem cell therapy candidates has been mechanistically linked to inherited, specific functionality in secreted exosome derivatives.

Even though exosomes are expected to enter clinical trials imminently, there has been a lack of manufacturing process development work that is needed to generate clinically relevant quantities of exosomes as trials progress towards larger patient numbers.

If manufacturing research is not undertaken now, then the advancement of exosomes as a new therapeutic platform will be slowed. Thus, there is an urgent need for technological advancements.

Here, we present process options for industrial and academic researchers to consider to translate exosomes into viable therapeutic candidates from a manufacturing perspective.

## Extracellular Vesicles: Biogenesis, Inherited Function, and Clinical Relevance

Living cells release vesicles into the local environment, and research into the potential therapeutic benefits of different **extracellular vesicle** (EV) types (see Glossary) has led to exciting discoveries resulting in the possibility of adopting EVs as new candidate therapeutic agents.

EV biogenesis occurs via several mechanisms [1–5], resulting in vesicles of different sizes and architectures. Broadly speaking, there are three main subclasses of EVs: (i) microvesicles, which are shed directly from the cell membrane and have a diameter range of 50–1000 nm; (ii) apoptotic blebs derived from dying cells, typically 50–4000 nm in diameter; and (iii) **exosomes**, which are smaller, with an approximate diameter range of 20–150 nm, although this varies between research groups [5–11]. Exosomes are released from multivesicular bodies (MVBs) rather than directly from the cell membrane via exocytosis, a feature that distinguishes these vesicles from other subclasses [4,12,13]. During this process, exosomes are loaded with various types of bioactive cargo (Figure 1), comprising protein and RNA molecules (including mRNA and miRNA) [5].

A growing body of research into stem cell therapy has revealed that the mode of action underlying the therapeutic effects of stem cells occurs largely via paracrine signaling [14–17]. This understanding has evolved based on the fact that implanted cells do not often engraft or persist long-term, but instead generate paracrine effects, which can be mediated by exosomes transmitting information into resident tissue cells. Indeed, postinjury tissue regeneration studies have revealed that the regenerative effect of exosomes can be as potent as that of parent cells in promoting regeneration and functional recovery in experimental animal models, including stroke [14], traumatic brain injury [15], pulmonary hypertension [16], and wound healing [17].

In this way, exosomes are effective communication vehicles that transfer bioactive proteins and genetic material between cells [18–20]. The exosome cargo ensures continued therapeutic

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effects long after the implanted cells have perished or migrated away from the target site (Figure 2).

## The Biological and Clinical Basis for EVs as Therapeutic Agents

There is widespread consensus that EVs have a biological signature that reflects the phenotype of the cells that produced them [21]. For this reason, the potential applications of EVs in a clinical context are diverse.

On the one hand, EVs have been proposed as diagnostic biomarkers of disease in cancers as diverse as ovarian cancer [22,23], glioblastoma [19], melanoma [24] prostate cancer [25], and colon cancer [26], based on unique miRNA profiles and other cargo that is transmitted with pathological effect. Similarly, they might be used as biomarkers of infectious disease, based on that fact that they transmit infection-specific elements. For example, exosomes isolated from Huh 7.5 cell lines infected with hepatitis C virus have been reported to infect primary human hepatocytes [27].

On the other hand, they can also act as potent mediators of cell signaling, which could be exploited for medicinal purposes. For example, they are able to transfer RNA and protein instructional cues from producing cells to other cells in the surrounding milieu [18]. This can have striking effects, as evidenced from experiments where EVs derived from mouse embryonic stem cells promoted the survival and expansion of mouse hematopoietic stem cells *in vitro*, while also upregulating transcription factors associated with pluripotency in recipient cells [28]. These findings also suggest that exosomes could be harvested, purified, and used as a biologic to control undesired or pathophysiological conditions.

This concept is further supported by *in vivo* studies. For example, exosomes isolated from indoleamine 2,3-dioxygenase-positive dendritic cells reduced inflammation in a mouse model of collagen-induced arthritis [29]. The exosomes, isolated using differential centrifugation, were $<$100 nm in size (as assessed by electron microscopy) and expressed typical exosome markers, such as CD81, hsc70, and CD80/86, as revealed by western blotting and fluorescence-activated cell sorting (FACS) [29].

Other studies have suggested the potential therapeutic application of exosomes in cardiovascular disease. For instance, two mouse models of cardiac ischemia/reperfusion injury (*in vivo* myocardial infarction and *ex vivo* Langendorff heart) showed that mesenchymal stem cell (MSC)-derived exosomes with a size range of 55–65 nm resulted in a 50% reduction in infarct size, measured as a percentage of the area at risk, compared with saline controls [30].

Recently, EVs were found to promote regeneration after stroke injury in both rat [31] and mouse [14]. In both models, functional recovery was accompanied by cellular and molecular evidence of neurogenic and angiogenic regeneration. For example, in the mouse model, MSC-derived EVs of undisclosed size were able to support neuronal survival and neurogenesis in postischemic tissue to a level similar to that of parent MSCs, as measured from the co-expression of markers of cell division and identity [14]. This also translated into improved motor coordination function in the animals.

With growing evidence that EVs such as exosomes might stimulate regeneration or modulate pathological conditions, there is a good rationale for pursuing the development of EVs as new potential therapeutic agents.

## Glossary

**Downstream processing:** the manufacturing steps after cell culture, which typically involve recovery, purification, washing, concentration, and formulation of the product.
**Dynamic bioreactor systems:** bioreactors that use agitation to ensure adequate mixing and mass transfer compared with static systems.
**Exosome:** an EV that is created in MVBs and then released from the cell into the extracellular environment via exocytosis.
**Extracellular vesicle (EV):** membrane-enclosed package of material that is generated via several distinct biological pathways.
**Flask bioreactors:** a modified form of cell culture flask with advanced functions, such as the separation of the liquid and air phases or compartmentalization to collect secreted products using membrane technology.
**Hollow-fiber bioreactor:** a 3D bioreactor that uses parallel bundles of semipermeable capillaries that allow the transfer of nutrients and gases to the cells residing in the extracapillary spaces.
**Impellers:** a rotating blade or paddle in a bioreactor that agitates the culture medium to ensure even mixing and distribution of nutrients.
**Mass transfer:** the net movement of mass from one place to another.
**Oxygen sparging:** introducing oxygen bubbles into the bioreactor to dissolve oxygen in the culture medium.
**Packed bed technologies:** bioreactor technologies that use a tube filled with particles that act as a physical substrate for cell attachment and growth. They allow perfusion of the culture media to distribute nutrients and oxygen.
**Shear stress:** a force experienced by cells in a bioreactor due to the flow of culture medium parallel to their surface.
**Tangential-flow filtration (TFF):** a method for separating and purifying biomolecules whereby the solution is passed tangentially across the filtration membrane rather than directly at it.
**Transmission surface plasmon resonance:** a technique commonly used in microfluidic applications that can detect adsorption of biological material to metal surfaces.

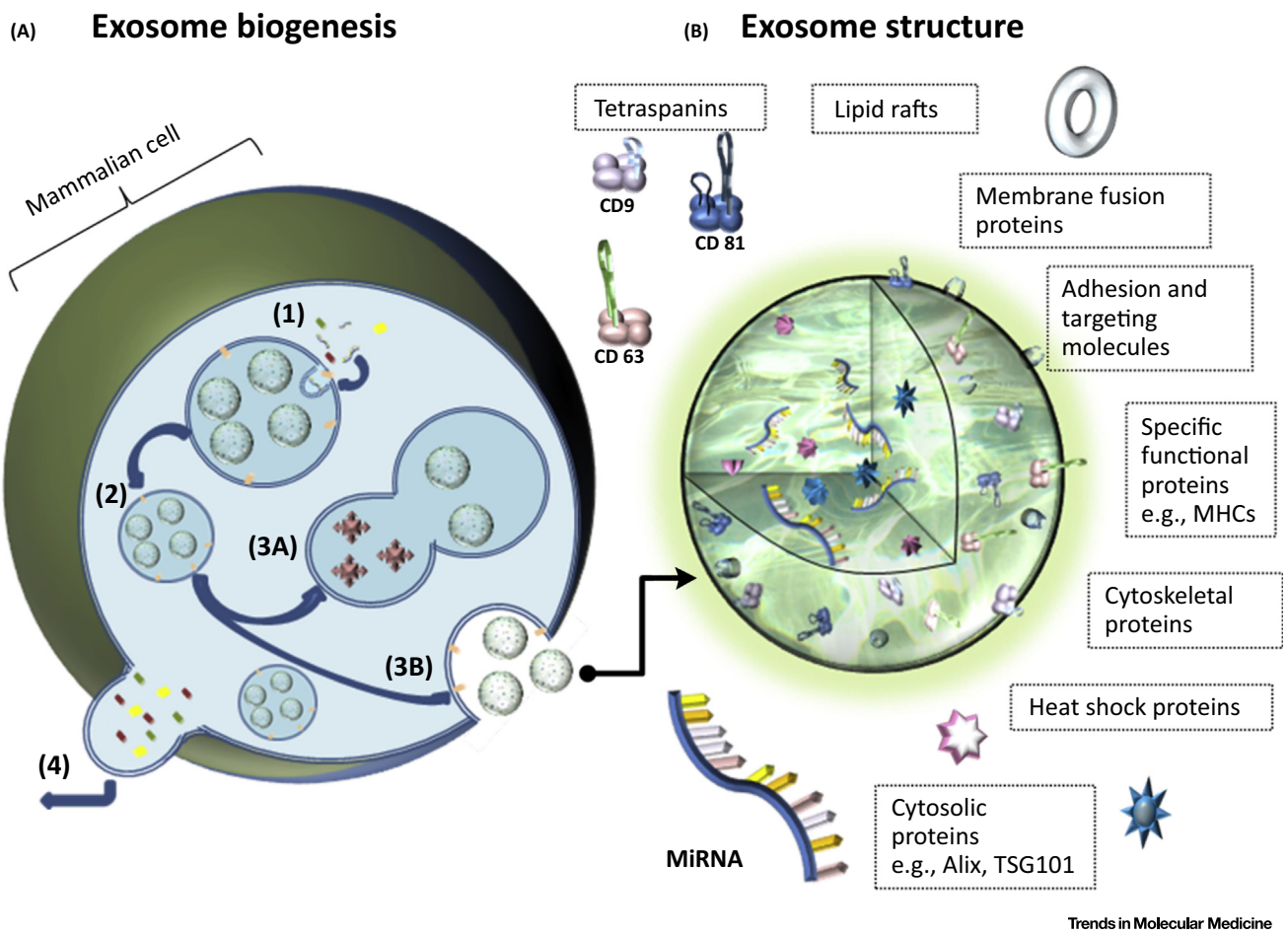


Figure 1. Exosome Biogenesis. Exosome biogenesis (A) begins when multivesicular bodies (MVBs) (1) bud inwards to form intraluminal vesicles (ILVs) that are loaded with genetic material and proteins (2). Next, MVBs fuse either with lysosomes (3A), which results in proteolytic degradation of exosomal contents, or with the plasma membrane (3B), resulting in the release of ILVs, now referred to as exosomes, into the extracellular environment. Nonexosomal vesicles bud directly from the cell membrane (4). Exosomes are typically in the size range of 20–150 nm and their structure (B) is complex. Tetraspanins (e.g., CD81, CD63, and CD9) and other transmembrane proteins, such as adhesion receptors, are present at the surface, while, internally, the cargo comprises an array of proteins (cytosolic, cytoskeletal, and growth factors) and miRNAs that convey specific functional cues.

However, exosomes are yet to be clinically validated because only a handful of studies have either been undertaken or are currently ongoing. These include the use of autologous, modified dendritic-derived EVs for maintenance immunotherapy [32,33], allogeneic MSC-derived EVs for the treatment of chronic kidney disease [34], type 1 diabetes mellitus (clinical trial NCT02138331), acute ischemic stroke (clinical trial NCT03384433), and autologous plasma-derived EVs for cutaneous wound repair (clinical trial NCT02565264). In addition, a single patient with graft versus host disease was treated with allogeneic MSC-EVs [35]. Existing data from these trials indicate that exosomes may have potential therapeutic value in several indications without having necessarily met the primary trial endpoint. However, across this small number of studies, a variety of purification methods were utilized, including filtration, ultracentrifugation, and PEG precipitation, which could impact the consistency of the final products. For example, across 32 preparations of exosomes generated for clinical use and purified using ultrafiltration/diafiltration followed by ultracentrifugation, the final exosomal protein quantity ranged from 99 to 26 648 μg [33].

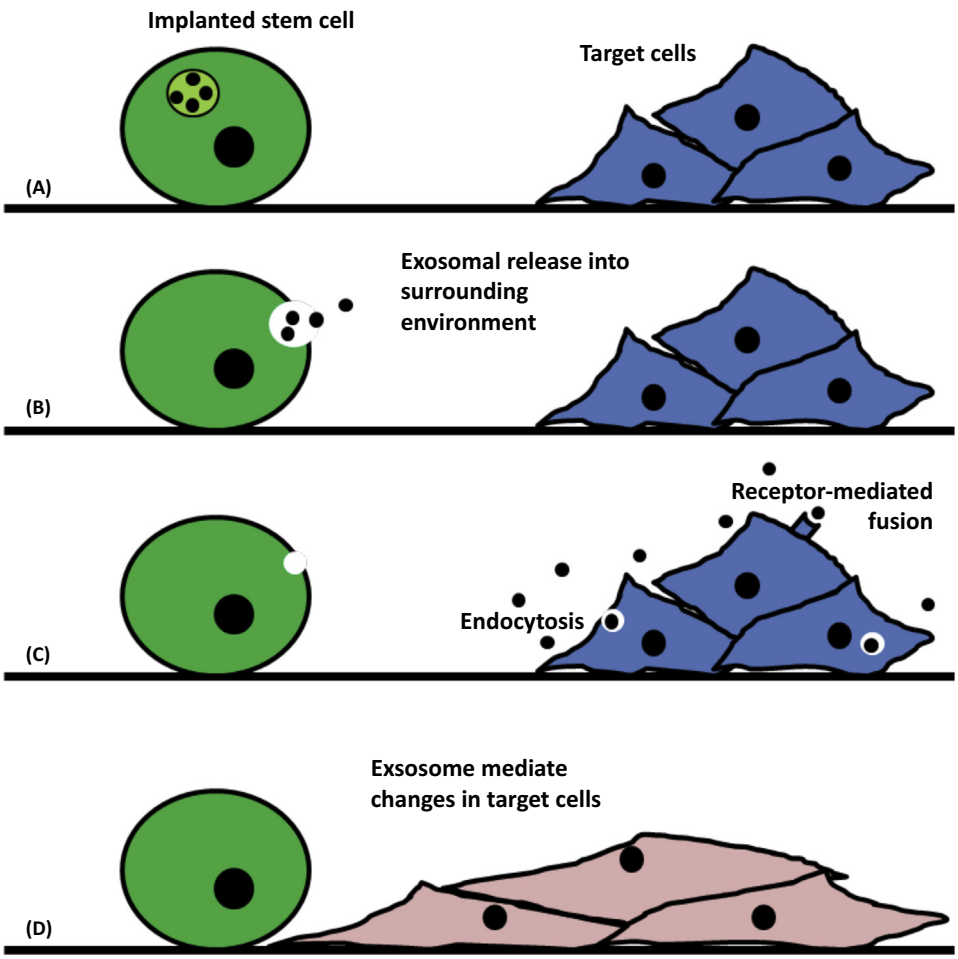


Figure 2. Exosomes and Stem Cell Transplantation. Diagrammatic representation of exosome function after *in vivo* stem cell transplantation. Implanted stem cells synthesize exosomes that convey functional characteristics of parental cells (A). Exosomes are then released by stem cells into the surrounding environment (B) and induce functional responses in adjacent resident tissue cells (C) that can modify their behavior, even resulting in sustained regenerative responses (D) after the stem cell has perished or exited the injury site.

Therefore, we hypothesize that only when manufacturing challenges have been addressed will it be possible to create greater consistency in the final product to advance these therapies into the clinic; the sooner these manufacturing challenges are addressed in the product development cycle, the faster patients may have access to them.

Broadly, these challenges may include: (i) a detailed characterization of exosome material to define target product attributes, including discrimination of nonexosomal artifacts and even exosome subpopulations; (ii) scalable cell culture methods for upstream production of exosomes; and (iii) scalable **downstream processing** for the isolation and purification of exosomes.

## Limitations of Cell Culture

Exosomes are secreted products of cells; thus, their manufacture is dependent on the ability to produce large quantities of cells in ways that do not alter the cell phenotype. Cellular changes

due to transitioning from conventional bench-scale cell culture using planar T-flasks to scalable cell culture platforms might alter the composition and function of the exosomes. Large-scale stem cell cultures are still a rate-limiting step for delivering stable and potent products at Phase III and on a market scale due to high development costs and regulatory and market uncertainty [36–41]. Accordingly, the opportunities for producing large quantities of stem cell-conditioned medium with which to undertake meaningful scale-up studies on exosome production are limited [42]. This was evident in a worldwide survey that showed that 77% of respondents used less than 100 ml of starting material, despite 83% of researchers using material generated from cell cultures [43].

Research efforts into scaling up cell culture have focused on technologies to maximize surface area, such as microcarriers in stirred bioreactors [44,45] or **hollow-fiber bioreactors** [46], which offer greater process control (Figure 3). The main technical limitation of these technologies is the need to control environmental parameters within the reactors, such that the phenotype of the cell (and derivative exosomes) does not change. When moving from static, planar cultures to dynamic, well-mixed 3D environments with high force generation (**impellers**, cavitation of bubbles from **oxygen sparging**), the risk of phenotypic alterations at the cellular level due to **shear stress** is still an issue. For example, T cell expansion was reduced when

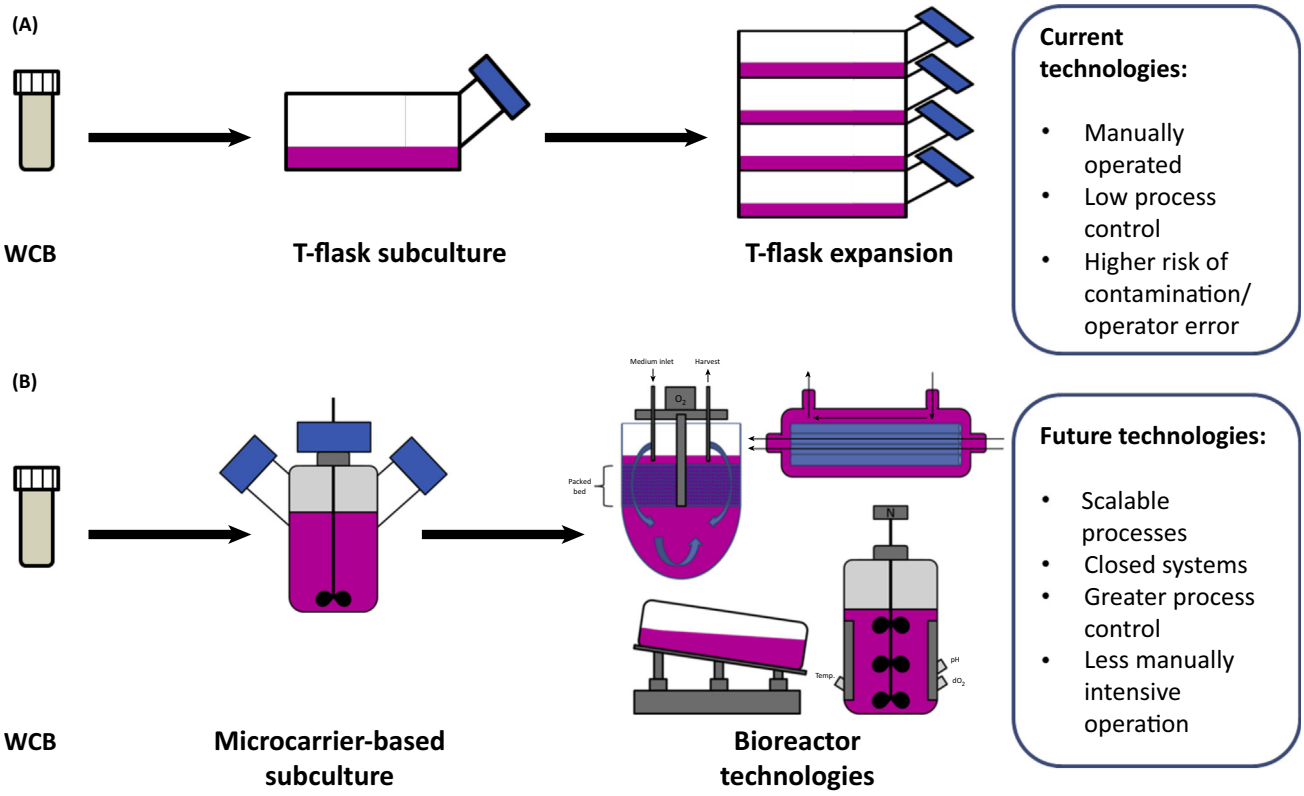


Figure 3. Upstream Processing of Stem Cells. Schematic showing the current laboratory scale methods used for upstream processing of stem cells (A). Cells are retrieved from the patient or from a working cell bank (WCB) and expanded predominantly using a T-flask platform. This leads to several significant pitfalls associated with current technologies. Development of new upstream processing is necessary (B) to scale up the production of large quantities of cells from the WCB and, therefore, large quantities of exosome product that can be made in a closed bioreactor system and with greater process control.

agitated at 180 rpm in bioreactors as a consequence of rapid downregulation of the interleukin-2 receptor [47]. In the case of MSCs, a prominent cell type for the production of candidate therapeutic exosomes, shear stress was found to induce mechanotransduction pathways involving p38 mitogen-activated protein kinase and extracellular signal-related kinase, which could lead to osteogenic differentiation [48]; these outcomes would likely change the exosome product, although this remains to be directly demonstrated.

At the extremes of an operating window, limiting cell death in these high-shear systems to minimize impurities derived from apoptotic blebs is of paramount importance. Apoptotic blebs overlap in size and might increase heterogeneity as well as reduce the potency of exosome products [49,50]; an example of this heterogeneity was shown in a study conducted on dendritic cell-derived apoptotic vesicles and exosomes, which showed that exosomal fractions had their own unique molecular composition and properties [51]. They might even induce undesirable cell signaling events, although this warrants further investigation.

Given that cells produce and secrete exosomes naturally, perfusion-based cultures (for example using hollow-fiber and **packed bed technologies)** should also be considered with the aim of providing adequate **mass transfer** in the cell culture. A key practical benefit to this approach is that these reactor systems can be designed and optimized to retain the exosome product within the culture compartment to yield a more concentrated conditioned medium, thereby reducing liquid handling requirements further downstream [52]. Here, there have been developments using novel **flask 'bioreactors',** such as the Integra CELLine systems [53], which can concentrate exosomes within a membrane compartment, allowing transfer of medium components over a prolonged period of time in culture. A caveat is that these flasks are still limited to being a scale-out approach and the harvest window is time limited because cells can undergo contact inhibition and changes in behavior at high densities; indeed, one particular study revealed that mouse adipose MSCs plated at a high seeding density (90%) exhibited altered expression of various genes important for cell-to-cell contact inhibition within 48 h [54]. However, if exosomes can be harvested before cultures are overconfluent, they might better conserve product parameters, given that, unlike **dynamic bioreactor systems**, these flask bioreactors may provide a similar mode of culture to planar T-flasks.

An additional limitation for scaling up cell cultures to produce exosomes is the continued heavy reliance on animal serum for optimal cell growth. For example, fetal bovine serum (FBS) is high in endogenous exosomes [55] and, if not removed before cell culture, process-related impurities stemming from FBS contaminants may make their way into the final drug product, which, from a regulatory standpoint for an injectable, is completely unfavorable. Therefore, xeno-free culture media components are desired, provided that they conserve comparable cell characteristics and exosome product attributes that might be expected to be of therapeutic grade. However, this task is not trivial. At the very least, exosome-depleted FBS should be characterized as a means of confirming that the stem cell-derived exosomes truly confer the functional properties ascribed to them.

A recent study on exosome production further highlighted the importance of culture reagents, notably FBS-containing versus serum-free medium. Specifically, both human and mouse neuroblastoma lines showed that switching from FBS-containing to serum-free medium left the resultant exosomes from both species unchanged in terms of biophysical and size characteristics [35]. However, the number of exosomes generated was increased when using serum-free reagents. While this may appear promising, further proteomic analysis showed that the serum-free exosomes contained reactive oxygen species and stress-related proteins,

whereas exosomes derived from cells cultured in serum-containing medium promoted higher levels of RNA-processing proteins. As a result, the switch from FBS-containing medium to serum-free medium appeared to cause a shift in exosomal biology, reflecting stress-induced phenotypic changes in culture [56]. These data illustrate how important culture conditions are in the manufacturing process, given that such changes can significantly modify the exosome product profile, which might in turn result in failure at the regulatory and/or clinical levels.

One significant advantage of generating exosomes as products, rather than using parent cells, is that the exosome-rich conditioned medium can be separated with ease from producer stem cells, because these cells are typically adherent. This overcomes one of the main challenges for adherent cell products, which need to be enzymatically detached from microcarriers and where harvesting and recovery are achieved with limited efficiency; this is partly due to the need for cell conservation while ensuring that damage from extended enzyme exposure is minimized [57]. Furthermore, with the advances in cell engineering and medical research, one may expect in the future more economically viable, exosome dedicated cell lines that might provide high expression of tailor-made exosomes.

## Downstream Processing for Efficient Purification

There are also significant downstream processing challenges to manufacturing exosomes. First, methods currently used to enrich exosomes from cell culture media are grandfathered in from the early viral purification industry, and operate via physical discrimination of target material from impurities. Here, four main isolation methods are used: size exclusion (based on typical exosomal diameters); sedimentation force or flotation density; (nonspecific) precipitation-based methods; and affinity-based capture.

The most commonly used method has historically been ultracentrifugation [43,58]. Two main variations of ultracentrifugation are used. The first uses a combination of different centrifugal forces to reduce contamination by cell debris and/or fragments (3000–10 000 g), then organelles and nonexosomal vesicles (10 000–20 000 g), before a final pellet of the exosomes is produced (100 000–120 000 g). The second discriminates exosomes from other vesicles via flotation using density gradients made from deuterium oxide ($D_2O$)/sucrose cushions or commercially available reagents, such as iodixanol [59]. Despite these protocols, co-isolation of nonexosomal vesicles and other particulate debris that share similar size and density is still observed.

From a manufacturing perspective, while it has been used to purify vaccines at commercial scales [60], ultracentrifugation has many limitations, which have seen a reduction in usage for alternate methods, such as filtration or chromatographic separation [61]. The reasons for this, which may also be applied directly to the future of exosome processing, are largely due to the high level of skill and manual labor required (gradient generation, sample balancing, and pellet resuspension, all of which must be performed to high levels of precision), the time-intensive nature of the processes, the associated costs of reagents and equipment, and the observations of losses in potency of labile products.

There are also significant limitations in interpreting process efficiency between different laboratories using different centrifuges. Indeed, exosome pelleting efficiency is dependent on several parameters defined by the centrifuges themselves (e.g., *k*-factor or rotor type), meaning that processes are only readily transferable if identical equipment and protocols are used [62,63]. When coupled with processing times that can extend to 72 h for routine small-scale operations, it is understandable that alternative process options have phased out ultracentrifugation in the viral and/or vaccine industries whenever possible.

As with any form of biologic manufacturing, any reagents added during the process need to be removed from the final product and, therefore, additional considerations must be given for adequate clearance of substances used, such as $D_2O$ or sucrose cushions. This leads to a requirement for additional pelleting steps, which increases operating costs, purification times, and product losses due to process inefficiencies and aggregation [64], not to mention losses in biological activity [65]. To address manufacturing and regulatory uncertainty here, further advancements are needed.

Nonspecific precipitation, typically using polyethylene glycol (PEG)-based solutions, is an alternative method to sediment exosomes without the need for expensive ultracentrifuges. This method can sediment exosomes at lower centrifugal forces (around 20 000 g), which can then be loaded into size-exclusion columns, although currently these columns are only commercially available as manually operated kits. However, these technologies may not be appropriate for larger scale production. By way of illustration, the large pore sizes of the resins used will likely present challenges related to pressure limitations and compression at larger scales. Moreover, the added need to remove PEG from the endproduct, especially for injectables [42], may require further processing and, therefore, ultimately, product losses. One study showed that it was possible to make columns rather than rely on kits and, because similar levels of purification were achieved, the convenience of the kits far surpassed that of the columns [66].

Another concern with these sedimentation processes is the co-isolation of nonexosomal vesicles, which can overlap in characteristics. These impurities must be identified, and be sufficiently depleted in a therapeutic to minimize safety risks to patients. Critically, one may also wish to enrich an exosome subpopulation to increase the efficacy of a therapeutic, which, with current technologies, is not possible using nonspecific precipitation and sedimentation alone.

Recently, there has been an increase in the use of **tangential-flow filtration** to concentrate exosomes from cell-culture media based on their size [42,67–69]. This process is more promising than the sedimentation methods listed above, due to tight and reproducible size distributions and the ease with which processes can be scaled and can facilitate product washes and buffer exchanges [52]. This makes tangential flow filtration attractive as a primary recovery method. Moreover, hollow fiber ultrafiltration coupled with microfiltration is a relatively gentle process that retains the structural and functional integrity of exosomes while enabling the removal of large particles and cell culture-derived proteins [61].

However, there are some issues, because ultrafilters are expensive and the co-isolation of material, such as serum proteins and DNA, from cell culture continues to be problematic. Excessive fouling leading to elevated pressure in the system, and consequent associated shear forces, could also be detrimental to the final preparation and must be carefully monitored.

All of the above downstream processing techniques are based on physical parameters and none have a way of completely discriminating exosomes beyond either size or density. This often leads to the co-isolation of nonexosomal vesicles or organelles with overlapping physical characteristics, resulting in insufficiently pure exosome preparations. This was revealed by comparing density gradient and standard ultracentrifugation to an immunoaffinity capture method, because the latter increased exosome-associated proteins by at least twofold relative to the ultracentrifugation approach [70]. This would become particularly troublesome if large-scale culture systems that lead to higher rates of cell death were to be used in the future. A remedy to this potential burden would be the development of scalable processes that use

methods of purification orthogonal to the current physical methods (i.e., which use the biochemical and biophysical characteristics of exosomes to discriminate from impurities via more selective processes). This need for reproducible and standardized platform technologies in the industry become apparent when literature searches for exosome purification yield varied and almost conflicting results with regards to which protocol is the most promising. Taking ultracentrifugation as an example, huge differences in efficiency of exosome recovery are reported across research groups [70–72] compared with commercial kits and affinity-based purification methods. In one such study, lab-scale commercial kits processing human serum samples up to a volume of 5 ml isolated an 80–300-fold higher yield of exosomes compared with ultracentrifugation [51].

Of the reported methodologies for exosome purification, immunoaffinity methods are perhaps the most promising but least reported to date in the literature [43]. The method often cited is based on antibody-conjugated magnetic beads, which can be used to pull out exosome populations from crude material. A study comparing exosome recovery from a human colon cancer cell line (LIM1863) using different methods [70] revealed that exosomes captured via immunoaffinity were superior in terms of expression levels of known exosomal markers, compared with ultracentrifugation and differential centrifugation. Moreover, the vesicles were more homogeneous (40–60 nm diameter) compared with those from ultracentrifugation (40–100 nm) and differential centrifugation (50–100 nm). Moreover, immunoaffinity isolation enabled the identification of novel molecules [ESCRT-III component VPS32C/CHMP4C, and the SNARE synaptobrevin 2 (VAMP2)] in exosomes for the first time [70]. This shows the potential of immunoaffinity isolation in terms of product characterization and isolation.

However, there are limitations when using beads. In the current format, scaling up becomes increasingly burdensome because mixing, mass transfer, and removal of beads via magnetic separation are achieved with limited efficiency at the larger scales and also requires specialist equipment [73]. However, the use of these beads at the laboratory scale suggests their potential in large-scale processing if the issues surrounding the introduction of process impurities are successfully overcome.

Realistically, the use of affinity methods is likely to be more economical and simpler to facilitate if antibodies are immobilized onto stationary phases, because, with a stationary phase, there is less opportunity for particulate impurities to be introduced, unlike those typically seen with beads. As such, further development of chromatographic steps that facilitate the specific capture of exosomes (or their subtypes) may be important.

Another chromatographic method shown to be effective in separating exosomes from other process impurities based on their characteristic negative charge is ion-exchange chromatography. A recent study demonstrated the applicability of chromatographic purification by use of a quaternary amine (QA) anion exchange column (AEx column) against the sucrose density gradient separation of amniotic fluid-derived exosomes [74]. The results indicated that the quality of the exosomes was superior from anion exchange purification over the more classical ultracentrifugation technique in terms of soluble impurity removal and the separation of CD marker-positive and -negative exosomes [74].

However, we posit that optimization of process conditions on a case-by-case basis is necessary because ion-exchange chromatography may still co-elute host cell DNA and albumin if improperly implemented, and be likely masked by the broad elution peaks of heterogeneous exosomes.

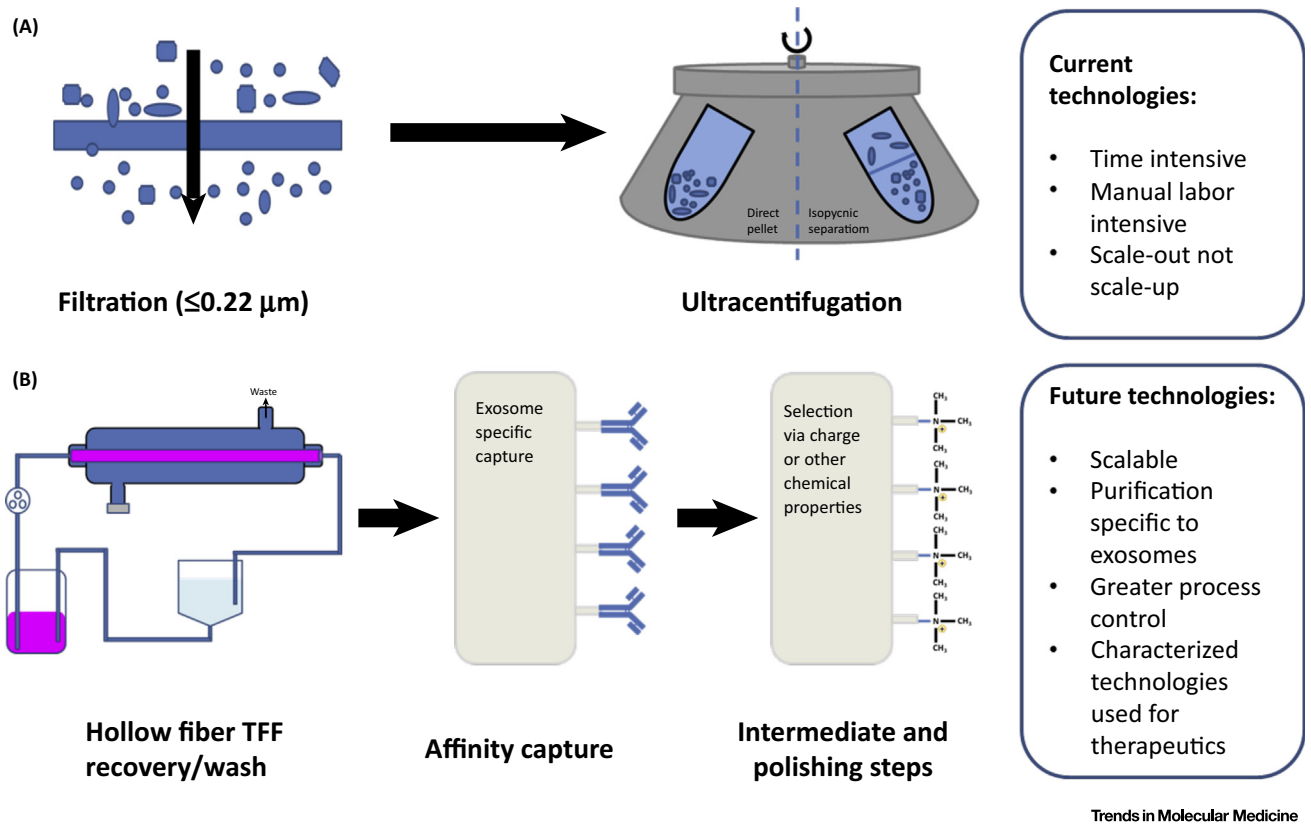


Figure 4. Downstream Processing of Stem Cells. Diagrammatic representation of the current laboratory-scale methods used for downstream processing of stem cell-derived exosomes (A). Crude conditioned media is concentrated using filtration and then ultracentrifugation methods are used to isolate exosomes on the basis of size and density. Future processing needs to be scalable and so tangential flow filtration (TFF), followed by affinity capture and final polishing steps, are most promising approaches for the delivery of high-purity exosome therapies (B).

A potentially beneficial advancement for chromatography would be to shift from traditional packed bed systems, which may not be appropriate for such particulate heavy feeds, to membrane or monolithic technologies with more open-pore structures that can accommodate exosome material while retaining separation power and increasing throughput. Increased throughput may be possible because higher flow rates can be used; this approach has already been adopted in the virus industry [75].

In principle, currently used methods for purifying exosomes need to be replaced with advanced platforms (Figure 4) and an ideal process for exosome purification should include a sequence of steps that comprises filtration-based recovery followed by chromatography-based purification; filtration-based recovery and concentration would deliver a product of defined size distribution and reduce the vast quantities of conditioned medium into a lower volume that is easier to process. Tangential flow filtration is a good candidate and has been shown to concentrate MSC-secreted exosomes up to 125-fold [30]. Further evidence for this shift in technologies is supported by a study where ultrafiltration and liquid chromatography (UF-LC) steps (in this case, size exclusion chromatography using Sephacryl columns) were tested against differential ultracentrifugation [69]. The results showed significantly higher yields using the UF-LC method relative to differential ultracentrifugation, without compromising the proteomic identity of EVs, while also showing that the biophysical properties were preserved. The authors also observed

an improved biodistribution of the EVs when injected into mice: fewer EVs accumulated in the lungs, likely due to the reduction of aggregation and damage to the exosomes during the UF-LC steps compared with ultracentrifugation [69].

We propose that sequential filtration followed by affinity-based chromatography that targets EV-specific surface proteins (e.g., CD81) offers the best chance of success in clinical development; the chromatographic steps should deplete non-EV DNA and culture medium-derived proteins, and finally, via the buffer exchange steps, allow washing and concentration of the product before formulation and secondary manufacture.

## The Analytics Challenge

Without doubt, advances in upstream cell culture and downstream processing will advance exosomes towards routine manufacture. However, equally critical, and underpinning these advances, is the capacity to measure and characterize the exosome product better than is currently achieved. It will be easier to address the process development and scale up of exosome products if the process is guided by a robust, regulatory accepted definition of what it is. The exosome community has already taken significant steps to provide a broad definition for exosomes and provide criteria for their identification. The International Society of Extracellular Vesicles (ISEV) has established a set of criteria for the proteomic identification of exosomes with a minimal list of requirements [76], namely, exosomes should (i) have transmembrane proteins to provide evidence of a membrane (e.g., tetraspanins, such as CD63, CD81, and CD9) [65,77]; (ii) have cytosolic proteins to provide evidence of membrane- or receptor-binding capacity (e.g., TSG101, Rab proteins, or annexins); (iii) be free of protein impurities from intracellular compartments not associated with the plasma membranes or endosomes (e.g., endoplasmic reticulum, Golgi, mitochondria, or nucleus); and (iv) be free of co-isolating extracellular proteins, such cytokines and serum components.

These, in combination with physical observations via electron microscopy and particle size distribution analysis, create a useful baseline. However, ultimately, more detailed characterization must be undertaken to describe exosomes in terms of functional capacity by mechanistically defining the action of key nucleic acid and protein signals on target cells, and by understanding exosome heterogeneity. In addition, if possible, mapping exosome subpopulations to define those harboring higher potency and/or defining unique components not present in other exosomes would be ideal. For example, a larger exosome may contain larger quantities of certain RNAs or proteins, or a smaller exosome may have a higher density of surface markers. Furthermore, assays need to be developed that detect exosomes with higher reliability and accuracy than at present. Examples of such steps have already been seen in the literature, for example using flow cytometry, which can enable the detection and semi-quantitative analysis of specific markers [78], as well as microfluidic tools allowing rapid sensing of exosomes using immunomagnetic capture targeting exosome markers, such as CD63 [58]. We posit that this microfluidic approach even offers the potential for development of in-line measurement technologies that can monitor exosome production during cell culture as a label-free surrogate measurement of the cells, and using exosome identity as a surrogate for cell identity and state. It might even be possible to isolate exosomes themselves using label-free tools. For example, microfluidic devices have been developed that utilize **transmission surface plasmon resonance** [59] or acoustic waves [60] to isolate exosomes from other vesicles and cells. While these tools may not fulfill the requirement of a large-scale purification platform, they might offer potential as label-free methods to isolate exosomes that can be subsequently characterized (e.g., via arrays of antibodies for on-chip profiling of exosome surface proteins) [59].

Finally, *in vitro* potency assays need to truly predict outcomes *in vivo*, which in turn will feed back to evolving product specifications to enable the development of exosomes as potential therapeutic agents.

## Viral Co-Isolation: A New Challenge on the Horizon?

In terms of product safety, as a therapy that is derived from mammalian cells, there is also the risk of co-isolating endogenous viruses. Naturally, if the live cells are being used as a therapy in their own right and the exosomal product is a secondary product, the screening of adventitious agents, such as viruses, would be a prerequisite and would lower risks of high titers entering the final product. Conversely, there is a risk that what is passably low, unobservable, or unscreened in a cell could be highly concentrated by downstream processing steps, many of which would be similar to those used for viral vaccine production (filtration, ultracentrifugation, precipitation/size exclusion, and even chromatographic technology if less-specific methods are used), due to the similarities in size and physical properties between viruses and exosomes. Furthermore, if, for example, dedicated cell lines for the production of exosomes for drug delivery or gene editing are created, mimicking recombinant protein and antibody production systems, one may find that proof of viral removal is absolutely necessary. At this point, one must scrutinize the current technologies available and find methods where an exosome may be separated from any viruses that may be present.

Issues surrounding this are apparent given that exosomal and viral identities are similar: the size ranges often overlap (thereby making viral filtration an unviable option) and, because both entities essentially comprise functional genetic material and surface proteins, chemical inactivation could damage the exosome as much as the virus in terms of disruption to functional surface proteins [79]. A common method of inactivation is that of exposure to low pH (3), typically during a chromatographic step: however, this method risks damaging exosomal surface proteins, or, if not strongly bound to the column, eluting the product altogether. Similarly, other techniques, such as ultraviolet (UV) inactivation, which aim to disrupt the nucleic acid sequences for viral attenuation, could also irreparably damage the exosome product. This poses a further challenge on the analytical spectrum because, even if exosomes could be shown to be taken up in *in vitro* quality control assays (due to the lack of damage to surface proteins), any damage to the internal genetic material may cause them to perform with limited or null activity biologically, which reinforces the need for suitable potency assays.

More complex procedures, such as heat treatment options, including pasteurization, dry heat, and vapor heat, can also be used for viral inactivation; however, while a single protein could be protected sufficiently by a protein stabilizer (also slightly protecting the virus) due to the size and make-up of exosomes, finding a way to maximize exosomal function while sufficiently removing virus might also be difficult, especially when taking into account the relatively complex optimization and implementation of these processes compared with UV or pH inactivation [80].

## Concluding Remarks

Exosomes are promising new candidate therapies and the recent explosion in research into their biology and function has caused global excitement. With several prominent preclinical studies showing potent effects of exosomes, in addition to emerging early clinical data, it is timely to address the bioprocessing challenges that underpin the manufacture of exosomes and other EVs. While phenomenal progress has been made in understanding the biological properties of exosome cargo, research must also focus on challenges related to achieving regulatory approval and their potential translational into the clinical setting.

### Outstanding Questions

How will researchers address the large-scale production of therapeutic exosomes from adherent cells that are challenging to adapt to scalable bioreactor technologies?

Are there subsets of exosomes that, when separated from others, can provide more potent products and that can additionally aid process development?

How can researchers produce exosome-specific multiplexed assays that combine measurements of size and surface protein expression, both of which individually can be mimicked by impurity background noise? Is there a way to specifically measure only exosomes?

What are other regulatory issues that may preclude the current methodologies from being clinically acceptable from a regulatory stand point?

Can researchers accurately predict how cell-culture impurities will change when scaling up in more dynamic systems or when moving to xeno-free culture systems, and have they done enough to ensure the removal of higher levels of those impurities that are undetectable at the small scale?

Box 1. Clinician's Corner

Exosomes are cell-secreted vesicles containing bioactive proteins and genetic material. Their specific cargo is reflective of the parent cell, and gives rise to their therapeutic effects.

Stem cell-derived exosomes have potential for use as drug candidates for a range of indications. However, to achieve this potential, scalable manufacturing processes are needed, both upstream and downstream.

Upstream processing needs to include scalable cell culture that can produce large quantities of secreted exosomes. Current bioreactor technologies are designed for suspension-adapted cells that are used to make antibodies or recombinant proteins. They are typically not suitable for the scalable expansion of adherent cells.

Downstream processing needs to transition from traditional ultracentrifugation methods to combinations of filtration and chromatographic-based methods that can achieve consistent and reproducible purification at scale.

Manufacturing science needs to be addressed early in the product development cycle so that exosomes can achieve a status as routine therapies more quickly and cost-effectively.

The most promising manufacturing approach to make in the first instance may be adoption of an advanced purification platform based on a two-step filtration-chromatography approach that can enable scalable and pure exosome products to be created. There are still many questions and hurdles to overcome (see Outstanding Questions and Box 1) to deliver exosomes as a new putative therapeutic tool for healthcare. These challenges will come in many forms: from scheduling and batch reproducibility, to process robustness and economic feasibility, along with thoroughly defining meaningful critical quality attributes for the product itself. It is vital that these issues are investigated fully in parallel with clinical validation studies before it will be possible to deliver exosomes to the clinic and to the patients who might benefit from them.

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