Molecules and Cells

Minireview

Manufacturing Therapeutic Exosomes: from Bench to Industry

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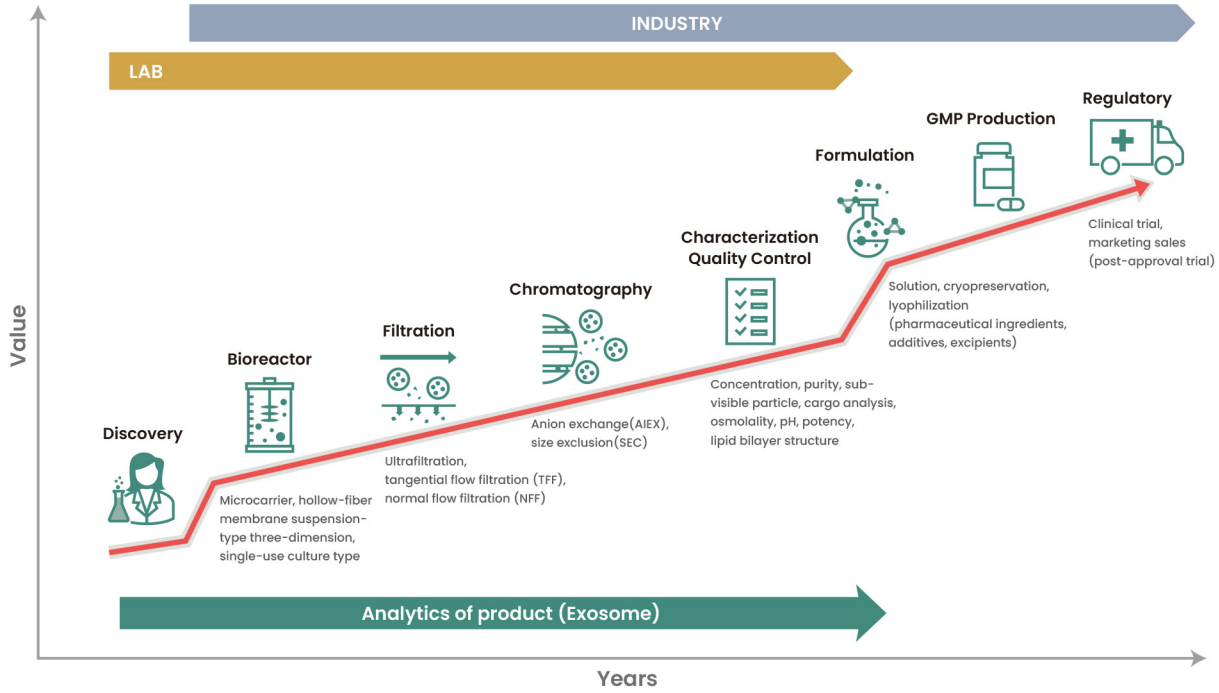
https://doi.org/10.14348/molcells.2022.2033
www.molcells.org



Process of manufacturing therapeutics exosome development for commercialization. The development of exosome treatment starts at the bench, and in order to be commercialized, it goes through the manufacturing, characterization, and formulation stages, production under Good Manufacturing Practice (GMP) conditions for clinical use, and close consultation with regulatory authorities.

Received 23 November, 2021; revised 8 February, 2022; accepted 14 February, 2022; published online 25 April, 2022

eISSN: 0219-1032

Exosome, a type of nanoparticles also known as small extracellular vesicles are gaining attention as novel therapeutics for various diseases because of their ability to deliver genetic or bioactive molecules to recipient cells. Although many pharmaceutical companies are gradually developing exosome therapeutics, numerous hurdles remain regarding manufacture of clinical-grade exosomes for therapeutic use. In this mini-review, we will discuss the manufacturing challenges of therapeutic exosomes, including cell line development, upstream cell culture, and downstream purification process. In addition, developing proper formulations for exosome storage and, establishing good manufacturing practice facility for producing therapeutic exosomes remains as challenges for developing clinical-grade exosomes. However, owing to the lack of consensus regarding the guidelines for manufacturing therapeutic exosomes, close communication between regulators and companies is required for the successful development of exosome therapeutics. This review shares the challenges and perspectives regarding the manufacture and quality control of clinical grade exosomes.

Keywords: characterization, exosome, manufacturing, purity, quality control

INTRODUCTION

In recent decades, the field of nanomedicine has experienced rapid advances in therapeutic applications (Mitchell et al., 2021). Among the novel nanoparticles, extracellular vesicles (EVs) (de Boer et al., 2021) have gained considerable attention for their role as delivery vehicles for bioactive molecules to recipient cells (Nam et al., 2020; Zhao et al., 2020). EVs are lipid vesicles secreted by almost all cell types and are categorized based on their biological properties and biogenesis pathways (Raposo and Stoorvogel, 2013; Thery et al., 2018; 2002). Exosomes, or small EVs, are composed of a phospholipid bilayer, with sizes ranging from 50 to 200 nm. They are generally termed lipid vesicles and are generated by inward invagination from endosomes (Pegtel and Gould, 2019). Microvesicles (or ectosomes) are formed by direct outward budding of vesicles from the plasma membrane, with sizes ranging from 0.1 to 1 μm (Doyle and Wang, 2019). Apoptotic bodies are large EVs that encapsulate parts of dying cells, such as intact organelles, micronuclei, and chromatin remnants (Battistelli and Falcieri, 2020). Exosomes, a type of EV, are intercellular communication messengers that carry various genetic or bioactive molecules. Therefore, exosomes have received immense attention from academia and the drug industry as a new paradigm of drug delivery. Currently, approximately 50 clinical trials have assessed the safety and efficacy of either naïve or engineered exosomes across different therapeutic areas.

The establishment of appropriate cell culture and exosome purification methods requires careful consideration, since exosome quality and productivity could be greatly affected by the conditions of parental cells and exosome purification methods. In addition, thorough exosome quality control is needed to minimize batch-to-batch variance due to EV heterogeneity. In this review, we discuss the major challenges regarding therapeutic exosome manufacture and the perspectives for successfully developing clinical-grade exosomes.

UPSTREAM CELL CULTURE

To date, exosomes have been produced5 from stem cells or immune cells in clinical trials, including bone marrow mesenchymal stem cells, adipose tissue-derived stem cells, and monocyte-derived dendritic cells (Mendt et al., 2019; Näslund et al., 2013; Zhang et al., 2020a). A flask-based static system in the cell culture chamber can be used for lab-scale culture of these originator cells; however, bioreactor systems, such as microcarriers, hollow-fiber membranes, and microfiber-bed types, are commonly used for large-scale culture (Chen et al., 2011; Valkama et al., 2018). Attempts to increase culture capacity are ongoing; however, increasing the productivity of exosomes generated from adherent cells, such as stem cells, is very limited.

In addition to stem cells and immune cells, the HEK293 cell line, which is commonly used for the production of therapeutic proteins, has also been used to produce exosomes for therapeutic use (Malm et al., 2020; Song et al., 2021; Yim et al., 2016). Compared to stem cells, large-scale culturing conditions for HEK293 cell lines are relatively well established, such as the appropriate dissolved oxygen (DO) level, pH, temperature, and culture media type. Additionally, genetic manipulation of HEK293 cells is relatively easy, which is advantageous for exosome engineering. Although the original HEK293 cell line is an adherent cell line, numerous HEK293-derived cell lines, such as 293-F, 293-H, freestyle 293-F, and Expi293F, have been established through media adaptation. Suspension cultures of 293-F and 293-H cell lines are currently used to produce biologics, such as rhFVIII (NUWIQ) and rFVIIIFc (ELOCTATE), respectively (Malm et al., 2020). Additionally, a three-dimensional stirred-tank bioreactor can be applied for the clinical- or commercial-scale bioproduction of exosomes. In practice, adherent-type two-dimensional and suspension-type three-dimensional cultures in both single-use culture systems are used to produce stable exosomes at a 1,000-2,000 L industrial scale (Cao et al., 2020; Thippabhotla et al., 2019).

DOWNSTREAM EXOSOME PURIFICATION

Exosome size ranges from 50 to 200 nm, and many exosome purification methods separate exosomes from various EVs based on their size and density. One of the most commonly used methods, normal flow filtration (NFF), a molecular weight cutoff system based on membrane pore size, utilizes vacuum or centrifugal forces to filter the culture media. NFF is often used in lab-scale early development to purify exosomes from cell culture media. However, its application to industrial-scale bioprocesses is highly limited due to prolonged processing time and limited scalability. In addition, these purification methods are inadequate for producing high-purity exosomes. Therefore, alternative purification steps that yield high purity are required for further drug development.

Exosome concentration

Ultracentrifugation (UC) is the gold standard method for separating exosomes from biological culture fluids. Exosomes can be separated from cell culture media between 100,000 and 200,000 × *g* by UC. Although UC is the most commonly used method for exosome separation in academia, it is not suitable for clinical trials because of its low productivity (Konoshenko et al., 2018). UC is often associated with incomplete sedimentation or aggregation of exosomes and contamination from macromolecules, such as cellular debris or proteins, which lower yield and purity (Konoshenko et al., 2018). Additional purification steps, such as microfiltration or sucrose gradient UC, can be used to increase exosome purity. However, these techniques are limited in their ability to produce clinical-grade exosomes for scaled-up manufacturing processes.

Tangential flow filtration (TFF), unlike NFF, uses a permeable membrane filter and tangential fluid flow to effectively concentrate materials of a specific size. TFF can concentrate exosomes from culture media with a higher recovery yield than UC by eliminating macromolecules or aggregates under 15 nm with significantly improved batch-to-batch consistency (Busatto et al., 2018). Many studies have compared UC and TFF in terms of their efficiency, quality, and applicability for exosome production (Andreu et al., 2016; Busatto et al., 2018; Lobb et al., 2015). Busatto et al. (2018) used MDA-MB-231 breast cancer cell culture media and showed that exosome concentration efficiency was 100 times higher with TFF than with UC (10^{10} EV/10^6 cells for TFF vs 10^8 EV/10^6 cells for UC), and the removal of albumin was improved 40-fold with TFF, compared to UC.

Other potential techniques to produce clinical-grade exosomes include the separation of EVs using super absorbent polymer (SAP) beads. SAP beads absorb small molecules, including water, via nanosized channels, but they expel and concentrate EVs (Yang et al., 2021). Yang et al. (2021) showed that SAP beads drastically enriched EVs with high purity in human urine and culture medium in a single step. Therefore, this method can be used to improve large-scale EV production and high-throughput analysis.

Exosome purification

Chromatography is widely used for exosome purification. Anion exchange chromatography (AIEX) is a chromatographic method, which uses the negative electrostatic properties of the exosome surface (glycan/phospho/sulfo group) to bind to an anion-exchange matrix for further purification and recovery. AIEX is a suitable option for scalable purification of exosomes with high purity (Kosanović et al., 2017). According to a previous report (Heath et al., 2018), exosomes from various cell culture media (HEK293T, H1299, HCT116, Expi293F, etc.) were efficiently separated and purified using a monolithic AIEX column using 250-765 mM NaCl. Exosomes purified with AIEX had a purity and yield similar to exosomes separated with UC without any impurities, such as bovine serum albumin, which were present in exosomes separated using ultrafiltration (Heath et al., 2018). In addition, efficiency was improved by reducing the time to separate and purify exosomes to less than 3 h, compared to UC, which takes a whole day. Another advantage of AIEX is its ability to remove non-ionic surfactants, such as poloxamer 188, polysorbate 20, and polysorbate 80, which are commonly used to enhance the solubility of media components in chemically-defined concentrated media (Martinelle et al., 2010). These non-ionic surfactants can also be further concentrated during ultrafiltration processes, such as TFF and diafiltration. We also confirmed that AIEX is effective in removing excess non-ionic surfactants from the concentrated cell culture medium during large-scale EV separation (data not shown).

Size-exclusion chromatography (SEC) is another chromatography method that is frequently used for EV purification. SEC can be used to address the limitations of protein contamination, exosome aggregation, and lengthy production processes of other methods. Böing et al. (2014) separated and purified exosomes from plasma using a Sepharose CL-2B column. Bind-elute SEC (BE-SEC) has recently been used to improve scalability by enabling loading up to a 100-fold increased volume of samples and significantly higher flow rates, compared with SEC. To produce clinical-grade exosomes, a combination of different separation/purification techniques should be used instead of a single-step method. Corso et al. (2017) proved that the combination of TFF and BE-SEC is more effective in purifying exosomes from myoblast C2C12 cell culture media than a single-step purification process. Oeyen et al. (2018) showed that urinary exosomes can be purified using the TFF-SEC combination method. ILIAS Biologics, a South Korea-based biopharmaceutical company that develops engineered exosomes using the EXPLOR® technology, also employs a combination of different separation/purification techniques to recover pure exosomes (Fig. 1).

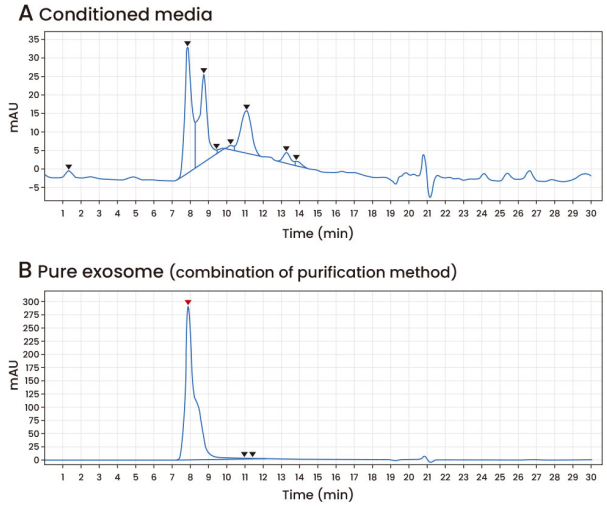


Fig. 1. Purity of EV. Chromatograms of EV in conditioned media (A) and after combination of purification method (B). The peak (red arrow) with a retention time of 7-9 min stands for the exosome particles, while the rest peaks are impurities of small size. For high-purity exosomes, various separation methods must be combined and purified.

EXOSOME QUALITY CONTROL

Many considerations should be made to optimize exosome quality, such as optimizing the originator cell line, culture and harvest conditions, separation and purification methods, and stability (Théry et al., 2006; Zhang et al., 2020b). In this section, we focus our discussion on the analytical methods and criteria for exosome quality control.

Analytic methods for assessing exosome quality

Exosome characteristics must be analyzed in order to determine the quality of the produced exosomes. General characterization includes measuring exosome particle number per volume using nanoparticle tracking analysis (NTA), the presence of positive/negative exosome markers using immunoblotting, and the size and structure of the lipid bilayer using cryo-electron microscopy (Cryo-EM) (Table 1) (Gurunathan et al., 2019; Kurian et al., 2021; Szatanek et al., 2017; Thery et al., 2018). Assays detecting originator cell-specific markers or common exosome markers, such as proteins, lipids, or nucleic acids, can be used to analyze exosome identity. In most cases, exosome-specific proteins have been identified using western blotting or FACS with a specific antibody (Yang et al., 2019). Recently, exosomes have been highlighted as potential drug carriers (Song et al., 2021; Yim et al., 2016). These therapeutic cargo molecules require quantitative or qualitative characterization. In addition, other components that comprise exosomes should be profiled, because they contain many different proteins, lipids, and nucleic acids from their host cells (Choi et al., 2013; 2020). Exosome profiling results can be used to predict genotoxicity and/or carcinogenicity by analyzing the proteins or nucleic acids that are responsible for inducing such effects. The safety profile of a drug is one of the most critical aspects of drug discovery. Thorough profiling of exosomes, including proteins, lipids, and nucleic acids, as well as conducting animal toxicology studies, will be critical for predicting any unforeseen safety issues.

Criteria for exosome quality control

Exosome quality is greatly affected by originator cell type, culture conditions, and purification processes. To commercialize exosome therapeutics, quality control criteria must be established to accurately assess their quality and verify their consistent manufacture. Standardized protocols or guidance for exosome quality attributes are needed regardless of the originator cell line type and culture conditions (Doyle and Wang, 2019; Thery et al., 2018). The attributes and acceptance criteria should include identity, strength, and purity based on exosome properties (Table 2).

Unlike small molecules or monoclonal antibody drugs, where each component of the final drug product is identified, exosomes comprise various component types, which complicates the clear definition of the extent to which each component constitutes an impurity. In previous research, exosome purity was assessed by measuring the total particle number of exosomes per total protein (pn/μg) or by measuring the amount of proteins of a specific size (Huang and He, 2017; Webber and Clayton, 2013). Both methods have limitations in confirming purity, because they analyze exosome purity based on size or amount of protein. NTA measures the Brownian motion of each particle by size to measure exosome concentration, and it is commonly used to measure the total exosome particle number. However, NTA results fluctuate highly depending on each manufacturer's specifications and equipment operation conditions (Bachurski et al., 2019). In addition, because impurities cannot be defined solely based on their size, it is not the best method to measure exosome purity. Size-exclusion high-performance liquid chro-

Table 1. Methods for EVs (exosome) characterization

Attributes	Methods
Particle number	Nanoparticle tracking analysis (NTA)
Exosome markers	
Positive markers	Immunoblotting
Tetraspanin (CD9, CD81)	
Lumen protein (Alix, TSG101)	
Negative markers	
Golgi protein (GM130)	
Endoplasmic reticulum protein (Calnexin)	
Nuclear protein (Lamin B1)	
Mitochondria protein (Prohibitin)	
Lipid bilayer structure	Cryo-electron microscopy (Cryo-EM)
Cargo loading	Immunoblotting
	Nano-flow cytometry
	Multiple reaction monitoring
	Enzyme-linked immunosorbent assay (ELISA)
	Polymerase chain reaction (PCR)
Proteomics	Liquid chromatography/tandem mass spectrometry (LC-MS/MS)
Lipidomics	LC-MS/MS
Transcriptomics	Transcriptome sequencing
	Small RNA sequencing

Table 2. Quality control of EVs (exosomes)

Attribute	Analysis factor	Methods
General properties	Appearance	General observation
	Osmolality	Osmometers
	Subvisible particles	Light obscuration particles count: high accuracy liquid particle counter
Identity	Active pharmaceutical ingredient	Immunoblotting, ELISA, PCR
Contents	Particle number	NTA
	Total protein	BCA protein assay
Purity	Purity	SE-HPLC
Impurities	Host cell protein	ELISA
	Host cell DNA	Residual DNA quantitative assay
Potency	Mechanism of action (MoA)	Based MoA assay
Safety	Mycoplasma	Culture method
	Bioburden	Membrane filtration
	Adventitious virus	*In vitro* assay (cytopathic effect, hemagglutination, hemadsorption)
	Sterility	Direct inoculation
	Endotoxin	Gel clot method, photometric method

matography (SE-HPLC) is another commonly used method to measure purity by analyzing the amount of proteins that may have different sizes than exosomes. Since SE-HPLC provides more accurate and consistent results than NTA, its use for measuring exosome purity is becoming a standard.

Host cell protein (HCP) and host cell DNA (HCD) analyses require attention when determining acceptance criteria for clinical/commercial exosome products. HCP and HCD are proteins and DNA originating from parental cells that exist outside the exosome product. During the manufacture of conventional biological products, the final product should be tested for any residual HCP and HCD, as these process-related impurities may impact the safety of the product by provoking unwanted immunogenicity. Therefore, current regulatory agencies have adopted strict guidelines and criteria for HCP and HCD analyses for biologics for therapeutic use (Bracewell et al., 2015; U.S. Food and Drug Administration, 2020; Valkama et al., 2018). As mentioned above, exosomes function by transferring information between different cells as physiological/pathological signals (Chitoiu et al., 2020; Veziroglu and Mias, 2020). The components of exosomes are considered part of the exosome itself. Therefore, it is crucial to analyze HCP and HCD outside of exosomes which does not exist within the exosome membrane. An ultracentrifugation method or a purification kit can be used to separate exosomes from impurities in the buffer; however, these methods still need more development to clearly separate between exogenous biomolecules and exosomes. To date, there is no clear way to define exosome HCD/HCP, and the methods for quantifying proteins and DNA derived from cells using HCP and HCD detection kits need to be further developed to measure process-related impurities.

PERSPECTIVES

The use of exosomes is a new approach for efficient intracellular delivery of bioactive molecules to target cells. Currently, scientific research on exosomes is mainly conducted in academia, where only a small quantity of exosomes is sufficient for accomplishing research purposes. However, several challenges remain in large-scale exosome production to support preclinical and clinical studies or commercial marketing beyond the laboratory scale used in academia. In this mini-review, we address exosome manufacturing challenges, including cell line development, upstream cell culture, and downstream purification processes in pre-clinical and clinical activities. First, a cell culture process in three-dimensional stirred-tank bioreactors should be developed to produce sufficient materials for therapeutic use and support clinical trials through market support via commercial-scale manufacturing. Second, traditional purification methods used in academia contain high process- and product-related impurities, limiting their application in commercial processes. More precise and scalable purification methods should be developed to produce high-purity exosomes for human use in clinical trials during drug development. We also addressed issues and solutions related to exosome characterization and quality control, since defining product properties of exosomes is complex challenging compared to that of traditional biologics, such as antibodies.

Although the following issues are not discussed in this mini-review, they should be considered from the perspective of drug development.

Formulation of exosome products in the early development stage may significantly improve their stability and establish prolonged shelf life. Additionally, an appropriate analytical matrix should be established to support the streamlined development of scalable manufacturing processes to produce exosome products. Recently, cell-derived nanovesicles (CDNs), also known as exosome-mimetic nanovesicles, have gained attention as nanovesicles that can be produced in a cost-effective and large-scale manner. CDNs are generated by subjecting cells to physical processes, such as extrusion, to produce nano-sized vesicles (Goh et al., 2017; Ilahibaks et al., 2019). While CDNs possess comparable advantages regarding scalable manufacturing, issues related to immunogenicity

due to the incorporation of unwanted cellular components within CDN and alteration of membrane conformation and composition by shearing process should be addressed for further clinical advances (Jo et al., 2014; Ou et al., 2021).

Criteria for establishing good manufacturing practice (GMP) facility for producing therapeutic exosomes are similar to those with other traditional biologics for cell and gene therapies. However, additional considerations should be made for exosome production, including conducting a virus clearance study related to exosome properties. Currently, only a limited number of contract manufacturing organizations worldwide are equipped to handle exosome products. Being aware of the fact that GMP manufacturing facilities must be prepared prior to Investigational New Drug (IND) submission for clinical trials, establishment of GMP facilities for exosome production will be crucial for development of exosomal therapeutics.

Many factors contribute to the prediction of drug safety. Since exosome therapeutics are a new drug class, and exosome-specific guidance for manufacturing and quality assessment has not been published, it is crucial for developers to communicate closely with regulators to proactively address any unexpected issues that may arise in biotherapeutic exosome development. A reasonable and practical regulatory guideline for exosomal drugs should be established through comprehensive discussions with the industry to prevent their inappropriate use.

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation (NRF) of Korea (2016M3A9B6945931) from Ministry of Science and ICT, Republic of Korea.

AUTHOR CONTRIBUTIONS

S.H.A., S.W.R., J.P., and C.C. constructed review. S.H.A., S.W.R., H.C., S.Y., J.P., and C.C. discussed review paper. S.H.A., S.W.R., H.C., S.Y., J.P., and C.C. wrote the paper. S.H.A., S.W.R., H.C., J.P., and C.C. had primary responsibility for final content. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

C.C. is the founder and shareholder, and H.C. is employee and minor shareholder, and J.P., S.H.A., S.W.R., and S.Y. are employee of ILIAS Biologics, Inc. The authors declare no additional financial interests.

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