Manufactured extracellular vesicles as human therapeutics: Challenges, Advances and Opportunities

Scott Estes[1,*], Konstantin Konstantinov[1], Jamey D. Young[2]

[1] Codiak BioSciences, Cambridge, MA, USA

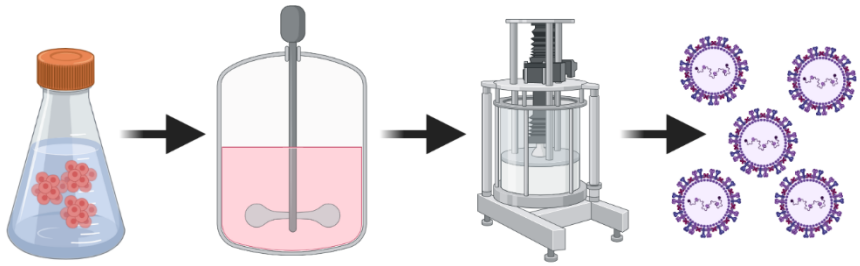
[2] Vanderbilt University, Dept. of Chemical and Biomolecular Engineering, Dept. of Molecular Physiology and Biophysics, Nashville, TN, USA

*To whom correspondence should be addressed: scott.estes@codiakbio.com

Abstract

Extracellular vesicles (EVs) have evolved across all phyla as an intercellular communication system. There are intrinsic advantages of leveraging this capability to deliver therapeutic cargo to treat disease, which have been demonstrated in numerous in vivo studies. As with other new modalities, the challenge has now shifted from proof of concept to developing reliable and efficient large-scale infrastructure to manufacture consistently pure and potent drug for broad-based patient access. This review focuses on how this challenge has been met with both existing and emerging technology platforms that are making impressive strides in the industrialization of EV manufacturing. In addition, we also highlight the gaps and opportunities that are beginning to be explored and addressed to hasten ushering in the era of therapeutic EVs.

Graphical Abstract



High-impact areas for research and development in the emerging EV biomanufacturing industry.

Introduction

Extracellular vesicles (EVs) are lipid-bound particles secreted by both prokaryotic and eukaryotic cells that contain a heterogenous cargo of proteins, nucleic acids and metabolites. Although the complexity of EV structure, function, and biosynthesis is not completely understood, EVs represent an important mechanism of intercellular communication and play significant roles in many physiological and pathological processes. While academic research into EV biology began in the 1980s, biotechnology industry involvement intensified nearly 25 years later. This long time lag was due to various factors, including the initial interpretation of EVs as cellular waste-removing vesicles, which diminished the perceived biological significance [1]. This view changed quickly after the publication of a few seminal papers [2–4] demonstrating that EVs are also information carriers capable of modifying the behavior of cells in distant organs [5] and even across species [6]. With this knowledge came the recognition that EVs could be exploited to deliver therapeutic payloads for a wide range of indications. While the promise

of therapeutic exosomes is still largely manifest at the preclinical stage, Table 1 highlights the burgeoning application of therapeutic EVs in the clinic.

EVs have several advantages over synthetic nanoparticle drugs and other cell-based therapies. These nanoparticles exhibit low levels of immunogenicity when derived from either autologous or allogeneic sources, thus limiting potential side effects. Furthermore, EVs deliver cargo to recipient cells more efficiently than artificial nanoparticles [7] and have been shown to traverse the blood-brain barrier [8]. Current estimates suggest that EVs can transfer between 10-30% of the RNA cargo to the cytosol of recipient cells, in contrast to lipid nanoparticle systems that are estimated to deliver only ~1-2% [9]. The size and membrane lipid composition facilitate fusion with target cells, where EVs can deliver the cargo while avoiding degradation. As a result, EVs have recently emerged as promising alternatives for delivery of nucleic acids [10,11], proteins [12,13], and small molecules [14] to recipient cells. EVs can be endogenously or exogenously loaded with a range of different therapeutic molecules, are highly biocompatible and non-toxic, and intrinsically express surface proteins capable of promoting increased blood circulation, tissue-targeted delivery, and cellular uptake [15,16].

The emergence of a new therapeutic modality is a rare event associated with tremendous opportunities, but also, certain challenges (**Figure 1**). In particular, due to the high complexity of EVs, the large-scale manufacturing of EVs is a difficult task that has recently taken a central stage in the EV field [17]. Historically, early methods for production of complex biologics have been established in academia, and later transferred to industry for further refinement and scale-up. While this might be perceived as the fastest path to commercialization, such methods are not always compatible with the demands of large-scale GMP manufacturing. The lessons learned from other modalities, such as AAV gene therapies, can be instructive in the manufacturing of EV therapeutics. The demand projections for AAVs vary broadly between clinical indications, in some cases increasing beyond 10^{14} viral genomes per dose. Such large numbers have resulted in major AAV manufacturing capacity challenges [18]. While clinical experience with EVs is still limited, recent reports suggest similar broadly varying dose patterns [19–21]. Hence, there is a critical need for basic science and engineering research that will lead to improvements in scalable and rapidly adaptable technologies for EV biomanufacturing. It is also important to learn from prior empirical approaches and advance toward the rational design of engineered EVs with defined and tunable therapeutic properties.

EV Engineering Approaches

Multiple approaches to enable the effective loading of different types of pharmacologically active molecules have been explored with varying degrees of efficiency. These engineering efforts can be broadly lumped into two classes; the engineering of cells to affect the intracellular loading of molecules by co-opting the cellular EV biosynthesis machinery (endogenous loading) or alternatively, exogenous

loading post EV purification. For effective loading of protein cargo, recent advances in the identification of specific EV markers such as PTGFRN and BASP1 [22] or oligomerizing the N-terminus of syntenin [23] have opened the door to classical genetic engineering to produce stably modified host cells in which proteins of interest are effectively loaded on or in EVs at high valency via genetic linkage to these fusion partners. An alternative genetic engineering approach that enables soluble EV cargo loading takes advantage of transient light induced dimerization of a therapeutic protein fused to a photoreceptor cytochrome and a corresponding light inducible dimerization domain fused to a tetraspanin. EVs loaded with a transcriptional repressor using this technology have been shown to be efficacious in animal models of sepsis [24] and have been approved to initiate a healthy volunteer safety trail in Australia. From a manufacturing perspective, endogenous loading is the most straightforward path to developing engineered EV based therapeutics as no API (active pharmaceutical ingredient) needs to be combined with the EV carrier during drug product manufacturing.

Similar approaches for the endogenous loading of nucleic acids, particularly RNAs, has not been as straightforward although recent work [25] have identified a series of consensus sequences termed “EXOmotifs” present in miRNAs that are enriched in extracellular vesicles relative to the cytoplasma. When said motifs were engineered into miR-37c, a miRNA normally not enriched in EVs, the authors, using a transwell format, showed a fifteen-fold increase in the delivery of the EXOmotif engineered miR-37c to the recipient cells relative to wild type miR-37c. In a similar strategy, Reshke et al. [11] integrated siRNA sequences into pre-miR-451 hairpins that selectively load into EVs. These engineered EVs reduced expression of target genes in the liver, intestine and kidney of mice at siRNA doses that are at least tenfold lower than the doses typically delivered via lipid nanoparticles. Whether these approaches will be broadly applicable to a variety of potential host cell lines and capable of loading an efficacious level of therapeutic miRNAs or siRNAs remains to be proven.

As noted above, exogenous loading methods have been developed out of necessity to engineer EVs post-purification for molecules not readily amenable to endogenous loading. Many studies have been published utilizing electroporation in an attempt to introduce small molecules and nucleic acids into EVs. However, the Schwan equation predicts that permeabilizing exosomes of 50-200nm diameter requires field strengths significantly higher than that achievable with commercially available electroporation systems designed for cell electroporation [26]. In addition, it has been shown that aluminum electrodes shed aluminum hydroxide particles when pulsed [25]. These particles will be difficult to distinguish from vesicles by the standard nanoparticle tracking analysis and can influence the cellular uptake of biological nanoparticles [26]. Consistent with this observation, a similar finding demonstrated that electroporation of siRNA resulted in severe aggregation of the siRNA and little effective loading into the lumen of the EVs [27]. Despite these obstacles, apparent successes have recently been published for a range of molecules such as the chemotherapeutic agent Doxorubicin [28] as well as miRNAs [29,30]. Regardless of the ability to overcome the technical challenges of electroporating small vesicles, there is currently the

additional limitation of scale. Although commercially available flow electroporation systems are available, these systems have volume restrictions that would make it impractical to generate large GMP batches of electroporated EV drug product.

Orthogonal approaches for exogenous loading have been successfully developed that capitalize on the recognition that efficacious EV mediated delivery of cargo can be achieved not only when loaded within the vesicle but also when attached to the surface. Conjugating a cholesterol anchor and linker to different classes of nucleic acid therapeutics such as siRNA [31] and antisense oligonucleotides [32] have been shown to enable loading of upwards of 2000-3000 molecules per vesicle and effective knockdown of the target gene. An alternative approach for loading RNA onto the surface of vesicles has also recently been published [33] and is based upon the identification of a phosphatidylserine binding motif in the GAPDH enzyme that has an affinity for EVs. When this motif was fused to dsRNA binding domains and the resulting fusion protein incubated with siRNA, the authors demonstrated the loading of approximately 500-700 siRNA molecules per vesicle and knock down of the target gene in vivo.

EV sources

A major factor influencing the EV manufacturing process is the EV source. Most reported studies have been focused on EVs of human origin derived from bulk sources (such as blood or plasma, [34,35]), primary mesenchymal stem cells (MSCs) [36], induced pluripotent stem cells (iPSCs) [8], immortalized primary cells (such as MSC-MYC [37]), or established cell lines such as HEK-293 (Human Embryonic Kidney cells) and CAP (CEVEC Amniocyte Production cells; CEVC Pharmaceuticals GmbH). While each of these sources has its advantages and disadvantages, immortalized cell lines are of primary interest for large-scale EV manufacturing and will likely find broad application. Such cell lines facilitate the construction of engineered EVs with advanced therapeutic properties, and have fewer barriers to scale-up [22]. The production of EVs for therapeutic purposes from various non-human sources is also attracting significant attention. Examples are microbial culture [38], bovine milk [39,40], various plants [41], and marine microalgae [42]. The main advantage of these sources is the broad availability and low cost. At present, however, the therapeutic applications are still limited, likely due to immunogenicity concerns specifically for parenteral administration.

Upstream EV manufacturing

For natural EV sources (human blood/plasma, bovine milk, plants), upstream manufacturing is not needed. On the other hand, the production of EVs in cell culture (human, microbial, microalgae) requires cultivation under optimized conditions in bioreactors of appropriate type and scale. Most current applications utilize primary or immortalized human cells. Examples of the former are MSCs producing

native EVs with promising applications in tissue regeneration [43,44]. Primary cells are most often anchorage-dependent, usually grown in two dimensional culture systems at low cell density. Due to the limited productivity and scalability, the 2D systems are increasingly being replaced with 3D bioreactors, such as stirred tanks with microcarriers, packed-bed systems, and hollow-fiber cartridges [45]. In parallel, the traditional (EV depleted) serum-containing media formulations are being replaced with serum-free media (enriched with growth factors [46]) of improved safety, performance and cost. However, even with the recent technological advancements, EV productivity from primary cells is limited, mainly due to low bioreactor cell densities [47].

Utilization of established human cell lines (HEK-293, CAP, etc.) grown in suspension mitigates these productivity constraints. Cultivation of these cell lines is compatible with the most advanced bioprocessing technology available today, including fed-batch or, particularly, high-density perfusion processing using chemically defined media, which can yield high EV productivity and is compatible with existing biomanufacturing infrastructure [48]. Likewise, impressive EV productivity, process robustness and low cost can be achieved in industrial bioreactors growing microbial cells, either wild-type or engineered. Most recently, the application of marine microalgae to large-scale EV production has been reported [42]. Good performance has been reported for scalable photobioreactors using ultra-low-cost media based on seawater. However, despite the excellent safety and nutritional values of microalgae, large demand for such EVs does not yet exist. The opportunity for microalgae EV engineering will likely broaden their therapeutic application [49].

Downstream EV manufacturing

While upstream EV manufacturing strongly depends on the EV source, downstream methods have broader applicability to different types of EVs. These methods have evolved historically as a variety of individual unit operations [50], which are often combined in sequences to achieve better EV purity. In general, most methods start with a cell separation step using differential centrifugation, filtration, or a combination of both [48,51,52]. Regardless of their broad acceptance, the centrifugation methods are slow, laborious, and not compatible with large-scale GMP manufacturing. For these reasons, new methods based on chromatography (size exclusion, ion exchange, hydrophobic interaction, affinity, multi-mode) and filtration (depth, tangential flow, dead end) are being actively developed [50,51]. Applications of advanced new purification methods based on field flow fractionation [53], nanoscale lateral displacement [54], acoustics [34], or affinity separation [55,56] are increasingly reported. The latter is facilitated by decorating the EV surface with desired proteins, which can be a convenient targets for affinity ligands [56,57].

High-impact areas for further development

Further advancement of EV manufacturing undoubtedly requires research in many directions. A few high-impact areas are outlined below.

- **Rational cell line engineering for high EV productivity and potency.** The cellular pathways involved in EV synthesis are complex and not well understood. Therefore, exploiting host cell line engineering to improve EV productivity and product quality is still in its infancy and will be enhanced by better understanding of EV biosynthesis and assembly pathways and bottlenecks [10]. Research in this area will help identify specific targets amenable to host cell engineering, yielding high performance cell lines for EV manufacturing
- **Cell culture media development.** Most current media formulations have been designed for high productivity of monoclonal antibody or recombinant proteins. Considering the composition of EVs, it is likely that the nutritional requirements of EV producing cells are not fully met by the existing formulations. Understanding and mapping these requirements into new media formulations could significantly boost EV productivity [57].
- **Affinity chromatography.** The recent progress with synthetic affinity ligands has potential in the design of a new generation of chromatography media with high EV specificity and capacity. Chromatography steps utilizing such media will deliver high purity, yield, and shorter purification trains [55,56].
- **Novel EV separation methods based on microfluidics and acoustics.** This technology is being developed for the separation of various particles, recently including EVs [34]. This purely physical approach has the additional advantages of being compatible with long-term continuous operation under aseptic conditions. Major current challenge is scalability, which could be tackled with the application of massively parallel microfluidics technology.
- **Application of integrated continuous biomanufacturing.** Recent studies have demonstrated the compatibility of this technology with several new therapeutic modalities [58]. The reported implementations are based on hollow fibers [45] and high-density stirred tank bioreactors [59,60], including 500L Good Manufacturing Practice (GMP) manufacturing of engineered EVs for different clinical indications [48]. Integration of the up- and downstream unit operations into a continuous production system is an attractive opportunity offering additional advantages [57].
- **Analytical methods for single EV characterization.** The analytical needs in the EV field are enormous at all stages, including basic research, product and process development, and GMP manufacturing. Of specific interest are methods for single EV characterization, needed for understanding the structure-function relationships and natural EV heterogeneity [61].

Conclusions

The broad clinical potential of EVs and the opportunities for precise engineering points to a bright future for EV therapeutics [43,62]. As with cell therapies [63,64], the exciting progress with EV engineering has enriched the EV clinical paradigm. It has allowed moving beyond the limitations of identifying what clinical indications could benefit from the intrinsic properties of native EVs to rationally engineering EVs with specific properties for targeted clinical indications. This approach has also eliminated the limitations of producing EVs from cell types with poor manufacturability attributes that significantly constrain the ability to manufacture large quantities of drug. As a result, significant progress has been made with the development of large-scale EV manufacturing processes [64]. As illustrated in **Figure 2**, a single 500L GMP campaign can yield 10^{17}-10^{18} purified EVs enabling large-volume applications, such as vaccines. Even with these strides, EVs remain a new emerging modality with much untapped potential for further multifold productivity and potency enhancement. This potential will be realized via continued investment and research in cell line and EV engineering, media optimization, up/downstream process development, all supported by novel analytical methods.

Acknowledgements

JDY was supported by the NSF Future Manufacturing program under grant number MCB-2036809. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

References Cited

1. Ratajczak MZ, Ratajczak J: **Extracellular microvesicles/exosomes: discovery, disbelief, acceptance, and the future?** *Leukemia* 2020, **34**:3126–3135.

2. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO: **Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells**. *Nat cell Biol* 2007, **9**:654–659.

3. Skog J, Würdinger T, van Rijn S, Meijer DH, Gainche L, Curry WT, Carter BS, Krichevsky AM, Breakefield XO: **Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers**. *Nat cell Biol* 2008, **10**:1470–1476.

4. Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, Dvorak P, Ratajczak MZ: **Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal**

transfer of mRNA and protein delivery. *Leukemia* 2006, **20**:847–856.

5. Choi H, Choi Y, Yim HY, Mirzaaghasi A, Yoo J-K, Choi C: **Biodistribution of Exosomes and Engineering Strategies for Targeted Delivery of Therapeutic Exosomes**. *Tissue Eng Regen Med* 2021, **18**:499–511.

6. Bleackley MR, Samuel M, Garcia-Ceron D, McKenna JA, Lowe RGT, Pathan M, Zhao K, Ang CS, Mathivanan S, Anderson MA: **Extracellular Vesicles From the Cotton Pathogen Fusarium oxysporum f. sp. vasinfectum Induce a Phytotoxic Response in Plants**. *Front Plant Sci* 2020, **10**:1610.

7. Heusermann W, Hean J, Trojer D, Steib E, von Bueren S, Graff-Meyer A, Genoud C, Martin K, Pizzato N, Voshol J, et al.: **Exosomes surf on filopodia to enter cells at endocytic hot spots, traffic within endosomes, and are targeted to the ER**. *J Cell Biol* 2016, **213**:173–184.

8. Upadhya R, Madhu LN, Attaluri S, Gitaí DLG, Pinson MR, Kodali M, Shetty G, Zanirati G, Kumar S, Shuai B, et al.: **Extracellular vesicles from human iPSC-derived neural stem cells: miRNA and protein signatures, and anti-inflammatory and neurogenic properties**. *J Extracell Vesicles* 2020, **9**:1809064.

9. Piffoux M, Volatron J, Silva AKA, Gazeau F: **Thinking quantitatively of rna-based information transfer via extracellular vesicles: Lessons to learn for the design of rna-loaded evs**. *Pharmaceutics* 2021, **13**.

10. Kojima R, Bojar D, Rizzi G, Hamri GC, El-Baba MD, Saxena P, Auslander S, Tan KR, Fussenegger M: **Designer exosomes produced by implanted cells intracerebrally deliver therapeutic cargo for Parkinson's disease treatment**. *Nat Commun* 2018, **9**:1305.

11. Reshke R, Taylor JA, Savard A, Guo H, Rhym LH, Kowalski PS, Trung MT, Campbell C, Little W, Anderson DG, et al.: **Reduction of the therapeutic dose of silencing RNA by packaging it in extracellular vesicles via a pre-microRNA backbone**. *Nat Biomed Eng 2020 41* 2020, **4**:52–68.

12. Sterzenbach U, Putz U, Low LH, Silke J, Tan SS, Howitt J: **Engineered Exosomes as Vehicles for Biologically Active Proteins**. *Mol Ther* 2017, **25**:1269–1278.

13. Zhang X, Xu Q, Zi Z, Liu Z, Wan C, Crisman L, Shen J, Liu X: **Programmable Extracellular Vesicles for Macromolecule Delivery and Genome Modifications**. *Dev Cell* 2020, **55**:784-801.e9.

14. Jang SC, Economides KD, Moniz RJ, Sia CL, Lewis N, McCoy C, Zi T, Zhang K, Harrison RA, Lim J, et al.: **ExoSTING, an extracellular vesicle loaded with STING agonists, promotes tumor immune surveillance**. *Commun Biol 2021 41* 2021, **4**:1–17.

15. Elsharkasy OM, Nordin JZ, Hagey DW, de Jong OG, Schiffelers RM, Andaloussi SE, Vader P: **Extracellular vesicles as drug delivery systems: Why and how?** *Adv Drug Deliv Rev* 2020, doi:10.1016/j.addr.2020.04.004.

16. O'Brien K, Breyne K, Ughetto S, Laurent LC, Breakefield XO: **RNA delivery by extracellular vesicles in mammalian cells and its applications**. *Nat Rev Mol Cell Biol* 2020, **21**:585–606.

17. Paolini L, Monguió-Tortajada M, Costa M, Clos-Sansalvador M, Cronemberger A, Gimona M, Kaparakis-Liaskos M, Konstantinov K, Lim S-K, Meisner-Kober N, et al.: **Large-scale production of Extracellular Vesicles: Report on the MassivEVs ISEV workshop.** *J Extracell Vesicles* 2022, **In press**.

18. Schaffer D: **Directed evolution of novel AAV vectors for clinical gene therapy**. In *Advancing Manufacture of Cell and Gene Therapies VII*. . Engineering Conferences International; 2022.

19. Lenzini S: **Establishing a Working Range for Effective MSC-EV Dose**. *RoosterBio Blog* 2021,

20. Willis GR, Kourembanas S, Mitsialis SA: **Toward Exosome-Based Therapeutics: Isolation, Heterogeneity, and Fit-for-Purpose Potency**. *Front Cardiovasc Med* 2017, **4**:63.

21. Gupta D, Zickler AM, El Andaloussi S: **Dosing extracellular vesicles.** *Adv Drug Deliv Rev* 2021, **178**:113961.

22. Dooley K, McConnell RE, Xu K, Lewis ND, Haupt S, Youniss MR, Martin S, Sia CL, McCoy C, Moniz RJ, et al.: **A versatile platform for generating engineered extracellular vesicles with defined therapeutic properties**. *Mol Ther* 2021, **29**:1729–1743.

23. Gupta D, Wiklander OPB, Görgens A, Conceição M, Corso G, Liang X, Seow Y, Balusu S, Feldin U, Bostancioglu B, et al.: **Amelioration of systemic inflammation via the display of two different decoy protein receptors on extracellular vesicles**. *Nat Biomed Eng* 2021, **5**:1084–1098.

24. Choi H, Kim Y, Mirzaaghasi A, Heo J, Kim YN, Shin JH, Kim S, Kim NH, Cho ES, In Yook J, et al.: **Exosome-based delivery of super-repressor IκBα relieves sepsis-associated organ damage and mortality**. *Sci Adv* 2020, **6**.

25. Garcia-Martin R, Wang G, Brandão BB, Zanotto TM, Shah S, Kumar Patel S, Schilling B, Kahn CR: **MicroRNA sequence codes for small extracellular vesicle release and cellular retention**. *Nature* 2022, **601**:446–451.

26. Marszalek P, Liu DS, Tsong TY: **Schwan equation and transmembrane potential induced by alternating electric field**. *Biophys J* 1990, **58**:1053–1058.

27. Kooijmans SAA, Stremersch S, Braeckmans K, de Smedt SC, Hendrix A, Wood MJA, Schiffelers

RM, Raemdonck K, Vader P: **Electroporation-induced siRNA precipitation obscures the efficiency of siRNA loading into extracellular vesicles**. *J Control Release* 2013, **172**:229–238.

28. Lennaárd AJ, Mamand DR, Wiklander RJ, EL Andaloussi S, Wiklander OPB: **Optimised Electroporation for Loading of Extracellular Vesicles with Doxorubicin**. *Pharmaceutics* 2021, **14**:38.

29. Yan C, Chen J, Wang C, Yuan M, Kang Y, Wu Z, Li W, Zhang G, Machens H-G, Rinkevich Y, et al.: **Milk exosomes-mediated miR-31-5p delivery accelerates diabetic wound healing through promoting angiogenesis**. *Drug Deliv* 2022, **29**:214–228.

30. Zhao Z, Shuang T, Gao Y, Lu F, Zhang J, He W, Qu L, Chen B, Hao Q: **Targeted delivery of exosomal miR-484 reprograms tumor vasculature for chemotherapy sensitization**. *Cancer Lett* 2022, **530**:45–58.

31. Haraszti RA, Miller R, Didiot M-C, Biscans A, Alterman JF, Hassler MR, Roux L, Echeverria D, Sapp E, DiFiglia M, et al.: **Optimized Cholesterol-siRNA Chemistry Improves Productive Loading onto Extracellular Vesicles**. *Mol Ther* 2018, **26**:1973–1982.

32. Kamerkar S, Leng C, Burenkova O, Jang SC, McCoy C, Zhang K, Dooley K, Kasera S, Zi T, Sisó S, et al.: **Exosome-mediated genetic reprogramming of tumor-associated macrophages by exoASO-STAT6 leads to potent monotherapy antitumor activity.** *Sci Adv* 2022, **8**:eabj7002.

33. Dar GH, Mendes CC, Kuan W-L, Speciale AA, Conceição M, Görgens A, Uliyakina I, Lobo MJ, Lim WF, EL Andaloussi S, et al.: **GAPDH controls extracellular vesicle biogenesis and enhances the therapeutic potential of EV mediated siRNA delivery to the brain**. *Nat Commun* 2021, **12**:6666.

34. Mengxi W, Yingshi O, Zeyu W, Rui Z, Po-Hsun H, Chuyi C, Hui L, Peng L, David Q, Ming D, et al.: **Isolation of exosomes from whole blood by integrating acoustics and microfluidics**. *Proc Natl Acad Sci* 2017, **114**:10584–10589.

35. Park O, Choi ES, Yu G, Kim JY, Kang YY, Jung H, Mok H: **Efficient Delivery of Tyrosinase Related Protein-2 (TRP2) Peptides to Lymph Nodes using Serum-Derived Exosomes.** *Macromol Biosci* 2018, **18**:e1800301.

36. Baek G, Choi H, Kim Y, Lee HC, Choi C: **Mesenchymal Stem Cell-Derived Extracellular Vesicles as Therapeutics and as a Drug Delivery Platform**. *Stem Cells Transl Med* 2019, **8**:880–886.

37. Lai RC, Yeo RWY, Padmanabhan J, Choo A, de Kleijn DP V, Lim SK: **Isolation and Characterization of Exosome from Human Embryonic Stem Cell-Derived C-Myc-Immortalized Mesenchymal Stem Cells.** *Methods Mol Biol* 2016, **1416**:477–494.

38. Deatherage BL, Cookson BT: **Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life.** *Infect Immun* 2012, **80**:1948–1957.

39. Adriano B, Cotto NM, Chauhan N, Jaggi M, Chauhan SC, Yallapu MM: **Milk exosomes: Nature's abundant nanoplatform for theranostic applications**. *Bioact Mater* 2021, **6**:2479–2490.

40. Marsh SR, Pridham KJ, Jourdan J, Gourdie RG: **Novel Protocols for Scalable Production of High Quality Purified Small Extracellular Vesicles from Bovine Milk**. *Nanotheranostics* 2021, **5**:488–498.

41. Kim J, Li S, Zhang S, Wang J: **Plant-derived exosome-like nanoparticles and their therapeutic activities**. *Asian J Pharm Sci* 2022, **17**:53–69.

42. Picciotto S, Barone ME, Fierli D, Aranyos A, Adamo G, Božič D, Romancino DP, Stanly C, Parkes R, Morsbach S, et al.: **Isolation of extracellular vesicles from microalgae: towards the production of sustainable and natural nanocarriers of bioactive compounds**. *Biomater Sci* 2021, **9**:2917–2930.

43. Cheng L, Hill AF: **Therapeutically harnessing extracellular vesicles**. *Nat Rev Drug Discov* 2022, doi:10.1038/S41573-022-00410-W.

44. Yeo RWY, Lai RC, Tan KH, Lim SK: **Exosome: A Novel and Safer Therapeutic Refinement of Mesenchymal Stem Cell:** *http://dx.doi.org/105772/57460* 2013, doi:10.5772/57460.

45. Whitford W, Ludlow JW, Cadwell JJS: **Continuous Production of Exosomes**. *Genet Eng Biotechnol News* 2015, **35**:34.

46. Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, Smuga-Otto K, Howden SE, Diol NR, Propson NE, et al.: **Chemically defined conditions for human iPSC derivation and culture**. *Nat Methods* 2011, **8**:424–429.

47. Lenzini S: **Extracellular Vesicle/Exosome Upstream Process Development: Maximizing Productivity to Accelerate Clinical Adoption**. *RoosterBio Blog* 2022,

48. Finn, PhD J, Konstantinov, PhD K: **Exosome-based therapeutics: ready for prime time**. *Cell Gene Ther Insights* 2020, **6**:597–605.

49. Sproles AE, Fields FJ, Smalley TN, Le CH, Badary A, Mayfield SP: **Recent advancements in the genetic engineering of microalgae**. *Algal Res* 2021, **53**:102158.

50. Liangsupree T, Multia E, Riekkola M-L: **Modern isolation and separation techniques for extracellular vesicles**. *J Chromatogr A* 2021, **1636**:461773.

51. Nordin JZ, Lee Y, Vader P, Mäger I, Johansson HJ, Heusermann W, Wiklander OPB, Hällbrink M, Seow Y, Bultema JJ, et al.: **Ultrafiltration with size-exclusion liquid chromatography for high yield isolation of extracellular vesicles preserving intact biophysical and functional properties**. *Nanomedicine Nanotechnology, Biol Med* 2015, **11**:879–883.

52. Jeppesen DK, Hvam ML, Primdahl-Bengtson B, Boysen AT, Whitehead B, Dyrskjøt L, Orntoft TF, Howard KA, Ostenfeld MS: **Comparative analysis of discrete exosome fractions obtained by differential centrifugation.** *J Extracell vesicles* 2014, **3**:25011.

53. Sitar S, Kejžar A, Pahovnik D, Kogej K, Tušek-Žnidarič M, Lenassi M, Žagar E: **Size Characterization and Quantification of Exosomes by Asymmetrical-Flow Field-Flow Fractionation**. *Anal Chem* 2015, **87**:9225–9233.

54. Wunsch BH, Smith JT, Gifford SM, Wang C, Brink M, Bruce RL, Austin RH, Stolovitzky G, Astier Y: **Nanoscale lateral displacement arrays for the separation of exosomes and colloids down to 20 nm**. *Nat Nanotechnol* 2016, **11**:936–940.

55. Reiter K, Aguilar PP, Wetter V, Steppert P, Tover A, Jungbauer A: **Separation of virus-like particles and extracellular vesicles by flow-through and heparin affinity chromatography**. *J Chromatogr A* 2019, **1588**:77–84.

56. Filipović L, Spasojević M, Prodanović R, Korać A, Matijaševic S, Brajušković G, de Marco A, Popović M: **Affinity-based isolation of extracellular vesicles by means of single-domain antibodies bound to macroporous methacrylate-based copolymer**. *N Biotechnol* 2022, **69**:36–48.

57. Konstantinov K: **Industrialization of the GMP manufacture of exosome therapeutics and opportunities for further multifold process productivity increase**. In *Advancing Manufacture of Cell and Gene Therapies VII*. . Engineering Conferences International; 2022.

58. Granicher G: **Integrated end-to-end MVA viral vector production**. In *Advancing Manufacture of Cell and Gene Therapies VII*. . Engineering Conferences International; 2022.

59. Whitford W, Guterstam P: **Exosome manufacturing status.** *Future Med Chem* 2019, **11**:1225–1236.

60. Martin S, Estes S: **Manipulating exosome biogenesis to enhance productivity of recombinant cell lines producing engineered exosome therapeutics**. In *27th ESACT Meeting*. . European Society for Animal Cell Culture Technology; 2022.

61. Chiang C, Chen C: **Toward characterizing extracellular vesicles at a single-particle level**. *J Biomed Sci* 2019, **26**:9.

62. Wiklander OPB, Brennan MÁ, Lötvall J, Breakefield XO, El Andaloussi S: **Advances in therapeutic applications of extracellular vesicles.** *Sci Transl Med* 2019, **11**.

63. Lipsitz YY, Bedford P, Davies AH, Timmins NE, Zandstra PW: **Achieving Efficient Manufacturing and Quality Assurance through Synthetic Cell Therapy Design.** *Cell Stem Cell* 2017, **20**:13–17.

64. Moody J: **Disruptive technologies for cell therapy manufacturing**. In *Advancing Manufacture of Cell and Gene Therapies VII*. . Engineering Conferences International; 2022.

Annotated References

* of special interest

** of outstanding interest

** (Reshke, 2020) Reports an approach where siRNA sequences can be endogenously loaded into EVs by integration into pre-miR-451 hairpins that are selectively enriched in EVs. By loading EVs with siRNA targeting GFP and then administering them intravenously to a transgenic mouse that expresses GFP ubiquitously, the authors assessed *in vivo* tropism and knockdown activity in a variety of organs.

* (Jang, 2021) Describes the development of exoSTING, an EV that is exogenously loaded with an immunomodulatory cyclic dinucleotide (CDN) via simple incubation with sufficiently high concentrations to allow for passive loading. It was found that exoSTING was ~100–200-fold more potent than free CDN in human peripheral blood mononuclear cells, resulting in significant improvements in antitumor activity in preclinical models of melanoma (when administered intratumorally) and hepatocellular carcinoma (when administered intravenously).

** (Dooley, 2021) Demonstrated that highly enriched EV proteins were effective engineering scaffolds for high density display of proteins with therapeutic potential on the surface or lumen of EVs via genetic engineering of the production cell line.

* (Gupta, 2021) Showed that fusing cytokine receptors containing oligomerization domains to the N-terminal fragment of syntenin enabled effective display of these decoy receptors on the surface of EVs. These engineered EVs were shown to be potent in multiple in vivo models of inflammation.

* (Garcia-Martin, 2022) Identified RNA sequence motifs that result in either cellular or EV enrichment. Authors showed inclusion of a EV motif increased miRNA delivery and subsequent target gene knock down in recipient cells.

* (Cheng, 2022) Provides a comprehensive summary of the clinical applications of EVs of various types.

* (Liangsupree, 2021) Well-structured review of EV purification technologies and development trends.

Tables and Figures

Table 1: Summary of clinical trials currently enrolling and/or treating patients with EV based therapeutics (As of May 2022). https://www.clinicaltrials.gov/

Study Identifier	Sponsor	Indication	Phase	Source of EVs	Native or Engineered	Therapeutic
NCT05127122	Direct Biologics	Covid Induced ARDS	III	Bone Marrow MSCs	Native	Undefined
NCT04969172	Tel-Aviv Sourasky Medical Center	Covid-19	II	HEK293	Engineered	CD24
NCT05216562	Dermama Bioteknologi Laboratorium	Covid-19	II	Undefined MSCs	Native	Undefined
NCT04592484	Codiak Biosciences	Solid Tumors	I/II	HEK293	Engineered	STING agonist
NCT03384433	Isfahan University	Acute Ischemic Stroke	I/II	Undefined MSCs	Native	Undefined
NCT04544215	Ruijin Hospital	Pulmonary Infection	I/II	Adipose MSCs	Native	Undefined
NCT04388982	Ruijin Hospital	Alzheimer	I/II	Adipose MSCs	Native	Undefined
NCT04213248	Sun Yat-sen University	GVHD Dry Eyes	I/II	Umbilical MSCs	Native	Undefined
NCT05156229	Codiak Biosciences	CTCL	I	HEK293	Engineered	IL-12
NCT05375604	Codiak Biosciences	Liver Cancer	I	HEK293	Engineered	STAT6 ASO
NCT04652531	University of Turin	Venous Ulcers	I	Autologous Sera	Native	Undefined
NCT04276987	Ruijin Hospital	Coronavirus Pneumonia	I	Adipose MSCs	Native	Undefined
NCT01294072	University of Louisville	Colon Cancer	I	Undefined Plant	Engineered	Curcumin
NCT05261360	Eskisehir Osmangazi University	Meniscal Injury	I	Autologous Synovial MSCs	Native	Undefined
NCT03608631	M.D. Anderson Cancer Center	Pancreas Cancer	I	Undefined MSCs	Engineered	kRAS siRNA
NCT01668849	University of Louisville	Oral Mucositis	I	Grapes	Native	Undefined

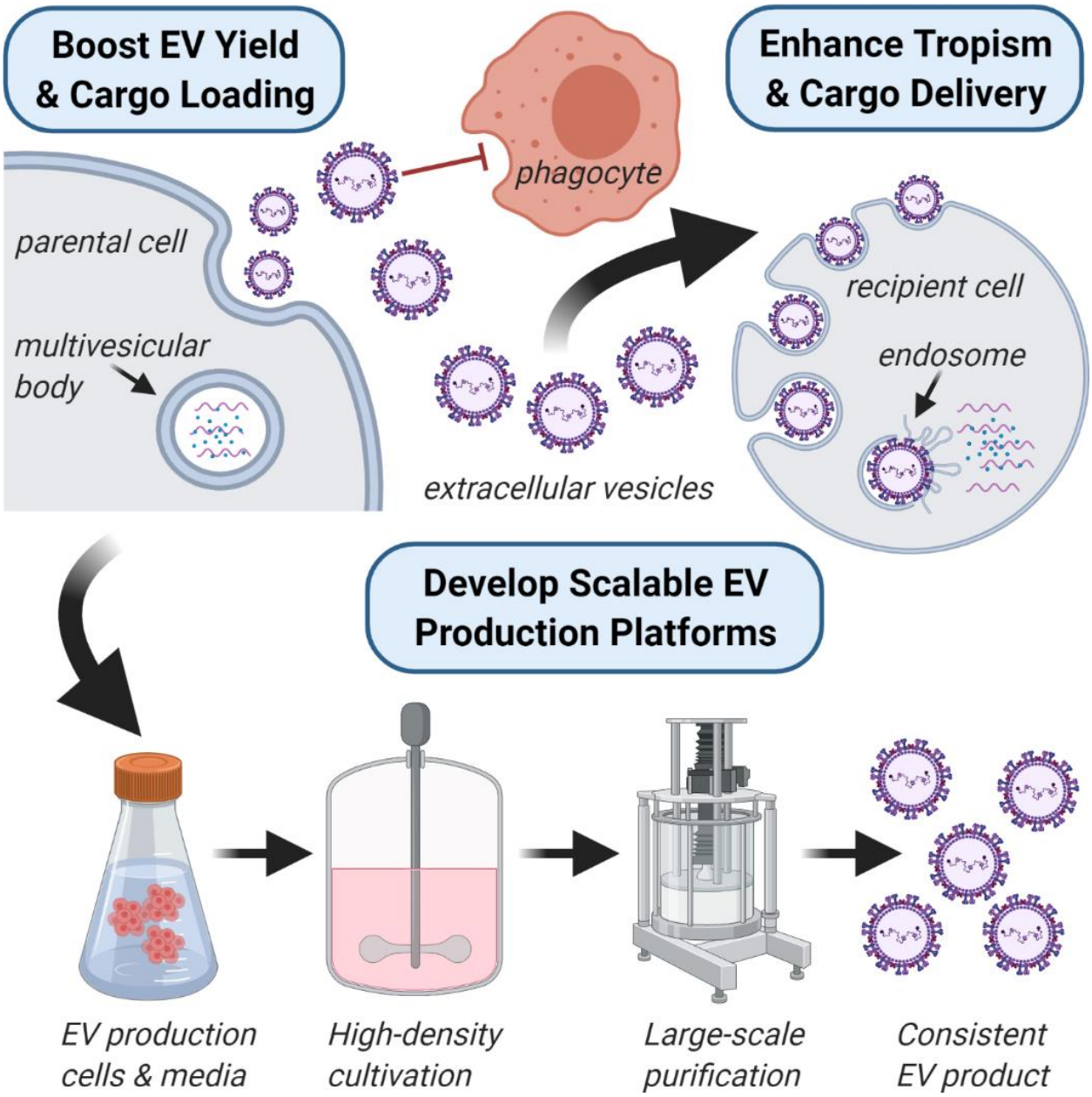


Figure 1. Challenges and opportunities for large-scale EV biomanufacturing. Unlocking the tremendous therapeutic potential of EVs requires advances in (i) understanding cellular mechanisms that control EV biosynthesis, cargo loading, and secretion, (ii) identifying properties of EVs that regulate the *in vivo* trafficking, biodistribution, and cargo delivery, and (iii) developing efficient solutions to cultivate EV-producing cells at high density and to purify EVs with specific features or contents.

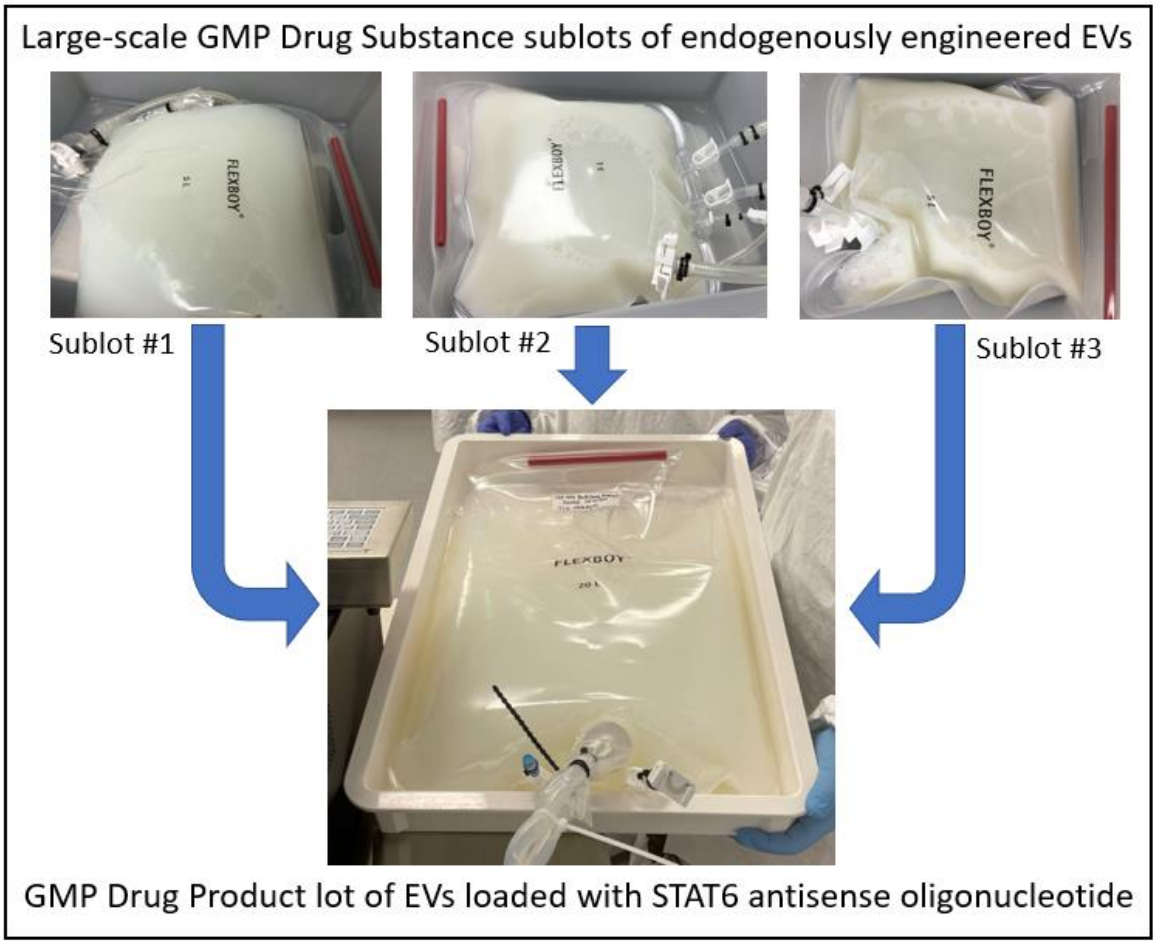


Figure 2. Large-scale GMP production of engineered EVs using a 500L high-density perfusion bioreactor and filtration/chromatography-based purification train. EVs are both endogenously (PTGFRN) and exogenously (STAT6 antisense oligonucleotide) engineered. Drug Substance of highly concentrated PTGFRN-EVs is stored as three sublots in 5L bags. Drug Product is produced by loading these EVs with STAT6 antisense oligonucleotide, resulting in a single lot stored in a 20L bag, ready for vial fill. The EV material is produced to support an immune-oncology clinical program sponsored by Codiak BioSciences [31]. Such 500L perfusion runs yield 10^{17}-10^{18} Drug Substance EVs, with a significant potential for a further multifold productivity increase.