

Dear Raluca Flavia Ghiman,

Thank you very much for giving us the opportunity to revise our manuscript entitled “Engineering of MSC-derived exosomes: a promising cell-free therapy for osteoarthritis” (manuscript ID: membranes-1813840). We would also like to thank the reviewers for their insightful questions and suggestions. We have carefully read the comments and revised our manuscript accordingly. Our point-to-point responses are listed as follows. We hope that this revised version can be acceptable for publication in *Membranes*. Please do not hesitate to contact us if there are any further questions.

Yours sincerely,

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Point-to-point responses to reviewers:

Reviewers 1#: In this review, Cheng et al discuss in depth various interdependent lines of research revolving around the use of exosomes in the treatment of osteoarthritis, including exosome biogenesis, detection, separation, characterization, production, modification, loading, and therapy. The review is well written, thorough and comprehensive – an effortless and enjoyable read.

Suggestions:

1. The title and introduction focus solely on exosomes, but EV-related OA research extends to other components of the secretome (see e.g. <https://pubmed.ncbi.nlm.nih.gov/31689923/>). Authors should probably briefly outline the broader picture, then explain their specific focus on exosomes.

Response: Thank you for your suggestion, we have reviewed the related literatures and added the following text to the introduction: “*Similar to MSCs, the use of their secretome (growth factors, cytokines, chemokines, enzymes, lipids, nucleic acids and transcription factors) and extracellular vesicles (either microvesicles or exosomes) has pleiotropic effects on OA arthrosis, such as immunomodulatory, regenerative, anti-catabolic, and chondro-protective properties[12].*” (page 2, line 52-55, highlighted in yellow) The recommended literature was cited as reference 12.

2. Section 2.3.1 states that UC is ‘suitable for scale-up separation’, but that’s almost the opposite of general opinion in my experience. The authors should perhaps better explain what they mean or revise the sentence.

Response: We accepted the suggestions and revised “UC is suitable for scale-up separation” to “*UC is widely used among researchers[50]. However, UC is suitable for large sample volumes but not for the small samples from clinical practice due to the time-consuming process and high cost of instruments [51].*” (page 4, line 155-157, highlighted in yellow)

3. Section 2.3.2 states that SEC ‘fails to separate exosomes from lipoproteins’. That’s true, but all single-step techniques based on density or size fail in this respect, including UC. Authors might add this consideration to sections 2.3.1 and 2.3.2, in the context of e.g. <https://pubmed.ncbi.nlm.nih.gov/28963190/>

Response: Thank you for your valuable comments. We agreed with the suggestions. After reviewing the relevant literatures, we added this consideration and mentioned a feasible technique in the sections 2.3.1 and 2.3.2. The recommended literature was cited as reference 52.

Changes in the manuscript:

(1) On page 4 line 157-159, we have added “*When it comes to plasma-derived exosomes, coisolation with lipoproteins is a major issue because of similar size and density [52].*” (highlighted in yellow)

(2) On page 4-5 line 165-169, we have added that NTA “*fails to separate exosomes from lipoproteins like UC [52,56]. To overcome the limitation of one-step isolation procedure based on density or size, a two-step technique combining density cushion*

separation followed by SEC can efficiently separate exosomes from lipoproteins and plasma proteins [56].” (highlighted in yellow)

4. In the same section, the authors state that “Compared to UC, SEC combined with UF generated up to 58 times more exosomes”, but I think that this result is highly linked to the specifics of reference 55 and does not have a general validity.

Response: We accepted the suggestion and deleted the specific data. That means we changed “*Compared to UC, SEC combined with UF generated up to 58 times more exosomes*” to “*Compared to UC, SEC combined with UF generated more exosomes*”. (page 5, line 170-171, highlighted in yellow)

5. In section 2.3.5, it might be worthwhile to add a mention to DLD (deterministic lateral displacement), see e.g

<https://pubs.rsc.org/en/content/articlelanding/2018/lc/c8lc01017j>

Response: Thank you for your advice. We reviewed the literatures and added the following content to the revised manuscript: “*Deterministic lateral displacement (DLD) is a simple, rapid, scalable and automatable technology for exosomes isolation [67]. For instance, Joshua et al. developed an integrated nanoscale deterministic lateral displacement (nanoDLD) chip containing 1024 parallel arrays capable of isolating serum and urine exosomes with flow rates up to 900 µL per hour [67].*” (page 5, line 189-193, highlighted in yellow) The recommended literature was cited as reference 67.

6. Section 2.4.1 might mention the fact that NTA cannot usually detect particles below 50nm in diameter. Other techniques worth mentioning in this context are dynamic light scattering and atomic force microscopy, see e.g.

<https://pubs.acs.org/doi/full/10.1021/acs.analchem.9b05716>

Response: Thank you for the comments. According to the suggestions and literatures, we added the recommended technologies to the section 2.4.1. The recommended literature was cited as reference 71.

Changes in the manuscript:

(1) On page 5 line 207-208, we have added descriptions for NTA: NTA “*cannot detect exosomes with the size of <50 nm in diameter [69]*”. (highlighted in yellow)

(2) On page 5 line 208-210, we have added descriptions about dynamic light scattering: “*Dynamic light scattering (DLS) is able to detect small particles (>5 nm), which is suitable for measuring monodisperse particles but not for complex exosome samples with large size range [70].*” (highlighted in yellow)

(3) On page 5 line 210-212, we have changed “*This method is unable to distinguish exosomes from protein aggregates or nanoparticles because it relies on particle-induced scattered light rather than particle morphology.*” to “*However, NTA and DLS are unable to distinguish exosomes from protein aggregates or nanoparticles because they rely on particle-induced scattered light rather than particle morphology.*” (highlighted in yellow)

(4) On page 5 line 216-218, we have added descriptions about atomic force microscopy: “*Atomic force microscopy (AFM) is a more efficient alternative for simultaneous nanomechanical and morphological analysis of exosomes[71].*” (highlighted in yellow)

7. In section 5 it might be worth to mention other OA-related studies involving EVs, such as e.g. <https://www.nature.com/articles/s41598-020-80032-7>

Response: Thank you for the comments. According to the suggestions, we have reviewed previous literatures and added other studies in the section 5 and 5.1. The recommended literature was cited as reference 177.

Changes in the manuscript:

(1) On page 12 line 479-482, we have added “*It is worth noting that other sources of exosomes play an important role in OA progression or treatment, including but not limited to chondrogenic progenitor cells [167], OA subchondral bone [168], platelet-rich plasma [169], and vascular endothelial cells [170].*” (highlighted in yellow)

(2) On page 13 line 532-534, we have cited the recommended literature and added “*ADSC-derived exosomes could also affect gene expression and protein release of both chondrocytes and synoviocytes, weakening IL-1 β -induced inflammatory response [177].*” (highlighted in yellow)

8. I’m quite sure it’s a problem linked to pdf conversion, but the small lettering in figure 1 was barely readable in the form I’ve received it.

Response: Thank you for your kind feedback. We have divided the original figure into two figures, and presented the text in a larger size and in bold font. The modified figures are on page 4 and 9 respectively:

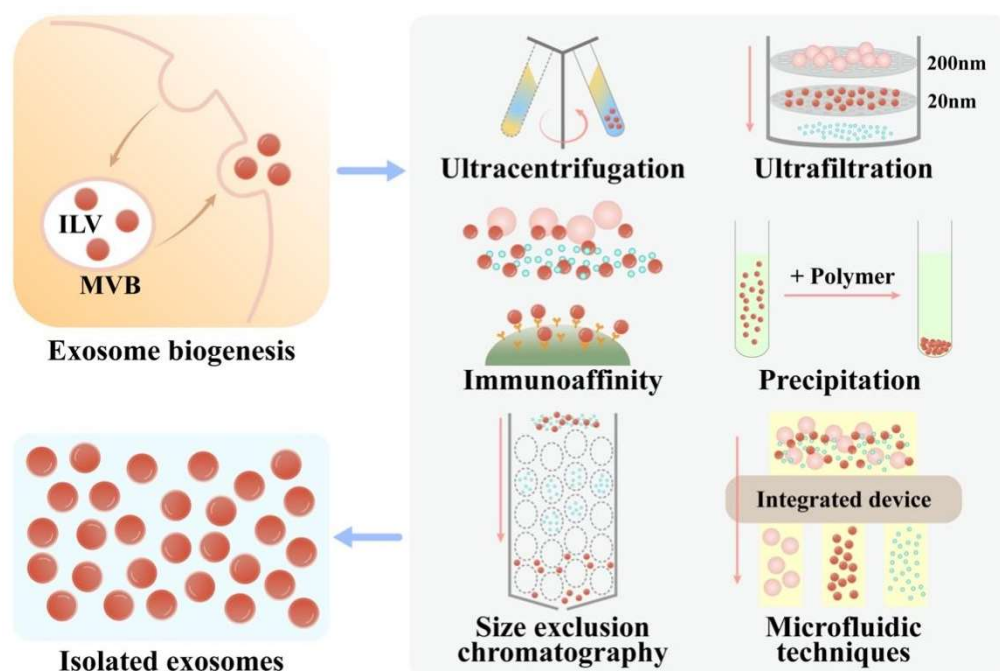
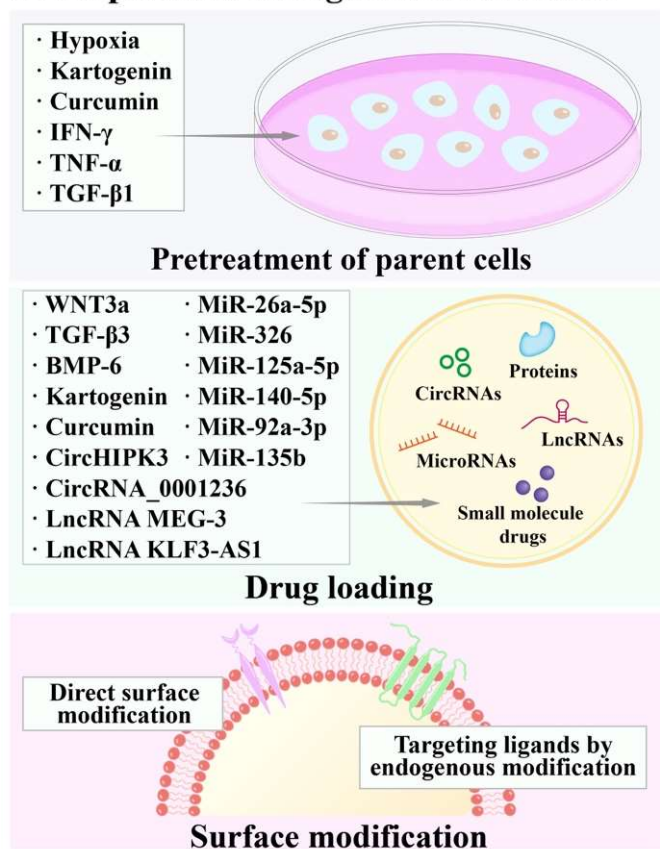


Figure 1. Methods for exosomes isolating. ILV, intraluminal vesicles; MVB, multivesicular bodies

A. Preparation of engineered exosomes



B. Delivery for OA treatment

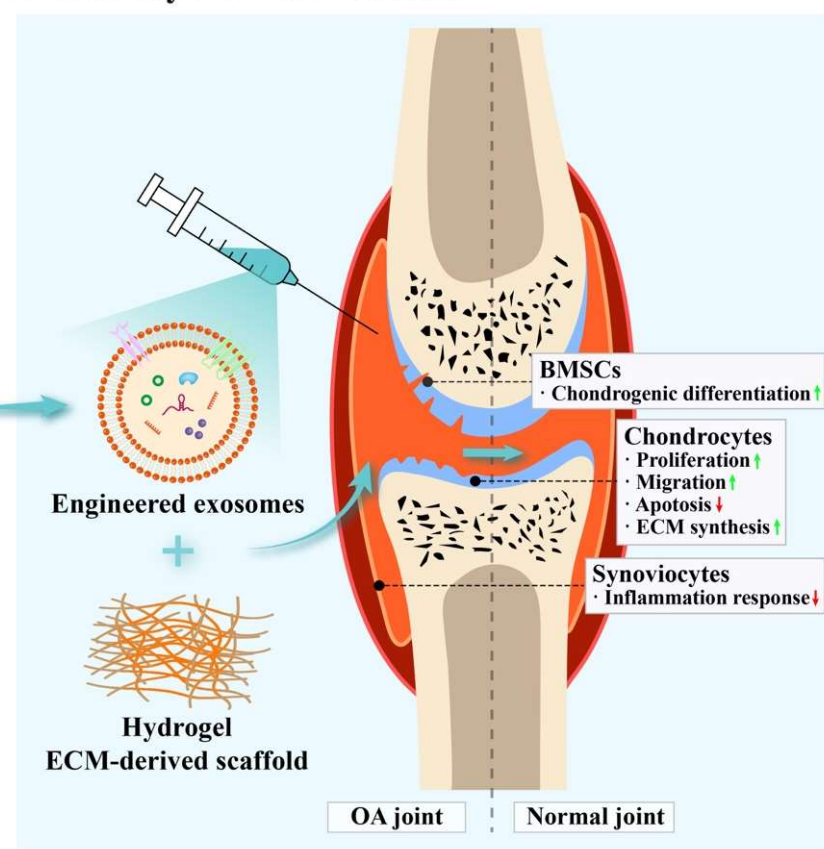


Figure 3. Processing and application of engineered exosomes for Osteoarthritis (OA) treatment. A) Preparation techniques for engineering exosomes. The processing methods include pretreatment of parent cells, drug loading and surface modification. B) Application of engineered exosomes for OA. The engineered exosomes are administrated to the OA joint through intra-articular injection or scaffold implantation and exert their regulatory effects on bone marrow mesenchymal stem cells (BMSCs), chondrocytes and synoviocytes. As a result, the exosome-based therapy can suppress OA progress and accelerate cartilage regeneration to restore OA joint.

Authors 2#: This is an interesting manuscript in which the authors review the utility, as well as limitations, of exosomes for osteoarthritis treatment. In summary, it contributes to update the state of the art on this topic.

Suggestions:

1. However, the authors have not included citations to relevant, recent papers (both reviews and primary research articles) that have been published on this issue. E.g. reviews PMID 31753036 PMID 32398951, or PMID 10.1097/JCMA.0000000000000570, as well as other primary articles. Please include these cites and discuss the novelty of the present manuscript compared to these recent reviews.

Response: Thank you for the valuable comments. We have carefully gone through recent literatures, cited and summarized the relevant findings in the discussion section and highlighted our novelty. The recommended literatures were cited as reference 226, 227 and 228.

Changes in the manuscript:

(1) On page 17 line 736-744, we have added *“There have been several reviews describing the pathogenic roles and therapeutic potential of exosomes in OA, and summarizing related engineering strategies, which have provided solid evidences that exosomes can serve as a promising cell-free therapy for OA [226-228]. Given the hurdles encountered in the clinical translation of exosomes, we focus more on detailed techniques for large-scale exosome production and preparation of engineered exosomes, and summarize treatment ideas including not only drug loading in exosomes, but also the assistance of biological materials. The purpose is to review the current technical methods and therapeutic strategies, and strive to promote the realization of clinical application of exosome in OA.”* (highlighted in yellow)

Authors 3#: The article providing insights into exosome-based cell-free therapy for the treatment of OA

Suggestions:

1. What are specific different approached that enhance the engineered exosomes for Osteoarthritis.

Response: Transfection by lentivirus, incubation, repeated freeze-thaw procedure and electroporation are commonly used to enhance the engineered exosomes for osteoarthritis, but these methods also apply to exosome engineering for....

2. Please add one table to summarize the clinical trials related to the manuscript and how exosomes engineering is good over the naive??

Response: Thank you for the valuable comments. According the suggestions, we searched for clinical trials related to exosome therapies for osteoarthritis on Pubmed and clinicaltrials.gov, and ultimately obtained four related clinical trials.

Changes in the manuscript:

(1) On page 18 line 796-804, we have added the discussion: *“To date, no clinical trials have been completed to evaluate exosome-based therapy for OA. There are a few clinical trials in progress (Table 1). Accumulating preclinical trials have shown that engineered exosomes have strengthened biological functions and targeting ability, confirming their therapeutic potential for OA, but lack of clinical trial validation. Insufficiency of clinical evidences has seriously hindered the translation of exosome-based therapy from labs to clinical application. Moreover, despite significant progress in understanding the biology of exosomes, the functional mechanism and the safety of MSC-Exos need further study. Researches on exosome-based therapy for OA are still at an early stage, more clinical trials can be expected in the future.”* (highlighted in yellow)

(2) On page 18 line 805-808, we have added table 1 as follow (highlighted in yellow):

Table 1. Clinical trials related to secretome therapies for OA.

Study	Official title	No. of patients	Interventions	Study design	Estimated time	Phase	NCT number
1	A Phase I Study Aiming to Assess Safety and Efficacy of a Single Intra-articular Injection of MSC-derived Exosomes (CelliStem®OA-sEV) in Patients With Moderate Knee Osteoarthritis (ExoOA-1)	10	Exosomes	Interventional, Single Group Assignment, None (Open Label)	2021.10-2023.10	Phase 1	NCT05060107
2	Umbilical Cord Derived Wharton's Jelly for Treatment of Knee Osteoarthritis	12	Umbilical Cord-derived Wharton's Jelly ¹	Interventional, Single Group Assignment, None (Open Label)	2022.01-2023-12	Early Phase 1	NCT04719793
3	Comparative Effectiveness of Arthroscopy and Non-Arthroscopy Using Mesenchymal Stem Cell Therapy (MSCs) and Conditioned Medium From Mesenchymal Stem Cell Culture (MSCs) for Osteoarthritis With Controlled Randomization in Phase I/II	15	Mesenchymal Stem Cells with Arthroscopy Mesenchymal Stem Cells without Arthroscopy Conditioned Medium without Arthroscopy	Interventional, Randomized, Parallel Assignment, None (Open Label)	2020.08-2024.12	Phase 1 Phase 2	NCT04314661
4	Secretome From Mesenchymal Stem/Stromal Cells on Human Osteochondral Explants: Cocktail of Factors Secreted by Adipose-derived Stromal Cells (ASC) for the Treatment of Osteoarthritis and/or for Articular Regeneration	24	ASC secretome ²	Observational, Cross-Sectional	2021.04-2022.12		NCT04223622

¹ Growth factors, cytokines, hyaluronic acid and extracellular vesicles including exosomes are all present in large quantities.

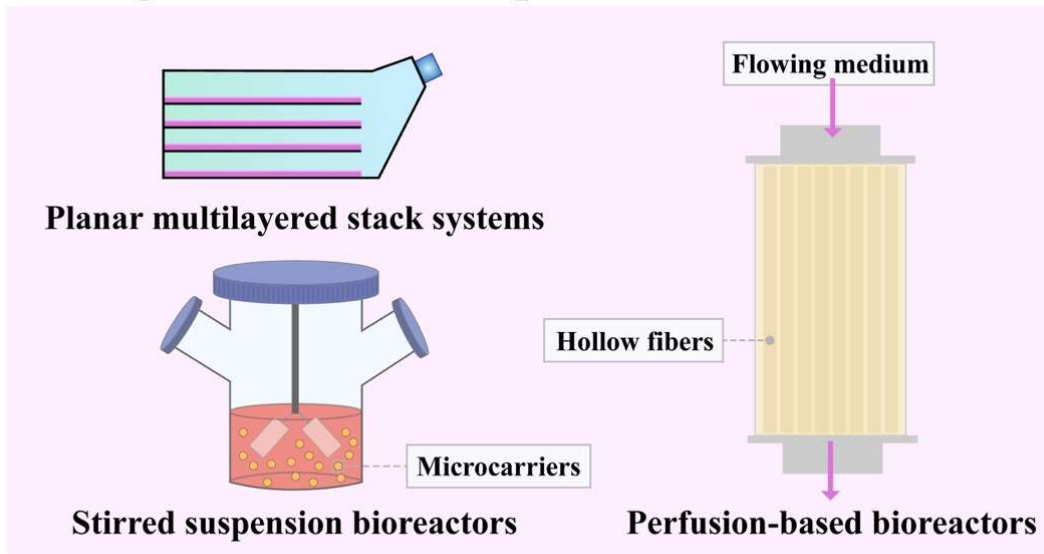
² Either complete conditioned medium or extracellular vesicles.

As for the advantages of engineered exosomes compared to natural exosomes, on page 2 line 70-75, we showed “*Although exosomes have their own benefits in terms of tissue regeneration, they still have disadvantages such as low yield, weak function, and low targeting, which fail to meet quantity and quality needs for disease treatment. A series of engineering approaches have been developed to overcome the above limitations of natural exosomes and advance exosome therapy into clinical practice.*” (blue font)

3. If possible also add one more figure and abstract figure.

Response: Thanks for the comments. We have added one figure on page 6 to briefly summarize techniques for large-scale exosome production. The figure is named “Figure 2” as following:

A. Large-scale cell culture platforms



B. Increasing single cell secretion of exosomes

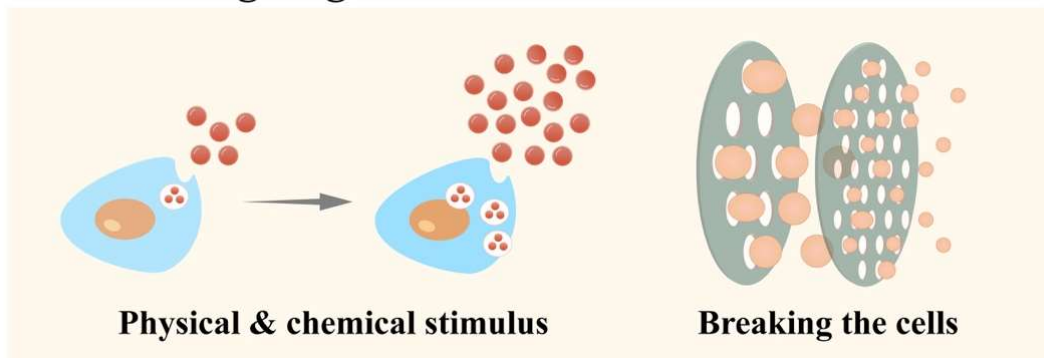


Figure 2. Techniques for large-scale exosome production. A) Large-scale cell culture platforms. They include planar multilayered stack systems, stirred suspension bioreactors and perfusion-based bioreactors (e.g., hollow fiber bioreactor). B) Increasing single cell secretion of exosomes. The methods mainly include stimulating the cells (e.g., hypoxia) and disrupting cell membranes (e.g., extrusion).

4. Please make it clear that what is the difference between MSC derived exosomes and benefits over the other sourced exosomes.

Response: Thank you for the comments. According to the suggestions and retrieved literatures, we added the comparison and discussion between MSC-derived exosomes and other sourced exosomes: “*Different sourced (e.g., immune cell, blood cell, neural cell) exosomes have their unique biological functions and tendency to cure certain diseases, on the basis of their specific properties and cargoes [225]. Compared to other sources, MSC is a popular choice because of the convenience of isolation and specialized biological functions [32]. As pluripotent and prolific producers of exosomes, MSCs can produce exosomes containing bioactive components related to self-renewal and differentiation [225]. It is also essential to*

continue exploring the clinical potential of other sourced exosomes.” (page 16-17, line 728-735, highlighted in yellow)

5. This paper briefly describes the current literature. I suggest to added more analysis and future directions. How it advance the current field.

Response: Thank you for the comments. We have added more analysis and future directions in the discussion part.

Changes in the manuscript:

(1) On page 17 line 776-777, we have added “*A new direction is to take advantage of nanoparticles [233,234].*”

(2) On page 18-19 line 810-815, we have added “*Future studies should also pay attention to the establishment of standards on production, storage, and transportation of exosomes, and to find appropriate doses and the optimal time windows for administration. With the in-depth exploration of the mechanism of exosomes in OA treatment, the required cargoes can be assembled into engineered exosomes according to the individual joint conditions of patients, to achieve personalized treatment and precision medicine.*”

(3) On page 19 line 816-817, we have added “*In conclusion, despite the challenges, MSC-derived engineered exosome-based cell-free therapy holds great promise in the treatment of OA and deserve further research.*”