Techniques for increasing the yield of stem cell-derived exosomes: what factors may be involved?

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Exosomes are nano-scale extracellular vesicles secreted by cells and constitute an important part in the cell-cell communication. The main contents of the exosomes include proteins, microRNAs, and lipids. The mechanism and safety of stem cell-derived exosomes have rendered them a promising therapeutic strategy for regenerative medicine. Nevertheless, limited yield has restrained full explication of their functions and clinical applications. To address this, various attempts have been made to explore the up- and down-stream manipulations in a bid to increase the production of exosomes. This review has recapitulated factors which may influence the yield of stem cell-derived exosomes, including selection and culture of stem cells, isolation and preservation of the exosomes, and development of artificial exosomes.

exosome, stem cell, yield, therapy

Introduction

Exosomes were discovered about 50 years ago, since then research to decipher their underlying mechanisms has been thriving (Anderson, 1969; Crawford, 1971). All cells can generate this type of extracellular vesicles (EVs) with an average diameter of 100 nm (usually in a range between 40 and 160 nm) and a cup shape under an electronic microscope (Mehryab et al., 2020). The exosomes comprise a myriad of bioactive compounds, including proteins, microRNAs, lipids, and DNA segments (Ferguson and Nguyen, 2016), which play a crucial role in the interplay of diverse cells (Kowal et al., 2014; Kalluri and LeBleu, 2020). The exosomes are generated and derived from a special bilayer organelle called multivesicular body (MVB). The formation of MVB includes several phases: (1) germination, starting with the formation of an incurvate structure at specific loci on the cell membrane; (2) formation of early-sorting endosomes (ESEs), which results in its outside-in plasma membrane orientation; (3) late-sorting endosomes (LSEs), which are matured from the ESEs when precursors of the exosomes, namely intraluminal vesicles (ILVs), are germinating, and (4) transformation to mature MVBs with ILVs lying at inside. Notably, as the formation of the exosomes is associated with specific sorting mechanisms, the compounds in such vesicles are divergent from other EVs, e.g., microvesicles, microparticles and other large vesicles, with respect to membrane proteins, lipids, and inclusions (Figure 1) (Trajkovic et al., 2008; Kowal et al., 2014; Kalluri and LeBleu, 2020). Such characteristics can also help with the discrimination of the exosomes from other vesicular structures.

Stem cell-based therapies have been tailored for the treatment of a broad range of diseases (Ratajczak et al., 2006; Bruno et al., 2019; Zakrzewski et al., 2019). Over 1,200 clinical trials involving utilization of mesenchymal stem

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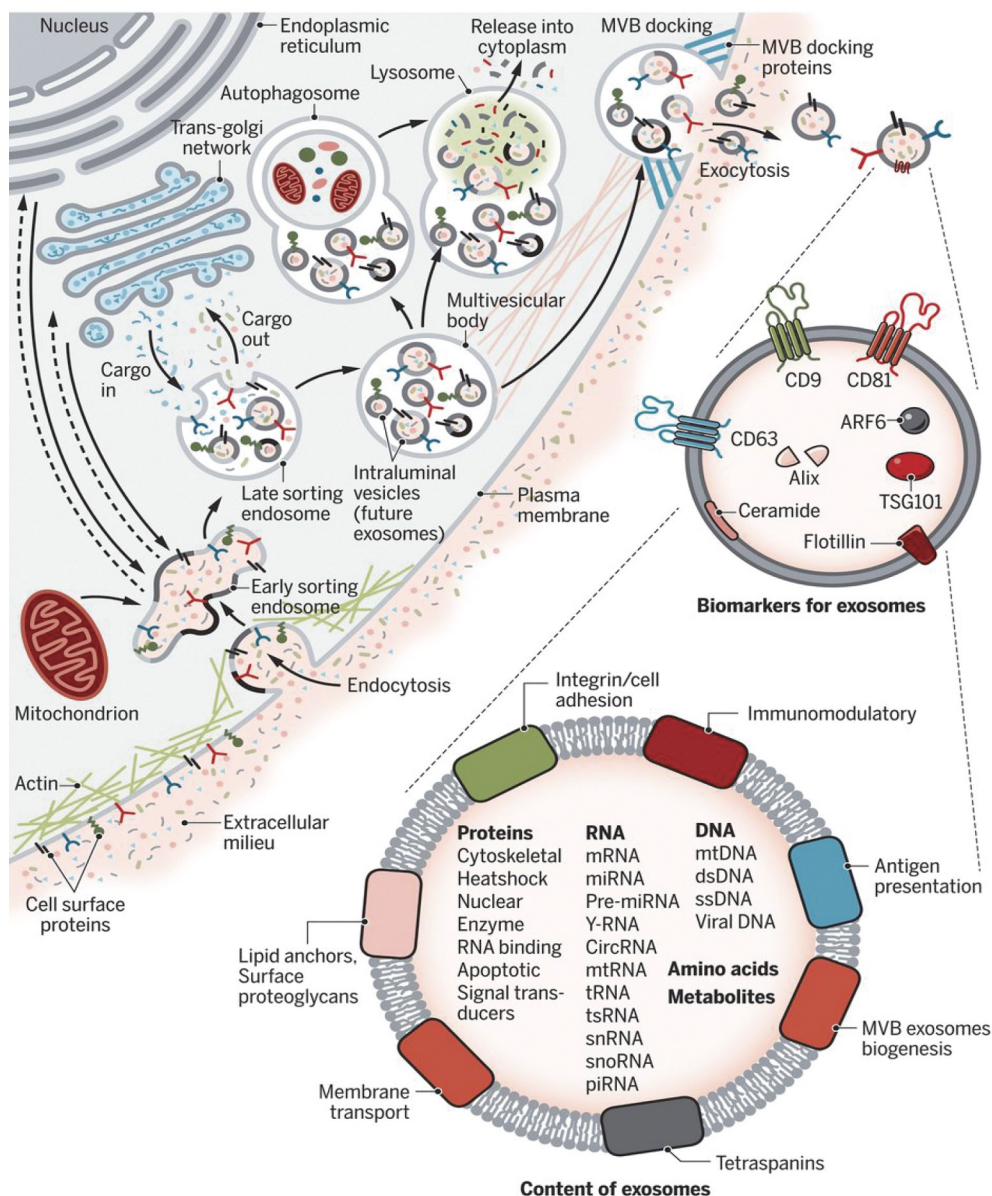*Corresponding author (email: xiehuiqi@scu.edu.cn)

Figure 1 Biogenesis and identification of exosomes. Exosomes originate from plasma membrane, and can be followed by fusion with the endoplasmic reticulum, trans-Golgi network, and mitochondria. The process goes through several stages and involves numerous pathways including Rab GTPases and ESCRT. The exosomes contain membrane and luminal constituents, including CD9, CD63, CD81, flotillin, TSG101, ceramide, and Alix, which can be used as molecular markers (Kalluri and LeBleu, 2020).

cells (MSCs) have so far been registered in ClinicalTrials. gov (Phinney and Pittenger, 2017). Inspiringly, apparent benefit and safety have been noted in most of these trials. Stem cell-derived exosomes possess favorable pharmacokinetic property, biocompatibility, and tissue-targeting ability owing to their bilayer structures and constituents of mRNAs, microRNAs, cytokines, chemokines, and immunomodulatory compounds (Mendt et al., 2019; Harrell et al., 2019; Haraszti et al., 2019; Fernández-Francos et al., 2021). Moreover, the ability of the exosomes to suppress inflammation, regulate cell proliferation, and promote damaged tissue repair has been corroborated (Harrell et al.,

2019; Massa et al., 2020), for example, in the skin (An et al., 2021), muscle and bone (Nakamura et al., 2015; Hao et al., 2017; Mianehsaz et al., 2019), nerve (Tsintou et al., 2021), heart (Bahardoust and Baghoi-Hosseinabadi, 2021), liver (Zhao et al., 2020), kidney (Ishiy et al., 2020), lung (Xu et al., 2020), immune system (Burrello et al., 2016), cancer (Sharma, 2018), and virus infection (Jamshidi et al., 2021) (Figure 2). Introduction of the exosomes into biomaterials, such as exosome-laden hydrogel (Wang et al., 2019a; Wang et al., 2019b), exosome-coated scaffold (Zhai et al., 2020; Kyung Kim et al., 2021), and exosome-based drug delivering vectors (Barile and Vassalli, 2017; Mehryab et al., 2020), has

also become hot topics in regenerative medicine research. Compared with stem cell-based therapies, exosome-based therapies are more promising owing to their better delineated mechanisms. Moreover, the merits of resolution of the tumorigenicity, toxicity, and immune rejection associated with cell therapy, along with the convenience to obtain the exosomes without damages to the cells, have further expanded the design and clinical application of exosome-based therapies (Kuriyan et al., 2017; Mendt et al., 2019).

Nevertheless, the feasibility of exosome-based therapies is still limited, which may be attributed to the trivial amount of the exosomes by individual cells and lack of efficient extracting strategy (Colao et al., 2018; Kalluri and LeBleu, 2020). As discovered, a dose of 800 μg kg^{-1} exosomes is required to render a notable clinic therapeutic effect (Lee et al., 2021). However, only about 10 μg exosomes can be harvested from 1 mL of 2D static stem cell culture medium by ultracentrifugation (Patel et al., 2019; Z. Wu et al., 2021), which suggested that there is still a long way to go before real clinical application. During the last decade, research devoted to enhancing the production of exosomes has increased. Strategies to improve the yield of exosomes have included up- and downstream manipulations (Lai et al., 2010; Colao et al., 2018). Upstream manipulations included selection of stem cells with high productivity (Haraszti et al., 2018), addition of soluble factors (Wang et al., 2020), modification of culture condition (Zhu et al., 2018), and application of high-density cell culture techniques (Kim et al., 2018). Downstream manipulations comprised development of a diverse range of separation methods including gradient centrifugation, precipitation of polymers, ultrafiltration, exclusion chromatography, immunoaffinity adsorption, and microfluidics (Li et al., 2017; D. Yang et al., 2020). As another downstream manipulation, storage technologies have also been improved to facilitate the exosome-based therapy (Charoenviriyakul et al., 2018). Furthermore, artificial exosomes have also been explored for producing exosomes with low cost, high output, and homogenous quality (García-Manrique et al., 2018). In this review, we have systematically summarized the strategies to increase the yield of stem cell-derived exosomes, with an aim to provide reference and inspiration for further research in this field.

Selection of stem cells

The generation and secretion of the exosomes are under complex regulation. Accordingly, the amount and properties of the exosomes are divergent due to their diverse origins, which may lead to discrepant therapeutic outcomes (Haraszti et al., 2016; Charoenviriyakul et al., 2017; Alvarez-Viejo, 2020). Therefore, rigorous selection of appropriate stem cells is crucial for enhancing the yield of the exosomes.

MSCs, which are well defined by the International Society for Cell and Gene Therapy (Dominici et al., 2006), are the most widely used stem cells for exosome studies. The MSCs can be further classified into several subcategories based on their source of derivation including bone marrow, adipose tissue, umbilical cord, skin, dental pulp, and menstrual blood (Lopez-Verrilli et al., 2016; Alvarez-Viejo, 2020). Among these, bone marrow mesenchymal stem cells (BMSCs), adipose mesenchymal stem cells (ADSCs), and human umbilical mesenchymal stem cells (HUMSCs) are most widely studied. As mentioned above, the constituents and therapeutic efficacy of the exosomes derived from disparate stem cells are different (Table 1) (Lopez-Verrilli et al., 2016; Börger et al., 2017; Ji et al., 2019; Alvarez-Viejo, 2020). The divergent ability of exosome secretion by different stem cells may in part account for this phenomenon. As discovered, HUMSCs can secrete exosomes by approximately 4-fold in quantity compared with BMSCs and ADSCs, while concurrently with the largest size (Haraszti et al., 2018). Meanwhile, such exosomes are also stable when cultured in serum-free medium, which is advantageous for massive production (Haraszti et al., 2019).

Other types of stem cells have also been studied, though their secreting capacity and therapeutic efficacy have not been fully explored (Yuan et al., 2018). It would be valuable to obtain comparative data on exosomes from other stem cells such as the urine-derived stem cells (Chen et al., 2018), dental pulp stem cells (Faruqu et al., 2020), and other newly discovered stem cells.

Culture methods of stem cells

The yield of exosomes can be influenced by modification of the culturing condition of stem cells, which may include adjustment of culture medium components, environment parameters or even modes of cultivation. Such factors may in turn influence the properties of exosomes and alter the productivity of the stem cells by regulating their behaviors (Table 2).

Soluble factors

A feasible way to enhance the secretion of exosomes of stem cells is by addition of certain soluble cytokines directly into the culture medium. A myriad of bioactive cytokines, including lipopolysaccharide (LPS) (Ti et al., 2015), N-methyldopamine (Wang et al., 2020), noradrenaline (Wang et al., 2020), and adiponectin (Kita and Shimomura, 2020), have been tried. In such a context, the property and therapeutic efficacy of exosomes may also be changed. Of note, some Chinese medicine, e.g., Suxiao Jiuxin pills, has been shown to contain biomolecules that can elevate the magni-
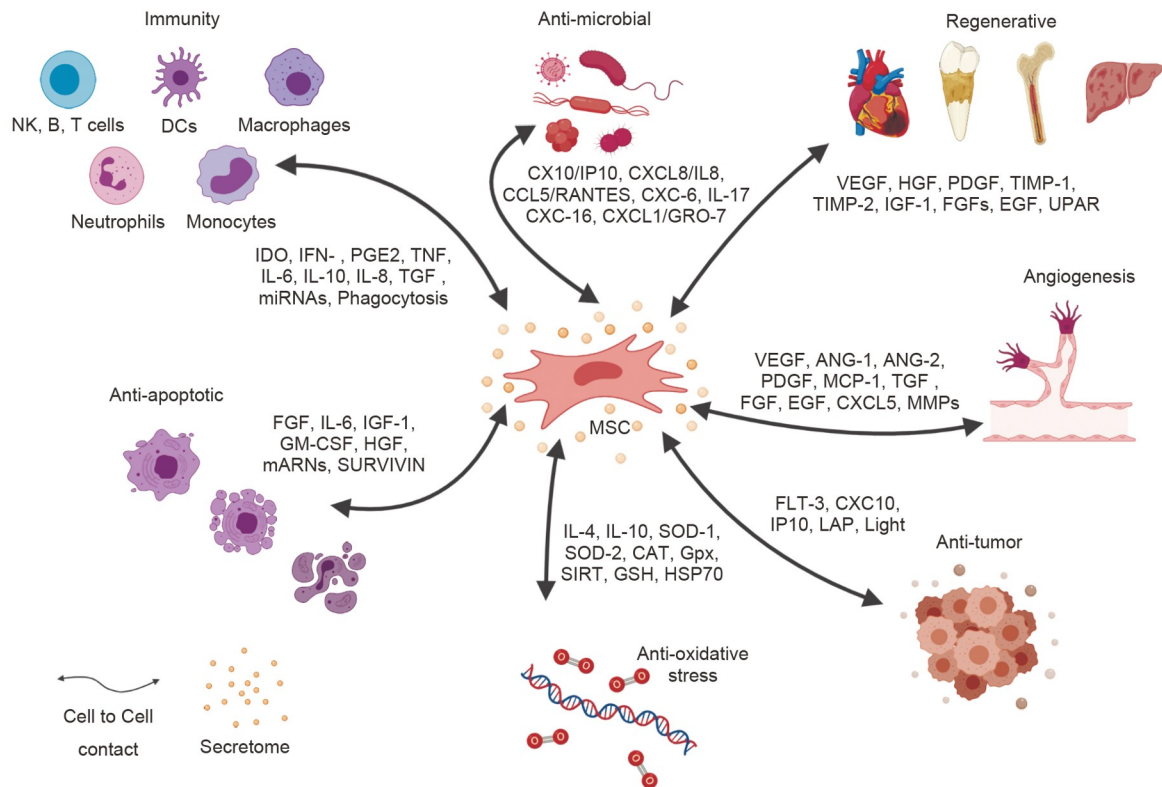
Figure 2 MSC-derived exosomes contain various bioactive compounds and have shown therapeutic benefits in many areas (Fernández-Francos et al., 2021).

Table 1 Comparison of exosomes derived from different types of stem cells

Cellular origin	Comparison method	Exosome comparison results	Applications	Reference
MSCs derived from human umbilical cord (HUMSCs), adipose tissue (ADSCs) and bone marrow (BMSCs)	Proteomic and lipidomic changes in exosome composition in the absence of serum	HUMSCs composition did not show changes, while BMSCs and ADSCs exosomes proteins have decreased	Optimizes conditions to improve the delivery of exosomal cargo and used these optimized exosomes to find molecules which may affect exosomal activity	Haraszti et al., 2019
BMSCs, ADSCs, HUMSCs	Compared the yield and diameter of exosomes isolated by ultracentrifugation	Wharton's Jelly-derived mesenchymal stem cells produce the most exosomes	Produces exosomes by tangential flow filtration in combination with 3D MSC cultures	Haraszti et al., 2018
U87 glioblastoma cells, Huh7 hepatocellular carcinoma cells and BMSCs	High-resolution lipidomic and proteomic analyses	The protein patterns of exosomes were more different from their cells of origin compared with that of MVs	Comprehensively analyses the protein and lipid composition of exosomes, MVs and source cells in 3 different cell types	Haraszti et al., 2016
ADSCs, HUMSCs and BMSCs	Reviewed the clinical and pre-clinical applications of three most used exosomes	Updated research addressing the therapeutic potential of exosomes	Examines the therapeutic potential of exosomes obtained from sources most used in cell therapy	Alvarez-Viejo, 2020
Menstrual MSCs (MenSCs), HUMSCs, BMSCs and chorion MSCs	Compared the effect on neuritic outgrowth in primary neuronal cultures	MenSC exosomes had a similar effect to BM-SC exosomes on neurite outgrowth in dorsal root ganglia neurons	Raises the potential of MenSCs as therapeutic conveyors in neurodegenerative diseases	Lopez-Verrilli et al., 2016
BMSCs and dental pulp stem cells (DPSCs)	Stimulated peripheral blood mononuclear cells and CD4$^+$ T cells with BMMSCs-E and DPSCs-E respectively	Capabilities of DPSCs-E were greater than BMMSCs-E	Compares the immunomodulatory capacity of BMMSCs-E and DPSCs-E	Ji et al., 2019

tude of exosome secretion by stem cells (Ruan et al., 2018).

Unfortunately, even though the soluble factors have shown the validity in influencing the exosome secretion by stem cells, there is still a long way to go before large-scale ap-

plications due to certain limitations including potential physiological status alternation and internalization of the agents into the parent cells (Silva et al., 2015; Zou et al., 2019). Hence, researchers have been reluctant to add soluble

Table 2   Comparison of cell culture condition

Methods	Cellular origin	Fold increase	Characteristic alternation	Reference
		Soluble factors		
N-methyldopamine and nore-pinephrine	BMSCs	3-fold	No significant alternation in the structure, composition and therapeutic effect of exosomes	Wang et al., 2020
LPS	HUMSCs	1.37-fold	Let-7b was upregulated, which enhanced Immunotherapeutic effect	Ti et al., 2015
Adiponectin	Endothelial F2T cells	3-fold	Adiponectin was present in exosomes	Obata et al., 2018
Suxiao Jiuxin pills	Cardiac MSCs	3-fold	Rab27a, SYTL4, Rab27b proteins and the small GTPases were upregulated	Ruan et al., 2018
		Chemical/physical stimulation		
Serum-deprived stress	BMSCs, ADSCs, HUMSCs	No significant improvement	Exosome activity was improved, while MVs activity was impaired	Haraszti et al., 2019
Hypoxia stress	BMSCs, ADSCs,	–	Therapeutic molecular including miRNA (miR-216a-5p, miR-125b, etc.) and proteins are upregulated	Zhu et al., 2018; Liu et al., 2020; Geßner et al., 2021
Flow or stretch stimulation	Dental MSCs, ADSCs, skeletal muscle cells	Up to 40.7-fold, differ between the cells	Isolated EVs showed typical structure, whereas 216 proteins were differentially expressed	Guo et al., 2021
Mechanical stimulation caused by a rotary cell culture system	HUMSCs	4-fold	Exosome composition, especially lncRNA H19, was upregulated, which enhanced the therapeutic effect	L. Yan et al., 2021
High frequency acoustic irradiation stimulating	U87-MGcells	8–10-fold	The EVs size distribution of stimulated cells consisted primarily of exosomes	Ambattu et al., 2020
		3D Culture		
3D spheroid culture	BMSCs	2–3-fold, decreased with increasing 3D spheroid size	Not mentioned	Kim et al., 2018
3D-printed scaffold-perfusion bioreactor system	Human dermal microvascular endothelial cells	10,000-fold by NTA, 14-fold by CD63 ExoELISA and 6.5-fold by BCA	Total protein content per exosome showed significant decrease	Patel et al., 2019
Engineered 3D tissue and flow bioreactor	Dental MSCs, ADSCs, skeletal muscle cells	Up to 40.7-fold (differ between the cells)	Isolated EVs showed typical structure, whereas 216 proteins were differentially expressed	Guo et al., 2021
Microcarrier-based 3D culture	HUMSCs	20-fold	Proteomics analysis showed 357 high-abundance proteins detected in all exosome variants and 21–369 low-abundance proteins unique to an exosome variant	Haraszti et al., 2018
		Biomaterials		
Nitric oxide-releasing polymer	Human placenta-derived MSCs	No significant improvement	MiR-126 was upregulated, which enhanced the pro-angiogenic activity of exosomes	Du et al., 2017
Li-incorporated bioactive glass ceramic	BMSCs	No significant improvement	MiR-130a was upregulated, which enhanced the pro-angiogenic activity of exosomes	Liu et al., 2019
PLGA nanoparticle	BMSCs	2-fold	Specific antioxidants or tissue regeneration factors may be increased	Park et al., 2020
Bioglass	BMSCs	2-fold	The ability of promoting ECs vascularization was enhanced	Z. Wu et al., 2021

factors for preserving the potential of the culture medium. In fact, the main purpose of adding soluble cytokines to the culture medium was to delineate the mechanisms by which the secretion of exosomes is regulated.

Chemical/physical stimulation

Cells may alter their characteristics to adapt to changes of their environment, and such alternations can be partially reflected by their secretions. As tissue generators, stem cells have been proven to be activated when tissue damage signals are triggered. Based on this rationale, researchers are inspired to create the damage-mimetic micro-environment chemically or physically during stem cell cultivation, with an aim to obtain exosomes with boosted production and therapeutic functions (Pusic et al., 2016; Yin et al., 2019).

Chemical stimulations such as hypoxia can influence the secretion of exosomes by the stem cells. Exosomes isolated

from the MSCs treated with hypoxia usually exhibited an enhanced therapeutic effect (Zhu et al., 2018; Liu et al., 2020; Geßner et al., 2021). Serum deprivation was another novel means to stimulate the secretion of exosomes by the stem cells, as substances in the culture serum may also to some extent interfere with the secretion (J. Li et al., 2015; Haraszti et al., 2019). In addition, mechanical forces such as flow and stretching factors have also been shown to impact the secretion of stem cells via bioreactors, which elevated the production of EVs by up to 37-fold (Guo et al., 2021; Yan et al., 2021). Furthermore, Ambattu et al. demonstrated that stimulating cells with high-frequency ultrasound could result in an 8–10-fold increase in the yield of exosomes (Ambattu et al., 2020). However, whether such changes are attributable to the fragmented debris of cell membrane remains to be determined. Of note, the aforementioned stimulations may also impact the phenotypes of the source cells (Haraszti et al., 2019).

3D culture

Along with the development of upstream manipulation techniques, researchers have tried 3D culture for enhancing the yield of exosomes. The 3D culture can maximize the culturing area and incessantly apply a shear force (Colao et al., 2018). An early method was the hanging-drop in a 3D spheroid culture (Kim et al., 2018). However, the efficiency of this method was stagnated after a gain by only 2–3-fold. Patel et al. increased the yield of exosomes by nearly 100-fold by culturing the cells on a 3D printed hollow fibrillar scaffold with a complementary perfusion system (Patel et al., 2019). However, subsequent experiments have found that the morphology and component of exosomes were significantly altered. The membrane proteins were critically decreased through such a process. Worse still, the fabrication procedure of this 3D-printing scaffold was too complex to be widely adapted.

Microcarrier-based suspension culture is so far the most suitable platform for 3D stem cell cultivation and has been extensively used for commercialized production (Tavassoli et al., 2018; Koh et al., 2020). The method was firstly applied in 2015 for the extraction of the exosomes (Jarmalavičiūtė et al., 2015). Researchers noted that, compared with the other two types of MSCs (BMSCs and ADSCs), HUMSCs derived from Wharton's jelly had the highest yield of exosomes, and the output could be further increased by approximately 20-fold when a microcarrier suspension culture system was applied, or even 140-fold when a tangential flow filtration system was introduced (Haraszti et al., 2018). This work was subsequently adapted by Xu et al. for the development of an exosome-based therapy (Ling et al., 2020). Furthermore, a bioreactor system invented by Tsinghua University has proven to be convenient for large-scale production of stem

cell-derived exosomes (Yan et al., 2020). Nevertheless, the culturing parameter and shear stress during suspension cultivation should be carefully evaluated in order to limit cell death and phenotype transformation (Becquart et al., 2016).

Biomaterials

The materials of culture substrate may provide another avenue for increasing the yield of stem cell-derived exosomes. Typical examples include NO-releasing polymer (Du et al., 2017), lithium-incorporated bioactive glass ceramic (Liu et al., 2019), ferroferric oxide coated PLGA nano-particles (Park et al., 2020) and bioglass (Z. Wu et al., 2021). In addition to the productivity, biomaterials could also improve the therapeutical efficacy of exosomes by forming a specific micro-environment to interact with the cells, which may to some extent determine the fate of stem cells (Novoseletskaya et al., 2019). Although many biomaterials have been demonstrated to influence the paracrine of stem cells (Peng et al., 2017), definite validations regarding the exosomes are scarce. Nevertheless, biomaterials and cultivation technologies could be combined, for instance, by extracellular matrix (ECM)-derived microcarriers, to further enhance the secretion of exosomes by stem cells (Turner and Flynn, 2012; Kornmuller et al., 2017).

Isolation of exosomes

In addition to the aforementioned upstream manipulations, isolation techniques are also critical to increase the yield of exosomes. Currently available techniques for the extraction of exosomes are mainly based on their chemical, physical, and immunological properties, and are adapted from the methods used for the extraction of virus and microvesicles, such as ultracentrifugation, polymer precipitation, ultra-filtration, exclusion chromatography, immunoaffinity chromatography, and microfluidics. To date, technology and facility designed exclusively for the extraction of exosomes are scarce (Li et al., 2017; Lane et al., 2017; D. Yang et al., 2020). Similar to upstream manipulations, the selection of extracting methods may impact not only the yield of exosomes, but also their characteristics such as size, structure, and biofunction (Rekker et al., 2014) (Table 3). Hence, isolation methods should be carefully selected based on the aim of research as well as characteristics of the method and sample. Notably, with proper combination, up- and downstream manipulations could be integrated in a multiplicative manner to increase the total yield of exosomes substantially.

Centrifugation and sedimentation

Differential ultracentrifugation, or ultracentrifugation, has

Table 3   Exosome isolation techniques

Isolation technique	Principle	Sample volume	Process time	Purity	Recovery	Reference
Differential ultracentrifugation	Sedimentation rate	100s of mL	3–6 h	Low	5%–20%	Tauro et al., 2012; Nordin et al., 2015
Gradient ultracentrifugation	Density, size and shape	~1 mL	20–24 h	High	10%–40%	Tauro et al., 2012
Polymer precipitation	Sedimentation rate	>100 μL	2–18 h	Low	5%–30%	Y. Tang et al., 2017; Börger et al., 2020
Ultrafiltration	Size	~10 mL	1–3 h	Moderate	~30%	Nordin et al., 2015
Tangential flow filtration	Size	100s of mL	1–2 h	Moderate	~80%	Busatto et al., 2018
Size-exclusion chromatography	Size	~1 mL	1–2 h	Moderate	40%–80%	Gámez-Valero et al., 2016; Moleirinho et al., 2019
Immunoaffinity capture	Surface marker expression	~100 μL	18–20 h	Very high	>90%	Foroni et al., 2020
Microfluidics-based techniques	Surface marker expression, size, density, etc.	~100 μL	1–3 h	High	40%–90%	Liu et al., 2017; Han et al., 2021

remained the standard method for extracting exosomes. The rationale behind this technique is that cell fragments, apoptotic bodies, and other large-size components in the culture medium can be separated according to their respective volume, density, and morphology (Witwer et al., 2013; Zarovni et al., 2015). Despite that ultracentrifugation has a high productivity and is easy to use, its clinical translation is limited due to low specificity, time-consuming, requirement of expensive device, difficulty in extracting exosomes from small-sized sample, and the causally occurred fusion of the extracted exosomes.

Density-gradient centrifugation can overcome the drawback of lacking specificity in ultracentrifugation by a special density-gradient medium method, which can be further classified into two categories, i.e., the isopycnic and moving-zone density-gradient centrifugation (D. Yang et al., 2020; Li et al., 2017). For isopycnic density-gradient centrifugation, the medium (usually comprised of sucrose and iodixanol) was set in a tapered density gradient from the bottom to the top. By this way, the dissolved substances with similar density will stay in a same layer, thus separating the exosomes from other compounds with large density discrepancy (Greening et al., 2015; D. Yang et al., 2020). Nevertheless, it is still difficult to separate the EVs with similar densities, resulting in impurity of exosomes.

In order to isolate the exosomes from other compounds with similar densities such as MVs, a moving-zone density-gradient centrifugation technique was developed, where a centrifugal medium with a density lower than other solvents was used. All dissolved substances in the system are incessantly moving during centrifugation (Li et al., 2017; D. Yang et al., 2020). Therefore, by carefully calculating the parameters of the exosomes and other compounds (such as MVs) and setting the centrifugation time, the exosomes could be isolated according to their disparate size, mass as well as density (D. Yang et al., 2020). Even though such

procedures are sophisticated, tedious, and with limited handling capacity compared with the ultracentrifugation method, it has the advantages of high specificity, broad application, and higher output, which have rendered it a suitable alternative for extracting special exosomes from media with limited sample availability, e.g., plasma and other biofluids (Greening et al., 2015).

Of note, polymer precipitation has been reported to efficiently increase the sedimentation rate of exosomes, which could decrease the centrifugal force required for extracting exosomes. The polymers can absorb the water on the surface of exosomes, making them easier to precipitate even at a lower centrifugation speed, thus producing exosomes with simpler equipment in less time. Furthermore, low cost and convenience to manipulate are also merits of polymer precipitation (Zeringer et al., 2015). Unfortunately, soluble proteins may also be precipitated during this process, thus affecting the purity of extracted exosomes (Lane et al., 2017). To this end, researchers are endeavored to improve the sample capacity of polymer precipitation, for instance, by introduction of PEG (Börger et al., 2020) and protamine (Deregibus et al., 2016). Currently, the majority of commercially available extracting kits, such as ExoQuick and Total Exosomes Isolation, are adapted from such methods (Ryu et al., 2020).

Size-based technique

For the tiny size of the exosomes, it is feasible to separate them from proteins and other large EVs. Ultrafiltration is a method for extracting the exosomes by their difference in size (Zeringer et al., 2015; Cardoso et al., 2021). As reported, ultrafiltration technique not only can filter out small-size compounds to obtain relatively pure exosomes, but also has the advantages of rapidity, stability, automation, and higher recovery, which may further expand its application in the

future (Heinemann et al., 2014). However, EVs with a larger size may rupture owing to the powerful stress during filtration, which may release other inclusions to contaminate the exosomes. Meanwhile, blockade and rupture of the filter membrane are often inevitable (Batrakova and Kim, 2015).

As a result, tangential flow filtration (TFF) strategy, a method originally used for extracting viruses and proteins, was adapted to prevent the filtrated particles from damage, possibly by allowing the dissolved substances to tangentially filtrate through the membrane. During the TFF, samples can be re-circulated to repeat the process several times to improve the recovery of exosomes (Haraszti et al., 2018; Busatto et al., 2018; Han et al., 2021). Given the advantage of high sample capacity and automation, and the ability to isolate exosomes with high purity, it is possible to produce stem cell-derived exosomes on an industrial scale by using TFF.

Exclusion chromatography technology has also been tested for it can gently isolate the exosomes without ultra-centrifugation (Lai et al., 2010; Cardoso et al., 2021). Despite the low recovery, the purity of exosomes obtained in this way is comparable to that by density gradient ultra-centrifugation, while structure disruption caused by the centrifugal force may be avoided (Lobb et al., 2015). Nevertheless, it is difficult for such method to separate the exosomes from other vesicles or particles with similar diameters such as MVs and protein polymers.

Immune-based technique

Accurate identification of the copious surface markers such as CD9, CD63, CD81, and phosphoryl serine (Kosaka et al., 2013; Montecalvo et al., 2013) is critical for the development of specific antibodies and receptors to bind with such protein markers to capture the exosomes. As reported, exosomes extracted from 450 µL medium by immunoaffinity was equal to the amount extracted from 2.5 mL medium by ultra-centrifugation (Zarovni et al., 2015; Y. Tang et al., 2017). Apart from immunoaffinity column chromatography, magnetic pellets with immobilized antibodies have also been widely utilized for the separation and validation of the exosomes (Clayton et al., 2001; McBride et al., 2017). Immunoaffinity methods may suit exosome research perfectly by generating a high purity product with a good recovery rate. On the other hand, drawbacks such as high cost and sophisticated manipulation have limited its application on a larger scale (Foroni et al., 2020). To further improve the efficiency of extracting exosomes by immunoaffinity extraction, a method based on immunoassay upon mass spectrometry has been developed (Ueda et al., 2014).

Microfluidics

Among the newly developed strategies for extracting and analyzing the exosomes, microfluidics has been the mostly studied and adapted. By this means, the extracting device can precisely isolate the exosomes based on their physical and chemical properties. Other isolation strategies, such as tangential flow filtration (Han et al., 2021), can be integrated with the microfluidic chip and greatly improve the efficiency and specificity of exosome extraction, in particular for small-sized samples (Liu et al., 2017; Contreras-Naranjo et al., 2017).

The strategies of microfluidics used for the extraction of exosomes have included not only the surface antigen, density and size recognition, but also elastic lift force (Liu et al., 2017), ultrasonic waves (Lee et al., 2015), and electrophoretic manipulations (Ayala-Mar et al., 2019), whilst the dissociation strategies can be classified into contact type and non-contact type (D. Yang et al., 2020). Based on such technology, multilayer micro-nano fluid technology was developed to further improve the efficiency of exosome extraction (Sundaram et al., 2020). Due to its low sample handling capacity, microfluidic technologies have often been applied to analyze exosomes from small-sized samples. Nevertheless, integration of exosome manipulations with highly automated mode is still worthy to explore.

Others

For the resemblance between the viruses and macromolecules in exosomes, technologies used for the extraction of viruses have been used for the extraction of exosomes. These included flow field-flow fractionation (Kang et al., 2008; Zhang and Lyden, 2019), static water filtration dialysis (Musante et al., 2014), etc. As mentioned above, a PEG precipitation technique, originally used for virus extraction, has demonstrated a high efficiency to extract exosomes from several liters of culture medium at a time (Börger et al., 2020). Furthermore, considering the similarity between exosomes and other nanoparticles, it is feasible to extract exosomes in any laboratory equipped with facilities for the extraction of proteins and viruses.

To overcome the shortcoming of individual method, combined techniques have been tested. For instance, the isopycnic gradient ultracentrifugation and ultrafiltration can be combined, for which the isopycnic gradient ultra-centrifugation is efficient to separate particles with different densities, while the ultrafiltration can subsequently purify the exosomes according to their size. Zhang et al. (2021) have developed a method called immunomagnetic sequential ultrafiltration, which consists of a tangential flow filtration step and a magnetic-bead antibody-based EV capture step. The combination of ultrafiltration and size-exclusion chromatograph is also a novel strategy worthy of consideration (Nordin et al., 2015; Cardoso et al., 2021).

Storage of exosomes

Storage of exosomes also bears a great deal of importance. Without eligible storage technology, long-term storage cannot be achieved. In this circumstance, exosomes have to be produced whenever a demand emerges, which can severely increase the burden to the packaging, transportation, and preparation. Therefore, it is necessary to develop a method to store the exosomes in a relatively gentle condition with easy access.

Ambient temperature and time are recognized as the most crucial elements affecting the viability and quantity of the exosomes stored, as bioactive proteins in the exosomes will gradually degrade with the increase of temperature and storage time (Oosthuyzen et al., 2013; Welch et al., 2017; Jeyaram and Jay, 2017; Qin et al., 2020). By contrast, membrane proteins, DNA and RNA in the exosomes seem to be insignificantly affected by the storage temperature (Zonneveld et al., 2014; Ge et al., 2014; Sarker et al., 2014; Jin et al., 2016). As discovered, –80°C is the optimal temperature for the storage of exosomes, at which the loss of bioactive proteins and exosomes can be efficiently suppressed (Jeyaram and Jay, 2017). However, the properties of exosomes will still degrade to some extent during the storage (Maroto et al., 2017). Wu et al. have systemically explored change of the property of the exosomes stored at various temperatures, and found –80°C to be the optimum temperature for the long-term storage (J. Wu et al., 2021).

To enhance the stability of exosomes, certain additives were introduced into the stock solution. For instance, addition of certain protease inhibitors into the storage solution has proven to be beneficial to protect the compounds in exosomes from degradation (Oosthuyzen et al., 2013). Furthermore, combination of thawing (Zhou et al., 2006) with certain preservative agents such as DMSO, sucrose, and mycose (Tegegn et al., 2016) are also helpful for improving the recycle rate of the exosomes. Researchers even proposed an assessment system to evaluate the degradation of the EVs stored under various conditions, which has provided a precious reference for research in this field (Richter et al., 2019).

Although exosomes have shown a fare stability when stored at –80°C, this temperature may be unrealizable in most factories during the fabrication and transportation of exosomes. A more convenient and efficient storage condition is therefore required. Lyophilization, which is commonly used for the preservation of bioactive compounds, has been adopted for the storage of exosomes (El-Nesr et al., 2010). Although this method has carried a risk of disrupting the membrane of exosomes, it still represents an optimum solution for the storage of the exosomes when protective agents are introduced (Bahr et al., 2020). Moreover, as discovered, the exosomes could be stored for more than a week at room temperature with minimal loss in quantity and bioactive compounds with the introduction of cryoprotectant mycose (Charoenviriyakul et al., 2018). The efficacy of such technique was found to be similar to that with storage at –80°C. Furthermore, spray drying, a method often used for preparation and storage of unstable drugs, may also be considered for the storage of exosomes (Bahr et al., 2020).

Artificial exosomes

As aforementioned, exosomes are bioactive compounds with complex components and can act as nanoscale tools for cell communication. Such characteristics have conferred them with excellent targeting ability and biocompatibility (van der Meel et al., 2014; Yáñez-Mó et al., 2015; Surman et al., 2019). As discovered, a diversity of membrane proteins, e.g., CD47, a marker highly expressed by cancer cells for evading the immunophagocytosis, is the main contributor to the therapeutic effect of exosomes (Kamerkar et al., 2017). Incorporation of gold particles on exosome membranes can confer them with an ability to escape immunophagocytosis (Van Deun et al., 2020). Accordingly, by producing stem cell-derived exosomes artificially, the steps and/or the time taken by the stem cells to secrete exosomes may be saved, thereby increasing the yield of exosomes. To produce more homogenous exosomes on a large scale, artificial exosomes have been considered as biomolecules as they are relatively clear and can be controlled to some extent (Figure 3).

Genetic engineering

Gene-editing technique represents as a novel and powerful means for modifying the exosomes derived from stem cells (Li et al., 2018). Researchers have improved the therapeutic outcome of certain diseases by over-expressing bioactive compounds such as miR-181-5p (Qu et al., 2017), miR-214 (Shi et al., 2021), and miR-155-5p (Damasceno et al., 2020) in the MSCs. Sterzenbach et al. have fabricated a drug vector to penetrate the blood-brain barrier by modifying the ESCRT-associated genes (Sterzenbach et al., 2017). To make the stem cell-based exosomes as an optimal drug carrier, importation of some unmodified bioactive compounds (such as miRNAs) directly into the isolated exosomes by $CaCl_2$ or electroporation has also been explored (Zhang et al., 2017; Liu and Su, 2019).

Although gene-modified strategy has substantially advanced our understanding of the secretion and synthesis of exosomes, the exploration to increase the yield of exosome by such techniques is still in its infancy (Kowal et al., 2014; Kalluri and LeBleu, 2020). Various pathways, e.g., the ESCRT protein and Rab GTPase, have been found to play a critical role in the synthesis and secretion of exosomes (Katzmann et al., 2001; Ostrowski et al., 2010; Li et al.,
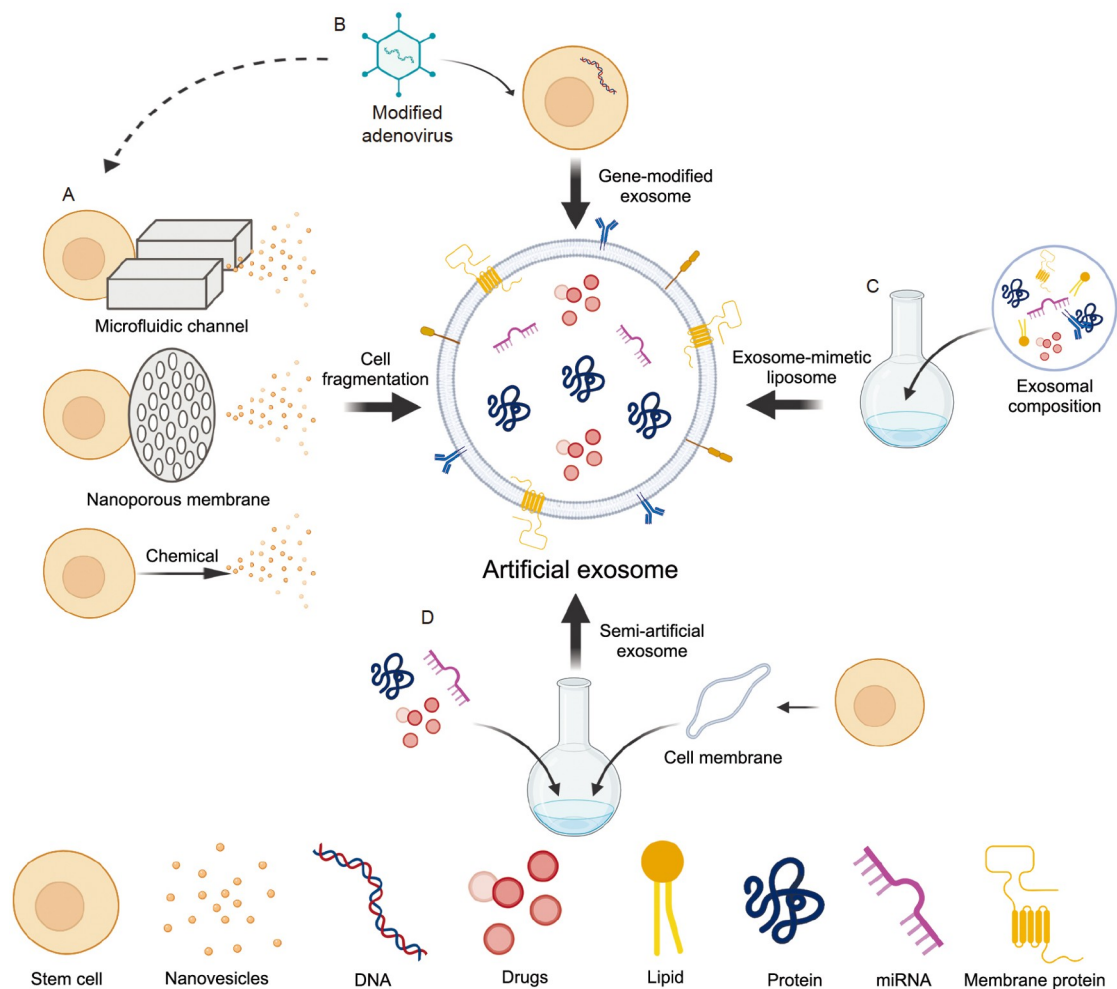
Figure 3   Artificial exosomes can be fabricated by various strategies. A, Fragmentation of the stem cells by mechanical force or chemical methods for producing artificial exosomes. B, Gene-editing techniques for producing artificial exosomes. C, Materials with appropriate physical and chemical properties are selected to produce artificial exosomes. D, Cell membrane and package of drugs and bioactive proteins are incorporated for producing artificial exosomes.

2018). Recently, researchers have also made an endeavor to upregulate the expression of genes associated with exosome secretion, including the ESCRT-III-Associated Protein Alix (Ji et al., 2021), HIF-1, and mTORC1 (Zou et al., 2019). Meanwhile, study of the culture condition has also identified other pathways such as Rab GTPase (Ruan et al., 2018; Z. Wu et al., 2021) and YAP (Guo et al., 2021) associated with exosome releasing. Such findings could be leveraged in combination to produce stem cells with an exosome-productive genotype, in this way the application of exosomes may be extensively expanded. However, certain compounds, such as ceramide (Dreux et al., 2012) and cholesterol (Strauss et al., 2010), not only are correlated with exosome synthesis, but also have influences on other intracellular metabolic activities. For this reason, the consequences elicited by gene-editing are still hard to predict. More importantly, exosomes and virus share certain similarities in multiple layers, thus the risk of contaminating the exosomes with undesirable virus genes is inevitable (Colao et al.,

2018). Such shortcomings should be overcome prior to application of such techniques for massive exosome yielding.

Cell fragmentation

Fragmentation of the cells into nanovesicles with a size equal to that of exosomes has also turned out to be a promising strategy (García-Manrique et al., 2018). Copious membrane proteins and special lipids are typical features of exosomes (Haraszti et al., 2019). All membrane proteins and lipids of the source cells could be utilized for the manufacture of exosomes (Goh et al., 2017). Accordingly, microfluidics can squeeze the cells into a hydrophilic channel to fragment them into exosome-analogous nanovesicles, by which abundant miRNA and surface proteins may be produced (Jo et al., 2014). Furthermore, cutting the cells with silicon nitride blades during their passage through the microfluidic channels can generate a more homogenous batch of exosomes (Yoon et al., 2015). Such cells can be further fragmented to

nanovesicles by replacing the blade with centrifugation or electricity to force them to cross a nano-membrane (Jo et al., 2014; Lee et al., 2020). Other chemical methods such as radioimmunoprecipitation analysis have also been used to produce nanovesicles with a size similar to that of exosomes (Martinelli et al., 2020). Notably, combination of the afore-mentioned techniques with gene expression modifications, such as miRNA overexpression, has also been tested (Z. Yang et al., 2020).

Exosome mimetic liposome

The methods for fabricating artificial exosomes are diverse. Liposome, an artificially-made vesicle composed of lipid and cholesterol, possesses sound biocompatibility and drug loading capacity. However, clinical applications of liposomes are scarce due to their undesirable immunogenicity and unsatisfactory stability, biocompatibility, targeting ability, and absorbability (Antimisiaris et al., 2018; Guimarães et al., 2021). Such drawbacks can be largely overcome by fabricating an exosome-liposome complex, by which the advantages of two can complement each other (Haraszti et al., 2019; Sakai-Kato et al., 2020). In theory, artificial exosomes with their laden nucleic acid antigen can be employed to target dendritic cells by the self-assembly of microemulsion micelle. In such artificial exosomes, lecithin, choles-

terol, dioleoacylphosphatidy-lethanolamine (DOPE), DC-Chol, and NBD-PE are leveraged to fabricate the lipid membranes, while the monoclonal antibody DEC205 and fetal bovine serum (BSA) are used to fabricate the surface proteins (K. Li et al., 2015). Furthermore, a pulsed jet method has also been developed to produce nanovesicles with lipid compositions analogous to those of exosomes (Kamiya et al., 2021). Although chemical synthesis of exosomes is still in its infancy, their prospect for the management of intractable diseases such as autoimmunity and tumors has already attracted much attention (Schiffelers et al., 2012; Vázquez-Ríos et al., 2019). Meanwhile, particular moieties of the naturally derived exosomes have also been used as raw material to synthesize artificial exosomes and to study their underlying mechanisms (Haraszti et al., 2019).

Semi-artificial exosome

Semi-artificial exosomes may also be used for improving the function and yield of stem cell-derived exosomes. By fusing extracellular vesicles with drug-laden liposomes (by co-incubating with PEG or without), researchers have successfully fabricated a hybrid exosome with outstanding cargo capacity, absorbability, and targeting ability, which may ultimately yield better therapeutic outcomes (Piffoux et al., 2018; Lin et al., 2018). The biocompatibility and targeting
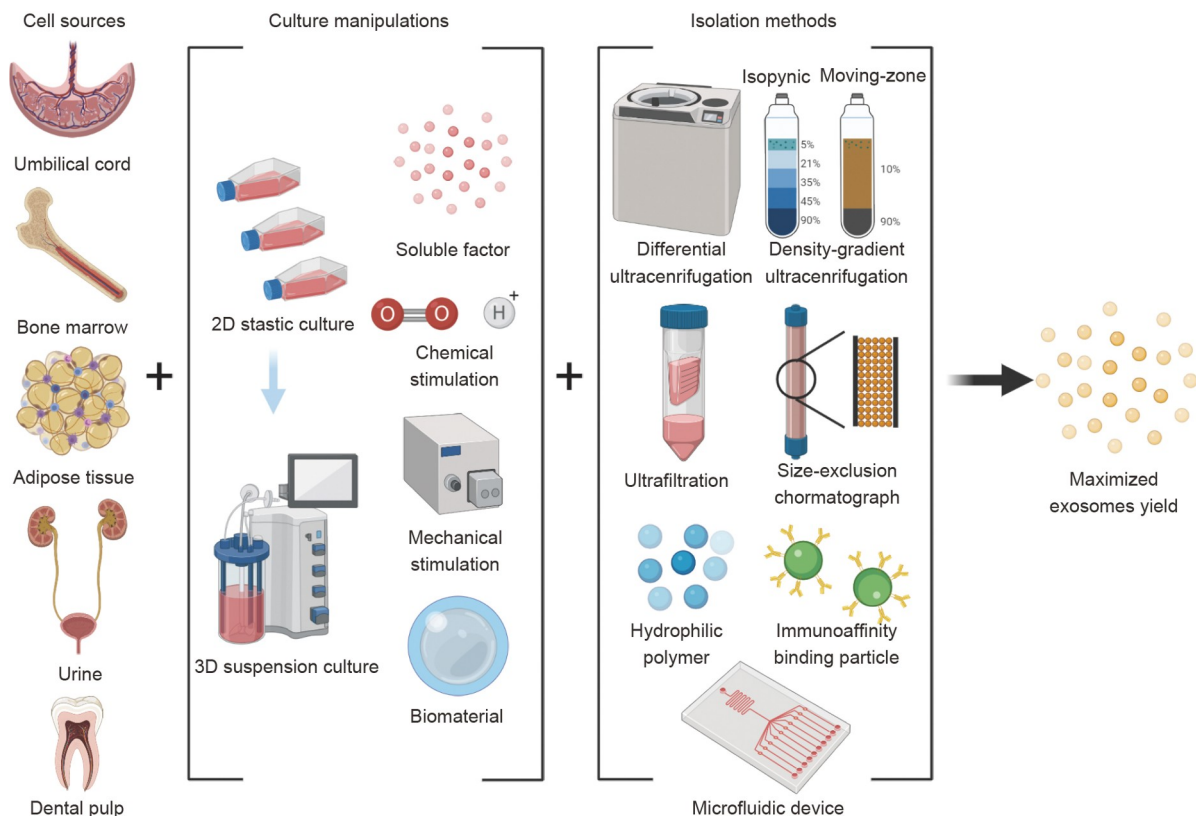

Figure 4 Methods that can maximize the yield of exosomes, in which the cell sources, culture manipulations, and isolation techniques can be adopted to increase the yield of exosomes.

ability of such nanovesicles may also be substantially improved by, for instance, encapsulating drug-laden liposomes with MSCs-derived plasma membranes (Toledano Furman et al., 2013; Yao et al., 2020) or filling the PLGA microparticles with bioactive compounds (Luo et al., 2017). Researchers have developed a system which has incorporated the secretome of myocardial cells into the PLGA particles and covered such particles with cardinal cell membranes. Subsequent experiments demonstrated that such artificial exosomes are highly efficient in treating cardiac infarction (J. Tang et al., 2017). Encapsulation of monoclonal antibody coupled with nanovesicles into the stem cell membranes has also proven to be feasible to render the exosome-mimetic particles with sound biocompatibility and targeting ability (Li et al., 2018). The technique can be further combined with genetic editing to improve the property of semi-artificial exosomes. Compared with fully artificial exosomes, semi-artificial exosomes have the advantage of requiring simpler facility. By combining their respective advantages, the application of such techniques may be further expanded.

Conclusion and outlook

Research on stem cell exosomes has attracted much attention as such nanovesicles hold great promise for biotherapy, though the majority of them are still in the preclinical phase due to the low yield of exosomes. As recapitulated in this review, factors such as cell source, culture condition and method, and isolation and storage can all have a great influence on the yield as well as the property of exosomes. A standard guideline for the manufacture is therefore needed to reduce the heterogeneity of exosomes whilst increase their yield.

Strategies to improve the yield of exosomes may be identified through combination of currently available methods. For instance, 3D microcarrier-based suspension culture may increase the output of exosomes when certain soluble factors and/or bioactive materials which are used as a biological microcarrier are applied (Haraszti et al., 2018; Patel et al., 2019) (Figure 4). Furthermore, full or semi-artificial exosomes may also be tested for massive production due to their relatively controllable composition and clear therapeutic pathway (García-Manrique et al., 2018; Surman et al., 2019). In the future, with various portfolios of aforementioned strategies combined, an optimal way to produce the desirable exosomes may be identified.

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