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PERSPECTIVE ARTICLE

Bioprinting of exosomes: Prospects and challenges for clinical applications

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Abstract

Three-dimensional bioprinting (3DBP) is an additive manufacturing technique that has emerged as a promising strategy for the fabrication of scaffolds, which can successfully recapitulate the architectural, biochemical, and physical cues of target tissues. More importantly, 3DBP offers fine spatiotemporal control and high submicron scale resolution, which can be leveraged for the incorporation and directional gradient release of single or multiple biomimetic cues, including cell-derived exosomes (EXOs). EXOs are extracellular vesicles that originate from the endosomal compartment of various cell types, with sizes ranging from 30 to120 nm. They act as cell mediators and contain discrete cell constituents, including growth factors, cytokines, lipid moieties, nucleic acids, metabolites, and cell surface markers, depending on the cell type. Essentially, owing to their therapeutic potential, EXOs derived from mesenchymal stem cells (MSCs) have been recently investigated in several clinical trials for the treatment of various conditions, including cancer, diabetes, dry eyes, periodontitis, and acute ischemic stroke. The 3DBP strategy of EXOs is especially useful in tissue engineering and regenerative medicine applications, as tissues can be biofabricated to closely mimic the complex microarchitecture and developmental profiles of native heterogeneous tissues for restoring biological functions. Moreover, EXOs can be manipulated to carry exogenous cargo such as genes or proteins of therapeutic interest, confer multifunctional attributes, and further enhance their tissue regenerative potential. However, significant challenges, including the selection of appropriate bioink, pattern resolution, engineering-defined exosomal gradient, spatial presentation and modulation of EXO release kinetics, as well as EXO stability and storage conditions, must be addressed for the successful translation of therapeutic grade EXOs to clinical settings. In this review, we highlight the recent advances and offer future perspectives on the bioprinting of EXOs as regenerative biotherapeutics for the fabrication of complex heterogeneous tissues that are suitable for clinical transplantation.

Keywords: Exosomes; 3D bioprinting; Bioink; Tissue engineering

1. Introduction to exosomes

Cell-secreted lipid-bound extracellular vesicles can be classified into three subtypes, including exosomes (EXOs), microvesicles, and apoptotic bodies, each of which have their own unique set of characteristics based on their biogenesis, release pathways, size,

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content, and function[1]. The biogenesis of EXOs occurs through a series of events that is initiated through the formation of primary endocytic vesicles facilitated by the inward budding of the plasma membrane[2]. The fusion of multiple endocytic vesicles results in the formation of early endosomes that mature into late endosomes and eventually multivesicular bodies (MVBs), which are subsequently released into the extracellular space[2]. EXOs have been found to play a significant role in intercellular communication[3] and a wide gamut of physiological processes, including tissue repair[4,5], maintenance of stem cell phenotype[6], immune response[7], and pathological processes, such as cardiovascular disease[8], ocular conditions[9], neurodegeneration[10], and autoimmune disorders[11,12]. Over the past decade, EXOs have garnered widespread attention as emerging diagnostic biomarkers of diseases[2] and as potential cell-free nanotherapeutics for the treatment of various pathological conditions[13,14].

EXOs have long served as novel biomarkers in clinical diagnostics on account of their contents, including proteins and nucleic acids, such as deoxyribonucleic acid (DNA), messenger ribonucleic acids (mRNAs), and microRNAs (miRNAs), which serve as reliable predictors of disease progression[2]. Studies have shown that in certain types of cancers, tumor cells release EXOs at a significantly higher amount compared to normal cells[15,16]. Moreover, the exosomal cargo released by unhealthy cells possesses a unique expression signature that is specific to a particular pathological condition[17]. Owing to this distinctive attribute, EXOs have been widely considered crucial for clinical diagnosis of various pathological conditions, including cancer and other neurodegenerative, infectious, and metabolic diseases[14]. More recently, EXOs have also shown great potential as regenerative biotherapeutics[4,5], immunomodulatory factors[18,19], and drug delivery vehicles[20] due to their unparalleled ability for intracellular crosstalk. For example, EXOs derived from cardiac progenitor cells have been shown to modulate gene expression in cardiac fibroblasts and endothelial cells, exerting a cardioprotective and pro-angiogenic effect in infarcted hearts *in vivo*[4]. In another instance, the internalization of bone marrow mesenchymal stem cell (BMMSC) EXOs by chondrocytes harvested from patients with osteoarthritis demonstrated that tumor necrosis factor-alpha-induced inflammatory effects were inhibited, while the production of collagen (Col) II and proteoglycans (PGs) was upregulated by these cells in vitro[21]. The EXO-mediated transfer of microRNA-23b-3p that is secreted from mechanically stimulated Schwann cells has also been shown to promote neurite outgrowth in vitro and enhance axonal regeneration in a sciatic nerve injury rat model *in vivo*[22]. Furthermore, studies have revealed that MSC-derived EXOs exert anti-inflammatory effects by suppressing macrophage activation through the inhibition of nuclear factor kappa B (NF-kB) signaling cascade to modulate foreign body responses to implanted biomaterials *in vivo*[18,19]. More recently, EXOs have been engineered to deliver exogenous therapeutic moieties to recipient cells for use as targeted drug delivery vehicles in various applications, including cancer[23,24]. Moreover, EXOs have been investigated as biotherapeutics in numerous clinical trials for addressing a wide range of conditions[14]. Additionally, several companies are actively investigating clinical grade EXOs for the treatment of various clinical indications, as presented in Table 1.

Table 1. List of major companies developing therapeutic EXOs for various clinical indications

Company	Product	Cell source	Indication	Clinical trial
Aegle Therapeutics	AGLE 102	BMMSCs	Dystrophic epidermolysis bullosa	Phase 1/2a
Exopharm	Plexaris	Platelets	Wound healing	Phase 1
United Therapeutics	UNEX-42	BMMSCs	Bronchopulmonary dysplasia	Phase 1
Direct Biologics	ExoFlo	BMMSCs	ARDS	Investigational new drug
Organicell	Zofin	Perinatal	COPD, COVID-19, osteoarthritis	Phase 1/2
	exoIL-12	Engineered MSCs	Cutaneous T-cell lymphoma	Phase 1
Codiak Biosciences	exoSTING		Solid tumors	Phase 1
	exoASO-STAT6		Myeloid-rich cancers	Phase 1
Avalon Globocare	AVA-201	Engineered MSCs	Oral cancers	Phase 1
Evox Therapeutics	DeliverEx platform with transmembrane protein cargo	Engineered MSCs	Niemann–Pick disease type C	Pre-clinical
Ilias Biologics	ILB-202	Engineered MSCs	Acute inflammatory diseases	Phase 1
Capricor	CAP-2003	Cardiospheres	Duchenne muscular dystrophy	Pre-clinical

Abbreviations: ARDS, acute respiratory distress syndrome; BMMSC, bone marrow-derived mesenchymal stem cells; COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 2019; MSC, mesenchymal stem cells.

2. Exosomes as cell modulators in regenerative medicine

The therapeutic potential of cell-derived EXOs, which includes their anti-inflammatory, immunosuppressive, pro/anti-angiogenic, and anti-fibrotic properties, has been actively exploited for various applications of regenerative medicine[13]. For example, Zhang *et al.* demonstrated that EXOs can promote the proliferation of dermal fibroblasts and epidermal keratinocytes, inhibit apoptosis, and activate the protein kinase B (Akt) pathway to promote cutaneous wound healing through a rat skin burn model[25]. Shabbir *et al.* showed that MSC-EXOs enhanced the migration of normal adult fibroblasts and diabetic chronic wound fibroblasts, isolated from a diabetic patient with non-healing ulcer, in vitro[26]. EXOs have been found to induce angiogenesis and activate several key signaling pathways, such as Akt, STAT3, and ERK, which are known to induce the expression of various growth factors, including hepatocyte growth factor, insulin growth factor 1, nerve growth factor, and stromal cell-derived factor 1, all of which are conducive for wound healing and tissue regeneration[26]. In a similar study, Geiger *et al.* demonstrated that fibrocyte-derived EXOs exhibited pro-angiogenic properties and induced the migration and proliferation of diabetic keratinocytes to accelerate wound closure in diabetic mice *in vivo*[27]. Additionally, miRNAs present in secreted EXOs have been shown to exert cardioprotective effects by enhancing the functionality of cardiomyocytes, preventing fibrosis, and promoting angiogenesis in ischemic cardiac muscles after myocardial infarction (MI)[28]. Wang *et al.* showed that subretinal delivery of retinal pigment epithelium-derived EXOs ameliorated photoreceptor loss and enhanced visual responsiveness in an N-methyl-N-nitrosourea-induced mouse model of retinal degeneration[29]. Along similar lines, Yu *et al.* demonstrated that the intravitreal administration of MSC-derived EXOs inhibited apoptosis, suppressed inflammatory responses, and downregulated monocyte chemoattractant protein-1 (MCP-1) to improve visual acuity and recovery in a laser-induced retinal injury mouse model[30]. Yet, another study revealed that the intravenous administration of BMMSC EXOs in a focal cerebral ischemia mouse model improved neurological functions through long-term neuroprotection and the induction of angiogenesis[31].

In addition to MSC-derived EXOs, there are mounting data to suggest that EXOs produced by immune cells can also be availed for tissue regenerative applications. For instance, EXOs derived from polarized macrophage populations can be manipulated to negatively or positively regulate bone regeneration[32]. Particularly, EXOs released from M1 macrophages have been shown to reduce osteogenic differentiation of MSCs by inhibiting bone morphogenetic protein (BMP) 2, BMP9, and runt-related transcription factor 2 (RUNX2) expressions, while EXOs from M2 macrophages increased osteoconductive gene expression in MSCs compared to controls[32]. Along similar lines, M2 macrophage-derived EXOs which are rich in miR-501 have been found to promote myotube formation in C2C12 cells in vitro and pubococcygeal muscle regeneration in a stress urinary incontinence animal model *in vivo*[33]. Additionally, EXOs from M2 macrophages enriched with miR-590-3p have been found to promote colonic epithelial cell proliferation in a dose-dependent manner in vitro and improve the wound-healing ability of epithelial cells upon administration in a dextran saline sulfate-induced colitis murine model *in vivo*[34]. Likewise, EXOs from B cells have been shown to regulate bone homeostasis during fracture healing by inhibiting excessive osteogenic activity in a mouse fracture model[35]. There is also evidence showing that EXOs released from dendritic cells (DCs) and regulatory T cells play a crucial role in exerting cardioprotective effects following MI[36-39]. Another study found that EXOs secreted from DC mediated $CD4^+$ T-cell activation and improved cardiac function in mice post MI[36]. Along similar lines, EXOs produced by regulatory T cells have been found to inhibit the proinflammatory function of effector T cells[35], induce a tolerogenic phenotype in DCs[38], and promote M2 macrophage polarization[39], thereby fostering a microenvironment conducive for cardiac repair and regeneration. Furthermore, EXOs released from dendritic epidermal T cells, which are T cells in the skin that possess a dendritic-like shape, have been found to promote the proliferation of epidermal stem cells and accelerate wound re-epithelialization in a murine excision wound injury model *in vivo*[40].

There is a growing line of evidence showing that EXOs play an active role in extracellular matrix (ECM) remodeling and in directly influencing cell binding and migration into tissue matrix[41-43]. More specifically, it has been demonstrated that cells continuously endocytose ECM molecules and re-secrete them on the exofacial surface of EXOs[44], which confer unique ECM-modulating properties to EXOs that can be exploited for orchestrating tissue regeneration and wound healing. In regard to this, several ECM molecules, including fibronectin (FN), glycosaminoglycans (GAGs), proteoglycans (PGs), hyaluronic acid (HA), and enzymes, such as proteases and glycosidases, have been identified on the exofacial surface of EXOs[41,43]. Studies have shown that FN-coated EXOs, which are involved in the endocytosis of integrin αvβ1–fibronectin complex, interact with heparan sulfate (HS) PGs on the cell membrane to facilitate EXO uptake[45]

or bind with cellular integrin receptors to promote directional cell migration via focal adhesion formation[44]. Additionally, the binding of FN-coated EXOs with laminin or Col fibrils in the ECM offers enhanced cell adhesion dynamics for augmenting cell adhesion and migration[46,47]. Likewise, HSPGs have been demonstrated to facilitate the uptake of EXOs by recipient cells[48]. Exofacially bound HA, secreted by BMMSCs, has been shown to contribute toward ECM reorganization, tissue regeneration, and the regulation of EXO interactions with target cells[49]. Furthermore, EXO surface-associated enzymes, such as matrix metalloproteases (MMPs), have been implicated to facilitate EXO mobility, release immobilized growth factors and signaling mediators, as well as influence cell migration within the ECM matrix via regulated matrix degradation[50]. With regard to their tissue regenerative potential, BMMSC EXOs have been demonstrated to negate proteolytic activity by attenuating MMP2 expression coupled with elevated expression of tissue inhibitors of MMP1/2 and imparting elastic matrix regenerative benefits in an abdominal aorta aneurysm rat model *in vivo*[51]. In another study, adipose MSC (AdMSC) EXOs reduced MMP1, MMP13, and ADAMTS-5 (a disintegrin and metalloproteinase with thrombospondin motif 5) expressions in human chondrocytes, thereby preserving chondrocyte-rich ECM and preventing cartilage degeneration[52,53]. As another exemplar, weekly intra-articular injections of EXOs derived from immortalized human embryonic MSCs promoted cartilage repair and regeneration through the upregulation of Col II deposition and GAGs in an osteochondral defect rat model *in vivo*[54].

Despite the favorable effects in different pathological conditions, one of the main challenges associated with the systemic delivery of EXOs is their rapid clearance from the circulation (plasma half-life of 2–4 min) by virtue of their accumulation in parenchymal organs, such as liver, lung, and spleen[55]. Hence, efforts have been made to facilitate the sustained release of EXOs from implantable biomaterial scaffolds to improve the bioavailability of EXOs and enhance their therapeutic outcomes. With regard to this, Shafei *et al.* loaded EXOs into alginate (Alg)-based hydrogel scaffolds to enhance angiogenesis and Col synthesis as well as to improve wound closure in a full-thickness wound excision rat model[56]. In another study, Alg scaffolds loaded with human umbilical cord MSC EXOs were employed to repair bone defects in rats *in vivo*[57]. EXOs secreted from cardiomyocyte-derived induced pluripotent stem cells, which have been loaded into engineered hydrogel foam that is composed of type I Col, facilitated cardiac regeneration in infarcted hearts[58]. Chen *et al.* discovered that endothelial progenitor cell-derived EXOs from a shear thinning hydrogel, composed of adamantine and β-cyclodextrin-modified hydroxyapatite, improved angiogenesis and functionality following MI[59]. Shi *et al.* showed that gingival MSC-derived EXOs loaded into a silk/chitosan hydrogel were found to improve wound healing in a diabetic skin defect rat model[60]. These studies demonstrated that the controlled release of EXOs from biomaterial scaffolds could serve as novel therapeutic platforms for inducing and promoting tissue injury repair and regeneration *in vivo*.

3. Three-dimensional bioprinting

Recently, three-dimensional bioprinting (3DBP), an additive manufacturing technique, has emerged as a promising strategy for engineering intricate tissue scaffolds for various biomedical applications[61,62]. Indeed, cell-laden gelatin- or fibrin-based bioinks printed alongside an embedded vasculature, and subsequently functionalized with endothelial cells to create a perfusable vascular network within a 3D perfusion chip have been shown to yield soft heterogeneous tissues at centimeter scale that can be maintained for long periods of time[63,64]. In this transformative approach, it is quite eminent that scaffolds can be fabricated through precise layer-by-layer deposition of materials to yield biologically relevant constructs with intricate geometries[61]. More importantly, the fine spatiotemporal control and high submicron scale resolution offered by 3DBP allow for the incorporation and directional gradient release of single or multiple biomimetic cues over a sustained period of time, which is amenable for tissue engineering and the applications of regenerative medicine[61]. This is particularly advantageous, as tissues biofabricated using this methodology can closely mimic the physical, biochemical, and complex developmental profiles of native heterogeneous tissues *in vivo*[62].

Therefore, biomaterial scaffolds fabricated with specific growth factor patterns and gradients conducive for tissue development have been employed through various bioprinting approaches[65]. These approaches have been investigated for the development of bone, cartilage, nervous, and vascular tissues[66-68]. For example, in order to mimic the bone and cartilage layers at the osteochondral interface, a highly porous nanocomposite scaffold with defined microarchitecture and spatiotemporal patterning of growth factors has been engineered through stereolithography technique[66]. Results have shown that the fabricated osteochondral scaffold promotes human bone marrow-derived MSC attachment and proliferation, but more importantly, induces chondrogenic and osteogenic differentiation of seeded MSCs *in vitro*. In addition, the differentiated cells significantly upregulated glycosaminoglycan production, Col II synthesis, and calcium deposition compared to the control samples.

In another study, therapeutic growth factors, vascular endothelial growth factor (VEGF), and bone morphogenetic protein 2 (BMP2) were incorporated as spatiotemporally defined patterns in implants using extrusion-based bioprinting for simultaneous induction of angiogenesis and osteogenesis[67]. The results demonstrated that implants with distinct spatial presentation of VEGF promoted an increase in vessel invasion compared to implants that have been homogenously loaded with VEGF. Furthermore, bioprinted implants with both spatial VEGF gradients and defined BMP2 localization accelerated bone defect healing with minimal heterotopic bone formation, suggesting that spatiotemporally defined growth factor delivery can be employed for the regeneration of large bone defects *in vivo*. In a different study, extrusion-based 3D-printed scaffolds with physical (microgrooves) and biochemical (spatiotemporal gradients of nerve growth factor and glial cell line-derived neurotrophic factor) cues were employed to provide axonal guidance and chemoattractant/chemokinetic function for neuroregeneration[68]. In a rat model of nerve injury, the 3D-printed scaffolds facilitated nerve regeneration across a 10-mm nerve gap and demonstrated functional restoration of the regenerated nerve 12 weeks after implantation *in vivo*.

Concurrently, these studies show the potential of 3DBP as a promising methodology for spatiotemporal patterning of growth factors, cell modulators, and therapeutic moieties to tightly regulate tissue development for engineering complex functional tissues for tissue transplantation or drug screening applications.

4. Bioprinting of exosomes for regenerative therapy

Although gradient patterning of growth factors offers biomimetic path-specific biochemical cues for tightly regulated tissue regeneration, the supraphysiological release of protein therapeutics has been shown to induce adverse effects without significant therapeutic benefits. For instance, major adverse events such as heterotopic ossification, osteolysis, infection, and cancer have been implicated with INFUSE® bone graft, which consists of a Col sponge, loaded with recombinant human BMP2[69,70]. Alternatively, directional gradient release of EXOs at the target site can be availed to modulate tissue regeneration and function. The advantages of employing EXOs over recombinant therapeutic growth factor (GF) proteins are multifold. As EXOs are naturally produced in the body, they possess unique membrane proteins that facilitate the internalization by recipient cells[71]. Furthermore, as EXOs are native to the body, they are associated with low immune responses and thus safe even at high dosages for clinical applications[72]. Moreover, as EXOs directly activate signaling processes to regulate cell function via the gene transcription machinery, they act upstream of protein synthesis[73], and thus can be leveraged to modulate tissue repair and regeneration. With the above considerations, the 3DBP of cell-derived EXOs offers great potential in the engineering of implantable constructs for the localized delivery of EXO-based therapeutics with precise spatiotemporal control.

As a proof-of-concept model, EXOs have been incorporated in a bioink to engineer scaffolds with spatially well-defined patterns for promoting cell differentiation *in vitro*[74]. With regard to that, EXOs derived from different macrophage subsets have been incorporated into scaffolds fabricated via inkjet-based bioprinting, and their influence on C2C12 mouse myoblasts has been investigated[74]. In order to facilitate the bioprinting process, glycerol was used as an additive in the bioink to reduce EXO agglomeration, increase the viscosity of the bioink, and also serve as a humectant to reduce solvent evaporation at the tip of the nozzle. Cellular uptake studies showed that fluorescently labeled ECM-bound EXOs were readily taken up by C2C12 cells within 15 min, suggesting that a bioprinted solid-phase ECM environment did not affect EXO membrane integrity for effective delivery of cargo into the cell cytoplasm. More importantly, ECM-bound EXOs derived from proinflammatory M1 macrophages demonstrated spatial inhibition of myogenesis, whereas EXOs from pro-regenerative M2 macrophages promoted a microenvironment that spatially induced myogenesis in a dose-dependent manner *in vitro*. These results corroborate the evidence showing the compatibility of 3DBP with EXOs and the potential use of the spatially defined patterns of EXOs to spatially trigger intended biological functions.

In another study, BMMSC EXOs were incorporated in a decellularized cartilage ECM/gelatin methacrylate bioink and 3D printed using the dynamic projection stereolithography technique to yield EXO-functionalized scaffolds[75]. The final bioprinted structure, which possessed radially oriented channels to promote cartilage repair and regeneration, was crosslinked in the presence of a photoinitiator, lithium acylphosphinate, and 405-nm wavelength visible light[75]. Subsequent implantation of these scaffolds in a rabbit osteochondral defect model demonstrated that the EXOs released from the bioprinted scaffolds rescued cartilage mitochondrial dysfunction, promoted chondrocyte migration, and supported M2 macrophage polarization, thereby facilitating the regeneration of cartilage *in vivo*[75]. Although this study did not aim to spatially distribute EXOs in the bioprinted structure, it provided evidence that the 3DBP of cell-derived EXOs can be used for tissue regeneration.

In a different approach, engineered gene-activated EXOs were grafted onto acellular 3D-printed porous polycaprolactone (PCL)-based scaffolds for vascularized bone remodeling *in vivo*[76]. More specifically, the gene encoded for VEGF protein was exogenously loaded into EXOs derived from chondrogenic progenitor cell line ATDC5 and anchored onto the surface of PCL scaffolds fabricated via extrusion-based bioprinting through a flexible linker to confer dual functions: induction of osteogenic differentiation and promotion of vascularization *in vivo*. Surface modification was carried out on the 3D-printed PCL scaffolds using 10% 1,6-hexanediamine solution to yield amino group-coated scaffolds, to which an exosomal anchor peptide, CP05, was covalently tethered via EDC/NHS (1-(3-dimethylaminopropyl)-3-ethylcarbonamide hydrochloride/n-hydroxysuccinimide) chemistry with a graft efficiency of approximately 27%. Finally, the CP05-modified PCL scaffolds were incubated with EXOs carrying the VEGF plasmid DNA to engineer EXO-activated PCL bone scaffolds. Micro-computed tomography data showed that the EXO-activated PCL scaffolds demonstrated evidence of newly-formed bone that had integrated well with the native bone tissue 12 weeks after implantation in a rat radial defect model. Additionally, hematoxylin and eosin staining confirmed the presence of newly-formed blood vessels, while immunofluorescence staining demonstrated a positive staining for the angiogenic marker CD31. These findings suggest that there is potential use of functional-engineered EXOs tethered to well-designed acellular scaffolds in the treatment of segmental bone defects.

In a different strategy, lyosecretome, a freeze-dried formulation of MSC secretome that is known to contain EXOs and secreted proteins, directly adsorbed onto the surfaces of 3D-printed PCL scaffolds or incorporated in an Alg bioink and co-printed along with PCL was evaluated as a potential scaffold prototype for bone tissue engineering[77]. In this study, AdMSCs harvested from the adipose tissues of humans were employed, and a cryoprotectant, mannitol, was added to the conditioned media prior to the freeze-drying process to preserve the integrity of EXO and stabilize the secreted proteins. A rapid release of EXOs and proteins was observed from PCL scaffolds employing the adsorption approach, while a controlled release of EXOs and proteins was observed in composite scaffolds composed of PCL and alginate hydrogel. In addition, the release of these bioactive factors can be fine-tuned by altering the composition and crosslinking density of the Alg hydrogel.

Cumulatively, results from these studies (Table 2) indicate that EXOs immobilized in a solid-phase bioink ECM environment or surface-functionalized onto bioprinted scaffolds maintain physical integrity and

Table 2. List of studies combining bioprinting with EXOs for potential tissue regenerative applications

Bioactive factor	Exogenous cargo	Bioink composition	Bioprinting technique	Exosome presentation	Exosome function	Model	Potential application	Ref.
Macrophage-derived EXOs	-	Not mentioned	Inkjet	Spatial distribution	M1 EXOs: spatial inhibition of myogenesis M2 EXOs: spatial promotion of myogenesis	*In vitro*	Skeletal myogenesis	[74]
BMMSC EXOs	-	Gelatin methacrylate/decellularized porcine cartilage ECM	Stereolithography	Homogenous bulk distribution	Restore chondrocyte mitochondrial dysfunction Enhance chondrocyte migration Promote M2 macrophage polarization	*In vivo* Rabbit osteochondral defect model	Cartilage repair and regeneration	[75]
Chondrogenic progenitor cell line (ATDC5) EXOs	Gene encoded for VEGF	PCL	Melt extrusion	Surface grafting via covalent linkages	Increase osteogenesis and angiogenesis	*In vivo* Rat radial defect model	Segmental bone defects	[76]
AdMSC secretome	-	PCL PCL/Alg	Melt extrusion Melt/pneumatic extrusion	Surface adsorption Homogenous bulk distribution	Promote osteoinductivity Induce cell binding, proliferation, and differentiation of cells	*In vitro*	Bone tissue regeneration	[77]

Abbreviations: AdMSC, adipose-derived mesenchymal stem cells; Alg, alginate; BMMSC, bone marrow-derived mesenchymal stem cell; ECM, extracellular matrix; EXO, exosome; M1 EXOs, exosomes derived from M1 macrophage phenotype; M2 EXOs, exosomes derived from M2 macrophage phenotype; MSC, mesenchymal stem cells; PCL, polycaprolactone.

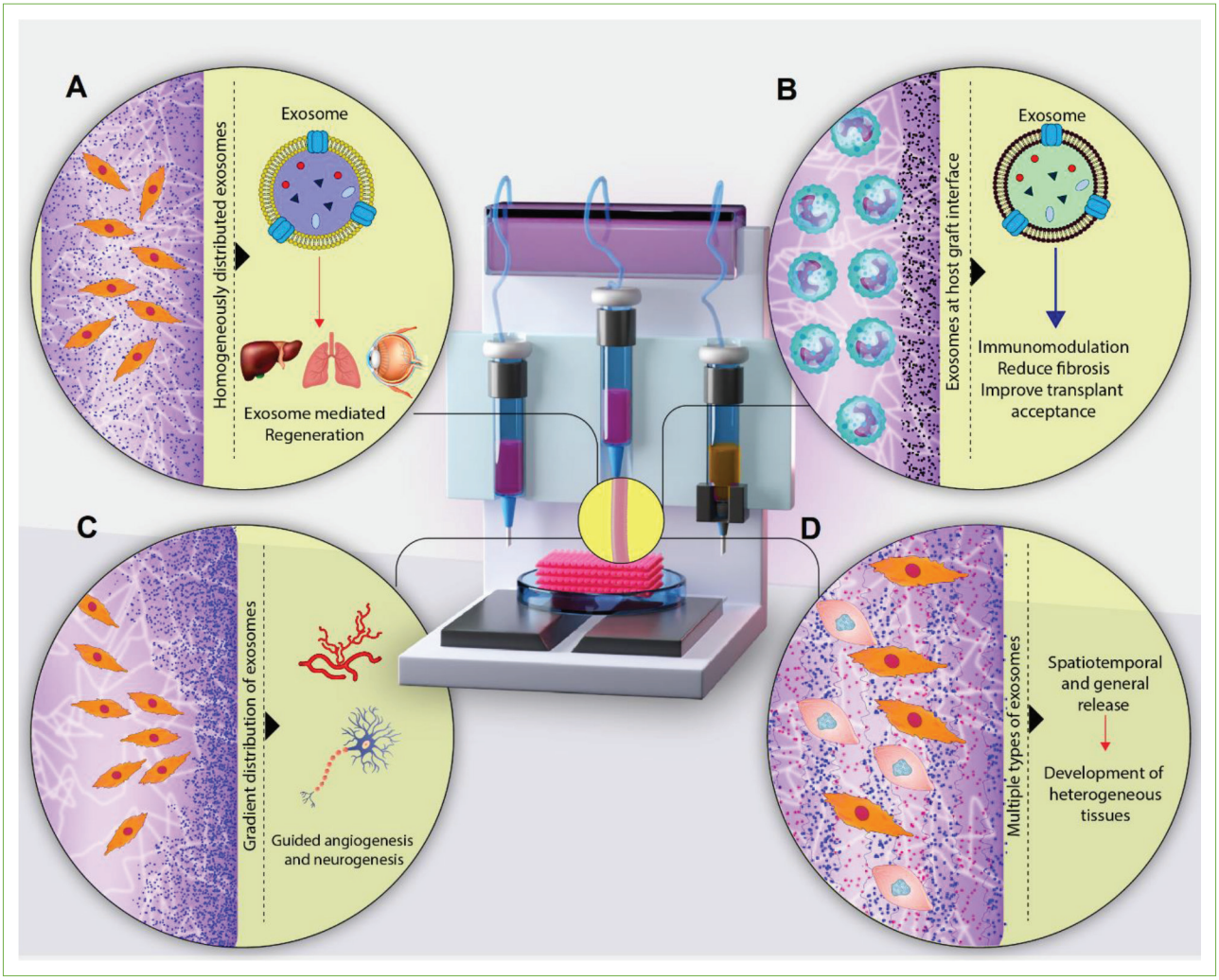

Figure 1. Bioprinting of cell-derived exosomes for tissue engineering and regenerative medicine applications. (A) Homogenous distribution of EXOs in bioprinted scaffolds can be used for EXO-mediated tissue regeneration. (B) EXOs localized along the walls of bioprinted scaffolds can be availed to promote immunomodulation and mitigate fibrosis at the host graft interface to improve implant outcomes following transplantation. (C) EXOs incorporated in well-defined spatiotemporal patterns can be employed for guided angiogenesis and neurogenesis to yield maturated biofabricated tissues *ex vivo*. (D) Gradient distribution and controlled release of multiple EXO types from bioprinted scaffold can be utilized for the guided development of heterogeneous tissues *ex vivo*.

retain biological activity, thus showing potentiality for the fabrication of implantable constructs that could modulate immune responses or stimulate tissue regeneration upon transplantation *in vivo*. Furthermore, the results also demonstrated that EXOs can be efficiently manipulated to carry exogenous cargo, such as genes or proteins of therapeutic interest, and bestow multifunctional attributes to further enhance and augment their tissue regenerative potential.

Additionally, 3DBP can be used for the fabrication of complex heterogeneous tissues that are suitable for clinical transplantation via spatial representation of EXOs for the induction and promotion of a controlled targeted tissue regenerative microenvironment *in vivo*. As a case in point, EXOs that are homogenously distributed inside bioprinted scaffolds can be exploited for controlled release applications to mediate tissue regeneration (Figure 1A), while EXOs that are localized to the walls of bioprinted scaffolds can be availed to help promote immunomodulation and mitigate fibrosis at the host–graft interface, so as to improve implant outcomes after transplantation (Figure 1B). Likewise, bioprinted scaffolds incorporating EXOs in well-defined spatiotemporal patterns can be employed for guided angiogenesis and neurogenesis to yield maturated, functionally relevant biofabricated tissues with agreeable neovascularization *ex vivo* (Figure 1C). These engineered tissues can then be easily integrated and anastomosed with

host vasculature upon transplantation *in vivo*. Moreover, the gradient distribution and controlled release of multiple EXO types from bioprinted scaffold encapsulating various cell types can be utilized for the guided development of heterogeneous tissues in numerous regenerative medicine applications (Figure 1D). However, one important aspect that needs to be considered for the sustained release of EXOs from bioprinted scaffolds is the dosage of EXOs at the target site, which should produce the intended therapeutic effect over a sustained period of time. In this context, several studies have revealed that the desirable time course of action for therapeutic EXOs depends on the end clinical application. To cite an instance, EXOs released from engineered hydrogels over a duration of 4–21 days induced angiogenesis, stimulated re-epithelialization, and promoted wound closure in chronic diabetic wounds[78-80]. In a similar situation, the sustained release of stem cell- or progenitor cell-derived EXOs from hydrogel scaffolds over a period of 21 days promoted myocardial regeneration in infarcted hearts[58,59,81].

5. Future perspectives

Bioprinting presents a unique opportunity to spatially and temporally pattern cell-derived EXOs at high resolution for engineering biologically relevant tissues. Additionally, this approach yields scaffolds with gradient release profiles of different exosomal types that can be used for the synchronized development of complex tissues composed of various cell types. This is critical for the fabrication of heterogeneous tissues with desired phenotype and function. However, despite the promising potential of 3DBP, there are several challenges to be addressed in printing EXO-laden bioink formulations to form scaffolds for tissue engineering applications. Firstly, the appropriate selection of bioprinting technique is crucial for the optimal bioprinting of EXOs. For instance, 3DBP approaches such as stereolithography and selective laser sintering are often excluded, as they are associated with high operating temperatures, hazardous chemical solvents, and extended use of high-intensity ultraviolet (UV) irradiation[82], all of which might significantly affect the biological function of bioprinted EXOs. On the other hand, extrusion, inkjet, or laser-assisted bioprinting approaches are often employed for bioprinting of growth factors and cell modulators, as they are relatively gentle and do not compromise on the bioactivity of encapsulated biotherapeutics[65,82]. The preferred 3DBP technique should be capable of achieving high printing resolution to systematically create defined exosomal gradients within the scaffold. This is essential for the spatial presentation and modulation of different EXO release kinetics to induce neotissue formation, which can effectively mimic the complex microarchitecture of native heterogeneous tissues for the restoration of biological function. Secondly, the choice of bioink is another important factor that needs to be considered, as it should not negatively impact the physical, chemical, or biological attributes of encapsulated EXOs. The selected bioink should provide a compatible microenvironment that is conducive for maintaining the stability and preservation of the intended biological function of EXOs. For example, the selection of an appropriate shear thinning bioink is favored for extrusion-based bioprinting, as it will significantly reduce the magnitude of shear stresses exerted on EXOs during the printing process, which might otherwise adversely affect its biological activity.

From the viewpoint of EXOs, few considerations remain for successful translation and clinical application of cell-derived EXOs as regenerative therapeutics[83]. Firstly, the source of producer cells for EXO production should be carefully examined because the choice of cells determines the quantity, functional activity, and target clinical application of EXOs. For instance, EXOs derived from corneal stromal stem cells have been shown to be enriched with anti-angiogenic factors, and thus can be used to engineer avascular tissues, such as the cornea[84], whereas EXOs derived from BMMSCs or AdMSCs have been shown to contain high levels of pro-angiogenic factors that can be used for vascular tissue repair and regeneration[85]. Likewise, MSC-EXOs derived from different tissue sources, including bone marrow, umbilical cord, menstrual blood, and chorion, promote neurite outgrowth in varying degrees[86]. Furthermore, the age and physiological state of the cells should also be considered to ensure reproducible EXO quality (cargo composition) with minimal batch-to-batch variability. Besides, the choice of the bioreactor system, be it the stirred tank bioreactor or hollow fiber bioreactor, plays a crucial role in the large-scale production of EXOs for clinical use, which is still in its infancy; this is attributed to the influence of cell culture parameters on EXO yield and cargo composition[83]. Particularly, through the application of fluid shear stress[87] or hypoxic culture conditions[88], physical stimulation has been shown to increase EXO quantity and confer enhanced therapeutic attributes, such as pro-angiogenic, immunomodulatory, and neuroprotective effects, which are advantageous for promoting vascularization in tissues *in vivo*.

Additionally, careful consideration needs to be given to the isolation, purification, and characterization of EXOs for the production of clinical grade EXOs. Although there is no consensus or standardized protocols for these methods, the International Society of Extracellular Vesicles has laid out the minimal set of information required for studies on EXOs[89]. With regard to this, ultracentrifugation (UC), wherein centrifugal forces of 100,000–200,000 × g

are imposed on biological culture fluids to separate EXOs, remains the gold standard for isolation and concentration of EXOs[23,90]. However, this technique is not amenable for scaled-up manufacturing processes, as it leads to EXO aggregation and is often characterized by low EXO yield and purity[23,90]. In contrast, tangential flow filtration (TFF), which involves the use of a permeable membrane filter and tangential fluid flow to separate and purify biomolecules of specific sizes, shows potential for large-scale EXO production, as it has demonstrated consistent production between batches and an improvement in quality, but more importantly, it is 100-fold more efficient in isolating EXOs compared to standard UC[91-93]. Size-exclusion chromatography (SEC) is another frequently employed technique used for EXO purification. It addresses limitations associated with UC, including the elimination of protein or cell debris contamination and the prevention of EXO aggregation. It is also a viable option for large-scale EXO separation and purification[90]. While UC, TFF, and SEC isolate and purify EXOs based on size and/or density, these techniques do not inherently possess the ability to purify specific EXO subpopulations or engineered EXOs loaded with therapeutic factors[23]. Hence, immune capture approaches, such as affinity chromatography that is suitable for up-scaled production of EXOs, can be availed for purification of EXO subpopulations or engineered EXOs[94,95]. However, it should be highlighted that the elution of intact EXOs is a challenge for chromatography-based purification methods. No single EXO isolation/purification step has been proven efficient; hence, a combination of different isolation/purification techniques has been employed[90]. For instance, a combination of TFF and bind-elute SEC protocols has been demonstrated to be more effective in purifying EXOs from C2C12 myoblast cultures compared to a single-step purification technique[96]. Along similar lines, the TFF-SEC combination method was shown to be efficient in the isolation of EXOs from urine[97]. More notably, clinical trials employing therapeutic EXOs routinely utilize a combination of TFF with UC purification protocols[23]. Collectively, these observations suggest that careful consideration is required for choosing the ideal combination of protocols for the optimal isolation and purification of various therapeutic EXOs.

Furthermore, there is a dearth of high-throughput methodologies to accurately assess EXOs quantitatively and qualitatively, so as to determine their purity, dosage, and potency. Nevertheless, rapid advances are being made with the advent of novel technologies that could accelerate the clinical translation of exosome-based therapeutics. Conventional characterization studies that evaluate the quality of produced EXOs typically include the determination of particle quantity and concentration using nanoparticle tracking analysis, visualization of particles by electron microscopy to determine the size and structure, quantification of total protein content via bicinchoninic acid assay, analysis of proteome by mass spectrometry, and identification of specific positive and negative exosomal surface markers using immunoblotting and flow cytometry techniques[14,90]. In addition, engineered EXOs need to be assessed quantitatively and qualitatively to characterize exogenously loaded therapeutic cargo via mass spectrometry, and their potency must be evaluated using relevant functional assays[14]. Furthermore, other exosomal components that originate from producer cells, such as nucleic acids, proteins, and lipids, should be thoroughly investigated to understand and prevent unwarranted issues associated with immunogenicity, genotoxicity, and/or carcinogenicity[90]. The standardization of these methods is paramount for establishing safety and efficacy profiles that are critical for the successful clinical development and translation of produced EXOs. Hence, the quality control and acceptance criteria used to assess the quality and consistency of EXO production should be inherently based on identity, purity, safety, and efficacy of therapeutic EXOs[90].

Finally, there is also a lack of understanding on the impact of storage conditions on EXO stability and bioactivity[83]. This understanding is imperative to discern the effects of storage-mediated changes on EXO size, number, cargo profiles, cellular uptake behavior, and bioactivity because these attributes inherently define the therapeutic attributes of produced EXOs. As storage at 4°C affects the biological activity and protein content of EXOs[98], the current consensus for EXO storage appears to be -80°C[14,23,99]. However, as different EXO types or subpopulations may demand different storage conditions, it becomes crucial to optimize the storage conditions for each EXO-based therapeutic[23]. Furthermore, factors such as the constituents and pH of storage buffer, number of freeze-thaw cycles, and storage container material also play crucial roles, as they may alter the characteristics of therapeutic EXOs[100]. Recent studies have demonstrated that the addition of cryoprotectants in EXO storage formulations, such as the Food and Drug Administration (FDA)-approved excipient trehalose, significantly improves the stability of EXOs[101,102]. Another promising approach is the lyophilization of EXOs, which has been shown to increase the stability and the shelf life of freeze-dried EXOs[99,103].

From the regulatory point of view, therapeutic EXOs need to be manufactured in a current good manufacturing practice (cGMP) facility in accordance with the adhered regulations for manufacturing traditional biologics, such as recombinant proteins and antibodies[90]. One important

aspect concerning this is the sterilization of produced EXOs. As a biological entity, EXOs contain many components, including nucleic acids, such as DNA, mRNAs, miRNAs, proteins, lipids, etc., which are not compatible with conventional steam, radiation, or gas-based sterilization techniques[23]. These techniques have the propensity to alter or inactivate the therapeutic cargo, thereby affecting the safety and efficacy of therapeutic EXOs. The 0.2 μm sterile filtration technique is the standard technique adopted for manufacturing EXOs[23]. However, this technique is not feasible for therapeutic particles bigger than 200 nm or those that tend to undergo agglomeration. Additionally, for commercial EXO products, residual protein or DNAs from producer cells that exist outside EXOs are still considered to be a component of EXOs, and thus part of the product itself[90]. Therefore, it is essential to separate these unbound impurities that might compromise the safety and efficacy of produced EXOs. Other than that, EXO therapeutics should be tested for bacterial endotoxins, mycoplasma, and infectious viral load, including human immunodeficiency virus I and II, hepatitis B and C viruses, cytomegalovirus, Epstein–Barr virus, and parvovirus. Finally, as EXOs are considered to be a new class of drugs, investigators should engage closely and proactively interact with regulatory agencies to streamline protocols for the development of EXO-based therapeutics[90].

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Conflict of interest

The authors declare no known conflict of interest.

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References

1. Doyle LM, Wang MZ, 2019, Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. *Cells*, 8: 727.

   https://doi.org/10.3390/cells8070727

2. Lin J, Li J, Huang B, *et al*., 2015, Exosomes: Novel biomarkers for clinical diagnosis. *Sci World J*, 2015: 657086.

   https://doi.org/10.1155/2015/657086

3. Bang C, Thum T, 2012, Exosomes: New players in cell-cell communication. *Int J Biochem Cell Biol*, 44: 2060–2064.

   https://doi.org/10.1016/j.biocel.2012.08.007

4. Li N, Rochette L, Wu Y, *et al*., 2019, New insights into the role of exosomes in the heart after myocardial infarction. *J Cardiovasc Transl Res*, 12: 18–27.

   https://doi.org/10.1007/s12265-018-9831-z

5. Kim YG, Choi J, Kim K, 2020, Mesenchymal stem cell-derived exosomes for effective cartilage tissue repair and treatment of osteoarthritis. *Biotechnol J*, 15: e2000082.

   https://doi.org/10.1002/biot.202000082

6. Hur YH, Feng S, Wilson KF, *et al*., 2021, Embryonic stem cell-derived extracellular vesicles maintain ESC stemness by activating FAK. *Dev Cell*, 56: 277–291.e6.

   https://doi.org/10.1016/j.devcel.2020.11.017

7. Feng Z, Zhou J, Liu Y, *et al*., 2021, Epithelium- and endothelium-derived exosomes regulate the alveolar macrophages by targeting RGS1 mediated calcium signaling-dependent immune response. *Cell Death Differ*, 28: 2238–2256.

   https://doi.org/10.1038/s41418-021-00750-x

8. Zamani P, Fereydouni N, Butler AE, *et al*., 2019, The therapeutic and diagnostic role of exosomes in cardiovascular diseases. *Trends Cardiovasc Med*, 29: 313–323.

   https://doi.org/10.1016/j.tcm.2018.10.010

9. Liu J, Jiang F, Jiang Y, *et al*., 2020, Roles of exosomes in ocular diseases. *Int J Nanomedicine*, 15: 10519–10538.

   https://doi.org/10.2147/ijn.S277190

10. Hill AF, 2019, Extracellular vesicles and neurodegenerative diseases. *J Neurosci*, 39: 9269–9273.

    https://doi.org/10.1523/jneurosci.0147-18.2019

11. Castaño C, Novials A, Párrizas M, 2019, Exosomes and diabetes. *Diabetes Metab Res Rev*, 35: e3107.

    https://doi.org/10.1002/dmrr.3107

12. Tavasolian F, Moghaddam AS, Rohani F, *et al.*, 2020, Exosomes: Effectual players in rheumatoid arthritis. *Autoimmun Rev*, 19: 102511.

https://doi.org/10.1016/j.autrev.2020.102511

13. Phinney DG, Pittenger MF, 2017, Concise review: MSC-derived exosomes for cell-free therapy. *Stem Cells*, 35: 851–858.

https://doi.org/10.1002/stem.2575

14. Perocheau D, Touramanidou L, Gurung S, *et al.*, 2021, Clinical applications for exosomes: Are we there yet? *Br J Pharmacol*, 178: 2375–2392.

https://doi.org/10.1111/bph.15432

15. Baran J, Baj-Krzyworzeka M, Weglarczyk K, *et al.*, 2010, Circulating tumour-derived microvesicles in plasma of gastric cancer patients. *Cancer Immunol Immunother*, 59: 841–850.

https://doi.org/10.1007/s00262-009-0808-2

16. Bebelman MP, Janssen E, Pegtel DM, *et al.*, 2021, The forces driving cancer extracellular vesicle secretion. *Neoplasia*, 23: 149–157.

https://doi.org/10.1016/j.neo.2020.11.011

17. Whiteside TL, 2017, Exosomes carrying immunoinhibitory proteins and their role in cancer. *Clin Exp Immunol*, 189: 259–267.

https://doi.org/10.1111/cei.12974

18. Mohammadi MR, Rodriguez SM, Luong JC, *et al.*, 2021, Exosome loaded immunomodulatory biomaterials alleviate local immune response in immunocompetent diabetic mice post islet xenotransplantation. *Commun Biol*, 4: 685.

https://doi.org/10.1038/s42003-021-02229-4

19. Fan L, Guan P, Xiao C, *et al.*, 2021, Exosome-functionalized polyetheretherketone-based implant with immunomodulatory property for enhancing osseointegration. *Bioact Mater*, 6: 2754–2766.

https://doi.org/10.1016/j.bioactmat.2021.02.005

20. Liang Y, Duan L, Lu J, *et al.*, 2021, Engineering exosomes for targeted drug delivery. *Theranostics*, 11: 3183–3195.

https://doi.org/10.7150/thno.52570

21. Vonk LA, van Dooremalen SFJ, Liv N, *et al.*, 2018, Mesenchymal stromal/stem cell-derived extracellular vesicles promote human cartilage regeneration in vitro. *Theranostics*, 8: 906–920.

https://doi.org/10.7150/thno.20746

22. Xia B, Gao J, Li S, *et al.*, 2020, Mechanical stimulation of Schwann cells promote peripheral nerve regeneration via extracellular vesicle-mediated transfer of microRNA 23b-3p. *Theranostics*, 10: 8974–8995.

https://doi.org/10.7150/thno.44912

23. Elsharkasy OM, Nordin JZ, Hagey DW, *et al.*, 2020, Extracellular vesicles as drug delivery systems: Why and how? *Adv Drug Deliv Rev*, 159: 332–343.

https://doi.org/10.1016/j.addr.2020.04.004

24. Arrighetti N, Corbo C, Evangelopoulos M, *et al.*, 2019, Exosome-like nanovectors for drug delivery in cancer. *Curr Med Chem*, 26: 6132–6148.

https://doi.org/10.2174/0929867325666180831150259

25. Zhang B, Wang M, Gong A, *et al.*, 2015, HucMSC-exosome mediated-Wnt4 signaling is required for cutaneous wound healing. *Stem Cells*, 33: 2158–2168.

https://doi.org/10.1002/stem.1771

26. Shabbir A, Cox A, Rodriguez-Menocal L, *et al.*, 2015, Mesenchymal stem cell exosomes induce proliferation and migration of normal and chronic wound fibroblasts, and enhance angiogenesis in vitro. *Stem Cells Dev*, 24: 1635–1647.

https://doi.org/10.1089/scd.2014.0316

27. Geiger A, Walker A, Nissen E, 2015, Human fibrocyte-derived exosomes accelerate wound healing in genetically diabetic mice. *Biochem Biophys Res Commun*, 467: 303–309.

https://doi.org/10.1016/j.bbrc.2015.09.166

28. Moghaddam AS, Afshari JT, Esmaeili S-A, *et al.*, 2019, Cardioprotective microRNAs: Lessons from stem cell-derived exosomal microRNAs to treat cardiovascular disease. *Atherosclerosis*, 285: 1–9.

https://doi.org/10.1016/j.atherosclerosis.2019.03.016

29. Wang Y, Zhang Q, Yang G, *et al.*, 2021, RPE-derived exosomes rescue the photoreceptors during retina degeneration: An intraocular approach to deliver exosomes into the subretinal space. *Drug Deliv*, 28: 218–228.

https://doi.org/10.1080/10717544.2020.1870584

30. Yu B, Shao H, Su C, *et al.*, 2016, Exosomes derived from MSCs ameliorate retinal laser injury partially by inhibition of MCP-1. *Sci Rep*, 6: 34562.

https://doi.org/10.1038/srep34562

31. Doeppner TR, Herz J, Görgens A, *et al.*, 2015, Extracellular vesicles improve post-stroke neuroregeneration and prevent postischemic immunosuppression. *Stem Cells Transl Med*, 4: 1131–1143.

https://doi.org/10.5966/sctm.2015-0078

32. Kang M, Huang CC, Lu Y, *et al.*, 2020, Bone regeneration is mediated by macrophage extracellular vesicles. *Bone*, 141: 115627.

https://doi.org/10.1016/j.bone.2020.115627

33. Zhou M, Li B, Liu C, *et al.*, 2021, M2 Macrophage-derived exosomal miR-501 contributes to pubococcygeal muscle regeneration. *Int Immunopharmacol*, 101: 108223.

https://doi.org/10.1016/j.intimp.2021.108223

34. Deng F, Yan J, Lu J, *et al.*, 2021, M2 macrophage-derived exosomal miR-590-3p attenuates DSS-induced mucosal damage and promotes epithelial repair via the LATS1/YAP/β-Catenin signalling axis. *J Crohns Colitis*, 15: 665–677.

https://doi.org/10.1093/ecco-jcc/jjaa214

35. Zhang H, Wang R, Wang G, *et al.*, 2021, Single-cell RNA sequencing reveals B cells are important regulators in fracture healing. *Front Endocrinol (Lausanne)*, 12: 666140.

https://doi.org/10.3389/fendo.2021.666140

36. Liu H, Gao W, Yuan J, *et al.*, 2016, Exosomes derived from dendritic cells improve cardiac function via activation of CD4(+) T lymphocytes after myocardial infarction. *J Mol Cell Cardiol*, 91: 123–133.

https://doi.org/10.1016/j.yjmcc.2015.12.028

37. Okoye IS, Coomes SM, Pelly VS, *et al.*, 2014, MicroRNA-containing T-regulatory-cell-derived exosomes suppress pathogenic T helper 1 cells. *Immunity*, 41: 89–103.

https://doi.org/10.1016/j.immuni.2014.05.019

38. Tung SL, Boardman DA, Sen M, *et al.*, 2018, Regulatory T cell-derived extracellular vesicles modify dendritic cell function. *Sci Rep*, 8: 6065.

https://doi.org/10.1038/s41598-018-24531-8

39. Hu H, Wu J, Cao C, *et al.*, 2020, Exosomes derived from regulatory T cells ameliorate acute myocardial infarction by promoting macrophage M2 polarization. *IUBMB Life*, 72: 2409–2419.

https://doi.org/10.1002/iub.2364

40. Liu M, Liu Z, Chen Y, *et al.*, 2022, Dendritic epidermal T cells secreting exosomes promote the proliferation of epidermal stem cells to enhance wound re-epithelialization. *Stem Cell Res Ther*, 13: 121.

https://doi.org/10.1186/s13287-022-02783-6

41. Al Halawani A, Mithieux SM, Yeo GC, *et al.*, 2022, Extracellular vesicles: Interplay with the extracellular matrix and modulated cell responses. *Int J Mol Sci*, 23: 3389.

https://doi.org/10.3390/ijms23063389

42. Rackov G, Garcia-Romero N, Esteban-Rubio S, *et al.*, 2018, Vesicle-mediated control of cell function: The role of extracellular matrix and microenvironment. *Front Physiol*, 9: 651.

https://doi.org/10.3389/fphys.2018.00651

43. Buzás EI, Tóth E, Sódar BW, *et al.*, 2018, Molecular interactions at the surface of extracellular vesicles. *Semin Immunopathol*, 40: 453–464.

https://doi.org/10.1007/s00281-018-0682-0

44. Sung BH, Ketova T, Hoshino D, *et al.*, 2015, Directional cell movement through tissues is controlled by exosome secretion. *Nat Commun*, 6: 7164.

https://doi.org/10.1038/ncomms8164

45. Purushothaman A, Bandari SK, Liu J, *et al.*, 2016, Fibronectin on the surface of myeloma cell-derived exosomes mediates exosome-cell interactions. *J Biol Chem*, 291: 1652–1663.

https://doi.org/10.1074/jbc.M115.686295

46. Shen AR, Zhong X, Tang TT, *et al.*, 2020, Integrin, exosome and kidney disease. *Front Physiol*, 11: 627800.

https://doi.org/10.3389/fphys.2020.627800

47. Hoshino A, Costa-Silva B, Shen T-L, *et al.*, 2015, Tumour exosome integrins determine organotropic metastasis. *Nature*, 527: 329–335.

https://doi.org/10.1038/nature15756

48. Christianson HC, Svensson KJ, van Kuppevelt TH, *et al.*, 2013, Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proc Natl Acad Sci U S A*, 110: 17380–17385.

https://doi.org/10.1073/pnas.1304266110

49. Arasu UT, Kärnä R, Härkönen K, *et al.*, 2017, Human mesenchymal stem cells secrete hyaluronan-coated extracellular vesicles. *Matrix Biol*, 64: 54–68.

https://doi.org/10.1016/j.matbio.2017.05.001

50. Shimoda M, Khokha R, 2017, Metalloproteinases in extracellular vesicles. *Biochim Biophys Acta Mol Cell Res*, 1864: 1989–2000.

https://doi.org/10.1016/j.bbamcr.2017.05.027

51. Sajeesh S, Broekelman T, Mecham RP, *et al.*, 2020, Stem cell derived extracellular vesicles for vascular elastic matrix regenerative repair. *Acta Biomater*, 113: 267–278.

https://doi.org/10.1016/j.actbio.2020.07.002

52. Woo CH, Kim HK, Jung GY, *et al.*, 2020, Small extracellular vesicles from human adipose-derived stem cells attenuate cartilage degeneration. *J Extracell Vesicles*, 9: 1735249.

https://doi.org/10.1080/20013078.2020.1735249

53. Li JJ, Hosseini-Beheshti E, Grau GE, *et al.*, 2019, Stem cell-derived extracellular vesicles for treating joint injury and osteoarthritis. *Nanomaterials (Basel)*, 9: 261.

https://doi.org/10.3390/nano9020261

54. Zhang S, Chu WC, Lai RC, *et al.*, 2016, Exosomes derived from human embryonic mesenchymal stem cells promote osteochondral regeneration. *Osteoarthr Cartil*, 24: 2135–2140.

https://doi.org/10.1016/j.joca.2016.06.022

55. Huang J, Xiong J, Yang L, *et al.*, 2021, Cell-free exosome-laden scaffolds for tissue repair. *Nanoscale*, 13: 8740–8750.

https://doi.org/10.1039/D1NR01314A

56. Shafei S, Khanmohammadi M, Heidari R, *et al.*, 2020, Exosome loaded alginate hydrogel promotes tissue regeneration in full-thickness skin wounds: An *in vivo* study. *J Biomed Mater Res A*, 108: 545–556.

https://doi.org/10.1002/jbm.a.36835

57. Yang S, Zhu B, Yin P, *et al.*, 2020, Integration of human umbilical cord mesenchymal stem cells-derived exosomes with hydroxyapatite-embedded hyaluronic acid-alginate hydrogel for bone regeneration. *ACS Biomater Sci Eng*, 6: 1590–1602.

https://doi.org/10.1021/acsbiomaterials.9b01363

58. Liu B, Lee BW, Nakanishi K, *et al.*, 2018, Cardiac recovery via extended cell-free delivery of extracellular vesicles secreted by cardiomyocytes derived from induced pluripotent stem cells. *Nat Biomed Eng*, 2: 293–303.

https://doi.org/10.1038/s41551-018-0229-7

59. Chen CW, Wang LL, Zaman S, *et al.*, 2018, Sustained release of endothelial progenitor cell-derived extracellular vesicles from shear-thinning hydrogels improves angiogenesis and promotes function after myocardial infarction. *Cardiovasc Res*, 114: 1029–1040.

https://doi.org/10.1093/cvr/cvy067

60. Shi Q, Qian Z, Liu D, *et al.*, 2017, GMSC-derived exosomes combined with a chitosan/silk hydrogel sponge accelerates wound healing in a diabetic rat skin defect model. *Front Physiol*,

https://doi.org/8:10.3389/fphys.2017.00904

61. Gu Z, Fu J, Lin H, *et al.*, 2020, Development of 3D bioprinting: From printing methods to biomedical applications. *Asian J Pharm Sci*, 15: 529–557.

https://doi.org/10.1016/j.ajps.2019.11.003

62. Gao B, Yang Q, Zhao X, *et al.*, 2016, 4D bioprinting for biomedical applications. *Trends Biotechnol*, 34: 746–756.

https://doi.org/10.1016/j.tibtech.2016.03.004

63. Kolesky DB, Homan KA, Skylar-Scott MA, *et al.*, 2016, Three-dimensional bioprinting of thick vascularized tissues. *Proc Natl Acad Sci U S A*, 113: 3179–3184.

https://doi.org/10.1073/pnas.1521342113

64. Liu X, Wang X, Zhang L, *et al.*, 2021, 3D liver tissue model with branched vascular networks by multimaterial bioprinting. *Adv Healthc Mater*, 10: 2101405.

https://doi.org/10.1002/adhm.202101405

65. Bittner SM, Guo JL, Mikos AG, 2018, Spatiotemporal control of growth factors in three-dimensional printed scaffolds. *Bioprinting*, 12.

https://doi.org/10.1016/j.bprint.2018.e00032

66. Castro NJ, O'Brien J, Zhang LG, 2015, Integrating biologically inspired nanomaterials and table-top stereolithography for 3D printed biomimetic osteochondral scaffolds. *Nanoscale*, 7: 14010–14022.

https://doi.org/10.1039/c5nr03425f

67. Freeman FE, Pitacco P, van Dommelen LHA, *et al.*, 2020, 3D bioprinting spatiotemporally defined patterns of growth factors to tightly control tissue regeneration. *Sci Adv*, 6: eabb5093.

https://doi.org/10.1126/sciadv.abb5093

68. Johnson BN, Lancaster KZ, Zhen G, *et al.*, 2015, 3D printed anatomical nerve regeneration pathways. *Adv Funct Mater*, 25: 6205–6217.

https://doi.org/10.1002/adfm.201501760

69. Epstein NE, 2013, Complications due to the use of BMP/INFUSE in spine surgery: The evidence continues to mount. *Surg Neurol Int*, 4: S343–S352.

https://doi.org/10.4103/2152-7806.114813

70. Shields LB, Raque GH, Glassman SD, *et al.*, 2006, Adverse effects associated with high-dose recombinant human bone morphogenetic protein-2 use in anterior cervical spine fusion. *Spine (Phila Pa 1976)*, 31: 542–547.

https://doi.org/10.1097/01.brs.0000201424.27509.72

71. Shenoda BB, Ajit SK, 2016, Modulation of immune responses by exosomes derived from antigen-presenting cells. *Clin Med Insights Pathol*, 9: 1–8.

https://doi.org/10.4137/CPath.S39925

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

https://doi.org/10.1016/j.addr.2021.113961

73. Zheng D, Huo M, Li B, *et al.*, 2020, The role of exosomes and exosomal microRNA in cardiovascular disease. *Front Cell Dev Biol*, 8: 616161.

https://doi.org/10.3389/fcell.2020.616161

74. Yerneni SS, Whiteside TL, Weiss LE, *et al.*, 2019, Bioprinting exosome-like extracellular vesicle microenvironments. *Bioprinting*, 13: e00041.

https://doi.org/10.1016/j.bprint.2019.e00041

75. Chen P, Zheng L, Wang Y, *et al.*, 2019, Desktop-stereolithography 3D printing of a radially oriented extracellular matrix/mesenchymal stem cell exosome bioink for osteochondral defect regeneration. *Theranostics*, 9: 2439–2459.

https://doi.org/10.7150/thno.31017

76. Zha Y, Li Y, Lin T, *et al.*, 2021, Progenitor cell-derived exosomes endowed with VEGF plasmids enhance osteogenic induction and vascular remodeling in large segmental bone defects. *Theranostics*, 11: 397–409.

https://doi.org/10.7150/thno.50741

77. Bari E, Scocozza F, Perteghella S, *et al.*, 2021, 3D bioprinted scaffolds containing mesenchymal stem/stromal lyosecretome: Next generation controlled release device for bone regenerative medicine. *Pharmaceutics*, 13: 515.

https://doi.org/10.3390/pharmaceutics13040515

78. Wang C, Wang M, Xu T, *et al.*, 2019, Engineering bioactive self-healing antibacterial exosomes hydrogel for promoting chronic diabetic wound healing and complete skin regeneration. *Theranostics*, 9: 65–76. 10.7150/thno.29766

79. Tao SC, Guo SC, Li M, *et al.*, 2017, Chitosan wound dressings incorporating exosomes derived from microRNA-126-overexpressing synovium mesenchymal stem cells provide sustained release of exosomes and heal full-thickness skin defects in a diabetic rat model. *Stem Cells Transl Med*, 6: 736–747.

https://doi.org/10.5966/sctm.2016-0275

80. Guo SC, Tao SC, Yin WJ, *et al.*, 2017, Exosomes derived from platelet-rich plasma promote the re-epithelization of chronic cutaneous wounds via activation of YAP in a diabetic rat model. *Theranostics*, 7: 81–96.

https://doi.org/10.7150/thno.16803

81. Han C, Zhou J, Liang C, *et al.*, 2019, Human umbilical cord mesenchymal stem cell derived exosomes encapsulated in functional peptide hydrogels promote cardiac repair. *Biomater Sci*, 7: 2920–2933.

https://doi.org/10.1039/c9bm00101h

82. Murphy SV, Atala A, 2014, 3D bioprinting of tissues and organs. *Nat Biotechnol*, 32: 773–785.

https://doi.org/10.1038/nbt.2958

83. Maumus M, Rozier P, Boulestreau J, *et al.*, 2020, Mesenchymal stem cell-derived extracellular vesicles: Opportunities and challenges for clinical translation. *Front Bioeng Biotechnol*, 8.

https://doi.org/10.3389/fbioe.2020.00997

84. Eslani M, Putra I, Shen X, *et al.*, 2017, Corneal mesenchymal stromal cells are directly antiangiogenic via PEDF and sFLT-1. *Invest Ophthalmol Vis Sci*, 58: 5507–5517.

https://doi.org/10.1167/iovs.17-22680

85. Hoang DH, Nguyen TD, Nguyen H-P, *et al.*, 2020, Differential wound healing capacity of mesenchymal stem cell-derived exosomes originated from bone marrow, adipose tissue and umbilical cord under serum- and xeno-free condition. *Front Mol Biosci*, 7: 119.

https://doi.org/10.3389/fmolb.2020.00119

86. Lopez-Verrilli MA, Caviedes A, Cabrera A, *et al.*, 2016, Mesenchymal stem cell-derived exosomes from different sources selectively promote neuritic outgrowth. *Neuroscience*, 320: 129–139.

https://doi.org/10.1016/j.neuroscience.2016.01.061

87. Piffoux M, Nicolás-Boluda A, Mulens-Arias V, *et al.*, 2019, Extracellular vesicles for personalized medicine: The input of physically triggered production, loading and theranostic properties. *Adv Drug Deliv Rev*, 138: 247–258.

https://doi.org/10.1016/j.addr.2018.12.009

88. Zhang X-F, Wang T, Wang Z-X, *et al.*, 2021, Hypoxic ucMSC-secreted exosomal miR-125b promotes endothelial cell survival and migration during wound healing by targeting TP53INP1. *Mol Ther Nucleic Acids*, 26: 347–359.

https://doi.org/10.1016/j.omtn.2021.07.014

89. Lener T, Gimona M, Aigner L, *et al.*, 2015, Applying extracellular vesicles based therapeutics in clinical trials – an ISEV position paper. *J Extracell Vesicles*, 4: 30087.

https://doi.org/10.3402/jev.v4.30087

90. Ahn SH, Ryu SW, Choi H, *et al.*, 2022, Manufacturing therapeutic exosomes: From bench to industry. *Mol Cells*, 45: 284–290.

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

91. Andreu Z, Rivas E, Sanguino-Pascual A, *et al.*, 2016, Comparative analysis of EV isolation procedures for miRNAs detection in serum samples. *J Extracell Vesicles*, 5: 31655.

https://doi.org/10.3402/jev.v5.31655

92. Busatto S, Vilanilam G, Ticer T, *et al.*, 2018, Tangential flow filtration for highly efficient concentration of extracellular vesicles from large volumes of fluid. *Cells*, 7.

https://doi.org/10.3390/cells7120273

93. Lobb RJ, Becker M, Wen SW, *et al.*, 2015, Optimized exosome isolation protocol for cell culture supernatant and human plasma. *J Extracell Vesicles*, 4: 27031.

https://doi.org/10.3402/jev.v4.27031

94. Kosanović M, Milutinović B, Goč S, *et al.*, 2017, Ion-exchange chromatography purification of extracellular vesicles. *Biotechniques*, 63: 65–71.

https://doi.org/10.2144/000114575

95. Greening DW, Xu R, Ji H, *et al.*, 2015, A protocol for exosome isolation and characterization: Evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. *Methods Mol Biol*, 1295: 179–209.

https://doi.org/10.1007/978-1-4939-2550-6_15

96. Corso G, Mäger I, Lee Y, *et al.*, 2017, Reproducible and scalable purification of extracellular vesicles using combined bind-elute and size exclusion chromatography. *Sci Rep*, 7: 11561.

https://doi.org/10.1038/s41598-017-10646-x

97. Oeyen E, Van Mol K, Baggerman G, *et al.*, 2018, Ultrafiltration and size exclusion chromatography combined with asymmetrical-flow field-flow fractionation for the isolation and characterisation of extracellular vesicles from urine. *J Extracell Vesicles*, 7: 1490143.

https://doi.org/10.1080/20013078.2018.1490143

98. Maroto R, Zhao Y, Jamaluddin M, *et al.*, 2017, Effects of storage temperature on airway exosome integrity for diagnostic and functional analyses. *J Extracell Vesicles*, 6: 1359478.

https://doi.org/10.1080/20013078.2017.1359478

99. Yuan F, Li YM, Wang Z, 2021, Preserving extracellular vesicles for biomedical applications: Consideration of storage stability before and after isolation. *Drug Deliv*, 28: 1501–1509.

https://doi.org/10.1080/10717544.2021.1951896

100. Chen YS, Lin EY, Chiou TW, *et al.*, 2020, Exosomes in clinical trial and their production in compliance with good manufacturing practice. *Ci Ji Yi Xue Za Zhi*, 32: 113–120.

https://doi.org/10.4103/tcmj.tcmj_182_19

101. Budgude P, Kale V, Vaidya A, 2021, Cryopreservation of mesenchymal stromal cell-derived extracellular vesicles using trehalose maintains their ability to expand hematopoietic stem cells in vitro. *Cryobiology*, 98: 152–163.

https://doi.org/10.1016/j.cryobiol.2020.11.009

102. Bosch S, de Beaurepaire L, Allard M, *et al.*, 2016, Trehalose prevents aggregation of exosomes and cryodamage. *Sci Rep*, 6: 36162.

https://doi.org/10.1038/srep36162

103. Charoenviriyakul C, Takahashi Y, Nishikawa M, *et al.*, 2018, Preservation of exosomes at room temperature using lyophilization. *Int J Pharm*, 553: 1–7.

https://doi.org/10.1016/j.ijpharm.2018.10.032