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REVIEW

Extracellular Vesicle-Integrated Biomaterials in Bone Tissue Engineering Applications: Current Progress and Future Perspectives

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Abstract: With an aging population and increased life expectancy, the clinical burden of bone-related disorders, especially large bone defects, continues to grow, underscoring the urgent need for effective regenerative strategies. Effective bone regeneration is essential not only for restoring skeletal structure and function but also for improving patients' quality of life and reducing the socioeconomic burden associated with prolonged recovery or surgical failure. Bone tissue engineering has emerged as a promising approach for healing large bone defects. Traditionally, stem cells, biomaterial scaffolds and growth factors have been considered the three essential elements of bone tissue engineering. However, stem cell-based therapies face several significant challenges, including ectopic tissue formation, malignant transformation, cell embolism, and immune rejection. In recent years, extracellular vesicles (EVs) have gained significant attention as an advanced alternative to stem cells and a novel cell-free therapy for bone regeneration due to their inherent advantages, such as low immune-rejection, excellent biocompatibility, significant bioactivity and high feasibility for carrying bioactive molecules or drugs. This review provides a comprehensive overview of the current state and future potential of EV-based strategies in bone tissue engineering. We first review the sources of parent cells for EVs applied in bone tissue engineering and the roles and potential mechanisms of EVs in bone regeneration. We then discuss the various modification strategies employed to enhance the therapeutic potential of EVs. Additionally, we summarize strategies for integrating EVs with various biomaterial scaffolds, with a specific focus on the latest advances in achieving controlled and sustained release of EVs from scaffolds at bone defect sites. Collectively, this review aims to offer key insights into the translational potential of EV-functionalized biomaterials and guide future directions in the development of next-generation bone regenerative therapies.

Keywords: bone tissue engineering, extracellular vesicles, biomaterials, mesenchymal stem cells

Introduction

Bone defects caused by trauma, infection and tumor resection are a common clinic scenario in orthopaedic practice.[1] The bone is a rigid organ that not only protects various internal organs, but also participates in hematopoiesis, storing minerals, supporting our body's physical structure and maintaining mobility.[2] Given the important roles of bone, it is crucial to develop effective methods to repair and reconstruct bone defects. Currently, the gold standard for treating large bone defects is autologous bone grafting. However, this strategy faces many problems, including potential donor site complications, limited autografts, and risk of potential graft failures.[3] Over the past few decades, bone tissue engineering has emerged as a promising strategy for treating large bone defects. This approach generally involves the integration of a three-dimensional (3D) biocompatible scaffold that provides shape and mechanical strength, seed cells with osteogenic potential, and molecular signals that can induce osteogenic differentiation and vascularization.[4] An ideal cellular source for bone tissue engineering approaches should be non-immunogenic, non-tumorigenic, and posses potent proliferative and osteogenic potentials. Among the various cell types evaluated for bone tissue engineering applications, mesenchymal stem cells (MSCs) are considered one of the most promising cellular sources due to their easy acquisition, potent proliferative and osteogenic potentials, low

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immunogenicity, and well-defined osteogenic differentiation pathway.[5] However, there are still many concerns about the potential risks associated with MSC transplantation, including ectopic tissue formation, malignant transformation, cell embolism, and immune rejection.[6] Additionally, even when MSCs are successfully administered, variables such as donor age, the number of in vitro expansion passages, culture conditions, and transplantation procedures may adversely affect their therapeutic efficacy.[7]

In recent years, numerous studies have demonstrated that the therapeutic effects of MSCs in tissue repair are primarily mediated by paracrine factors, rather than through direct differentiation into parenchymal cells to restore or replace the damaged tissue. Among these paracrine factors, extracellular vesicles (EVs) play a pivotal role.[8] According to the most recent consensus from the International Society for Extracellular Vesicles (ISEV2023), EVs are lipid bilayer-enclosed particles released by cells that lack the ability to self-replicate.[9] Once considered cellular debris, EVs are now recognized as critical mediators of intercellular communication, capable of delivering bioactive cargo to recipient cells or interacting with cellular receptors via surface proteins. Traditionally, EVs have been classified into three main subtypes based on their size, origin, and biogenesis: exosomes, microvesicles, and apoptotic bodies. Exosomes are formed through the inward budding of endosomal membranes, microvesicles (also referred to as ectosomes) arise from the outward budding of the plasma membrane, and apoptotic bodies are generated during cellular fragmentation in apoptosis (Table 1).[10] However, current isolation methods are unable to reliably distinguish EV subtypes based on their biogenesis, and universal molecular markers for definitive classification remain lacking. Consequently, ISEV discourages the use of biogenesis-based terminology unless specific EV subpopulations have been rigorously isolated and characterized. Despite the continued prevalence of the term "exosomes" in the literature, ISEV recommends the use of the broader, more inclusive term "EVs".[9]

During EV formation, they selectively package proteins, nucleic acids, and lipids from their parent cells, functioning as 'signaling complexes'. They transmit biological information through direct membrane fusion, endocytosis or ligand–receptor interactions, thereby influencing the behaviors of the recipient cells.[11] The precise biological function of EVs is a reflection of their parent cells and the local microenvironment.[12] Stem cell-derived EVs have gained prominence in regenerative medicine research, as numerous studies have demonstrated that they are the key mediators of the biological functions of their parent cells.[13] EVs offer several compelling advantages, including eminent bioactivity, stability, low immune-rejection, desirable biocompatibility, and high feasibility for modularized customized modification.[14] Although stem cells have long been considered a major component of bone tissue engineering strategies, EVs are emerging as an advanced substitute, capable of recapitulating the therapeutic potentials of stem cells while avoiding the potential risks associated with in vivo stem cell administration.[15] In recent years, considerable research has focused on the use of EVs in bone tissue engineering, though their development remains in the early stages. One major challenge is that free EVs do not achieve durable retention and controlled release at defect sites, leading to the combination of scaffolds as carriers for EVs.[16] An increasing number of studies have explored the use of various bioactive scaffolds integrated with EVs for bone tissue engineering applications. The purpose of this review is to discuss the roles of EVs in bone regeneration and their application in bone tissue engineering. We specifically

Table 1 The Three Classical Types of EVs

Type	Size	Morphology	Cellular Origin	Markers	Biogenesis	Common Isolation Methods
Exosomes	40–150 nm	Cup-shaped	Most cell types	CD63, CD9, CD81	Multivesicular endosome	Ultracentrifugation, Density gradient, Affinity chromatography; Size-exclusion chromatography
Microvesicles	150–1000 nm	Cup-shaped	Most cell types	Annexin A1, ARF6	Plasma membrane shedding	Ultracentrifugation, Affinity chromatography; Size-exclusion chromatography
Apoptotic bodies	1–5 μm	Heterogeneous	All cell types	Annexin V, PS	Apoptosis	Centrifugation, Filtration, Flow cytometry

focus on current strategies for integrating EVs with various bioactive scaffolds and the latest advances in achieving controlled and sustained release of EVs from the scaffolds at bone defect sites.

The Roles of EVs in Bone Regeneration

Bone regeneration is a complex and highly orchestrated biological process involving dynamic intercellular communication among MSCs, bone-forming osteoblasts, bone-resorbing osteoclasts, osteocytes, immune cells, and other cell types.[17] It is now well established that EVs play a crucial role in mediating these cellular interactions within the bone microenvironment. Several comprehensive reviews have thoroughly discussed the roles of EVs and their interactions with recipient cells during bone regeneration.[18,19] In this section, we elaborate on the sources of parent cells for EVs used in bone tissue engineering and elucidate the roles and potential molecular mechanisms of EVs in bone regeneration.

Parent Cells of EVs

Numerous studies have investigated the use of both natural and engineered EVs derived from various parent cells in bone tissue engineering. Among theses parent cells, MSCs stand out as the predominant cellular source of EVs. Additionally, EVs derived from bone cells, immune cells, and endothelial cells are also employed in bone tissue engineering applications. A summary of studies on the application of EVs derived from different parent cell sources in bone regeneration is provided in Table S1 (supplementary data).

MSCs

Researchers have utilized EVs secreted by MSCs derived from diverse tissues, such as bone marrow (BMSCs),[20–36] adipose tissue (ASCs),[37–44] umbilical cord (UCMSCs),[45–47] induced pluripotent stem cells (iPS-MSCs),[48,49] dental tissues (DMSCs),[50–57] and synovial membranes (SMSCs),[58] in combination with biomaterials to promote bone regeneration in preclinical animal models.

MSCs derived from different tissues offer distinct advantages. Among them, BMSCs are one of the most thoroughly researched and utilized MSC types in both academic and practical settings. As a vital cellular component within the bone microenvironment, their ability to proliferate, migrate, and differentiate into osteoblasts is crucial for successful bone regeneration.[59] Studies have shown that BMSC-derived EVs (BMSC-EVs) enhance osteogenic differentiation of both MSCs and osteoblasts in vitro.[20,25,28,60–62] Furthermore, when integrated with biomaterials, BMSC-EVs can significantly accelerate bone regeneration at bone defect sites in vivo.[20,25,26,28,60,62]

ASCs are abundantly distributed throughout the body and are easily accessible, making them a highly practical source of parent cells for EVs.[37] Research has shown that ASCs produce a greater quantity of EVs compared to BMSCs. Although a considerable body of studies have demonstrated that ASC-derived EVs (ASC-EVs) can accelerate bone regeneration when integrated with biomaterials in vivo,[37–42] there is still some controversy regarding their osteogenic potential. For instance, studies by Li et al and Liu et al found that only EVs from osteogenically induced ASCs were capable of promoting osteogenic differentiation of BMSCs in vitro. Conversely, EVs from non-osteogenically induced ASCs did not exhibit the ability to enhance osteogenic differentiation of BMSCs.[37,63] However, research by Gandolfi et al, Kim et al and Xing et al reported that EVs from non-osteogenically induced ASCs enhanced the osteogenic potential of ASCs, BMSCs and MC3T3-E1 cells (murine calvariae preosteoblast cell line) in vitro.[39,41,42] One possible explanation for this disparity is that Li et al and Liu et al evaluated the osteogenic potential of ASC-EVs under 2D cell culture conditions, whereas Gandolfi et al, Kim et al and Xing et al assessed it using MSCs seeded on scaffolds in a 3D culture environment.

UCMSCs are derived from postnatal waste tissues. Compared to MSCs derived from other tissue origins such as BMSCs and ASCs, UCMSCs possess higher self-renewal capacity, lower immunogenicity and fewer ethical concerns.[64] Studies have shown that EVs secreted by UCMSCs (UCMSC-EVs) can promote osteogenic differentiation of BMSCs, mouse osteoblast progenitor cells and MC3T3-E1 cells in vitro.[45–47] Additionally, when combined with biomaterials, UCMSC-EVs have been shown to enhance bone regeneration at bone defect sites in vivo.[45–47]

iPS-MSCs combine the advantages of both iPSs and MSCs. Even after 40 passages in vitro, iPS-MSCs retain their self-renewal capacity and do not exhibit tumorigenic risk.[65] Researchers have demonstrated that EVs secreted by iPS-

MSCs (iPS-MSC-EVs) can promote osteogenic differentiation of BMSCs in vitro and enhance bone regeneration when combined with scaffolds in vivo.[48,49]

Human DMSCs derived mainly from dental pulp (DPSCs), gingiva (GMSCs), apical papilla (SCAPs) and periodontal ligament (PDLSCs) are promising candidates for bone regeneration due to their minimally invasive tissue collection methods and strong osteogenic differentiation potential.[66] Studies have demonstrated that EVs secreted by DPSCs (DPSC-EVs),[51,52] GMSCs (GMSC-EVs),[53–55] SCAPs (SCAP-EVs)[50] and PDLSCs (PDLSC-EVs)[56,57] can promote osteogenic differentiation in vitro and accelerate bone regeneration at bone defect sites in vivo.

Currently, there is no consensus on the optimal parent cells for EVs in bone tissue engineering applications. Li et al conducted a comparative analysis of the osteogenic potential of BMSC-EVs, ASC-EVs, and EVs secreted by SMSCs (SMSC-EVs). Their findings indicated that ASC-EVs exhibited superior effects in enhancing migration, proliferation, and osteogenesis of BMSCs in vitro, as well as promoting bone regeneration in a mouse model in vivo, compared to BMSC-EVs and SMSC-EVs.[58] However, a study by Liu et al reported that BMSC-EVs showed stronger osteogenic potential compared to ASC-EVs.[63] The inconsistency between studies may be attributed to differences in EV concentrations and pretreatment conditions of the parent cells.

Bone Cells

Osteoblasts are the primary functional cells responsible for bone formation, playing a key role in the secretion, synthesis, and mineralization of the bone matrix.[67] Mizukami et al demonstrated that EVs secreted by mature primary osteoblasts promoted osteogenic differentiation of mouse mesenchymal stromal cells in vitro. Furthermore, local administration of EVs encapsulated in a gelatin hydrogel at bone defect sites significantly enhanced bone healing in a mouse femoral bone defect model.[68] MC3T3-E1 is an osteoblast precursor cell line derived from mouse calvaria. Previous studies have demonstrated the osteo-inductive potential of EVs derived from osteogenically differentiated MC3T3-E1 cells. These EVs have also been utilized in combination with scaffolds or hydrogels to improve bone regeneration in vivo.[69,70]

Osteoclasts play a crucial role in maintaining bone homeostasis, primarily through their function in resorbing the bone matrix.[67] Studies have shown that EVs derived from osteoclasts significantly upregulate expression of osteogenic markers and promote mineralization in MSCs in vitro. When integrated with biomaterials, osteoclasts-derived EVs have been demonstrated to enhance bone regeneration in vivo.[71,72]

Immune Cells

Immune cells play a pivotal role in bone regeneration. Numerous studies have highlighted the influence of immune cells, such as T cells, B cells, and macrophages, on MSC-mediated bone regeneration. Optimizing the host immune microenvironment has been demonstrated to improve stem cell-based bone regeneration.[73,74] Within bone defect sites, MSCs can modulate immune cell functions through EVs, while immune cells can, in turn, impact MSC differentiation by releasing EVs.[14,15]

Macrophages are immune-regulating cells that play pivotal roles in both initiating and resolving inflammation through polarization into various phenotypes. M1 macrophages are characterized by their pro-inflammatory phenotype, while M2 macrophages exhibit an anti-inflammatory phenotype. Promoting the polarization of macrophages toward the M2 phenotype has been shown to effectively enhance bone angiogenesis and accelerate bone healing.[75] Peng et al demonstrated that EVs secreted by M2 macrophages not only promoted polarization of macrophages toward the M2 phenotype but also enhanced osteogenic differentiation and mineral deposition in BMSCs. When encapsulated in a multifunctional DNA-based hydrogel, these M2 macrophage-derived EVs accelerated the healing of alveolar bone defects in vivo.[76] In a separate study, Wei et al used EVs derived from BMP2-pretreated RAW 264.7 cells (a murine macrophage cell line) to modify titanium nanotube implants, thereby promoting osteogenesis in BMSCs.[77]

In addition to macrophage-derived EVs, those secreted by polymorphonuclear leukocytes (PMNs) and dendritic cells have also been integrated with biomaterials to enhance bone regeneration. Wang et al demonstrated that PMN-derived EVs (PMN-EVs) promoted the proliferation and osteogenic differentiation of BMSCs in vitro. BMSC-based cell sheets integrated with PMN-EVs significantly accelerated bone regeneration in a rat calvarial defect model.[78] In another study, Cao et al demonstrated that EVs from mature dendritic cells promoted the proliferation and osteogenic differentiation of BMSCs in vitro and enhanced BMSC-mediated bone regeneration in a rat femoral defect model in vivo.[79]

Other Cells

In addition to MSCs, bone cells and immune cells, EVs secreted by other cell types, such as chondrogenic progenitor cells, endothelial cells and Schwann cells, have also been utilized in combination with biomaterials to enhance bone regeneration. ATDC5 is a chondrogenic progenitor cell line known for its significant capacity for osteogenic differentiation.[80] Zha et al introduced vascular endothelial growth factor (VEGF) plasmid into EVs secreted by ATDC5 cells (ATDC5-EVs) through electroporation. This approach achieved dual functions: promoting osteogenic differentiation of BMSCs and enabling controlled delivery of the VEGF gene. These engineered EVs were subsequently integrated with 3D-printed porous bone scaffolds, which effectively improved vascularized bone regeneration in vivo.[81]

Evidence has suggested that EVs secreted by vascular endothelial cells have the potential to induce bone remodeling.[82] Lin et al successfully enriched programmed cell death ligand 1 (PD-L1) in EVs derived from genetically modified human umbilical vein endothelial cells (HUVECs). These PD-L1-overexpressing EVs were demonstrated to suppress T cell activation and induce osteogenic differentiation of BMSCs when pre-cultured with T cells in vitro. Furthermore, incorporating HUVEC-derived EVs into an injectable hydrogel significantly accelerated fracture healing in a murine model.[83]

Schwann cells are typically observed in peripheral nerves, and EVs derived from Schwann cells (SC-EVs) have been proved to effectively promote nerve regeneration.[84] A study by Wu et al discovered that SC-EVs also play a direct role in bone regeneration. Their research demonstrated that SC-EVs promoted the migration, proliferation, and osteogenic differentiation of BMSCs in vitro. Additionally, when combined with titanium alloy scaffolds, SC-EVs improved bone regeneration in vivo.[85]

Function and Potential Mechanisms of EVs in Bone Regeneration

In the previous section, we introduced the sources of parent cells of EVs applied in bone tissue engineering applications. It is evident that EVs, particularly those derived from MSCs, play pivotal roles in promoting bone regeneration. Extensive research has shown that the beneficial effects of EVs in bone regeneration are primarily due to their ability to induce osteogenic differentiation, facilitate angiogenesis, and modulate immune responses.[15] It is well established that the biological characteristics and functions of EVs are determined by their cargoes, which encompass a diverse array of bioactive molecules inherited from their parent cells, including proteins, nucleic acids, and lipids.[11,12] The lipid bilayer membrane of EVs acts as a protective barrier, shielding their cargoes from degradation by extracellular proteases, nucleases, and other enzymes. By transferring their cargoes between cells, EVs mediate genetic alteration in target cells, leading to cell fate change. Several previous reviews have already examined the mechanisms through which EVs contribute to bone regeneration.[19,86] In this section, we will elaborate on the roles and potential mechanisms of EVs in bone regeneration, focusing on their ability to induce osteogenic differentiation, promote angiogenesis and regulate immune responses.

Promoting Osteogenesis

As previously mentioned, numerous studies have demonstrated that EVs released by MSCs and some other cells can directly enhance the osteogenic differentiation of MSCs, osteoblasts, and osteoprogenitor cells.[20,25,45,46,50,60–62,82,85] The process of bone regeneration, involving the osteogenic differentiation of MSCs into mature osteoblasts and their subsequent mineralization, is intricately regulated by various miRNAs.[87] MiRNAs are small non-coding RNAs (containing about 18–22 nucleotides) that regulate gene expression at the post-transcriptional level by binding to target mRNAs and inducing their degradation and/or translational inhibition.[88] Among the various molecules contained in EVs, miRNAs have garnered significant attention due to their regulatory roles in gene expression.[89] EVs have been shown to be enriched with osteogenesis-related miRNAs. For instance, Qin et al extracted small RNAs from BMSC-EVs and subjected them to miRNA/small RNA-sequencing analysis, finding that three miRNAs critical for osteogenesis (miR-196a, miR-206 and miR-27a) were highly enriched in BMSC-EVs. Functional tests demonstrated that all these three miRNA mimics exhibited osteogenic effects, with miR-196a exhibiting the highest potency.[25] Guo et al demonstrated that BMSC-EVs promoted osteogenic differentiation of BMSCs by suppressing the expression of WWP1, an inhibitor of osteoblast differentiation, through the delivery of miR-19b-3p.[33] Chen et al analyzed miRNAs contained in ASC-EVs and found that among the top 30 most highly enriched miRNAs in the EVs, five miRNAs (miR-21, miR-199b, miR-10a, miR-10b and miR-let-7f) were reported to be involved in maintaining bone homeostasis or promoting stem cell osteogenic differentiation.[38] Jing et al demonstrated that the enhanced osteogenesis

induced by SCAP-EVs was mediated through the highly expressed miRNA-150-5p. Further bioinformatic analysis predicted that miRNA-150-5p might facilitate osteogenesis by regulating the PI3K-Akt, Wnt, and MAPK signaling pathways.[50] Hu et al found that UCMSC-EVs enhanced calcium deposition and endothelial network formation, promoting both osteogenic differentiation and angiogenesis by delivering miR-23a-3p, which activated the PTEN/AKT signaling pathway.[47] Wang et al conducted small RNA sequencing analysis of DPSC-EVs and identified the highly expressed miR-1246 as a potential key regulator of DPSC-EVs in promoting bone tissue regeneration.[90] In another study by Cao et al, the promoting effect of EVs derived from mature dendritic cells on proliferation and osteogenesis of BMSCs was demonstrated to be mediated by highly expressed miR-335. Further investigation revealed that miR-335 was transferred to BMSCs by EVs and inhibited the Hippo signaling pathway by targeting large tongue suppressor kinase 1 (LATS1).[79]

Furthermore, researchers have discovered that miRNA profiles within EVs change depending on the stage of osteogenic differentiation. Zhai et al demonstrated that EV released from MSCs exposed to longer osteogenic differentiation time showed enhanced osteogenic potential compared to that of MSCs exposed to shorter osteogenic differentiation time. Specifically, osteogenic miRNAs (miR-503-5p, miR-146a-5p, miR-129-5p, and miR-483-3p) were upregulated and anti-osteogenic miRNAs (miR-133a-3p, miR-32-5p, and miR-204-5p) were downregulated in EVs derived from late-stage osteogenic cultures (day 10 and day 15), compared to those from early-stage osteogenic cultures (day 0 and day 4). Further bioinformatic analysis suggested that these differentially expressed miRNAs might activate the PI3K/Akt and MAPK signaling pathways.[91] In line with this finding, Pishavar et al observed a similar trend in EVs derived from placental stem cells (PSCs). They found that miR-10, miR-27a, and miR-192, which have been reported as late markers of osteogenesis, were upregulated in EVs derived from late-stage osteogenic cultures.[61]

Other researchers compared miRNA profiles within EVs secreted by MSCs derived from different tissue origins. Liu et al found that only three miRNAs were significantly different (1 up-regulated: miR-23b-3p; 2 down-regulated: miR-199a-3p and miR-214-3p) between osteogenically induced BMSC-EVs and ASC-EVs. All three miRNAs were reported to be involved in regulating osteogenic and/or adipogenic differentiation. They further concluded that the osteo-inductive potential of BMSC-EVs was attributed to multiple miRNAs (let-7a-5p, let-7c-5p, miR-31a-5p, and miR-328a-5p), which targeted Acvr2b/Acvr1, regulating the competitive balance of Bmpr2/Acvr2b towards Bmpr-elicited Smad1/5/9 phosphorylation.[63]

Recent research has revealed that long non-coding RNAs (lncRNAs) can be transferred to target cells via EVs. These lncRNAs act as competing endogenous RNAs, binding and sequestering miRNAs to prevent them from interacting with their target mRNAs.[92] For instance, Yang et al demonstrated that BMSC-EVs carrying lncRNA MALAT1 significantly enhanced osteogenic activity and mitigated osteoporosis symptoms in ovariectomized mice by acting as a miR-34c sponge, leading to the upregulation of SATB2 expression.[93] Behera et al identified that lnc-H19 was enriched in BMSC-EVs and functioned as an activator of the ANGPT-Tie2 axis. By serving as a miRNA-106a sponge, lnc-H19 promoted both BMSC osteogenesis and endothelial angiogenesis.[94] Additionally, Qi et al reported that EVs derived from osteogenically-induced BMSCs transferred lncRNA-ENSRNOG00000056625, which acted as a sponge to sequester miR-1843a-5p, preventing its binding to Mob3a. This interaction promoted YAP dephosphorylation and nuclear translocation, ultimately alleviating senescence-related phenotypes, and enhancing proliferation and osteogenic differentiation of senescent BMSCs.[30]

In addition to nucleic acids, the abundant proteins encapsulated in EVs also play a vital role in cell-to-cell communication. Li et al performed quantitative proteomics to compare the protein profiles of BMSC-EVs, ASC-EVs, and SMSC-EVs. They discovered that proteins associated with regulation of actin cytoskeleton, focal adhesion, extracellular matrix (ECM)-receptor interaction, PI3K-Akt signaling pathway, and cAMP signaling pathway were more abundant in ASC-EVs compared to BMSC-EVs and SMSC-EVs, which could account for the enhanced osteogenic potential of ASC-EVs.[58] Al-Sharabi et al compared the protein profiles of EVs derived from osteogenically induced MSCs with those from non-osteogenically induced MSCs. The findings revealed that the upregulated differentially expressed proteins in EVs derived from osteogenically induced MSCs were mainly involved in pathways related to wound and bone healing. In contrast, the upregulated proteins in EVs derived from non-osteogenically induced MSCs appeared to be involved in pathways related to EV formation and biogenesis.[28] Ge et al performed proteomic analysis of EVs derived from osteoblast cell line MC3T3. Their findings indicated that proteins encapsulated in EVs from

osteoblasts were highly enriched in osteogenesis-associated signal pathways, including integrin signaling, eukaryotic initiation factor 2 signaling, and mTOR signaling pathways.[95]

In conclusion, EVs promote osteogenesis in MSCs and osteoblasts by delivering their bioactive cargoes, particularly nucleic acids and proteins. This effect is likely mediated through the modulation of key signaling pathways, including PI3K/Akt, BMP/Smad, Wnt/β-Catenin, AMPK, and Hippo. However, the precise mechanisms underlying EVs' osteogenic activity remain incompletely understood. Further research is needed to elucidate the detailed mechanisms and related downstream signaling cascades.

Enhancing Angiogenesis

Improving vascularization of the implants remains a significant challenge in bone tissue engineering. In cases of large bone defects, the implanted seed cells are typically positioned several hundred microns away from the nearest capillary supply, leading to hypoxia and subsequent apoptosis of the seed cells, which compromises the efficacy of the implants.[96] Therefore, promoting angiogenesis has consistently proven to be an effective strategy to facilitate bone regeneration.[97] Numerous studies have shown that MSCs derived from various tissues (such as bone marrow, adipose tissue, placenta, and umbilical cord) secrete EVs that not only enhance osteogenesis but also exhibit potent pro-angiogenic effects. In vitro experiments have demonstrated that MSC-EVs can significantly enhance the proliferation, migration, and tube formation of endothelial cells.[98–101] Additionally, in animal models, biomaterials integrated with MSC-EVs have been shown to effectively promote bone regeneration by enhancing vascularization at bone defect sites.[50,60,102] Furthermore, direct injection of MSC-EVs into bone fracture sites has been reported to accelerate fracture healing through improved vascularization.[103]

The pro-angiogenic effects MSC-EVs are closely associated with their encapsulated bioactive cargoes. MSC-EVs are enriched with angiogenesis-promoting biomolecules, including VEGF, angiogenin, basic fibroblast growth factors (bFGF), and angiopoietin-1 (ANG-1). Notably, levels of VEGF, angiogenin, monocyte chemotactic protein-1 (MCP-1) and the receptor-2 for VEGF (VEGF-R2) are even higher in EVs than in their parent MSCs.[99] In addition to these angiogenic factors, EV-encapsulated miRNAs also play an important role in their pro-angiogenic effects. For instance, Gong et al demonstrated that EVs secreted by the MSC line C3H10T1/2 enhanced the proliferation, migration and angiogenesis of HUVECs by delivering miR-30b, which downregulated the expression of DLL4. DLL4 is a membrane-bound ligand from the Notch signaling family that plays a negative regulatory role in vascular sprouting and vessel branching.[104] Wang et al reported that miR-210, which targeted the angiogenesis-related gene Efna3, was enriched in BMSC-EVs. EVs collected from BMSCs with silenced miR-210 exhibited significantly reduced pro-angiogenic effects both in vitro and in vivo.[105] Other studies reported that ASC- EVs exerted pro-angiogenic effects via the transfer of miR-125a and miR-31, which targeted DLL4 and hypoxia-inducible factor-1α (HIF-1α), respectively, in recipient endothelial cells.[106] Additionally, Jing et al found that miR-126-5p, highly expressed in SCAP-EVs, was transferred to HUVECs to enhance expression of angiogenic genes such as VEGF and ANG-1.[50]

The pro-angiogenic effects of EVs can be further enhanced by hypoxia pretreatment of the parent cells. Liu et al found that EVs released by hypoxia-treated UCMSCs promoted angiogenesis of HUVECs in vitro and bone fracture healing in vivo through miR-126 and the SPRED1/Ras/Erk signaling pathway. Hypoxia preconditioning resulted in elevated miR-126 levels in UCMSC-EVs through HIF-1α activation.[103] Additionally, certain biomaterials can amplify the angiogenic potential of EVs. For instance, Liu et al demonstrated that lithium-containing biomaterials upregulated the expression of miR-130a in BMSC-EVs, which resulted in the downregulation of the PTEN protein and activation of the AKT pathway, thereby enhancing the proliferation, migration, and tube formation of HUVECs.[107] In summary, EVs can enhance bone regeneration by delivering their encapsulated pro-angiogenic biomolecules to endothelial cells and promoting vascularization.

Regulating Immune Responses

As mentioned above, immune cells play a pivotal role in bone regeneration. Typically, a large number of immune and inflammatory cells are located in the microenvironment of bone defect sites. The implanted biomaterials are often recognized as foreign substances by the host immune system, triggering a cascade of immune responses.[20] Studies have

shown that prolonged exposure to pro-inflammatory cytokines can lead to chronic inflammation, resulting in fibrous encapsulation around the bone graft and osseointegration failure.[108] Therefore, focusing solely on direct osteogenesis while neglecting the immune reactions caused by biomaterials and seed cells is insufficient for constructing an ideal tissue-engineered bone graft. Only mild inflammatory responses are beneficial for bone regeneration.[109]

EVs can modulate immune responses by interacting with immune effector cells, including T cell, B cells, macrophages, and other immune cells, through mechanisms such as membrane fusion, ligand–receptor interactions and delivery of bioactive cargoes.[110] Growing evidence indicates that MSC-EVs possess immunosuppressive functions. They have been shown to inhibit immune cell activation, promote the expression of anti-inflammatory factors and reduce inflammatory responses.[111] For instance, MSC-EVs can suppress the proliferation of T cells and B cells, promote the conversion of T cells into regulatory T cells, inhibit dendritic cell maturation, and attenuate the function of natural killer cells.[112] Additionally, MSC-EVs have been found to facilitate tissue damage repair by modulating the M1/M2 polarization of macrophages localized at the site of tissue injury.[113,114] In a study performed by Fan et al, both in vitro and in vivo results demonstrated that BMSC-EVs promoted macrophage M2 polarization via the NF-κB pathway. Moreover, EV-functionalized scaffolds created a more favorable immune microenvironment for bone regeneration compared to scaffolds without EV modification.[20] Consistent with this finding, Li et al observed that ASC-EVs inhibited inflammation and promoted the polarization of M1 macrophages to M2 macrophages. Their data further indicated that ASC-EVs exerted their immunomodulatory effects through miR-451a, which targeted the macrophage migration inhibitory factor (MIF).[115]

In summary, the roles of EVs in bone regeneration encompass three main aspects: firstly, they directly promote osteogenic differentiation of MSCs and osteoblasts; secondly, they stimulate angiogenesis, thereby accelerating vascularized bone regeneration; and thirdly, they modulate the inflammatory response at the site of injury, creating a favorable immune environment for bone regeneration. As previously discussed, the beneficial effects of EVs are primarily attributed to their encapsulated bioactive molecules, particularly miRNAs and proteins. Table 2 summarizes the miRNAs enclosed in EVs that are implicated in bone regeneration.

Table 2 MiRNAs Enclosed in EVs That are Involved in Bone Regeneration

Cargoes of EVs	Parent cells	EVs	Therapeutic Roles and Possible Mechanisms	References
miR-196a, miR-27a, miR-206	Human BMSCs	Exosomes	Promote osteogenic differentiation of osteoblasts and expression of osteogenic genes	[25]
miR-19b-3p	Rat BMSCs	Exosomes	Promotes osteogenic differentiation of BMSCs by suppressing the expression of WWP1	[33]
miR-21	Human ASCs	Microvesicles	Promotes osteogenic differentiation of hMSCs by targeting Smad7-Smad1/5/8-Runx2	[38]
miR-let-7f	Human ASCs	Microvesicles	Accelerates osteogenic differentiation of hMSCs by targeting Axin2	[38]
miR-10a	Human ASCs	Microvesicles	Restores the differentiation capability of hMSCs by inhibiting KLF4	[38]
miR-10b	Human ASCs	Microvesicles	Promotes osteogenic differentiation of hMSCs by targeting SMAD2	[38]
miR-199b	Human ASCs	Microvesicles	Promotes osteogenic differentiation of hMSCs by suppressing GSK-3b/b-catenin	[38]
miR-150-5p	Human SCAPs	Exosomes	Promotes osteogenic differentiation of preosteoblasts, potential involved signaling pathways: Wnt, PI3K-Akt, MAPK	[50]
miR-23a-3p	Human UCMSCs	Exosomes	Promotes osteogenic differentiation of BMSCs by targeting PTEN/AKT signaling pathway	[47]

(*Continued*)

Table 2 (Continued).

Cargoes of EVs	Parent cells	EVs	Therapeutic Roles and Possible Mechanisms	References
miR-1246	Human DPSCs	Exosomes	Promotes osteogenic differentiation of BMSCs	[90]
miR-335	Rat dendritic cells	Exosomes	Promotes osteogenic differentiation of BMSCs by targeting LATS1	[79]
miR-let-7a-5p, miR-let-7c-5p, miR-328a-5p, miR-31a-5p	Rat BMSCs	Exosomes	Promote osteogenesis by targeting Acvr2b/Acvr1	[63]
miR-146a-5p, miR-503-5p, miR-483-3p, miR-129-5p	Human MSCs	Exosomes	Promote osteogenesis by targeting PI3K/Akt and MAPK signaling pathways	[91]
miR-30b	Murine MSC line C3H10T1/2	Exosomes	Promotes angiogenesis both in vitro and in vivo by suppressing the expression of DLL4	[104]
miR-210	Murine BMSCs	Exosomes	Promotes angiogenesis both in vitro and in vivo by suppressing the expression of Efna3	[105]
miR-125a	Human ASCs	Exosomes	Promotes angiogenesis both in vitro and in vivo by suppressing the expression of DLL4	[106]
miR-31	Human ASCs	Microvesicles	Promotes angiogenesis both in vitro and in vivo by targeting HIF1-1α	[106]
miRNA-126-5p	Human SCAPs	Exosomes	Promotes the expression of angiogenic genes in HUVECs, potential involved signaling pathways: MAPK, ErBb, Ras	[50]
miR-126	Human UCMSCs	Exosomes	Promotes angiogenesis both in vitro and in vivo by targeting HIF1-1α	[103]
miR-130a	Rat BMSCs	Exosomes	Promotes angiogenesis both in vitro and in vivo by activation of AKT signaling pathway	[106]
miR-451a	Human ASCs	Exosomes	Inhibits inflammation and promotes the polarization of M1 macrophages to M2 macrophages by targeting MIF	[115]

EV-Biomaterial Delivery System in Bone Tissue Engineering

In The Roles of EVs in Bone Regeneration, we introduced the sources of parent cells for EVs applied in bone tissue engineering and elucidated the roles and potential molecular mechanisms of EVs in bone regeneration. EVs are emerging as an ideal candidate for developing cell-free therapies for bone regeneration due to their inherent advantages, such as low immune-rejection, stability, biocompatibility, and high feasibility for modularized customized modification.[14] However, one of the major challenges in applying EVs to bone tissue engineering is that free EVs do not allow for durable retention at the defect site.[16] To address this issue, researchers have proposed an excellent solution: combining EVs with biomaterial scaffolds to achieve sustained aggregation and controlled release of EVs at the defect site. To date, numerous studies have successfully combined EVs with biomaterial scaffolds, demonstrating the strong potential of these scaffolds for efficient EV loading and release modulation.[15] Traditionally, biomaterial scaffolds, stem cells, and growth factors have been considered the three essential elements for bone tissue engineering.[116] With the development of EV-integrated scaffolds, this three-basic-element model has the potential to be simplified to two-basic-element model: biomaterial scaffolds and EVs.[117] However, the use of EVs alone is often insufficient for complete tissue regeneration, especially in challenging scenarios like large bone defects.[118] Therefore, various techniques have been applied to engineer EVs to enhance their therapeutic potential in bone regeneration. These engineering methods can generally be divided into two main categories: endogenous engineering strategies and exogenous engineering strategies.[15,119] Endogenous engineering strategies involve modifying the parent cells of EVs, while exogenous engineering strategies involve modifying EVs after they have been isolated from their parent cells. Additionally, the way in which EVs are integrated with biomaterial scaffolds plays a critical role in determining the

bone regenerative potency of these EV-loaded scaffolds.[119] In this section, we elaborate on the methods for engineering EVs and strategies for integrating EVs with biomaterial scaffolds.

Endogenous Engineering of EVs

In Parent Cells of EVs, we have introduced the diverse sources of parent cells for EVs applied in bone tissue engineering. It is well established that EVs exert their therapeutic effects in a content-dependent manner, with proteins, mRNAs, and/or microRNAs playing pivotal roles in their biological functions. As miniature versions of cells, the contents of EVs are determined not only by the type of parent cells but also by their culture conditions.[120] Furthermore, studies have shown that modifying parent cells before EV isolation can alter the contents and biological functions of EVs.[121] In this section, we discuss methods for engineering EVs at the cellular level through physical manipulation, pre-conditioning treatment and genetic engineering of parent cells, as illustrated in Figure 1.

Physical Manipulation of Parent Cells

Mechanical loading of the skeleton system is crucial for bone development, growth, and maintenance. Piezo1 is a mechanotransducer that confers mechanosensitivity to bone-forming osteoblasts, thereby regulating mechanical load-induced bone formation and remodeling.[122,123] He et al demonstrated that EVs derived from BMSCs pretreated with Yoda1, a Piezo1 agonist, exhibited enhanced osteogenic capabilities compared to control EVs. Moreover, these Yoda1-pretreated BMSC-derived EVs, when incorporated into hydrogels, displayed significantly improved bone regenerative capacity in both subcutaneous ectopic bone formation model in nude mice and rat calvarial bone defect model.[32]

Extruding physical forces directly onto cells is a commonly used methods for the physical modification of parent cells. The traditional method of isolating EVs via differential centrifugation is widely recognized as time-consuming, labor-intensive, and producing EVs with low purity and yield, which somewhat limits their potential for large-scale clinical application.[124] To address these deficiencies in the production of natural EVs, researchers have utilized cell-derived plasma membrane fragments to produce EV mimetics (EMs) via lipid self-assembly. EMs, which closely resemble EVs in shape and content, can be fabricated by serially extruding cells through polycarbonate membrane filters with decreasing pore sizes using a microextruder.[125] This extrusion technique yields 100–200 times more EMs than the conventional differential centrifugation method, significantly improving production efficiency and substance loading.[126] Compared to natural EVs, EMs exhibit similar size, structure, surface markers, and can be modified using both endogenous and exogenous engineering strategies. To date, EMs derived from various parent cells, including MSCs, iPSC-ECs, ATDC5 cells, and others, have been effectively utilized in bone tissue engineering.[118,124,127,128] Although EMs are not naturally secreted by parent cells, they are considered more promising vesicles than EVs for gene and drug delivery due to their higher yields, less time-consuming isolation process, and relatively simpler cargoes.[124] Therefore, in this review, we also include EMs when discussing the application of EVs in bone regeneration.

In addition to exerting physical forces to parent cells, researchers have discovered that magnetic scaffolds can influence the contents of EVs. Zhu et al found that modification of hydroxyapatite scaffolds with magnetic nanoparticles reduced the levels of certain proteins, such as reactive oxygen species, ubiquitin, and ATP in osteoclast-derived EVs, while increasing Rho kinase levels. This modification weakened the negative impact of EVs on osteogenic cells and promoted bone regeneration.[129]

Pre-Conditioning of Parent Cells

Pre-conditioning of parent cells can enhance the biologic activity of EVs derived from them. Researchers have utilized various methods including osteogenic induction, hypoxia pretreatment, and stimulation with growth factors, cytokines and other biochemical reagents to optimize the pro-osteogenic efficacy of EVs. Although the relationship between cell state and EV production remains incompletely understood, pre-conditioning of parent cells offers advantages such as operational simplicity, low cost, preservation of EV structure, and elimination of additional purification steps, highlighting its potential for scalable manufacturing.

Osteogenic Induction

Pre-culturing parent cells in osteogenic induction medium is an effective strategy to enhance the osteogenic potential of

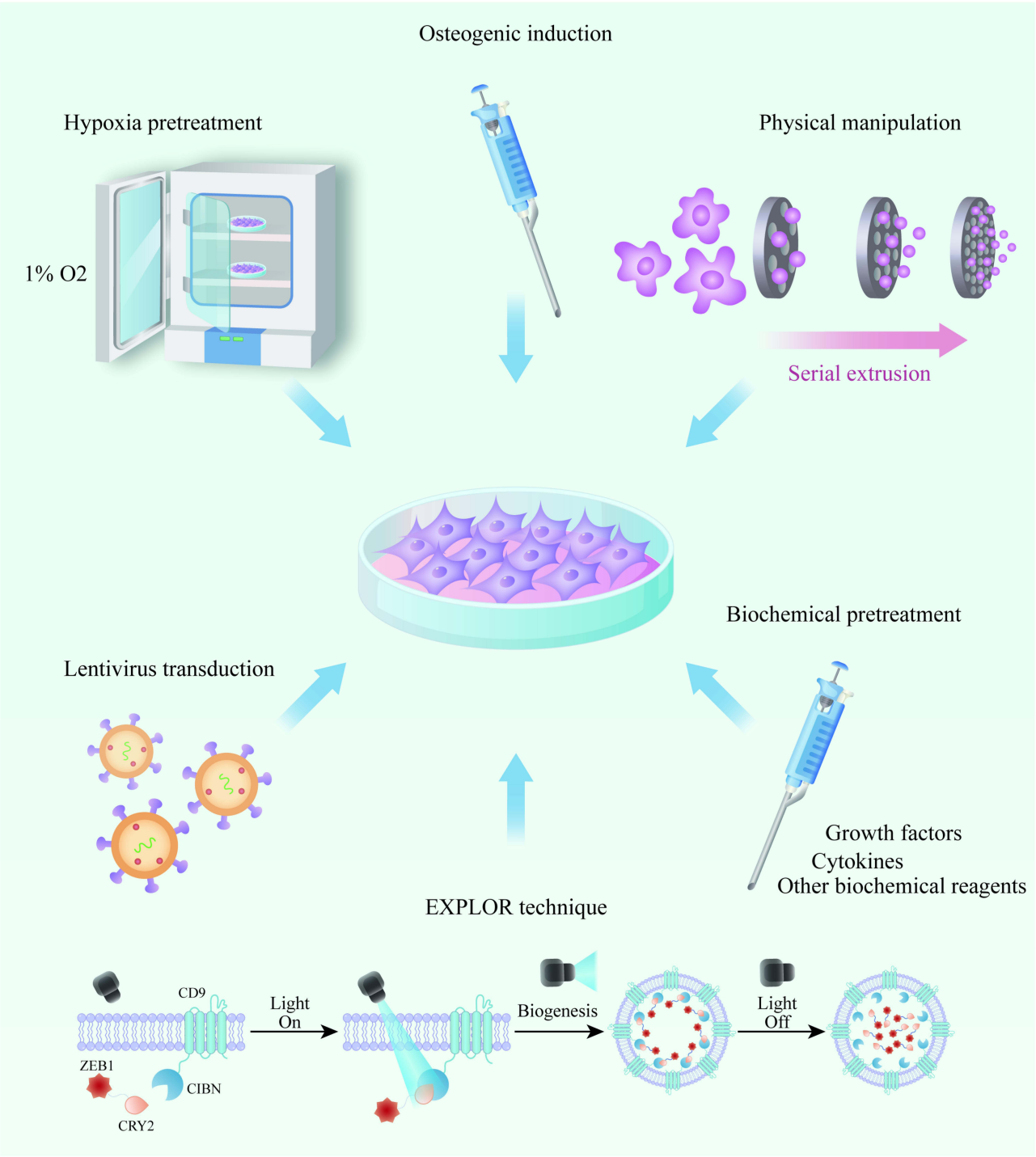

Figure 1 Schematic summary of strategies for endogenous engineering of EVs at the cellular level, including physical manipulation, pre-conditioning treatment (including osteogenic induction, hypoxic exposure and biochemical pretreatment) and genetic engineering of parent cells (such as lentivirus transduction and the EXPLOR technique). Adapted from *Biomaterials*, Volume 283, Tao SC, Li XR, Wei WJ, et al, Polymeric coating on beta-TCP scaffolds provides immobilization of small extracellular vesicles with surface-functionalization and ZEB1-Loading for bone defect repair in diabetes mellitus, Page 121465, Copyright 2022, with permission from Elsevier.[117]

EVs. Studies have shown that EVs derived from osteogenically-induced MSCs are more effective in promoting osteogenic differentiation of MSCs in vitro and bone regeneration in vivo compared to EVs derived from undifferentiated MSCs.[28] Additionally, several studies have reported that EVs secreted by non-osteogenically induced ASCs lack osteogenic potential, whereas EVs secreted by osteogenically induced ASCs significantly promote osteogenic

https://doi.org/10.2147/IJN.S522198

differentiation of BMSCs.[37,63] Furthermore, researchers have demonstrated that EVs induce osteogenic differentiation of MSCs in a stage-dependent manner. Specifically, EV released from MSCs exposed to longer osteogenic differentiation time induce osteogenic differentiation more efficiently than those released from MSCs exposed to shorter osteogenic differentiation time.[61,91] Taken together, these findings suggest that osteogenic induction is one of the most cost-effective and practical methods for enhancing the osteogenic potential of EVs.

Hypoxia Treatment

Preconditioning parent cells in a hypoxic environment is another widely applied pretreatment strategy to enhance the osteogenic potential of EVs. It is well established that MSCs naturally reside in hypoxic conditions.[130] Cells respond to hypoxic environment by expressing HIF-1α, an important regulator of bone repair and regeneration that mediates key biological processes such as angiogenesis and osteogenesis.[131,132] Hypoxic pretreatment not only stimulates MSCs to secrete more EVs, but also enhances the angiogenic potential of EVs both in vitro and in vivo.[100,103] In a study by Liu et al, hypoxic preconditioning of UCMSCs was shown to enhance the pro-angiogenic potential of EVs by transferring functional miR-126 to HUVECs, resulting in the down-regulation of SPRED1. Furthermore, they demonstrated that hypoxia preconditioning amplified the therapeutic efficacy of EVs, thereby accelerating bone fracture healing in a murine femoral fracture model.[103] Zhuang et al also discovered that EVs derived from hypoxia-pretreated BMSCs promoted the proliferation, migration, and angiogenesis of HUVECs and ultimately enhanced vascularized bone regeneration in a rat calvarial bone defect model. Mechanistically, hypoxia induced overexpression of miR-210-3p in EVs, which inhibited EFNA3 expression and subsequently activated the PI3K/AKT pathway.[133] In another study by Cui et al, EMs were generated from hypoxia-preconditioned endothelial cells derived from human induced pluripotent stem cells (hiPSCs) through an extrusion approach. Although these EMs did not directly induce osteogenic differentiation of BMSCs, they exhibited potent pro-angiogenic activities and re-educated bone marrow endothelial cells to secret cytokines that promoted osteogenic differentiation of BMSCs and alleviated the pro-inflammatory microenvironment dominated by M1 macrophages in osteoporotic bones.[127] In addition to hypoxic pretreatment of parent cells, researchers also use chemicals that stabilize the expression of HIF-1α to mimic hypoxia in cells under normal oxygen levels. As mentioned earlier, cells respond to hypoxic treatment by expressing HIF-1α, which regulates several hypoxia-responsive genes. Under normoxic conditions, HIF-1α is proteasomally degraded by the iron-containing prolyl hydroxylase (PHD) enzyme. Dimethyloxaloylglycine (DMOG) is a small angiogenic molecule that inhibits PHD enzyme, thereby stabilizing HIF-1α.[134] Liang et al found that EVs derived from BMSCs preconditioned with a low dose of DMOG promoted neovascularization via the AKT/mTOR pathway and enhanced bone regeneration in critical-sized bone defects in rats.[22]

Biochemical Pretreatment

Preconditioning parent cells with various growth factors and cytokines can further enhance the osteogenic potential of EVs. BMP-2 is a well-established osteogenic factor and the only osteo-inductive growth factor approved by the Food and Drug Administration.[52] Wei et al demonstrated that EVs from BMP2-stimulated macrophages induced osteogenic differentiation of BMSCs more efficiently than control EVs and enhanced the bio-functionality of titanium nanotubes.[77] Previous studies indicated that inflammatory mediators could induce the regenerative capacity of MSC-EVs.[135] In a study by Lu et al, tumor necrosis factor-α (TNF-α), a major inflammatory factor, was used as a preconditioning agent to mimic the acute inflammatory phase upon bone injury. Their study showed that TNF-a preconditioning enhanced the osteogenic potential of ASC-EVs by increasing their Wnt-3a content.[136] Sun et al found that pretreatment of BMSCs with lipopolysaccharide (LPS), a potent inducer of inflammatory cytokine release, significantly enhanced the immunoregulatory potential of EVs by increasing EV secretion and promoting the polarization of macrophages from M1 to M2 phenotype.[137]

Beyond the biochemical agents mentioned above, researchers have employed other agents, such as dexamethasone,[31] tauroursodeoxycholic acid,[44] hydrogen peroxide,[138] and strontium-substituted calcium silicate ceramics,[139] to pretreat MSCs and enhance the therapeutic potential of EVs in bone regeneration. Furthermore, rather than using a single biochemical agent, researchers have explored the synergistic effects of multiple agents on parent cells to further enhance the therapeutic potential of EVs. For instance, Man et al investigated the synergistic effects of the hypoxia mimetic agent deferoxamine (DFO) and the DNA methyltransferase inhibitor 5-azacytidine (AZT) on the therapeutic efficacy of

BMSC-EVs for bone repair.[132] DFO is an iron chelating agent that induces cellular hypoxia by inhibiting the activity of PHD, thereby stabilizing HIF-1α expression.[140] AZT is a DNA methyltransferase inhibitor that promotes osteogenic differentiation of MSCs.[141] Interestingly, the demethylation of HIF-1α is essential for its stabilization during hypoxia, enhancing its transcriptional activity.[132] Their study demonstrated that inducing hypomethylation during hypoxia synergistically enhanced osteogenesis in BMSCs, thereby increasing the pro-angiogenic and osteogenic potentials of their secreted EVs.

Genetic Engineering of Parent Cells

The strategy of loading EVs with therapeutic molecules via genetic modification of the parent cells can improve the therapeutic efficacy of EVs. By utilizing biological tools, such as viral vectors or plasmids, parent cells can be genetically engineered to increase the expression of endogenous molecules, which are subsequently encapsulated within their derived EVs through the cell's biomolecular synthesis mechanisms. Endogenous cargoes are usually miRNAs and proteins with therapeutic benefits.

Lentivirus and adenovirus transduction are commonly used by researchers to genetically modify parent cells in bone tissue engineering applications. For instance, Huang et al established a stable BMSC line that constitutively overexpresses BMP2 via lentivirus transduction.[27] They discovered that EVs derived from these BMP2-overexpressing BMSCs retained the general physical and biochemical characteristics of naïve BMSC-EVs in terms of size distribution, surface marker expression and endocytic properties. However, these EVs showed significantly enhanced bone regenerative potential compared to naïve BMSC-EVs in a rat calvarial bone defect model in vivo. Interestingly, despite BMP2 being constitutively expressed in the parental cells, BMP2 protein was absent as a constituent of the secreted EVs. Further investigations revealed that the enhanced pro-osteogenic potential of EVs was partly attributed to altered EV cargoes, including miRNAs that potentiate the BMP2 signaling cascade. Subsequently, the same research group utilized EVs derived from BMP2-overexpressing BMSCs in combination with hydrogels to repair rat calvarial bone defects.[142] In another study by Fan et al, EMs were obtained from MSCs in which the expression of noggin, a natural BMP antagonist, was downregulated by transduction of lentivirus particles encoding noggin shRNA.[118] They demonstrated that EMs from noggin-suppressed MSCs significantly accelerated bone healing compared to naïve EMs in a rat calvarial bone defect model. Mechanistic studies revealed that the enhanced osteogenic potential of EMs from noggin-suppressed MSCs was mediated via inhibition of miR-29a. Meanwhile, Chen et al generated a stable ASC line in which miR-375, a positive regulator in the osteogenic differentiation of MSCs, was upregulated by lentivirus transduction.[40] Administration of EVs from miR-375-overexpressing ASCs improved osteogenic differentiation of BMSCs in vitro and enhanced bone regeneration in a rat model of calvarial bone defect in vivo. In another study, Li et al transfected BMSCs with adenovirus carrying triple point-mutations (amino acids 402, 564, and 803) in the HIF-1α coding sequence.[24] The mutant HIF-1α effectively maintained cellular expression under normoxic conditions. They discovered that EVs derived from these mutant HIF-1α-modified BMSCs exhibited enhanced pro-osteogenic and pro-angiogenic potential in vitro. Furthermore, injecting these EVs into the necrotic region significantly accelerated bone regeneration and angiogenesis in a rabbit model of steroid-induced avascular necrosis of the femoral head. Another group demonstrated that EVs derived from mutant HIF-1α-modified BMSCs, in combination with β-tricalcium phosphate (β-TCP) scaffolds, effectively repaired critical-sized bone defects by promoting bone regeneration and neovascularization.[21]

Besides viral transduction, chemical transfection methods are also utilized by researches to genetically modify parent cells. As mentioned earlier, excessive infiltration or persistence of inflammatory cells contributes to chronic inflammation and hinder bone regeneration. Studies have shown that TIM3, a membrane protein encoded by Havcr2, promotes the polarization of M2 macrophages, which in turn secrete cytokines that suppress inflammation. Lu et al transfected BMSCs with Havcr2 overexpression plasmid with a commercial DNA transfection reagent and obtained engineered EVs that highly expressed TIM3. Their results demonstrated that these TIM3-overexpressing EVs, when encapsulated in hydrogel, accelerated bone regeneration in a mouse calvarial bone defect model by modulating the immune microenvironment and mitigating the adverse effects of excessive inflammation.[29] In a separate study, Liu et al generated BMSC-EVs overexpressing miR-20a, a microRNA known for its potent pro-osteogenic potential, by transfecting BMSCs with miR-20a mimics using a commercial transfection reagent. Their study showed that these BMSC-EVs overexpressing miR-20a

significantly promoted the migration and osteogenesis in BMSCs in vitro and improved titanium alloy scaffold osteointegration in osteoporotic rats.[143]

Recently, researchers have developed an innovative technique for loading proteins into exosomes using optogenetics, known as EXPLOR (exosomes for protein loading via optically reversible protein-protein interactions) technology.[144] This technique exploits the natural process of exosome biogenesis and optogenetically controlled reversible protein-protein interactions, enabling precise, reversible and efficient delivery of the target proteins into exosomes. To load target proteins into exosomes, two vectors expressing fusion proteins are introduced into parent cells: CIBN (a truncated version of CIB1) conjugated with an exosome-associated tetraspanin protein CD9, and CRY2-conjugated cargo protein. A single pulse of 488-nm laser irradiation triggers the rapid translocation of CRY2-conjugated cargo proteins from the cytosol to the plasma membrane and the membrane of multivesicular bodies (MVBs), where CIBN-conjugated CD9 proteins are co-localized. During the process of endogenous biogenesis, the cargo proteins are incorporated into exosomes. Upon removal of the illumination source, the cargo proteins are detached from the CD9-fused CIBN, resulting in their release into the exosomes' intraluminal space.[145] Zinc Finger E-Box Binding Homeobox 1 (ZEB1), which is highly expressed in $CD31^{hi}$ $endomucin^{hi}$ endothelial cells, promotes angiogenesis-dependent bone formation and is considered a very promising therapeutic molecule.[146] To efficiently load ZEB1 into exosomes, Tao et al transfected SMSCs with two vectors expressing fusion proteins: a CIBN-CD9 expression vector and a CRY2-ZEB1 expression vector, and then utilized the EXPLOR technique to load ZEB1 into SMSC-derived exosomes.[117] Further experiments demonstrated that ZEB1 could confer multiple biological functions to exosomes, including promoting angiogenesis and osteogenesis while inhibiting osteoclastogenesis. Scaffolds functionalized with these engineered exosomes were shown to enhance vascularized bone regeneration in a diabetic rat model of cranial defect.

In summary, Endogenous Engineering of EVs outlines strategies for engineering EVs at the cellular level, including physical manipulation, pre-conditioning treatments, and genetic modification of parent cells. These approaches are grounded in the premise that alterations in parent cells are reflected in their secreted EVs. Physical and pre-conditioning methods are relatively simple to implement and can produce EVs enriched with therapeutic molecules. Genetic engineering of parent cells, on the other hand, enables targeted modification of specific components, thereby enhancing the therapeutic potential of the resulting EVs. Although manipulating parent cells is generally more feasible than directly modifying EVs, some researchers remain concerned that key aspects of EV biogenesis-particularly membrane formation and cargo selection-are still not fully understood. As a result, these strategies function as a form of "black box modification", with outcomes that are sometimes difficult to predict.[121]

Exogenous Engineering of EVs

In Endogenous Engineering of EVs, we introduced methods for the endogenous engineering of EVs at the cellular level through pre-conditioning treatments, physical manipulation and genetic engineering of parent cells. In this section, we discuss strategies for the exogenous modifying of EVs following their isolation. EVs are considered a new generation of natural nanoscale delivery systems due to their high biocompatibility, small size, low immunogenicity and capability of crossing the blood–brain barrier.[147] Current methods for exogenous engineering of EVs include mechanical extrusion, incubation, sonication, electroporation, recurrent freezing and thawing, and direct EV surface modification through covalent or non-covalent interactions.[148] Among these techniques, electroporation and surface modification are commonly employed in bone tissue engineering applications.

Electroporation

Electroporation is a widely used technique for loading exogenous cargo into EVs. It is a passive approach that introduces target drugs, proteins, and nucleic acids into EVs by creating transient pores in the EV membrane, allowing these molecules to diffuse inside.[15] However, careful optimization of experimental parameters-such as voltage, electroporation buffer composition, and electric field frequency-is essential. Improper conditions may lead to drug loss, membrane rupture, or compromise of EV functionality. Moreover, the current efficiency of exogenous cargo loading into EVs remains difficult to precisely control, posing challenges for accurate prediction and quantification.[121] Zha et al electroporated VEGF plasmids into ATDC-EVs.[81] Application of these modified ATDC5-EVs, in combination with biomaterial

scaffolds, significantly promoted vascularized bone regeneration in a rat radius defect model. The same research group also introduced VEGF plasmids into EMs extracted from ATDC5 cells, achieving a transfection efficiency of approximately 20%, thus demonstrating the feasibility of exogenous modification of EMs.[124] In another study, Cui et al harnessed the triple effects of the Schnurri-3 (Shn3) gene-encoded Shn3 protein, which include inhibiting bone differentiation, promoting osteoclast activity, and suppressing type H blood vessel formation. They loaded small interfering RNA-Shn3 (siShn3) into EVs derived from iPS-MSCs via electroporation with a loading efficiency of 18%. These siShn3-modified EVs showed stable physical characterizations and enhanced anti-osteoporotic effect in a bilateral ovariectomy-induced bone loss mouse model.[149]

Surface Functionalization Strategies of EVs

Researchers have developed various membrane surface modification strategies aimed at displaying specific therapeutic or targeting moieties on the EV surface to enhance their therapeutic efficacy, targeting precision, or active transport in vivo. These strategies can be categorized into non-covalent techniques (such as electrostatic binding, hydrophobic insertion, anchoring peptides, and aptamer-based modification) and covalent methods, including click chemistry.

Multivalent Electrostatic Interaction

Polyethyleneimine (PEI) is a biocompatible cationic polymer, considered as one of the most promising non-viral gene transfer vectors. It combines with plasmid DNA (pDNA) to form a PEI/pDNA complex through electrostatic interactions.[150] When complexed with nucleic acids, PEI can induce osmotic swelling, known as the proton-sponge effect, which facilitates the release of endosomal contents without the need for additional endosomolytic agents.[55] EVs contain proteins and negatively charged phospholipids, such as phosphatidylserine, which result in a negatively charged surface.[151] Diomede et al complexed GMSC-EVs with PEI through electrostatic interactions.[55] They found that PEI modification not only enhanced the internalization of EVs by target cells through proteoglycan binding but also increased the efficacy of EVs by promoting the release of intracellular contents through the proton-sponge effect. These PEI-complexed EVs, when combined with PLA scaffolds, showed improved bone healing with superior osteogenic properties compared to non-functionalized EVs. Subsequently, the same group coated PDLSC-EVs with PEI to further enhance the therapeutic potential of EVs in bone regeneration.[56] In another study, Liang et al sequentially coated EVs with PEI and human BMP2 plasmids (phBMP2) using layer-by-layer self-assembly to create an EVs–PEI/phBMP2 nonviral gene vector (Figure 2A).[151] Scaffolds coated with EVs–PEI/phBMP2 significantly promoted bone repair in a rabbit model of femoral condyle defect. Additionally, their findings suggested that the presence of EVs in EVs–PEI/pDNA complexes could mitigate the excessive positive charge of PEI, thereby reducing its cytotoxicity.

Modification by Anchoring CP05

The CP05 peptide (CRHSQMTVTSRL) presents a simple approach for expressing targeting or therapeutic moieties on the EV surface by binding with high affinity to the second extracellular loop of CD63, a tetraspanin abundantly expressed on EV membranes. Acting as an excellent connecting linker, CP05 enables the direct conjugation of targeting or therapeutic moieties to EVs, thereby avoiding the low loading efficiency associated with conventional methods such as transfection, electroporation, and lyophilization. Moreover, this surface conjugation approach preserves the native size and morphology of EVs and does not alter their in vivo biodistribution.[148] The tri-amino acid sequence, arginine-glycine-aspartate (RGD) is the minimum recognition sequence required for cell attachment and is one of the most extensively studied adhesive peptides in biomaterial research.[156] Studies have shown that RGD-derived peptides enhance the activation of BMPR/Smad signaling pathway to promote osteogenesis.[157] To load RGD-derived peptide onto EVs (EVs_{RGD}), Ma et al designed a fusion peptide RGD-CP05 by connecting GRGDSPC with CP05 through a linker. Additionally, to facilitate the colonization of EVs_{RGD} onto titanium scaffolds, they introduced a titanium-binding peptide (TBP), which specifically binds to titanium surface, and developed another fusion peptide TBP-CP05, enabling EVs_{RGD} to adhere to 3D printed titanium implants (Figure 2B). Their research demonstrated that EVs_{RGD} harnessed the synergetic effects of EVs and RGD, enhancing osteogenic differentiation of BMSCs in vitro, and resulting in successful osseointegration around the titanium implants in vivo.[152]

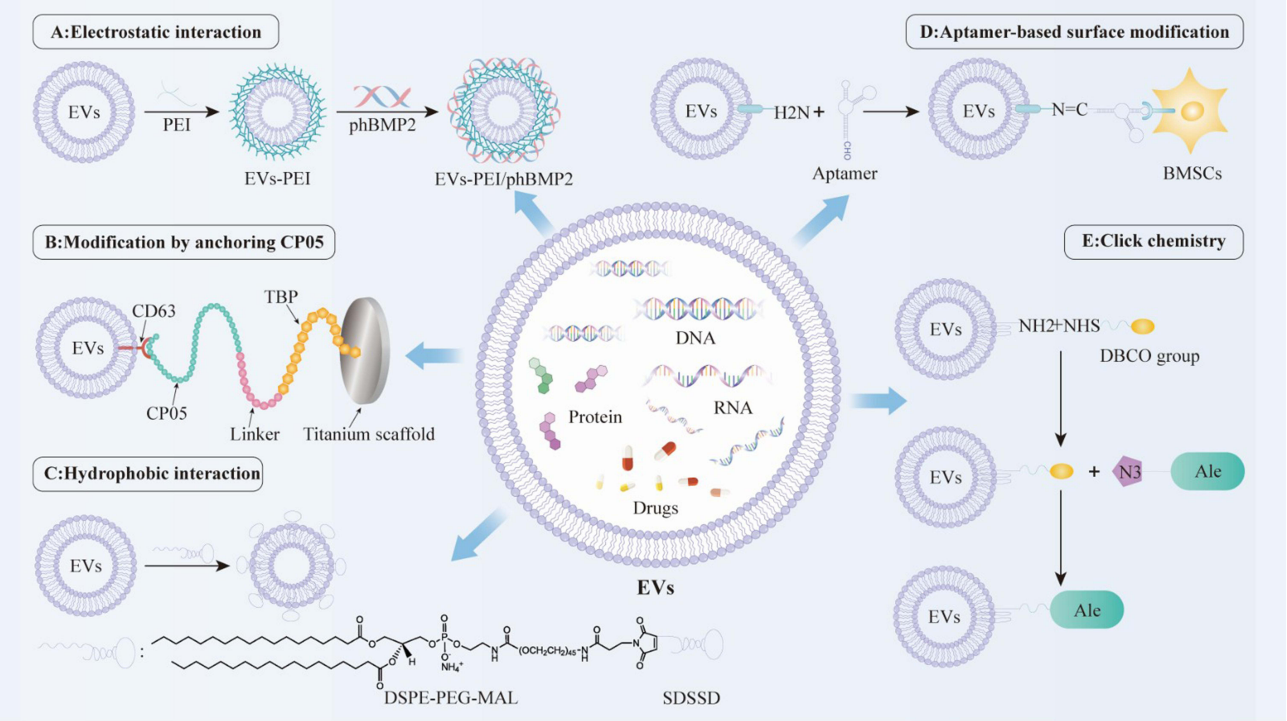

Figure 2 Schematic summary of strategies for EV surface modification. (**A**) Multivalent electrostatic interaction: positively charged polyethyleneimine (PEI) and negatively charged human BMP2 plasmids (phBMP2) were sequentially coated on the negatively charged membranes of EVs via electrostatic interactions, forming EVs–PEI/phBMP2 complexes. Used with permission of Royal Society of Chemistry, from Mesenchymal stem cell-derived microvesicles mediate BMP2 gene delivery and enhance bone regeneration, Liang Z, Luo Y, Lv Y, Volume 8, Edition 30, 2020; permission conveyed through Copyright Clearance Center, Inc.[151] (**B**) Modification by anchoring CP05: The CP05 peptide specifically binds to the tetraspanin CD63 marker on EV membrane, while the titanium-binding peptide (TBP) specifically targets the titanium surface. EVs are directed to the titanium surface through a fusion peptide TBP-CP05. Used with permission from Royal Society of Chemistry, Synergetic osteogenesis of extracellular vesicles and loading RGD colonized on 3D-printed titanium implants, Ma S, Li X, Hu H, et al, Volume 10, Edition 17, 2022; permission conveyed through Copyright Clearance Center, Inc.[152] (**C**) Hydrophobic interaction: the bone-targeting-peptide SDSSD, modified with DSPE-PEG-Mal, is effectively anchored to the EV membrane through hydrophobic interaction between the diacyllipid tail and the EV phospholipid layer. Adapted from Zou J, Shi M, Liu X, et al. Aptamer-functionalized exosomes: elucidating the cellular uptake mechanism and the potential for cancer-targeted chemotherapy. *Anal Chem*. 2019;91(3):2425–2430. Copyright 2019 American Chemical Society.[153] (**D**) Aptamer-based surface modification: the 5′-end of a BMSC-specific aptamer is modified with an aldehyde group, allowing it to react with the amino groups of EV membrane proteins to produce aptamer-functionalized EVs. Used with permission from Royal Society of Chemistry, Aptamer-functionalized exosomes from bone marrow stromal cells target bone to promote bone regeneration, Luo ZW, Li FX, Liu YW, et al., Volume 11, Edition 43, 2019; permission conveyed through Copyright Clearance Center, Inc.[154] (**E**) Click chemistry: alendronate (Ale) modified with an azide group (Ale-N3) is conjugated with EV membranes modified with an alkynyl group (EVs-DBCO) using click chemistry. Adapted from Wang Y, Yao J, Cai L, et al. Bone-targeted extracellular vesicles from mesenchymal stem cells for osteoporosis therapy. *Int J Nanomed*. 2020;15:7967–7977.[155]

Hydrophobic Interaction

The diacyllipid insertion method provides a straightforward approach for displaying targeting or therapeutic moieties on the surface of EVs. In this method, targeting or therapeutic agents are conjugated with a hydrophobic diacyllipid tail, facilitating their effective anchoring to the EV membrane. This anchoring occurs through the hydrophobic interaction between the diacyllipid tail and the phospholipid layer of the EV membrane.[153] Cui et al modified a bone-targeting peptide SDSSD (Ser, Asp, Ser, Ser, Asp) with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]-maleimide (DSPE-PEG-Mal). The diacyllipid-modified peptide was then anchored to the EV membrane via hydrophobic interaction (Figure 2C). Intravenous administration of these SDSSD-modified EVs in mice significantly increased their accumulation in bone tissue, while reducing their distribution in other organs, allowing for precise delivery of therapeutic cargoes to osteoblasts in vivo.[149] In a separate study by Wu et al, a pentapeptide, cysteine–arginine–glutamic acid–lysine–alanine (CREKA), was conjugated with DSPE-PEG-Mal. The diacyllipid-modified CREKA was then inserted into the membrane of ASC-EVs to obtain CREKA-functionalized EVs, which are capable of targeting fibrin, a natural fibrous network formed by large precursor protein fibrinogen following virtually all forms of tissue damage including bone injury. These CREKA-functionalized EVs enhanced the bone repair substantially in a rat femoral defect model, owing to their improved fibrin-binding and retention capacity.[43]

Aptamer-Based Surface Modification

Aptamers, screened through Systematic Evolution of Ligands by Exponential Enrichment (SELEX), are single-stranded DNA/RNA oligonucleotides that exhibit high affinity and specificity for target molecules due to their unique three-dimensional structures. These DNA/RNA oligonucleotides have been employed to recognize diseased tissues and selectively deliver therapeutic agents.[158] Aptamer-functionalized, drug-loaded EVs have been extensively explored in cancer therapy.[153] Luo et al used the cell-SELEX technique to select a BMSC-specific aptamer.[154] They confirmed its specificity by incubating it with various bone-derived cell types. In their study, the 5′-end of the aptamer was modified with an aldehyde group, enabling it to react with the amino groups of EV membrane proteins to form a stable Schiff base. The aldehyde-modified aptamer was then incubated with BMSC-EVs overnight at 4 °C to produce aptamer-functionalized EVs. In vitro experiments demonstrated that EVs conjugated with aptamers were specifically taken up by BMSCs (Figure 2D). When intravenously injected into a postmenopausal osteoporosis mouse model induced by ovariectomy, these BMSC-specific aptamer-functionalized EVs significantly increased their accumulation in the limbs and enhanced bone regeneration in vivo.

Click Chemistry

Click chemistry is a highly efficient chemo-selective conjugation technique that operates under mild conditions, allowing target molecules to be bound to the EV membrane. It has gradually emerged as a mainstream approach for the chemical modification of EVs.[121] In this method, an alkyne group is introduced to EVs via a condensation reaction using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide-N-hydroxysuccinimide (EDC-NHS). These alkyne-modified EVs are then covalently conjugated to the azido groups of the targeting moieties. Click chemistry reactions are highly efficient and do not affect the size or integrity of EVs, nor do they impact the uptake of EVs by target cells. Compared to non-covalent modification techniques mentioned above, click chemistry-being a covalent binding approach-offers more stable conjugation between targeting moieties and EVs, thereby minimizing the risk of modification loss during long-term storage or repeated freeze-thaw cycles.[148] Despite limitations such as incomplete byproduct removal and complex modification procedures, these challenges are expected to be progressively addressed through future optimization of large-scale production methods. Wang et al modified alendronate (Ale), which specifically targets bone tissue through hydroxyapatite, with an azide (N3) group, while the EV membrane was functionalized with an alkynyl (DBCO) group. The Ale-N3 and EVs-DBCO were subsequently coupled using copper-free "click chemistry" (Figure 2E). These Ale-conjugated EVs demonstrated strong bone-targeting ability and effectively stimulated bone regeneration in a rat model of ovariectomy-induced osteoporosis.[155]

In Exogenous Engineering of EVs, we discuss strategies for exogenous modification of EVs after their isolation. Compared to modifying parent cells, direct modification of EVs offers certain inherent advantages. It does not rely on the mechanisms of EV biogenesis, thereby avoiding potential side effects associated with the aforementioned "black box" process. Moreover, for patient-derived EVs, it may represent the only viable modification option. However, post-isolation modification also presents challenges, including time-consuming and labor-intensive procedures, significant EV loss, difficulty in removing by-products and impurities, and the risk that improper selection of targeting peptides or anchoring proteins may disrupt EV endocytosis or lead to premature degradation of targeting moieties. Therefore, meticulous post-modification handling and rigorous validation experiments are essential to ensure successful EV modification.[121]

Integration of EVs with Biomaterials

Achieving durable retention and controlled release of EVs at defect sites remains a significant challenge in bone tissue engineering. The intrinsic properties of biomaterials, the methods used to integrate EVs with these materials, and the surface modifications of biomaterials all influence the local release of EVs. In Endogenous Engineering of EVs and Exogenous Engineering of EVs, we summarized the endogenous and exogenous strategies for modifying EVs in bone tissue engineering applications. In this section, we will elaborate on recent advances in strategies for integrating EVs with biomaterial scaffolds.

Post-Solution Absorption

Post-solution absorption is one of the earliest and most utilized methods for integrating EVs with biomaterial scaffolds. This approach involves simply incubating scaffolds in an EV solution or applying an EV suspension onto the scaffolds, allowing for the physical adsorption of EVs by the scaffolds.[119] Through this method, EVs have been integrated with various biomaterial scaffolds, including ceramic materials (such as β-TCP, hydroxyapatite, and mesoporous bioactive glass (MBG)),[48,63,129] synthetic polymers (such as PLA, polycaprolactone (PCL), and poly(lactic-co-glycolic acid) (PLGA)),[37,39,159] natural polymers (such as silk fibroin and collagen),[41,62] metals (such as titanium and its alloys),[85] and natural bone materials (such as decellularized bone matrix (DBM)).[160] Although post-solution absorption is widely used, its primary drawback is the inability to achieve durable retention and sustained release of EVs at the defect site. For instance, Zhang et al modified β-TCP scaffolds with EVs secreted by iPSC-MSCs through post-solution absorption, demonstrating that the EV/scaffold constructs significantly accelerated bone regeneration in a rat model of calvarial bone defect. However, EVs loaded onto β-TCP scaffolds exhibited an initial burst release, with over 60% of EVs released within the first day in vitro, and nearly all EVs released by the fifth day.[49] Similarly, Wu et al observed a comparable EV release profile with β-TCP scaffolds modified with EVs secreted by stem cells derived from exfoliated deciduous teeth.[102] In another study, Kim et al fabricated porous silk fibroin scaffolds using a freeze-drying method and subsequently coated the scaffolds with ASC-EVs through post-solution absorption. This study did not observe an initial burst release of EVs; instead, approximately 70% of EVs were released from the scaffolds in a sustained manner over seven days.[41] The disparities between studies suggest that the release profiles of EVs from scaffolds are closely related to the inherent characteristics of the scaffolds.

Some researchers have explored lyophilization of the EV/scaffold constructs after coating the scaffolds with EVs through post-solution absorption. For example, Liu et al applied BMSC-EV concentrate to hierarchical MBG scaffolds and subsequently lyophilized the EV-loaded scaffolds. The scaffold-loaded EVs showed a rapid release in the first week, followed by a sustained, slow release thereafter, with release percentages of approximately 30% on day 1, less than 50% on day 2, 71.40% on day 14, and 75.42% on day 28.[63] These findings suggest that it is possible to prolong the release duration of EVs from the scaffolds by lyophilizing the EV/scaffold constructs.[119] While most studies allow the scaffolds to remain stationary after applying the EV suspension, some studies facilitate the absorption of EV suspension through agitation. For instance, Diomede et al coated PLA scaffolds with GMSC-EVs by immersing the scaffolds in the EV suspension under agitation for 48 hours.[55] Further investigation is needed to determine whether agitation can enhance the amount of EVs that adhere to the scaffolds.

In summary, although post-solution absorption is the most widely used method for integrating EVs with scaffolds, relying solely on physical absorption is insufficient for retaining EVs over the desired bone regeneration period, which typically takes several weeks. To ensure that EVs continue to exert their biological efficacy at the bone defect site, more sustainable and controllable delivery methods need to be developed.[119]

Surface Modification Strategies of Biomaterials

Surface-Modifying Agents

Proteomic studies have revealed that the membrane of EVs is abundant in tetraspanins, lactadherin (LA), integrins, and lysosome-associated membrane protein-2b (Lamp-2b), which are crucial for mediating cell adhesion and targeting.[148] Additionally, it has been reported that MSC-EVs can bind to several ECM proteins such as fibronectin and type I collagen.[62] Leveraging this characteristic of EVs, researchers have coated biomaterial scaffolds with various surface-modifying agents, including peptides, ECM proteins, and functional polymers, to enhance EV adhesion to the scaffolds and prolong the release duration of EVs. For instance, Xie et al coated DBM scaffolds with fibronectin before applying an EV suspension.[160] Their results showed that after repeated washing with PBS, EVs remained evenly distributed on the surface of porous DBM scaffolds.

Polydopamine is recognized as a promising molecule for anchoring both synthetic and biological substances or forming an adhesive layer on diverse substrates in tissue engineering applications.[161] Li et al modified PLGA scaffolds with polydopamine to enhance EV adhesion and achieved a slow, sustained release profile of EVs.[37] Their results showed that approximately 165.72 ± 15.4 μg of EVs per scaffold could be incorporated onto each polydopamine-modified PLGA scaffold, with about 28.19 ± 9.2% of EVs still retained in the scaffold after 8 days. In contrast, PLGA scaffolds without polydopamine modification showed significantly lower EV loading (about 73.6 ± 22.4 μg of EVs per scaffold), with

nearly complete depletion of EVs within 4 days. In another study, Xing et al fabricated a bioactive silk fibroin/PCL composite electrospun scaffold functionalized with ASC-EVs using a polydopamine coating approach. The findings indicated that EVs were effectively anchored to the surface of electrospun nanofibers and were released in a sustainable manner through the polydopamine-mediated immobilization strategy, with approximately 10% of the EVs remaining on the scaffold after 9 days.[42] Similarly, other researchers have utilized polydopamine to modify titanium nanotubes, as well as PCL, PLA, and other composite scaffolds.[34,36,77,159] Their findings indicate that polydopamine-modified surfaces provide a convenient, effective, and reliable carrier for the slow, sustained release of EVs.

Poly-L-lysine is a polycationic polymer composed of lysine monomers, recognized for its ability to bind to DNA, cell membranes, and negatively charged proteins.[162] Zhai et al modified titanium scaffolds with poly-L-lysine by incubating the scaffolds in a poly-L-lysine solution overnight before applying the EVs.[91] Previous studies have shown that the surface of EVs is negatively charged,[151] which facilitates their attachment to poly-L-lysine-modified titanium scaffolds through electrostatic interactions. EVs loaded onto poly-L-lysine-modified titanium scaffolds exhibited a sustained release in vitro, with about 25% of EVs retained in the scaffolds after 50 hours. In another study, Zhuang et al modified gelatin scaffolds with poly-L-lysine to enhance the attachment of EVs derived from hypoxia-pretreated BMSCs through electrostatic interactions. They demonstrated that EVs loaded onto these poly-L-lysine-coated scaffolds exhibited sustained release during the first week after implantation, with approximately 30% of EVs retained in the scaffolds after 7 days.[133]

As previously mentioned, the CP05 peptide selectively binds to CD63, a tetraspanin highly expressed on the surface of EVs. Zha et al used the CP05 peptide to modify PCL scaffolds, enhancing EV grafting efficiency.[81] First, a positively charged amine group (-NH2) was introduced to PCL scaffolds using 1,6-hexanediamine. The amino group-functionalized scaffolds were then immersed in a CP05 peptide solution to graft CP05 onto the scaffolds. Their findings demonstrated that CP05-modified PCL scaffolds exhibited significantly higher affinity for EVs compared to unmodified control scaffolds, achieving a graft efficiency of 41.7% (wt/wt). In another study, Ma et al designed a fusion peptide to link CP05 with heptaglutamate (EEEEEEE, E7), a hydroxyapatite-binding domain which enables various bone regeneration agents to target diverse hydroxyapatite-containing materials. They demonstrated that modifying EVs with these fusion peptides significantly increased the loading efficiency of EVs onto hydroxyapatite scaffolds and slowed the releasing rate of EVs from the scaffolds. Furthermore, hydroxyapatite scaffolds functionalized with BMSCs-EVs via these fusion peptides exhibited good biological activity and safety, and promoted bone regeneration in a rat calvarial bone defect model.[35]

Polyetheretherketone (PEEK) is widely utilized in bone tissue engineering due to its excellent mechanical properties, radiolucency, and chemical resistance.[163] To enable sustained release of EVs from PEEK scaffolds, Fan et al modified PEEK scaffolds with tannic acid.[20] Tannic acid is rich in polyphenol groups, which facilitates the attachment of EVs to PEEK scaffolds via reversible hydrogen bonding.[164] EVs loaded onto unmodified PEEK scaffolds exhibited a rapid burst release in vitro, with nearly 80% of EVs released within the first day, and almost complete release within three days. In contrast, EVs loaded onto tannic acid-modified PEEK scaffolds demonstrated a controlled release, with only about 20% of EVs released on the first day and sustained EV release over 14 days.

Polymer Coating Spheres

Swanson et al innovatively applied droplet microfluidics technology to encapsulate EVs within PLGA-PEG-PLGA triblock microspheres, which were subsequently physically adsorbed onto poly(l-lactic acid) (PLLA) scaffolds using a solution absorption method.[165] They discovered that EVs encapsulated in these PLGA-PEG-PLGA triblock microspheres exhibited a slow and sustained release profile, with only about 18% released within the first day and detectable for up to 10 weeks in vitro. The released EVs maintained their spherical shape, size, membrane integrity, and bioactivity. Additionally, PLLA scaffolds functionalized with EV-encapsulated microspheres significantly accelerated bone regeneration in a murine model of calvarial bone defect. Their findings demonstrated that EVs can be encapsulated within biodegradable polymer vehicles through microfluidic droplet generation and polymeric self-assembly while preserving their cargoes and biological activities. Furthermore, EVs were released from the polymeric microspheres in a controlled manner, with release kinetics tunable by adjusting the polymer composition.

Avidin-Biotin System

The avidin-biotin system is one of the strongest noncovalent biological interactions, making biotin modified surfaces an ideal platform for capturing target substances including proteins, drugs, and cells.[166] Chen et al innovatively applied this system to facilitate the self-assembly of ASC-EVs onto titanium surfaces, as illustrated in Figure 3.[38] In their study, a polypyrrole film doped with biotin was electrochemically fabricated on a titanium surface, allowing for the subsequent grafting of streptavidin. Biotin-labeled hASC-EVs were then immobilized onto the biotin-doped polypyrrole titanium surface via the avidin-biotin system. This approach increased the quantity of ASC-EVs anchored to the titanium surface by 185-fold compared to pristine

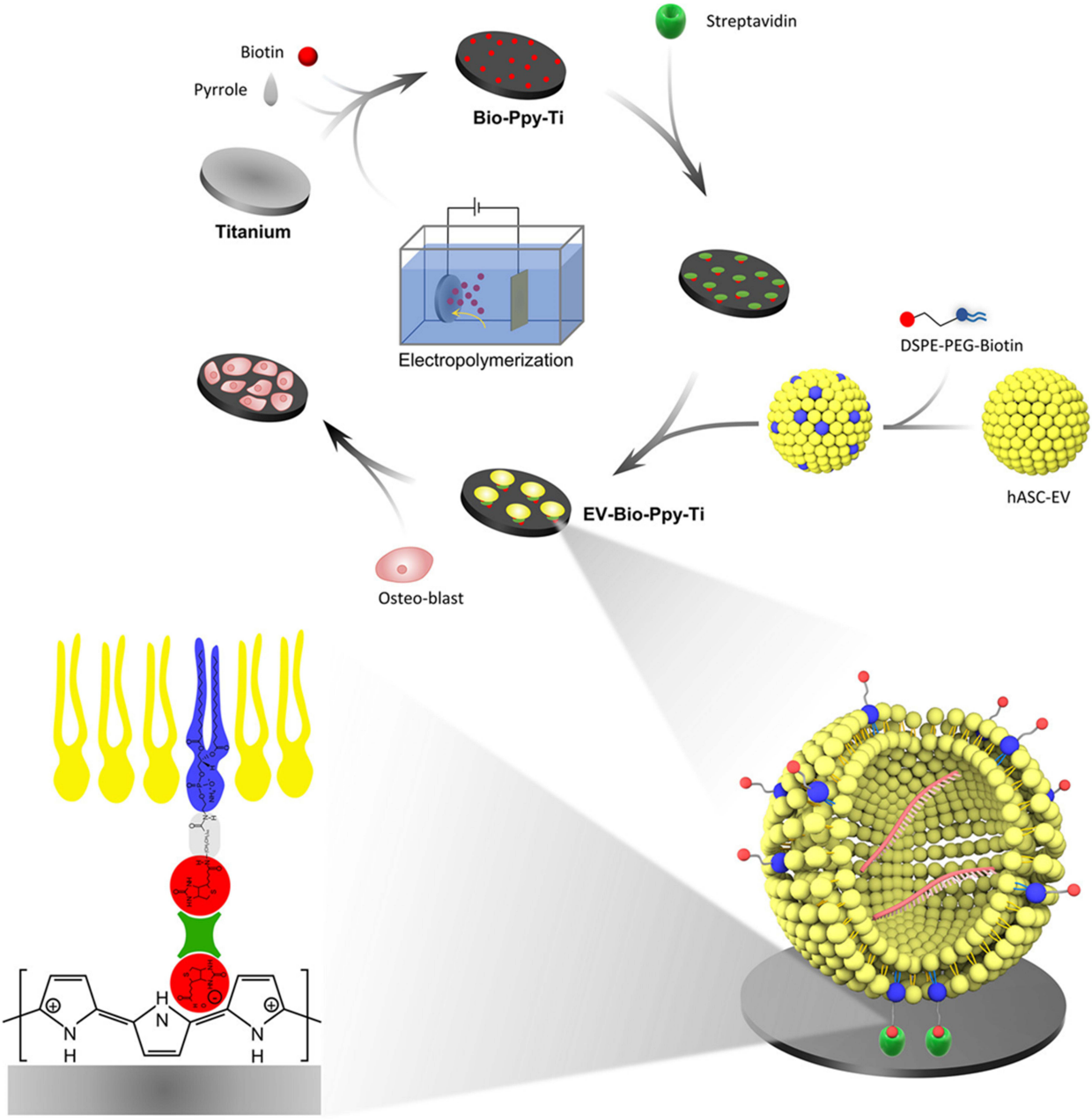

Figure 3 Schematic illustration of the procedures for fabricating self-assembled EV-functionalized biotin-doped polypyrrole titanium through the avidin-biotin system. First, a biotin-doped polypyrrole film (Bio-Ppy-Ti) is electrochemically deposited onto the titanium surface via an electrochemical potentiostatic method. Next, biotin-labeled EVs are immobilized on the surface of Bio-Ppy-Ti through the strepavidin-biotin interaction to form EV-Bio-Ppy-Ti. Finally, the osteodifferentiation of osteoblasts cultured on EV-Bio-Ppy-Ti is investigated to evaluate its osteoinductive properties. Reproduced from Chen L, Mou S, Li F, et al. Self-assembled human adipose-derived stem cell-derived extracellular vesicle-functionalized biotin-doped polypyrrole titanium with long-term stability and potential osteoinductive ability. *ACS Appl Mater Interfaces.* 2019;11(49):46183–46196. Copyright 2019 American Chemical Society.[38]

titanium after 30 seconds of ultrasonic agitation, with EVs remaining stable on the surface for 14 days at 4°C. Compared to pristine titanium, EV-functionalized biotin-doped polypyrrole titanium demonstrated enhanced cell compatibility and osteoinductivity for osteoblasts in vitro, as well as improved bone regeneration ability in an in vivo ectopic bone formation model. Zha et al also utilized the avidin-biotin system to modify biomaterials for bone tissue engineering applications.[124] They employed coaxial electrospinning to create a core-shell nanofiber film using soft chitosan and hard PLA. The chitosan in the outer layer of the nanofiber provided abundant amino groups that interacted with the carboxyl groups of biotin, enabling the chitosan/PLA nanofibers to be biotinylated for subsequent streptavidin grafting. ATDC5 cells were incubated with DSPE-PEG-biotin and processed through sequential extrusion to produce biotinylated EMs. Finally, these biotinylated EMs were tightly bound to the core-shell nanofiber film via the avidin-biotin system.

In summary, surface modification of biomaterials to enhance EV adhesion and enable sustained release represents a relatively straightforward and cost-effective strategy with considerable potential for clinical translation. Future studies should focus on identifying more biocompatible and versatile surface-modifying agents for scaffold functionalization and on developing advanced strategies to achieve more precise and controllable EV release.

EV-Loaded Hydrogels

Hydrogels are commonly used as delivery vehicles for EVs. During the cross-linking process, EVs become encapsulated within the pores of hydrogels. Upon in vivo implantation, EVs are released into the surrounding microenvironment as the hydrogel degrades.[119] Compared to other biomaterials such as ceramics, synthetic polymers, and metals, hydrogels generally exhibit a more sustained release of EVs in vivo, due to their high porosity, excellent hydrophilicity, and optimal pore size for EV entrapment. However, because of their limited mechanical strength, hydrogels are mainly used in non-load-bearing regions as bone tissue engineering scaffolds.[167] To overcome this limitation, researchers often combine hydrogels with other materials to enhance their mechanical properties, creating composite hydrogel scaffolds.[168] This section will review recent research progress on EV-loaded hydrogels in bone tissue engineering, with a focus on the development of composite hydrogels and various hydrogel modification strategies.

Hydroxyapatite, which closely resembles the physical structure of natural bone minerals, is commonly integrated into hydrogels to create composite scaffolds in bone tissue engineering.[169] Yang et al incorporated hydroxyapatite into a hyaluronic acid-alginate hydrogel to enhance its mechanical property.[46] This composite hydrogel demonstrated a slower release profile of EVs compared to the pure hyaluronic acid-alginate hydrogel. After 14 days of continuous in vitro measurement, 71.20 ± 2.64% of EVs were released from the composite hydrogel, whereas 84.81 ± 4.91% of EVs were released from the pure hydrogel. The faster EV release from the pure hydrogel was attributed to its higher swelling ratio and degradation rate. UCMSC-EVs combined with this composite hydrogel exhibited enhanced bone regeneration in a rat model of calvarial bone defect. Similarly, Chen et al used hydroxyapatite to improve the mechanical properties of hydrogels.[40] They developed a composite hydrogel composed of thiol-modified hyaluronic acid, hydroxyapatite, and thiol-modified heparin to encapsulate EVs secreted by ASCs overexpressing miRNA-375. This composite hydrogel showed a slow and controlled release of EVs in vitro, with about 95% of EVs released after 14 days. Wang et al developed a more complex composite hydrogel containing hydroxyapatite, silk fibroin, chitosan, and difunctionalized polyethylene glycol.[45] This composite hydrogel leverages the benefits of each component while mitigating their individual drawbacks, resulting in a self-healing material with superior mechanical properties and plasticity. EV release analysis showed that only 78.22 ± 0.36% of the EVs were released over 30 days in vitro. The combination of UCMSC-EVs with this composite hydrogel effectively promoted bone healing in a rat model of femoral bone defect. Besides hydroxyapatite, researchers have also incorporated DBM, β-TCP and MBG nanoparticles into hydrogels to improve the mechanical properties of the composite hydrogels, yielding promising experimental outcomes.[32,33,170]

To prolong the delivery and release of EVs from hydrogels, Wu et al developed a thermosensitive injectable hydrogel composed of chitosan and β-glycerophosphate.[60] The composite hydrogel remained in a liquid state at room temperature but transitioned into a gel upon reaching body temperature. EVs encapsulated in the thermosensitive hydrogel exhibited a controlled release profile, with 80% of EVs released by day 8 and a deceleration in the release rate thereafter. Upon injection into the bone defect site, the thermosensitive hydrogel solidified in situ at body temperature, enabling a sustained and controlled release of EVs as the hydrogel gradually degraded. Xu et al also developed a thermosensitive hydrogel using poly(N-isopropylacrylamide) (PNIPAAm) and black phosphorus. PNIPAAm hydrogels

exhibit "thermal deswelling" properties with a low critical solution temperature (LCST). When the temperature is below the LCST, the hydrogels are hydrophilic, absorbing water and swelling. When the temperature exceeds the LCST, the hydrogels become hydrophobic, releasing bound water molecules and shrinking by 80 to 90%. Black phosphorus generates localized heat upon near-infrared (NIR) light irradiation, thereby increasing the hydrogel temperature. In their study, BMSC-EVs were encapsulated within these black phosphorus-modified PNIPAAm hydrogels. The authors demonstrated that controllable NIR light irradiation could trigger the reversible cascade reaction in the hydrogels, leading to the controlled release of EVs along with water molecules. Both in vitro and in vivo studies showed that the modified hydrogels significantly enhanced the proliferation and osteogenic differentiation of BMSCs, as well as improved bone repair in a rat calvarial bone defect model.[171]

As mentioned earlier, EV membranes are rich in integrins, making the RGD peptide sequence, which is a highly conserved integrin recognition motif, a popular choice for modifying various biomaterials.[172] Huang et al developed a photocrosslinkable alginate hydrogel with RGD peptides attached to its backbone.[142] Their study demonstrated that the inclusion of RGD peptides significantly enhanced EV retention by slowing the release of EVs from the hydrogel. After seven days of in vitro monitoring, the RGD-modified hydrogel had released only about 20% of the encapsulated EVs, compared to nearly 60% from the unmodified alginate hydrogel. Furthermore, EVs released from the RGD-modified hydrogel maintained a size distribution more similar to that of the original EVs, compared to those released from the unmodified hydrogel. Importantly, the addition of RGD peptides did not negatively impact the osteogenic activity of EVs. The RGD-modified hydrogel not only extended the release duration of EVs but also facilitated host cell attachment and proliferation via the unbound RGD domains within the alginate, providing dual benefits.

DNA hydrogels, which are three-dimensional polymer networks primarily composed of DNA, offer superior biocompatibility, biodegradability, programmability, and controllable assembly and disassembly, compared to other polymer hydrogels. The inherent structural diversity, sequence specificity, and precise targeting abilities of DNA molecules allow DNA hydrogels to precisely respond to various stimuli, including pH, light, enzymes, heat, ions and small biomolecules.[173] Jing et al innovatively used DNA hydrogels as EV delivery vehicle, they designed a PEG/DNA hybrid hydrogel system capable of controlled EV release, triggered by the pathological cue metalloproteinase-9 (MMP-9) in response to the dynamic microenvironment of diabetes, as illustrated in Figure 4.[50] To create this system, single DNA strands S1 and S2 were copolymerized with an 8-arm vinyl sulfone (VS)-functionalized PEG to form PEG-DNA conjugates, designated as PS-1 and PS-2. The hydrogel gelation occurred via the formation of a three-stranded DNA structure by combining PS1, PS2, and an MMP-9 DNA aptamer-linker, with EVs being incorporated during the DNA hybridization process. Upon injection of the hydrogel into the pathological defect site, MMP-9 present in the diabetic microenvironment triggered the separation of the DNA aptamer-linker from PS-1 and PS-2, inducing the disintegration of the DNA hydrogel and the subsequent release of EVs. In vitro, the hydrogel exposed to MMP-9 for 14 days achieved a cumulative EV release of 92.2 ± 4.2%, significantly higher than the 62.3 ± 6.4% observed in PBS. Furthermore, the released DNA aptamers during hydrogel degradation acted as MMP-9 antagonists, neutralizing its harmful effects and thereby protecting the surrounding tissues. The efficacy of the EV-loaded PEG/DNA hybrid hydrogel system for bone healing was further validated in a mandibular bone defect model in diabetic rats. This study offers valuable insights into the design of bioresponsive EV-delivery hydrogel systems for on-demand, site-specific administration of EVs driven by pathology cues. In another study by Peng et al, M2 macrophage-derived EVs with immunomodulatory effects and silver nanoclusters with anti-bacterial properties were incorporated into DNA hydrogels. The results demonstrated that the composite hydrogel significantly enhanced EV retention for at least 7 days following administration in a mouse model of diabetic alveolar bone defect. The sustained release of both EVs and silver nanoclusters significantly accelerated alveolar bone healing by regulating macrophage polarization and promoting the expression of proliferative and osteogenic factors.[76]

3D bioprinting technology is an advanced biofabrication strategy that precisely arranges biologics such as living cells and ECM components, along with biocompatible materials, into a predetermined 3D hierarchical structure to create artificial multicellular tissues and organs.[174] Chen et al introduced an innovative approach by using BMSC-EVs instead of BMSCs as the primary component of bioink.[23] They incorporated EVs into a composite of decellularized cartilage ECM and gelatin methacrylate (GelMA) hydrogel. GelMA is a photosensitive material that undergoes free-radical crosslinking in the presence of photoinitiators when exposed to UV light (360–380 nm), resulting in the formation of

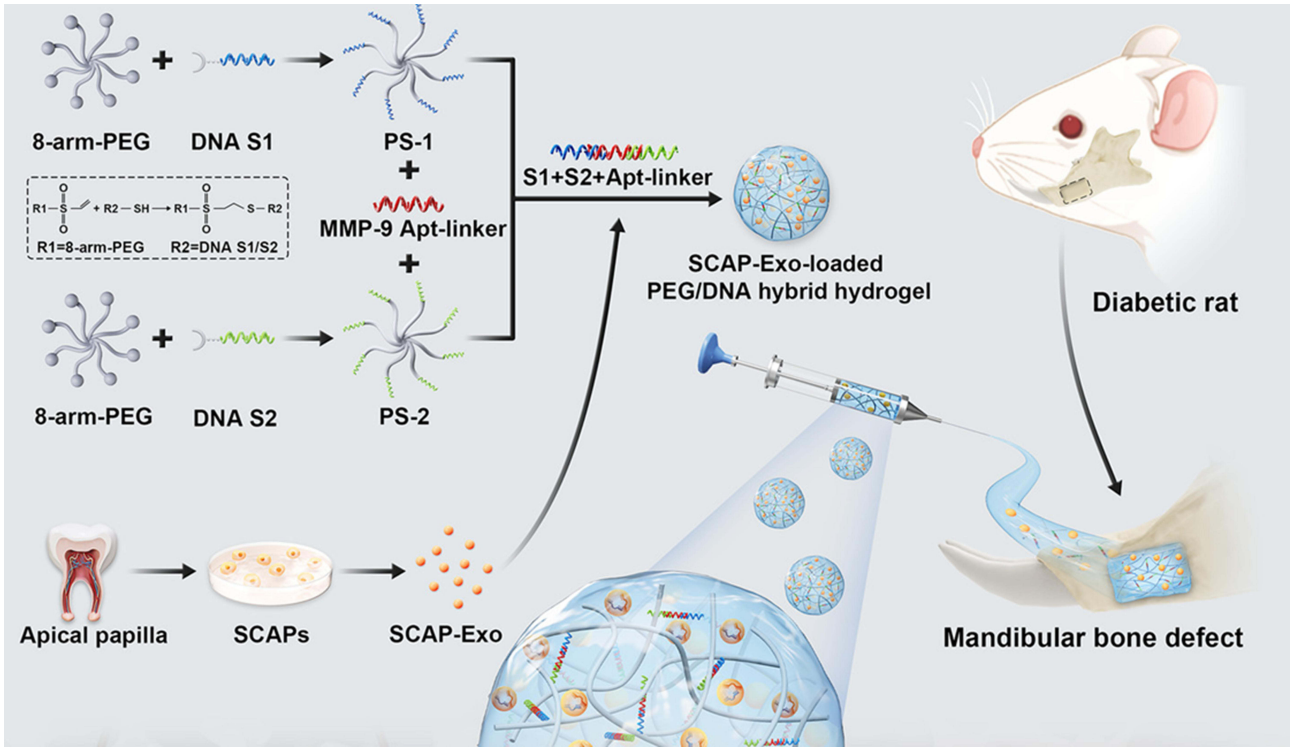

Figure 4 Schematic illustration of the synthesis process for a polyethylene glycol (PEG)/DNA hybrid hydrogel system designed for the controlled release of DMSC-EVs in the treatment of diabetic bone defects. First, single DNA strands S1 and S2 are copolymerized with 8-arm vinyl sulfone (VS)-functionalized PEG to form PEG-DNA conjugates, designated as PS-1 and PS-2. These are then mixed in equal proportions with an MMP-9 aptamer-linker solution to form a stable hydrogel structure under suitable conditions, with EVs being introduced concurrently during the DNA hybridization process. Once the hydrogel is injected into the pathological defect site, the presence of MMP-9 triggers the separation of the DNA aptamer-linker from PS-1 and PS-2, leading to the disintegration of the DNA hydrogel and subsequent release of EVs. Reproduced from Jing X, Wang S, Tang H, et al. Dynamically bioresponsive DNA hydrogel incorporated with dual-functional stem cells from apical papilla-derived exosomes promotes diabetic bone regeneration. *ACS Appl Mater Interfaces*. 2022;14(14):16082–16099. Copyright 2022 American Chemical Society.[50]

a chemically stable three-dimensional gelatin scaffold.[175] A stereolithography-based 3D printer was employed for bioprinting the ECM/GelMA/EV composite. Utilizing user-defined computer-aided-design files, the printer generated a virtual mask and directed visible light onto the bioink, rapidly crosslinking it to produce scaffolds with radially oriented 3D architectures. The resulting 3D-printed scaffold demonstrated a sustained release of EVs in vitro, retaining over 56% of the EVs within the structure by day 14. In vivo fluorescence imaging further revealed that, compared to EVs in PBS, the 3D-printed scaffold significantly enhanced EV retention for at least 7 days. When implanted in a rabbit model of osteochondral defect, the 3D-printed scaffolds effectively promoted the regeneration of cartilage and subchondral bone. This study highlights the potential of using EVs as substitutes for living cells as primary components of bioink for 3D bioprinting, demonstrating significant application potential in tissue and organ regeneration.

In summary, the excellent biocompatibility and tunable physicochemical properties of hydrogels make them an ideal platform for EV delivery. Compared to scaffolds composed of ceramics, synthetic polymers, or metals, hydrogels offer more precise control over the loading and release of EVs by enabling modulation of parameters such as porosity, swelling behavior, surface charge, and degradation rate, thereby enhancing therapeutic efficacy. In addition, certain hydrogels possess inherent capabilities to regulate cell adhesion, proliferation, and differentiation.[176] However, their intrinsically limited mechanical strength has, to some extent, restricted their broader application in bone tissue engineering. To address this limitation, considerable efforts have been made to develop various composite hydrogels that reinforce mechanical strength while preserving the desirable features of traditional hydrogels. Moreover, owing to their excellent rheological properties, hydrogels are well-suited for integration with 3D printing technologies, enabling the fabrication of scaffolds with precisely controlled architecture, porosity, and mechanical properties that effectively load EVs.

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Conclusions and Future Perspectives

Advancement in EV research has highlighted both natural and engineered EVs as promising tools in bone tissue engineering. This review summarizes the various sources of parental cells for EVs used in bone tissue engineering and explores the potential mechanisms by which EVs facilitate bone regeneration. MSCs derived from various tissues are the most frequently used parental cells for EV production in this field. EVs derived from MSCs not only enhance osteogenic differentiation but also support bone regeneration through mechanisms such as promoting angiogenesis and modulating immune responses. Nonetheless, the precise mechanisms by which EVs facilitate bone regeneration, including the key signaling molecules involved and their downstream pathways, remain unclear and require further investigation. It is postulated that EVs mediate bone regeneration through the concerted actions of their diverse internal cargoes. Nevertheless, most existing studies have primarily focused on the roles of miRNAs and proteins within EVs, suggesting that the underlying mechanisms by which EVs mediate bone regeneration are far from fully elucidated. Further research is needed to identify and characterize the various bioactive components in EVs that contribute to osteogenic differentiation, angiogenesis and modulation of inflammation and their downstream pathways.

In this review, we also discuss various endogenous and exogenous modification strategies for modifying EVs to enhance their therapeutic potential in bone tissue engineering. While these modification strategies have proven effective in both in vitro and in vivo experiments, caution is warranted regarding potential adverse effects that may arise from alterations to the membrane structure and compositional content of EVs. Additionally, we elaborate on strategies for integrating EVs with various biomaterial scaffolds. Despite the promising bone-regenerative effects observed in animal models when combining EVs with biomaterials, achieving a stable, sustained, and controlled release of EVs at the injury site remains a major challenge. Conventional approaches, such as physical adsorption of EVs onto biomaterial scaffolds, do not effectively ensure a controlled and sustained release of EVs. Previous research has focused on modifying biomaterial scaffolds with various polymers to prolong the release duration of EVs. Future studies should aim to develop more effective loading strategies that ensure long-term preservation, bioactivity protection, and sustained release of EVs at bone defect sites.

Furthermore, there still are some critical technical issues that need to be addressed before large-scale clinical applications of EVs in tissue engineering. First, isolation of EVs is an essential factor that can significantly affect EVs. Conventional EV isolation methods primarily exploit size-based differences to separate EVs from cellular debris and protein aggregates. Widely used techniques include differential centrifugation, density gradient centrifugation, size-exclusion chromatography, polymer-based precipitation, and immunomagnetic separation.[177] However, they are generally low-throughput and lack the specificity required for the selective isolation of defined EV subpopulations. In recent years, emerging technologies have enabled more refined separation of EV subpopulations. These include flow cytometry, microfluidic platforms, and field-flow fractionation. These advances offer improved purity and resolution but remain constrained by limited sample throughput.[121] Beyond improving purity and specificity, increasing EV yield per unit time appears to be a more pressing challenge for clinical translation. The industrial adaptation of traditional techniques-such as the development of large-scale ultracentrifugation systems, the design of more user-friendly commercial EV extraction kits, and the isolation of EMs via cell extrusion through sequentially smaller pore-size membrane filters-represents a promising direction for the scalable production of therapeutic EVs. Progress in this area will require interdisciplinary collaboration among experts in biology, materials science, and engineering to drive forward the clinical application of EV-based therapies. Apart from the isolation process, the processes involved in EV purification, preparation, preservation, quantification, and characterization remain unstandardized, contributing to significant batch-to-batch heterogeneity and impeding direct comparison among studies. Moreover, potential microbial contamination during isolation, along with the inclusion of exogenous serum components and abnormal vesicles, may result in serious consequences. To address these issues, the establishment of rigorous and standardized criteria for purity, as well as robust technical specifications and quality control systems, is essential to ensure the reproducibility and reliability of future investigations. In this context, further exploration into Good Manufacturing Practice (GMP)-compliant manufacturing processes is urgently required to enable the stable and scalable production of homogeneous EVs, which remains a critical barrier to their clinical translation. Lastly, but equally important, the safety of EVs and EV-biomaterial composites in vivo remains

a critical concern. While EVs are generally considered to offer superior biocompatibility and enhanced biosafety compared to conventional stem cell therapies, the full composition of EVs, the interactions between their various components, as well as the key regulatory molecules and downstream signaling pathways, are not yet fully understood. This lack of comprehensive understanding could lead to unpredictable or undesirable biological effects. Furthermore, it is crucial to determine whether different EV modification strategies, or modifications to the parent cells, could potentially cause adverse effects in vivo that have previously been overlooked. Additionally, uncertainties regarding the biodistribution and bioelimination of EVs in the bloodstream and in various pathological microenvironments, along with the uncertain optimal therapeutic dose of EVs, may present significant risks. Therefore, comprehensive animal studies and preclinical research are necessary before EVs can be safely and effectively translated into clinical practice.

Despite the considerable challenges that impede the large-scale clinical translation of EVs, several clinical trials (eg NCT05520125, NCT04849429, and NCT04281901) are either ongoing or have been completed to evaluate the safety and therapeutic efficacy of EV-based interventions for bone-related diseases. These ongoing efforts highlight the growing recognition of EVs as a promising candidate capable of modulating complex regenerative processes. As our understanding of EV biology and their underlying mechanisms continues to advance, and with ongoing progress in bioengineering, materials science, and nanotechnology, EVs are anticipated to play an increasingly transformative role in bone tissue engineering. In particular, the development of engineered EVs with enhanced targeting capacity, sustained release kinetics, and tailored bioactivity, in combination with advanced functional biomaterials, may provide unprecedented opportunities to overcome current therapeutic limitations and accelerate the clinical translation of next-generation bone regenerative strategies.

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Disclosure

The authors declare that they have no competing interests.

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