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*Review*

# Beyond Traditional Medicine: EVs-Loaded Hydrogels as a Game Changer in Disease Therapeutics

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**Abstract:** Extracellular vesicles (EVs), especially exosomes, have shown great therapeutic potential in the treatment of diseases, as they can target cells or tissues. However, the therapeutic effect of EVs is limited due to the susceptibility of EVs to immune system clearance during transport in vivo. Hydrogels have become an ideal delivery platform for EVs due to their good biocompatibility and porous structure. This article reviews the preparation and application of EVs-loaded hydrogels as a cell-free therapy strategy in the treatment of diseases. The article also discusses the challenges and future outlook of EVs-loaded hydrogels.

**Keywords:** extracellular vesicles; hydrogel; cell-free therapy

## 1. Introduction

Traditional cell therapies like stem cell transplantation hold tremendous potential for treating diseases such as myocardial infarction [1,2]. However, these cell therapies may encounter various limitations, including low stability in the storage and transportation of living cells, potential risks of tumorigenicity and immunogenicity, and high cost of treatment [3–6]. To overcome these limitations, cell-free therapy (CFT) provides an alternate exciting approach [7–11] in which bioactive molecules such as protein, mRNA, and miRNA can perform the primary functions of cells [12,13].

The bioactive molecules for CFT can be extracted from cell culture media [14,15]. During the cell cultivation process, cells release extracellular vesicles (EVs) into the surrounding environment. EVs are sub-micron-sized particles with a phospholipid membrane and contain molecules from cells, including proteins, nucleic acids, and soluble small molecules [16,17]. EVs play a crucial role in CFT [18]. The three main types of EVs are exosomes (40–160 nm), microvesicles (100–1000 nm), and apoptotic bodies (50–5000 nm) [19,20]. Researchers have discovered that mesenchymal stem cells (MSCs) secrete a form of EVs containing miRNA associated with tumors [21]. These EVs can influence the behavior of tumor cells [22] and have potential applications in cancer treatment [23].

However, EVs are subject to rapid clearance, which presents an obstacle for the application of the therapeutic EVs in CFT. For example, EVs isolated from melanoma cells (B16BL6) are rapidly cleared within approximately 2 min after intravenous injection in mice [24]. During the transportation process in the bloodstream, EVs are easily cleared by macrophages [25] and tend to accumulate in the liver, spleen, and lungs, rather than at the lesion sites [26]. To solve this problem, researchers have used hydrogels to protect EVs from the clearance of immune cells [25], successfully deliver EVs and persistently keep them at lesion sites [27]. EVs-loaded hydrogel offers several advantages in disease treatment, including a high loading rate of EVs, sustained release of EVs, and cryopreservation stability [28].

In this work, we review the production and applications of EVs-loaded hydrogels in disease therapy. We will describe the detailed process of synthesizing EVs-loaded hydrogels, the critical biomolecules that contribute to their therapeutic efficacy, and their

specific applications in treating various diseases, such as myocardial infarction, intervertebral disc degeneration, osteoarthritis, bone deficiency, diabetic ulcers, and nerve injury. Additionally, we critically assess the significance and potential limitations of using these hydrogels in disease therapy, offering an insightful evaluation of their role in advancing medical treatments.

## 2. Synthesis of EVs-Based Hydrogels

The synthesis of EVs-based hydrogels is shown in Figure 1. First, EVs are isolated, typically by ultracentrifugation from cell culture medium supernatants. Then, the isolated EVs are assembled into injectable hydrogels or hydrogel patches by in situ polymerization or adsorption.
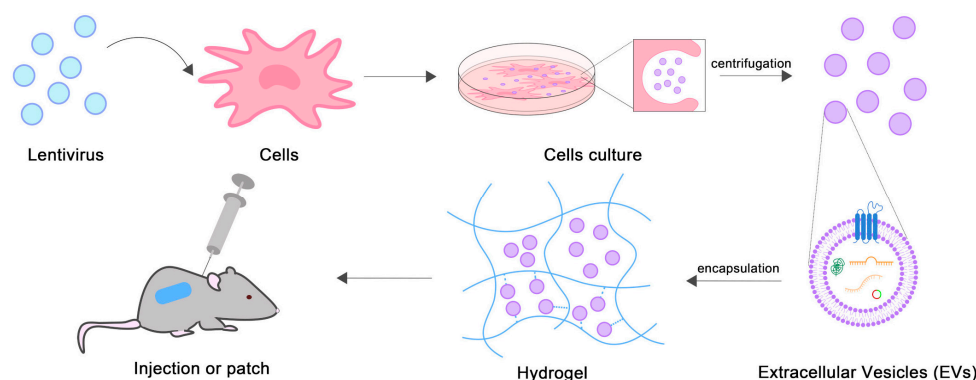


**Figure 1.** Schematic diagram of EVs-loaded hydrogels synthesis.

### 2.1. Acquisition of EVs

EVs can be classified into native EVs, engineered cell EVs, and post-modified EVs based on the source of their components [29]. There are three major pre-treatment methods for the acquisition of therapeutic EVs. The most primitive method is the isolation of EVs directly from the source cells without any means of intervention [30]. For example, MSCs are able to differentiate into a variety of cell types, including bone, cartilage, fat, etc. [1,31,32]. The therapeutic effect of EVs isolated from MSCs is similar to that from MSCs transplantation [30]. Xia et al. [33] isolated EVs derived from umbilical cord mesenchymal stem cells (UCMSCs) for bone repair. Sun et al. [34] isolated EVs from bone marrow mesenchymal stem cells (BMSCs) to promote epiphyseal plate damage restoration.

Another common approach is to induce parental cells by hypoxia to obtain engineered cell EVs enriched with specific biomolecules [35]. Researchers have shown that the gene regulation patterns of cells are significantly different under hypoxia induction [36], and there are hypoxia activation and repression genes [37–39]. Bai et al. [40] used hypoxia-induced BMSCs to isolate EVs for the treatment of myocardial infarction. Zuo et al. [41] isolated EVs from hypoxia-induced BMSCs for cartilage regeneration. The isolation of EVs after lentiviral transfection of cells is also a method of interest to researchers [42]. Zhao et al. [43] incubated BMSCs with retroviruses, and the resulting EVs overexpressed miR-29b-3p, reducing scarring during wound healing. Li et al. [44] transfected UCMSCs with pCDH virus, up-regulated the expression of 5′-nucleotidase (CD73) in EVs, and attenuated inflammation after spinal injury.

In addition, the unique protein- and carbohydrate-decorated surface of EVs makes EVs a promising natural nanocarrier [45,46]. Exogenous RNA, proteins, and small molecules can be packaged in EVs by methods such as co-incubation, electroporation, freeze-thawing, ultrasound, extrusion, etc. [47–49]. As a nanocarrier, EVs can protect the molecules of interest from immune clearance, facilitate the molecules to cross biological barriers, and enhance the binding specificity of the molecules to the target tissues [50].

### 2.2. Package of EVs on Hydrogels

EVs can be loaded into hydrogels through in situ polymerization or adsorption. The interactions between the polymer backbone of hydrogels and EVs include van der Waals forces, hydrogen bonding, and electrostatic interaction, which enhance the stability of the EVs in the hydrogel and thus prolong the residence time of the EVs in vivo [51]. The in situ polymerization method involves the co-mixing of a gel precursor solution and an EVs solution. The polymerization conditions must be mild, e.g., 365 nm UV irradiation for 1 min [52], and polymerization at room temperature [53] or 37 °C [54]. Alternatively, hydrogels can also be prepared first, and then mixed with the EVs solution to allow the adsorption of EVs into hydrogels [55]. The most often used hydrogel materials for EVs delivery include calcium alginate (Ca-Alg) [53,56], arginine-glycine-aspartate (RGD) [57–59], hyaluronic acid (HA) [55,60–62], chitosan (CS) [54,63–66], and gelatin methacrylate (GelMA) [34,52,67,68]. The synthesis of the hydrogel has been well documented in many reviews [69–79], and is not described in the current work.

Compared with the interaction between EVs and the polymer backbone of hydrogels in the common physical encapsulation, the integrin present on the surface of the EVs' membrane [80] has much stronger binding affinity with RGD peptides [81], which can increase the stability of EVs in the hydrogels presenting RGD in the polymer backbone. As a result, the functional lifetime of the EVs can be increased [59]. In addition, the dynamic hydrogen bonding between the DA of the dopamine-grafted gelatin (GelDA) gel and the surface amines of EVs can promote the attachment of EVs to the hydrogel network, leading to the development of heat-sensitive or ROS-sensitive composite gels for precise EVs release in therapy [82].

### 3. Bioactive Molecules in EVs Used for Disease Treatment

### 3.1. Protein

In EVs, most proteins are secreted by parent cells in a soluble form and embedded in the surface phospholipid bilayer [83], and applied to intercellular communication [84]. The lack of relevant proteins may lead to unregulated intercellular signal transduction or obstacles in transportation, thereby triggering numerous diseases. Treatment of related diseases often requires restoring protein function [85]. Biological functional enzymes or therapeutic proteins are susceptible to protein degradation and poor cellular uptake during transport, and EVs are ideal delivery vehicles for proteins [86].

### 3.2. mRNA

The safety and conversion efficiency of mRNA through chemical modification in vitro synthesis were demonstrated in the widespread use of mRNA as a vaccine in the 2019 Novel Coronavirus Disease (COVID-19) pandemic [87,88]. This successful case has sparked widespread interest in using mRNA in both the application of vaccines and other medical needs [89,90]. mRNA can be encapsulated into isolated EVs and then endocytosed into recipient cells to express targeted proteins [91].

### 3.3. miRNA

A key active ingredient in achieving therapeutic effects in EVs is miRNA [92]. miRNA is a 19–24 nucleotide long non-coding RNA that regulates gene expression by targeting the 3′-untranslated region (3′-UTR) of mRNA [93]. Various distinct cellular expression processes can be regulated by a single type of miRNA [94]. In different forms of EVs secreted by various cells, or even one type of cells, there may be different carried miRNAs, exerting distinct regulatory effects. Compared to drugs that act on a single gene, miRNA possesses a stronger biomolecule capability to selectively regulate multiple genes [95]. Importantly, researchers have found that, to enhance its therapeutic effects, it is possible to engineer the enrichment or design modifications of miRNAs contained in EVs through interventions on parent cells such as lentiviral transduction [29,43,96].

### 3.4. circRNA

In some situations, miRNAs may be degraded by RNA exonucleases. In contrast, circular RNA (circRNA), a covalently closed, single-stranded, circular RNA [97], exhibits excellent environmental resistance and stability [98,99]. In the absence of coding for proteins, circRNA regulates the behaviors of cells by modulating the expression of miRNAs or downstream proteins [100]. CircRNA has been shown to play an important role in a variety of cancers [101]. CircRNAs are predominantly located in the nucleus and can be packaged in EVs for transport in circulation [102].

### 3.5. Antagomir

Antagomirs, sometimes referred to as anti-miRNA [103], are microRNA inhibitors with a chemical modification of homologous miRNA. The modification makes antagomirs more resistant to degradation and more effective in inhibiting miRNA expression [104,105]. The difference between antagomir and normal RNA is the complete 2′-O-methylation of three parts, namely the ribose part, the thiosulfate bond part, and the 3′-end cholesterol part [105–107]. Antagomir has shown cutting-edge promise in treating fluorescent tumors, as it does not act on tumor cells in a cell-by-cell toxic manner, but rather prevents them from initiating metastasis [108].

## 4. The Application of EVs-Loaded Hydrogels in Disease Treatment

### 4.1. Myocadial Infarction

The non-proliferative nature of adult cardiomyocytes leads to the fibrosis of damaged myocardium [109]. Researchers have pointed out that EVs secreted by mesenchymal stem cells (MSCs) [110], cardiac progenitor cells (CPCs) [111], and induced pluripotent stem cells (iPSCs) [112,113] carry important molecules in heart-specific processes, which can promote angiogenesis, inhibit apoptosis and fibrosis, reduce cardiac ischemic injury [114,115], and protect the cardiac function of ischemic heart disease [116] while avoiding the tumorigenicity and high tendency of arrhythmia brought about by stem cell therapy or cardiomyocyte therapy.

Gordana et al. [117] assembled EVs secreted by induced cardiomyocytes (iCMs) into the hydrogel patches to form cardiac patches, and this process was achieved by the in situ polymerization of collagen within a gelfoam mesh at 37 °C. The patches can continuously release iCM-EVs for up to 21 days in vitro and rat myocardial infarction models, and iCM-EVs are enriched with numerous heart-specific miRNAs, including miR-1 and miR133a, which can inhibit the rational hypertrophy of heart disease and reduce the size of myocardial infarction (Figure 2).

Ji et al. [57] encapsulated RGD hydrogels enriched in hypoxia-inducible factor-1α (HIF-1α)-overexpressing UCMSCs-derived engineered EVs (HIF-1α-EVs). The hydrogels inhibited the elevation of interleukin-6 (IL-6) and connexin 43 (conx43) in the region of myocardial infarction, which accelerated the recovery of cardiac function, reduced infarct size, and inhibited cardiomyocyte apoptosis.

Mehdi et al. [53] loaded two synthetic miRNA mimics, miR-126 and miR-146a, into the EVs secreted by adipose-derived mesenchymal stem cells (ADSCs) and assembled the EVs into injectable Ca-Alg hydrogels by in situ polymerization. miR-126 regulates the PI3K/AKT signaling pathway by upregulating vascular endothelial growth factor receptor 2 (VEGFR2), Protein kinase B (AKT), and the mechanistic target of rapamycin (mTOR), and inhibiting the expression of Sprouty-related EVH1 domain containing 1 (SPRED-1), thereby promoting cell migration and proliferation. miR-146a plays an anti-inflammatory role by inhibiting the expression of Interleukin-1 receptor-associated kinases (Irak-1) and tumor necrosis factor receptor-associated factor 6 (Traf6) genes, reducing pro-inflammatory cytokines. The composite hydrogel has been shown to reduce the size of myocardial infarction and fibrosis and promote angiogenesis in cardiac tissue.
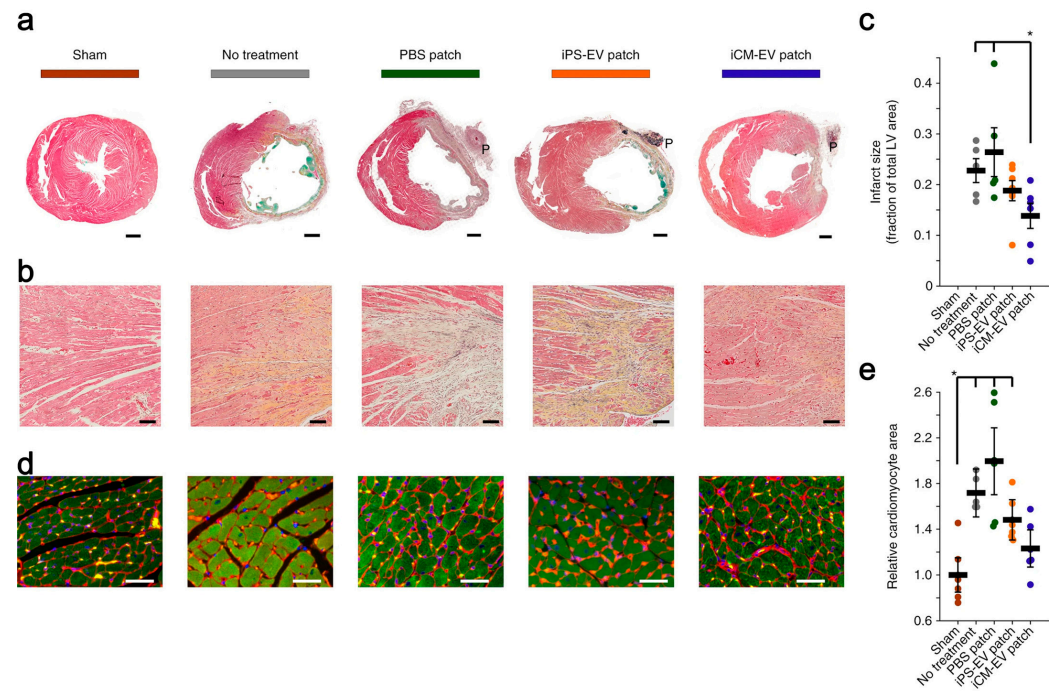
**Figure 2.** iCM-EVs-collagen gelfoam patches reduced infarct size and pathological hypertrophy. (**a**) Transverse cardiac sections after treatment with patches. Scale bars: 1 mm; (**b**) high-power images of the infarct border zone. Scale bars: 100 μm; (**c**) infarct size as a percentage of the total area of the left ventricle (LV). * $p < 0.05$ by two-tailed *t*-test; (**d**) sections were stained with wheat-germ agglutinin (red), troponin (green), and DAPI (blue). Scale bars: 50 μm; (**e**) relative cardiomyocyte area quantified. * $p < 0.05$ by two-tailed *t*-test. [117].

### 4.2. Intervertebral Disc Degeneration

Intervertebral disc degeneration (IDD) is usually caused by the aging of intervertebral disc nucleus pulposus stem cells (NPSCs) [118]. In degenerated tissues, there is dysfunction in the surviving NPSCs [119], so it is difficult to achieve satisfactory therapeutic results by intervening in cell death or using stem cell therapy alone. Mesenchymal stem cell (MSCs)-based tissue engineering has the potential to treat cartilage defects [120], and MSCs secrete EVs rich in miRNAs associated with cartilage regeneration, demonstrating great therapeutic potentials due to their high stability, readily available, and abundant sources [121].

Shao et al. [58] isolated miR-3594-5p-enriched EVs from the medium of BMSCs, and combined them with RGD complex decellularized nucleus pulposus hydrogel (RGD-DNP) through the integrin expressed on the EVs membrane. The gel exhibited excellent cell-integration ability, and this study demonstrated for the first time that miR-3954-5p can effectively slow down cellular aging by targeting the 3′-UTR of the homeodomain-interacting protein kinase 2 (HIPK2) mRNA to reduce its expression.

Ye et al. [55] demonstrated, for the first time, the role of M2c-type macrophages (M2c) in the immune regulation of IDD tissues. After the lyophilization of the HA hydrogel, the EVs enriched with miR-124-3p released by M2c were loaded into a hydrogel through adsorption. The gel was implanted into the degenerative site of the tail vertebral disc in rats, and EVs were continuously released in vivo for 28 days. miR-124-3p down-regulated cartilage intermediate layer protein 2 (CILP-2) in NPCs, indirectly promoted the expression of extracellular matrix (ECM) protein (collagen type II (ColII) and aggrecan), and inhibited the expression of metalloproteinases (matrix metalloproteinase 13 (MMP13) and ADAM metallopeptidase with thrombospondin type 1 motif 5 (ADAMTS5)), while enhancing the conduction of the TGF-β pathway. The hydrogel improved the metabolism of the NPCs matrix, which was conducive to the long-term treatment of IDD.

In addition, more and more research has shown that abnormal intracellular reactive oxygen species (ROS) levels are associated with NPSCs aging and are also a significant cause of IDD [122–124]. IDD can be slowed down by controlling the source ROS levels. Wang et al. [82] isolated the EVs with high expression of glutaredoxin3 (GLRX3) by treating BMSCs with hypoxia, and developed an injectable dopamine-grafted gelatin (GelDA) and aldehyde-functionalized chondroitin sulfate (ACS) composite hydrogel (GDC) for the delivery of EVs. GLRX3 inhibited the expression of P16INK4a, P21Cip1, and various cytosenescence-related factors including MMP13, interleukin-1β (IL-1β), and IL-6. The hydrogel has been shown to slow down mitochondrial damage in the rat IDD model to alleviate the aging of NPSCs, thereby slowing down IDD (Figure 3).
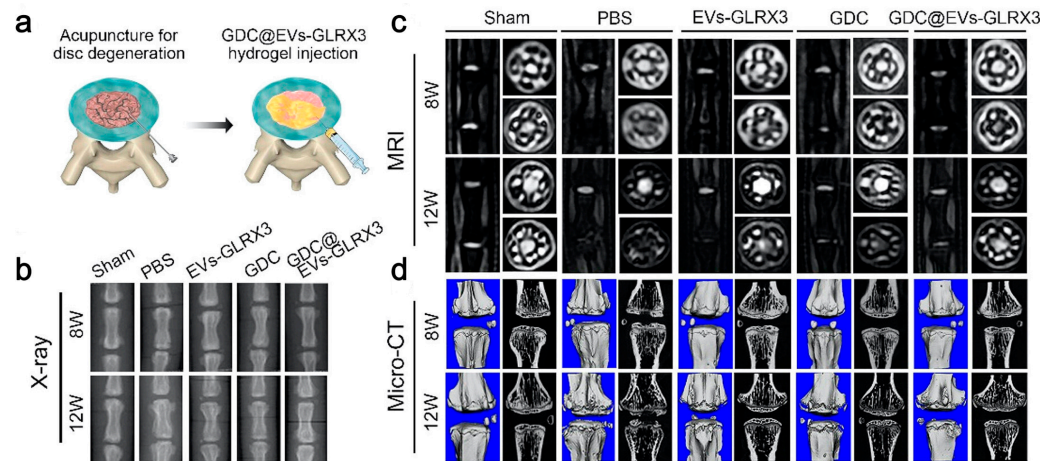


**Figure 3.** GLRX3-EVs-GDC slowed down disc degeneration. (**a**) The hydrogel was transplanted into the degenerative intervertebral disc; (**b**–**d**) representative images of X-ray, MRI, and Micro-CT of the discs at postoperative weeks 8 and 12 [82].

### 4.3. Osteoarthritis

Osteoarthritis (OA) is caused by changes in the composition or structure of any component of the joint, including cartilage fissures, chondrocyte apoptosis, etc. [125]. In addition, the articular cartilage cannot repair itself, and once damaged, it will continue to deteriorate [126]. OA is the leading cause of disability worldwide [127,128]. The role and mechanism of articular chondrocytes (ACs) and BMSCs in cartilage repair have been extensively studied [129–131], and their mechanisms of action typically involve EVs.

Cui et al. [61] isolated EVs secreted by subcutaneous adipose tissue-derived stem cells (ScASCs), and prepared injectable composite hydrogels of hydroxyacrylate polyethylene glycol diacrylate (HB-PEGDA) and mercaptoylated hyaluronic acid (SH-HA) (HB-PEGDA/SH-HA) in droplet-based microfluidic devices as an effective sustained-release carrier for EVs. miR-99a-3p is overexpressed in the EVs to inhibit the expression of ADAMTS4 and promote ECM repair. The composite hydrogel can be used for long-term treatment of OA.

Sun et al. [62] obtained EVs enriched with engineered miR-445 from transforming growth factor β3 (TGFβ3) pretreated BMSCs. The EVs were loaded into the gelatin–fibrinogen–HA–glycerol composite hydrogels by in situ polymerization, which were injected into rat knee joints. miR-445 regulates chondrogenesis and treats OA by targeting the SOX11/FOXO signaling pathway, reducing the expression of SRY-related HMG-box transcription factor 11 (SOX11), further enhancing the transcription of Forkhead box protein O1 (FOXO1), and upregulating the expression of SRY-related HMG-box transcription factor 9 (SOX9).

Sleep is beneficial for cartilage repair [132], and circRNAs are involved in the pathogenesis of OA [133]. Guo et al. [134] successfully isolated circRNA3503-loaded EVs using melatonin (MT)-induced synovium mesenchymal stem cells (SMSCs). In this work,

poly(D_L-lactide)-b-poly(ethylene glycol)-b-poly(D_L-lactide) (PDLLA-PEG-PDLLA) tri-block copolymer gel (PLEL) was synthesized by in situ polymerization and used as the EV carrier for the first time. By inhibiting the expression of hsa-miR-181c-3p and hsa-let-7b-3p, circRNA3503 indirectly promotes the expression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and SOX9, thereby promoting chondrocyte renewal and treating OA (Figure 4).
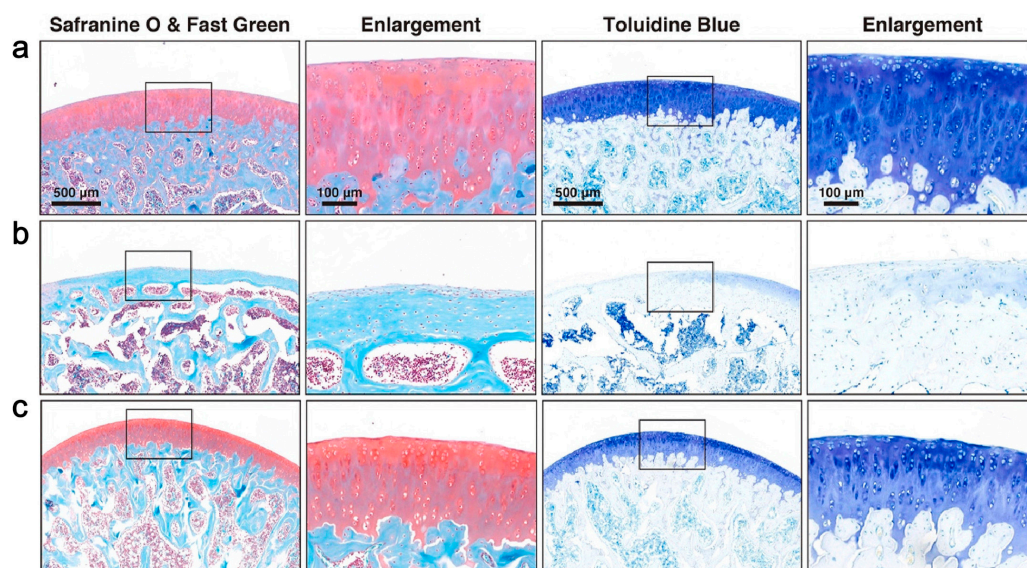


**Figure 4.** CircRNA3505-EVs-PLEL promoted the prevention of osteoarthritis. Histologic analysis with safranin O & fast green and toluidine blue staining for different groups: (**a**) normal; (**b**) osteoarthritis; (**c**) CircRNA3505-EVs-PLEL-treated (scale bar: 500 μm for low magnification and 100 μm for enlargement) [134].

*4.4. Bone Deficiency*

Bones could self-repair and regenerate after damage, and scar-free healing is achieved through the synergistic action of stem cells, progenitor cells, macrophages, etc. [135,136]. Despite this, the nonunion of fractures remains numerous and requires therapeutic interventions to promote bone repair and regeneration [137–139]. However, some growth factors such as recombinant human bone-forming proteins (rhBMPs) and platelet-derived growth factors (PDGFs) have been disappointing in their clinical and preclinical efficacy [140]. A growing body of research suggests that BMSCs play a crucial role in bone remodeling by secreting EVs [141,142].

Liu et al. [143] found that the EVs secreted by hypoxic pretreated BMSCs contained a large number of biglycans (Bgn). Liu and coworkers developed an injectable hydrogel composed of polyethylene glycol/polypeptide (PEG/PP) copolymer and mixed with EVs. The hydrogel could continuously release EVs for up to 3 weeks in a rat skull defect model. Bgn upregulates a variety of osteogenic properties-related genes including bone morphogenetic protein-2 (Bmp2), alkaline phosphatase (Alp), osteocalcin (Opn), Osteocalcin (Ocn), etc. Bgn also activates the PI3K/AKT signaling pathway and significantly promotes osteoblast differentiation.

Lee et al. [144] reported a cellular nanoelectroporation technique for the delivery of plasmids of Bmp2 and vascular endothelial growth factor A (VEGF-A) to human adipose-derived mesenchymal stem cells (hAdMSCs) and a large amount of mRNA was loaded in EVs secreted by plasmid-transfected hAdMSCs. By in situ polymerization, an EVs-loaded PEGylated poly (glycerol sebacate) acrylate (PEGS-A) injectable hydrogel was synthesized and the EVs were delivered locally in a controlled manner. In a rat model of a femoral defect, mRNA was efficiently expressed to achieve efficient angiogenesis and bone regeneration with less accumulation in other organs (Figure 5).
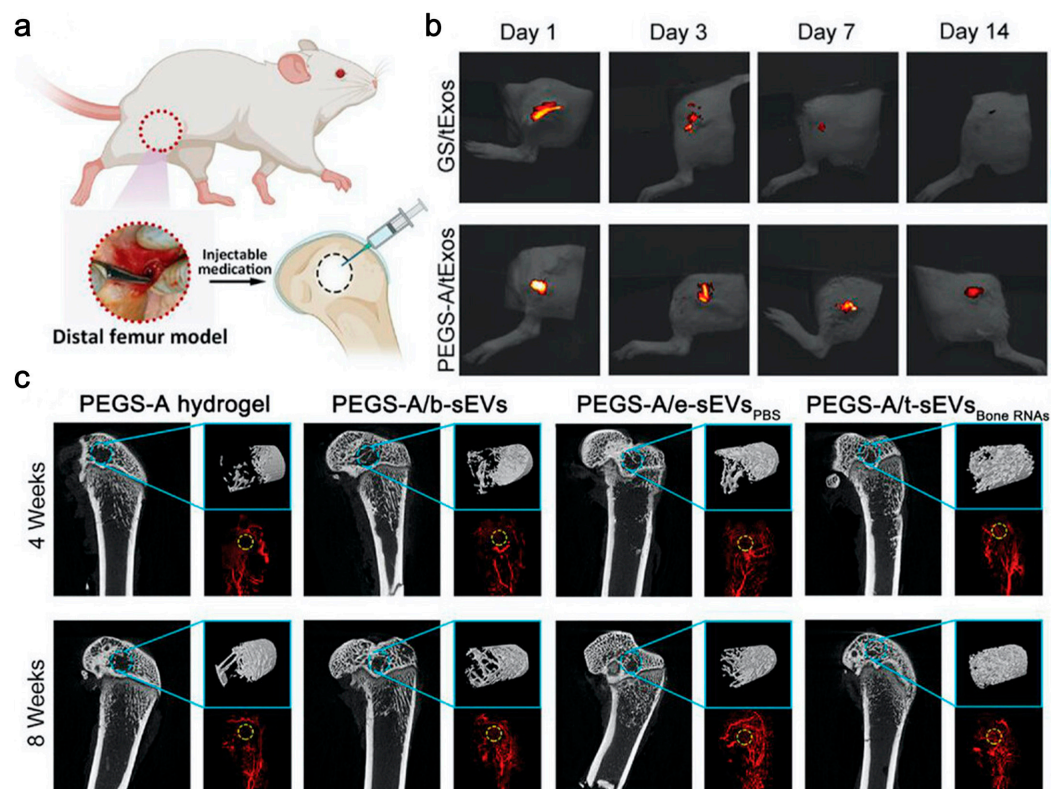
**Figure 5.** Bone RNAs-EVs-PEGSA accelerated bone regeneration and angiogenesis. (**a**) Injection treatment in rats with a critical-size femoral defect; (**b**) Fluorescence imaging of PKH26-labeled EVs delivered by gelatin sponge (GS) and PEGS-A in vivo; (**c**) Micro-CT images of new bone and vessel formation at weeks 4 and 8 [144].

Based on the clinical evidence of traumatic brain injury (TBI) and accelerated bone healing, Bai et al. [145] proposed that EVs released by the damaged neurons were rich in miR-328a-3p and miR-150-5p, associated with bone formation, directly targeting the 3′UTR of forkhead box protein O4 (FOXO4) or calcineurin B-like protein (CBL) to promote bone formation. The skull defects were almost completely repaired after 3 months of injection of methacrylated glycol chitosan (MeGC) hydrogel carrying these EVs into a rat model of skull defects.

*4.5. Diabetic Ulcer*

About 20% of patients with diabetes have diabetic ulcers (DU) [146], the most common chronic wounds worldwide [147–149]. Incurable ulcers can seriously affect the patient's quality of life, causing significant physical and psychological suffering. This refractory wound is caused by a range of therapeutic mechanisms, such as recurrent infections, delayed angiogenesis, impaired leukocyte function, and obstructed migration of keratinocytes, fibroblasts, and endothelial progenitor cells [150,151].

Zhou et al. [67] loaded EVs from BMSCs into a dopamine-modified GelMA hydrogel with tissue adhesion by in situ polymerization. The hydrogel increased the expression of IL-6, the cluster of differentiation 31 (CD31), and TGF-β in a diabetic rat skin wound model, significantly accelerating the wound closure rate and promoting healing.

Cui et al. [52] pretreated ADSCs with hypoxia, and separated and embedded their EVs on GelMA hydrogels by in situ polymerization. The EVs were rich in circ-Snhg11, which played a key role in wound healing, and by downregulating the expression of miR-144-3p, enhanced the expression of downstream nuclear factor erythroid 2-like 2 (NFE2L2) and HIF1α, enhancing the migration, proliferation, and revascularization of vascular endothelial cells (ECs) for the treatment of diabetic wounds.

Based on the pathological features of abnormal hyperplasia of vascular endothelial cells in infantile hemangioma (IH) [152], Sha et al. [54] isolated CD133-positive Hemangioma stem cells (HemSCs) from IH and obtained the EVs from culture supernatants. They modified CS with hyaluronic oligosaccharides (oHA) to synthesize a thermosensitive hydrogel that served as a carrier for the EVs. In the EVs, the miR-7 family is highly expressed to promote angiogenesis, and miR-21 as well as miR-221 are highly expressed to promote endothelial cell proliferation. As a result, the composite hydrogel promoted wound healing (Figure 6).
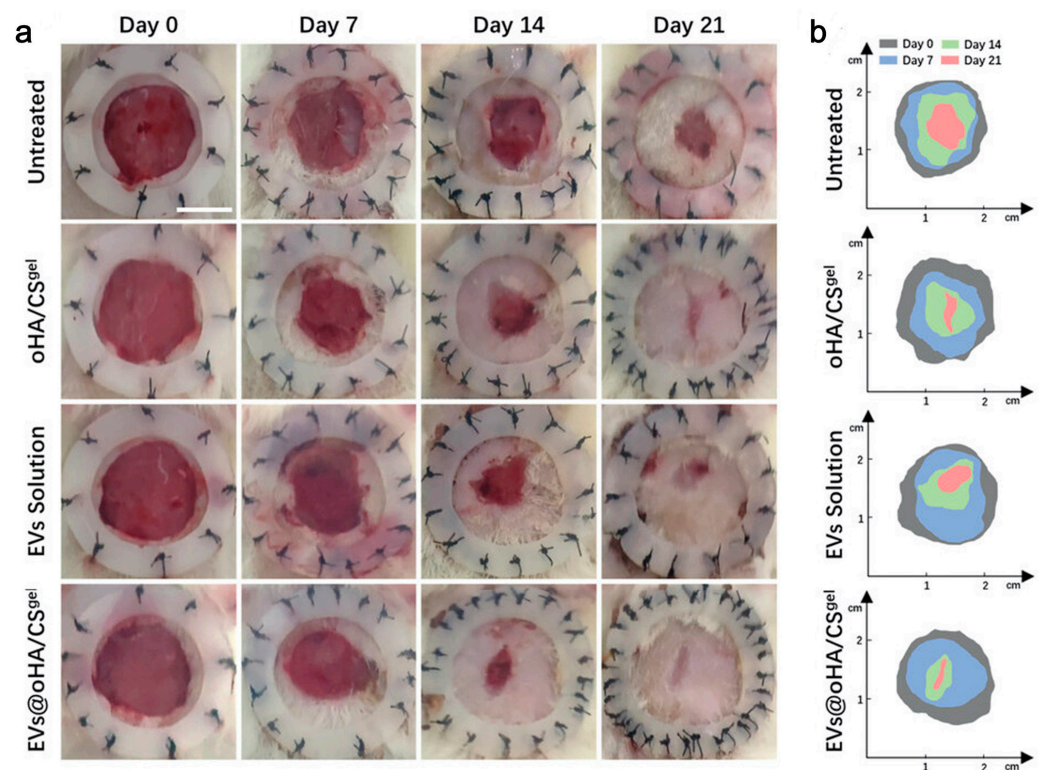


**Figure 6.** miR-7/21/221-EVs-oHA/CS accelerated wound healing of diabetic wounds. (**a**) Wound images on days 0, 7, 14, 21 after the first treatment; (**b**) schematic diagram of wound healing [54].

*4.6. Nerve Injury*

Peripheral nerve injury (PNI), a disruption of bioelectrical communication between the spinal cord and the innervated body, can lead to chronic pain, muscle wasting, disability, and paralysis [153–155] and is a significant clinical challenge. Autologous nerve transplantation is currently a common strategy for the repair of PNI, but there are still shortcomings such as defects in donor tissue and cumbersome surgery [156]. Spinal cord injury (SCI) within the central nervous system is also one of the most devastating neurological diseases, with an estimated 180,000 new cases of SCI occurring each year worldwide [157] leading to long-term disability and complications such as neuroinflammation and oxidative damage [158,159]. Effective SCI remediation methods are still a huge challenge. Methods that utilize natural or synthetic materials as implantable nerve catheters or nerve scaffolds are promising in promoting nerve cell growth [160,161].

Han et al. [56] isolated NT-3 mRNA-rich EVs by transfecting ADSCs with lentivirus and encapsulated them in Ca-Alg hydrogels by in situ polymerization. The gel stably delivered NT-3 mRNA to targeted cellular SCs, effectively expressing neurotrophic factor 3 (NT-3) protein, and promoting peripheral nerve regeneration and functional recovery.

Qi et al. [162] used a commercially available injectable polyethylene glycol ether and polyethylene glycol triblock copolymer hydrogel (PLGA-PEG-PLGA) as a carrier for miR-138-5p modified UCMSCs EVs. The hydrogel was formed by dissolving PLGA-PEG-PLGA in a PBS solution containing EVs. In the SCI rat model, miR-138-5p decreased

neuronal apoptosis by increasing the expression of nuclear factor erythrocyte 2-related factor 2 (Nrf2) protein thereby decreasing the expression of Kelch-like ECH-associated protein 1 (keap1), and it also played an anti-inflammatory role by decreasing the expression of NOD-like receptor thermal protein domain-associated protein 3 (NLRP3), thereby downregulating caspase-1. As a result, the EV-loaded hydrogel promoted the recovery of neurological function.

Ning et al. [163] developed a double network gel of gelatin methacrylate and polypyrrole cross-linked tannins (GMP), loaded with EVs from BMSCs by adsorption. The EVs highly expressed miRNAs associated with axonal regeneration, myelination, and anti-inflammatory effects, and inhibited the expression of the proteins p-IKKα/β, p-IκBα, and p-P65, promoted the polarization of M2 microglia, increased the expression of axon-associated protein neurofilament (NF) and growth-associated protein-43 (GAP43), and promoted axon growth and the formation of synaptic networks (Figure 7).
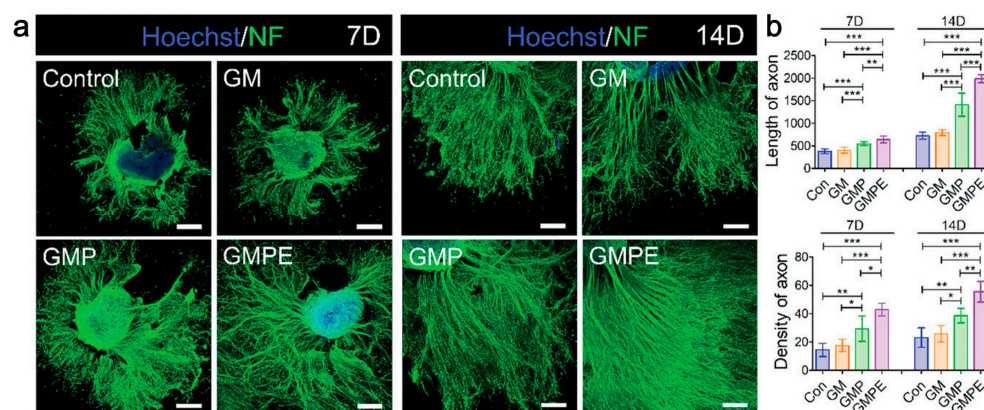


**Figure 7.** miRNA-EVs-GMP promoted axon growth and the formation of synaptic networks. (**a**) Immunofluorescence images of NF-positive axons in dorsal root ganglions (DRGs) cultured in hydrogels; (**b**) yhe density and length of axons were quantified. Statistical differences were determined using an ANOVA with Bonferroni's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) [163].

### 4.7. Others

Malignancies are associated with high morbidity and mortality worldwide, and immunotherapy is an effective strategy for the treatment of malignant cancers [164], including adoptive cell immunotherapy, cancer vaccines, small molecule inhibitors, etc. [165]. Efforts have been underway for decades in the development of therapeutic cancer vaccines [166], and vaccines work primarily by activating systemic anti-tumor responses, whether hematologic or solid [167]. Yang et al. [168] developed a nano-clay GelMA hydrogel vaccine. The hydrogel was loaded with chemokine 21 (CCL21a) and tumor cell-derived EVs, which contained granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA and surface-modified sonosensitizer chlorin e6 (Ce6). CCL21a was responsible for recruiting tumor cells into the hydrogel, leading to EVs-induced immunogenic cell death (ICD) in cancer cells. Ce6-enhanced tumor cell phagocytosis of tumor cells in sonodynamic therapy. The hydrogel vaccine was shown to elicit effective anti-tumor immunity in colon and breast cancer mice.

Muscle wasting affects about 15% of people over the age of 65 [169] and is characterized by a decline in muscle function and mass [170]. Muscle wasting is usually caused by a loss of the self-renewal capacity of Schwann cells (SCs). Some miRNAs, such as miR-1 [171] and miR-133 [172], have been shown to play an important role in promoting muscle regeneration, while other miRNAs are pathogenic to muscle wasting, such as miR-29b [173] and miR-628 [174]. Xu et al. [175] showed that miR-467a-3p and miR-874-5p inhibited the differentiation of SCs and the formation of muscle tissue, respectively. They overexpressed TSG101 to target SCs on the BMSCs-EVs surface, and transported antagomiR-467a-3p and antagomiR-874-5p, respectively, to construct two engineered EVs. Sodium alginate (SA)

and Pluronic F-127 (PF127) were used to form a layered injectable hydrogel. With the EV loaded in the hydrogel, the EVs containing two antagomirs were released in vivo for the treatment of muscle atrophy (MA).

Postoperative pericardial adhesion (PPA) is a fibrous connection between the epicardium and the thoracic cavity [176,177]. PPA reduces the surgical field of view and prolongs the operative time during cardiac surgical reoperation [178]. PPA also limits left ventricular diastolic filling, leading to myocardial infarction and, in severe cases, sudden death [179]. The molecular mechanism of PPA has not been fully elucidated, and effective methods to prevent PPA are urgently needed. Wu et al. [180] encapsulated the EVs from iCMs in a hyaluronic acid–g–(2-aminoethyl methacrylate hydrochloride–dopamine) (HAD) hydrogel by in situ polymerization under 365 nm of UV irradiation (7 mW/cm$^2$) for 10 s. The EVs-loaded hydrogel inhibited Nrf2, alleviating oxidative stress in primary cardiomyocytes, and downregulated interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and IL-6, exerting anti-inflammatory effects. In addition, HAD hydrogel acted as a polyanion trap to prevent PPA by neutralizing MSR-1 scavenger receptors and inhibiting the recruitment of GATA6+ macrophages to adhesion sites [181].

Acute kidney injury (AKI) occurs in approximately 32% of patients admitted to the intensive care unit (ICU) and 24% of patients undergoing cardiac surgery [182], and there is currently no effective treatment to prevent AKI, so there is an urgent need for new renal-protective therapies [183]. Chen et al. [59] developed an RGD hydrogel as a scaffold for the EVs of human placenta-derived mesenchymal stem cells (hP-MSCs) by in situ polymerization. miR-let-7a-5p in EVs was key to reducing renal apoptosis and enhance autophagy in AKI by targeting inhibition of caspase-3 (CASP3) mRNA and ras-related GTP binding D (RragD) mRNA, thereby exerting renal protective effects.

With the improvement of life quality, EVs have also attracted increasing interest from the beauty industry. Dermatologists and cosmetologists are committed to skin renewal and repair, and anti-wrinkle is one of the top research priorities [184,185]. HA-based hydrogels are used to develop new fillers that are highly effective in repairing the damaged dermal microenvironment [186]. Park et al. [187] extracted EVs from ADSCs and conjugated them to HA hydrogels by adsorption. miRNA-let-7b-5p and miR-24-3p in the EVs induced overexpression of CD301b in macrophages and promoted the proliferation of fibroblasts, demonstrating the anti-aging potential of the EV-loaded HA hydrogel.

## 5. Implications, Limitations and Future

In recent years, EVs have attracted a lot of attention for CFTs [188], which can be isolated from human cells, body fluids, milk, bacteria, etc. [189–191]. Hydrogels have a good biocompatibility, excellent biodegradability, and a high loading capacity, and can be designed as tissue patches or intravenous and subcutaneous injectable agents [192,193]. Not surprisingly, hydrogels have become an excellent EVs delivery platform, which can control the release of EVs through swelling [194] or degradation [195] and solves the problems of short intravenous half-life of EVs, rapid immune clearance, and liver accumulation [196]. Composite hydrogels containing EVs derived from different cells can be used as an effective CFT in many diseases such as myocardial infarction and osteoarthritis (Table 1).

**Table 1.** Applications of EV-loaded hydrogel in the treatment of diseases.

| Origins of EVs | Preprocessing Method | Biological Molecules | Hydrogels | Target Disease | Therapeutic Effect | Reference |
|---|---|---|---|---|---|---|
| iCMs | | miR-1, miR133a | collagen | MI | Inhibit the rational hypertrophy of heart disease and reduce the size of myocardial infarction | [117] |
| | | N/A | HAD | PPA | Prevent PPA | [180] |
| UCMSCs | Cells transduced with an HIF-1α-overexpressing lentivirus | HIF-1α | RGD | MI | Accelerate the recovery of cardiac function, reduce infarct size, and inhibit cardiomyocyte apoptosis | [57] |
| | EVs loaded with miRNA mimics | miR-138-5p | PLGA-PEG-PLGA | SCI | Promote the recovery of neurological function | [162] |
| ADSCs | EVs loaded with miRNA mimics | miR-126, miR-146a | Ca-Alg | MI | Reduce the size of myocardial infarction and fibrosis and promote angiogenesis in cardiac tissue | [53] |
| | | miR-99b-3p | HB-PEGDA/SH-HA | OA | Accelerate cartilage repair | [61] |
| | Cells transduced with plasmids of Bmp2 and VEGF-A | Bmp2 mRNA and VEGF-A mRNA | PEGS-A | BD [1] | Achieve efficient angiogenesis and bone regeneration | [144] |
| | Cells treated with hypoxia | circ-Snhg11 | GelMA | DU | Promote wound healing in diabetes | [52] |
| | Cells transduced with NT-3 plasmid | NT-3 mRNA | Ca-Alg | PNI | Promote peripheral nerve regeneration and functional recovery | [56] |
| [187] | | miRNA-let-7b-5p and miR-24-3p | HA | wrinkles | Reduce wrinkles | [187] |

**Table 1.** *Cont.*

| Origins of EVs | Preprocessing Method | Biological Molecules | Hydrogels | Target Disease | Therapeutic Effect | Reference |
|---|---|---|---|---|---|---|
| BMSCs | | miR-3594-5p | RGD-DNP | IDD | Slow down IDD | [58] |
| | Cells treated with hypoxia | GLRX3 | GDC | IDD | Slow down IDD | [82] |
| | Cells treated with TGFβ3 | miR-445 | GFHG [2] | OA | Accelerate cartilage repair | [62] |
| | Cells treated with hypoxia | Bgn | PEG-PP | BD | Accelerate bone regeneration | [143] |
| | | N/A | GelMA | DU | Accelerate the wound closure rate and promote healing. | [67] |
| | | miRNAs | GM-PPy-TA | SCI | Promote axon growth and the formation of synaptic networks | [163] |
| | EVs loaded with antagomirs | antagomiR-467a-3p and antagomiR-874-5p | Alg-PF127 | MA | Promote the formation of muscle tissue | [175] |
| BMDMs | Cells induced M2c Polarization by IL-10 | miR-124-3p | HA | IDD | Slow down IDD | [55] |
| SMSCs | Cells treated with MT | circRNA3503 | PDLLA-PEG-PDLLA | OA | Promote cartilage repair | [134] |
| TBI | | miR-328a-3p and miR-150-5p | MeGC | BD | Accelerate bone re-generation | [145] |
| HemSCs | | miR-7, miR-21 and miR-221 | oHA- CS | DU | Promote wound healing | [54] |
| tumor cells | Cells transfected with pcDNA3.1(-)-GM-CSF plasmid | GM-CSF mRNA | Nano clay-GelMA | Malignancies | Achieve effective anti-tumor immunity | [168] |
| hP-MSCs | | miR-let-7a-5p | RGD | AKI | Prevent AKI | [59] |

[1] Bone deficiency. [2] Gelatin-fibrinogen-hyaluronic acid-glycerol composite hydrogel.

However, there are still significant challenges before the hydrogels loaded with EVs can be translated into clinical practice. The first group of challenges are associated with the hydrogels. The stability and EV-release characteristics of hydrogels are affected by the hydrogels' synthesis conditions and in vivo tissue temperature [197]. The hydrogels based on natural biomaterials often have low mechanical strength [198,199]. The residual monomers or crosslinkers in the hydrogels may present biosafety issues [200]. Therefore, new hydrogel materials need to be designed and synthesized. In addition, the large-scale production of some hydrogels is needed [201]. The second group of challenges are associated with EVs. The purity of the EVs, commonly isolated by ultracentrifugation, needs to be improved [30,202]. The heterogeneity in the source of EVs will lead to different therapeutic effects [203]. EVs are stored at –80 °C, and repeated freeze–thaw processes may lead to the deterioration of bioactive molecules [204]. Finally, in the preparation of EVs-loaded hydrogels by in situ polymerization method, in order not to affect the activity of EVs, the optimal reaction conditions of the in situ polymerization still need to be explored [205].

As the field of hydrogel materials continues to advance, challenges associated with EVs-loaded hydrogels are progressively being addressed [206]. For example, by selecting suitable hydrogel materials that cater to specific release characteristics, the controlled release of EVs can be finely tuned. This includes options for short-term and long-term release, as well as tailored approaches like continuous or pulsatile release modes, thereby expanding the versatility and applicability of these systems in therapeutic scenarios [74]. Hydrogels with higher ester bonds such as 8-Arm-Poly(ethylene glycol)-mono(2-acryloyloxyethyl) succinate (8-Arm-PEG-MAES) exhibit faster swelling and degradation [207], resulting in a faster release of EVs. Additionally, by modifying the ratio of hydrophobic networks, the hydrophilicity of the hydrogel network can be adjusted, enabling control over the swelling rate, and thereby realizing a time-regulated release of EVs [208]. The passively controlled release of EVs can be achieved through stimuli-responsive hydrogels, such as isoguanosine-phenylboronic acid-guanosine (isoGPBG) pH-responsive hydrogels that rely on Schiff base bonds [209] and hydrazine or aldehyde-modified hyaluronic acid (HA) enzyme-sensitive hydrogels containing enzyme-sensitive peptides [210]. Furthermore, the separation and purification of EVs can be efficiently accomplished using microfluidic technology, which allows for the effective segregation of EVs from small sample volumes and their classification and extraction based on size [211–213]. In the preparation of EVs-loaded hydrogels, the adsorption in pre-formed hydrogels is more advantageous over in situ polymerization, because it is much easier to remove the residual monomers, cross-linked agents, initiators, toxic metal ions and other impurities from the pre-formed hydrogels [163,214–216].

In addition to accurate drug release and targeted treatment in disease treatment, EVs-loaded hydrogels are also useful in the fundamental research in life science, such as intercellular interactions, signal transmission, molecular communication, etc. [217,218].

For future preclinical studies, two important directions include identifying the optimal cell source of the EVs and developing new composite hydrogel materials. It is also crucial to evaluate the benefit–risk ratio of each EVs-loaded hydrogel, including production cost, potential contamination in the production, long-term safety and efficacy, etc. Overall, EVs-loaded hydrogels hold tremendous promise in cell-free therapy strategies.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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