Biomaterials Translational

Exosome-loaded biomaterials for tendon/ligament repair

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Key Words:
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From the Contents

ABSTRACT

Exosomes, a specialised type of extracellular vesicle, have attracted significant attention in the realm of tendon/ligament repair as a potential biologic therapeutic tool. While the competence of key substances responsible for the delivery function was gradually elucidated, series of shortcomings exemplified by the limited stability still need to be improved. Therefore, how to take maximum advantage of the biological characteristics of exosomes is of great importance. Recently, the comprehensive exploration and application of biomedical engineering has improved the availability of exosomes and revealed the future direction of exosomes combined with biomaterials. This review delves into the present application of biomaterials such as nanomaterials, hydrogels, and electrospun scaffolds, serving as the carriers of exosomes in tendon/ligament repair. By pinpointing and exploring their strengths and limitations, it offers valuable insights, paving the way the future direction of biomaterials in the application of exosomes in tendon/ligament repair in this field.

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Introduction

Exosomes are extracellular vesicles (EVs) ranging from 30 to 150 nm in diameter. They are crucially involved in intercellular communication, facilitating the transfer of bioactive lipids, nucleic acids, and proteins between cells, ultimately triggering biological responses in receptor cells.[1] Exosomes can be released by various cells, including fibroblasts, immunocytes (such as T cells, B cells, dendritic cells, etc.), adipocytes, and tumour cells, and are also found in physiological fluids such as blood, urine, tears, saliva, and ascites.[2, 3] Exosomes contain many components, including proteins, lipids, and nucleic acids (mRNA, microRNA, and other non-coding RNAs). They also contain specific molecules, such as cytoskeletal proteins (actin, tubulin, etc.), adhesion molecules, the endosomal sorting complex required for transport proteins, transport/binding proteins, and heat shock proteins. In addition to serving as biomarkers during exosome biogenesis, these molecules also participate in the biological functions of exosomes.[4, 5]

Exosome biogenesis primarily involves plasma membrane invagination, multivesicular body formation, and finally exosome secretion.[6] First, the cell plasma membrane undergoes endocytosis and invagination to form early endosomes. Subsequently, the early internal constitutional membrane invaginates to form early vesicular bodies, which fuse with the cell membrane and release exosomes into the extracellular matrix (ECM). Notably, the cytosolic contents of the donor cells are loaded into exosomes during exosome synthesis, secreted outside the cell via exocytosis, and then transferred to recipient cells.[7]

Currently, exosome separation and isolation are primarily achieved through conventional methods such as ultracentrifugation, size exclusion chromatography, and ultrafiltration. Among these, ultracentrifugation is the most

commonly utilised technique for purifying exosomes. It uses high-speed spinning to precipitate and isolate uniform sized vesicles from a sample.[8] Density gradient ultracentrifugation, a more specialised form of ultracentrifugation, is particularly suitable for processing large-volume samples. Nevertheless, centrifugal forces may compromise the structural integrity of exosomes. On the other hand, size exclusion chromatography employs a column filled with porous polymer beads to precisely separate exosomes based on their diameters. In addition to preserving the normal morphology of exosomes, this approach prevents shear force-induced disruption. However, despite its precision, size exclusion chromatography may involve more complex operations.[9] Alternatively, ultrafiltration offers excellent retention of exosome biological activity. It enables the selective isolation of exosomes through ultrafiltration membranes, offering simplicity and efficiency. However, exosomes may obstruct the ultrafiltration membrane pores, reducing separation efficiency.[10] Additionally, adhesion might occur between exosomes trapped within the membrane, ultimately diminishing the yields. It is also noteworthy that employing new technologies such as microbeads, kit extraction, microfluidic chips, and thermophoresis could enrich exosomes faster and more efficiently.[11, 12]

Exosomes and their clinical applications have been extensively studied since their discovery. Exosome research spans multiple disciplines and has yielded valuable insights. For instance, exosomes have been shown to stimulate skin cell proliferation, improve Alzheimer's disease treatment, and promote periodontal regeneration, among other positive clinical impacts.[13-15] Furthermore, exosomes have been documented to significantly impact tendon and ligament repair. For instance, it was recently reported that exosomes could restore the regenerative and repair capabilities of aged and damaged tendon-derived stem cells (TDSCs), facilitating ligament remodelling and reducing scarring.[16, 17]

Besides alleviating inflammation and regulating angiogenesis, exosomes can also act on other mechanisms to promote tendon and ligament repair.[18, 19] Notably, although exosome application has significantly improved the tendon/ligament repair outcomes in animal studies, there are still some limitations in this field.[20] First, exosome stability and half-life *in vivo* are limited, potentially limiting therapeutic efficacy. Additionally, ensuring that exosomes can accurately target and maintain effective concentrations in the injured area is crucial for realising their therapeutic potential, but achieving this goal without suitable carriers is challenging. Furthermore, tendon and ligament repair is a complex biological process requiring the precise regulation of multiple biological signals. In this regard, relying solely on exosomes may not yield the optimal therapeutic effects.[21] To overcome these limitations and maximise the therapeutic potential of exosomes, the use of biomaterials becomes a necessary strategy.

Rapid advancements have been witnessed in biomedical engineering in recent years, and biomaterials have been widely explored and applied to improve exosome bioavailability. Enhancing researchers' understanding of the inherent characteristics of various biomaterials involved in exosome delivery could help them make better selections, further advance toward the fusion utilisation of different materials based on their distinct features, and construct more efficient exosome delivery systems. In this regard, this article summarises the applications, advantages, shortcomings, and prospects for developing biomaterials to enhance exosome utilisation efficiency and bioavailability. These biomaterials can improve exosome stability, targeting ability, and persistence in multiple ways, enhancing their biological effects *in vivo*. For instance, exosomes embedded in hydrogels are significantly stabilised and retained at the injury site and the stability of their content is also positively impacted under physiological conditions.[22] Furthermore, utilising advanced techniques such as three-dimensional (3D) bioprinting to fabricate 3D scaffolds encapsulating exosomes and other bioactive molecules helps in maintaining the structure, stability, and functionality of these molecules for extended periods, resulting in superior physiological effects.[23] Hydrogel microspheres, which are highly customisable and biocompatible, have also been extensively explored and applied in drug delivery and tissue engineering. Notably, their porous structure and excellent sustained-release capabilities effectively protect exosomes and promote their stable delivery *in vivo*. Furthermore, hydrogel microspheres can regulate exosome release rate and direction, resulting in precise targeting and sustained release to the target tissue, thus enhancing exosome bioavailability.[24] Additionally, electrospun fibre technology could yield micro/nanofibres with high surface area and porosity, making them suitable for fabricating carrier materials. Encapsulating exosomes in fibres through electrospinning could enhance their uniform distribution and stability within the scaffold, increasing their presence time and bioavailability *in vivo*. Electrospun fibres also possess excellent biocompatibility and mechanical properties, making them ideal for supporting and processing exosomes.[25] Focusing on the combination of biomedical engineering and clinical medicine, this review systematically summarises the biomaterials currently used to improve exosome bioavailability. Specifically, a systematic literature search was conducted in PubMed using "exosomes", "biomaterials", "nanomaterials", "hydrogels", "hydrogel microspheres", and "electrospinning" as the keywords. The search was limited to studies published in English language peer-reviewed journals. The primary selection criteria were studies that focused on exosome-biomaterial interactions, with an emphasis on their application in tissue engineering and regenerative medicine. We only included articles relevant to this study's main theme and excluded reviews, commentaries, and unrelated studies.

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Application of Exosome-Loaded Nanomaterials in Tendon/Ligament Repair

Exosome solutions are usually injected directly into damaged bone tissues to achieve therapeutic effects. Difficult to retain in the target area and are prone to leakage or clearance by the body, limiting their biological usefulness.[26] Due to their appropriate degradation rates, nanomaterials, as typical carrier materials used in biomedical engineering, can regulate the release dose and time of exosomes without interfering with their biological functions, making them ideal exosome carriers.[27]

Nanomaterials can be categorised into three groups based on their different spatial scales of biomaterial structures: nanoscale (≤ 100 nm), submicron-scale (100 nm–1 μm), and microscale (≥ 1 μm).[28] Nanomaterials are typically defined as materials comprising particles, aggregates, or aggregates in the unbound state, with nanoparticles (NPs) as the basic structural unit, and other examples including nanomaterial scaffolds, nanoneedles, and so on.[29-31] Notably, NPs are usually classified based on material compositions, which could include lipids, polymers, proteins, or metal-based particles.[29] Nanotechnology-based exosome delivery systems combine exosomes with NPs, liposomes, or polymeric micelles. Given their small size, large surface area to volume ratio, and targeted ligand features, NPs can improve exosome delivery efficiency, ensure their continuous release, and minimise off-target deliveries, thus improving treatment outcomes.

Nanomaterial scaffolds that can efficiently and continuously load exosomes have recently gained significant interest in biomedical engineering, particularly with the emergence of photo-crosslinked and 3D bioprinted scaffolds.[30] These photo-crosslinked scaffolds undergo polymerisation triggered by ultraviolet/visible light irradiation-stimulated photoinitiators. Gelatin and chitosan are commonly used as synthetic materials in this process. Gelatin-based scaffolds could remarkably promote cellular proliferative differentiation and directional migration, while chitosan-infused scaffolds are highly biocompatible.[32] Furthermore, 3D bioprinted materials are meticulously modified in their surfaces and structural configurations, enabling precise control over the distribution of exosomes within the materials, thus regulating their structural integrity and functional activity.[33]

Exosomes have recently been discovered to exert anti-inflammatory effects, but their rapid clearance and poor preservation could limit their viability in this regard. Increased inflammation and oxidative stress have previously reported in arthritis-induced bone damage sites.[34] According to research, mesenchymal stem cell (MSC)-derived exosomes can alleviate arthritis-induced inflammation via modulating pro-inflammatory cytokines (such as interleukin (IL)-1β, IL-6, and IL-8) and nutritional factors (such as epidermal growth factor). For instance, He et al.[35] used bone marrow-MSC (BM-MSC)-derived exosomes to treat a rat osteoarthritis (OA) model by direct injection. This treatment significantly attenuated IL-1β-induced type II collagen A1 (*COL2A1*) and aggrecan (*ACAN*) downregulation and matrix metalloproteinase 13 (*MMP-13*) and a disintegrin and metalloproteinase with thrombospondin motifs (*ADAMTS*) upregulation, resulting in an anti-inflammatory effect. Researchers have introduced nanomaterials as carriers to further enhance exosome delivery and uptake by cells.

According to research, transforming growth factor-β-carrying TDSC-derived exosomes can activate the transforming growth factor-β SMAD family member 2/3 (Smad2/3) and extracellular signal-regulated kinase 1/2 (ERK1/2) signalling pathways.[36] Yu et al.[37] embedded BM-MSC-derived exosomes into fibronectin and injected them into rat patellar tendon defect areas. According to the results, these exosomes could be released from fibronectin and retained in the defect area, leading to the upregulation of tendon-related genes such as tenomodulin (*Tnmd*) and mohawk homeobox (*Mkx*), thus enhancing tenomodulin and type I collagen expression while promoting TDSC proliferation and migration *in vivo* (**Figure 1A**).[37] Furthermore, Shi et al.[38] embedded MSC-derived exosomes into fibronectin and administered it in the defect area of the patellar tendon. They discovered significant CD146-positive TDSC proliferation, downregulation of apoptotic cells and C-C chemokine receptor type 7-positive pro-inflammatory macrophages, and upregulation of anti-inflammatory mediators.[38]

The quality of tendon-bone healing is a major concern in orthopaedics, and current efforts are focused on safely and effectively achieving tendon-bone healing, shortening the healing time, and enhancing bone strength.[39] Proper tissue engineering techniques can facilitate inward bone growth into the tendon-bone interface. Additionally, exosomes can promote tendon-bone healing in rotator cuff models.[40] Various nanomaterials have also been proven to promote tendon-bone healing. For instance, calcium silicate NP-modified natural fish scale remarkably promoted transitional tissue repair in rat and rabbit tendon-bone repair models, more positively impacting tendon-bone interface repair.[41] Both traditional and novel nanomaterials have been continuously demonstrated to exert significant promotional effects on tendon-bone healing (**Figure 1B**, and **C**).[42] However, research on using nanomaterials as stem cell exosome carriers to promote tendon-bone healing still requires further enrichment.

The nanoplatforms currently available in nanomedicine are typically categorised into three main groups: organic nanosystems, inorganic nanosystems, and organic-inorganic hybrid nanosystems. Given their wide variety of structures, compositions, morphologies, physicochemical properties, and biological effects, these platforms greatly enrich the diversity of nanomedical applications.[43] However, they also show significant heterogeneity when interacting with the complex *in vivo* environment. Additionally, their potential mechanisms of interaction remain unclear. They may also exhibit certain levels of systemic toxicity and immunogenicity.[44] Furthermore, compared to novel materials like hydrogels, their biocompatibility, stability, and targeting specificity are relatively inferior. As a result, further research is required to fully elucidate their underlying mechanisms and improve nanomaterial biocompatibility, stability, and targeting capabilities in tendon/ligament repair.

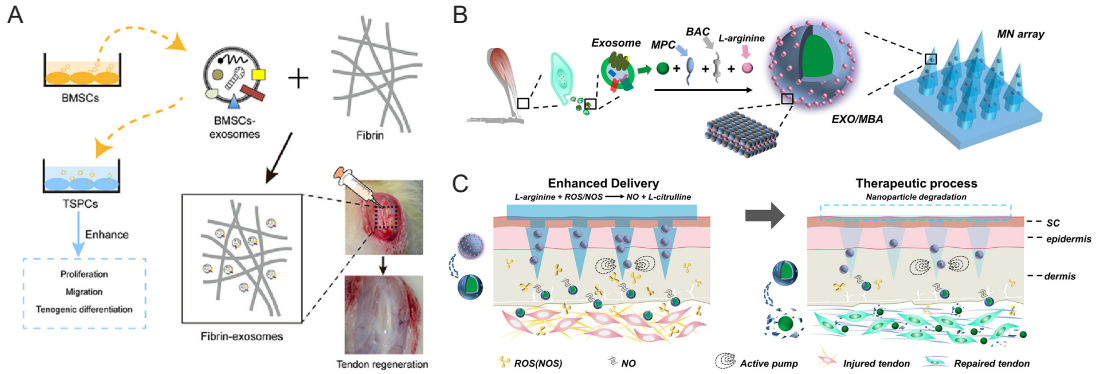

Figure 1. Application of nanomaterials loaded with exosomes in tendon/ligament repair. (A) BMSC-derived exosomes promote tendon regeneration by enhancing the proliferation and migration of endogenous tendon stem/progenitor cells. A was reprinted from Yu et al.[37] Copyright 2020 Acta Materialia Inc. (B) The fabrication procedure of EXO-loaded microneedle array driven by a nitric oxide nanomotor. (C) Healing process of achilles tendinopathy following the application of EXO-loaded microneedle array driven by a nitric oxide nanomotor. B and C were reprinted with permission from Liu et al.[42] Copyright 2021 American Chemical Society. BAC: benzalkonium chloride; BMSC: bone marrow mesenchymal stem cell; EXO: exosomes; MBA: methacryloyloxyethyl phosphorylcholine-N,N-bis(acryloyl) cystamine-ammonium persulfate; MN: microneedle; MPC: mesenchymal progenitor cell; NO: nitric oxide; NOS: nitric oxide synthase; ROS: reactive oxygen species; SC: stem cell; TSPC: tenogenic differentiation of tendon stem/progenitor cell.

With continuous iterations and advancements, novel nanomaterials such as two-dimensional inorganic nanosheets and magnetic NPs have exhibited remarkably enhanced stability and targeting specificity. Magnetic nanomaterials can also leverage their unique physical properties. Specifically, when combined with biocompatible but non-directional hydrogels, they can enhance drug delivery via anisotropy while retaining excellent hydrogel biocompatibility. To address nanomaterial heterogeneity, researchers can explore the surface engineering of nanomaterials, which involves chemical modifications of their outer membranes. For example, coupling reactions or lipid assembly could be used to display synthetic ligands, reducing host rejection reactions.[45] Furthermore, multiple *in vitro* cytotoxicity NP studies involving different cell lines, incubation times, and colourimetric assays were conducted to address the systemic toxicity of nanomaterials. These studies offer additional references and assurance that exosomes could be delivered safely using nanomaterials. Moreover, these advancements present promising research directions for the future development of nanomaterial-based exosome delivery systems to be used in tendon/ligament repair, offering improved stability, targeting specificity, and safety profiles.

Application of Exosome-Loaded Hydrogels in Tendon/Ligament Repair

Hydrogels, a class of macromolecular polymers comprising a network of crosslinked polymer molecular chains and exhibiting immunogenicity properties comparable to the ECM, could be used to reduce chronic inflammation and toxicity and create a conducive environment for cell growth and tissue regeneration.[46] Hydrogels could be synthesised via chemical, physical, and enzymatic crosslinking, with physical and chemical crosslinking as the more commonly used approaches currently.[47, 48] Physical crosslinking involves hydrogel formation between molecules through inter-ion interaction, hydrogen bonding, and crystallisation and is generally a reversible process. Notably, the use of toxic organic solvents and small molecule crosslinking agents could be avoided in physical crosslinking, offering the advantages of good biocompatibility and degradation. However, this process exhibits a weak interaction force between hydrogel molecules and defects of low mechanical strength and stability.[49] On the other hand, chemical crosslinking involves interlinking macromolecules with covalent bonds. Generally, introducing a crosslinking agent facilitates compound addition and condensation reactions, leading to the formation of chemical crosslinks between molecules, ultimately yielding a firm hydrogel network. Although this approach offers better mechanical strength and stability, it is prone to producing monomer residues and is also associated with insufficient biosafety.[50]

Traditional exosome delivery involves direct injection of the relevant solution into the injury site, often resulting in short retention durations and uneven distribution of effective doses over time.[51] Given their hydrophilicity and crosslinking properties, hydrogels can more effectively regulate drug release, addressing the limited retention durations and uneven distribution of exosome solutions in direct injections. In this regard, utilising hydrogel materials for exosome delivery holds great promise for ensuring sustained release processes.

Hydrogel materials, as exosome carriers, could also serve as biological scaffolds and drug delivery devices, enabling the creation of anti-inflammatory microenvironments *in vivo*. For example, Guan et al.[52] introduced aldehyde-functionalised chondroitin sulfate into methacrylated hydrogels, creating a double-network framework hydrogel with ECM compatibility (GMOCS). Furthermore, when combined with rat bone marrow stromal cell (BMSC)-derived exosomes, > 80% of exosomes were released unhindered from a hydrogel network over a 14-day observation period. Further studies also demonstrated that exosome-loaded GMOCS hydrogels could

regulate macrophage polarisation at the injury site, inducing a shift from the M1 to the M2 phenotype and upregulating anti-inflammatory factors, thus exerting anti-inflammatory effects (**Figure 2A**).[52] Similarly, Jiang et al.[53] reported that using acellular cartilage ECM scaffolds loaded with human umbilical cord Wharton's jelly-derived MSC exosomes effectively promoted macrophage polarisation toward the M2 phenotype, inhibiting inflammation within the joint cavity, thus promoting articular cartilage regeneration. Furthermore, Zhang et al.[54] incorporated platelet-rich plasma-derived exosomes into thermosensitive hydrogels, enabling continuous exosome release for 28 days. This approach also effectively promoted mouse BM-MSC and chondrocyte proliferation and migration, enhanced BM-MSC chondrogenic differentiation, and attenuated IL-1β-induced chondrocyte apoptosis and degeneration by inhibiting the nuclear factor kappa B and signal transducer and activator of transcription signalling pathways, thereby exerting anti-inflammatory effects and delaying and ameliorating spontaneous OA progression (**Figure 2B**).[54]

Wang et al.[55] utilised a fibrinogen gel loaded with adipose-derived stem cell-derived exosomes (ASC-Exos) to treat partial thickness rotator cuff tear (PTRCT) in rabbits to explore the efficacy of biomedical engineering non-surgical treatment in promoting tendon healing of partial thickness rotator cuff tear. At 12 weeks post-surgery, the tendon thickness in the ASC-Exos/fibrinogen group (1.63 ± 0.19 mm) was significantly greater than that in the control and fibrinogen groups, indicating that the fibrinogen gel loaded with ASC-Exos effectively promoted tendon regeneration and partial thickness rotator cuff tear healing on the bursal side.[55] Additionally, Lu et al.[56] constructed a gelatin methacryloyl (GelMA) hydrogel substrate incorporating platelet-derived exosomes and demonstrated that it can promote pluripotent stem cell differentiation into tendon cells, enhance TDSC proliferation and regeneration, and promote tendon repair (**Figure 2C**). Similarly, Zhang et al.[57] explored the effects of the GelMA hydrogel loaded with TDSC-derived EVs on tenocyte-derived stem cell proliferation, migration, and differentiation *in vitro*. According to the results, the GelMA hydrogel, as an exosome carrier, ensured the long-term slow release of TDSC-derived EVs at the injury site, significantly improving the histological score of regenerated tendons via tendon gene upregulation and excessive ossification inhibition, thus improving the tissue mechanical properties. Additionally, in a previous study, microRNA sequencing showed miR-145-3p upregulation, presenting a novel therapeutic approach for the local treatment of Achilles tendon injuries using EVs (**Figure 2D**).[57] Notably, ECM-derived scaffolds have also shown great potential in tendon repair and reconstruction. Cui et al.[58] used collagen-binding domains to bind TDSC-derived EVs to collagen and firmly coupled them with decellularised bovine tendon slices to fabricate scaffolds with biofunctional properties. The decellularised bovine tendon slices scaffold provided adequate tensile strength, creating a mechanical microenvironment for tendon repair and TDSC-derived EVs to exert paracrine effects, balancing matrix synthesis and degradation in damaged tendons, ultimately promoting tendon repair.[58]

Poor healing at the tendon-bone interface is a significant contributor to joint injuries and subsequent retears following ligament repair. Zhang et al.[59] utilised hydrogel as a carrier to inject BMSC-secreted exosomes obtained from rats of different ages into injured rat rotator cuffs to address the challenge of high retear rates in rotator cuff tears (RCTs). According to the results, the hydrogel loaded with BMSC-derived exosomes promoted ECM remodelling, osteogenic differentiation, and angiogenesis, accelerating tendon-bone interface healing in RCTs. Moreover, BMSC-derived exosomes obtained from younger individuals demonstrated better prognostic outcomes.[59] Cai et al.[60] employed a sodium alginate hydrogel loaded with kartogenin pretreated human BMSC-derived exosomes to treat chronic RCT. Compared to the control group, more mature and orderly arranged collagen fibres were observed at the tendon-bone interface at 8 weeks, effectively promoting cartilage formation, as well as collagen and attachment-regenerated tissue maturation.[60] Additionally, Shi et al.[61] explored the role of BMSC-derived exosomes in modulating inflammation and enhancing tendon-bone healing during tissue repair. During tendon-bone healing after applying exosome-loaded hydrogels at the injury site, the hydrogel + exosome group induced M2 macrophage polarisation, modulated the inflammatory factor content at the healing site, and enhanced tendon-bone healing biomechanical properties while promoting fibrocartilage regeneration at the tendon-bone interface.[61] Furthermore, Zhang et al.[62] explored the effects of hypoxia on exosome quality. Specifically, they conducted *in vivo* and *in vitro* experiments using adhesive hydrogel as a carrier, ultimately demonstrating that periarticular injection of hypoxia-cultured BMSC-derived exosomes around the anterior cruciate ligament reconstruction graft could accelerate tendon-bone tunnel integration after anterior cruciate ligament reconstruction by improving the microstructure of the surrounding graft bone.[62]

Although exosome-carrying hydrogel materials have recently been used extensively in bone or cartilage repair and ligament reconstruction, some limitations remain.[63] Firstly, hydrogels, as traditional bioengineering materials, lack adequate strength and are prone to permanent rupture. Hydrogels' natural tensile strength and stiffness, as well as mechanical and biomechanical properties, are still unsatisfactory. Double network hydrogels and polyionic complexes are some of the strategies that have been used to enhance the mechanical properties of polyelectrolyte hydrogels. The former introduces a soft, tenacious secondary network to significantly enhance the toughness of the polyelectrolyte network. On the other hand, the latter introduces a second polyelectrolyte with an equal amount of opposite charges, forming a high-density ionic bond with the polyelectrolyte network as a sacrificial bond, thus enhancing the hydrogels' mechanical properties.[64] Second, due to the intricate physiological microenvironment within the human body, extensive trials are required to evaluate the biocompatibility and degradability of these scaffold materials before clinical translation.[65] According to research, ultraviolet-triggered surface-catalysed initiator radical polymerisation could facilitate the creation of multilayer hydrogels with distinct compositions and stratified characteristics when

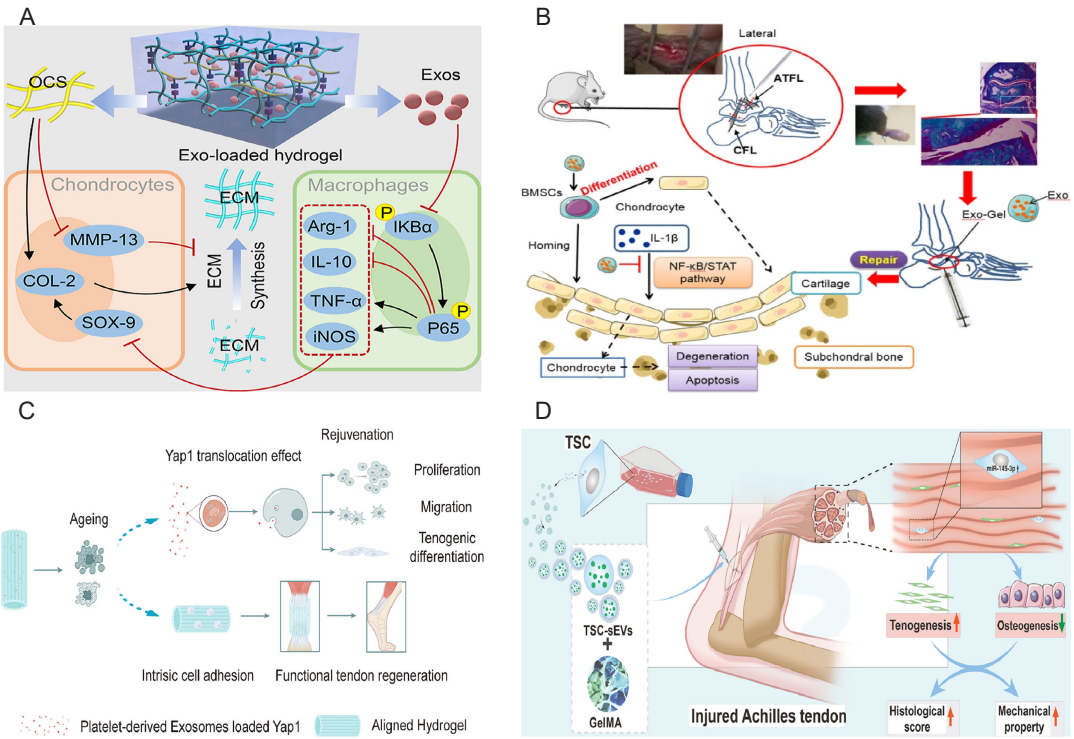

Figure 2. Application of exosome-loaded hydrogel in tendon/ligament repair. (A) Mechanism by which the GMOCS double network framework hydrogel regulates macrophage polarisation at the injury site. Reprinted from Guan et al.[52] (B) Mechanism by which thermosensitive hydrogel loaded with plasma-derived exosomes exert anti-inflammatory effects. Reprinted from Zhang et al.[54] (C) The overall experimental process through which the GelMA hydrogel loaded with platelet-derived exosomes promotes tendon repair. Reprinted from Lu et al.[56] Copyright 2023 Acta Materialia Inc. (D) Analysis of the process through which the GelMA hydrogel loaded with extracellular vesicles of tendon stem cells promotes the healing of injured achilles tendon. Reprinted from Zhang et al.[57] Copyright 2023 Acta Materialia Inc. Arg-1: arginase-1; ATFL: anterior talofibular ligament; BMSC: bone mesenchymal stem cell; CFL: calcaneal fibular ligament; COL-2: type II collagen; ECM: extracellular matrix; Exo: exosome; Gel: gelatin; GelMA: gelatin methacryloyl; GMOCS: gelatin methacryloyl OCS; IL-10: interleukin-10; IL-1β: interleukin-1 beta; iNOS: inducible nitric oxide synthase; IκBα: inhibitor of nuclear factor kappa-B alpha; MMP-13: matrix metalloproteinase 13; NF-κB: nuclear factor kappa B; OCS: aldehyde-functionalized chondroitin sulfate; sEV: small extracellular vesicle; SOX-9: SRY-box transcription factor 9; STAT: signal transducer and activator of transcription; TNF-α: tumor necrosis factor alpha; TSC: tendon stem cell; Yap1: yes-associated protein 1.

applied iteratively. Fine-tuning the properties of each layer can improve the overall biocompatibility of the hydrogels.[66] Moreover, optimising hydrogel materials, gaining a deeper understanding of the exosome release dynamics, and developing more sophisticated hydrogel materials and exosome delivery systems remain significant challenges during clinical translation.

To enhance hydrogel performance, crosslinking materials (such as nanoclays) can be incorporated into viscoelastic hydrogel scaffolds through electrostatic interactions and covalent bonding, resulting in higher ultimate strength and compression modulus compared to standalone hydrogel scaffolds.[67] In the future, hydrogel materials will be used not only as scaffolds and carriers for exosome delivery through local injection but also as microneedles and dressings,[68,69] further leveraging their excellent biocompatibility and sustained release properties. Moreover, expanding hydrogel use to include research across a wider range of organs and systems will further highlight their enormous clinical potential.

Application of Exosome-Loaded Hydrogel Microspheres in Tendon/Ligament Repair

Traditionally, hydrogels are formed by crosslinking molecules into 3D volumes. Notably, hydrogels' external dimensions are at the millimetre scale or larger, with a nanometer scale mesh size. Although bulk hydrogels can be equipped with a micrometre-scale porosity, they are not suitable for all applications, especially when injections or smaller sizes are required. Once prepared, bulk hydrogels must be shaped and then surgically implanted, often requiring surgical incisions. Given their smaller size and more injectable qualities (particulate nature and small particle size), hydrogel microspheres could be used as an alternative.[70]

Hydrogel microspheres have recently shown promising applications in bioengineering owing to their unique physical and chemical properties. Their remarkably high surface-to-volume ratio and high porosity allow them to optimise cellular interactions, nutrient exchange, and metabolic waste removal while facilitating their functional modification. These properties can also promote efficient loading and sustained

release of biologically active EVs such as exosomes.[71, 72] Given their small size, high surface-to-volume ratio, high porosity, and injectability,[73] hydrogel microspheres can efficiently encapsulate exosomes, facilitating their targeted delivery and controlled release to specific cells or tissues via minimally invasive injection. Therefore, in addition to effectively protecting exosomes from degradation and rapid clearance, encapsulating exosomes into hydrogel microspheres also significantly enhances their therapeutic efficacy. Moreover, hydrogel microspheres could be used in granular hydrogel forms to create microporous scaffolds to promote cell infiltration, or they can be embedded in large hydrogels to construct multistage structural materials. These modifications could be achieved through batch emulsification, microfluidic emulsions, lithography, electrohydrodynamic spraying, and mechanical fragmentation.[70] Electrospun scaffolds with conductive properties are commonly used in biomedical engineering, and the underlying mechanism entails creating an electric field between the deposition needle and the collector. During this process, conductive and volatile solvent selection, which depends on polymer compatibility, is crucial. Furthermore, once collected on the rotating mandrel, the fibre can be easily aligned, and the collector can accurately control the degree of alignment by adjusting the rotation speed. Under ideal conditions, the strong electrostatic force overcomes the tension on the tip surface when the polymer solution is pushed through the tip, triggering the formation of the polymer jet. Additionally, the solvent evaporates rapidly as the jet moves to the ground or to the negatively charged collector, causing the polymer fibres to gather orderly in their respective layers. With continuous advancements in biomaterials science, multiple protocols have been used to make more polymers compatible with electrospinning technology.[74]

There are limitations to stand-alone exosome therapy, including poor targeting and the inability of the content of loaded molecules to reach the therapeutic concentration. In this regard, applying exosome-loaded hydrogel microspheres can prolong the duration of exosomes *in vivo* and enhance their anti-inflammatory effects. Wan et al.[75] treated OA using a novel photo-crosslinked spherical GelMA hydrogel that contains engineered exosomes modified with cartilage-affinity peptide (CAP). According to the results, the hydrogel significantly suppressed inflammation-related gene expression and OA-associated immune response, enhancing joint preservation, thus demonstrating its targeting and sustained therapeutic effects (**Figure 3A**).[75] Furthermore, Chen et al.[76] integrated CAP-modified liposomes containing the mitophagy activator (ursolic acid) into hyaluronic acid methacrylate hydrogel microspheres using HM@WY-Lip/UA (hyaluronic acid methacrylate hydrogel microspheres with WYRGRL-decorated liposomes loaded with urolithin A) microfluidic technology, achieving subcellular therapy for arthritis via dysfunctional mitochondria elimination. Moreover, aberrant fibroblast growth factor (FGF) signalling pathway silencing contributed to joint malformation and OA.[76] Chen et al.[77] also effectively activated the *FGF18* gene in OA chondrocytes at the genomic level using hybrid exosomes containing CAP and loaded with *FGF18*-targeting gene editing tools (CAP/*FGF18*-hyEXO). Subsequently, these exosomes were encapsulated in methacrylate-modified hyaluronic acid hydrogel microspheres through microfluidics and photopolymerisation to create an injectable microgel system with a self-regenerating hydration layer (CAP/*FGF18*-hyEXO@HMs). Besides offering sustained lubrication against frictional wear, this system also demonstrated synergistic effects in promoting cartilage regeneration and reducing inflammation.[77]

Tendon injury, a common complication in athletes, is characterised by a slow healing process that often results in tendon degeneration. Although the high incidence of iatrogenic complications in tendon repair surgery has prompted the exploration of therapeutic interventions to enhance healing, a standardised clinical treatment method for improving tendon healing remains elusive.[78] To address this issue, researchers are not only working to improve surgical techniques, but are also exploring additional bioengineering approaches and materials to promote healing and tendon regeneration. Tendon regeneration involves extensive tendon cell proliferation and migration.[79] Ren et al.[80] revealed that exosomes can promote tendon cell proliferation and differentiation, accelerating tendon regeneration. Furthermore, encapsulating exosomes in hydrogel microspheres could enhance their effectiveness in treating tendon injuries.[80] Additionally, Jin et al.[16] demonstrated that stem cell-secreted exosomes obtained from human exfoliated deciduous teeth carry abundant anti-ageing signalling factors, and their local delivery via hydrogel microspheres can reduce senescent cells and ectopic ossification, structurally and functionally rescuing the endogenous tendon regeneration and repair capacity of aged rats, thus highlighting their therapeutic potential in age-related diseases. On the other hand, Cai et al.[81] used Smad3-small interfering RNA NP-encapsulated MMP2 loaded into degradable GelMA microspheres to inhibit fibroblast proliferation and prevent tendon adhesion, presenting a safe and effective approach to reduce adhesion tissue formation post-tendon repair surgery and promote tendon regeneration. These studies fully leveraged the functions of exosomes and hydrogel microspheres' carrier role, impacting multiple stages and directions of tendon regeneration.

The tendon-bone interface is highly susceptible to injuries and difficult to restore owing to its limited regenerative capacity. Compared to tendon-to-tendon and bone-to-bone healing, tendon-bone healing is more complex and slower. Previous research demonstrated that exosome-loaded hydrogel microspheres can promote tendon-bone healing in various aspects, improving the efficacy of anterior cruciate ligament reconstruction surgery. Yang et al.[82] designed exosome-carrying microfluidic chip-manufactured injectable hydrogel microspheres sensitive to MMP-1, which could spatiotemporally control new blood vessel formation and promote bone healing (**Figure 3B**). Additionally, Zhao et al.[83] suggested that developing hydrogel microspheres containing various active substances, mainly exosomes, can accelerate the healing process, facilitate blood vessel formation at the tendon-bone interface during the early healing stages, and promote fibrocartilage formation (**Figure 3C**). Although these findings indicate that injecting hydrogel microspheres loaded with cell-derived exosomes could be a promising therapeutic strategy for tendon-bone healing, further validation through clinical trials is required.

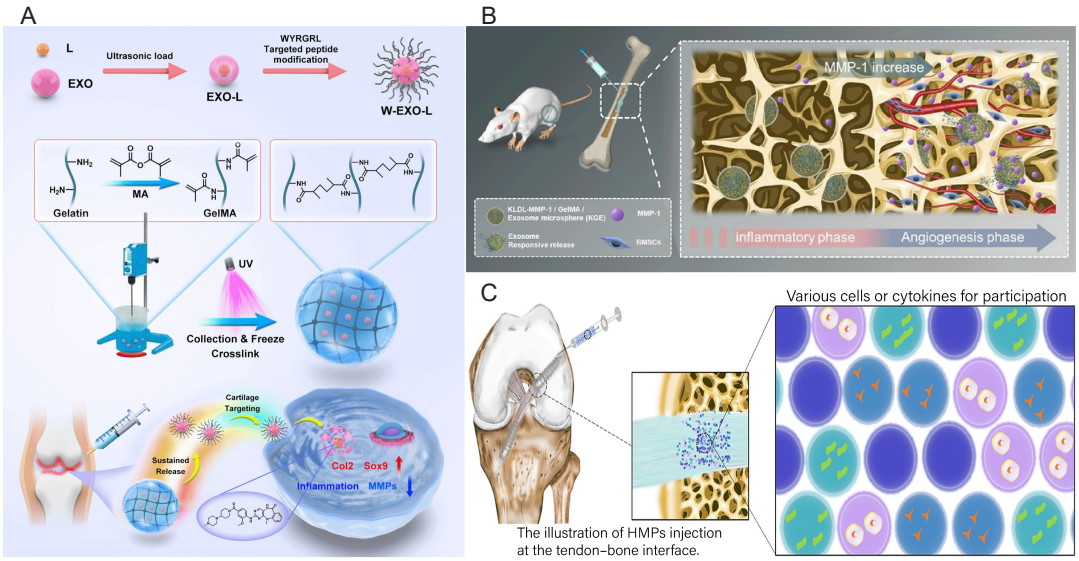

Figure 3. Application of exosome-loaded hydrogel microspheres in tendon/ligament repair. (A) Injectable photo-crosslinked spherical hydrogel for osteoarthritis treatment encapsulating engineered exosomes with targeting peptides. Reprinted from Wan et al.[75] (B) Injection of MMP-1-sensitive microspheres with exosome release function for spatiotemporal control to promote neovascularisation and bone healing. Reprinted from Cai et al.[81] Copyright 2021 Wiley-VCH GmbH. Reproduced with permission. (C) Illustration of injection at the tendon-bone interface with multifunctional HMPs loaded with various cells or cytokines involved in different healing stages. Reprinted from Zhao et al.[83] Copyright 2021 Wiley Periodicals LLC. Reproduced with permission. BMSC: bone marrow mesenchymal stromal cell; Col2: type II collagen; EXO: exosome; GelMA: methacrylate gelatin; HMP: hydrogel microsphere; KLDL: KLD12-linker-D-lysine; L: LRRK2-IN-1, a small molecular inhibitor, with inhibits leucine-rich repeat kinase 2 (LRRK2); MA: methacrylate; MMP: matrix metallopeptidase; Sox9: SRY-box transcription factor; UV: ultraviolet; WYRGRL: an exosome surface-conjugated collagen II-targeting peptide.

Bioengineering-based strategies for improving patient prognosis could somewhat compensate for the shortcomings of traditional surgical methods. Exosome-loaded hydrogel microspheres have shown promising preliminary results for ligament and tendon repair, but further exploration and validation are still required, especially through clinical trials to assess their safety and effectiveness. There are also some limitations to their application. For instance, the preparation of high-quality exosome-loaded hydrogel microspheres remains challenging, technically complex, and low-yielding. There is also a need to further explore the most suitable microsphere sizes as a unified reference standard, and to develop technical specifications in biomedical engineering. Additionally, in-depth investigations into the optimal application methods and treatment timing for different injury types and severities are required. Moreover, research on the safety and toxicity of exosome-loaded hydrogel microspheres is still limited, necessitating further experimental and clinical studies.

Notably, several research and promotional aspects pertaining to hydrogel microspheres could be explored in the future. First, the preparation technology should be optimised further to reduce technical difficulties, improve product quality, augment key parameters, form a unified industry standard, and improve exosome loading stability and effectiveness. Second, in-depth research and comparison of their application effects in various types and severities of ligament or tendon injuries are necessary. Specifically, appropriate usage methods and timing should be determined, and corresponding treatment protocols should be established. Finally, efforts should be focused on strengthening research into their safety and biological toxicity in order to provide more reliable evidence for clinical application.

Application of Exosome-Loaded Electrospun Scaffolds in Tendon/Ligament Repair

Electrospinning, a classical fibre fabrication method, can produce fibres with sizes ranging from micrometres to nanometres. Some of its advantages include procedural simplicity, cost-effectiveness, adjustable fibre diameters, and controllable fibre structures,[25] which make it suitable for biomedical engineering. Electrospun nanofibre scaffolds have a high surface area-to-volume ratio and large porosity, which enhance drug loading capacity. Additionally, nanofibre scaffolds have a micro-nano structure on the surface, which promotes cell attachment, proliferation, and migration, facilitating exosome-cell interactions. Moreover, electrospun nanofibre scaffolds protect exosomes from premature release or degradation *in vivo*, ensuring their functional stability. It is also noteworthy that a controlled release of exosomes can be achieved by adjusting the pore structure and surface properties of electrospun nanofibre scaffolds, ultimately improving their therapeutic efficacy and reducing their side effects (**Figure 4A** and **B**).[84, 85]

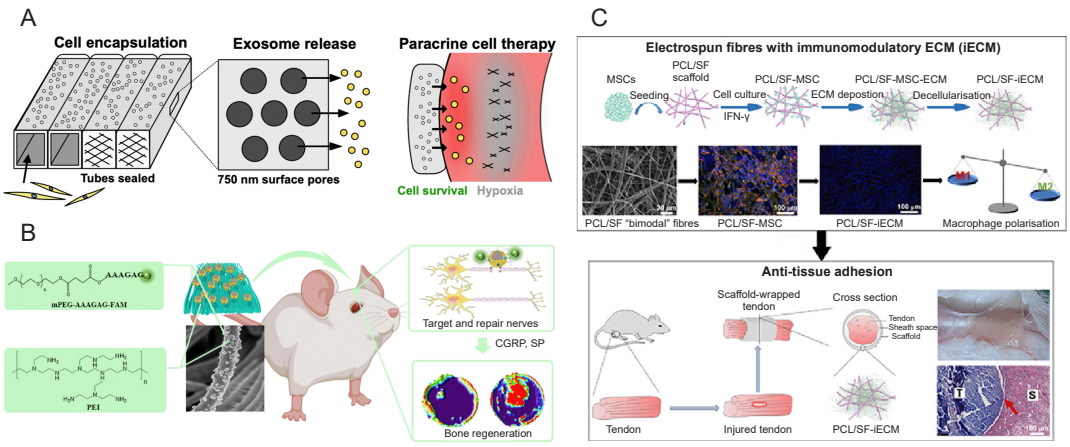

Figure 4. The broad applications of exosome-loaded electrospun nanofibre scaffolds in the medical field. (A) Porous scaffold for mesenchymal cell encapsulation and exosome-based therapy of ischaemic diseases. Reprinted from Czosseck et al.[84] (B) Aptamer engineered exosomes loaded onto biomimetic periosteum to promote angiogenesis and bone regeneration by targeting injured nerves via JNK3 MAPK pathway. Reprinted from Su et al.[85] (C) MSC-derived immunomodulatory extracellular matrix functionalised electrospun fibres to alleviate foreign body reaction and tendon adhesion. Reprinted from Dong et al.[86] Copyright 2021 Acta Materialia Inc. CGRP: calcitonin gene-related peptide; ECM: extracellular matrix; FAM: fluorescein amidite; IFN-γ: interferon-γ; JNK3: c-Jun N-terminal kinase 3; MAPK: mitogen-activated protein kinase; mPEG: methoxy polyethylene glycol; MSC: mesenchymal stem cell; PCL: polycaprolactone; PEI: polyethylenimine; S: scaffold; SF: silk fibroin; SP: substance P; T: tendon.

Electrospun scaffolds with conductive properties are commonly used in biomedical engineering, and the underlying mechanism entails creating an electric field between the deposition needle and the collector. During this process, conductive and volatile solvent selection, which depends on polymer compatibility, is crucial. Furthermore, upon collection on the rotating mandrel, the fibre can be easily aligned, and the collector can accurately control the degree of alignment by adjusting the rotation speed. Under ideal conditions, the strong electrostatic force overcomes the tension on the tip surface when the polymer solution is pushed through the tip, triggering the formation of the polymer jet. Additionally, the solvent evaporates rapidly when the jet moves to the ground or to the negatively charged collector, causing the polymer fibres to gather orderly in their respective layers. With continuous advancements in biomaterials science, many protocols have been used to make more types of polymers compatible with electrospinning technology.[74]

The molecular contents of exosomes possess anti-inflammatory properties, and electrospinning can regulate their release rate, resulting in effective anti-inflammatory effects. Exosomes have been used alongside electrospinning technology for myocardial regeneration. Specifically, incorporating exosomes into electrospun scaffolds can enhance MSC wound healing ability and collagen production while upregulating the anti-inflammatory M2 macrophage phenotype, greatly reducing the initial immune response and alleviating inflammation.[87] Furthermore, Li et al.[88] prepared an electrospun material loaded with exosomes (sEVs@DSPE-PLLA, defined as small EVs immobilised onto electrospun fibres functionalised with distearoylphosphatidylethanolamine-polyethylene glycol 5000-NH_2 and made of poly(L-lactic acid) and discovered that it could alleviate inflammation and promote macrophage polarisation toward an anti-inflammatory phenotype. This anti-inflammatory effect could be achieved through gene expression regulation and cytokine secretion. Additionally, sEVs@DSPE-PLLA exerted a promotional effect on fibroblast and keratinocyte proliferation and migration, facilitating new tissue formation during wound healing (**Figure 5A**).[88] Moreover, Wang et al.[89] developed a nanofibre scaffold for delivering ASC-Exos, which showed significant anti-inflammatory effects. Overall, exosome-loaded electrospun scaffolds may exert anti-inflammatory effects by regulating inflammation, blocking inflammatory cell infiltration, promoting anti-inflammatory cell activation, and modulating the immune balance.

Additionally, exosome-loaded electrospun scaffolds could somewhat improve muscle-tendon regeneration. Dong et al.[86] designed a poly(ε-caprolactone)/silk fibroin composite fibre scaffold and functionalised it with MSC-derived exosomes and found that it could alleviate *in vitro* reactions, reduce fibrous capsule formation, and promote polarisation of M2 macrophages, effectively reducing tendon adhesions, thereby promoting tendon regeneration (**Figure 4C**). On the other hand, Zhang et al.[90] prepared MSC-derived exosome-loaded patches using a dynamic wet-spinning system and then conducted *in vitro* and *in vivo* studies to assess their effects on rabbit tendon cell activity and chronic RCT healing. According to the results, the patches significantly promoted tendon cell proliferation and migration, which is crucial for tendon regeneration.[90] Overall, exosome-loaded electrospun scaffolds can promote tendon healing via various mechanisms, including promoting cell proliferation and migration, modulating inflammatory responses, promoting tissue regeneration, and lowering tendon adhesions.

Exosome-loaded electrospun materials could improve tendon-bone interface healing.[91] Du et al.[92] prepared biomaterials with exosome release functionality by loading exosome carriers onto electrospun materials. Subsequently, they evaluated the impact of these biomaterials on tendon-bone interface healing through *in vivo* and *in vitro* experiments. According to the results, these exosome-loaded electrospun materials could release bioactive factors, promote nerve, blood vessel, and bone tissue regeneration, and somewhat improve tendon-bone interface healing (**Figure 5B**).[92] Furthermore, Su et al.[93] prepared fibre scaffolds using electrospinning technology and then fixed exosomes on them. Subsequently, they performed *in vivo* and *in vitro* experiments to evaluate the effect of the prepared exosome-loaded scaffolds on tendon-bone cell activity and tendon-bone interface healing. According to the results, the exosome-repair scaffolds significantly promoted tendon-bone cell proliferation and migration, greatly enhancing tendon-bone interface healing (**Figure 5C**).[93] Overall, exosome-loaded electrospun scaffolds could enhance tendon-bone interface healing through multiple pathways, including promoting cell proliferation and migration, modulating inflammatory responses, and promoting angiogenesis and matrix regeneration.

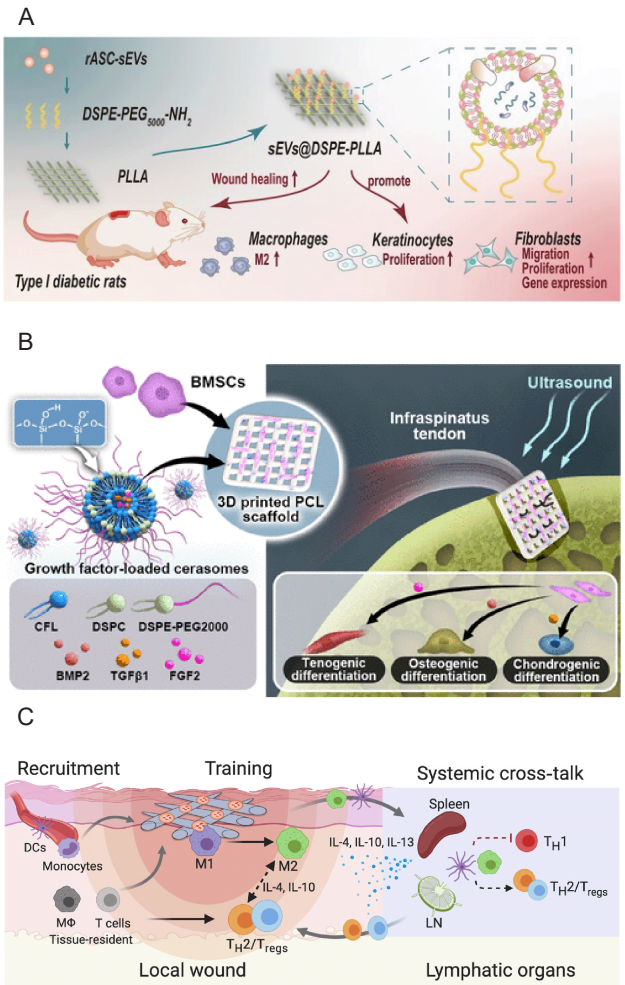

Figure 5. The application of electrospun scaffolds loaded with exosomes in tendon/ligament repair. (A) Mechanism of action of sEVs@DSPE-PLLA. Reprinted from Li et al.[88] (B) A diagram showing the mechanism of PCL scaffold preparation and utilization. Reprinted from Du et al.[92] (C) Immunomodulatory mechanisms involved in tissue repair by Exo-PEF materials. Reprinted from Su et al.[93] 3D: three-dimensional; BMP2: bone morphogenetic protein 2; BMSC: bone marrow mesenchymal stem cell; CFL: calcaneofibular ligament; DC: dendritic cell; DSPC: distearoylphosphatidylcholine; DSPE: distearoylphosphatidylethanolamine; FGF2: fibroblast growth factor 2; IL: interleukin; LN: lymph node; MΦ: macrophage; PCL: polycaprolactone; PEF: polyethylenimine-modified electrospun fibre; PEG2000: polyethylene glycol 2000; PEG5000: polyethylene glycol 5000; PLLA: poly-L-lactic acid; rASC: rat adipose-derived mesenchymal stem cell; sEV: small extracellular vesicle; TGFβ1: transforming growth factor beta 1; T_H: T helper cell; T_{regs}: regulatory T cell.

While electrospinning offers promise for tendon/ligament repair, its long-term effectiveness and safety require further validation. The technology lacks sufficient long-term clinical data to definitively demonstrate its lasting benefits. Secondly, the material properties of electrospun fibres may create concerns regarding biocompatibility, such as *in vitro* reactions and tissue rejection, which may affect their long-term stability and safety in the human body. To improve the biocompatibility

of electrospun scaffolds, several strategies have been proposed to exert control over fibre morphology. This can be achieved by modulating several parameters during the electrospinning process, which directly alter the features of the prepared fibres, including their diameter, porosity, and alignment. Such control is designed to align with the specific requirements of various biomedical applications.[94] We can further improve the strength and stability of these fibers using various post-processing techniques. Some of these techniques include heating the fibers, chemical crosslinking, and stretching them mechanically. These interventions are designed to improve the fibres' structural attributes, which in turn enhances their biocompatibility profile. In addition, although electrospinning technology can be an effective approach for producing carriers with micro to nano scale fibre diameters, precise control over fibre structure remains a significant challenge. In clinical applications, delivery of electrospun materials loaded with exosomes to sites of ligament or tendon injury may also pose technical challenges, especially for localised treatment of deep tissue injuries. Moreover, the use of organic solvents, which can be toxic to humans, in the electrospinning process may leave residues with the potential to alter the biological effects of exosomes.[95] Finally, the lack of standardised preparation methods for electrospun materials loaded with exosomes has resulted in variations among different research groups, potentially affecting the comparability and reproducibility of their clinical applications.

Electrospun scaffolds loaded with exosomes hold great promise for the future, but further development and refinement are necessary to fully realize their potential. Future improvements in the application of electrospun scaffolds loaded with exosomes in tendon/ligament repair primarily include enhancement of biocompatibility and degradability of carrier materials, optimising the fabrication process to enhance the consistency and stability of fibre structure, and exploring new functional modification methods to promote the interaction between the carrier and exosomes. In addition, comprehensive clinical studies are needed to confirm their long-term effectiveness and safety in patients.

Discussion

This comprehensive review provides a nuanced examination of the current research landscape of exosomes and biomaterials in ligament and tendon repair. Although previous studies have investigated the potential of exosomes and biomaterials individually, our work sheds light on the exciting possibility that combining these approaches can create a powerful synergy, leading to improved tissue regeneration.

In this review, we have highlighted several gaps that need to be further investigated. One notable gap is the need for more targeted research to identify the exact mechanisms underlying the interaction between exosomes and specific biomaterials. Moreover, clinical trials are needed to explore the efficacy of different biomaterials in delivering exosomes.

Although exosome-biomaterial combinations have shown promising results, several challenges limit its clinical translation. Issues such as scalability, cost-effectiveness, and regulatory considerations are major challenges that need to be addressed to realize the full therapeutic potential of these novel approaches. Furthermore, researchers should aim to improve the long-term safety and biocompatibility of biomaterials.

The data reviewed here indicate that exosomes and biomaterials can be used to formulate innovative therapeutic strategies not only for ligament and tendon repair but also for a wide range of tissue injuries. Furthermore, the customizable nature of these approaches opens up exciting possibilities for personalised medicine and targeted therapies.

In future, interdisciplinary collaboration is advocated to resolve the remaining challenges in the field. Partnerships between researchers in biological fields, materials science, medicine, and engineering are encouraged to drive innovation and accelerate the clinical translation of research findings. Moreover, leveraging emerging technologies such as artificial intelligence and machine learning can optimise treatment outcomes and tailor therapies to individual patient needs (**Figure 6**).

The aim is to translate the promise of exosome-biomaterial therapies into effective treatments for patients. However, bridging the gap between lab research and real-world application remains a hurdle. To achieve this, collaboration between researchers, industry professionals, and regulatory agencies is essential. Working together will help navigate the complex path of bringing these therapies to the clinic. Prioritising patient-centered approaches that conform to regulatory standards will improve the safety and effectiveness of these groundbreaking therapies in clinical settings.

Conclusion and Outlook

Although the field of biomedical engineering has witnessed significant progress in the exploitation of exosome technology, there are still many challenges. This review demonstrates that combining exosomes with various biomaterials can repair ligaments and tendons. This convergence is expected to promote the development of regenerative medicine. By examining the intricate interactions between exosomes and biomaterials, we not only reveal new tissue repair mechanisms but also highlight important avenues for future research. The key lies in enhancing the integration of biomaterials with exosomes and fully leveraging the delivery capabilities of biomaterials. Beyond examining the inherent properties of biomaterials, such as minimising harm to the body, enhancing their capacity to carry therapeutic molecules, and improving their binding affinity to exosomes, we must also consider how these biomaterials interact with cells over relevant timescales. These modifications will improve the efficacy of therapeutic strategies for implementing personalised ligament or tendon regeneration. Combining exosomes with biomaterials holds promise for improving ligament and tendon repair outcomes. The utility of biomedical engineering in clinical settings remains an active area for further research, and it is expected to emerge as a powerful tool for tissue repair.

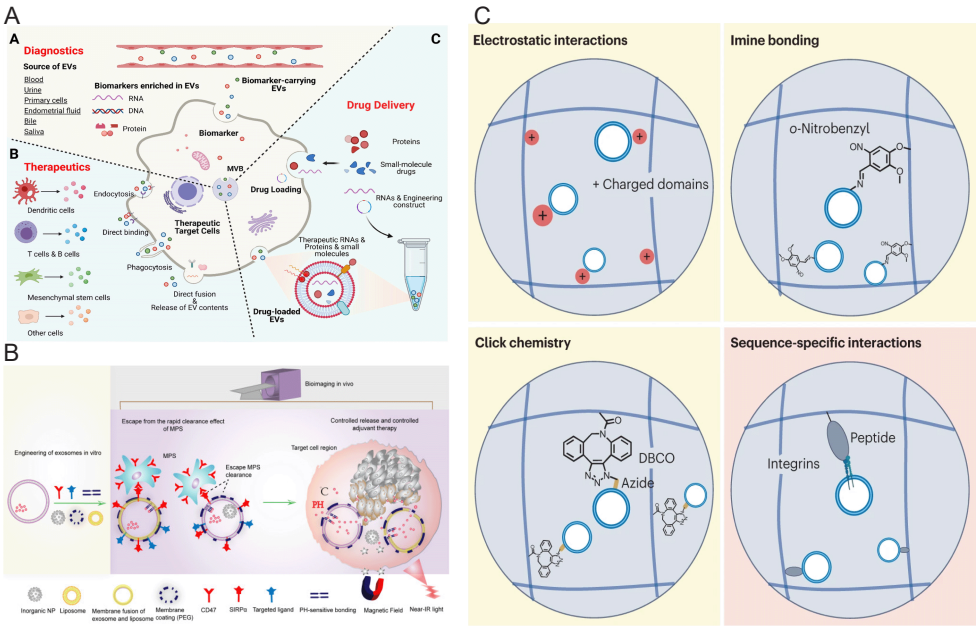

Figure 6. Future applications and prospects of exosomes-loaded biomaterials. (A) Potential applications of EVs. Reprinted from Escudé Martinez de Castilla et al.[96] Copyright 2021, with permission from Elsevier B.V. (B) Strategies for developing exosome-based multifunctional nano-delivery platforms. Reprinted from Zou et al.[65] (C) Biomaterial strategies for promoting EV retention. Reprinted by permission from Debnath et al.[97] Copyright 2023 Springer Nature Limited. C: cell; DBCO: dibenzocyclooctyne; EV: extracellular vesicle; IR: infrared; MPS: mononuclear phagocytic system; NP: nanoparticle; PEG: polyethylene glycol.

Author contributions

HW led the writing of the manuscript. YG, YG, HW, YJ, JZ, DS and GZ contributed to the writing and revisions of the manuscript. LW, and YK supervised the project. All authors approved the final version of the manuscript.

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Conflicts of interest statement

The authors declare no competing interests.

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