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Injectable hydrogels for sustained delivery of extracellular vesicles in cartilage regeneration

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ABSTRACT

Extracellular vesicles (EVs) are a population of small vesicles secreted by essentially all cell types, containing a wide variety of biological macromolecules. Due to their intrinsic capabilities for efficient intercellular communication, they are involved in various aspects of cellular functioning. In the past decade, EVs derived from stem cells attracted interest in the field of regenerative medicine. Owing to their regenerative properties, they have great potential for use in tissue repair, in particular for tissues with limited regenerative capabilities such as cartilage. The maintenance of articular cartilage is dependent on a precarious balance of many different components that can be disrupted by the onset of prevalent rheumatic diseases. However, while cartilage is a tissue with strong mechanical properties that can withstand movement and heavy loads for years, it is virtually incapable of repairing itself after damage has occurred. Stem cell-derived EVs (SC-EVs) transport regenerative components such as proteins and nucleic acids from their parental cells to recipient cells, thereby promoting cartilage healing. Many possible pathways through which SC-EVs execute their regenerative function have been reported, but likely there are still numerous other pathways that are still unknown. This review discusses various preclinical studies investigating intra-articular injections of free SC-EVs, which, while often promoting chondrogenesis and cartilage repair *in vivo*, showed a recurring limitation of the need for multiple administrations to achieve sufficient tissue regeneration. Potentially, this drawback can be overcome by making use of an EV delivery platform that is capable of sustainably releasing EVs over time. With their remarkable versatility and favourable chemical, biological and mechanical properties, hydrogels can facilitate this release profile by encapsulating EVs in their porous structure. Ideally, the optimal delivery platform can be formed *in-situ*, by means of an injectable hydrogel that can be administered directly into the affected joint. Relevant research fulfilling these criteria is discussed in detail, including the steps that still need to be taken before injectable hydrogels for sustained delivery of EVs can be applied in the context of cartilage regeneration in the clinic.

1. Introduction

Articular cartilage is a supporting connective tissue within joints that provides a smooth surface with a low friction between the ends of long bones [1–3]. It is mainly composed of slow-dividing chondrocytes (5–10% of total cartilage mass) that maintain the extracellular matrix (ECM) of the tissue, a firm gel containing collagens, proteoglycans and matrix proteins [3]. Unlike most bodily tissues, cartilage is avascular and alymphatic and has no neural supplies. Chondrocytes rely solely on the diffusion capacity of the required nutrients through the ECM. This

Abbreviations: AdMSC, Adipose-derived MSC; AFSC, Amniotic fluid-derived stem cell; BMSC, Bone marrow-derived MSC; cGMP, Current Good Manufacturing Practice; CRISPR, Clustered Regularly Interspaced Palindromic Repeats; CPC, Chondrogenic progenitor cell; ECM, Extracellular matrix; ESC, Embryonic stem cell; EV, Extracellular Vesicle; ICRS, International Cartilage Repair Society; ISEV, International Society of Extracellular Vesicles; MMP, Matrix metalloproteinase; MSC, Mesenchymal stem cells; OA, Osteoarthritis; OARSI score, Osteoarthritis Research Society International score; PEG, Poly(ethylene glycol); RA, Rheumatoid arthritis; SC-EV, Stem-cell derived EV; SEC, Size-exclusion chromatography; SMSC, Synovium-derived mesenchymal stem cell; TFF, Tangential Flow Filtration; UCSC, Umbilical cord stem cell; UTR, Untranslated region.

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reduced access to nutrients means that although cartilage is capable of transmitting heavy loads for a lifetime, it hardly recovers after injury or disease. In healthy individuals, the articular cartilage is maintained in joint homeostasis [2,3], but in case of illness the precarious balance between anabolic and catabolic activities of proteolytic enzymes is lost, which can slowly deteriorate cartilage tissue [2–4].

Rheumatic diseases, with osteoarthritis (OA) and rheumatoid arthritis (RA) being the most common disorders, represent a class of disabling conditions characterised by cartilage deterioration [2]. It is estimated that worldwide over 300 million individuals are living with OA [5], while RA has an incidence of 0.5 to 1% [5,6]. Herein, not only does the prevalence increase with age, also the experienced physical burden worsens over time [6–8]. Rheumatic diseases are considered the leading cause of years lost to disability among older adults, with progressive pain, significant functional disability and a reduced quality of life [1,6,7]. Due to rising obesity rates and an overall aging population, studies have predicted a vast increase in the prevalence of rheumatic diseases over the coming decades [6,8]. With currently only symptomatic treatment available, rheumatic diseases put an enormous strain on the healthcare system. Drugs that delay, stop or reverse disease progression have not yet obtained regulatory approval, although a few promising phase III clinical trials are currently ongoing [2,7,8]. One of such is the investigation whether intra-articular injections of platelet-rich plasma are as effective as hyaluronic acid or corticosteroids in maintaining knee joint mobility and reducing pain levels in patients with OA (ClinicalTrials.gov Identifier: NCT04980105). Although no results have been posted yet, it is not expected that cartilage will be fully regenerated after treatment, so patients would greatly benefit from a novel therapy that increases the self-healing capability of cartilage in the affected joints [4].

In this regard, the therapeutic potential of stem cells has captivated the attention of many [4,9–11]. These cells inherently take part in tissue repair and regeneration with their large self-renewal capacity and trans-differentiation properties. Mesenchymal stem cells (MSCs) are of particular interest, as multipotent stem cells are able to differentiate into several cell types that include chondrocytes, osteoblasts and adipocytes, among others [12]. Yet despite overwhelming initial potential, stem cell therapy has encountered some major limitations, including: 1) the necessity for a prolonged presence of stem cells with phenotypic stability at the site of injury, while <1% of transplanted cells reach or remain at target tissues [13]; 2) the safety concern of uncontrolled proliferation of transplanted stem cells, leading to unwanted tissue formation at the therapeutic site; 3) the potential for tumorigenesis and mutagenesis; 4) the risk of immunogenic complications and consecutive rejection of the stem cells; and 5) the high cost and expertise that comes with appropriate handling, storage, transportation and transplantation methods to prevent reduction of the stem cell treatment success rate [11,12]. For in-depth information on the challenges of conventional OA treatment, and research advances and limitations of stem cell treatment for regenerative medicine, readers are referred to recent reviews by Loo et al. [14] and Jiang et al. [15].

Part of these limitations to stem cell therapy could be overcome by the use of extracellular vesicles [16,17]. There is a growing body of evidence showing that the paracrine factors including EVs released by MSCs facilitate tissue repair, rather than the trans-differentiation of the transplanted MSCs themselves [10,12,18]. EVs are secreted by essentially all cell types, and relay signals from one cell to another [19–21]. They are a heterogeneous group of small vesicles with a phospholipid bilayer that encapsulate a wide variety of biological macromolecules from the parental cell. The fact that EVs play a role in intercellular communication through transport of sugars, lipids, proteins and nucleic acids makes them attractive not only for applications in drug delivery, but also regenerative medicine [22]. Their exceptional potential for regenerative use originates from their innate ability of transporting the paracrine factors that induce tissue repair [4,12,17]. However, clinical application is still hampered, partially due to their short half-life and low delivery efficiency. A delivery platform that provides local sustained release of EVs should therefore be designed. Herein, biocompatible hydrogels are of high interest. These materials composed of three-dimensional polymer networks capable of absorbing and retaining large amounts of water have been investigated in depth [3,23].

This review aims to provide an overview of research conducted in the area of injectable hydrogels for the sustained delivery of EVs in cartilage regeneration. To this end, the role of EVs in the regeneration of cartilage will be discussed, after which the use of different biomaterials for the sustained delivery of EVs is considered. Lastly, these two components are combined to investigate what has been researched thus far in the field. This elaborate overview allows for assessing whether EV-encapsulating hydrogels have potential in cartilage regeneration in humans, and which challenges still need to be overcome to enable their clinical application.

2. Extracellular vesicles in regenerative medicine

2.1. Biogenesis and role of extracellular vesicles in intercellular communication

In the past decades it has become clear that EVs play an important role in intercellular communication by transporting biological macromolecules from donor cells to recipient cells [20,21]. They are secreted from essentially all cell types as a heterogeneous population of vesicles that differ in size, cargo and membrane protein and lipid composition [24]. This discrepancy likely occurs due to the various pathways of EV biogenesis and intracellular cargo sorting machinery that are used to produce EVs. For instance, exosome biogenesis generally happens via inward budding of the endosomal membrane to form multiple vesicles inside, each with a diameter of approximately 30 to 150 nm [19,20]. These small vesicles are then secreted into the extracellular environment by fusion of the multivesicular body with the plasma membrane. Microvesicles on the other hand are a subpopulation of larger EVs, with a diameter of 50 to 1000 nm. They are formed entirely at the site of the plasma membrane, where cytosolic cargo is sorted inside the EVs before contractile machinery releases the microvesicles via membrane scission [19–21]. After secretion, the EV subpopulations that can be found in the extracellular environment together represent the phenotypic state of the parental cell [21,24]. A homogeneous culture of the same cell type can thus produce a wide array of different EVs, that are hypothesized to have different functions, though there are current technical challenges in separating the different EV subsets. Fig. 1 displays the heterogeneity of SC-EVs after secretion [25]. Some are suspected to act as mediators in pathological processes such as cancer or autoimmunity [26], and EVs in the synovium are implied to play a role in the pathophysiology of RA [17]. Contrastingly, EVs have also strongly been implicated to drive physiological and regenerative processes, such as wound healing and tissue repair [12,17,21].

Although the use of stem cells has been implicated in regenerative medicine for a few decades, evidence has been found that alongside the differentiating and self-renewal capacity of stem cells, stem cell-derived EVs also play an important role. While it has not been fully elucidated how SC-EVs are responsible for the regenerative effects in damaged tissues, it is hypothesised that they are an essential part of the paracrine effectors released by stem cells [27]. EVs have been shown to relay signals from cell to cell and exert their biological function in two ways: 1) they bind to the recipient cell where they interact with surface ligands on the plasma membrane to activate intracellular signalling cascades, or 2) they transfer their intraluminal cargo, likely through uptake by endocytic pathways followed by endosomal escape [19,20,28]. Both processes are supported by scientific literature, but the general consensus is that the mechanism is dependent on EV subpopulation (i.e. size, membrane protein composition and cargo content), EV donor cell source and phenotypic state of the recipient cell type [29].

In the past decade, a growing body of evidence has shown that the

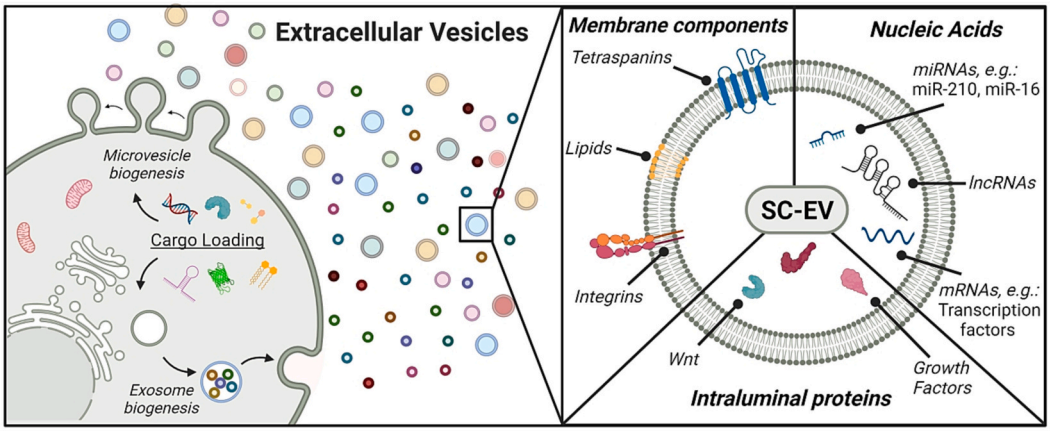

Fig. 1. Schematic representation of stem cell-derived extracellular vesicle formation. Different pathways of EV biogenesis including microvesicle and exosome generation lead to a widely heterogenic population of vesicles in terms of size, membrane composition and cargo. Examples of natural membrane components, as well as intraluminal cargo proteins and nucleic acids are depicted.

internalisation of EVs is a process dependent not only on the subpopulation and membrane composition of EVs, but that the cell type of the recipient cells is also implicated, as it appears that similar EVs are taken up through varying pathways in different cell types [29]. Correspondingly, protein or nucleic acid cargo may exhibit a distinct biological action when comparing the effects in different cell lines.

Due to the highly efficient manner in which EVs transport fragile RNA macromolecules, they are of interest in drug delivery of nucleic acid-based therapies [22]. For example, RNA therapeutics show great promise for disease modification, but their major limitation is the delivery to the site of action in the body. For one, they are unable to cross the hydrophobic cell membrane due to their negative charge [30,31]. Additionally, RNases that are ubiquitously present in the cellular environment break down exogenous RNA, leading to a very short half-life. Over the past few decades, numerous synthetic drug delivery systems have been designed to improve the pharmacokinetic and pharmacodynamic properties of biological drugs [32]. Still, due to limited targeting ability, rapid clearance from the body and potential for immunotoxicity and accumulation in the liver and spleen, synthetic nanoparticles still face many challenges in the transport of RNA therapeutics [22,32,33].

The intrinsic properties of EVs may overcome some of these limitations. As natural carrier systems, they have no inherent toxicity, and they use endogenous cellular machinery to produce, sort and encapsulate the desired cargo [22,34]. During transport in the extracellular environment, fragile EV cargo is protected from enzymatic degradation [29]. EVs have been shown to cross not only the cell membrane, but also intracellular and tissue barriers, such as the blood-brain barrier [22,35]. These features make EVs an interesting prospective nanocarrier for biological therapeutics, and worthwhile investigating for that purpose. Additionally, EVs have advantages over conventional gene therapy vectors such as lenti- or adeno-associated viruses (AAV) because of their low immunogenicity and reduced risk of mutagenesis. For example, therapies employing AAV viral vectors are limited to a single administration, whereas EVs can be used as a treatment multiple times [36].

2.2. EV-mediated tissue regeneration

2.2.1. Importance of EV donor and recipient cell type

The therapeutic properties of EVs in the context of regenerative medicine have been investigated in numerous preclinical studies. Since EVs represent the phenotypic state of their parental cells, it is likely that the EV donor cell source is an important factor in EV-mediated tissue regeneration. Yin et al. reviewed the use of different SC-EVs in diseased or damaged physiological systems, such as the neurological, respiratory, musculoskeletal and cardiological systems, liver, eyes, skin and kidneys [37]. Current research is mainly focused on the use of EVs derived from multipotent mesenchymal stem cells (MSC-EVs), though also (induced) pluripotent stem cells (iPSC-EVs), embryonic stem cells (ESC-EVs) and other adult stem cells are being tested to some extent [37]. Herein, MSCs can be obtained from various human tissues, specifically adipose tissue (AdMSC-EVs), bone marrow (BMSC-EVs), human umbilical cord (UCSC-EVs) or the synovial fluid (SMSC-EVs). In the application of stem cell therapy for cartilage regeneration, Tuan et al. reviewed the advantages and disadvantages for the use of each [4]. For example, while AdMSCs are easily accessible for isolation and ex-vivo expansion, their chondrogenic capacity is limited and they produce low levels of collagen type II. In contrast, BMSCs display a high level of collagen type II production, but the cell harvesting is more invasive. SMSCs have demonstrated the highest chondrogenic capacity, but considering the retained fibroblastic characteristics of these cells, there are risks for implementation in cartilage regeneration [4]. The main drawbacks may be mitigated when using MSC-EVs, but the effects of MSCs harvested from different tissues in the repair of cartilage have only been discussed in detail for whole cell-therapy. While Zhu et al. compared iPSC-MSC-EVs and SMSC-EVs in an OA mouse model [38], to our knowledge, there has been no comprehensive review studying more than two tissue sources. Considering how different studies test different EV dosages, it is difficult to assess which EV source is more potent in cartilage regeneration [39]. However, a recent study found that the tissue source of MSCs affects the bioactivity of MSC-EVs in glioblastoma, indicating that their functionality may indeed differ depending on the tissue source [40]. This knowledge should be taken into consideration for the application of MSC-EVs in cartilage regeneration. Although translation of whole cell-therapy effects to therapeutic SC-EVs could open new avenues for treatment of rheumatic diseases, the question which subpopulation of isolated SC-EVs has the largest regenerative effect remains unanswered. Most studies focus on investigating exosomes [41], but a comprehensive comparison of regenerative properties of EV subpopulations based on size or other characteristics have not been executed. Similarly, preconditioning of the MSCs prior to EV isolation may be relevant for increasing their regenerative potency, but more research is required to decipher the correct conditions for the appropriate disease model [39].

2.2.2. Natural MSC-EV cargo for tissue regeneration

One of the most interesting and important parameters in EV-mediated tissue regeneration is the EV cargo that facilitates tissue repair. It was found that MSC-EVs contain many different proteins [42] and a plethora of nucleic acids that include at least 60 types of micro RNA (miRNA) [43]. Since then, more proteomic analyses of MSC-EVs have been conducted, which were summarised by Qiu et al., who

identified over 4000 unique proteins [44]. Van Balkom et al. combined several proteomics studies to distinguish a unique protein signature of 22 hallmark proteins in MSC-EVs [45]. However, for most MSC-EV cargo components, the mechanistic details that underly their function in regeneration remain to be elucidated. As was mentioned by Velot et al., EV-related research is often limited by the choice of researchers to characterise EV-mediated effects, and not their content, membrane composition or the cargo responsible for said effects [46].

Many proteins on the EV surface are involved in intracellular cargo sorting, EV transport throughout the extracellular environment or the docking and uptake into the target cell [19]. The intraluminal and membrane cargo of MSC-EVs is thought to affect cell growth, migration and proliferation, immunomodulation, ECM modelling and tissue reorganisation [12]. The most well-known proteins carried by MSC-EVs involved in regenerative medicine are growth factors, polypeptides with the inherent ability to induce and promote cell growth and proliferation [47,48]. In cartilage regeneration, they have been found to enhance chondrogenesis, modulate the joint immune response and assist in treatment of cartilage defects [4]. Examples of MSC-EV-proteins involved in regeneration are members of the transforming growth factor (TGF) superfamily, fibroblast growth factor (FGF) family, insulin-like growth factor (IGF), interleukin-6 (IL-6) and platelet-derived growth factor (PDGF) [41,44]. MSC-EV-associated Wnt proteins and Wnt-related molecules are not only involved in the control of cartilage and bone homeostasis, but also regulate cartilage repair [49]. Herein, some have stimulating and others have inhibiting effects on chondrogenesis, growth plate assembly and columnar formation, hypertrophy, mineralisation and perichondral bone formation, as reviewed by Usami et al. in 2016 [49].

MSC-EVs may also stimulate tissue regeneration through transport of regenerative RNAs. An example herein is the induction of stem cell-like characteristics in target cells through transfer of messenger RNAs (mRNAs) encoding for stem cell-associated transcription factors. Ratajczak et al. found that treatment with ESC-EVs lead to an increase in early pluripotent (Oct-4, Nanog and Rex1) and early hematopoietic stem cell marker expression (Scl, HoxB4 and GATA 2) in hematopoietic progenitor cells [50]. Furthermore, MSC-EVs delivered proteins such as Wnt3 and Hedgehog, which are primarily expressed by stem cells [50,51]. The increase in concentration of these MSC-EV-delivered proteins was found to enhance target cell survival and proliferation.

BMSC-EVs transfer mRNA for the insulin-like growth factor-1 receptor to cisplatin-damaged proximal tubular epithelial cells (PTECs), thereby increasing tubular cell sensitivity to IGF-1 and enhancing cell proliferation. Co-incubation of damaged PTECs with IGF-1 and BMSC-EVs ameliorated cellular injury even further, providing a mechanism for the BMSC-EV-associated renoprotection [52].

In the context of cartilage regeneration specifically, upregulation of anti-apoptotic proteins and downregulation of catabolic genes was found to promote cartilage repair [53,54]. The factors regulating this mechanism have only been investigated in the context of renal regeneration by Lindoso et al. However, it is possible that their results can be extrapolated to the observed results in cartilage regeneration. They uncovered a pathway involving MSC-EV mediated transport of miRNAs and their transcriptional modulation [55]. miRNAs are noncoding pieces of RNA with a length of around 22 nucleotides, which have important functions in post-translational gene regulation [56]. In their study, PTECs exhibited changes in miRNA levels after incubation with MSC-EVs, which ultimately resulted in protection from cell death through downregulation of caspase-3, caspase-7, SHC1 and SMAD4 mRNA expression, which correlate with apoptosis, hypoxia and cytoskeleton reorganisation [55].

Cartilage regeneration by MSC-EVs is promoted through their chondroprotective nature. By EV-mediated downregulation of the expression of matrix degrading enzymes (e.g. matrix metalloproteinase (MMP)-1, MMPS-12 and IL-1b), cartilage tissue repair has been facilitated. However, the molecules behind this effect remain unknown, though it is thought to involve increased expression of IκBa, which inhibits the NFκB signalling pathway [57]. Similarly, evidence was found that MSC-EVs increased production of collagen type II, thereby preventing chondrocyte hypertrophy and improving ECM quality [58].

The overview of MSC-EV cargo components involved in tissue regeneration given above is not complete, but further detailed discussion of all relevant proteins and nucleic acids for this purpose is beyond the scope of this review. Keshtkar et al. give a broader overview of the encapsulated paracrine effector molecules through which MSC-EVs aid in tissue regeneration [59]. For an extensive summary about miRNAs involved in cartilage regeneration specifically, the reader is referred to the recent review by Foo et al. [41]. In the following sections, multiple EV-cargoes that have not been mentioned above will be discussed for their potential therapeutic efficiency in cartilage regeneration, as they have been investigated *in vivo*.

3. EV-engineering to enhance natural characteristics

In the past few years, the use of EV engineering for the application as nano-vehicles in biotherapeutic delivery has gained a lot of interest. EVs already naturally carry proteins and nucleic acids, but by employing techniques that allow for selective EV enrichment of compounds of interest, the possibilities for EV cargo seem limitless. EVs can be engineered in a multitude of ways, which can be generally divided in endogenous or exogenous modifications. Endogenous modification involves re-engineering of the EV-producing cell to increase biogenesis of EVs with the required characteristics. This can happen through genetic manipulation of the parental cell source, or through loading of the parental cell with drugs, proteins or other components prior to EV isolation. Environmental alterations such as hypoxia or mechanical stimulations during cell culture to achieve EV enrichment of specific cellular components can also be considered endogenous engineering of EVs. Exogenous engineering utilises mechanisms to introduce therapeutic agents into EVs after secretion, therein omitting the modification of the parental cell source of EVs. It can be divided in post-isolation cargo-loading and post-isolation EV modification. Fig. 2 illustrates the possible approaches for EV-engineering that can be applied to enrich EVs for the components of interest. In the following sections, each of these approaches will be explained by means of a few examples.

3.1. Genetic manipulation of the parental cell source

Prior to EV isolation, the parental cell source can be manipulated genetically to overexpress certain targets. While the techniques involved in the overexpression are usually similar, the goal of the overexpression can differ significantly. For example, researchers can increase cellular production of certain miRNAs, mRNAs or soluble proteins, with the aim to stochastically load more of these targets into EVs. However, it is also possible to modify expression of targets that are involved in EV biogenesis and cargo loading, to enrich EVs for a particular subpopulation. Lastly, overexpression of proteins or peptide sequences can also serve to target EVs towards a specific tissue in the body including cartilage.

3.1.1. Stochastic loading

Wei et al. published a study in which they used a lentiviral construct to overexpress the miRNA-181a in human UCSCs [60]. It was determined with qPCR that in both UCSCs and UCSC-EVs, the transduction had succeeded in upregulating the expression of miRNA-181a compared to a negative control lentivirus [60].

Che et al. temporarily overexpressed miRNA-143 in BMSCs using lipofectamine-mediated transfection [61]. EVs isolated from these BMSCs had upregulated expression of miRNA-143, and were found to suppress proliferation, migration and invasion of prostate cancer cells, while enhancing the apoptosis rate, through downregulation of the miRNA-143 target TFF3 [61].

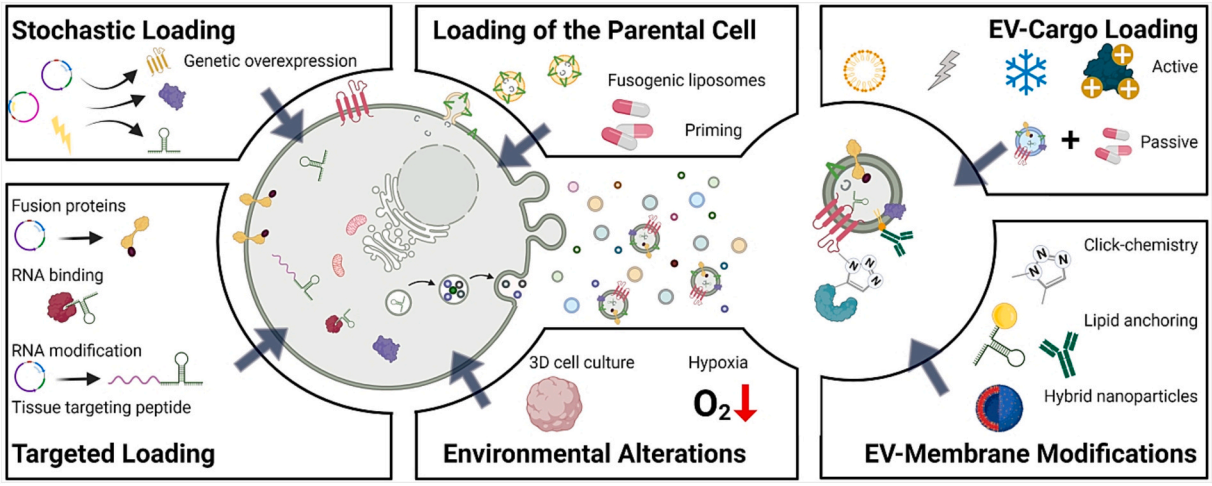

Fig. 2. Schematic overview representing different methods for EV engineering. Cells can be genetically manipulated for stochastic or targeted loading (left), stimulated exogenously through environmental alterations or have compounds delivered for EV integration (middle). This will result in EVs that are enriched with the desired components. After EV-isolation, cargo can be loaded into EVs in an active or passive manner (right), or the EV-surface can be modified with various components.

Kosaka et al. overexpressed the protein nSMase2 in HEK293T cells with the aim to increase the production of miRNA-containing EVs [62]. nSMAse2 is involved in the synthesis of ceramide, a lipid that is implicated in one of the EV biogenesis pathways [19,20]. Inhibition of nSMAse2 lead to similar expression levels of endogenous miRNA-16 and exogenous miRNA-146a in cells, but both miRNA levels in EVs were reduced with approximately 60%. Consistently, nSMAse2 overexpression increased miRNA secretion in EVs [62]. Since nSMAse2 exerts its function mostly by increasing EV release, but not by actively recruiting specific cargo into EVs, stochastic loading of EVs with certain targets can be influenced by up- or downregulation of nSMAse2.

3.1.2. *Targeted loading*

Targeted loading employs (over)expression of certain constructs that increase the loading efficiency of intended target cargo into EVs. mRNAs can be enriched into EVs by modifying the 3′-untranslated region (3'UTR) with a 25 nucleotide-long sequence. Bolukbasi et al. showed that mRNAs that are generally enriched in microvesicles compared to their parental glioblastoma cell line, have variants of this 25 nucleotide sequence [63]. Herein, the presence of a CTGCC sequence was crucial for this targeting 'zipcode', and a binding site for miRNA-1289 on the stem-loop structure further increased the mRNA enrichment in microvesicles. By designing zipcode-EGFP plasmids, EGFP mRNA was found to be significantly enriched in microvesicles [63].

Bonacquisti et al. had a similar approach [64]. From a large designed library, they selected an 8-nucleotide RNA-motif, which had a high sorting efficiency into exosomes. They then fused this RNA-motif to fluorogenic agents to track the movement of the RNA in the cell, and to determine how it was sorted into EVs [64]. While this engineering method does not have direct applications to regenerative medicine, it does provide insight in the mechanisms of RNA-cargo sorting and release, which in turn can be applied to loading EVs with therapeutic RNAs in the future.

Besides Bolukbasi et al. and Bonacquisti et al., more researchers have aimed to improve RNA loading into EVs [65]. Particularly, an engineering strategy that combines attachment of MS2 domains to EV membrane proteins with the structural modification of designed RNA, has been found to increase RNA loading into EVs. MS2 domains originate from the coat protein of the RNA bacteriophage MS2, and are able to bind RNA with a specific stem-loop structure. Promising results have been reported that show increased integration of RNA into EVs by allowing coupling of RNA to the genetically modified proteins with MS2 domains, but the RNA release kinetics from the MS2 domain once inside the EV or recipient cell, still require optimisation [65]. Hung et al. employed this system by engineering intended cargo RNAs with an MS2-stem loop, and fusing MS2 protein dimer to EV-associated CD63 to incorporate cargo RNA into the EV lumen [66]. Additional expression of the viral protein VSV-G increased the RNA sorting to up to 40-fold compared to the cytosol. Although this system was deemed effective in enriching EVs for small RNAs, the RNA cargo was found to be quickly degraded upon internalisation in recipient prostate cancer cells, demonstrating the limiting step in EV-mediated RNA transfer [66]. The above-mentioned techniques for increasing RNA sorting into EVs are not solely applicable to a single type of RNA. Indeed, because of the versatile methods to enrich EVs for large or small RNAs, they can be widely applied in regenerative medicine, adaptable for the desired target RNA.

Overexpression to increase endogenous loading can not only be applied to RNAs, but also to proteins. By tethering cargo of interest to fusion proteins that are often sorted into EVs, these linked components can be enriched in EV cargo. Dooley et al. designed a versatile platform that can be used to upregulate a wide variety of molecules in and on EVs [67]. By fusing GFP to various scaffold proteins that are preferentially packaged into EVs (either a tetraspanin, lipid anchor or single-pass transmembrane protein), it was determined that the PTGFRN and BASP1 showed the highest levels of GFP per EV. In fact, even specific truncated forms of these proteins were able to package GFP into the EV lumen with high efficiency due to their resistance against cleavage by EV protease ADAM10. Dooley et al. were also able to fuse cytokines and antibody-fragments to the extraluminal side of truncated and full-length PTGFRN. These particular fusions provide the option for cellular or tissue targeting of EVs, for example towards arthritic cartilage that could benefit from tissue regeneration. BASP1 was found to enable EV luminal loading of proteins with a size between 14 and 168 kDa [67]. By incorporating proteins implicated in cartilage regeneration, this technique could be used to engineer EVs as cartilage repairing agents.

While making use of CRISPR Cas gene technology could be yet another great opportunity for regenerative medicine, a drawback of the Cas9 protein is that it is only marginally incorporated into EVs naturally. Even though Cas9 sorting into EVs can benefit from targeted loading through fusion to a scaffold, a particular challenge is to release the Cas9 from the scaffold. Gee et al. designed a platform (NanoMEDIC) that incorporates chemical-induced dimerization of FRB-SpCas9 fusion protein with VSVG-fused FKB12-Gag in the presence of a rapamycin analogue to recruit Cas9 protein into EVs [68]. By also expressing a packaging signal

and two self-cleaving riboswitches, small guide RNA (sgRNA) can be tethered and released selectively into the EVs through targeted loading. In this engineered system, they determined that FRB and SpCas9 had to be fused on the N-terminal site of SpCas9 for increased gene editing functionality, likely because this construct disassociated more readily in target cells to release FRB-SpCas9 from VSVG-FKB12-Gag. While the platform was then tested for its gene editing capability in an *in vivo* Duchenne muscular dystrophy model, the advanced level of EV engineering shows potential for more applications [68]. In a similar approach, Yim et al. employed the protein-protein interaction between photoreceptor cryptochrome 2 (CRY2) and truncated CIBI protein to load cargo proteins into EVs [69]. Irradiation with blue light facilitated attachment, which was reversed upon removing the light source, thereby releasing the cargo protein. In this construct, CRY2 was fused to mCherry as the cargo protein, and CIBI was fused to EV-associated tetraspanin CD9. Besides mCherry, the authors also explored the technique with functional cargo proteins Bax, Cre, and IκB [69]. This technique could be adapted to incorporate proteins with regenerative capabilities into EVs, to be subsequently applied in tissue regeneration.

Yao et al. published a study in which they described a mechanism to enrich Cas9 ribonucleoprotein in EVs through targeted loading [70]. By inserting an RNA aptamer into the sgRNA sequence and fusing an aptamer binding protein to EV-associated CD63, they observed an increased loading of the sgRNA. Due to the intrinsic affinity between Cas9 protein and sgRNA, increased loading of sgRNA also enriched EVs for Cas9. The additional expression of VSV-G protein enhanced functional delivery of the complex by helping the Cas9 protein go through endosomal escape in recipient cells [70]. The increased loading of Cas9 protein can be beneficial not only in genetic diseases, but also in cartilage regeneration, for example by temporarily knocking down gene expression for genes or miRNAs involved in osteoarthritis.

3.1.3. Tissue and cellular targeting employing stochastic or targeted loading

Particularly useful for clinical application is the EV-engineering that aims to increase EV tropism. A variety of proteins are supposedly involved in the targeting of EVs towards certain organs, but the specific roles of most proteins remain unknown. Integrins (ITGs), proteins generally expressed on the cellular and EV membranes facing the extracellular environment, have been often implicated to be involved in the docking and uptake of EVs due to their interaction with a variety of both cellular and extracellular matrix proteins [19,20,71,72]. Besides that, integrins are implicated in the targeting of exosomes towards specific organs. Hoshino et al. published a paper in 2015 that showed strong evidence for the involvement of EV integrin patterns in metastatic organotropism of cancer [73]. For example, EVs with ITGα6, ITGβ1 and ITGβ4 were present abundantly on lung-tropic exosomes, whereas ITGβ5 was associated with liver-tropism, and ITGβ3 was expressed in exosomes isolated from cells with known brain-tropism [73]. In a study investigating the involvement of various cellular and EV-associated proteins, de Jong et al. showed that $ITG\beta_1$ was involved in functional delivery of sgRNA [74].

A perhaps more straightforward method for targeting toward specific tissues would be by engineering the EV membrane surface to present tissue targeting moieties. Alvarez-Erviti et al. designed a methodology to achieve tissue-specific delivery of therapeutic small interfering RNA (siRNA)-containing EVs [35]. Their general approach consisted of a muscle- or neuron-targeting peptide fused to an EV-associated protein. They engineered dendritic cells to temporarily express a construct containing Lamp2b fused to the targeting peptide prior to EV-isolation. After loading the EVs with siRNA through electroporation (see Post-isolation EV-cargo loading), they compared siRNA activity between Lipofectamine 2000-mediated transfection and incubation with modified EVs. They found similar siRNA delivery in murine muscle and neuronal cells, which decreased protein expression significantly [35].

Cheng et al. employed the fusion protein technique to produce EVs with multiple targeting moieties [75]. They designed a construct to functionally anchor antibody fragments against CD3 and epidermal growth factor receptor (EGFR) in the EV membrane, through tandem fusion with the transmembrane domain of the platelet-derived growth factor receptor. Herein, αCD3 targeted naïve T-cells, and αEGFR would locate EGFR-expressing tumour cells. Simultaneously, they expressed another fusion protein construct, to include the Programmed Cell Death Protein 1 (PD-1) and OX40L molecules on the EVs as well, both with the final aim to increase T-cell activation and induce targeted cancer immunotherapy [75].

A similar approach was investigated by Kooijmans et al., who linked EGFR-targeting nanobodies to a signal peptide for decay-accelerating factor (DAF). DAF is a glycosylphosphatidylinositol (GPI)-anchored protein that is naturally sorted into EVs with high efficiency, and by using a signal peptide derived from this protein, the nanobody was functionally anchored into the EV membrane at the site of a lipid raft [76]. Being able to target therapeutic EVs to the site of the damage is particularly useful for regenerative medicine and cartilage repair. Treatments can be given intravenously to improve patient comfort, or the EV retention can be improved primarily at the desired location in the body. Additionally, with improved targeting capabilities, the effective dose could be lowered, making the transition to clinical application easier.

3.2. Loading of the parental cell

Another method of packaging EVs with a component of interest, is to load the parental cells with said components. This has been applied with chemotherapeutic drugs, which were sorted into EVs by priming murine BMSCs with high concentrations of paclitaxel [77]. It was found previously that the used BMSCs have a remarkable resistance against paclitaxel, making this method appropriate for priming [78]. The EVs that were isolated through differential centrifugation, showed anti-tumour activity against a prostate cancer cell line, with an IC_{50} in cell viability at a protein concentration between 0.047 and 0.095 mg/mL. This effect was largely due to the paclitaxel associated with the EVs, at 11.68 ng paclitaxel per mg of proteins, although non-primed BMSC-EVs too showed 30–40% loss of tumour cell viability at a protein concentration of 0.38 mg/mL [77].

Lee et al. described a methodology using membrane fusogenic liposomes carrying various components to equip EVs with fluorophores, drugs, lipids or chemicals [79]. The parental MSC line was loaded with lipophilic and hydrophilic agents by allowing liposomes to fuse with the cellular membrane. With confocal microscopy it was shown that lipophilic dye was localised in the plasma membrane, whereas hydrophilic dye was transferred to the cytosol. The EVs that were isolated afterwards showed colocalization of both dyes. In a similar manner, they were able to enrich EVs with azide-lipids on the EV-membrane, to allow for a versatile platform that could incorporate different components inside and outside of EVs using copper-free click chemistry (see Post-isolation EV modification) [79]. Loading of the parental cell as an EV-engineering technique can be essential for incorporating unnatural proteins, lipids or drugs as EV-cargo. Encapsulating them into EVs through established cellular pathways as opposed to physical methods will still improve the component's pharmacokinetic profile in comparison to not encapsulating them, while not disturbing EV-integrity. These techniques thus have plenty putative uses in regenerative medicine.

3.3. Environmental alterations

Environmental alterations such as hypoxia or a 3D instead of 2D culturing method prior to EV isolation, can affect cargo content of EV subpopulations. EVs isolated from a 3D cell culture were shown to both have a vastly increased EV production of up to 7.5-fold higher [80], and display a different small RNA and protein profile compared to 2D cell culture [81].

The effect of hypoxia on EV content and functionality has been

investigated in multiple instances. MSC-EVs isolated after hypoxia were shown to have increased miRNA-210 expression compared to normoxic EVs, which was found to have cardioprotective effects after myocardial infarct *in vitro* and *in vivo* [82]. In another study, hypoxia led to the production of hUCSC-EVs with enrichment for miR-146a-5p, causing a lung protective effect in asthmatic mice [83]. An extensive review about the effects of hypoxia on EV-production and functionality was written by Bister et al. [84].

3.4. Post-isolation EV-cargo loading

Loading of EVs with cargo post-EV-isolation has been commonly used in the past few years. This has led to the development of a variety of methods to enrich EVs for RNA, low molecular weight drugs or dyes after isolation. An example is passive loading, in which incorporation of hydrophobic compounds into the EV membrane relies on passive diffusion during highly optimised co-incubation. This technique has been applied for various drugs, including paclitaxel [85], curcumin [86] and celastrol [87], but also for siRNA conjugated with hydrophobic cholesterol [88].

EV-cargo loading through electroporation subjects a mixture of EVs and intended cargo to a high voltage electric charge, inducing the formation of transient pores in the EV-membrane. This procedure temporarily permeabilises the EVs, to allow incorporation of new components into the EV lumen [89]. Doxorubicin, a chemotherapeutic drug with high cardiotoxicity, has been shown to have enhanced *in vitro* potency when encapsulated in EVs utilising a highly optimised electroporation protocol [90,91]. Albeit controversial due to the potential for siRNA precipitation during electroporation which causes overestimation of the siRNA-amount packaged in EVs [92], the post-isolation loading technique has been applied to load EVs with nucleotides. An example herein is the study by earlier mentioned Alvarez-Erviti et al., who loaded brain-targeting EVs with siRNA to knockdown a therapeutic target for Alzheimer's disease in wild-type mice [35]. Linear exogenous DNA smaller than 1000 base pairs could also easily be incorporated into EVs with electroporation, yielding hundreds of DNA molecules per vesicle on average, although the authors mentioned that they failed to observe functional gene delivery using this method [93].

Another method by which exogenous loading can be achieved is by chemical interruption of the EV-membrane with the help of surfactants. By co-incubation of EVs, intended payload and the detergent saponin, the EV-membrane is temporarily permeabilised [89]. Porphyrins with different degrees of hydrophobicity were loaded into EVs using saponin, which allowed an up to 11 fold higher incorporation than passive loading [94].

Rapid freeze-thawing cycles can also be used to encapsulate exogenous components. It should be noted that this technique can result in a damaged EV-membrane, which may cause a reduction in the EV-functionality [95]. Nonetheless, this approach was applied to incorporate the potent antioxidant catalase in monocyte- or macrophage-EVs, yielding enriched EVs that showed a good uptake profile *in vitro* [96].

The above described techniques for exogenous loading of EVs have been relatively well-established, but novel methods for increased loading of components into EVs are continuously being developed. A recently reported method utilises synthetically reprogrammed proteins containing an enhanced amount of positively charged amino acids, to increase cellular uptake of negatively charged DNA species. These so-called 'positively supercharged' proteins were created by modulating the lysine/arginine ratio of the exposed surface of GFP, and associated with the EV-membrane after co-incubation [97].

Unlike many active loading strategies where biotherapeutic cargoes are loaded using specific EV-associated proteins or sequences, many post-isolation loading strategies are not limited to the EV subpopulations that are associated with these moieties. As such, it is hypothesized that EV-cargo loading after isolation presumably has the advantage that cargo is not incorporated in specific subpopulations, as would likely happen when parental cells selectively sort components into specific EVs. Instead, varying subpopulations could be enriched with the desired component.

3.5. Post-isolation EV-modification

In addition to loading EVs with new components in the lumen, the EV-surface can also be modified after isolation. An exemplary method herein is click-chemistry. By administering unnatural metabolic precursors (e.g. azide-containing sugars or lipids) to EV-producing cells, these moieties are incorporated into EV membranes through glyco- or lipoproteins. Through azide-alkyne cycloaddition, perhaps better known as click chemistry, several studies have investigated attaching fluorescent dyes or proteins with a reactive group to EVs. For example, the earlier mentioned study by Lee et al. managed to incorporate azide-containing lipids into cellular membranes, using fusogenic liposomes. They then employed copper-free click chemistry to attach fluorescent dyes, which allowed them to track *in vitro* EV cell uptake, and *in vivo* biodistribution of these engineered EVs [79]. Di et al. approached EV-associated click chemistry in a different manner, by installing maleimide moieties on the EV-surface through hydrophobic insertion [98]. Showing good reactivity to thiol-containing functional groups, maleimide acts as a handle for click chemistry, connecting to thiol-containing fluorescent dyes, magnetic particles and gold nanoparticles.

By conjugating siRNA to cholesterol (cc-siRNAs), O'Loughlin et al. managed to load the therapeutic RNA into EVs using an optimised but scalable method involving co-incubation of cc-siRNA and EVs in a ratio of 15:1. This method facilitated concentration-dependent, siRNA-mediated knockdown *in vitro* [99].

Lipids could also be used to anchor antibodies or nanobodies to the EV-surface after EV-isolation. Kooijmans et al. generated recombinant fusion proteins linked to EGFR-targeting nanobodies to induce targeting towards EGFR-overexpressing cancer cells [100]. By making use of the phosphatidylserine-binding domains of lactadherin, the fused nanobodies had a high affinity for phosphatidylserine on the EV membrane, and readily associated to them without affecting EV size, integrity or functionality, while enhancing the uptake into target cells [100].

Membrane-surface engineering was also applied by Antes et al., who aimed to target EVs towards injured tissue by embedding the EV-surface with a streptavidin-conjugated anchor [101]. Streptavidin was coupled to glycerol-phospholipid-PEG conjugates, which were anchored into EVs upon co-incubation to allow for binding of biotinylated compounds. They demonstrated that this platform could bind various types of biotinylated molecules, including but not limited to cell-specific peptides and tissue-targeting antibodies [101].

Aside from EV engineering to adapt the cargo of EVs either via exogenous or endogenous loading, there are other ways to modify natural EV characteristics. In recent years, more research has been conducted to combine the favourable properties of synthetic liposomes and natural EVs into a hybrid nanoparticle [102]. Liposomes are capable of encapsulating and transporting therapeutics involving RNA interference, but have limitations in terms of delivery efficiency. The intrinsic properties of EVs overcome these hurdles, but have a relatively low loading capacity for exogenous cargo, even when utilising EV-engineering. Evers et al. studied hybrid nanoparticles formed from SKOV3-EVs, liposomes and siRNAs labelled with Alexa Fluor 647 [102]. They found that these hybrids had a limited toxicity in HEK293T, U87-MG and SKOV3 cells, and are able to deliver siRNA, though with a reduced potency compared to liposomes in SKOV3 and HEK293T cells. More interesting for the purpose of regenerative medicine is that the hybrids, formed from liposomes and cardiac progenitor cell-derived EVs, retain the regenerative properties found in EVs alone [102]. Sato et al. engineered similar hybrid nanoparticles by fusing the EV membrane with liposomes using a freeze-thaw method [103]. They isolated EVs from HER2-overexpressing cells to enrich the fused liposome-EVs by stochastic loading, thereby showing that genetic modification can be

combined with membrane engineering, creating a novel platform for rational EV design for drug delivery [103].

By using post-isolation EV functionalisation with biotherapeutics, the regenerative potential of MSC-EVs in the treatment of rheumatic diseases could be further optimised. Taking into account that the techniques described above have a high degree of versatility, there are many possible applications even within cartilage regeneration, by coupling different siRNAs, proteins or compounds to EVs.

4. EV-isolation and scale up

In the past few decades, many researchers have ventured into the field of extracellular vesicles. This wide interest has led to a large amount of high-impact publications that propose new roles for EVs in health and disease; introduce mechanisms for biogenesis or uptake; or show new methodologies for EV-engineering or EV-isolation, among other topics. This has resulted in variety of different experimental systems, parental cell sources and instruments used for isolation, causing a degree of heterogeneity that could hamper comparison between experiments [104,105]. The International Society of Extracellular Vesicles (ISEV) has therefore published a position statement in 2014 and 2018 to provide an overview of methods and criteria for EV-isolation and analysis, based on current best-practise [104,106]. While there is no general consensus of optimal isolation conditions, the following section will briefly explain the most-used EV-isolation methods and give a short review on their yield, purity, functionality and upscaling potential to industrial isolations for clinical application.

4.1. High yield, low purity

There is a precarious balance between yield and purity of isolated EVs. Methods utilising polyethylene glycol (PEG)-precipitation as the major EV-isolation procedure yield the highest amount of extracellular material, but this also includes material of a non-vesicular nature, resulting in 'impure' EVs [39,104,106]. In PEG-precipitation, PEG will act as a solvent 'sponge', thereby reducing availability of aqueous solution for all dissolved components. By increasing the concentration of PEG, the effective EV and protein concentrations in the medium will also increase, until solubility for one of the components is exceeded [107]. Precipitated EVs and other components can then be collected through centrifugation. Commercially available EV-isolation kits often make use of PEG precipitation to separate EVs from contaminants based on their solubility [39].

Another high recovery, low specificity EV-isolation method is one-step ultracentrifugation. In this method, conditioned medium is collected and centrifuged at high speed for a lengthy amount of time. Because there is no filtration or low-speed centrifugation step prior to ultracentrifugation, this technique will pellet all vesicular and protein material, including cell debris, leading to many impurities.

4.2. Intermediate yield, intermediate purity

Unlike PEG-precipitation or one-speed ultracentrifugation, methods such as size-exclusion chromatography, differential ultracentrifugation and ultrafiltration have an intermediate recovery and purity. Large cellular debris is likely removed from the conditioned medium, but isolations might include free proteins that contaminate the EVs. Perhaps the technique most commonly used for the isolation of smaller EVs, is differential ultracentrifugation [39,108]. This method is based on separating EVs from contaminants present in cultured medium by serial centrifugation steps using various speeds. The lower speeds function to remove cells and larger cellular debris, while the lengthy, high-speed centrifugation will isolate the EVs [105]. Between centrifugation steps, the supernatant is collected and transferred to a new tube to avoid carry-over of contaminants. Considering that large EVs (e.g. microvesicles) and cargo-heavy exosomes are pelleted at lower speeds, differential ultracentrifugation primarily isolates small EVs, although perfect separation is as of yet not realistic [105].

Size-exclusion chromatography (SEC) separates EVs from proteins and other contaminants based on their size. A SEC column is usually filled with a porous polymer material as the stationary phase that allows the contaminated sample to run through at different speeds, based on their size [39,105,109]. Particles with a small size (e.g. proteins) enter the pores of the matrix during chromatography, thereby slowing down. EV particles on the other hand, will elute faster due to their larger size and their subsequent inability to enter the pores of the matrix. However, complete separation of EVs and proteins is unlikely, as the sizes of protein aggregates and smaller EVs can overlap.

Similar to SEC, ultrafiltration also isolates EVs based on their size [39]. Generally, EVs are separated from contaminants through a series of filtering steps utilising membranes with different pore sizes. Herein, small particles are allowed to pass through the filter, whereas large particles are excluded. Tangential flow filtration (TFF), a type of ultrafiltration that employs a cross-flow mode, has an advantage over regular ultrafiltration in the sense that due to the horizontal flow, the odds of membrane clogging are much smaller [39]. However, in both regular ultrafiltration and TFF there is a possibility that EVs adhere to the filter membrane, thereby reducing recovery rates [105].

4.3. Low yield, high purity

Techniques such as density gradient centrifugation or immune capture can recover specific EV subtypes with high specificity, though generally only with a low yield compared to the total amount of EVs present in the starting material [104,106]. Density gradient ultracentrifugation is a method that is able to separate specific subpopulations of EVs from contaminants (cells, cellular debris, other EV subpopulations), based on different floating densities. The density gradient is usually established with sucrose or iodixanol solutions that have varying densities. After adding sample to the gradient, ultracentrifugation for at least 16 h will facilitate separation of EV populations [110], though some EV subpopulations have been shown to take a longer time to reach equilibrium density [111,112]. Most EVs have a buoyant density of 1.23 to 1.16 g/L [108].

Because of the distinct protein expression of EV subpopulations, specific EV classes can be isolated by means of immunoaffinity [39,105]. EV isolations can be specifically enriched or depleted for a certain population, using antibodies against surface markers that are bound to microbeads or other matrices. Under low-speed centrifugation or the application of a magnetic field, EVs bound to the beads can then be specifically isolated [105]. However, while this method leads to highly enriched EV populations, the recovery is generally very low compared to the total amount of EVs present in the conditioned medium. Moreover, using antibodies targeting specific proteins may result in the isolation or exclusion of specific EV subpopulations.

Another approach to obtain EVs with a high specificity, is through the combination of different methods described above [104,106]. Examples are the combination of ultrafiltration and SEC [113], or ultrafiltration followed by one-step ultracentrifugation. Unfortunately, increasing the purity of EVs during isolation often goes hand in hand with reduced yield and loss of EV functionality [39,105,106]. However, it is still under debate whether this loss in functionality is caused by EV damage or the removal of specific subpopulations, protein corona, or functional contaminants.

4.4. Functionality and potential for upscaling

Not only the yield and purity of the EVs are affected by the method of isolation, also their protein composition and functionality may change depending on the technique used. For example, centrifugations at high speed during EV-isolation can induce aggregation of EVs of different phenotypes, as was shown by flow cytometry and electron microscopy

with receptor-specific gold labelling by Linares et al. [114]. A finding of similar consequence was reported by Mol et al., who compared the functionality of cardiomyocyte progenitor cell-derived EVs isolated using ultracentrifugation or SEC. They reported a higher functionality of SEC-EVs, possibly resulting from the high shear forces that the EVs are subjected to during isolation with ultracentrifugation, which might lead to vesicle damage and subsequent loss of functionality [109]. Concordantly, SEC is considered an isolation method that, while time-consuming and labour-intensive, provides minimal damage to isolated EVs, as was shown in the context of UCSC-EVs by Monguió-Tortajada et al. [115]. This allowed them to retain their inhibitory function on T-cell proliferation, contrary to UCSC-EVs isolated by differential ultracentrifugation. Additionally, SEC is relatively easy to scale-up, although this requires including a method to concentrate the EV-containing solution (e.g. TFF) due to the limited sample volume that can be applied to SEC. Nonetheless, the preserved functionality and potential for upscaling makes SEC a promising technique for clinical applications in the future.

While PEG precipitation is considered a crude and non-specific technique [107], a study has demonstrated that this technique preserves EV-associated proteins during isolation better than differential ultracentrifugation [116]. However, while not labour-intensive and easily scalable, the many co-isolated contaminants hamper this method of EV-isolation for therapeutic applications. Contrastingly, isolations using ultrafiltration allow for more control over the environmental conditions during purifications, which is much more promising [108].

5. EV-therapy for cartilage regeneration

In the past decade, the application of therapeutic MSC-EVs for utilisation in tissue regeneration has become an increasingly popular field of research. So far, MSC-EV-mediated cartilage repair has been studied both *in vitro* and *in vivo*, resulting in a variety of review articles that provide an overview of the field [117–120]. The following sections will summarise relevant papers published since 2018, which have investigated the use of therapeutic EVs for the purpose of cartilage regeneration *in vivo* without the use of a scaffold. Herein, articles are separated based on the contribution of EV-engineering in the EV formulation intended for therapeutic applications. Aside from the obtained outcomes, special attention will be paid to the animal model, parental cell source of EVs, EV isolation method, distinct EV characteristics and limitations of the study, as summarised in Table 1.

5.1. EV-therapy without EV-engineering

He et al. demonstrated that BMSC-EVs are able to prevent cartilage damage and relieve knee pain in a rat model for OA [121]. Male rats were given an intra-articular injection with sodium iodoacetate to mimic the effects of OA in the knee. BMSC-EVs were isolated via ultracentrifugation and injected intra-articularly once weekly, starting one week after establishing OA. After six weeks, the pain levels of rats were evaluated before knee joints were collected for further study. EV-treatment was found to significantly relieve pain, prevent further cartilage damage and promote ECM synthesis compared to disease control. Supplemental *in vitro* studies showed that BMSC-EVs were able to attenuate the effects of inflammatory IL-1β-mediated up- and down-regulation of catabolic and anabolic markers, respectively. Additionally, BMSC-EVs improved chondrocyte proliferation and migration after IL-1β-treatment, and also increased growth factor and proliferation marker expression. Though the results of this study were promising, the required weekly injections and the unknown time span of protective effects are potential limitations which should be investigated [121].

Zavatti et al. compared the therapeutic effects of amniotic fluid stem cells (AFSCs) to AFSC-EVs in a rat model of OA [122]. OA was established three weeks after intra-articular injection of 2 mg/100 μL/knee monoiodoacetate. AFSCs were isolated from the amniotic fluid of pregnant women in the 16th or 17th week of their pregnancy, and expanded before EV isolation with EV isolation kit. Rats were injected intra-articularly with vehicle control (phosphate buffered saline (PBS)), AFSCs or AFSC-EVs (100 μg) twice, with a period of ten days between injections. They demonstrated that three weeks post-treatment, AFSCs and AFSC-EVs treated rats showed a pain threshold comparable to healthy control and significantly different from OA control rats. Corresponding results were found upon H&E staining of the collected joints, where AFSC-EV-treated OA joints displayed near complete tissue repair. In AFSC-treated joints, a few superficial fissures remained. Overall, authors concluded a superior therapeutic effect of AFSC-EVs compared to AFSCs, though they name the required repetition of EV injection as a limitation [122].

Due to the activated platelets in activated platelet-rich plasma (PRP), there is an abundant presence of growth factors and cytokines. Liu et al. devised a way to compare the therapeutic effect of PRP-EVs and activated PRP in an *in vivo* model of OA [123]. Six weeks after surgically establishing OA in rabbits, the animals were treated by intra-articular injection for six weeks with one-week intervals. Immunohistochemistry analysis demonstrated that PRP-EVs reversed the OA-mediated reduction in collagen II and Runt-related transcription factor 2 (RUNX2) protein expression, induced cartilage repair and overall inhibited OA progression to a higher extent than activated PRP. The chondrocyte count and OARSI score confirmed these observations, showing that while OA is not fully ameliorated with PRP-EVs, there is a significant improvement of joint condition compared to OA control and activated PRP-treated animals. Supplementary *in vitro* experiments showed a decrease in pro-inflammatory Tumour Necrosis Factor alpha (TNF-α) after PRP-EV or activated PRP treatment, potentially through the Wnt5/β-catenin pathway [123].

In an *in vitro* model of cartilage inflammation, SMSC-EVs were found to largely reverse the IL-1β-induced chondrocyte apoptosis and inflammation through EV-mediated transport of the miRNA-26a-5p [124]. By targeting the phosphatase and tensin homolog phosphatase (PTEN), miRNA-26a-5p likely exerts its function by compensating for the PTEN upregulation in chondrocytes caused by IL-1β. These observations were confirmed *in vivo*, where Lu et al. simulated OA by complete transection of several ligaments and meniscus in the knee joint in rats. A comparison of regular SMSC-EVs and SMSC-EVs with a knockdown of miRNA-26a-5p demonstrated that the isolated EVs aid cartilage regeneration by reducing inflammation and apoptosis in treated rats compared to defect control. However, the contribution of miRNA-26a-5p appears to significantly enhance these effects [124].

Aside from miRNAs, long non-coding RNAs (lncRNAs) are also implied as effectors in cell-to-cell communication [125]. To verify whether the exosomal lncRNA KLF3-AS1 from MSC-EVs plays a role in cartilage regeneration *in vivo*, Liu et al. used MSC-EVs from cells with knocked down expression of lncRNA KLF3-AS1. *In vitro*, lncRNA KLF3-AS1 knockdown inhibited the intrinsic abilities of MSC-EVs to upregulate aggrecan and collagen II, and downregulate MMP-13 and RUNX2 expression in chondrocytes exposed to IL-1β. Correspondingly, rats with a collagenase-induced cartilage defect that were intra-articularly injected once with regular MSC-EVs, displayed less severe cartilage degradation compared to cartilage defect controls, and MSC-EVs with the KLF3-AS1 knockdown. These parameters were studied in terms of Mankin score, percentage of proliferating cell nuclear antigen (PCNA) positive cells, and mRNA and protein expression of Col2A1, aggrecan, MMP-13 and RUNX2 [125].

5.2. EV-therapy including endogenous EV-engineering

EV-engineering through stochastic loading in the context of cartilage regeneration was applied by Mao et al., who overexpressed miRNA-92a-3p in human BMSCs and normal and OA primary human chondrocytes [126]. They found that miRNA-92a-3p expression was elevated in EVs isolated from chondrogenic BMSCs, while on the other hand, miRNA-

Table 1
Overview of relevant research conducted research since 2018 with regards to free EV-therapy for cartilage regeneration.

Reference	Animal model	EV cell source	Isolation method	EV engineering	Route of administration, dosage	Comparison
He et al., 2020 [121]	Rat, injection of sodium iodoacetate.	Rat BMSCs	Ultracentrifugation		IA injection with 40 μg/100 μL. Weekly for 6 weeks.	1. Healthy control 2. Disease control 3. OA + EVs
Zavatti et al., 2020 [122]	Rat, injection of mono-iodoacetate	Human AFSCs from women pregnant 16–17 weeks	Centrifugation and EV-isolation kit		IA injection with 100 μg/50 μL on days 21 and 31.	1. Disease control 2. OA + cells. 3. OA + EVs
Liu et al., 2019 [123]	Rabbit, surgical destabilisation	Rabbit PRP	EV-isolation kit		IA injection, 100 μg/mL, once a week for six weeks.	1. Healthy control + saline 2. Disease control + saline, 3. OA + PRP-EVs, 4. OA + activated PRP
Lu et al., 2021 [124]	Rat, surgical destabilisation	Human SMSCs	Ultracentrifugation		IA injection with 30 μL, 10^11 particles/mL on days 7, 14 and 21	1. Healthy control, 2. Disease control, 3. OA + EV formation inhibitor, 4. OA + EVs, 5. OA + EV-sham-miRNA 6. OA + EV-miRNA-inhibitor
Liu et al., 2018 [125]	Rat, collagenase II induced arthritis (COIA)	Human MSCs	EV-isolation reagent and centrifugation		Single injection, unknown dosage.	1. Healthy control, 2. Disease control, 3. OA + PBS, 4. OA + MSC-EVs, 5. OA + MSC-si-KLF3-AS1-EVs
Mao et al., 2018 [126]	Mouse, collagenase VII induced arthritis (COIA)	Human BMSCs	Ultracentrifugation	miRNA-92a-3p enrichment by induction of MSC chondrogenesis	Injection with 15 μL 500 μg/mL on days 7, 14 and 21.	1. Healthy control, 2. Disease control, 3. MSC-EVs, 4. MSC-92a-3p-EVs,
Wang et al., 2018 [127]	Rat, surgical destabilisation	Rat MSCs	EV-isolation kit	miRNA-135b enrichment by induction of MSC chondrocyte differentiation with TGF-B1	IA injection 100 μL, 10^11 particles/mL	1. OA + MSC-EVs, 2. OA + TGF-B1 MSC-EVs, 3. OA + TGF-B1 MSC-NC-EVs, 4. OA + TGF-B1-miR135b inhibitor-EVs
Pan et al., 2021 [128]	Rat, collagenase II induced arthritis (COIA)	Human MSCs	EV-isolation kit	lncRNA malat-1 enrichment by lentiviral overexpression in hMSCs	IA injection, 40 μg/100 μL, once a week for six weeks.	1. Healthy control, 2. Disease control, 3. OA + hMSC-EVs, 4. OA + hMSC-malat-1-EVs
Rong et al., 2021 [129]	Rat, surgical destabilisation	Rat BMSCs	Differential, filter- and density gradient (ultra) centrifugation	Hypoxic pre-treatment of BMSCs prior to isolation, enrichment of miRNA-216a-5p	Injection with 200 μL (200 μg total protein of Evs)	1. Disease control 2. OA + BMSC-EVs, 3. OA + Hypoxic-EVs, 4. OA + miRNA-NC Hypoxic-EVs, 5. OA + miRNA KD hypoxic-EVs,
Yan et al., 2020 [80]	Rabbit, cartilage defect (CD)	Human UCSCs	Differential (ultra) centrifugation	Cells grown in a 3D culture in a hollow fibre bioreactor	IA injections, 10^10 particles/mL, 500 μL, once weekly, 4 weeks	1. Defect control + PBS, 2. CD + 2D-exosomes, 3. CD + 3D-exosomes
Wang et al., 2020 [130]	Mouse, surgical destabilisation	Mouse Chondrogenic progenitor cells, from CBA mice or MRL/MpJ mice	Differential (ultra) centrifugation	Organism level, MRL/MpJ 'superhealer' mice	IA injection, 8 μL 10^10 particles/mL in PBS. Once weekly, from week 4 to week 7 after surgery	1. Healthy control, 2. Disease control, 3. OA + control-CPC-EVs, 4. OA + MRL/MpJ-CPC-Evs
Song et al., 2021 [131]	Rat, collagen-induced arthritis (COIA)	Mouse macrophages	Differential (ultra) centrifugation	Loaded with EGCG by sonication	IV injection on day 30, 32, 34, 36, 38, 40, 42, 44, 46, 48.	1. Disease control, 2. OA/RA + EGCG, 3. OA/RA + EV-EGCG
Li et al., 2021 [132]	Rat, full-thickness cartilage defect model	Rat AdMSCs	Differential centrifugation, PEG precipitation and ultracentrifugation	Conjugated to chitosan oligosaccharides by passive loading with shaking	IA injection, 100 μg, once a week, 8 times.	1. Healthy control, 2. Defect control, 3. CD + Chitosan oligosaccharide, 4. CD + AdMSC-EVs, 5. CD + EVs-COS
Topping et al.	Mouse, antigen induced RA	Human polymorphonuclear leukocytes (neutrophil)	Differential centrifugation	Modified with injured cartilage-targeting	IV injection, 6.0 *10^5 EVs, 22 days after start of RA induction.	1. Healthy control 2. Disease control 3. RA + EVs

(continued on next page)

Table 1 (*continued*)

Reference	Animal model	EV cell source	Isolation method	EV engineering	Route of administration, dosage	Comparison
2020 [133]				antibody, anti-TNF and anti-IL10		4. RA + EV-antiROS-CII-antiIL10 5. RA + EV-antiROS-CII-antiTNF/ IL10
Zhang et al., 2019 [135]	Rat, monosodium iodoacetate	Human ESC-MSCs	TFF concentration		IA injections, 100 μg/50 μL. once weekly, 2 4 or 8 weeks.	1. Disease control 2. OA + EVs, 3. Healthy control
Zhang et al., 2018 [53]	Rat, osteochondral defect	Immortalised E1-MYC 16.3 human EMSC-derived MSCs	Size fractionation, TFF concentration		IA injection, 100 μg/100 μL, after surgery, then once weekly for 2, 6 or 12 weeks.	1. Defect control 2. CD + MSC-EVs, (both groups after 2 weeks, 6 weeks and 12 weeks)

Footnote 1: MSC = Mesenchymal Stem Cell, AdMSC = adipose tissue-derived MSC, AFSC = Amniotic fluid-derived MSC, BMSC = Bone marrow-derived MSC, ESC-MSC = Embrionic stem cell-derived MSC, SMSC = Synovial fluid-derived MSC, USCS = Umbillical cord-derived MSC, PRP = Platelet-rich plasma, IA = Intra-articular, IV = Intravenous, OA = Osteoarthritis, CD = Cartilage Defect, COIA = Collagenase-induced OA.

92a-3p was vastly downregulated in OA chondrocytes-EVs compared to healthy chondrocyte-EVs. Through lipofectamine-mediated transfection to induce overexpression or downregulation of the miRNA, it was determined that miRNA-92a-3p in EVs was involved in regulating cartilage development during chondrogenesis of MSCs. By targeting the 3'UTR of WNT5A mRNA, and thereby downregulating WNT5a expression, MSC-miRNA-92a-3p-EVs were able to inhibit cartilage degradation in an *in vivo*, collagenase VII OA model [126]. The authors proposed that the engineered MSC-EVs inhibit the progression of early OA by maintaining COL2A1, Wnt5A, MMP13 and Aggrecan on levels compared to healthy control. For all parameters investigated *in vivo*, MSC-92a-3p-EVs were considered superior to regular MSC-EVs [126].

Based on the notion that TGF-β1 is an important player in cartilage repair in OA, Wang et al. isolated EVs from rat-MSCs treated with TGF-β1 to determine whether a specific miRNA was implied in the regeneration mechanism [127]. After establishing an enrichment of miRNA-135b in the MSC-EVs, they found a relation with the downregulation of the transcription factor Sp1 in recipient chondrocytes, which enhanced chondrocyte viability. This mechanism was validated *in vivo*, where it was shown in a rat model of OA that MSC-EVs with miRNA-135b were responsible for a reduction in cartilage degeneration [127].

The lncRNA named metastasis-associated lung adenocarcinoma transcript 1 (lnc-malat-1) has been implicated in inflammatory regulation, proliferation of chondrocytes and reduction of ECM degradation [128]. Pan et al. used lentiviral modification of human MSCs to overexpress this lncRNA, from which EVs were isolated. *In vitro*, lncRNA-EVs were found to inhibit chondrocyte apoptosis and inflammation induced by IL-1β, and increase proliferation speed compared to model control. Pan et al. then induced OA by injection of collagenase II in the knee joint cavity of rats, which was treated with PBS, MSC-EVs or MSC-lncRNA-EVs three weeks after establishing OA. Similarly to the *in vitro* results, OA symptoms were relieved after 6 injections with either EV formulation, where lncRNA-enriched MSC-EVs displayed a superior profile over regular MSC-EVs. The authors concluded that while the effects of lncRNA-malat-1 on cellular function and protein expression should still be studied in detail, MSC-lncRNA-EVs could offer a new therapeutic opportunity for OA prevention and treatment [128].

Rong et al. studied EVs isolated after hypoxic pre-treatment of rat BMSCs, where they found that an hypoxic environment elevated the release of small EVs [129]. *In vitro*, these hypoxic BMSC-EVs were taken up into chondrocytes more easily compared to BMSC-EVs without hypoxic pre-treatment (normoxic EVs), which possibly contributed to EV-mediated promotion of chondrocyte proliferation and migration. These results were confirmed in a rat OA model triggered by surgical destabilisation of the medial meniscus, which was treated by intra-articular injection of BMSC-EVs four weeks after establishing OA. Consecutive histological and immunohistochemical analysis of the knee joints four weeks post-treatment showed that hypoxic EVs and normoxic EVs were both enhancing cartilage repair compared to PBS control, but hypoxic EVs were remarkably superior. Hypoxic EVs were enriched with miRNA-216a-5p, which might facilitate the found upregulation of collagen II and Sox9, and decrease in MMP13 expression. As miRNA-216a-5p knockdown in hypoxic EVs were found to inhibit chondrocyte proliferation and migration and lessen apoptosis inhibition compared to regular hypoxic EVs, this miRNA in particular is implied to act as a mediator in cartilage regeneration [129].

Human UCSCs grown in a 3D cell culture hollow fibre bioreactor produced more UCSC-EVs compared to 2D-grown cells [80]. Rabbits with an induced cartilage defect were subjected to a total of four weekly intra-articular injections in the knee, containing either 2D-EVs or 3D-EVs. Both showed signs of neo-tissue formation and integration with surrounding tissues, although 3D-EVs demonstrated better histological outcomes. Yet, upon comparison based on the International Cartilage Repair Society (ICRS) macroscopic assessment for gross appearance of the joints, the disease control and 2D-EVs were not deemed statistically different. However, a particular difference in intraluminal cargo between the 2D-EVs and 3D-EVs was not mentioned, and the *in vivo* studies lack a healthy control group. These limitations complicate making conclusive statements [80].

Free EV therapy for cartilage regeneration using EV-engineering on an organism level has also been studied. MRL/MpJ 'superhealer' mice are an inbred strain of mice where tissues have extraordinary regenerative properties after injury or disease. Wang et al. aimed to find out whether EVs isolated from the chondrogenic progenitor cells (CPC-EVs) isolated from MRL/MpJ or regular control mice were better for treatment of OA [130]. Potentially due to an upregulation of miRNAs-148a-3p, −221-3p and −222-3p, or downregulation of miRNAs-let-7b-5p, −22-3p and -125a-5p in MRL-EVs compared to control-EVs, the MRL-EVs were indicated to be superior. This assessment was based on the higher amount of proteoglycan in cartilage, a significantly lower OARSI score, and an overall less severe joint wear and mild cartilage matrix loss. Both EV types managed to increase collagen II and aggrecan expression, and decrease collagen I presence. However, none of the miRNAs were separately investigated for their contribution in cartilage regeneration, so it remains unknown whether individual up- or down-regulation provides an incentive for therapeutic applications [130].

5.3. Free EV-therapy utilising exogenous EV-engineering

In December 2021, Song et al. published a paper where they used EV engineering after EV isolation to load mouse macrophage-EVs with epigallocatechin gallate (EGCG), which is thought to have antibacterial, antioxidant and immune-enhancing functions [131]. EGCG was loaded into the macrophage-EVs by repeated sonication on ice. In a rat model of collagen-induced RA, they showed that after ten intravenous injections over a time span of 20 days, the EGCG-EVs significantly decreased

redness and swelling of the ankle joints compared to PBS control, similar to intra-articular injections with EGCG. However, the therapeutic effect of intra-articular EGCG alone was inferior to EGCG-EVs, as was also confirmed by histological analysis. EGCG-EV-treated mice displayed only mild inflammation and a smooth cartilage surface in the joints, potentially via downregulation of apoptotic proteins partially induced by EGCG. Nonetheless, despite the promising results, the therapeutic effect of EGCG-EVs was not compared to control EVs, which hampers determining the additional functionality of EGCG [131].

The therapeutic effect of AdMSC-EVs conjugated to chitosan oligosaccharides for the purpose of cartilage regeneration after a complete cartilage defect in rats, was tested by Li et al. in 2021 [132]. EVs were isolated from AdMSCs by medium collection, followed by differential centrifugation, PEG-precipitation and ultracentrifugation, after which they were conjugated to chitosan oligosaccharides (COS) by passive loading under the influence of mild shaking. Because COS have been reported to promote bone tissue regeneration, and were shown to improve cartilage damage, the authors hypothesised that conjugation of COS to AdMSC-EVs would promote their regenerative effects, potentially by increasing chondrocyte viability and migration in the cartilage. The therapeutic effects of COS-EVs, COS and EVs separately, as well as a healthy and disease control were compared after 8 treatment injections. EVs and COS-EVs displayed the ability to regain healthy expression levels of collagen I and II, as determined by immunohistochemistry. While COS, EVs and COS-EVs all showed cartilage injury alleviation, COS-EVs were found to have superior treatment effects. The authors suggest that COS-EVs improve cartilage tissue repair in OA by suppressing apoptosis and increasing chondrocyte viability and migration through gene-regulation specific for chondrocytes, osteoblasts, apoptosis and the Akt/PI3K pathway. The exact mechanisms through which COS promotes initiation of these pathways remains unknown [132].

Topping et al. engineered human neutrophil-derived EVs to present a novel antibody specific against damaged arthritic cartilage that can occur in RA [133]. Neutrophil-derived EVs were chosen for their anti-inflammatory properties [134]. These cells were first stimulated with TNFα to induce EV formation, which were then isolated by differential centrifugation. Thereafter, the EVs were enriched with antibodies through a method employing lipid-cushioned sonication upon which they were purified again to remove unbound antibody. The authors set up a mouse-model of antigen-induced RA, where they induced inflammation in one knee, while using the contralateral knees as controls. They showed that the antibody-conjugated EVs localised primarily to the RA knee joint after intravenous (IV) administration, and had increased retention compared to control EVs. Further fortification of the EVs with antibodies against IL-10 and TNFα resulted in a therapeutic EV able to reduce knee swelling and inflammation in the RA joint after IV administration. The isolated knee joints after mouse sacrifice exhibited reduced expression of pro-inflammatory TNF, IL-1β and IL-6, as well as MMP13 and ADAMT5 genes. Additionally, the knee joints showed histological improvements for the antibody-conjugated EV-treated mice [133].

5.4. Limitations and research opportunities of free EV-therapy

To provide more clarity about the optimal treatment regimen of free EV-therapy, Zhang et al. investigated the pain suppressing and cartilage healing properties of EMSC-derived EVs over time [135]. OA was induced in rats fourteen days prior to weekly intra-articular injections of PBS or EMSC-EVs. Within two weeks, EMSC-EV-treated rats already showed a significant decrease in pain compared to PBS-treated rats, which further improved to levels similar to healthy control after 4 weeks. EMSC-EVs also altered early gene expression by reducing pro-inflammatory gene expression and inhibiting apoptotic, fibrotic and pain-related genes. Interestingly, genes involved in matrix regulation also differed due to EV-treatment. Significant differences between PBS and EV groups were revealed by histological analysis after four weeks of treatment, in favour of the latter. After eight weeks, EV-treated rats had a condylar structure and extracellular matrix that was effectively restored; experienced similar pain levels as the healthy control; and had a suppressed inflammation in the affected joint. With eight successive injections over a period of eight weeks, MSC-EVs were implied to have alleviated tissue injury and enhanced the repair of both cartilage and bone tissue [135].

These outcomes were in line with a previous study, where rats with an osteochondral defect were treated with human EMSC-derived EVs for 2, 6 or 12 weeks [53]. EMSC-EVs were found to initiate repair of the defects as early as two weeks, with formation of new cartilage tissue and deposition of s-GAG and collagen II in the extracellular matrix. Tissue repair was maintained in EV-treated rats over time, where collagen VI deposition plateaued at six weeks, but collagen II formation continued increasing. After twelve injections, the EV-treated rats showed smoothly formed cartilage that integrated fully with host older cartilage, whereas PBS-treated rats had only fibrous repair tissues with limited collagen II and increased presence of collagen I. EMSC-EVs were also found to reduce apoptosis and enhance proliferation of PCNA-positive cells, while increasing M2 macrophage infiltration, thereby decreasing the inflammatory response [53].

Based on the previously mentioned observations and reported results, it is evident that free EV therapy has the potential to promote chondrogenesis; suppress inflammation of the joint; and show protective functions against apoptosis and matrix degradation. However, while EV-engineering can lead to enrichment of components that enhance the intrinsic regenerative properties of EVs, some recurring limitations remain. A major drawback of free EV-therapy is the need for multiple administrations before tissue repair is sufficient, as was demonstrated by Zhang et al. [53,135]. Herein, it must be noted that these studies did not include control groups comparing the effects of EV-therapy over time with less (frequent) injections. Nevertheless, above mentioned studies indicate that cartilage regeneration benefits from a prolonged presence of therapeutic EVs in the joint. However, multiple injections do not only increase treatment burden, the intra-articular injections themselves also cause repeated local discomfort [135]. Utilising a biocompatible scaffold that provides a sustained delivery of therapeutic EVs within the joint after a singular injection would therefore be an alternative worthwhile investigating.

6. EV-encapsulating hydrogels as a platform for sustained EV-delivery

EVs are interesting nanocarriers for the transport of regenerative agents, but a few hurdles need to be overcome before therapeutic EVs can be applied in a clinical setting. The current most pressing limitation of EV-mediated therapy is the need for a prolonged presence of EVs at the location of injury. In free-EV therapy, this need translates to multiple injections over the course of a few weeks, which is not only a disadvantage in terms of patient compliance and comfort, but also causes a varying concentration over time. Consequently, EV-concentrations will strongly fluctuate between two consecutive administrations, partially due to the very short reported circulatory half-life of EVs [136,137]. Presumably, tissue repair and regeneration benefit from a constant presence of EVs, which could be achieved by making use of a biocompatible delivery system that slowly releases EVs at the location of injury to maintain a stable concentration over time. The following sections will provide an explanation of the fundamental requirements of biomaterials for the purpose of tissue engineering and how EVs can be incorporated without compromising biological activity.

6.1. Polymer-based biomaterials as scaffolds in tissue engineering

Scaffolds in the context of tissue engineering are materials that have been designed to promote suitable interactions between cells, thereby

contributing to the regeneration of damaged or diseased tissue. There are a variety of fundamental requirements for scaffolds in tissue repair [138,139]: 1) First and foremost the scaffold should be biocompatible, as this is vital for maintaining the essential cellular activities in the native tissue, needed for optimal repair. The cells surrounding the location of the implanted or injected scaffold should not be hampered in their proliferation and natural processes, so the scaffold should possess appropriate surface properties and topography. 2) For patient convenience, the scaffold can be made of biodegradable materials so that removal of the scaffold is unnecessary. However, attention should be paid to appropriate degradation rates and the prevention of cytotoxic degradation by-products. 3) The presence of interconnected pores with a suitable size range enables the infiltration and incorporation of cells, EVs or biomolecules in the scaffold. In the section "Incorporation of EVs into hydrogels", this aspect will be discussed in more detail. Additionally, the pores should facilitate sufficient oxygen and nutrient supply to ingrowing cells, as well as the clearance of waste products [140]. 4) The mechanical characteristics of the scaffold should ideally match the mechanical properties of the native tissue. An important aspect herein is the thickness of the scaffold, which should allow for satisfactory load-bearing capacities. 5) Lastly, convenience for implementation should not be forgotten. Since injectable biomaterials that form in-situ do not require a surgical procedure, they have an important advantage over other biomaterials [138,139]. Depending on the cellular environment and function of the tissue the scaffold is intended to mimic, the features of all aforementioned requirements should be adapted. For example, skin functions as a protective barrier between the internal and external environment and is also involved in thermoregulation by allowing for water vapor transmission [141]. In case of severe burn injuries or other conditions that require vast regeneration of the skin, wound healing can be promoted by use of tissue engineering, but this procedure would require a sterile, adhering scaffold with low toxicity and immunogenic response that can fulfil these demands [141]. Tissue repair of the heart after a myocardial infarct requires a biomaterial with good mechanical strength and flexibility that can endure the considerable volume changes that occur when a heart is beating. For this purpose, biomaterials made of collagen derived from natural and synthetic sources have been investigated previously [142].

As cartilage has an intricate structure with a complex interplay of cellular, chemical and immune components in an extracellular matrix, scaffolds intended for aiding in cartilage regeneration need specialised qualities. Articular cartilage functions to provide stable movement without friction within joints, and is capable of carrying heavy weights without vulnerability to compression and shear. It is therefore essential for the fabricated cartilage scaffold to have similar mechanical properties to articular cartilage, as investigated by Robinson et al. [143] and reviewed by Armiento et al. [144].

Polymer-based biomaterials used as scaffold for tissue engineering are versatile materials because their chemical, physical and mechanical characteristics can be precisely tailored by making relatively small changes in the polymer composition [138,139]. Currently, these biomaterials are mostly made of natural polymers (polysaccharides and proteins), or synthetic polymers (e.g. polyesters and polyurethanes). The natural polymers, including hyaluronic acid, collagen and fibrin, generally exhibit a biocompatible profile with suitable biodegradability. They have the advantage that they are naturally found in the extracellular matrix of many tissues. This abundancy in the human body ensures that they elicit minimal inflammatory or immune responses, and promote cellular processes related to tissue regeneration [139]. However, materials based on natural polymers have some shortcomings with regards to their applicability in cartilage regeneration specifically, as they often display poor mechanical properties and therefore present challenges in precisely shaping the scaffold for individual use [138,139]. Materials based on synthetic polymers on the other hand, can be designed to match the physical, chemical and mechanical properties of the injured tissue of the patient. Unfortunately, while the possibilities for synthetic polymers seem endless, personalised medicine comes with a large cost. Additionally, so far only a small number of biomaterials comprised of synthetic polymers have been approved for specific applications by the regulatory agencies (as reviewed by Abdulghani et al.) [139]. The solution herein could lie in the use of hybrid biomaterials, in which synthetic polymers are added to natural ones, to combine the advantages of both polymer classes and lighten the influence of their limitations. However, combinations do not solve the problem of market approval, as each biomaterial for a new application still needs to be granted approval separately, irrespective of the polymers used.

One of such versatile biomaterials that have the possibility for hybridisation between natural and synthetic polymers, are hydrogels. Hydrogels are networks of hydrophilic polymers that are crosslinked either physically or chemically to form a three-dimensional structure [12]. The spaces between the crosslinked polymeric chains can be filled with water molecules, resulting in the formation of a gel-like structure with a mass much higher than the dry weight of the polymers [145,146]. Depending on the chemical characteristics of the polymers that the hydrogel is comprised of, their properties differ, leading to differences in swelling ratio, porosity, rheological properties and applicability for drug delivery. Some hydrogels even have intrinsic mechanisms for self-healing in case of damage, which can significantly increase their durability. Self-healing can occur through both non-covalent and covalent interactions, e.g. electrostatic or hydrophobic interactions for the former, or disulphide or imine bonds for the latter. Besides conventional gel preparation, certain hydrogels can be formed in-situ, which makes them interesting injectable materials for approaches in clinical application for cartilage regeneration [12,138,145]. For instance, the thermoresponsive synthetic polymer PNIPAM is soluble in an aqueous solution below the lower critical solution temperature. Upon heating of the solution to temperatures higher than 32 °C, the polymer undergoes a reversible phase transition and precipitates. When making use of co-polymers where PNIPAM has integrated hydrophilic blocks into its structure (e.g. chitosan or hyaluronic acid) the polymer starts forming crosslinks upon raising the temperature, thereby fabricating a hydrogel [147]. Recently, photo- and pH-responsive hydrogel formations have also been reported, as reviewed by Chyzy et al. [145].

To illustrate how hydrogels can be used as a biomaterial in cartilage regeneration, this review will provide one of many representable examples in recent literature. Ma et al. devised a study to investigate a strontium alginate/chondroitin sulphate hydrogel for the purpose of cartilage tissue engineering, prepared in a 'one-pot reaction' with sodium alginate, chondroitin sulphate and strontium chloride [148]. Strontium acts as a crosslinker and thus increased the stiffness of the gel, but decreased the degree of swelling. The hydrogel was non-cytotoxic to chondrocytes, appeared to have anti-inflammatory properties and inhibited chondrocyte apoptosis *in vitro*, as deduced from reduced expression of MMP-9, B-cell lymphoma 2 and p53 genes. These observations were in line with the results from a rabbit cartilage defect model, where the cartilage appeared to be partially repaired on a macroscopic and histological level compared to disease control, four weeks after hydrogel implantation [148].

6.2. Incorporation of EVs into hydrogels

Despite the range of valuable properties that hydrogels exhibit and the hypothesis that they aid in cartilage regeneration on their own, one of the most interesting of hydrogel characteristics is their capability to act as a local depot for drug delivery [149]. Although especially the incorporation of stem cells into hydrogels has been a major focus in tissue engineering, research that aims to integrate EVs into hydrogels has also been conducted increasingly (for previous reviews, readers are referred to Riau et al. (2019) [11] and Akbari et al.(2020) [12]). For this purpose, there are additional criteria that a scaffold should meet. As mentioned previously, the pores of a hydrogel are important in this regard, where not only their presence but also their size is crucial. Pores

can be classified based on their diameter according to different definitions, but for the incorporation of biological entities such as cells, nanoparticles or EVs, the definitions proposed by Elbert et al. would be most applicable [150]. Herein, a hydrogel mesh size below 100 nm is nanoporous, between 100 and 1000 nm is microporous, and above 1 μm is macroporous. Considering the size of EVs generally lies between 50 and 150 nm, nano- and micropores are optimal in hydrogels used as an EV-release depot [21]. However, because in cartilage repair the aim is to induce chondrogenesis and matrix formation, it is important that the pores are also large enough to allow for ingrowth of chondrocytes. Osteoid ingrowth and bone tissue regeneration require a mesh size of respectively 40–100 μm and 100–350 μm, so it is likely that cartilage repair calls for similar characteristics [145].

Integration of biological entities in the structural basis of the hydrogel can enhance its functional properties. Through non-covalent interactions between the hydrogel and the desired cargo, components can be trapped within the hydrogel to ensure a sustained release [151]. For example, the inherent cationic properties of chitosan make scaffolds with this polysaccharide favourable for the delivery of anionic components, such as genes or low molecular weight drugs. By tuning the characteristics of the polymers to retain the desired cargo, the cargo release kinetics can be augmented. This has paved the way for so-called 'smart hydrogels' with the ability to release their content on demand (e.g. glucose-responsive hydrogels for modulation of insulin levels based on blood sugar levels) [149]. Upon environmental stimuli, the hydrogels control drug release by swelling, dissolution or degradation, which otherwise would only happen due to time-dependent degradation.

Perhaps of particular importance is the matter of EV stability once inside the hydrogel. Loss of EV functionality arises relatively quickly upon storage in various conditions, potentially due to the degradation of growth factors and miRNA inside EVs or membrane instability. Temperature, presence of excipients and isolation methods all affect their functionality, so it is not unreasonable to believe that the hydrogel composition is also of influence [95,152]. This hypothesis is in line with reports that in synthetic nanoparticle-hydrogel composites, the stability of nanoparticles can be enhanced by alteration of the amount of crosslinks, use of surfactants or adaptation of the chemical functionality of polymer side chains [151]. However, biological entities such as EVs likely respond differently. Chitosan-based hydrogels appear to possess stabilising qualities. Zhang et al. isolated EVs from human placenta-derived MSCs, and encapsulated them in a chitosan hydrogel with thermosensitive capabilities for treatment in ischemic injury [153]. In an *in vitro* simulation at 37 °C for 24 h, chitosan hydrogels enhanced the stability of EV-component miRNA-126, and reduced the degradation of proteins inside MSC-EVs compared to free MSC-EVs. In a scratch wound assay of human umbilical vein endothelial cells (HUVECs), the improved stability translated to an increased proangiogenic profile for hydrogel-encapsulated MSC-EVs compared to free MSC-EVs. These results were confirmed *in vivo*, where the loaded hydrogel also exhibited a satisfactory EV release profile of over 72 h [153].

However, the hereabove mentioned additional criteria (i.e. appropriate pore size, hydrogel functionality and retaining EV-stability) are all affected by gelation and EV-incorporation procedures. In general, there are two ways that can be used to integrate isolated EVs into a hydrogel, as illustrated in Fig. 3. In the first method, the polymers and EVs are mixed together, after which the gelation process is started by the addition of a crosslinker, or by activating an external trigger. The second procedure is more applicable in clinical settings. Polymers, crosslinkers and EVs are mixed together simultaneously in solution, which gives rise to the possibility for in-situ gelation by means of a dual chamber syringe that injects all three components at the site of injury. Taking into consideration that implantation of a gel in the joint for local cartilage regeneration through the first method requires open-knee surgery, in-situ gelation would be preferred [11].

In-situ biomaterial formation with EVs has already been achieved for diseases other than OA, for example by Yao et al. in the context of heart repair after myocardial infarction [154]. Using a dual chamber spray needle, MSC-EVs were mixed with fibrin in one chamber, with thrombin present in the other. Upon applying of all components on the heart, fibrin and thrombin started the gelation process, with MSC-EVs entrapped in the large pores. This system was found to significantly increase EV retention on the heart compared to free EV injection, and displayed increased tissue repair of the myocardium in both a rat and pig model [154].

Also Mol et al. developed an EV-containing hydrogel with the purpose to increase local EV-retention *in vivo* [155]. They designed a hydrogel composed of ureido-pyrimidinone (UPy) moieties on a PEG backbone. Cardiac Progenitor Cell-derived EVs were mixed with dissolved UPy-PEG polymers in high pH PBS (pH of 9), after which local gelation occurred upon injection into a pH-neutral environment. The encapsulated EVs were found to be released over a period of two weeks while retaining their functionality *in vitro*. Using fluorescently labelled

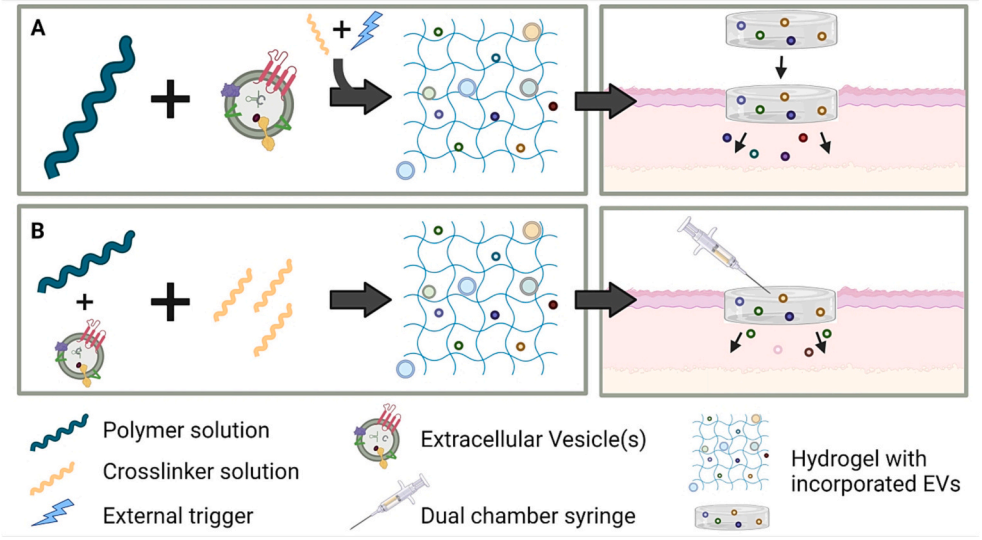

Fig. 3. Overview of two methods for EV-incorporation into hydrogels. (A) The EVs and polymers are mixed, after which a crosslinker and/or an external trigger (e.g. warmth, UV-light) starts the gelation process. (B) Polymers, crosslinker and EVs are added simultaneously in a dual chamber syringe to achieve in situ gelation at the target site.

EVs, it was shown in an mouse model that UPy hydrogels retain (part of) the EVs within their pores for at least three days, leading to an improved EV stability compared to injection of free EVs [155].

7. Injectable EV-encapsulating hydrogels for sustained cartilage regeneration

Encapsulating EVs in a hydrogel with similar characteristics to native tissue could combine the positive effect of both components in tissue repair. Hydrogels generally ensure a sustained retention time of EVs at the site of injury and provide opportunity for ingrowth of chondrocytes and extracellular matrix within the joint. Moreover, patient compliance can be increased by allowing for administration through a single intra-articular injection. In the following section, relevant conducted research in recent years will be described to provide a comprehensible review of the developments in the field. Herein, a distinction is made between studies that claim or have potential for administration via intra-articular injection, and those that have also investigated this *in vivo*. All papers have been summarised in Table 2. Particularly of interest are the hydrogel composition; EV source, isolation method and specific characteristics; animal model; dosage; time points; and *in vivo* outcomes.

7.1. EV-loaded hydrogels with potential for administration via local injection

Hu et al. isolated EVs from human UCSCs that were found to increase chondrocyte and BMSC viability, migration and proliferation *in vitro*, potentially through EV-induced mRNA upregulation of Sox9, collagen II and aggrecan in these cells [156]. Additionally, the UCSC-EVs caused enhanced deposition of glycosaminoglycans and collagen II in the extracellular matrix. These observations were thought to originate from EV-mediated delivery of miRNA-23a-3p, thereby activating the PTEN/AKT signalling pathway. For potential clinical application of these UCSC-EVs, Hu et al. designed a hydrogel composed of gelatin methacryloyl (GelMa) and nanoclay that would crosslink upon radiation with UV-light at 365 nm for 2 to 3 min. Herein, the laponite nanoclay

Table 2
Overview of relevant conducted research regarding injectable EV-encapsulating hydrogels for cartilage regeneration.

Reference	*Hydrogel composition*	*Crosslinker*	*EV source*	*EV-Isolation method*	*Animal model*	*Dosage*	*Time points*	*Comparison*
Hu et al., 2020 [156]	Gelatin methacryate with gelatin or nanoclay	UV-radiation (365 nm) for 2 or 3 min	Human UCSCs	Differential (ultra) centrifugation	Rat, CD	Implantation during surgery. 10 * 10^10 EVs/mL, volume not specified	12 weeks	1. Defect control, 2. CD + Hydrogel only, 3. CD + EV-Hydrogel, 4. healthy control.
Liu et al., 2017 [157]	o-nitrobenzyl alcoholl modified hyaluronic acid, gelatin	Photo-induced (395 nm, 1 min) imine crosslinking	Human iPSC-MSCs	Differential (ultra) centrifugation	Rabbit, CD	2 × 10^8 EVs in 20 μL hydrogel	12 weeks	1. Defect control, 2. CD + in-situ formed EV-hydrogel, 3. CD + in-situ formed hydrogel, 4. CD + implanted pre-formed EV-hydrogel, 5. CD + IA EV injection
Heirani-Tabasi et al., 2021 [158]	Chitosan-hyaluronic acid	EDC/NHS	Human articular chondrocytes, grown three-dimensionally in hydrogels	Differential (ultra) centrifugation	Rabbit, CD	30 μg EVs and 1.5^6 MSCs in 100 μL hydrogel	Studied at 4 and 24 weeks	1. CD + hydrogel, 2. CD + MSC-EVs, 3. CD + MSCs, 4. CD + MSC-hydrogel, 5. CD + EV-hydrogel, 6. CD + MSCs-EVs-hydrogel
Zhang et al., 2021 [159]	Alginate-dopamine, Chondroitin sulphate, regenerated silk fibroin	Horseradish peroxidase, H_2O_2	BMSCs	Differential (ultra) centrifugation	Rat, CD	100 μg EVs (protein) in 500 μL hydrogel,	Studied at 2, 6 and 12 weeks	1. Defect control, 2. CD + Hydrogel, 3. CD + EV-hydrogel
Tao et al., 2021 [160]	poly(D,L-lactide)-b-poly (ethylene glycol)-b-poly (D,L-lactide) (PLEL)	Thermo-sensitive	Human SMSCs overexpressing circRNA3503 via genetic overexpression.	Differential (ultra) centrifugation with sucrose density gradient	Rat, surgical destabilisation	Administration every 4 weeks, 2,0 *10^9 EVs in 100 μL hydrogel	24 weeks	1. Healthy control, 2. Disease control, 3. OA + PLEL@saline, 4. OA + PLEL@SMSC-EVs, 5. OA + PLEL@Wnt5a/b-dKO-sEVs, 6. OA + PLEL@circRNA3503-sEVs, 7. OA + PLEL@Wnt5a/b-dKO-circRNA3503-EVs
Yang et al., 2021 [161]	Furan-modified hyaluronic acid, poly(ethylene glycol)	Diels Alder reaction	Human iPSC-MSCs	Differential (ultra) centrifugation	Rat, surgical destabilisation	1 * 10^9 particles in 100 μL hydrogel	28 days	1. Healthy control, 2. Disease control + saline, 3. OA + hydrogel, 4. OA + single free EV injection, 5. OA + multiple free EV injections, 6. OA + EV-hydrogel

Footnote 2: MSC = Mesenchymal Stem Cell, BMSC = Bone marrow-derived MSC, SMSC = Synovial fluid-derived MSC, iPC-MSC = induced pluripotent stem cell-derived MSC, USCS = Umbillical cord-derived MSC, EV = extracellular vesicle, OA = Osteoarthritis, CD = Cartilage Defect.

functioned to improve the biological and mechanical properties of the GelMa hydrogel, allowing for UCSC-EV incorporation into the 100–200 nm pores by addition to the solution prior to gelation. Facilitated by the degradation of the GelMa/nanoclay hydrogel over time, EVs were found to be sustainably released over a period of 31 days. UCSC-EV morphology remained similar before and after encapsulation and release by the hydrogel. Although the authors state to have fabricated an injectable hydrogel with UCSC-EVs, this feature was not investigated *in vivo*. Instead, the hydrogel was implanted during surgery into a cartilage defect in rats. After 12 weeks, the EV-containing GelMa/nanoclay hydrogel had increased the ICRS score to healthy levels, increased collagen II deposition compared to disease control and hydrogel alone, and neo-tissue formation similar to native cartilage was observed [156].

In another study EVs isolated from human iPSC-derived MSCs were incorporated into a glue-like hydrogel composed of gelatin and hyaluronic acid modified with o-nitrobenzyl alcohol moieties [157]. The polymers and MSC-EVs were combined in solution, after which the hydrogel was crosslinked through imine bond formation under UV-radiation at 395 nm. The EV-hydrogel combination was able to release MSC-EVs over time, where 10% of the EVs were released after 14 days immersion in PBS. Specific stability testing of EV-cargo after hydrogel crosslinking and subsequent release was not conducted. However, in an *in vivo* rabbit model of full-thickness cartilage defect, the EV-loaded hydrogel showed therapeutic effects on cartilage regeneration. During surgery to establish the disease model, the hydrogel was formed *in-situ* via injection and subsequent photo-induced crosslinking in the cartilage defect. The scaffold therefore has potential for intra-articular injection, though a way must be found to allow for photo-induced crosslinking post-injection without a need for surgery. Analysis of the joints was executed twelve weeks after surgery, where it was shown that *in-situ* gelation was superior over implantation of the pre-formed hydrogel. The *in-situ* administered EV-loaded hydrogel caused the formation of new cartilage with a strong positive staining for glycosaminoglycans and collagen II, with only a limited presence of collagen I. *In-situ* forming hydrogels without MSC-EVs were also successful in repairing the cartilage defect, however, to a lesser extent. A singular injection of free MSC-EVs had only a very limited effect. These results imply that the hydrogel and MSC-EVs function in tissue repair synergistically. Implanted, pre-formed hydrogels caused a non-uniform distribution and ingrowth of chondrocytes, leading to an insufficient integration of old and new cartilage. From this study it could not be concluded to which extent the EV-loaded hydrogel repairs the cartilage, as a healthy control group was not included [157].

Heirani-Tabasi et al. developed a chitosan-hyaluronic acid hydrogel that was chemically crosslinked with Ethyl(demethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) [158]. Both MSCs and chondrocyte-EVs were entrapped in the same hydrogel, which was administered immediately after surgery in rabbits with an osteochondral defect. Herein, the chondrocyte-EVs functioned to prime the MSCs for chondrogenesis and cartilage regeneration. The combination of MSCs and chondrocyte-EVs in hydrogels was deemed more successful in increasing ICRS scores compared to incomplete combinations of any of these components. However, both hydrogel-MSCs and EV-primed hydrogel-MSCs were able to promote neo-tissue generation with similar characteristics to native cartilage, as deduced from histological analysis [158].

7.2. Injectable EV-loaded hydrogels for cartilage regeneration

Some studies have already been published reporting EV-encapsulating hydrogels that can be injected for the purpose of cartilage regeneration. In brief, scientists make use of a dual-chamber syringe containing a solution with polymer and EVs, and a solution with crosslinker, which are combined upon localised injection to start the gelation process *in situ*. Fig. 4 depicts a graphical illustration of the general approach.

In November 2021, Zhang et al. published a paper in which they fabricated a hydrogel that was inspired by mussel-based materials to obtain a hydrogel with capabilities to strongly adhere to wet tissues [159]. Composed of alginate-dopamine, chondroitin sulphate and regenerated silk fibroin, the hydrogel was designed to crosslink through a reaction catalysed by H_2O_2 and horseradish peroxidase (HRP), using a dual-chamber syringe [159]. This combination formed a hydrogel with pores of 10 to 40 μm in diameter that was only 40% degraded after 20 days incubation in PBS. BMSC-EVs were integrated evenly into the hydrogel before gelation by keeping them in solution with the polymers before crosslinking, as shown by confocal microscopy of fluorescently labelled EVs. *In vitro*, 85 to 90% of the EVs were sustainably released from the hydrogel in 14 days. These BMSC-EVs retained their structural integrity in the presence of HRP and H_2O_2, and in the same experiment they were found to recruit BMSCs into the adhesive hydrogel faster than the scaffold alone. The EV-encapsulating hydrogel allowed for BMSC infiltration and extracellular matrix mineralisation *in vitro*, aiding in differentiation of BMSCs to chondrocytes through increasing the cellular protein expression of various proteins, including collagen II. These results were validated in a rat model of a cartilage defect that was established through a surgical procedure. Morphological changes over time (before surgery, 2, 6 and 12 weeks after treatment) were studied by MRI scanning, histological analysis and gross observation of the joint. After 12 weeks, the ICRS scores of EV-hydrogel-treated rats were comparable to the healthy knee, and formed cartilage was more smooth and continuous than PBS- and hydrogel-treated rats. It is thought that these effects were mediated by chemokine signalling. Although the researchers presented strong evidence that the EV-encapsulating hydrogel is capable of aiding in regeneration of articular cartilage, the study is limited by the lack of a control group investigating the effects of a

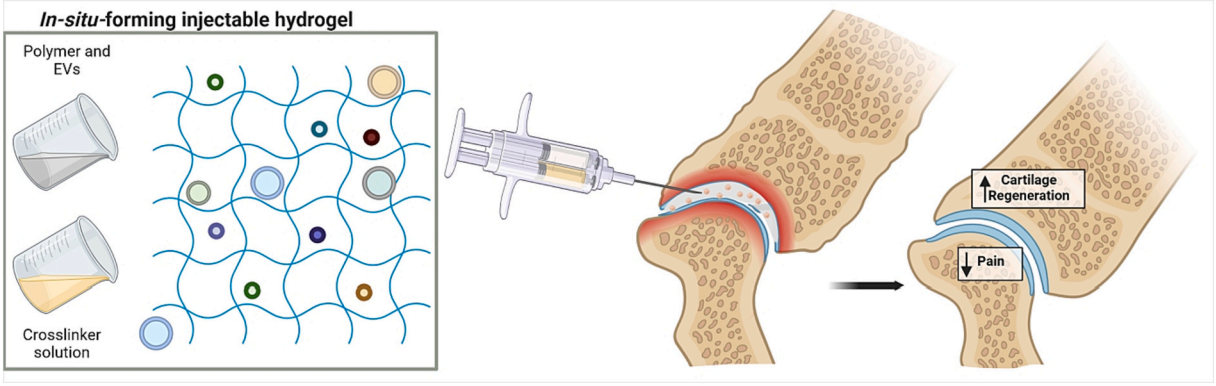

Fig. 4. Schematic overview of the injectable EV-loaded hydrogels for cartilage regeneration in vivo. Polymer and crosslinker solutions are added together with EVs, after which they are injected and crosslinked in-situ by means of a dual-chamber syringe.

singular intra-articular injection of free EVs [159].

Tao et al. investigated a thermosensitive hydrogel with encapsulated EVs overexpressing circular RNA 3503 (circRNA3503)[160]. This circRNA is upregulated during sleep, which coincides with improved cartilage repair. It is therefore suspected that circRNA3503 is involved in cartilage regeneration by acting as a sponge of hsa-miR-181c-3p and hsa-let-7b-3p, resulting in a decrease in inflammation-induced apoptosis and ECM degradation and an increase in ECM synthesis. In brief, human SMSCs were engineered to overexpress circRNA3503 and have a double knock-out for the proteins Wnt5a/b, to promote cartilage regeneration. The researchers of the study designed a thermosensitive triblock copolymer (poly(D,L-lactide)-b-poly(ethylene glycol)-b-poly(D,L-lactide), also: PLEL), to form a strong hydrogel *in-situ* at temperatures around 37 °C. When co-culturing the engineered SMSC-EVs with cells *in vitro*, the hydrogel was necessary for maintaining a high level of circRNA3503 in these cells over time (28 days versus 6 days), showing that the gel increased EV-cargo stability and released SMSC-EVs in a sustained manner. The function of the SMSC-Wnt5a/b-dKO-circRNA3503-EVs was evaluated *in vivo* in a rat OA model that was established by surgical transection of joint components. Rats were injected intra-articularly with the EV-containing hydrogels every four weeks, and downstream analysis of the knee joints occurred 24 weeks post-surgery. A strong feature of this study was the inclusion of a variety of control groups (healthy; OA; OA with PLEL@saline; OA with PLEL@SMSC-EVs; OA with PLEL@SMSC-Wnt5a/b-dKO-EVs; and OA with PLEL@SMSC-circRNA3503-EVs). This way it could be shown that the severe cartilage damage in the OA group was not alleviated by the hydrogel alone, and also not by the SMSC-EVs with a Wnt5a/b double knock-out. While the PLEL@SMSC-Wnt5a/b-dKO-circRNA3503-EVs treatment caused substantial prevention of OA disease progression and increased healthy cartilage formation, this effect was significantly weaker than the regenerative effect of PLEL@SMSC-circRNA3503-EVs without a double knock-out for Wnt5a/b [160].

Lastly, Yang et al. aimed to achieve controlled release of EVs isolated from iPSC-derived MSCs from a hydrogel composed of furan-modified hyaluronic acid and PEG [161]. The polymers and MSC-EVs were added together in a pre-gel solution where, upon injection into the articular cavity, a spontaneous Diels-Alder cycloaddition took place to crosslink the polymers. The MSC-EVs were evenly distributed in the hydrogel, as visualised by confocal microscopy after fluorescent labelling of the EVs. The *in vitro* EV release kinetics were then determined to be dependent on hydrogel degradation over time, though the cumulative release in the absence of HAnase was only around 15% after 16 days, where it appeared to stagnate. The released EVs had a regular morphology, but the hydrogel encapsulation and release compromised the EV functionality in promoting chondrogenesis and migration to a small extent when compared to freshly isolated EVs. To test whether despite the reduced functionality, administration of hydrogel-encapsulated EVs were superior over singular and multiple intra-articular EV injections, Yang et al. conducted an *in vivo* study in a rat model for OA. This comparison is especially useful because it shows the applicability of hydrogels as a scaffold for MSC-EVs in a potential future clinical application. A week after establishing OA, rats received intra-articular injection with their respective treatment. The rats receiving multiple MSC-EV injections were treated on day 7, 14, 21 and 28, and all rats were sacrificed 5 weeks after surgery for downstream analysis. The EV-loaded hydrogel had a similar relieving effect on the OARSI score as multiple MSC-EV injections, which both show significantly more cartilage regeneration than the OA control, hydrogel and/or a single MSC-EV administration [161].

8. Discussion and future perspectives

The discovery that MSC-EVs are important effectors of MSCs in the repair of damaged cartilage tissue, paved the way for a whole new field of research in regenerative medicine. Carrying biological macromolecules such as stem cell-associated proteins, growth factors and nucleic acids, MSC-EVs taken up by cells surrounding the site of injury appear to cause an alteration in gene expression of these cells. MSC-EVs thereby inherently contribute to chondrogenesis, increase chondrocyte migration and decrease apoptosis. The ECM is largely reformed upon treatment with MSC-EVs, often with levels of glycosaminoglycans and collagen II similar to healthy cartilage. By utilising EV-engineering, these regenerative qualities can be enhanced, for example by conjugating the EVs to particular components, or by loading EVs with therapeutic molecules. However, previous research identified a major drawback of simply injecting EVs in the joint cavity, as usually multiple administrations are required before the healing effect is significant. This need for multiple local injections likely originates partially from the short half-life of EVs *in vivo* [136,137]. As multiple EV administrations over the course of a few weeks seem to have more satisfactory results, it is hypothesised that cartilage regeneration benefits from a prolonged exposure to EVs. As versatile and biocompatible materials with the ability to encapsulate many different types of agents, hydrogels are often considered the platform of choice to incorporate EVs. Hydrogels are able to slowly release their contents to the surrounding tissues, thereby facilitating a sustained EV presence in the injured joint over time.

While preclinical studies with the aim to investigate various dosages and dosing regimens optimal for repairing cartilage tissue have been conducted [53,135], there remains a knowledge gap in what exactly is needed for MSC-EVs to elicit their regenerative functions in humans. Conditions that lead to satisfactory cartilage regeneration in rats or rabbits (including but not limited to the EV-cargo, dose and disease progression), may not have the same effect in humans. An accurate translation of preclinical results to predicted therapeutic effect in humans requires the use of appropriate disease models during animal studies [162]. This necessity is one of the technical obstacles in translational research. OA and RA are both highly heterogeneous diseases, so it can be argued that no single animal model fully represents either disease [163,164]. Contrastingly, animal models where arthritis is established through collagenase treatment have similarities to both diseases. Overall, although OA has been reported to occur naturally in rodents, rheumatism is most often induced chemically; through surgical destabilisation of the joint; or through artificial formation of a cartilage defect. With these methods it should be taken into account that while highly relevant in preclinical context, the therapeutic outcome in a clinical setting cannot always be accurately predicted [163,164].

To our knowledge, no human studies have been reported that investigate EV-encapsulating hydrogels in the context of cartilage regeneration on PubMed or ClinicalTrials.gov. However, when considering hydrogels and EVs separately, some trials have been registered. Of the five registered trials on ClinicalTrials.gov, three studies on hydrogels as a treatment in cartilage repair have been completed between four and six years ago, but no results were posted (NCT01879046, NCT01895959 and NCT04293861). Two other trials are still ongoing (NCT04840147 and NCT05186935). PubMed searches yield more study results, where hydrogels are considered as a delivery platform for stem cells [165], or as an add-on therapy next to microfractures [166]. For EVs in cartilage regeneration, two ClinicalTrial.gov studies are still ongoing (NCT05060107 and NCT04223622), and on PubMed a single study set-up is proposed to investigate the efficacy and safety of knee OA treatment with EV-containing Wharton's jelly [167].

Regardless of current investigations in humans, there are still a few challenges that need to be faced before EV-loaded hydrogels can be translated to clinical practice. Herein, the harvesting of the stem cells and the isolation of SC-EVs are of particular concern. Considering that MSCs have been investigated most often as the parental cell source of EVs in regenerative medicine, different isolation methods from human tissues have been well-established. It should be taken into account here that the regenerative function of MSCs is partially dependent on the specific tissue source and their phenotypic state, which may be true for

MSC-EVs as well. Comparative studies for MSC-EVs derived from different human tissues in terms of chondrogenic potential and overall regenerative capacity are therefore warranted. MSCs are present in a diverse range of human tissues, including bone marrow, adipose tissue and synovial fluid, but also the skin, dental tissues, and various tissues from the umbilical cord and placenta (as reviewed by Mushahary et al.) [168]. Although clinical application requires robust and standardised methods of isolation to obtain MSCs of high quality and regenerative potential, experts in the field worldwide have not reached consensus regarding the optimal isolation and characterisation methods [168].

The second set of challenges lies in the isolation and storage of MSC-EVs. MSCs are relatively easy to cultivate and expand, as they retain their self-renewal and differentiating capacity during upscaling [169]. This is favourable for further MSC processing, because an EV isolation for a single administration requires a large number of cells [106]. While there are endeavours to optimise EV isolation procedures to allow for industrial scale isolations, currently there is no single EV isolation method that is deemed scalable as well as satisfactory in terms of purity [22]. Bioreactors could be used for cells grown both in suspension and monolayers, but these methods are not widely used, and more research is still needed to determine the effects of these conditions on EV-cargo [170,171]. A step in the right direction is that the International Society of Extracellular Vesicles (ISEV) has published position statements with recommendations for the isolation and characterisation of EVs to ensure reproducibility of experiments [106], and to allow for the use of therapeutic EVs in human clinical trials [172]. Besides EV-isolations, the storage and need for cGMP compliance also need to be addressed [22]. There have been reports that MSC-EVs can be isolated in clinically relevant scales by using PEG precipitation, but this technique collects rather than purifies EVs, resulting in low EV purity [173]. As earlier discussed, there is a precarious balance between yield, purity and stability with the current EV-isolation methods, so it should still be investigated whether changing the EV purity affects the regenerative capabilities. Since the EV protein content, functionality and biodistribution *in vivo* may be affected by the isolation technique [109,113,114,135], this should be considered a priority.

As a relatively young field, there are still many EV-engineering approaches insufficiently explored. Such strategies could potentially increase the EV yield or enhance their functionality, and might improve functional cargo loading or lengthen retention times [22]. Moreover, MSC-EVs can aid in tissue regeneration in a multitude of ways but the detailed mechanisms remain largely unknown, which is needed to determine targets for EV-engineering purposes. As the downstream effector molecules of many intraluminal EV components (e.g. miRNAs) in both EV donor and recipient cells are not always clear, their up- or downregulation in cells could induce both positive and negative unforeseen effects.

Additionally, the incorporation of therapeutic agents into EVs and the formation of hybrid nanoparticles are interesting approaches that could be beneficial in treatment of OA and RA [174]. However, the issues with scalability and storage are more pressing, as they are pivotal limiting factors that need to be addressed before EV-based therapies can feasibly be considered for clinical applications. Simultaneously, it is unlikely that all EV-engineering approaches will have been exhausted before the first human clinical trials, considering the seemingly endless list of potential EV components.

All in all, the use of injectable EV-encapsulated hydrogels as a platform for sustained delivery of extracellular vesicles in the context of cartilage regeneration is a promising approach for treating rheumatic diseases. With an ageing population, the prevalence of OA is ever increasing and becoming a larger burden on society each year [5]. The field of EV research has matured in recent years to allow for clinical translation in the near future, but there are still some obstacles that need to be overcome, including but not limited to optimalisation of MSC- and EV-isolation and purification, as well as upscaling for industrial production. Nevertheless, the field holds a great amount of potential, meriting further investigation.

CRediT authorship contribution statement

Sanne M. van de Looij: Writing – original draft, Writing – review & editing, Visualization. **Olivier G. de Jong:** Writing – review & editing. **Tina Vermonden:** Writing – review & editing. **Magdalena J. Lorenowicz:** Writing – review & editing.

Data availability

No data was used for the research described in the article.

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