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Control and interplay of scaffold-biomolecules interactions applied to cartilage tissue engineering.

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Abstract

Cartilage tissue engineering based on the combination of biomaterials, adult or stem cells and bioactive factors is a challenging approach in regenerative medicine with the aim of achieving the formation of a functional neotissue stable in the long term. Various 3D scaffolds have been developed to mimic the extracellular matrix environment and promote cartilage repair. In addition, bioactive factors have been extensively employed to induce and maintain the cartilage phenotype. However, the spatiotemporal control of bioactive factor release remains critical for maximizing the regenerative potential of multipotent cells, such as mesenchymal stromal cells (MSCs), and achieving efficient chondrogenesis and sustained tissue homeostasis, which are essential for the repair of hyaline cartilage. Despite advances, the effective delivery of bioactive factors is limited by challenges such as insufficient retention at the site of injury and the loss of therapeutic efficacy due to uncontrolled drug release. These limitations have prompted research on biomolecule-scaffold interactions to develop advanced delivery systems that provide sustained release and controlled bioavailability of biological factors, thereby improving therapeutic outcomes. This review focuses specifically on biomaterials (natural, hybrid and synthetic) and biomolecules (molecules, proteins, nucleic acids) of interest for cartilage engineering. Herein, we review in detail the approaches developed to maintain the biomolecules in scaffolds and control their release, based on their chemical nature and structure, through steric, non-covalent and/or covalent interactions, with a view of their applications in cartilage repair.

Keywords: Cartilage, scaffold, controlled delivery, nucleic acids, growth factors

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Introduction

Articular cartilage lesions can significantly compromise the patient's quality of life due to pain and functional disability and represent a heavy burden for healthcare economy worldwide. Joint injuries have many possible origins including degenerative diseases and traumatic events. Due to its avascular nature and a low chondrocyte to extracellular matrix (ECM) ratio, damaged cartilage has limited self-healing capability(1,2). Surgical techniques such as microfracture, implantation of autologous chondrocytes or mosaicplasty attempt to repair the damaged cartilage. However, current methods do not allow achieving optimal biophysical properties, which results in accelerated matrix degradation and generally poor tissue quality on the long-term(3). Tissue engineering (TE) appears as a promising solution to restore the structure and the function of articular cartilage(4,5). It relies on the association of at least three different elements: cells, a supporting scaffold and biological factors(6). The ultimate objective of cartilage engineering is to generate a fully functional tissue produced by chondrocytes, the only mature cellular component of cartilage capable of secreting the ECM specific of hyaline cartilage(7,8).

Initial TE strategies have used chondrocytes combined with biofactors and a 3-dimensional construct to avoid chondrocyte dedifferentiation during the amplification phase *in vitro*(9,10). Besides, mesenchymal stromal cells (MSCs) are of particular interest owing to their ability to differentiate into chondrocytes under appropriate conditions(11,12). MSCs can be obtained from different sources including bone marrow, umbilical cord, adipose tissue and synovial membrane(13,14). However, bone marrow-derived MSCs represent the most promising source of MSCs used for cartilage engineering because of their superior chondrogenic potential(15,16). MSC-based therapies for cartilage engineering require the use of chondro-inductive biofactors including growth factors (GFs), peptides, or genetic material to help control or enhance cell differentiation, maintain the mature chondrocyte state, hence promoting cartilage repair. These factors differ in their mechanism of action but also in their functions during the process of cartilage repair. It is therefore important to precisely control the kinetics of action of these biomolecules in order to avoid unwanted adverse effects. For these reasons, controlled levels and spatio-temporal release of these molecules are essential to promote the formation of high-quality cartilage matrix, with the required biomechanical properties. To avoid repeated injections of biomolecules along the cartilage regeneration process, much work has been done to incorporate biomolecules into the constructs and control their delivery to resident or implanted cells.

The physical and biochemical properties of scaffolds are critical for the success of cartilage repair. A biomaterial has to be biocompatible, biofunctional to promote cell adhesion and integration into the host tissue, and biodegradable. Scaffolds should also have appropriate biomechanical properties to withstand external forces resulting from joint motion. Therefore, the architecture of the scaffold plays a key role in maintaining its stability while allowing cell impregnation, cell-cell interactions and free circulation of nutrients and cell wastes(5). Obviously, the chemical nature of the material forming the scaffold has a significant impact on the above properties.

In this article, we first provide an overview of the options for careful selection of the appropriate scaffold for cartilage TE. In a second part, we present the different biofactors currently used for cartilage TE and their regulatory role on both the differentiation of MSCs towards chondrocytes and the secretion of cartilage ECM by chondrocytes. Finally, we discuss the strategies

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used to functionalize scaffolds with biofactors and evaluate the impact of different approaches on the release kinetics of biofactors and their effects on chondrogenesis.

I. Scaffolds for cartilage tissue engineering: requirements and elaboration.

I.1 Criteria

In the field of cartilage TE, scaffolds play a pivotal role in establishing an ideal microenvironment for promoting cell adhesion, migration, proliferation and/or differentiation. This essential function relies on several key parameters, such as appropriate architecture, controlled degradability, mechanical properties, and biocompatibility. In fact, the structural characteristics of scaffolds, including their porosity, permeability, and interconnectivity, exert significant influence over the complex process of articular cartilage formation and subsequent tissue regeneration(17,18). Overall, the scaffold architecture must allow cell attachment and migration onto the scaffold while ensuring a proper interconnectivity(19). Since scaffolds act as temporary supports for tissue development, their controlled degradation is critical for the effective formation and integration of newly generated cartilage tissue within the surrounding endogenous tissue. It is also essential that the by-products released during the degradation process are non-toxic and easily eliminated from the body(20). Ideally, scaffolds should exhibit intrinsic mechanical properties similar to those of the native cartilage tissue such as tensile strength, toughness and stiffness. These parameters are important to promote integration and support continued tissue development after scaffolds implantation into the knee joint(21).

I.2 Materials

Materials used for cartilage TE must obviously be biocompatible and meet specific requirements of the targeted application. Numerous reviews have thoroughly examined the diverse compositions, structures, fabrication techniques, and characteristics of existing biomaterials(22–25).

Briefly, biomaterials can include either natural or synthetic polymers as well as hybrid materials combining both (Table 1). Natural polymers such as collagen(26,27), gelatin(28,29), hyaluronic acid (HA)(30,31), chitosan (CH)(32,33), chondroitin sulfate (CS)(34,35), fibrin(36) and alginate(37,38) are commonly employed in fabricating scaffolds for cartilage repair. Originating from natural sources, these materials exhibit high biocompatibility, bioactivity and possess properties close to those of native tissues making them suitable candidates. Unfortunately, most natural materials exhibit rapid degradation, which may compromise scaffold integrity. Extracted and processed biopolymers also exhibit limited mechanical strength, which may hinder their ability to support cells and promote tissue repair. Moreover, processing natural polymers into scaffolds may be challenging due to their susceptibility to changes during processing, such as chain scission or protein denaturation(5).

On the other hand, synthetic polymers have emerged as promising alternatives in TE, due to their tunable properties. Key synthetic polymers include polylactic acid (PLA)(39–42), polyglycolic acid (PGA)(43), poly-lactic-co-glycolic acid (PLGA)(44,45) , polycaprolactone (PCL)(46,47) and polyethylene glycol (PEG)(48,49). The chemical composition and structure of synthetic polymers make them scaffolds with highly customizable properties. In fact, they can be tailored to exhibit specific mechanical, physical and chemical properties such as stiffness, porosity, and degradation rate in order to meet the requirements of cartilage tissue repair. However, synthetic materials also exhibit some

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drawbacks, notably the lack of inherent bioactivity which hinders cell adhesion, proliferation, and differentiation. Tissue integration and functionality are consequently affected because the biomaterial does not fully replicate the biochemical and biomechanical properties and cues of native cartilage tissue(50). Moreover, harmful acidic degradation products may compromise biocompatibility and trigger an inflammatory response(20).

Obviously, the chemical nature of the material forming the supporting scaffold has a significant impact on their properties, and notably their ability to incorporate bioactive compounds. For instance, hydrophobic aliphatic polyesters (PLA, PGA, PLGA, PCL…) can be processed into porous scaffolds but with poorly hydrated solid walls, while hydrophilic polymers (collagen, gelatin, hyaluronic acid, PEG…) can form highly swollen hydrogels. Of note, the biophysical characteristics of the scaffolds resulting from their chemical composition can also be engineered to enhance or direct MSC differentiation. The role of substrate features such as mechanical properties, porosity or topology has been reviewed elsewhere(51,52) and will be addressed in the present article only when directly related to biomolecules interactions with the scaffold and their release profile.

I.3 Techniques

The desired scaffold architecture, mechanical properties and shapes can be achieved by selecting the appropriate scaffold fabrication method. A variety of fabrication methods allow for the production of scaffolds in the form of three-dimensional membranes, hydrogels, microspheres, sponges, or their combinations, thereby providing versatility to meet the diverse requirements of cartilage TE(53). Hydrogels, in particular, have attracted considerable attention as scaffolds for cartilage TE due to their structural and functional resemblance to the ECM(54). Hydrogel formation involves the development of hydrophilic polymer networks through chemical cross-linking, physical gelation, or self-assembly processes. These networks are capable of absorbing water and of swelling in aqueous solutions, which promotes the attachment, migration, differentiation, and proliferation of cells while effectively delivering growth factors and creating an appropriate microenvironment for nutrients(55)-(56). Scaffold fabrication techniques for cartilage TE include conventional and rapid prototyping methods (RP), which have been described in many reviews (Table 2)(57,58).

Conventional methods for scaffold fabrication are often constrained by limitations in compatibility and repeatability, and frequently rely on manual intervention, making them unsuitable for large-scale application. Among these methods, phase inversion is commonly employed for membrane preparation, and usually divided into thermally induced phase separation (TIPS) and non-solvent induced phase separation (NIPS). TIPS utilizes temperature manipulation in liquid-liquid or liquid-solid systems to achieve membranes with varied porosities, whereas NIPS involves immersing a polymer solution in a non-solvent solution, resulting in membranes with different porosities and pore sizes(59). The TIPS technique has been specifically applied to fabricate PLLA scaffolds with finely tuned pore dimensions, mimicking natural conditions conducive to efficient chondrogenesis(60). Additional techniques, such as freeze-drying, can also improve scaffold porosity and pore size. Freeze-drying, or lyophilization, involves freezing polymer solutions followed by solvent sublimation under vacuum, producing scaffolds with interconnected porous structures(61). Electrospinning is another widely used method due to its simplicity, rapidity, cost-effectiveness, and ability to generate nonwoven scaffolds with high porosity and interconnectivity(62). Electrospun nanofiber-based scaffolds are expected to be good candidates for osteochondral and cartilage repair but their outcome is mostly limited by spinnability issues of the biomolecules-containing aqueous solutions. Coaxial electrospinning offers a promising alternative, as only the shell solution, producing the outer part of the fibre, must exhibit

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good spinnability. In this technique, drugs, proteins, or other bioactive substances are incorporated into the fibre core through coaxial flow, resulting in a core-sheath structure. These biphasic nanofibers, capable of controlled biomolecule release, have been extensively studied for biomedical applications, including osteochondral regeneration(63–65). However, despite its effectiveness, it often lacks the ability to precisely control scaffold architecture and mechanical properties. Nevertheless, developments in biomaterial design have shown potential to overcome these limitations. For instance, electrospun scaffolds combining gelatin-chondroitin sulphate nanofibers with mechanically robust polycaprolactone (PCL) have been shown to successfully promote chondrogenesis without the need for differentiation media(66).

In contrast, advanced RP techniques, such as 3D printing including 3D bioprinting and selective laser sintering, allow intricate scaffold design with precise spatial control, enabling for the formation of complex structures layer by layer (LBL)(67). 3D bioprinting appears a promising approach to insert biomolecules at desired 3D locations in order to build a scaffold with spatiotemporal controlled biomolecule release properties(68–70). The modification of scaffold characteristics depends on various factors such as the ink type, and other parameters such as the printing temperature, needle size, layer density, and extrusion rate(67,71,72).

Additionally, the fabrication process must meet several critical criteria beyond its functionality, such as cost-effectiveness and scalability. Developing scalable manufacturing processes up to Good Manufacturing Practice (GMP) standards is also crucial for ensuring the successful translation of TE strategies into clinical practice.

II. Biomolecules for cartilage tissue engineering

Several biomolecules, including but not restricted to, growth factors, peptides, genetic material and small molecules, are described to mediate cellular proliferation, migration, and differentiation. These factors can interact with target cells and trigger a series of specific cellular activities. Here, we will focus on the main biomolecules involved in the development of cartilage and describe their role in regulating the processes of chondrogenesis and cartilage homeostasis (Table 3).

II.1 Small molecules

II.1.1 Kartogenin (KGN) is a non-toxic and stable small molecule, reported to promote collagen synthesis and enhance the chondrogenic differentiation of MSCs. During MSC chondrogenesis, KGN frees CBFβ, which then binds to the transcription factor RUNX1. This complex plays a crucial role in initiating the transcription of genes associated with cartilage ECM production(73). Together with other advantages, such as low immunogenicity(74), KGN shows significant promise in promoting cartilage regeneration(75). Furthermore, the absence of induction of genes related to hypertrophy and calcification was observed when KGN was applied on MSCs or chondrocytes(73,74). Although dosage and duration time still remain to be optimized, several studies have reported the beneficial effect of a continuous supply of KGN released from a scaffold on cartilage repair(76–79). For example, KGN-encapsulated PLGA microspheres provide sustained release of KGN, improving retention and enhancing therapeutic efficacy(79,80).

II.1.2 Curcumin is a yellow polyphenol pigment isolated from Curcuma longa (turmeric). It has been studied for its antioxidant and anti-inflammatory effects(81–83). Recently, high viability and phenotype maintenance of chondrocytes cultured in curcumin-containing silk scaffolds suggested a great potential for cartilage engineering(84). Although the role of curcumin in chondrogenic

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differentiation is not yet fully understood, it might indirectly induce the chondrogenic differentiation of MSCs thanks to its anti-apoptotic capacity via caspase-3 inhibition and its anti-inflammatory function on prostaglandins such as PGE2(85,86).

II.2.3 Glucosamine is a naturally occurring amino monosaccharide found in connective and cartilage tissues as a component of glycosaminoglycans (GAG). It helps maintain the strength, flexibility, and elasticity of these tissues and has been widely studied for osteoarthritis treatment as a stimulator of chondrocyte metabolism(87). The mechanism underlying its chondroprotective action remains incompletely understood but it is known to modulate GAG production, particularly hyaluronic acid (HA) and keratan sulfate (KS), by synovial cells and chondrocytes(88).

II.2.4 Icariin a prevalent flavonoid glycoside, stands as the principal pharmacological constituent of Herba Epimedium (HEP). HEP is commonly utilized as a traditional Chinese herbal remedy, with a history of extensive use in China, Japan, and Korea where it has been valued for its anti-rheumatoid, tonic, and aphrodisiac properties(89). Different studies suggested the relevance of using Icariin as an effective growth factor for cartilage TE by promoting chondrogenic differentiation and reducing hypertrophic markers(90–92). It has been shown that Icariin upregulates parathyroid hormone-related protein (PTHrP) and downregulates the expression of Indian Hedgehog homolog (IHH)(93), thereby reducing cartilage degradation and destruction(94).

II.2.5 Melatonin (MLT) is a ubiquitous molecule in nature. MLT has been shown to play several biological roles, including the promotion of hMSCs chondrogenic differentiation(95),(96) and chondrocyte function(97,98). This process is mediated through MLT membrane receptors 1 and 2, leading to BMP-2 expression and subsequent Smad1/5/8 phosphorylation, which are crucial steps in stem cell differentiation(96). MLT has also been shown to upregulate miR-526b-3p and miR-590-5p, which target Smad7, enhancing Smad1 phosphorylation and promoting the differentiation of hMSCs(99). MLT not only promotes cartilage formation but also prevents apoptosis and calcification of chondrocytes. MLT induces autophagy in chondrocytes by increasing Sirt1 expression and activity, which in turn inhibits the expression of pro-apoptotic proteins (Bax and cleaved caspase-3) and promotes anti-apoptotic proteins (Bcl-2), resulting in reduced chondrocyte apoptosis(100).

II.2.6 Ascorbic Acid also known as Vitamin C, plays a pivotal role in promoting chondrocyte proliferation and inducing chondrogenesis(101). Ascorbic acid acts as a cofactor in the formation of hydroxyproline and hydroxylysine within the collagen molecule thereby enhancing it stability, and promoting its synthesis(102). Many studies have demonstrated the effect of ascorbic acid on chondrocyte growth and ECM secretion, as exemplified by a significant increase of type II collagen secretion concomitant to matrix metalloproteinase-13 decrease(103).

II.2 Growth factors and other proteins

II.2.1 Transforming Growth Factor-β (TGF-β) plays critical roles in regulating MSC differentiation from early to terminal stages, including condensation, proliferation, commitment, maturation and terminal differentiation(104–106). TGF-β family includes TGF-β1, 2, 3, Activins (A and B), Inhibins (A and B), Bone Morphogenetic Proteins (BMP-1 to 20), Growth Differentiation Factors (GDFs) including Nodal, myostatin (GDF-8), and Mullerian-inhibiting substance (MIS). TGF-β members bind serine/threonine kinase type II receptors, which activate type I receptors anchored in the cell membrane through phosphorylation. The resulting signal transduction via R-Smads 2 or 3 to the nucleus modulates the

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responsive genes (Figure 1)(107,108). TGF-β1, 2, 3 are potent stimulators of proliferation and metabolism of chondrocytes through the secretion of cartilage ECM components including proteoglycans and type II collagen(109). Currently, TGF-β3 serves as a common and potent inductive molecule incorporated into various scaffolds to stimulate the chondrogenic differentiation of MSCs as it is considered to have a greater chondrogenic effect than TGF-β1(44,110,111).

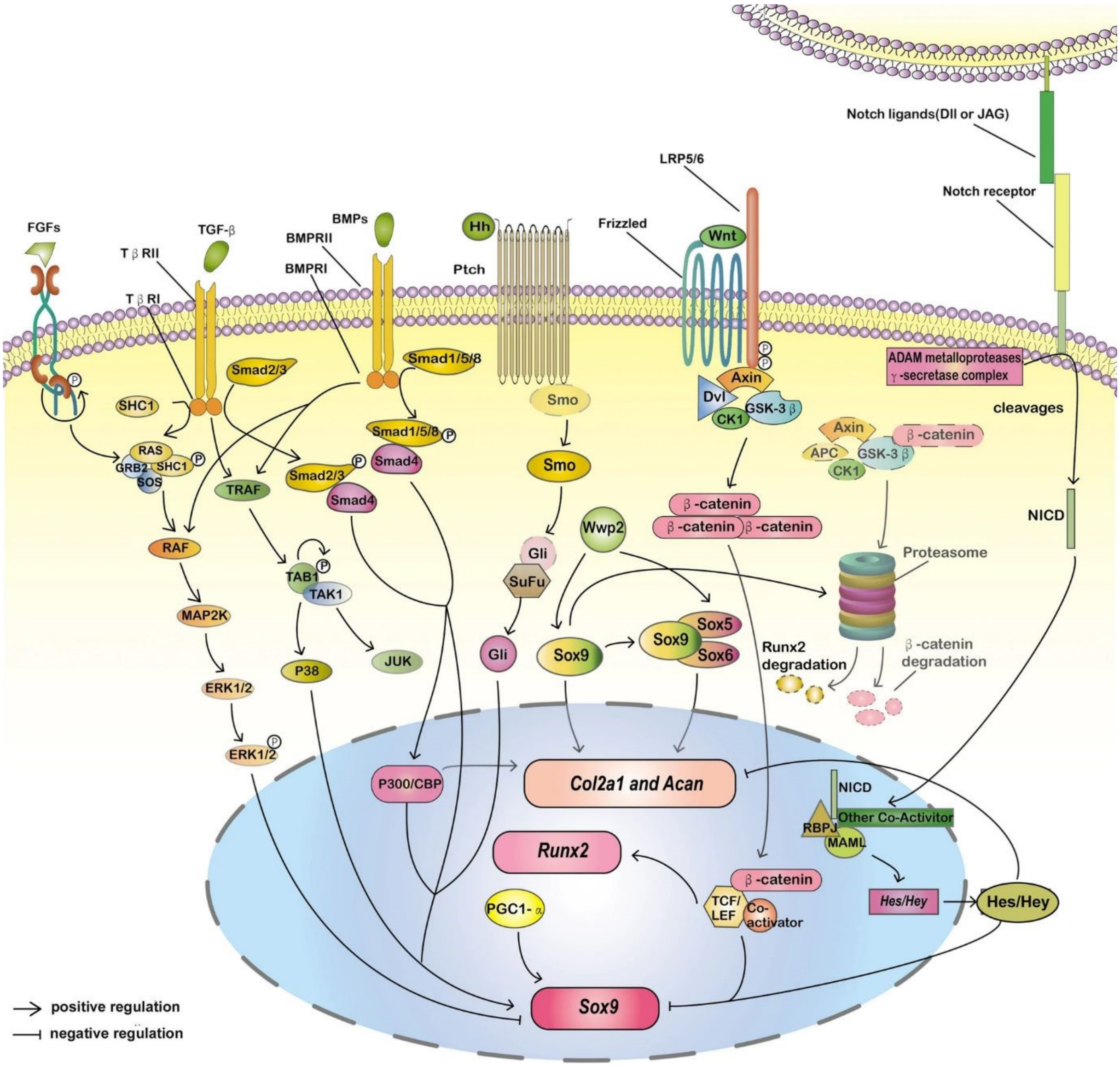

Fig. 1. Signalling pathways and proteins involved in the chondrogenic differentiation of mesenchymal stromal cells. Reproduced from ref. (112) with permission from Springer Nature, copyright 2022.

II.2.2 Bone Morphogenetic Proteins (BMPs) are members of the TGF-β superfamily. They can induce the differentiation of MSCs into chondrocytes and promote the synthesis of cartilage ECM(113). At least 30 different BMPs have been described, of which BMP-2, BMP-4, BMP-6 and BMP-7 have been the most widely studied in the field of cartilage TE(113). The different BMP isoforms act together or

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sequentially at all stages of differentiation(113,114). For example, BMP-2 and BMP-4 induce the differentiation of MSCs into chondrocytes, but only BMP-4 may inhibit hypertrophic terminal differentiation(115). Indeed, over-expression of BMP-4 suppresses the formation of hypertrophic chondrocytes during the *in vitro* differentiation of murine C3H10T1/2 mesenchymal progenitor cells(116). In addition, a BMP-4 loaded alginate gel has shown promising results in cartilage repair(117).

II.2.3 Insulin-Like Growth Factor (IGF) exists in two isoforms, IGF-1 and IGF-2, that have been shown to promote the proliferation of chondrocytes, and the secretion of cartilage ECM(118–120). More specifically, IGF-1 was shown to induce the proliferation of chondrocytes and the chondrogenic differentiation of MSCs through the IGF-1/PI3K/Akt and IGF-1/MAPK/ERK signalling pathways, whereas IGF-2 was described to enhance differentiation by priming MSCs through SOX9 regulation(120–122). These results suggest that both isoforms are required during the early phases of chondrogenic differentiation but IGF-1 can also act at a later stage to enhance the secretion of ECM(123,124).

II.2.4 Fibroblastic Growth Factors (FGFs) belong to a family of 22 highly homologous polypeptides involved in chondrocyte proliferation, joint development, and homeostasis of cartilage. Among them, FGF-2 or basic FGF (bFGF), which is recognized by its cognate receptor FGFR-1, is the most studied for its effect on chondrocytes and MSCs through multiple downstream signalling cascades, including PKCδ, NFκB, Ras-Raf-MAPK and PI3K/Akt pathways (Figure 1)(125). This growth factor is described for its capacity to enhance the proliferation of MSCs, to delay the loss of chondrogenic potential of MSCs, and also to maintain the chondrogenic potential of chondrocytes during expansion and the differentiation of MSCs by up-regulating SOX9(126–128). However, other studies have demonstrated that bFGF can promote chondrocyte catabolism via FGFR1/Ras/Raf/MEK1-2/ERK1-2 axis and inhibit the anabolic activity of IGF-1 and BMP-7(129). bFGF could also induce the formation of fibrocartilage, which is a poor alternative to hyaline cartilage(129–131). Therefore, based on these studies, the use of bFGF in cartilage TE is questionable. Nevertheless, improved healing of osteochondral lesions has been demonstrated in rabbits using a highly porous scaffold soaked with bFGF(132). However, an inverse dose response was observed, which might partly explain the controversial results reported so far. These results suggest that bFGF concentration might be an essential criterion for efficient cartilage repair and highlight the need for its tight controlled release by an optimized scaffold in order to maintain its beneficial properties.

II.2.5 Platelet-Derived Growth Factor (PDGF) is a dimer with a molecular weight of approximately 25 kDa. This cytokine released by platelets at injury sites promotes mesenchymal cell proliferation(133). Prolonged exposure to PDGF enhances cartilage ECM production, while suppressing the progression of cells along the endochondral maturation pathway(133). These observations suggest the possibility to use PDGF at the late stage of MSCs differentiation to avoid hypertrophic differentiation. In addition, some isoforms, such as PDGF-BB, have an interesting chemoattractant property that could be used to retain MSCs and chondrocytes in a scaffold after *in vivo* implantation, allowing the secretion of ECM at the injury site(134–136).

II.2.6 Parathyroid Hormone-related protein (PTHrP) is a member of the parathyroid hormone family secreted by MSCs, smooth muscle cells and some cancer cells. It is a 141 amino acid polypeptide, which acts as an endocrine, autocrine, paracrine, and intracrine hormone. Most of its biological functions are mediated by its amino terminus, including its role on cartilage(137,138). PTHrP is largely described to promote chondrogenesis by repressing hypertrophy through the transcriptional control of Runx2 activity by Gsa/cAMP/PKA-dependent signalling pathway (Figure 1)(139–142). However, PTHrP is also described to induce osteogenic differentiation of bone marrow-derived MSCs through upregulation of local factors, notably BMPs(143,144). In the context of MSCs-based cartilage TE, PTHrP

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has to be precisely controlled in order to take advantage of its anti-hypertrophic function without affecting the early steps of chondrogenesis.

II.3 Peptides

More recently, the use of peptides that can induce cellular responses such as cell recruitment, tissue integration and differentiation has been investigated in cartilage TE(145–147). Technological advances have reduced synthesis costs, making research and potential applications more accessible. For instance, the peptide "HAVDI" from the N-cadherin sequence, mimics the cell-cell interaction signal, which is key in facilitating MSC condensation, the initial step for chondrogenesis(148). This interaction enhances the chondrogenic potential of MSCs encapsulated in HA hydrogels(149). Another approach relies on the use of chemoattractive peptides to recruit endogenous MSCs. Although this strategy aims at increasing neotissue integration rather than cartilage engineering, it shows a great potential in cartilage repair. Indeed, scaffolds prepared from porcine acellular cartilage matrix functionalized with BMHP (Bone Marrow Homing Peptide) enhanced cartilage formation in full-thickness cartilage defects of rabbits(150). After a six-month period, defects were filled with neocartilage tissue that exhibited a smooth surface similar to native tissue. A similar approach used an injectable hydrogel functionalized with KLPP peptide to facilitate simultaneous recruitment of endogenous MSCs promoting interface integration and improving cartilage repair(147).

II.4 Nucleic acids

Nucleic acid-based strategies rely on the modulation of transcription factors or regulatory molecules in transfected cells *in vitro* or in the endogenous cartilage tissue in *ex vivo* strategies (144,151,152). A better understanding of MSC biology has allowed for the discovery of numerous nucleic acid molecules capable of influencing the chondrogenic differentiation of MSCs, as a complementary and powerful strategy for cartilage TE(153–157). Due to their diversity, nucleic acid-based therapies can target the entire differentiation process, from its induction to the maintenance of the quiescent stage of chondrocytes and the inhibition of hypertrophy(158–160). As an example, using RNA interference (RNAi) tools, the specific suppression of anti-chondrogenic factors could represent a promising approach for MSCs-based cartilage repair. Recently, the potential of siRNAs targeting the RUNX2 gene to inhibit the expression of hypertrophic markers after the chondrogenic differentiation of MSCs has been demonstrated(155,161). A siRNA targeting sonic hedgehog (SHH) can significantly attenuate cartilage degeneration and decrease the OA score in rats models(162). Other studies have reported the potential of microRNAs (miRNAs) to regulate the expression of genes involved in cartilage synthesis and homeostasis(163–166). MiRNAs are short, single-stranded RNA molecules 18 to 24 nucleotides long. They function as transcriptional repressors by binding to the untranslated region (UTR) of target messenger RNA (mRNA), decreasing the expression of target genes. Over 30 miRNAs present in human joint tissue are implicated in regulating cartilage homeostasis and the OA development. Among these, miR-140 has gained considerable interest and multiple miR140 targets have been identified and described(166). Notably, the inhibitory effect of miR-140 in chondrocyte hypertrophy has been shown to occur through the inhibition of histone deacetylase (HDAC)-4 and SMAD1(167,168).

II.5 Hypoxia-mimicking molecules

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It is well recognized that the chondrogenic differentiation of MSCs can be achieved by maintaining the cells under hypoxic conditions, thus simulating the native environment of articular cartilage(169). Additionally, hypoxic culture conditions have been shown to suppress the expression of markers associated with endochondral ossification through the activation of the PI3K/Akt/FoxO pathway(170). The secretion of ECM was found to be enhanced when using human articular chondrocytes pretreated with hypoxia prior to encapsulation in alginate hydrogels and implantation in a nude mouse model(171).

Hypoxia induces the expression and stabilization of hypoxia-inducible factor-1α (HIF-1α), a key regulator of the hypoxic response that plays a critical role in chondrocyte differentiation and survival *in vivo*. Under normoxia, HIF-1α is hydroxylated by prolyl-hydroxylase domain enzymes (PHDs) and the factor inhibiting HIF (FIH) hydroxylase, resulting in immediate ubiquitination and subsequent proteasomal degradation of the subunit. In a low-oxygen environment, the activities of PHDs and FIH are inhibited. HIF-1α accumulates in cells and binds to HIF-1β to form HIF-1, which then binds to HRE to participate in multiple signalling pathways by regulating the transcription of hundreds of genes, including those specific to cartilage. Currently, HIF signalling pathways mainly include: PI3K-Akt /HIF-1α, SENP1/HIF-1α, HIF-1α/BNIP3/Bcline-1, MAPK/HIF-1α(172) (Figure 1). Instead of hypoxia incubators or chambers, several hypoxia-mimetic agents have been employed to induce hypoxia. These molecules are not widely studied but their low cost and ease of use make them a promising class of potential tools for use in cartilage TE(173).

II.5.1 DMOG (Dimethyloxalylglycine) is a competitive inhibitor of hydroxylase enzymes, and its presence results in increased nuclear localization of HIF-1α, thereby promoting chondrogenic differentiation through the increased production of type II collagen and other extracellular matrix components(174). Recently, the sequential application of DMOG and PTHrP encapsulated in PLGA microspheres effectively mimicked the hypoxic microenvironment, thereby promoting chondrogenic differentiation with phenotypic stability(175).

II.5.2 DFO (Deferoxamine) is a chelating agent that is approved by the Federal Drug Administration for the treatment of iron excess(176). The activity of PHD is dependent on Fe^{2+} and O_2 concentrations. Consequently, a reduction in iron concentration results in decreased HIF-1α hydroxylation and its accumulation in cells. As a PHD inhibitor, DFO is a suitable agent to mimic hypoxic conditions and therefore represents a promising molecule with the potential to optimize biomaterials and existing TE techniques for tissue regeneration(177).

II.5.3 Cobalt chloride ($CoCl_2$) has the capacity to impede the degradation of HIF-1α protein, thereby inducing its accumulation and, consequently, inducing hypoxia. This characteristic is derived from the ability of Co^{2+} to inactivate FIH by substitution for Fe^{2+} in the iron-binding center of the enzyme(178,179). A $CoCl_2$ encapsulation into an alginate scaffold has been shown to promote chondrogenesis without the use of costly growth factors(180).

As described above, many biomolecules (Table 3) can be used to regulate cellular activity at different stages of chondrogenesis, depending on their function or the time of application. However, most of these active molecules have short-term action due to their rapid elimination or degradation after delivery. Therefore, they must be protected before being released in a controlled manner. Scaffold engineering to precisely fine-tune the spatiotemporal release of biomolecules is therefore being investigated to better control cell behaviour and *in fine*, improve cartilage TE efficacy(113).

III. Interactions and mechanisms involved in biomolecule delivery from scaffolds

III.1 Non-covalent interactions

As the field of cartilage TE has rapidly expanded, many three-dimensional and porous scaffolds are being considered as active biomolecules release systems. Typically, active molecules entrapped in polymer materials are released in the surrounding medium due to the combination of diffusion of the molecule through the matrix and matrix erosion. The proportion of these two contributions depends on the nature of the polymers, the hydration level of the matrix, its porosity and interactions, specific or not, between the active compounds and the supporting materials. As to diffusion, in the absence of a covalent bond between the biomolecules and the matrix, the retention rates and release kinetics mostly result from weak interactions and steric hindrance (Fig. 2, Table 4).

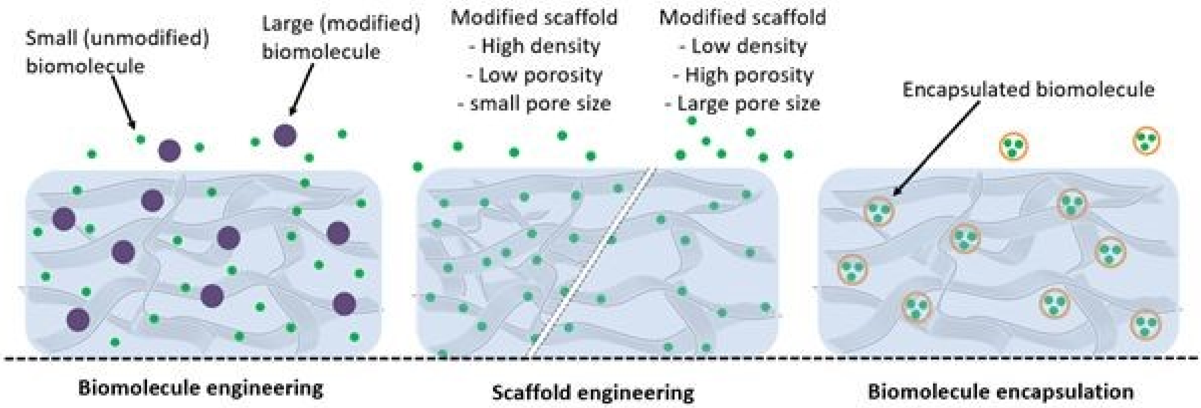

Fig. 2. Schematic representation of possible steric interactions between active molecules and scaffolds, and their modulation to alter biomolecule release kinetics. **Biomolecule engineering**: small, unmodified (green) and large or modified biomolecules (purple) interact differently within the scaffold matrix based on their size and structure. **Scaffold engineering**: The scaffold porosity and density are tuned to control biomolecule interactions: high-density scaffolds with low porosity and small pore size limit diffusion, while low-density scaffolds with high porosity and large pore sizes enhance molecule release. **Biomolecule encapsulation**: Biomolecules are effectively encapsulated in (nano-)particles within the scaffold, ensuring confinement and preserving functionality.

III.1.1 Steric hindrance is a physical barrier to the diffusion of active compounds or vector particles through hydrogels. The meshes of the scaffold create a steric obstruction, which hinders the diffusion of the biomolecules and delays their release. Steric interactions are involved when biomolecules are loaded or released by diffusion through and out of the matrix. In the case of particles used as reservoirs of biomolecules or small molecules, or in the case of vectors used to protect and deliver nucleic acids, the relevant dimensions are those of the carriers. The loading rate and delivery kinetics thus depend on the biomolecule or vector size but also on the chemical nature and structure of the matrix in which they are immobilized. Structural features include the morphology, density (concentration, crosslinking rate), porous structure (tortuosity, pore size) of the scaffold. As an example, more than 95 % of IGF-1 was released over a 28-day period, with gelatin microparticles cross-linked with 40 mM glutaraldehyde, while similar release values were obtained after only 6 days when using microparticles cross-linked with 10 mM glutaraldehyde(181).

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In order to control the release of siRNA, siRNA lipoplexes encapsulated in a gellan gum hydrogel exhibited a prolonged release over 60 days, while naked siRNA was released from the hydrogel within 48 hours(182). Similarly, the impact of steric interactions was evaluated in the case of siRNA-nanoparticles (si-NPs) loaded in a porous biodegradable polyester-polyurethane (PUR) scaffold(183). The si-NPs were formulated with a defined charge ratio between positively charged tertiary amines on the DMAEMA block of the polymer and negatively charged phosphate groups on the siRNA backbone (Figure 3). The diffusion and the release kinetics of encapsulated siRNAs and free siRNAs were compared. The release data demonstrated cumulative release of si-NPs approaching 80 % over 21 days, which is considerably slower compared to naked siRNAs completely released in three days. The release rates of naked and complexed siRNAs scaled with their hydrodynamic diameter and their diffusivity throughout the PUR matrix. This property is particularly useful for gene transfer strategies, since NPs preserve nucleic acid integrity and serve as a vector allowing genetic material to cross the cell membrane(153,184,185). Recently, we demonstrated that it was possible to obtain different siRNA diffusion profiles from a collagen hydrogel, depending on the size of the vector used(186). Interestingly, the inhibition profiles of the target gene Runx2 over time was correlated with the release kinetics, hence with the size, of the siRNA vector.

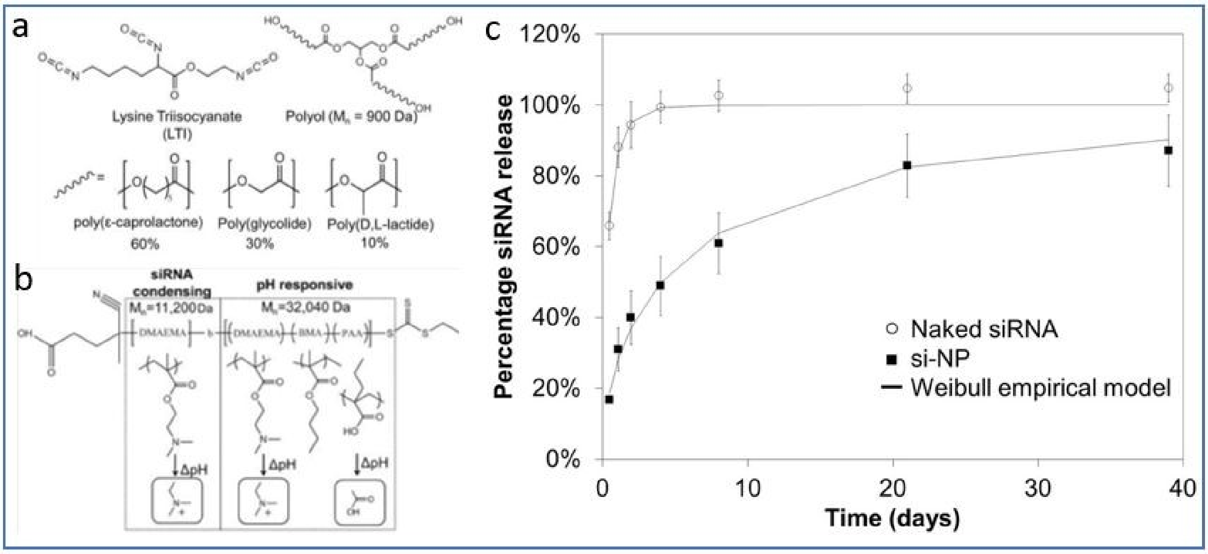

Fig. 3. Sustained local delivery of siRNA from an injectable scaffold. **(a)** Chemical structure of polyurethane precursors. Lysine Triisocyanate reacts with the –OH groups of the polyol to form urethane bonds and creates the PUR network. (**b**) Chemical structure of the micelle-forming, pH–responsive diblock copolymer used for siRNA packaging and intracellular delivery. The homo-2-(diethylamino)ethyl methacrylate (DMAEMA) block was designed for siRNA condensation due to the positive charge on the tertiary amines. The other block is pH-responsive and tuned for endosomal escape due to micelle destabilization and endosomolytic activity triggered by protonation of 2-propyl acrylic acid (PAA) and DMAEMA. **(c)** Naked siRNA is rapidly released with an initial burst of over 60 % at 12 hours and is entirely released after 3 days. Si-NPs exhibit a slower kinetic with a burst release of less than 20 % during the first 12 hours, followed by a sustained release reaching 80 % by 21 days. The Weibull empirical model equation best-fit was determined and is overlaid here for each data set. Reproduced from ref. 183 with permission from Elsevier, copyright 2012.

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III.1.2 Scaffold degradation. One major function of scaffolds applied to cartilage TE is to provide 3D support for chondrocytes, and thus to promote ECM secretion. However, the scaffolds are not intended to stay in the joint. Ideally, they should be gradually degraded and replaced by the newly created cartilage matrix. This is why scaffolds used in cartilage TE are generally made of biodegradable materials(187,188). Scaffold degradation is accompanied by a reduction of steric constraints and therefore plays an important role in the release of encapsulated biomolecules.

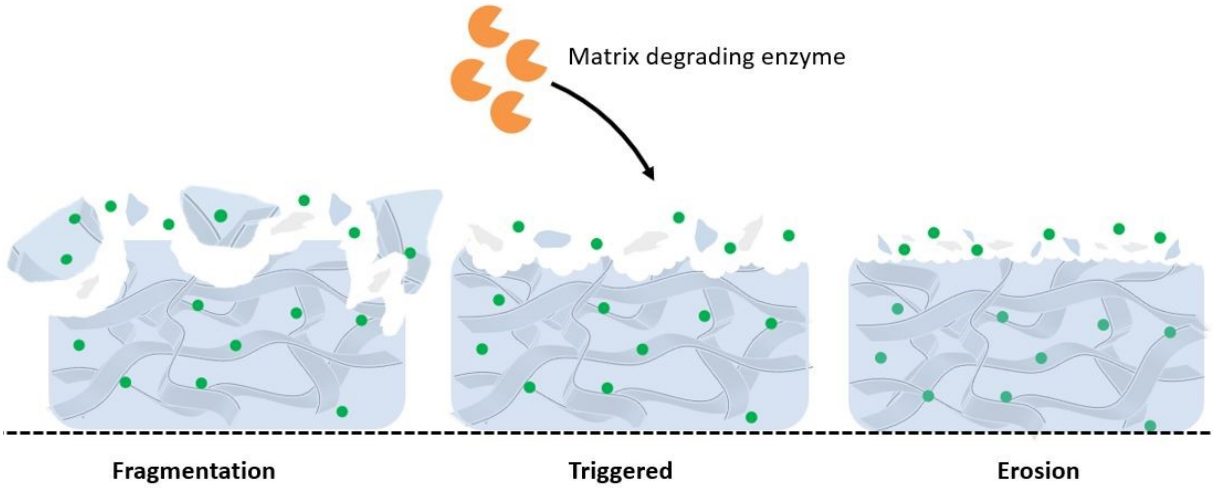

Fig. 4. Schematic representation of biomolecule delivery by scaffold degradation. **Fragmentation**: the scaffold breaks into smaller fragments, releasing encapsulated molecules into the surrounding environment. **Triggered degradation**: enzymatic activity selectively degrades specific regions of the scaffold, facilitating the release of biomolecules. **Erosion**: the scaffold undergoes gradual thinning and complete degradation, resulting in the sustained release of the remaining embedded biomolecules.

A multitude of factors can affect the rate of scaffold degradation *in vivo*, including the surrounding environment, the composition and structure of the biomaterial, and the physical loading to which the scaffold is subjected(189–191). Indeed, the degradation of a polymer scaffold involves chain cleavage processes induced for instance by hydrolysis, oxidation or photo-degradation(190). *In vivo*, scaffold degradation takes place in an aqueous biological environment where hydrolysis plays an important role, often promoted by enzymatic activities (proteases, esterases…). In the context of cartilage TE, the degradation of the natural polymer scaffold, comprised of collagen for instance, may be accelerated by matrix metalloproteases (MMPs) secreted by chondrocytes resulting in accelerated or triggered release of biomolecules(192). As an example, micelles containing miR-140 entrapped in MMP-sensitive microparticles composed of gelatin methacryloyl hydrogel have been developed(193). In the presence of MMPs, complete release of miR-140 from the microparticles was achieved after 5 days, compared to more than 14 days in the absence of MMPs (Figure 5). The authors exploited the presence of large amounts of MMPs in the OA joint capsule to degrade the scaffold, thus releasing the entrapped micelles loaded with miR-140. After *in vivo* injection, a notable reduction in osteophyte formation and OARSI score was demonstrated in a DMM-induced OA model. The group having received the micelles containing miR-140 entrapped in MMP-sensitive microparticles demonstrated the most favourable outcome with regard to GAG level, indicating optimal retention of cartilage thickness. In addition, the expression of COL2 was the highest while MMP13 expression exhibited an inverse correlation in this group. These findings collectively suggest that this MMP-sensitive microparticles have the potential to delay the degeneration of articular cartilage and the progression of OA.

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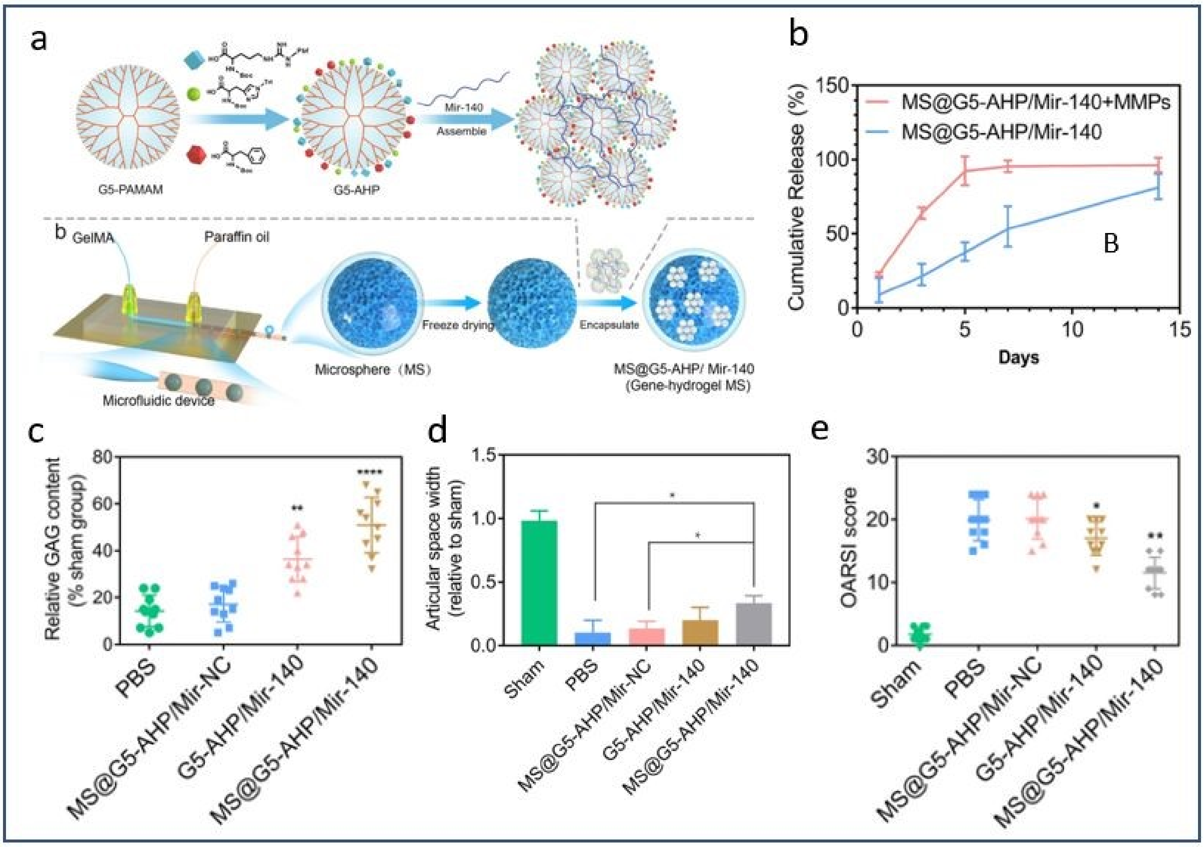

Fig. 5. MMPs triggered mir-140 release for local treatment of osteoarthritis **(a)** Synthesis of the multifunctional gene vector: arginine (A), histidine (H), and phenylalanine (P)-modified generation 5 (G5) polyamidoamine (G5-AHP), G5-AHP/miR-140 and G5-AHP/miR-140 after immobilization in gene-hydrogel microspheres (MS@G5-AHP/miR-140). **(b)** Cumulative release curve of MS@G5-AHP/miR-140. **(c)** Relative glycosaminoglycan (GAG) content after 12 weeks. **(d)** Articular space width in the medial compartment of the mouse knee joints 12 weeks after surgery. **(e)** OARSI score for each group after 12 weeks. Reproduced from ref. 193 with permission from Springer Nature, copyright 2022.

Similarly, MMP-13-responsive hydrogel microspheres were used to control the release of celecoxib-loaded liposomes in the context of OA(194). Compared to microspheres immersed in hyaluronidase only, the drug release efficiency reached 82 % on day 5 after immersion in the solution containing MMP13 and hyaluronidase, indicating a significantly accelerated release of celecoxib in the presence of MMP13 (Figure 6b). After inducing OA in rats via ACL transection and partial medial meniscectomy, intra-articular injection of these microspheres yielded significant reduction in cartilage degeneration(194) showed by lower OA score, higher joint space width and smaller osteophyte formation.

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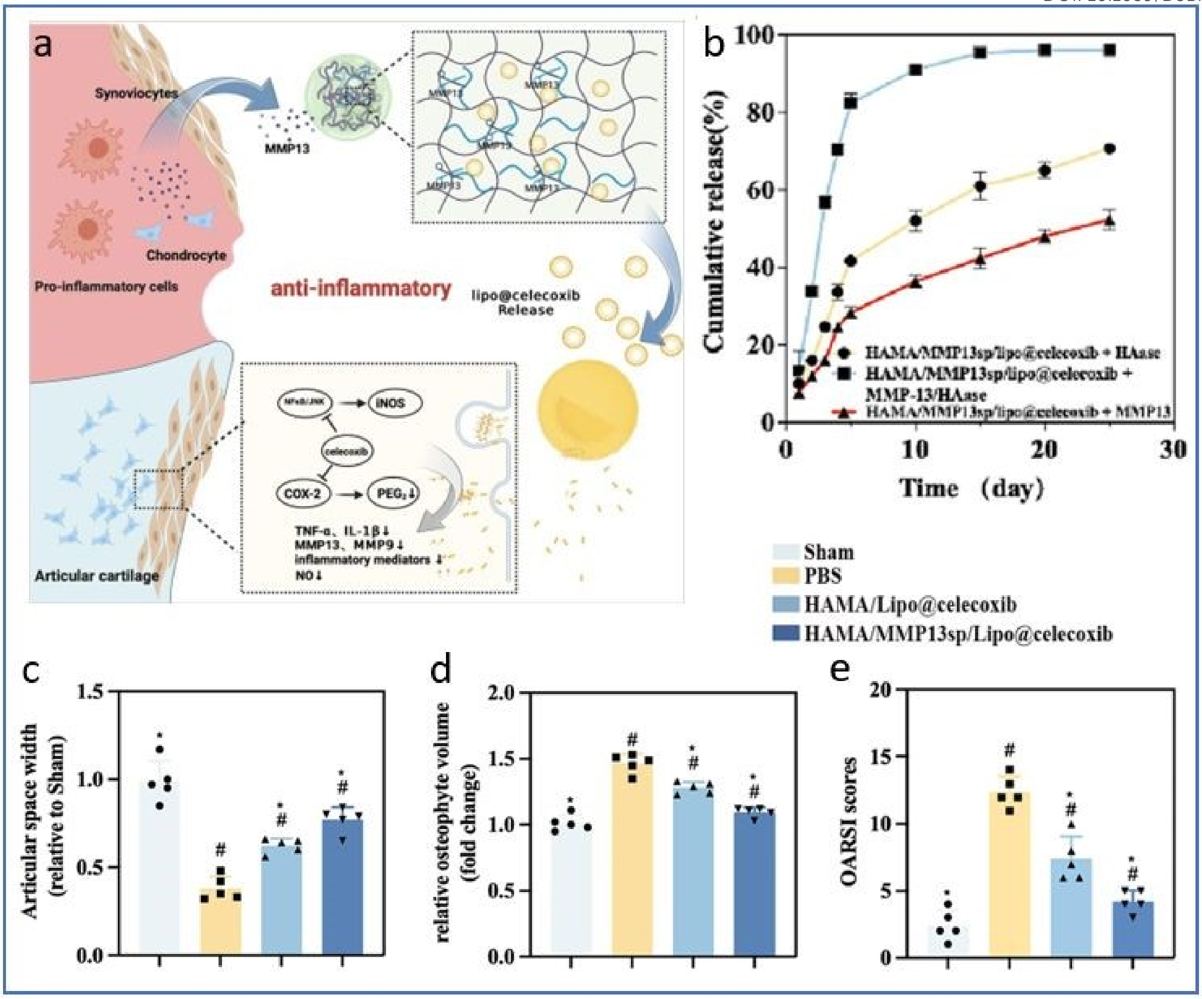

Fig. 6. MMP13 responsive hydrogel microspheres by precise delivery of celecoxib **(a)** Schematic representation of the responsive release of celecoxib from hydrogel microspheres induced by MMP13. **(b)** Drug release profiles of celecoxib in HAase, MMP13 and MMP13/HAase solution. **(c)** Measure of joint space width of the lateral knee joint compartment evaluated on X-rays. **(d)** Relative osteophyte volume measured by micro-CT. **(e)** OARSI scores determined on histological sections of knee joints. Reproduced from ref. 194 with permission from Elsevier, copyright 2024.

The rate of degradation depends also on the composition and structure of the biomaterial. For example, the release of curcumin from a gelatin scaffold can be prolonged by adding silk fibers to the scaffold(195). Another study showed that the degradation rate of a scaffold made of PLGA and PLA nanofibers could be adapted by varying the PLGA/PLA ratio: the higher the PLGA/PLA ratio, the faster the scaffold degrades(196). Finally, cartilage repair is also correlated with mechanical loading applied to the scaffold. Higher shear stresses resulted in early and fast release of sirolimus, with high cumulative drug release(197).

However, achieving precise control of biomolecule delivery solely by adjusting scaffold biodegradation remains limited. One reason is that, depending on the application, it might be required that the active molecule be released faster than the scaffold degradation rate, which should be consistent with the rate of neo-tissue formation. In contrast, the diffusion out of a highly solvated hydrogel is often very fast, hence retention of the active compounds through weak interactions or reversible covalent attachment to the matrix is needed. Different strategies have been explored to achieve optimal

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delivery profiles of single or multiple drugs(198). Comprehensive understanding of the interactions and release mechanisms underlying the drug delivery kinetics is key to providing new insights into cartilage repair.

III.1.3 Weak interactions. Although steric interactions between scaffolds and biomolecules are ubiquitous, other non-covalent interactions can be exploited to finely tune the spatio-temporal release of biomolecules. Electrostatic and hydrophobic non-covalent interactions are widely involved in the interactions between scaffolds and growth factors.

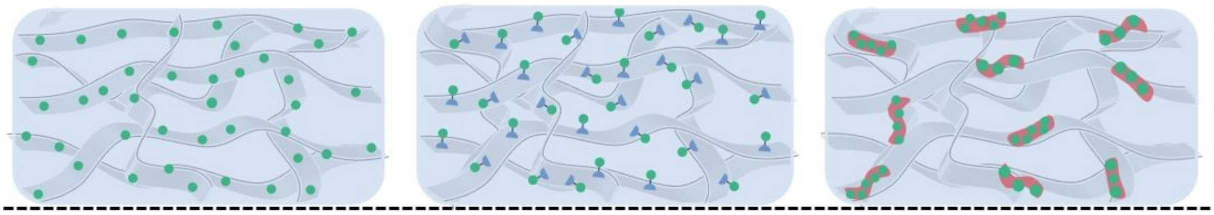

Fig.7. Schematic representation of weak interactions that can be used to control biomolecule delivery from scaffolds with (left) s**pontaneous natural interactions** between unmodified biomolecules and the scaffold, (middle) **modified biomolecules** functionalized to improve their affinity for the scaffold, thereby enabling a more sustained release and (right) **heparin semi-interpenetrated scaffold** where heparin (red) is dispersed into the scaffold, enabling stable and enhanced interactions with biomolecules.

Several natural macromolecules, abundant in the connective tissues of vertebrates (collagen, heparin, or hyaluronic acid) and largely used to elaborate scaffolds for TE, exhibit attractive interactions with diffusing active biomolecules. Collagen is the main component of cartilage ECM and among the most used materials for the construction of scaffolds in TE. Some GFs, including TGF-β1, bFGF and BMP-2 have been demonstrated to naturally possess a strong affinity for collagen and can bind to collagen-based scaffolds via ionic interactions. However, the release profiles usually feature an initial burst, which makes this approach unsuitable for controlled release(199,200). One possibility to enhance their binding to the scaffold and slow down their release is to engineer recombinant GF by adding a collagen binding domain (CBD) at one terminal end(201,202). Indeed, recombinant PTHrP expressed as a fusion with a CBD heptapeptide displayed higher collagen-binding capacity compared to the native PTHrP, with a dissociation constant two times lower(203). Applied to cartilage TE, this engineered peptide allowed for sustained release from a collagen scaffold and prolonged effect over several days. Reversely, peptides with a strong affinity for specific regions of GFs can be designed and bound to the scaffolds to control the GF presentation and activity(204). The modification of scaffolds with such peptides resulted in higher retention, less dissemination and better controlled release, thus reducing the amount of GFs required and providing a more cost-effective approach for TE applications(205–207). Several TGFβ1-binding peptides, including HSNGLPL, have been used to functionalize biopolymer scaffolds, with *in vitro* and *in vivo* applications(208–211). Similarly, the incorporation of heparin in scaffolds is widely used for drug delivery purposes(193,196,212–215). Indeed, positively charged amino acids of GFs can interact with GAGs through electrostatic interactions, particularly with the sulphate groups of heparan sulphate proteoglycans (HSPG) that are

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widely produced in the ECM of tissues(213,216–220). For instance, heparin was trapped as a semi-interpenetrated polymer within a crosslinked network or covalently bound to a polymer backbone by grafting tyramine, methacrylate, thiols or maleimide moieties to the heparin chains(221–225). Once trapped in the network of polymers or grafted onto the scaffold, electrostatic interactions established between heparin and GFs such as TGF-β or bFGF slowed down their release. Recently, heparin covalently conjugated to a hyaluronan hydrogel was shown to achieve sustained release of 60 % TGF-β1 after two weeks, whereas in the absence of heparin, 97 % of TGF-β1 was released over the same period(226). The system demonstrated remarkable efficacy in promoting chondrogenesis, as shown by a 19- and 32-fold increase of aggrecan and type 2 collagen expression respectively, after 21 days of differentiation when heparin was covalently conjugated to a hyaluronan hydrogel (Figure 8).

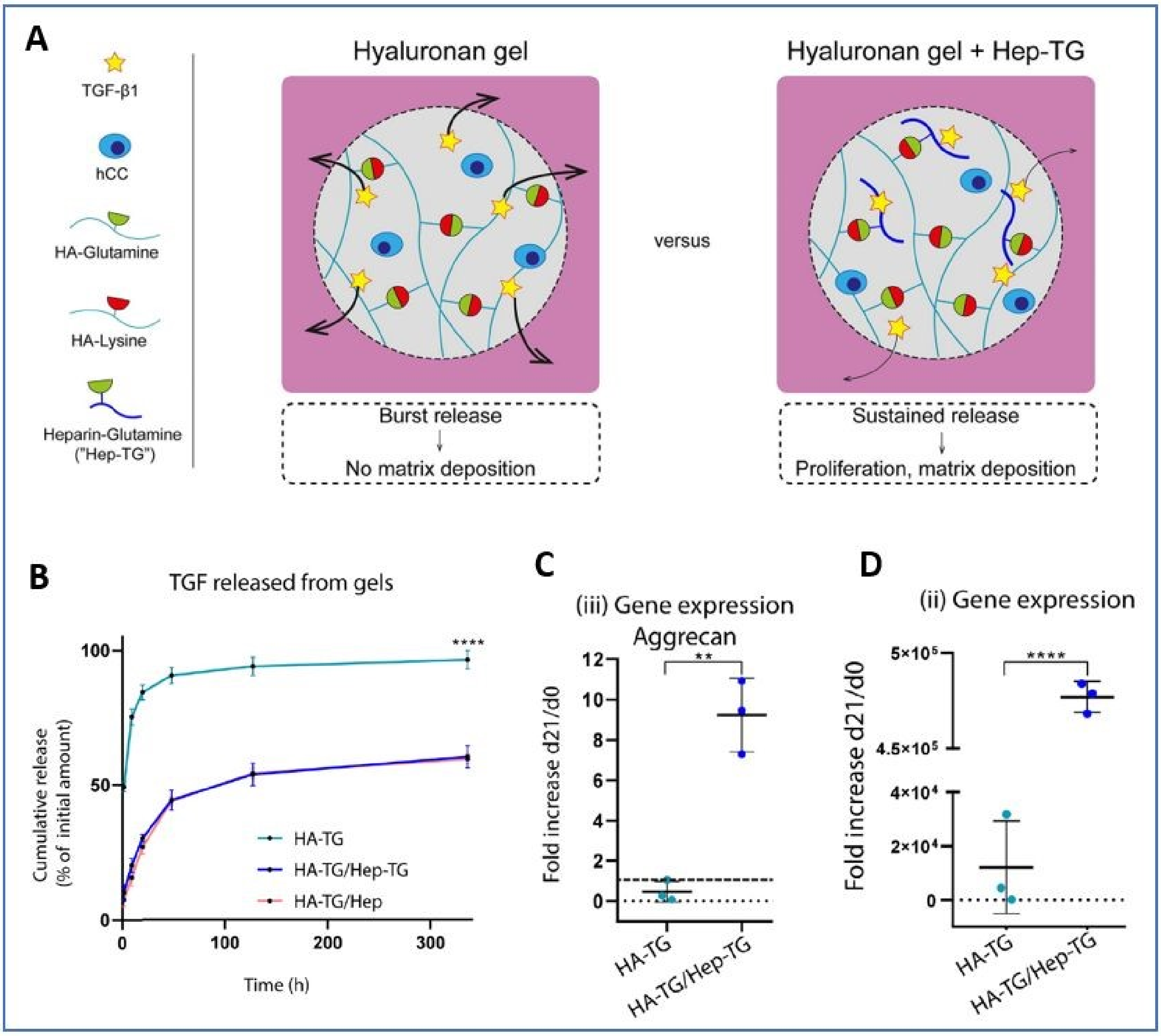

Fig.8. Injectable heparin-conjugated scaffold for local delivery of TGF-β1. **(A)** Schematic of the study design**. (B)** Cumulative TGF-β1 release from Hyaluronan gel (HA-TG), Hyaluronan gel + Heparin-Glutamine (HA-TG/Hep-TG) or Hyaluronan gel + Heparin (HA-TG/Hep) gels. **(C, D)** gene expression after 21 days of differentiation for (C) aggrecan and **(D)** collagen type II. Reproduced from ref. 226 with permission from *Elsevier*, copyright 2019.

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Interestingly, sulphated alginate, which is used in cartilage TE for its beneficial effects on chondrocyte proliferation and phenotype maintenance, can also act as a GAG analogue and interact with most GFs, thereby extending their therapeutic effect(227–231). This dual property of alginate was used to form a macro-porous alginate scaffold, where uronic acid units were sulphated to mimic heparin, and successfully loaded with TGF-β1 to enhance MSC chondrogenesis(232). Finally, electrostatic interactions can also be involved in particular for gene therapy applications using non-viral vectors for gene transfer(1,153,233,234). Naked nucleic acids bear a negative charge, while the vectors obtained by complexation with cationic polymers or lipids exhibit a net positive charge(183,235). For example, the incorporation of siRNA into positively charged copolymer micelles significantly slowed down the release from injectable polyurethane, which can be attributed in part to attractive electrostatic interactions between the vector and the scaffold matrix(183).

Hydrophobic interactions also largely participate to the retention of biomolecules into a scaffold. As an example, gelatin or gelatin-silk fibroin microspheres have been used for the sustained release of curcumin adsorbed into the microspheres for the treatment of OA(195). The slower release rate with gelatin-silk fibroin microspheres than gelatin microspheres was attributed to hydrophobic interactions between the curcumin and the hydrophobic domains of the silk fibroin, along with the lower degradation rate compared to pure gelatin microspheres. It is also possible to alter the interactions between proteins and scaffolds to control the biomolecule release kinetics. For example, PLGA microspheres have been used as pharmacologically active microcarriers for the delivery of TGF-β3 to promote the differentiation of MSCs into chondrocytes(236). In this study, PLGA microspheres were modified with poloxamer P188, resulting in a more hydrophilic scaffold and therefore allowing for larger and sustained release of TGF-β3 reaching 70 % at day 30. These PLGA microspheres modified with poloxamer P188, exerted a superior effect on chondrogenic differentiation compared to unmodified PLGA-TGF-β3.

III.2 Covalent Interactions

Covalent binding of biomolecules to scaffolds is commonly used to increase retention rates and significantly reduce the initial burst release often observed with non-covalent methods(237). Biomolecules can be directly grafted to the materials or attached via a linker. The immobilization strategies may involve existing chemical functions present on the scaffolds and biomolecules or require previous activation. Because biomolecules are usually not soluble or will be denatured in organic solvents, their bioconjugation requires the use of aqueous-based chemistry. To address these needs, various activation strategies are being developed, and the main biocompatible chemical immobilization systems adapted to cartilage TE are presented below (see also Table 4).

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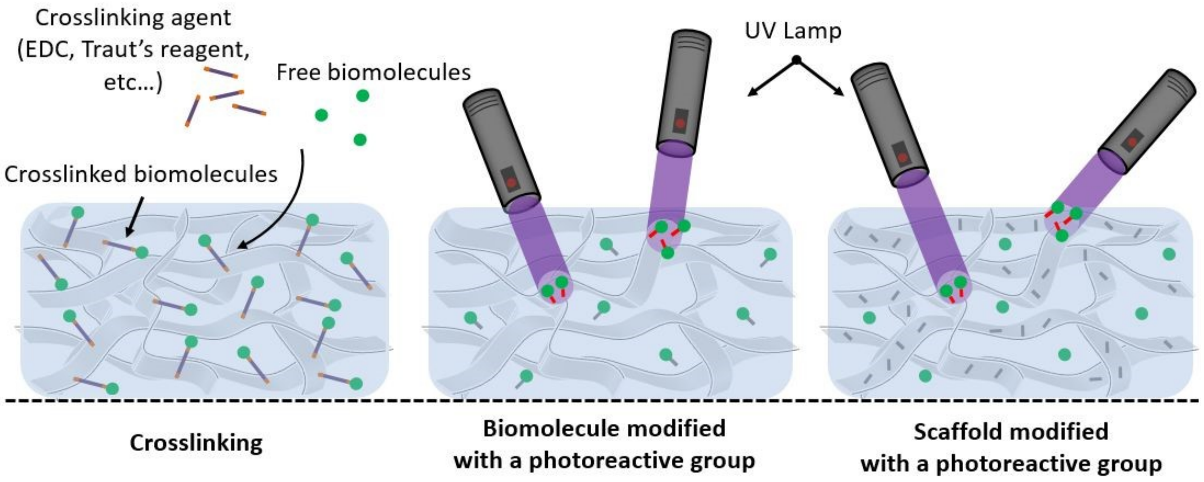

Fig. 9. Different strategies for chemical modifications of the scaffolds or biomolecules, and covalent grafting to control biomolecule delivery from scaffolds. **Crosslinking** of biomolecules to the scaffold using crosslinking agents such as EDC or Traut's reagent, leading to stable attachment in the scaffold. **Biomolecules modified with photoreactive groups** are immobilized onto the scaffold under UV irradiation, enabling localized and precise functionalization. **Scaffolds modified with photoreactive groups** allow immobilization of free biomolecules upon UV exposure, facilitating controlled biomolecule attachment.

III.2.1 Direct grafting. One of the most common methods to conjugate biomolecules is the formation of an amide bond between a carboxyl group of the biological factor and a primary amine on the scaffold matrix, or vice versa. The zero-length linker 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC), a water-soluble carbodiimide, is often used to activate the carboxyl groups(206,238). EDC is released after formation of the amide bond, leaving no additional atom between the biomaterial and the biofactor. EDC has been used to conjugate TGF-β1, TGF-β3, BMP-2 and BMP-4 to a variety of biomaterials(239–242). The release of TGF-β3 immobilized onto PLGA-gelatin-chondroitin sulphate-HA hybrid scaffold demonstrated a biphasic pattern characterized by a fast release of 14.5 % of initial loading during the first day followed by a plateau by day 7 to reach 29.5 % of release at 28 days. The release of TGF-β3 induced the chondrogenic differentiation of MSCs(240). To improve biomolecule immobilization, sulfo-N-hydroxysuccinimide (sulfo-NHS) is often combined to EDC to form a more stable amine-reactive sulfo-NHS ester intermediate, with improved reaction efficiency in aqueous media(205,237,243). The combination of EDC and NHS activation was used to conjugate KGN on chitosan-based nanoparticles (NPs) and microparticles (MPs), which resulted in higher *in vitro* chondrogenic differentiation of MSCs compared to control with unconjugated KGN(244). The *in vitro* release of KGN was compared between MPs and NPs, with 55 % of KGN released from MPs and 35 % released from NPs over a 50-day period (Figure 10). Micropellets of MSCs treated with kartogenin-conjugated MPs or NPs exhibited stronger Safranin-O and Alcian blue staining, indicating enhanced proteoglycan synthesis. *In vivo*, the administration of CHI-KGN NPs or CHI-KGN MPs resulted in a reduction of degenerative changes in rats with induced OA, reflected by a significant reduction of OARSI scores.

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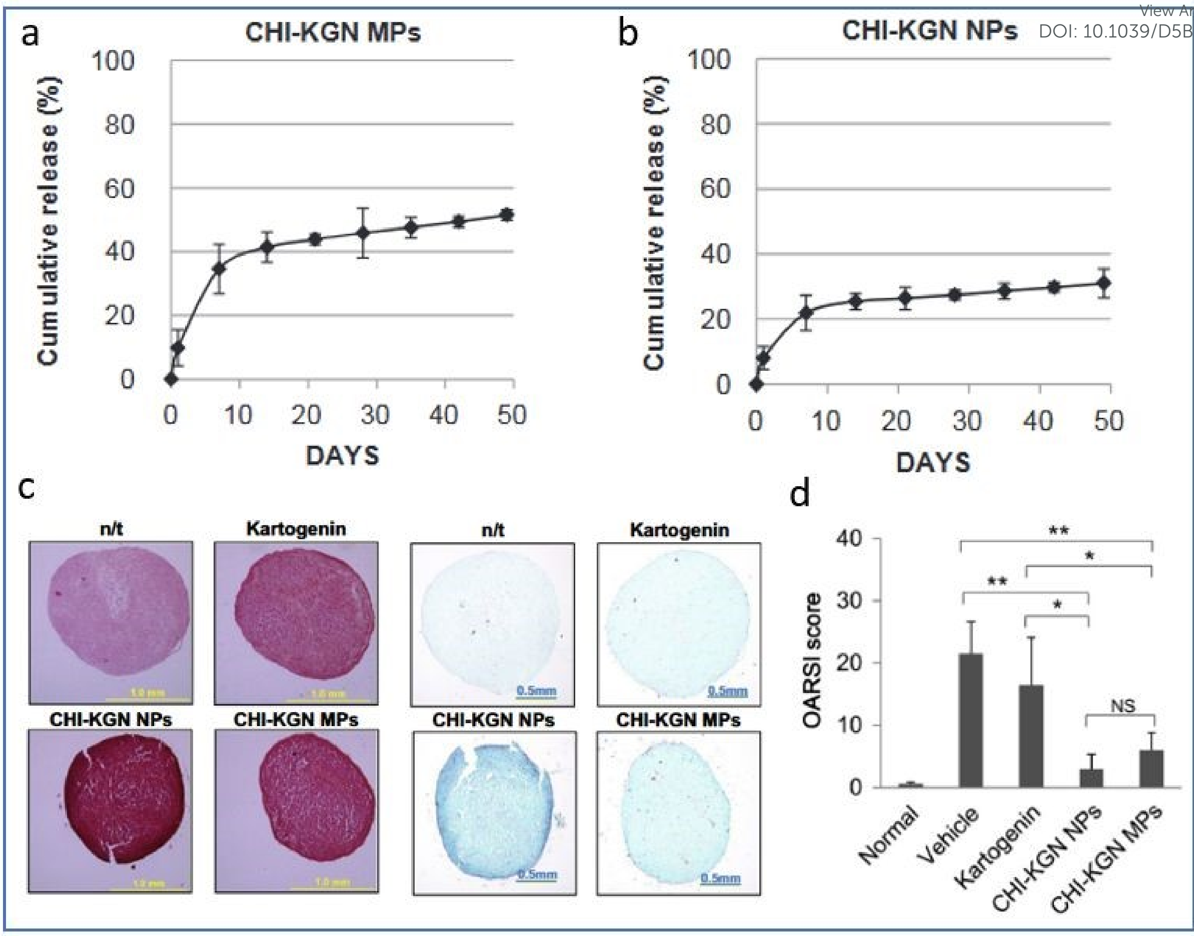

Fig. 10. Intra-articular delivery of kartogenin-conjugated chitosan nano/microparticles**. (a)** Cumulative release of KGN from kartogenin-conjugated chitosan microparticles (CHI-KGN MPs) and **(b)** nanoparticles (CHI-KGN NPs). **(c)** Safranin-O and Alcian-blue staining of cells micropellets cultured with or without kartogenin, CHI-KGN MPs or CHI-KGN NPs at 3 weeks. **(d)** OARSI scores from histological sections of medial tibial plateaus of rats injected with vehicle, KGN or implanted with the CHI-KGN MPs or CHI-KGN NPs at 14 weeks. Reproduced from ref. 244 with permission from Elsevier, copyright 2014.

However, immobilization on the scaffold and the various necessary steps required can affect the bioactivity of the drug. Carbodiimide coupling can interact with the amine groups present in the lysine residues or N-terminus of GF, as well as the carboxylic groups present in the aspartate or glutamate residues or C-terminus. This lack of selectivity may result in some bioactive functional groups being involved in grafting bond formation, potentially resulting in a loss of GF bioactivity(204). Similarly, the presence of numerous functional groups leads to the random immobilisation of GF, thereby affecting the accessibility of ligands to the corresponding cell receptors(205).

III.2.2 Click Chemistry represents a quick, selective, and high yield chemical conjugation method without the need for extensive post-processing to remove by-products(245,246). Click reactions offer the advantage of being nontoxic for cells and to be done in water or in complex biological environments avoiding multiple steps that can affect biomolecules/ drugs bioactivity(247). Among them, thiol-based click strategies are based on reactions with free thiols that are present in cysteine residues of native GFs. Alternatively, thiols can be introduced chemically on the primary amines of biomolecules, notably proteins, using 2-iminothiolane, also known as Traut's

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reagent(248,249). This chemical modification was applied for instance to the immobilization of TGF-β1 onto an acrylated poly(glycidol) (PG-Acr) hydrogel. Acrylate groups on the PG-Acr served both for hydrogel formation and conjugation with the thiolated GF via Michael-type addition. Grafting of TGF-β1 did not compromise hydrogel formation and improved chondrogenesis of encapsulated MSCs compared to gels with TGF-β1 simply impregnated without thiolation(250). The GAG/DNA and collagen/DNA ratios were approximately two times higher and the cartilage ECM was thicker when TGF-β1 was grafted. This clearly demonstrated the superior effect of covalent immobilization of TGF-β1 onto the hydrogels compared to the non-covalent incorporation.

Another major click chemistry reaction that is gaining a great deal of interest in the biomedical field is strain promoted azide-alkyne cycloaddition (SPAAC). For example, alkyne groups were added to BMP-2 and azides were added to a methoxy polyethylene glycol-polycaprolactone to facilitate attachment and *in fine* improve cell differentiation(251). In another example, hydroxyethyl cellulose (HEC) was modified to make it amenable to biorthogonal click chemistry. This largely available modified natural polymer has been considered for cartilage TE but it lacks reactive functions. A method based on a first esterification step with citric acid to introduce carboxylic functions (handles), which are used to introduce either azide or alkyne (DBCO) moieties has been developed. Alkyne- and azide-modified HEC are then mixed and reacted with SPAAC to form a biocompatible and biorthogonal HEC scaffold(252). The reactivity introduced on HEC can be exploited to immobilize biomolecules, for instance, N-terminal azide-modified GF. However, poorly controlled covalent modifications can negatively affect the activity of biomolecules. Therefore, the immobilization strategy should be appropriate to the chemistry of biomaterials, the availability of reactive groups in the biomolecule structure and the location of the reactive groups relative to the receptor binding domain(253,254).

III.2.3 Photo-Immobilization is another common method to immobilize biofactors on biomaterials. Photoinitiated reactions provide additional control over the location of biomolecule grafting sites within a 3D structure thanks to localized application of UV irradiation. The biomolecule is first functionalized with a photoreactive group such as benzophenone or azidophenyl(255,256). Then, the modified biomolecule is bound to the biomaterial upon exposure to light, typically UV(205,206,257). A major advantage of photo-immobilization over other immobilization methods lies in its ability to easily generate GF patterns. By using photomasks or laser scanning light sources, GFs can be immobilized in specific locations within both 2D and 3D matrices, which provides enhanced control over cell behaviour. BMP-2, PDGF and other GFs were photo-immobilized to promote osteogenesis or chondrogenesis(113,258–261). As an example, TGF-β1 was conjugated to acrylated PEG molecules (acryloyl-PEG-NHS) through the reaction of amine groups on GFs with succinimidyl groups on PEG(262). This PEG-TGF-β1 conjugate was combined with PEG-diacrylate and the mixture exposed to UV light to initiate the crosslinking reaction and form a hydrogel. Covalently immobilized TGF-β1 increased ECM production by vascular smooth muscle cells embedded in the PEG hydrogel. The production of collagen was significantly greater when TGF-β1 was tethered to the hydrogels than when soluble TGF-β1 was used. Furthermore, the Young's modulus, which reflects the stiffness of the scaffold, was significantly higher when TGF-β1 was tethered to the scaffolds.

III.3 Multi-Scaffolding System

Hydrogels are efficient biomaterials for TE as already widely described, but are limited for the long-term delivery of biomolecules mainly due to the lack of strong interactions to prevent or slow down

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the release of molecules (261,263–265). To overcome this limitation, drug-loaded MPs or NPs can be incorporated within the hydrogels. Such composite systems, owing to the wide range of chemical structures and properties available for the polymers or inorganic materials, are a strong alternative for the localized entrapment of bioactive cargo, and their controlled and sequential release in cartilage TE(266) (Figure 11).

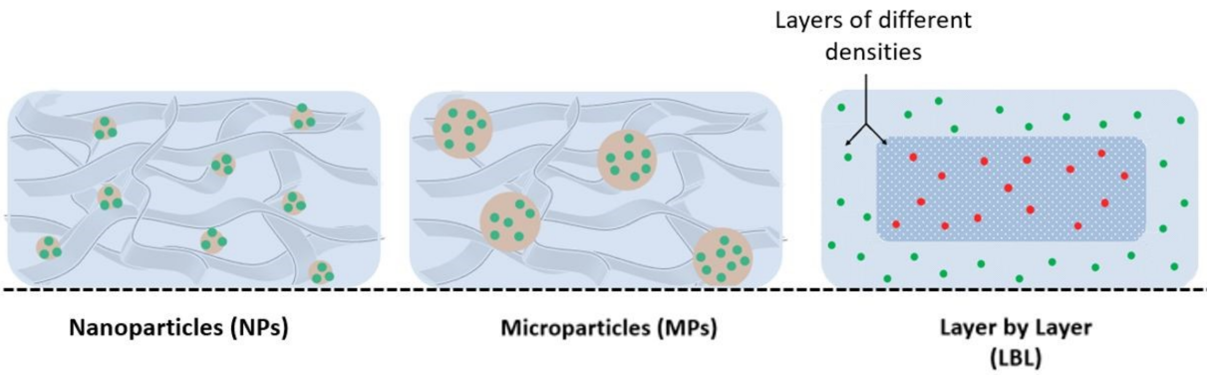

Fig. 11. Schematic representation of composite systems used to control biomolecules delivery from scaffolds. **Nanoparticles (NPs)** or **microparticles (MPs)** embedded within the scaffold matrix as localized biomolecule reservoirs**. Layer-by-layer (LBL)** assembly, creating stratified layers of varying densities for the precise spatial distribution and controlled release of biomolecules.

MPs and NPs display high surface area to volume ratios, small dimensions, high drug encapsulation efficiencies and the capacity to quickly respond to surrounding environmental stimuli, such as temperature, pH, magnetic fields or ultrasounds(267–269). In this field, the PLGA polymer is widely used due to its controllable degradation profile, ease of manufacture and FDA-approval for drug delivery in clinical applications(270). Recently, an injectable hydrogel system (Col-Apt@KGN MPs), which is a collagen-based scaffold containing the aptamer 19S (Apt19S) and PLGA-based MPs encapsulating KGN, was described to allow the sequential release of Apt19S and KGN(271) (Figure 12). The Apt19S was rapidly released from the hydrogel within 6 days, while KGN was slowly released for 33 days via the degradation of PLGA MPs. The Apt19S enabled the recruitment of endogenous MSC and KGN promoted their chondrogenic differentiation and cartilage-specific ECM secretion as confirmed by higher production of GAGs in the Col@KGN MPs and Col-Apt@KGN MPs groups at both 14 and 21 days. In a rabbit model of full-thickness cartilage defect, the Col-Apt@KGN MPs group demonstrated the most effective repair, with the regenerated tissue showing a smooth surface and uniform integration within the surrounding healthy cartilage and a ICRS score significantly superior compared to the other groups at 14 weeks.

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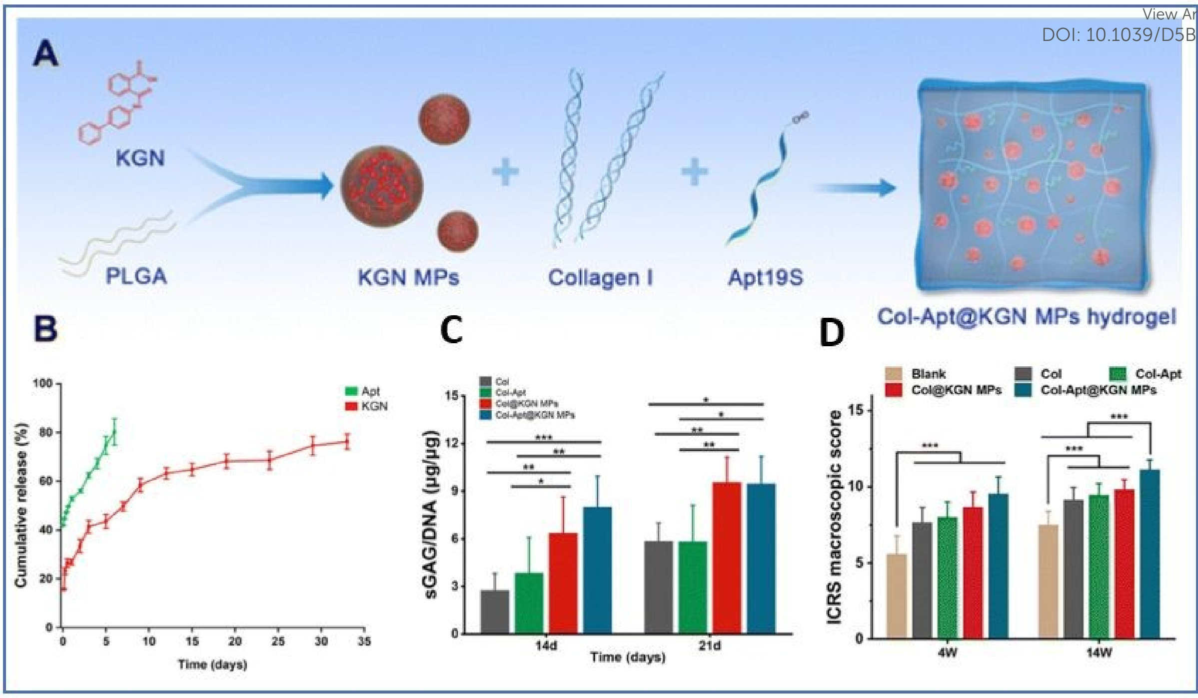

Fig. 12. Spatiotemporal sequential release hydrogel for cartilage regeneration (A) Fabrication of a collagen hydrogel incorporating I-Apt19S and KGN-loaded PLGA microspheres (Col-Apt@KGN MPs). (B) Cumulative drug release for 33 days *in vitro*. (C) Quantitative determination of the sGAG/DNA ratio at 14 days and 21 days. (D) ICRS macroscopic score evaluating cartilage repair. Reproduced from ref. 271 with permission from RSC, copyright 2008.

Another composite scaffold, consisting of an injectable chitosan/silk fibroin hydrogel and PLGA MPs, loaded respectively with SDF-1 and KGN, has been successfully used to obtain a sequential release of these two biomolecules for cartilage TE(272). The authors suggest that the burst release of SDF-1 (ca. 40 % at 24h) accounts for the recruitment of endogenous MSCs to the defect area. The slower and sustained release of KGN promoted the differentiation of MSCs into chondrocytes, hence favouring cartilage repair. This approach gave interesting results *in vivo*, after creating surgical lesions on rabbit knees.

Finally, polyionic complex NPs loaded with TGF-β2 were encapsulated in an alginate hydrogel impregnated with BMP-7. This resulted in a sequential delivery of the biological factors, BMP-7 being released faster than TGF-β2, with 80 % and 30 % of the GF released after 21 days of incubation respectively(273). Since the molecular weight of the polymer that forms the particles can affect the release kinetics of the encapsulated drug, this property was also evaluated in order to tune the release of different active compounds. *In vitro*, TGFβ3 loaded into low-molecular-weight PLGA (10 kDa)-based MPs exhibited a sustained release over 28 days, reaching approximately 96 % of the initial dose(274). In contrast, Levatinib, an anti-angiogenic small molecule, was released at a much slower rate from high-molecular-weight PLGA (100 kDa)-based MPs with only 40 % released after 56 days. Although TGFβ3 and Levatinib have distinct chemical natures, their dual release from respectively 10 kDa and 100 kDa PLGA MPs resulted in the significant downregulation of osteogenesis-related genes (BMP2, RUNX2, OPN, OCN, ALP) observed after 56 days.

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Processing and 3D structuration as described in section I.3 offer versatile strategies for manipulating drug loading and release profiles from scaffolds(139,275). Layer-by-Layer assembly is commonly used to construct porous scaffolds with good performance in avoiding GF loss of function, while achieving high sequestration rate under mild aqueous conditions and controlled delivery. A method for 3D printing of hydrogels with core-shell capsules sensitive to external stimuli was designed for the on-demand release of biomolecules(276). The capsules consisted of an aqueous core, which can be formulated to maintain the activity of payload biomolecules, here the Horse Peroxidase (HRP) protein used as a proof-of-concept and a PLGA shell that sterically holds the molecules inside the capsules. The shell is loaded with plasmonic gold nanorods (AuNR) that selectively disrupt the capsules when irradiated with a laser at a specific wavelength, therefore triggering the release of HRP with high spatiotemporal control(276). Similarly, TGF-β1-embedding core-shell nanospheres were fabricated via co-axial electrospraying of the GF along with PLGA and then mixed to a bioink composed of 10 % gelatin methacrylate (GelMA), 5% polyethylene glycol diacrylate (PEGDA) and a biocompatible photoinitiator(277). The MSCs-laden constructs were 3D bioprinted by stereolithography and the sustained release of TGF-β1 up to 21 days significantly improved the chondrogenic differentiation of encapsulated MSCs.

IV. Challenges & Future Perspectives

Despite considerable advances made optimising scaffold design and association with bioactive molecules, clinical translation has achieved only limited success in the treatment of articular cartilage defects. While pre-clinical experiments in small and large animal models have yielded promising results, further investigation is necessary to assess the clinical safety, reliability, and efficacy of TE strategy. With regard to the controlled release of drugs over space and time, the majority of research revolves around simulated conditions *in vitro.* However, there is a lack of clear evidence regarding the actual dosage and kinetics of growth factors release *in vivo*. It is necessary to create reliable assessment tools that can noninvasively track the growth factor delivery after implantation. Real-time monitoring in living organisms represents one of the main challenges that requires urgent consideration, in particular for cartilage TE.

A further issue that requires attention is the occurrence of hypertrophic or fibrotic neotissue overtime following scaffold implantation in cartilage repair strategies. It is of paramount importance to prevent hypertrophy or fibrosis to allow an appropriate integration between the implant and the surrounding endogenous tissues. To address this challenge, the incorporation of anti-hypertrophic or anti-fibrotic cues may enhance the stability and longevity of engineered cartilage, thereby advancing the field closer to developing functional cartilage repair therapies. It is therefore crucial to gain a deeper understanding of the precise timing and dosage for their application and to control their release from scaffolds. Concurrently, cell source modulation, genetic engineering and culture condition optimisation will be pivotal factors in the translation of TE approaches to clinical success.

The microenvironment-responsive release approach is emerging as a promising solution for controlling the timing of molecule release. The use of cleavable linkers, such as those sensitive to pH or proteases represents a significant opportunity to selectively release active molecules in response to changes in the nearby tissue environment thereby controlling the temporal and spatial availability of the specific factors for optimized tissue regeneration. Future strategies will undoubtedly benefit from evolving advances in monitoring, fabrication techniques and novel strategic pairings of biomolecules.

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Conclusion

Cartilage TE seeks to generate a neo-tissue that mimics the physiological function of native cartilage, offering a viable solution for cartilage repair. However, existing strategies often result in the deposition of an ECM with suboptimal biomechanical properties that degrade over time, poor integration in the host tissue or tissue fibrosis. To enhance cartilage regeneration using scaffolds containing MSCs or chondrocytes, various biomolecules have been incorporated into different types of biomaterials in order to boost their biological activity and reduce the need for repeated injections. These biofactors play crucial roles in differentiation processes or cartilage homeostasis and typically require a sustained release due to their rapid clearance *in vivo*. Moreover, the kinetics of their release must be tailored to the specific biofactor. Recent advances have successfully employed scaffolds as biomolecule reservoirs, ensuring a prolonged release of active molecules over weeks or even months, with promising outcomes for cartilage engineering. Innovations in biomaterials chemistry have further improved the control and retention of biomolecules within scaffolds, preserving their bioactivity. However, the majority of controlled release research is conducted *in vitro*, under simulated conditions, and the absence of real-time monitoring in living organisms remains a major limitation, hindering further progress in the field. It is clear that future strategies will greatly benefit from the integration of advanced monitoring technologies, innovative fabrication techniques, and the development of novel agent combinations. Additionally, cleavable linkers, responsive to pH or proteases, offer a promising approach for the selective release of active molecules in response to dynamic changes in the local tissue environment, representing a critical avenue for future research and application in cartilage TE.

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Table 1: Main materials used for Cartilage Tissue Engineering.

	Materials	Advantages	Disadvantages	Ref
Natural	Collagen	Biocompatibility Biodegradability Low immunogenicity Cell adhesion proliferation and differentiation	Low mechanical strength Low solubility Rapid Biodegradation Difficult to handle	(26,27)
	Gelatin	Biocompatibility Biodegradability Accessibility	Low mechanical strength Stability Poor mechanism properties Low solubility	(28,29)
	Hyaluronic acid (HA),	Biocompatibility Biodegradability Easy chemical modification Bioactivity	Cost; Low mechanical strength Fast degradation	(30,31)
	Chitosan (CH),	Biocompatibility Biodegradability Cell adhesion, proliferation and differentiation Anti-microbial activity	Low mechanical strength Low solubility	(32,33)
	Fibrin	Biocompatibility Costless Accessibility Cell adhesion and proliferation	Cost Low mechanical strength Fast degradation	(36)
	Alginate	Biocompatibility Biodegradability Accessibility Bioactivity	Low mechanical strength Limited strength Difficult to handle	(37,38)
Synthetic	PLA	Biocompatibility thermostability Thermoplasticity Degradability	Poor cell adhesion	(39–42)
	PGA	Avaibility Easy processing Biocompatibility	Acid release upon degradation Poor cell adhesion Fast degradation Mechanical Properties	(43)
	PLGA	Mechanical properties Controlled degradability	Acid release upon degradation	(44,45)
	PCL	Mechanical properties Biocompatibility Thermoplasticity Biodegradability	Poor cell adhesion Poor hydrophobicity	(46,47)
	PEG	Biocompatibility Biodegradability	Poor cell adhesion	(48,49)

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Table 2: Main scaffolds fabrication techniques for cartilage tissue engineering.

Techniques	Advantages	Disadvantage	Ref
Phase inversion	Versatility Compatibility Repeatability	Lack of pore interconnectivity control Processing conditions Applicability	(60,278)
Solvent casting and particle leaching	Easy processing Adjustable porosity	Cytotoxic solvent Low mechanical strength	(279,280)
Gas foaming	Costless Porosity control	Pore size distribution Lack of pore interconnectivity control	(281)
Electrospinning	Large scale production possibilities Repeatability Easy process	Limited range of polymers	(282–284)
Freeze-drying	Adjustable porosity and structure Greater interconnectivity of the porous structure	Energy demanding and time consuming Cytotoxic solvents	(285)
3D Bioprinting	High resolution High throughput capability Reproducibility Easy to use	Inkjet viscosity	(286–288)

Table 3: Main biomolecules used for cartilage engineering and their effect on chondrogenic differentiation.

Biomolecules	Type of biomolecule	Desired effect on MSCs chondrogenic differentiation	Adverse effects on differentiation	Ref
Kartogenin	Small molecule	Induces chondrogenesis / Enhances matrix production	/	(74,76,289–293)
Curcumin	Small molecule	Induces chondrogenesis	/	(85,86)
Glucosamine	Small molecule	Induces chondrogenesis	/	(87,88)
Icariin	Small molecule	Induces chondrogenesis / Inhibits hypertrophic differentiation	/	(89–91)
Melatonin	Small molecule	Induces chondrogenesis	/	(96,98)
Ascorbic Acid	Small molecule	Chondrocyte growth / Enhances cartilage matrix production	/	(101,102)
TGF-β1 and 3	Growth factor	Enhances proliferation/ Inhibits migration / Induces differentiation / maintains articular chondrocytes	Promotes hypertrophic differentiation	(104,105,253,262,275,289,294–298)

BMP-2	Growth factor	Enhances matrix production	Promotes hypertrophic differentiation Induces osteogenic differentiation	(299,300)
BMP-4	Growth factor	Induces chondrogenesis / Maintains chrondrocyte phenotype / Enhances matrix production / Inhibits hypertrophic differentiation	/	(115)
BMP-6	Growth factor	Induces chondrogenesis / Enhances matrix production / Inhibits hypertrophic differentiation	Promotes hypertrophic differentiation	(301,302)
BMP-7	Growth factor	Induces chondrogenesis / Enhances matrix production / inhibits hypertrophic differentiation	/	(299,303,304)
IGF-1	Growth factor	Induces chondrogenesis	/	(120)
IGF-2	Growth factor	Primes chondrogenic differentiation	/	(122)
FGF-2 (bFGF)	Growth factor	Maintains chondrogenic potential / Enhances matrix production	Promotes fibrocartilage formation	(126–131)
PDGF	Growth factor	Exerts chemotactic effects / induces proliferation / Induces chondrogenesis	/	(298,305,306)
PTHrP	Protein	Inhibits hypertrophic differentiation	/	(139–141,307)
Peptides	Amino Acid sequence	Recruit endogenous stromal cells	/	(147,150)
Nucleic acid	miRNA siRNA	Potential interest for all cartilage engineering steps	/	(153,158,159,308,309)

Hypoxia-mimicking agents	Small molecules	Induces chondrogenesis / inhibits hypertrophic differentiation	/	(169,170,310)

Table 4: Main entrapment mechanisms used for the controlled release of biomolecules involved in the chondrogenic differentiation of MSCs

Interaction type	Entrapment mechanism	Biomolecules	Refs
Non-covalent interactions	weak interactions	TGFβ1-2-3 / PTHrP / bFGF / BMP-2 / Curcumin / Chondroitin sulphate / Nucleic acid	173,179-182,188–293
	Steric entrapment	TGFβ1-2-3 / IGF / bFGF / PTHrP / BMPs / Kartogenin / Chondroitin sulphate / Curcumin / Nucleic acid	151-153,156,164-169
Covalent interactions	Crosslinking	TGF-β1-3 / BMP-2 / BMP-4 / Kartogenin	207-210,212
	Photo-Immobilization	BMP-2 / PDGF	95,217–221
	Click Chemistry	TGF-β1 / BMP-2	227-228
Multi-scaffolding system	Combination of several techniques	TGF-β1-2-3 / BMP-7 / Kartogenin / Curcumin	237-246,253-255

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No primary research results, software or code have been included and no new data were generated or analysed as part of this review.