Advanced Drug Delivery Reviews 192 (2023) 114647

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/adr

Protein-engineered biomaterials for cartilage therapeutics and repair

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ARTICLE INFO

Article history:
Received 5 June 2022
Revised 17 October 2022
Accepted 5 December 2022
Available online 9 December 2022

Keywords:
Peptides
Proteins
Biomaterials
Hydrogels
Drug Delivery
Cartilage
Osteoarthritis

ABSTRACT

Cartilage degeneration and injury are major causes of pain and disability that effect millions, and yet treatment options for conditions like osteoarthritis (OA) continue to be mainly palliative or involve complete replacement of injured joints. Several biomaterial strategies have been explored to address cartilage repair either by the delivery of therapeutics or as support for tissue repair, however the complex structure of cartilage tissue, its mechanical needs, and lack of regenerative capacity have hindered this goal. Recent advances in synthetic biology have opened new possibilities for engineered proteins to address these unique needs. Engineered protein and peptide-based materials benefit from inherent biocompatibility and nearly unlimited tunability as they utilize the body's natural building blocks to fabricate a variety of supramolecular structures. The pathophysiology and needs of OA cartilage are presented here, along with an overview of the current state of the art and next steps for protein-engineered repair strategies for cartilage.

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https://doi.org/10.1016/j.addr.2022.114647
0169-409X/

1. Introduction

Articular cartilage is a highly specialized tissue designed to facilitate movement of diarthrodial joints by reducing friction and providing lubrication during articulation [1]. Hyaline (glass-like) cartilage lines the surface of bones within an articulating joint, enclosed within a synovial, fluid-filled cavity. The combination of synovial fluid with the smooth surface of articular cartilage allows for almost frictionless movement between articulating surfaces. Whether through aging, wear and tear, various disease states, or trauma, breakdown of articular cartilage can lead to many forms of arthritis, a family of conditions that, while not fatal, are painful and debilitating. Osteoarthritis (OA), the most common type, is characterized by degeneration of the articular cartilage and subchondral bone, and is one of the leading causes of disability globally, with over 300 million cases in hip and knee joints worldwide, resulting in an estimated 9.6 million years of healthy life lost to disability (YLD) as defined by the World Health Organization (WHO) [2]. In the US alone, this results in \$80 billion in healthcare spending annually, for both ambulatory and inpatient care, and contributes to immense loss of economic activity and quality of life for patients who suffer from this condition long term [3]. Because articular cartilage is avascular, aneural, and alymphatic, the tissue does not naturally heal, and repair of cartilage tissue remains clinically elusive [4,5]. Early treatment focuses on pain management with non-steroidal anti-inflammatory drugs (NSAIDs) or steroidal injections, but these do not provide repair strategies, and end stage disease often necessitates total joint replacement [6]. Currently no disease-modifying OA drug (DMOAD) exists clinically, due in part to the lack of delivery systems for the joint [7]. Systemic delivery of drugs do not reach the joint or penetrate through the dense cartilage extracellular matrix (ECM) to target chondrocytes, and intraarticular administration of drugs results in rapid clearance from the synovial joint [8]. Osteochondral autografts, autologous chondrocyte implantation (ACI), and microfracture surgery are also surgical methods of small defect repair that have seen some clinical success but come with their own complications such as donor site morbidity, lack of sufficient autologous chondrocyte numbers, and the formation of fibrocartilage, a scar-tissue like cartilage that lacks the highly organized structure of healthy native cartilage [9,10]. Thus, biomaterials continue to be a major area of research for cartilage repair, either acting as a vehicle for drug delivery or a scaffold for tissue repair and regeneration [11].

A wide variety of polymeric materials have been investigated for cartilage therapeutic delivery and repair, including both synthetic (e.g., poly-ethylene glycol (PEG), polyvinyl alcohol (PVA)) and naturally derived materials (e.g., hyaluronic acid, collagen, alginate). Many are currently clinically available or under investigation for arthritis management, and while pain management has been somewhat successful in alleviating symptoms no biomaterial to date has successfully halted or reversed OA progression [12,13]. Protein-engineered materials are another class of materials that benefit from a high level of tunability, enabling their design to be highly application specific and reproducible [14]. Proteins are multifunctional macromolecules composed of sequential amino acids that are one of the fundamental building blocks of life, and advances in synthetic biology have enabled the use of rational design to develop novel materials by engineering proteins with biomimetic or bioinspired sequences and secondary structures. Synthetic biology enables the replication of natural peptide sequences, combinations of sequences inspired by various sources, and even completely novel sequences to provide virtually unlimited functionality. These materials can be stimuli responsive (e.g., temperature or pH sensitive), functional (biologically interactive with local cells and tissues), and viscoelastic due to the natural mechanical folding-unfolding of protein domains [15–17]. Biofunctionality can be engineered directly into the material, by way of matrix binding, cell signaling, and adhesive ligands. In comparison to polymer-based hydrogels, protein-based materials naturally degrade to amino acids which are nontoxic [18]. Furthermore, protein-based biomaterials benefit from their inherent sequence modularity. Protein-based materials may affect similar structure and function despite low sequence similarity [19]. While a protein may elicit immunogenicity, protein design tools have evolved to overcome these challenges by computational or rationally chosen mutations that can remove potential inflammatory response while conserving function [20–22]. The use of protein-based materials has historically been limited in orthopedic applications due mainly to the perception that mechanical properties of these materials are less robust than those of synthetic polymers, however advancing methods in protein and peptide engineering has begun to shift the paradigm [23]. In the following review we discuss the specific needs of cartilage repair and therapeutic delivery and assess the current state of protein-engineered materials for cartilage as well as discuss areas for future development.

2. Articular cartilage and joint health

Articular cartilage is a complex structure that itself exists in a complex system of numerous tissues (Figure 1). Articular joints, also known as synovial joints or diarthroses, feature the joining of bones and their overlying hyaline cartilage within a fibrous joint capsule. The joint cavity is filled with synovial fluid that functions to reduce the friction between the articulating hyaline cartilage of the joint. Cartilage does not have direct blood supply and receives nutrition and waste exchange via diffusion from the blood supply of the surrounding tissues and convection via joint movement. The main function of articular cartilage is to distribute load over a large area to minimize stresses experienced by the surrounding structures and to allow pain-free motion of the articulating surfaces with minimal friction. This function is entirely dependent on the tissue's biphasic nature: the fluid phase, consisting of water and salts, and the solid phase, comprised of various ECM molecules [24,25]. Water constitutes 65–85% of the total tissue weight and the movement of fluid in and out of the bulk of the tissue is responsible for controlling much of the tissue's viscoelastic behavior [26,27]. Cartilage ECM is composed primarily of type II collagen (75% by dry weight) and proteoglycans, most specifically aggrecan ($\sim$20–25% by dry weight) [26,28]. The glycosaminoglycans (GAGs) that make up larger proteoglycan molecules contain a high concentration of negatively charged sulfate and carboxyl groups, which attract water and maintain the high hydration and osmotic pressure of cartilage, thereby providing support for compressive loads [29,30]. Type II collagen, in turn, provides the tissue with high tensile strength and the ability to withstand shear stresses. The equilibrium compressive modulus of native articular cartilage varies between 0.3–1.5 MPa [26]. The combination of high compressibility and high porosity along with low permeability ensures consid-

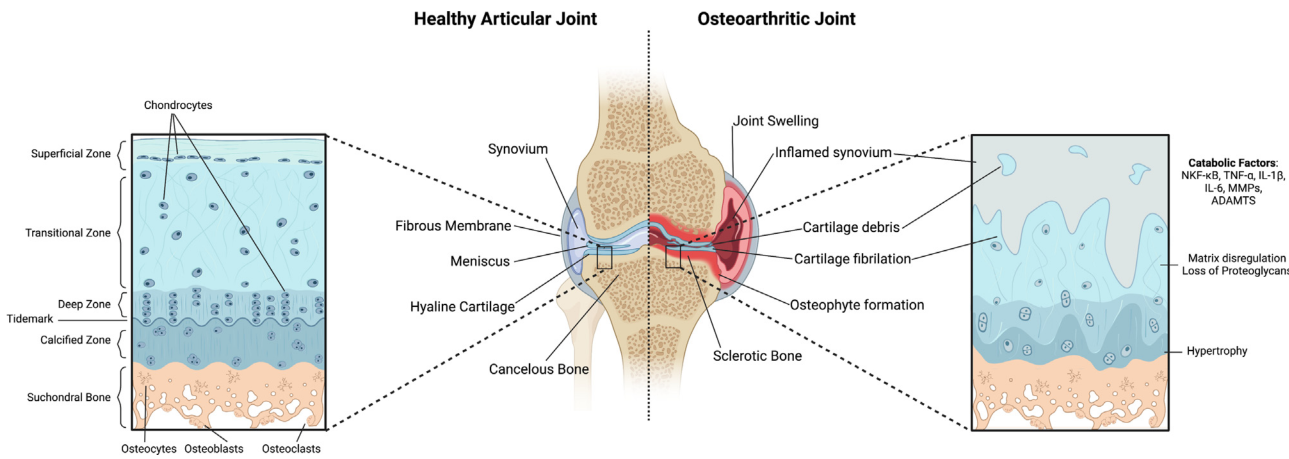

Fig. 1. Articular joint anatomy and make up. (A) Healthy articular joint constituents. Inset shows a cross-sectional layout of cells and ECM fibers in hyaline cartilage and the subchondral bone. (B) Osteoarthritis of the articular joint features swelling, cartilage degradation, and sclerosis of the bone. Inset shows breakdown and disorganization of the articular cartilage. (Not to scale) Created with BioRender.

erable volume flow rate at high fluid pressurization, an ideal effect for maintaining cartilage function during loading [1].

This ECM network is made up of a zonal architecture: the superficial layer, middle zone, and deep zone, below which are the tidemark, calcified cartilage, subchondral bone and cancellous bone (Figure 1A) [1]. The superficial layer contains a densely packed collagen II network in parallel sheets with low proteoglycan content ECM, providing high tensile stiffness and shear strength while protecting the deeper layers [24]. The transitional or middle zone contains larger and sparser collagen fibrils and a higher proteoglycan content. The deep zone has the highest proteoglycan density but the lowest concentration of water. Collagen fibers here run perpendicular to the cartilage structure and are embedded into the final calcified layer through the tidemark, providing resistance to shear. The deep zone also contains type X collagen, characteristic of endochondral ossification. The ECM of cartilage is further supported by minor components such as collagens type VI, IX, and XI, as well as elastin and fibronectin, and the glycoprotein lubricin. The complex, anisotropic structure of articular cartilage that gives the tissue its functionality also makes it difficult to restore, as repair strategies most often result in fibrocartilage that lacks the arrangement of hyaline cartilage.

Chondrocytes, the main cell of cartilage, make up only $\sim$ 2% of adult articular cartilage by mass. Mesenchymal stromal cells are sparsely present in the perichondrium, which surrounds the perimeter of cartilage tissue, and are more abundantly found in the marrow of subchondral bone. Because chondrocytes are embedded within the dense cartilage matrix, the ECM is largely responsible for regulating their metabolism and functions. Blood supply to the articular joints reaches the synovium but does not penetrate cartilage [31]. Thus, nutrient exchange depends on convective fluid flow between the synovium and articular cartilage or through subchondral blood vessels, which is limited by tissue calcification. The limited blood supply presents difficulties for systemic therapeutic delivery, which has led to an increased emphasis on intraarticular (IA) therapeutic delivery. IA injections, however, are complicated by rapid clearance within the synovial fluid, necessitating creative solutions for maintaining therapeutic levels within the joint space. In addition, therapeutic drugs must penetrate the dense and highly charged matrix in order to reach chondrocytes and downregulate catabolism and promote repair.

2.1. Osteoarthritis

OA is characterized as a chronic and progressive degeneration of articular cartilage and the underlying bone tissue, resulting in progressively worsening pain and disability. Because of the physical damage to cartilage tissue observed in OA, it has commonly been associated as a disease of "wear and tear" that results from altered mechanical loading of articulating joints.

Degeneration of the cartilage ECM is the hallmark of OA. The causes of OA are still unclear, although aging, obesity, both overuse and disuse have been implicated, along with metabolic factors such as diabetes or alcohol abuse. The molecular make-up of the cartilage changes, resulting in biomechanical changes and changes to cellular metabolism including the generation of cartilage degrading enzymes matrix metalloproteinase 13 (MMP13) and A disintegrin-like and metalloproteinase with thrombospondin type 1 motifs-4 (ADAMTS4) (Figure 1B) that drive progressive degeneration. Degradation of the collagen network and loss of proteoglycans are the primary markers of OA. Early OA shows disruption and fibrillation of the articular surface, leading to fissures and pitting. Damage to articular cartilage leads to overall thinning and reduction in joint space, as well as sclerosis of subchondral bone and the formation of osteophytes (bony protrusions) [32,33]. Synovitis, or inflammation of the synovium, often presents clinically in OA as painful joint swelling. The most common hypothesis of the cause of synovial inflammation is the presence of degraded cartilage fragments in the synovium, or damage-associated molecular patterns (DAMPs). Considered foreign bodies by synovial cells, DAMPs stimulate proinflammatory cytokine secretion, which has been found to be a critical mediator of the progression of OA.

2.1.1. Injury and posttraumatic osteoarthritis

Posttraumatic osteoarthritis (PTOA) is a subset of OA that results from sudden traumatic injury to the joint. Approximately 12% of the overall prevalence of OA is attributed to PTOA of the hip, knee, and ankle [34]. Acute injury to synovial joints and their contents, as well as chronic ligamentous instability can result in in PTOA. Ligamentous and meniscal injury in the knee lead to a 10-fold increased risk of developing OA compared to those without joint injury, and articular fracture can increase that risk more than 20-fold [35]. Acute trauma is followed by various phases of response: (1) inflammation and apoptosis, (2) matrix catabolism, and (3) an anabolic phase of limited cartilage remodeling and repair. Because a precipitating event can usually be identified, early intervention and prevention is highly desirable in cases of PTOA.

2.1.2. Inflammation and pathogenesis of cartilage degeneration:

Inflammation is increasingly being understood to be a mediating factor in both PTOA and OA [32,33,36]. The production of proinflammatory cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α) and inflammatory activation of chondrocytes is associated with the initial response to joint injury and is accompanied by the release of matrix metalloproteinases and matrix fragments that initiate catabolism in cartilage and suppression of matrix production [36]. These and other inflammatory cytokines remain elevated in OA joints and contribute to the continued pathophysiology of OA [37]. Both IL-1β and TNF-α stimulate catabolic responses in chondrocytes via the production of proteolytic enzymes such as MMPs −1, −3, and −13. Additionally, IL-1β and TNF-α have been shown to inhibit anabolic activity of chondrocytes; IL-1β reduces type II collagen and aggrecan expression, the two major ECM components of cartilage, while TNF-α similarly suppresses proteoglycan, link protein, and type II collagen by chondrocytes. Additionally, ADAMTS-4 has also shown upregulation induced by both IL-1β and TNF-α

IL-1β and TNF-α also perpetuate OA progression by inducing the production of other proinflammatory cytokines like IL-6 and IL-8, and inflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2). In addition, IL-1β and TNF-α induce the production of reactive oxygen species (ROS) like nitric oxide (NO) and downregulate the expression of antioxidant enzymes that would scavenge ROS, accelerating radical damage of cartilage. NO also further enhances the activity of MMPs and blocks anabolic activity in chondrocytes as well as promoting chondrocyte apoptosis.

2.2. Current therapy

Current nonsurgical treatment for OA is largely palliative [38]. Initial treatment focuses on pain management, with cyclooxygenase inhibitors like acetaminophen and non-steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen and naproxen [7]. Neither of these treatments address disease progression, however, and prolonged use is discouraged as they can have negative secondary effects. Intraarticular corticosteroid injections (i.e., dexamethasone, methylprednisolone) offer localized treatment and have shown success in reducing pain scores, as well as having anti-inflammatory and immunosuppressive effects. However, these have a short retention time in the articular space and can require multiple treatments to manage pain, and have not shown any long term effects on preventing OA progression.

Viscosupplementation, intraarticular injection of viscous fluids or hydrogels that make up the synovium that are lost in OA provide relief of symptoms by reducing friction, thus providing pain relief. Various formulations of lightly cross-linked HA hydrogels are currently approved for viscosupplementation, including Synvisc-ONE®, EUFLEXXA®, and MonoVisc®. HA viscosupplementation has shown to enhance ECM matrix protein synthesis and regulate inflammation [38]. Though viscosupplementation has shown positive results in managing OA pain, the elevated catabolism of the OA joint results in rapid degradation and the need for repeated intraarticular injections (every 6 months). Arthroplasty, more commonly known as joint replacement surgery, is the end-stage surgical solution for OA, and consists of major orthopedic surgery involving replacing the entire articulating joint with prostheses of metal alloys and polymers. While these surgeries provide reliable, long-term improvement in movement, pain, and quality of life, loosening can still occur, with incidence rates ranging from 10% to 70% [39]. This necessitates revision surgeries, particularly common in patients under 65 years of age for whom life expectancy and mobility exceeds the functional expectancy of the implant. Revision surgeries are more expensive, more technically difficult, and result in higher rates of complications and failure [40]. As the overall population ages and the average age of OA sufferers (particularly PTOA) decreases, the need for viable regenerative strategies has increased. For advanced cartilage defects, regenerative surgeries such as subchondral drilling and microfracture aim to access mesenchymal stromal cells (MSCs) from the underlying subchondral bone to aid in cartilage regeneration. This, however, results in the formation of fibrocartilage that does not replicate the mechanical function of healthy hyaline cartilage [9]. Autologous transplantation methods include mosaicplasty, the implantation of small cartilage "tiles" harvested from autologous non-loadbearing cartilage, ACI, and matrix-assisted ACI (MACI), which uses collagen or HA-based matrices seeded with autologous chondrocytes [41]. While these therapies have shown promise, donor site morbidity and lack of availability of cells can limit their success. Autologous cell harvesting requires time (6–8 weeks) for processing and expansion of cells sufficient for treatment, after which up to 8 weeks of restriction on motion is prescribed as the defect heals. The resulting tissue repair has shown sufficient success in the clinical setting, but does not provide a rapid, off-the-shelf solution, and is appropriate for osteochondral defects, but not an appropriate treatment mechanism for stopping or slowing the progression of the degenerative effects of osteoarthritis. In addition, the procedure can be more technically difficult and invasive, particularly where lesions are difficult to reach. MACI may provide some benefit by preventing loss of implanted cells to the lesion. Scaffold materials in use for MACI include hyaluronic acid based HYAFF®11, type I/III collagen membrane MACI®, and poly-L-lysine BioSeed®-C [42–44]. Short to mid-term outcomes have been promising, with 8–11% failure rate up to 11 years of follow up reported for ACI and MACI respectively, however one study reported a nearly 60% failure rate of MACI at 15 years follow up, suggesting that the long term resilience of the repaired cartilage is insufficient [45]. Next generation materials that are cell-instructive may be the solution to more lasting repair.

The search for DMOADs continues, aiming to either halt, slow, or reverse the progression of structural changes caused by OA. Current areas of investigation include targeting pro-inflammatory cytokines (NF-κB, IL-1β, TNF-α), matrix degrading enzymes (MMPs), or the Wingless/Integrated (Wnt) signaling pathway, all of which decrease matrix catabolism in OA patients, or regenerative strategies to counteract matrix loss [46,47]. Success has been limited largely by delivery mechanism: systemic drug delivery for therapeutics beyond pain management are limited in their effectiveness due to the lack of vasculature within cartilage, and carry a higher risk of adverse, off-target effects. Conversely, injectable therapeutics do not provide long-term relief as the combination of limited cartilage penetration and rapid clearance from the synovium limit their effectiveness [8]. Thus, a variety of biomaterial carriers such as hydrogels, micelles, and micro- and nanoparticles are under investigation to enhance the residence time of proposed DMOADs within the articular joint space. Increasing attention is being given to drug delivery into the bulk of cartilage tissue; rather than simply prolonging the residence time of drugs in the synovium, these drug delivery systems target the chondrocytes that are embedded deep within the matrix to a key mediator of matrix catabolism in OA [8]. Penetration into the cartilage matrix is hindered by the dense, highly anionic ECM, which prevents the influx of particles larger than 10 nm. Additionally, synovial turnover occurs rapidly, with most therapeutic molecules cleared within 24 hours via lymphatic drainage. This necessitates repeated injections that can lead to toxicity and other unwanted off target effects. Thus, the success of any potential DMOAD is largely dependent on biomaterials used to deliver it into the OA cartilage.

3. Biomaterials for cartilage repair

Biomaterials can address several needs for cartilage repair and regeneration as: delivery vehicles for therapeutic molecule delivery (i.e., sustained release drug depot, targeted nanoparticles); mechanical support systems (i.e., viscosupplementation, defect plug); and scaffolds for cell-driven tissue repair. Hydrogels are 3D polymer networks that exhibit viscoelastic behavior and high water content like healthy cartilage and are thus ideal materials for cartilage repair [13,48,49]. Hydrogels form networks by either physical or chemical crosslinking, and these crosslinks can be tuned to enhance properties like swelling, modulus, pore size and permeability [50]. Recent effort has especially focused on stimuli-responsive hydrogels. Thermally or chemically responsive hydrogels allow the ability to form *in situ* and conform to defect spaces or imbue the biomaterial with a trigger for drug release making them highly desirable for minimally invasive repair approaches. In addition to hydrogels, fibrous and porous scaffolds have been used for cartilage repair, particularly as they can mimic the fibrous collagen network of cartilage [51–53]. Electrospun fibers can be organized with controlled alignment and porosity to direct cell infiltration and alignment. Composite scaffolds comprised of fibrous networks combined with hydrogel phases are particularly appealing for recapitulating the zonal structure of cartilage for critical size and full thickness defects. The nature of the defect and the pre-formation of these scaffolds requires more complex surgeries for implantation.

3.1. Design considerations

Biomaterial therapeutic strategy for cartilage repair and OA must consider the fact that articular cartilage is an active, mobile, and load-bearing tissue. Factors such as lubrication of the joint, prevention of migration and integration with the host tissue, and response to load must all come into consideration. Additionally, cartilage ECM is dense and contains a high concentration of negatively charged proteoglycans, which can limit perfusion of many therapeutic molecules [28,54]. These considerations are outlined below and summarized in Box 1

Box 1 Design considerations for biomaterial design in cartilage therapeutics and repair.

Crosslinking density is a key factor that affects diffusion, biomechanics, degradation rate of the biomaterial and the behavior of encapsulated or migrating cells. The degree of crosslinking in hydrogels determines its compressive properties via the swelling ratio and the nutrient supply through the permeability [55]. Maintaining permeability values between 1.1–7.6 $\times$ 10^{-13} m^4/N.sec (average permeability of articular cartilage) may highly improve overall construct properties [24]. This should also be considered when delivering therapeutic payloads to cartilage. Macromer concentration also affects hydrogel permeability [56,57]. Similarly, lower crosslinking density also results in a larger mesh size, thus being more permissive to cellular biosynthesis and neomatrix distribution [58].

Regenerative strategies aim to drive local or delivered cells to repair damaged tissue. As such, the products of biosynthesis can be used as identifiers of successful differentiation and cell-mediated repair. For cartilage repair, chondrocytes or stem cells are stimulated to produce cartilage matrix components, namely type II collagen and aggrecan. Production of more type I cartilage is indicative of more osteogenic or other non-cartilaginous ECM. Type X collagen is a marker of hypertrophic chondrocytes, present mostly in the deep and subchondral zones of cartilage, and is generally not a desired product for articular cartilage engineering. Downregulation of catabolic molecules that drive cartilage degradation, such as ADAMTS4, MMP13, and nitric oxide synthase-2 (NOS2), are also key targets for halting and reversing damage caused by OA.

Hydrogels can be designed to be injectable and form *in situ*, a factor useful for both minimally invasive therapies as well as filling irregular void spaces created by tissue damage. Injectable materials must have sufficiently low viscosity to be injected via a fine gauge needle and must increase viscosity or begin crosslinking rapidly to form and be retained within the desired injection site, as well as retaining any accompanying therapeutics for encapsulation [59]. Cell-laden injectable materials must also be able to protect cells from shear-induced damage [60]. *In situ* formation of hydrogels using chemical crosslinking techniques that result in covalent bonds between polymer chains requires the use of crosslinking agents that can pose toxicity issues, however, chemical crosslinking can produce more robust hydrogels with higher moduli. In contrast, physical crosslinking involves weaker reversible bonds (ionic bonds, hydrogen bonds), and allows materials to exhibit shear thinning behavior, which is useful for injectable applications [61]. Physically crosslinked hydrogels can be stimuli-responsive, forming in response to changes in temperature, pH, and ionic concentration, which are useful triggers for injectable materials as the body has very specific cues.

Because of the nature of articulating joints, an injectable material must integrate or at least adhere to the tissue quickly to maintain its function. The ISO standard for gelation or curing time for injectable materials is 4–15 minutes (ISO Standard 5833:2002). Joint immobilization is possible while the material cures, but less desirable, particularly when considering outpatient procedures. Indeed, in the case of arthroscopic surgeries involving constant fluid irrigation, rapid curing and adhesion to the native tissue at the injury site can both prevent biomaterial migration and promote the integration of cell-mediated tissue regeneration. In particular, the use of aldehyde functionalized biomaterials that rapidly interact with native amino acid groups has been used to this end [59,62]. Cell-mediated integration is a longer process that takes on the order of weeks for appreciable deposition of relevant matrix components. Studies have shown that scaffold crosslinking must be able to be modified *in situ* to allow cell motility and infiltration into the scaffold as well as to create void space for neomatrix. This can be achieved either through physical crosslinks that are reversible and more permissive to cell growth and matrix deposition, or through controlled degradation, such as through the incorporation of enzymatically degradable chemical crosslinks [63,64].

Delivery of therapeutics to the articular space must consider transport within the joint. The first major hurdle is rapid clearance of small molecules from the synovium; indeed, most drugs have a half-life on the order of hours [65]. Ensuring the appropriate therapeutic concentration of drug is delivered to its target is necessary both to minimize the necessity for repeated injections as well as to avoid off-target effects of excess drug. Hydrogels can deliver loaded drugs, however, the retention is usually on the order of days because the mesh size of the hydrogel is so much larger than the encapsulated molecule. Methods such as covalent conjugation to the polymer network or affinity-based sequestration can retain drugs within the hydrogel or prolong their release, respectively. Liposomes, microparticles, and nanoparticles are all other methods of enveloping drugs in smaller packages and can extend drug residence within the joint on the order of weeks to months, depending on the size, charge, and composition [65]. This however targets the synovium but does not necessarily target cartilage or the chondrocytes buried deep within the tissue. Tissue penetration has recently garnered much interest in enhancing DMOAD efficacy [8,66,67]. A hydrodynamic diameter of $\leq$ 10 nm is necessary to penetrate the full thickness of cartilage [68]. In addition, the high fixed charge density (FCD) of cartilage (-170.0 mM) also hinders transport of therapeutics to the target chondrocytes [69]. A number of recent studies have attempted to address this problem by designing cationic carriers that can penetrate the anionic GAG network, or by using peptide sequences that target specific cartilage ECM molecules (such as type II collagen) to deliver drugs directly to chondrocytes [66,67,70,71].

3.2. Biomaterial sources

3.2.1. Naturally derived materials

Naturally derived hydrogels used for cartilage repair include animal-derived biopolymers like collagen (usually type II), and HA, which make up then native cartilage ECM [57,72,73]. These are widely used as they generally exhibit a lack of toxicity and benefit from bioactive interactions with local or delivered cells [51,74,75]. However, they also bear the potential for eliciting an immune response [76]. Other naturally derived polymers such as the polysaccharides alginate, chitosan and cellulose derivatives mimic the glycosaminoglycan rich native ECM [77–79]. Animal derived materials are susceptible to enzymatic degradation, which is a particular issue in the OA joint where catalytic enzymes are upregulated; plant derived polymers may be protected from enzymatic degradation but may still be susceptible to hydrolytic cleavage. The sourcing of naturally derived polymers from animal or vegetables results in variability that can hinder reproducibility. Naturally derived biomaterials typically also show poor mechanical properties and can require additional chemical crosslinking mechanisms, which can introduce cytotoxicity to the system [80].

3.2.2. Synthetic materials

Synthetic polymers such as poly-ethylene glycol (PEG) and poly (N-isopropylacrylamide) (PNIPAAM) benefit over naturally derived polymers in their enhanced mechanical properties, limited immunogenicity and bio-inertness [81,82]. As there is minimal variation between batches, these materials are well controlled and easily characterized. Some polymeric scaffolds like poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and their copolymer poly (lactic co-glycolic acid) (PLGA) are composed of ester linkages, making them hydrolytically degradable [83]. Nondegradable synthetic polymers can be readily modified with natural or naturally derived polymers or moieties to add specific bioactive features (i.e. tunable degradation, cell interactions) to the otherwise inert materials [81,84,85].

3.3. Protein-engineered biomaterials

Engineered protein-based materials represent a new frontier in possibilities for biomedical therapeutics. While previous work focused on naturally derived proteins (collagen, fibrin, etc.), they brought with them limited function and the risk of immunogenicity. Protein engineering has allowed for the ability for functional proteins to be safely integrated into the body and overcome the functional barriers provided by a native domain sequence. Protein engineering has often targeted optimization of protein/peptide repeats, the combination of multiple domains, domain mutagenesis, and the insertion of protein tags to expand or increase function [86,87]. These methods have resulted in modified protein-based biomaterials for drug delivery with improved mechanical integrity, drug retention and release, and additional functionality. These properties are critical to developing a protein-based material to effectively treat OA.

Advancing techniques in synthetic biology have generated renewed interest in protein-engineered hydrogels. Rational design can be used to control features like self-assembly and secondary structure of the protein-material, thus controlling adhesion, drug delivery and affinity binding to other molecules, and material mechanics [88–92]. These materials can be designed to incorporate cell- and ECM-binding domains to promote tissue integration and other bioactive functions [70,93,94]. Bioinspired proteins can be computationally designed to minimize immunogenicity, and the degradation products (amino acids) are non-cytotoxic. Protein-based materials have inherent viscoelasticity that results from the unfolding and refolding transitions of protein domains, which also allows them to be permissive to remodeling by cells [14,17,23]. Protein-engineered materials can also be designed to be stimuli-responsive (e.g., temperature or pH sensitive), a highly desirable feature for biomedical applications with highly specific environmental conditions [23,95–97]. Many of the advancements in protein-based materials for OA treatment involve the use of stimuli-responsiveness for controlled drug delivery (summarized in Table 1) [98–100]. Protein-based stimuli-responsiveness often involves the material undergoing conformational changes based on pH, temperature, or ionic strength. For instance, protein-based materials for OA have utilized a solution-to-gel (sol–gel) transition of a protein solution into a hydrogel at physiological conditions, namely from ambient temperature to body temperature, to create functional *in situ* hydrogels for OA drug delivery [101]. The stimuli-responsive nature of many protein-engineered materials has also led to the development of various self-assembling peptide (SAP) materials [102]. SAPs utilize the same thermodynamically favorable interactions that drive molecular assembly in nature, either mimicking molecules found in nature or creating new molecules by designing peptide sequences bottom-up to arrange in favorable secondary and tertiary structures. These dynamic systems are thus also ideal for designing cell-instructive scaffolding for regenerative engineering therapies (Table 2).

Protein- and peptide-engineered materials can be based on naturally occurring materials from a variety of sources such as mammalian proteins, other animal, or plant proteins. For example, elastin-like polypeptides (ELPs) [103] and cartilage oligomeric peptide coiled-coil domain (COMPcc) [104] derive their main sequences from proteins found in humans and other mammals, while silk-like polypeptides (SLPs) are derived from the silk of moths or spiders [105] and resilin is derived from the cuticles of a variety of arthropods [106]. The creative potential of protein-engineered materials is not limited to biomimetic structures, however. Engineered protein materials can achieve a wide variety of structures by rational design of the amino acid sequence, utilizing the hydrophobicity/hydrophilicity, charge, and size of the amino acid building blocks to engineer specific folding patterns, such as

Table 1
Protein-engineered materials for intraarticular drug delivery.

Family	Peptide Sequence	Active biomolecule	Model	Gelation	Notes	Ref
ELP	(VPG**V**G) and (VPG**V**G)$_{1}$(VPG**G**G)$_{7}$(VPG**A**G)$_{8}$	–	*In vivo* rat knee joint	Aggregating ELP T_t < 32 °C Nonaggregating ELP T_t > 50 °C	No therapeutic tested, ELP was investigated for biodistribution and half-life as proof of concept for future intraarticular drug delivery applications.	[112]
	((VPG**V**G)$_{5}$(VPG**G**G)$_{3}$(VPG**A**G)$_{2}$)$_{90}$ and (VPG**V**G)$_{30}$	IL-1Ra	*In vitro*	ELP-(V)$_{30}$-IL-1Ra: T_t = 34 °C, ELP-($V_5G_3A_2$)$_{90}$-IL-1Ra: 35 °C < T_t < 40 °C	Fusion peptide of ELP with IL-1Ra, Steric hinderance by the ELP portion of the fusion protein during association, but IL-1 receptor and ELP-IL-1Ra association was as stable as commercial IL-1Ra.	[113]
	VPG**K**G(VPG**V**G)$_{16}$ -102	IL-1Ra and/ or sTNFRII	*In vivo* murine fracture model	–	Chemically crosslinked ELP, encapsulated anti-inflammatory drug sustained release *in vitro* and *in vivo* enhanced local drug concentrations and extended intraarticular residence.	[121]
	[VPG**S**G]$_{16}$[VPG∫G]$_{15}$[VPG**A**G]$_{15}$ [VPG**V**G]$_{13}$[VPG**T**G]$_{13}$[VPG**I**G]$_{13}$ [VPG**L**G]$_{12}$[VPG**E**G]$_{9}$[VPG**R**G]$_{7}$[VPG**K**G]$_{6}$	Anti-TNF-α monoclonal antibody	*In vitro*	$T_t \sim$ 39 °C	ELP successfully fused to the heavy chain of the mAb and retained full binding and neutralization potential of TNF-α.	[122]
KLD-12	KLDLKLDLKLDL	TGF-β 1	*In vitro*	–	Biotin-streptavidin tethering vs adsorption of TGF-β 1 to peptide hydrogel showed adsorption promoted BMSC chondrogenesis over tethering. Sustained release of TGF-β 1.	[134,135]
	KLDLKLDLKLDLG**GRPKPQQFFGLM**	Substance P (SP)	*In vitro* and *in vivo* rat knee model	–	KLD12 fusion peptide with SP recruited endogenous circulating MSCs and promoted expression of anti-inflammatory cytokines.	[141]
	KLDLKLDLKLDL + (KLDL)$_{3}$-**GGDHLSDNYTLDHDRAIH**	Link protein N-terminal peptide (LPP)	*In vitro* and *in vivo* rabbit knee model	Frequency sweep showed gel like behavior. Combined KLD-12 and KLD-12-LPP hydrogel G' $\sim$ 150 Pa	1:1 combination of KLD-12 and KLD-12-LPP fusion peptide resulted in enhanced cellular infiltration and resultant chondrogenesis and tissue integration.	[142]
COMPcc^{SS}	MRGSH6GS**GDLAPQMLRELQETNAALQDVRELLRQQV KEITFLNTVMESDASGKLN**	BMS493	*In vitro*	–	Encapsulates and delivers pan-RAR inverse agonist via affinity binding while also sequestering ATRA via competitive binding. Reduced expression of MMP13 in chondrocytes.	[167]
ELP + COMPcc	MRGSH6GSKPIAASA-**[(VPGVG)$_{2}$VPGFG(VPGVG)$_{2}$]$_{5}$**-LEGSELA(AT)$_{6}$AACG-**DLAPQMLRELQETNAALQDVRELLRQQVKEITF LKNTVMESDASG**-LQA(AT)$_{6}$AVDLQPS	Atsttrin	*In vitro* and *in vivo* rabbit ACL transection model	Critical micelle temperature range 20-40 °C wit peak at 35 °C T_t = 20-50 °C depending on concentration (10 mg/mL to 1 mg/mL). Frequency sweep showed G' > G'' from 0.1 to 10 Hz	Combined block copolymer of COMPcc with ELP (E_5C)for intraarticular delivery of anti-inflammatory drug showed sustained release and increased effectiveness in promoting chondrogenesis and suppressing catabolism over drug alone.	[101]
S-GFPs	+9 GFP: MGHHHHHHGGASKGEELFTGVVPILVELDGDVNGHKFSV**R** GEGEGDATNGK LTLKFICTTGKLPVPWPTLVTTLTYGVQCFS**R**YPDHMK**R** HDFFKSAMPKGYVQ E**R**TISFKKDGKYKT**R**AEVKFEG**R**TLVN**R**IKLKG**R**DFKEKGNILGHKL**R**YNFNS HKVYITADKQKNGIKANFKI**R** HNVEDGSVQLADHYQQNTPIGDGPVLLPDNHY LSTQSALSKDPNEK**R**DHMVLLEFVTAAGITHGMDELYK Janus GFP: MGHHHHHHGSACELMVSKGEELFEGDVPILVELDGDVNGHEFSV**R** GEGEGD ATKGELTLKFICTTGELPVPWPTLVTTLTYGVQCFS**R**YPKHMKQHDFFKSAMP EGYVQE**R**TISFKDDGTYKT**R**AEVKFEGDTLVN**R**IELKGKDFKEKGNILGHKLE YNFNSHRVYITADK**R**KNGIKAEFKI**R**HNVKDGSVQLADHYQQNTPIG**R**GPVLL P**RR**HYLST**R**SALSKDPKEE**R**DHMVLLEFVTAAGIDHGMDELYK	–	*In vitro* explant model	–	Cationic supercharged GFPs, showed increased uptake and residence in cartilage explant culture, with increasing uptake correlated with decreasing net positive charge. Net-neutral Janus S-GFPs showed more favorable uptake when compared to other net-neutral S-GFPs, although not as successful as the cationic carriers. No therapeutic tested.	[199]

Table 2
Protein-engineered materials for cartilage tissue engineering.

Family	Peptide Sequence	Active biomolecule	Model	Mechanics	Delivery	Notes	Ref
ELP	(VPG**V**G)$_5$(VPG**G**G)$_3$(VPG**A**G)$_2$	–	*In vitro*	T_t = 35 °C |G*| = 0.08 kPa, comparable to PNIPAAM, hyaluronan, and collagen injectable materials but much softer than native tissue (440 kPa)	Injectable	*In vitro* culture of chondrocytes showed successful maintenance of morphology and synthesis of collagen and GAGs.	[111]
	(VPG**V**G)$_5$(VPG**G**G)$_3$(VPG**A**G)$_2$	–	*In vitro*	–	Injectable	*In vitro* culture of human adipose derived adult stem cells in the absence of exogenous chondrogenic supplements.	[115]
	ELP[KV6-112] and ELP[QV6-112]	–	*In vitro*	|G*| of crosslinked ELP = 0.26 kPa After 28 days of cell culture, |G*| = 1.8 kPa	Injectable, *in situ* crosslinking	Enzymatically cross-linked via transglutaminase. Supports *in vitro* matrix production by encapsulated chondrocytes.	[117]
	VPG**B**G(VPG**B**G)$_6$-224,	–	*In vivo* goat osteochondral defect	–	Injectable, *in situ* crosslinking	Chemically crosslinked ELP. 3-month data showed better histological grades than unfilled defects however by 6 months rapid degradation resulted in better histological grades on the unfilled defect.	[118]
SLP	RGDSRGDSLAPLSAPLST RGDSRGDSSKSTEVPTEV	–	*In vitro*	–	–	Chondrocytes grown on RGD containing fibroin were better able to synthesize cartilage matrix products over the wild-type fibroin.	[93]
SELP	MDPVVLQRRDWENPGVTQLNRAAHPPFASDPMG**AGSGAGAGS [(GVGVP)$_4$GKGVP(GVGVP)$_3$(GAGAGS)$_4$]$_{12}$ (GVGVP)$_4$ GKGVP (GVGVP)3 (GAGAGS)$_2$GAG**AMDPGRYQDLRSHHHHHH	–	*In vitro*	–	Injectable	Encapsulated MSCs produced chondrogenic matrix.	[127]
	MESLLP-{[(VPG**V**G)$_2$-VPG**E**G-(VPG**V**G)$_2$]$_{10}$-(VGIPG)$_{60}$-[V(**GAG-AGS**)$_5$G]$_2$-[(VPGIG)$_5$-AVTGRGDSPASSV]$_6$.		*In vitro* and *ex vivo* organ culture	Exhibited shear thinning behavior	Injectable	Pre-annealed hydrogel allowed β-sheets to preform prior to injection resulting in clinically relevant gelation time.	[128]
KLD-12	KLDLKLDLKLDL	–	*in vitro*	Equilibrium modulus = 26 kPa (~10% native tissue), Dynamic stiffness ~ 5% native tissues by 26 days. Higher cell seeding density and ITS resulted in equilibrium modulus = 93 kPa and dynamic stiffness = 1.28 MPa at 28 days (1/5 – 1/3 that of native human cartilage).	Injectable	encapsulated chondrocytes proliferated and maintained phenotype while producing biomechanically functional ECM.	[129]
	KLDLKLDLKLDL	–	*In vitro*	Sinusoidal dynamic compression: 2.5% strain amplitude, 0.3 Hz applied – mechanics of final constructs not measured	Injectable	Dynamic compression of chondrocyte-loaded KLD-12 constructs triggered increase in construct remodeling.	[130]
	KLDLKLDLKLDL and RADARADARADARADA	–	*In vitro*	–	Injectable	Encapsulated bovine BMSCs showed cell proliferation and chondrogenesis an increased cell-cell contact in KLD-12 and RADA16 hydrogels but not agarose.	[131]
	KLDLKLDLKLDL	TGF-β 1	*in vitro*	–	Injectable	Sustained release of adsorbed TGF-β 1 increased chondrogenesis of encapsulated BMSCs. Tethered TGF-β 1 had no effect.	[134,135]
	KLDLKLDLKLDL	TGF-β 1, dexamethasone, IGF-1	*In vivo* rabbit knee full thickness critical sized defect	–	Injectable	KLD without chondrogenic factors showed greatest repair of osteochondral defect.	[137]

Table 2 (*continued*)

Family	Peptide Sequence	Active biomolecule	Model	Mechanics	Delivery	Notes	Ref
	KLDLKLDLKLDL	–	*In vivo* equine trochlear defect	–	Injectable	Microfracture improved the quality of repair tissue while KLD improved the amount of filling and protected against radiographic changes.	[138]
	KLDLKLDLKLDL	PDGF-BB and HB-IGF-1	*In vitro* cartilage explant and *In vivo* rabbit knee osteochondral defect	–	Injectable	Trypsin pretreatment improved tissue integration with KLD scaffolds.	[139,140]
	KLDLKLDLKLDL + $(KLDL)_3$-**GG-DHLSDNYTLDHDRAIH**	Link protein N-terminal peptide (LPP)	*In vitro* and *in vivo* rabbit knee model	Frequency sweep showed gel like behavior. Combined KLD-12 and KLD-12-LPP hydrogel G' ~ 150 Pa	Injectable	1:1 combination of KLD-12 and KLD-12-LPP fusion peptide resulted in enhanced cellular infiltration and resultant chondrogenesis and tissue integration.	[142]
RADA16	RADARADARADARADA	–	*In vitro*	–	Injectable	Encapsulated chondrocytes maintained phenotype and produced relevant matrix	[144]
	RADARADARADARADA	Dexamethasone	*In vitro*	–	Injectable	Dex reduced aggrecanase activity.	[145]
	RADARADARADARADA	HB-IGF-1	*In vitro*	–	Injectable	Growth factor adsorption onto peptide prior to self-assembly stimulated matrix production in encapsulated chondrocytes.	[146]
	RADARADARADARADA	TGF-β 1 or HB-IGF-1	*In vitro*	Structures encapsulated with ADSCs, but not chondrocytes, achieved G* equivalent of chicken and calf cartilage	Injectable	RADA-GAG composites (Heparin, Chondroitin Sulfate, or Decorin) did not preferentially bind TGF-β 1 but did slow the release of HB-IGF-1. RADA-GAGs complexes promoted chondrogenic differentiation of ADSCs.	[147–149]
	RADARADARADARADA	–	*In vitro*	Tan(δ) of cellularized PCL/RADA scaffold was equivalent to chick and calf cartilage (gel like behavior) however G* of constructs was significantly lower than native cartilage	Surgical implant/ Not injectable	Redifferentiation of dedifferentiated chondrocytes and chondrogenesis of hMSCs was supported by the inclusion of RADA16 in composite PCL/RADA scaffolds vs PCL alone. Cellular interaction with RAD sequence suggested.	[152,153]
	$(RADA)_4$-GG-**PFSSTKT** or $(RADA)_4$-GG-**SKPPGTSS**	Bone marrow homing peptides: PFS or SKP	*In vivo* rabbit knee full thickness defect	Hardness, contact stiffness, and reduced modulus of composites with homing peptide were all equivalent to native cartilage	Surgical implant/ Not injectable	Acellular/decellularized cartilage matrix combined with homing peptide-functionalized RADA successfully recruited endogenous MSCs for cartilage repair	[157,158]
Resilin	M-MASMTGGQQMG-HHHHHHH-DDDDK-LDHMRTLS-(**AQTPSSKQFGAPQTPSSQFGAP**)$_5$-**KWADRHGGMR-GGTVYAVTGRGDSPASSGGG**-LE	–	*In vitro*	G* = 22 kPa, unconfined compression modulus = 2.4 MPa, on par with articular cartilage	Potentially injectable. T_t – 40.5 °C	Robust mechanical properties in shear and compression due to high crosslinking density. MSCs showed cytocompatibility and spreading; chondrogenesis was not assessed.	[172]
CMP	**POGPOGPOGPOGPOGPOG POGY**	–	*In vitro*	–	Photocrosslinked – not injectable	CMP-PEODA composite structures promoted sGAG and type II collagen deposition by encapsulated chondrocytes and MSCs while suppressing hypertrophic phenotype. compared to those cultured in PEODA gels alone. CMP provided collagen binding sites that enhanced hydrogel crosslinking	[176,177]
	KLDLKLDLKLDL-GG-**POGPOGPOGPOGPOGPOGPOG**	–	*In vitro* and *in vivo* mouse	–	Composite scaffold with	Self-assembling KDL12-CMP7 block copolymer was mixed with PLCL to form	[178]

(*continued on next page*)

Table 2 (*continued*)

Family	Peptide Sequence	Active biomolecule	Model	Mechanics	Delivery	Notes	Ref
			subcutaneous model		PLCL: implantable/not injectable	composite scaffold. CMP-containing constructs promoted chondrogenic expression by MSCs. CMP containing scaffolds also suppressed hypertrophic expression of type X collagen	
	pColdI: NHKVHHHHHHIEGRHMELGTLEGSEFKLVDLQSR Scl2.28: GQD GRN GER GEQ GPT GPA GPR GLQ GLQ GLQ GER GEQ GPT GPA GPR GLQ GER GEQ GPT GLA GKA GEA GAK GET GPA GPQ GPR GEQ GPQ GLP GKD GEA GAQ GPA GPM GPA GER GEK GEP GTQ GAK GDR GET GPV GPR GER GEA GPA GKD GER GPV GPA GKD GQN GQD GLP GKD GKD GQN GKD GLP GKD GKD GQN GKD GLP GKD GKD GQD GKD GLP GKD GKD GLP GKD GKD GQP GKP HA-binding: **CGGGRYPISRPRKR** CS-binding: **CGGGYKTNFRRYYRF** MMP7 sensitive: **CGGGPLELRAGGGC** Cyclic integrin binding: **GRGDSC**	–	*In vitro*	G' = 8 kPa, unconfined compressive modulus = 2.5 kPa	Potentially injectable? pH sensitive at 8.5	Scl2 blank slate proteins were modified with HA or CS binding peptides via acrylate functionalization and crosslinked with MMP-sensitive crosslinks. hMSCs in GAG-binding hydrogels showed increased chondrogenic differentiation,	[181]
	Scl2.28: GQD GRN GER GEQ GPT GPA GPR GLQ GLQ GLQ GER GEQ GPT GPA GPR GLQ GER GEQ GPT GLA GKA GEA GAK GET GPA GPQ GPR GEQ GPQ GLP GKD GEA GAQ GPA GPM GPA GER GEK GEP GTQ GAK GDR GET GPV GPR GER GEA GPA GKD GER GPV GPA GKD GQN GQD GLP GKD GKD GQN GKD GLP GKD GKD GQN GKD GLP GKD GKD GQD GKD GLP GKD GKD GLP GKD GKD GQP GKP MMP7 sensitive crosslinker: **PLELRA** Aggrecan-cleavable crosslinker: **RDTEGEARGSVIDR**	–	*In vitro*	G* = 7 kPa, unconfined compressive modulus = 4-5 kPa Dynamic mechanical analysis showed increasing compressive modulus with increasing frequency		hMSC-laden modified Scl2 constructs promoted chondrogenic matrix deposition and maintained mechanical integrity over 6 weeks. MMP7-sensitive RGD functionalization temporally controlled cell adhesion resulting in increased chondrogenic expression	[106,182]

KLDLKLDLKLDL (KLD-12) and RADARADARADARADA (RADA16), which both have positive charged/hydrophobic/negative charged/hydrophobic patterns and form β-sheets [107]. Non-canonical amino acids (NCAAs) can be utilized to expand the library, and thus structural potential of protein-engineered materials, even further [108]. The means of synthesizing protein-based materials makes them particularly amenable to incorporation of bioactive moieties, either through direct conjugation to the peptide sequence, or via intermolecular interactions. Cartilage tissue engineering, repair, and therapeutic delivery continues to be a hot topic for investigation, as the implications for successful therapies would improve the lives of millions. Protein-engineered materials hold the potential to address several unmet needs for cartilage repair. Here we will discuss the current state of protein-engineered materials for cartilage repair and discuss potential future areas of investigation. The following studies presented have been selected based on the primary material of use being protein or peptide derived and its use of synthetic biology and recombinant techniques in order to focus on the unique tools protein engineering provides the field of biomaterials and drug delivery. For this reason, naturally derived/extracted materials such as natural collagen/gelatin or silk fibroin are mentioned but are not elaborated on in detail.

3.3.1. Elastin-like polypeptides (ELPs)

Elastin is a naturally occurring component of the ECM, that, as its name suggests, provides elasticity to several tissues. Elastin is an important component of the ECM of articular cartilage, particularly in the superficial zone, playing a role in maintaining its mechanical integrity and elasticity [109]. ELPs are artificial polypeptides based on naturally occurring elastin, consisting of the pentapeptide sequence Val-Pro-Gly-Xaa-Gly, where Xaa (known as the guest residue) may be any amino acid except proline [110]. ELPs are soluble in aqueous solutions and display an inverse phase transition, in which they precipitate and form a gelatinous aggregate, or "coascervate," above a transition temperature (T_t) (Figure 2) [111]. Their thermoresponsive nature makes them highly appealing for injectable applications, including drug delivery and cartilage repair, where surgical intervention would likely further compromise joint mechanics.

ELPs were investigated for intraarticular drug delivery due to their ability to form a drug "depot" upon injection and aggregation at 37°C, after which disaggregation and natural degradation of the peptide biomaterial would allow the loaded drug to be released over time [112]. Two ELP formulations were studied: an "aggregating ELP" with guest residue Val (T_t < 32°C, MW 47 kDa) was compared to a "non-aggregating ELP" with guest residues Val/Gly/Ala at a ratio of 1:7:8 (T_t > 50°C, MW 61 kDa). The aggregating ELP exhibiting a 25-fold increase in half-life upon injection into the joint space compared to the nonaggregating ELP, demonstrating the tunability of the peptide system by modifying select residues within the peptide sequence [112]. This study focused on the ability of the aggregating ELP coascervate to be retained within the joint space versus the non-aggregating ELP, which was rapidly cleared from the joint space, but did not actually evaluate the retention of a potential therapeutic molecule. Researchers then developed a fusion protein between ELP and IL-1 receptor antagonist (IL-1Ra), which could be retained in the joint space as previously demonstrated, allowing IL-1Ra to maintain a potentially therapeutic presence in the joint space for weeks [113]. IL-1Ra competitively binds IL-1, downregulating the activity of MMPs and promoting collagen and glycosaminoglycan synthesis in chondrocytes. Recombinant IL-1Ra was successfully coupled to two ELP sequences investigated in the previously mentioned studies, both which featured T_t < 37°C [111,112]. Both fusion proteins showed association rate constants lower than commercial IL-1Ra, but equivalent dissociation rate constants, suggesting steric hinderance by the ELP portion of the fusion protein during association. Once complexed, however, the IL-1 receptor and ELP-IL-1Ra association was as stable as commercial IL-1Ra. Both fusion protein aggregates dissociated more rapidly and to a greater extent than ELP alone. IL-1 antagonist bioassays were performed using human lymphocytes, murine thymocytes, and human intervertebral disc cells; while bioactivity was preserved, fusion proteins were generally less active or required higher concentrations than commercially available IL-1Ra. Still, the fusion proteins were able to inhibit lymphocyte and thymocyte proliferation, as well as inhibiting IL-1 β upregulation of ADAMTS-4 and MMP-3 in intervertebral disc cells. Interestingly, collagenase proteolysis of the fusion proteins yielded a fragment with higher immunoreactivity and bioactivity than the whole fusion protein, suggesting that the bulky ELP domain partially inhibited the bioactivity of the fusion proteins. As collagenase was upregulated in OA, release of IL-1Ra from the fusion protein has the potential to be mediated by the disease state of the joint. A similar study was conducted by the same group with a TNF-α antagonist, soluble TNF receptor type II (sTNFRII), forming a fusion protein ELP-sTNFRII with similar results, although it was not investigated for OA treatment [114].

ELPs have also been investigated as potential scaffold materials for cartilage repair. Although they can be designed undergo a thermosensitive phase transition at physiologic temperature, they form coascervates rather than true hydrogels. These coacervates have shown to be able to support cartilaginous matrix synthesis and retention by encapsulated porcine chondrocytes and human adipose-derived adult stem cells (hADASCs) *in vitro* (Figure 2) [111,115]. Most notably, the ELP coacervate culture was able to promote chondrogenic differentiation of hADASCs in the absence of chondrogenic supplements dexamethasone and TGF-β, which are standard supplements critical for promoting chondrogenesis *in vitro* [116]. The ratio of type II collagen to sGAG accumulation in the ELP without chondrogenic supplementation was closer to the values of native cartilage than the constructs cultured in chondrogenic media. This, however, may be attributed to the short culture time (14 days). It is hypothesized that the phase transition of the ELP enabled cell-to-cell interactions that promoted chondrogenesis like the condensation of cartilage tissue that occurs during embryogenesis. The mechanics of the ELP constructs were not extensively investigated, however, which is a concern when

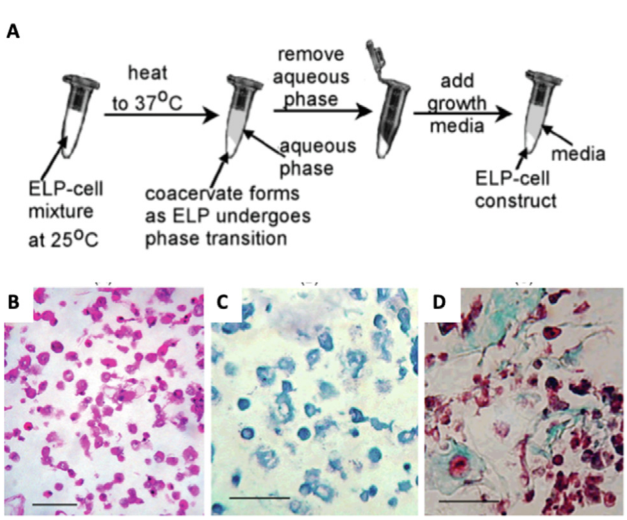

Fig. 2. Elastin-like polypeptides: (A) Thermoresponsive coascervate formation and culture and histological sections after 15 days of culture stained for (B) cell morphology (hematoxylin & eosin) (C) sGAG (Toluidine blue) and (D) collagen and ECM (Masson's trichrome) (scale bar = 50 μm). Reproduced and adapted with permission from reference [111] ACS Biomacromolecules.

designing scaffolds that will undergo loadbearing and mechanical stresses immediately after delivery. The dynamic shear modulus of the ELP scaffold (~80 Pa), was comparable to other hydrogels used for cartilage tissue engineering but was nearly 4 orders of magnitude less than that of articular cartilage (100–500 kPa). Indeed, the ability of ELP coacervates to slowly dissociate and become digested in the presence of collagenases and the relatively short time course of this dissolution (~1 week) that make it an appealing mechanism for drug delivery, makes an unmodified ELP a less than ideal scaffold material for cartilage tissue engineering. With that in mind, ELP hydrogels have been engineered to undergo enzyme-initiated crosslinking via tissue transglutaminase (tTG) by genetically encoding glutamine (Q) and lysine (K) amino acid residues into the ELP sequence [117]. The lysine-containing sequence exhibited a T_t of 54°C, much too high to be useful for physiologic applications, while the glutamine-containing sequence had a T_t of 29°C. When combined at an equimolar ratio, the T_t was 31°C, and in the presence of salts necessary for activation of tTG, the T_t decreased to 22°C, which could present potential difficulties for injectability. Free-standing ELP hydrogels were fabricated via tTG crosslinking, with the resulting dynamic shear stiffness approximately 3 times greater than the unmodified ELP coacervate; this further increased over 28 days of culture as encapsulated chondrocytes produced matrix. The utilization of a native enzyme as the crosslinking agent enabled this system to be an *in situ* forming hydrogel, ideal for minimally invasive therapies. However, the slow crosslinking kinetics of this system limit its potential for clinical translation.

In an effort to achieve clinical translation, researchers designed a lysine containing ELP that could be chemically crosslinked with a biocompatible amine-reactive crosslinker, β-[Tris(hydroxymethyl) phosphino] propionic acid [betaine] (THPP) [118]. ELPs crosslinked with this system had a compressive and shear stiffness between 10–50 kPa, and were crosslinked in under 5 minutes, much more relevant to clinical application [119]. Acellular ELP solution and THPP were used to fill critically sized goat osteochondral defects and analyzed for tissue repair at 3 and 6 months. At 3 months, ELP-filled defects scored higher than controls for biomaterial integration based on gross and histological grading, however by 6 months the ELP-filled defects had scores equivalent or poorer than the control. Positive outcomes of the scaffold implementation included evidence of cell infiltration and biomaterial resorption. Degradation of the ELP hydrogel was likely mediated by proteolysis, and the authors posit that using genetic engineering the ELP material can be tuned to have more favorable crosslinking, transition temperature, and degradation properties. The extreme defect studied here was not representative of most human pathology, but rather similar to microfracture augmentation treatment, and so it was not immediately clear if the ELP hydrogel would display similar results in a purely chondral lesion. Using artificial neural network analysis, authors sought to identify the relationships between ELP scaffold formulation parameters (crosslinking density, molecular weight, and concentration) on outcomes (mechanical properties, matrix accumulation, metabolite usage and production, and histological appearance); crosslinking density was the strongest predictor of outcomes [120]. Although mechanics and degradation rates of the ELP scaffolds produced thus far have come short in terms of tissue engineering of a load-bearing tissue like cartilage, utilization of a neural network was shown to be a powerful tool for future engineering of biomaterial scaffolds for cartilage tissue engineering.

Combining lessons from both previous areas of investigation, namely anti-inflammatory drug delivery via ELP carrier and crosslinking of ELP to form tissue engineering scaffolds for cartilage repair, researchers developed a chemically crosslinked ELP depot to deliver anti-inflammatory treatment to prevent PTOA [121]. Crosslinked with THPP as described earlier, ELP hydrogels were constructed with either 75 mg/mL of IL-1Ra, 25 mg/mL of sTNFRII, both, or none. Release profiles were characterized *in vitro* and *in vivo* and a closed articular fracture model in mice was used to assess the efficacy of the ELP-anti-inflammatory therapeutic in preventing PTOA. Drug release was detected *in vivo* up to 5–14 days post-injection for all the formulations, after which IL-1Ra or sTNFRII were not detectable. Sustained delivery of IL-1Ra alone was able to reduce the severity of PTOA related changes in cartilage and synovium post-fracture, while sTNFRII alone or with IL-1Ra had deleterious effects on the cartilage, synovium, and subchondral bone of the affected joint. Results of this study focused mainly on the efficacy of the anti-inflammatory treatment in preventing PTOA and the ability of the ELP to act as a sustained-release drug depot; tissue sections were not evaluated for the location of the ELP depot or its persistence in the intraarticular space before it completely degraded, nor was it evaluated for any evidence of cellular ingrowth. As intraarticular injections were made immediately post-fracture, there was presumably no specific cartilage defect being filled to evaluate in the same manner as the previously discussed crosslinked ELP study. Additionally, the mechanics of the construct and the role the construct would play in the mechanics of the articular joint were not addressed.

Recently another attempt was made at producing an ELP-anti-inflammatory fusion protein, fusing ELP sequences to the C-terminus of each heavy chain of an anti-TNF-α monoclonal antibody (mAb) [122]. ELP were successfully fused to the heavy chain of the mAb and retained full binding and neutralization potential of TNF-α. The fusion protein molecule formed aggregates above 39°C and became soluble at 37 °C; authors did not elaborate on how this would affect delivery or retention of the therapeutic, as this was a preliminary study. Future studies aim to look at the efficacy of the fusion ELP-mAb in effectively treating inflammation in the joint space.

3.3.2. Silk-like polypeptides (SLPs)

SLPs, as the name suggests, are derived from domesticated silk moth *Bombyx mori* (mulberry silk) or spider silk heavy chains. They encompass proteins composed of repeat units of silk-like protein blocks with sequence GAGAGS and are capable self-assembly [123–125]. The highly crystalline GAGAGS repeat regions are interspersed with less organized amino acid regions, resulting in highly elastic materials. SLPs are similar to ELPs, and are often used in combination to create silk-elastin-like proteins (SELPs), also capable of hydrophobic self-assembly driven by the formation of β-sheets [99]. The result is a protein biomaterial that is stimuli-responsive with high mechanical integrity. Naturally derived silk-based materials have been used for centuries as suturing materials, due to their strength and naturally low immunogenicity. Fazal and Latief recently reviewed the use of naturally derived silk fibroin materials for cartilage repair and treatment of joint injuries [126]. For the purposes of this review, here we will focus on the use of engineered SLPs and SELPs in cartilage repair.

One of the first examples of engineered silk protein for cartilage repair was performed by Kwambe *et al.*, where, using transgenic *B. mori* silkworms, they were able to produce silk fibroin incorporating a tandem repeat of the RGD sequence in order to investigate the effects of cell adhesion on cartilage formation in an *in vitro* chondrogenesis model [93]. Though increasing RGD-integrin mediated adhesion is generally understood to cause chondrocytes to lose their phenotype, here they found that chondrocytes grown on the RGD containing fibroin were better able to synthesize cartilage matrix products over the wild-type fibroin, suggesting that the RGD + fibroin material could promote moderate cell adhesion without inducing spreading and dedifferentiation. Though the study did observe a temporal effect of chondrocyte adhesive force on the fibroin scaffolds, they did not measure the stiffness of the scaffolds themselves to elucidate any further source of their results. They did, however, posit that RGD enhanced chondrocyte motility, and thus chondrogenesis, within the silk fibroin scaffolds.

SELP materials undergo an irreversible sol–gel transition that is dependent on the number and sequence of silk and elastin blocks. SELP-47 K is one such block copolymer that has been investigated as a potential injectable *in situ* forming scaffold for cartilage tissue engineering [127]. The sequence is composed of 4 silk-like blocks, 7 elastin-like blocks, and one modified elastin-like block that contains lysine (K) in the guest position $[(\mathrm{GAGAGS})_4(\mathrm{GVGVP})_7(\mathrm{GVG}\mathbf{K}\mathrm{P})_1]$. MSCs cultured in these scaffolds in the presence of TGF-β 3 show upregulation of chondrogenic markers and produce cartilage-like matrix. The irreversible sol–gel transition afforded by the silk blocks is presented as a property that can provide greater mechanical robustness to otherwise soft elastin coascervates, without the need for external crosslinking agents. However, the mechanics of the material has not been investigated. They hypothesized that increasing mesh size by increasing elastin blocks or decreasing silk blocks could also further enhance chondrogenesis by making the scaffold void space more permissive to collagen deposition.

In SELPs, the elastin component provides the thermogelling property, while silk motifs allow for the formation of β-sheets, increasing the elastic moduli of the materials. However, forming these structures takes a long time that is not feasible for clinical application as a therapeutic approach. Cipriani *et al.* produced SELP co-recombiners with an optimized thermal (preannealing) treatment to accelerate β-sheet formation while maintaining injectability (Figure 3) [128]. The result was a robust, fibrillar hydrogel that exhibited shear-thinning behavior, thus retaining its injectability, while forming a stable hydrogel at 37 °C within 15 minutes. The complex modulus was positively correlated with protein polymer concentration, while the effect of annealing time plateaus at 24–48 hours at which point the β-sheet formation is complete. Both *in vitro* studies with chondrocytes and an *ex vivo* organ culture model of a full-sized osteochondral defect demonstrated cell proliferation and chondrogenic matrix deposition. However, with a short culture time of 28 days, the amount of matrix produced predictably did not reach native values. Of particular note is that the *ex vivo* organ culture that included a "bone" phase resulted in more robust chondrogenic matrix deposition by the chondrocytes.

3.3.3. KLD-12

KLD-12 is another self-assembling peptide hydrogel that has been studied as a 3D scaffold for cartilage tissue engineering. This material self-assembles into fibrillar hydrogels when exposed to physiologic electrolyte concentrations such as immersion in phosphate buffered saline (PBS). Early studies encapsulating chondrocytes in this self-assembling hydrogel revealed that cells maintain viability, a rounded morphology typical of the chondrocytic phenotype, and synthesized proteoglycan and collagen on par with chondrocytes seeded in agarose hydrogels [129]. Mechanical testing of KLD-12 hydrogels showed that initial equilibrium modulus was less than 1 kPa. With the accumulation of matrix components during a culture period of 28 days, the cell-laden hydrogels reached an equilibrium modulus of 26 kPa, approximately 10 % that of native tissue, and a dynamic stiffness (1 Hz) reaching $\sim$ 120 kPa, 5 % of native tissue. When cultured with higher cell density (30×10^6 vs 15×10^6) and low serum medium, matrix production and subsequent mechanics increased even more, with an equilibrium modulus of 93 kPa and dynamic stiffness of 1.28 MPa at 28 days (1/5 – 1/3 that of native human cartilage). Under dynamic compression, chondrocyte-loaded KLD-12 constructs experienced an increase in MMP-9 and MMP-3 production and upregulation of several other MMPs and ADAMTs proteases, resulting in aggrecan fragmentation, potentially indicative of construct remodeling [130].

KLD-12 was also investigated for its ability to support chondrogenic differentiation from bone marrow derived stromal cells (BMSCs) [131]. In this study, bovine BMSCs were encapsulated in three types of hydrogels, KLD-12, RADA16 (another peptide-based hydrogel described below) or agarose and cultured with TGF-β 1 to stimulate chondrogenesis. Cell proliferation was observed in both the peptide hydrogels, along with extensive cell–cell contact, reminiscent of the condensation that occurs during embryogenesis that drives cartilage development [132,133]. This also resulted in more robust ECM production. Interestingly, both protein hydrogels also exhibited less rounded cell morphologies, which may have potentially been the cause of hydrogel compaction; the softer protein hydrogels ($\sim$1 kPa storage modulus) compared to agarose (10 kPa) may have been more permissive to mechanical pulling by the encapsulated cells, which in turn may be mediated by cell-scaffold interactions that were not present in the agarose gel. When pre-mixed with TGF-β 1 allowing the growth factor to adsorb onto the scaffold, KLD-12 scaffolds stimulated chondrogenesis of encapsulated BMSCs, while tethering of TGF-β 1 via biotin-streptavidin conjugation did not [134]. It was hypothesized that TGF-β 1 attraction to the KLD-12 scaffold was mediated by the negatively charged aspartic acid residues (D) [135]. A similar study using insulin-like growth factor-1 (IGF-1) showed that while soluble IGF-1 promotes cell proliferation and chondrogenic matrix production in KDL-12 scaffolds, neither adsorption nor tethering of IGF-1 had similar such effects [136].

In vivo studies of this particular scaffold system for cartilage repair have included a rabbit model of a critically sized cartilage defect and an equine trochlear defect model [137–139]. In the rabbit critically sized defect, KLD-12 scaffolds demonstrates the greatest repair in the absence of chondrogenic factors (TGF-β1, IGF-1, streptavidin, and dexamethasone) and allogenic BMSCs [137]. Surprisingly, the addition of BMSCs hinders the quality of the repair and encourages the growth of osteophytes. The full thickness

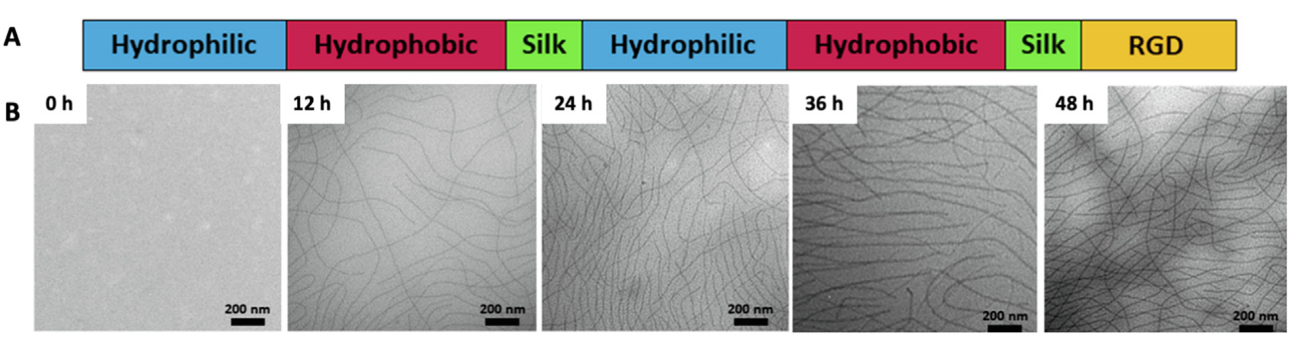

Fig. 3. A) Graphical scheme of the composition of silk-elastin like polypeptide $(\mathrm{EIS})_2$-$(\mathrm{I}_5\mathrm{R})_6$. B) Transmission electron micrographs of $(\mathrm{EIS})_2$-$(\mathrm{I}_5\mathrm{R})_6$ in water (0 h) and at various amounts of preannealing time. Reproduced and adapted with permission from reference [128] Copyright 2018, American Chemical Society.

defect accesses the underlying bone marrow, which provides a cell source to infiltrate the scaffold, like current abrasion arthroplasty and subchondral bone microfracture techniques. In a subsequent study, scaffolds combined with platelet-derived growth factor-BB (PDGF-BB) and IGF-1 have been applied to osteochondral defects in rabbits along with enzymatic pretreatment with trypsin; enzymatic pre-treatment enables the controlled release of proteoglycans from native cartilage, allowing space for newly synthesized repair tissue to integrate with the damaged host cartilage [139,140]. In the equine model, KLD treatment and microfracture (clinical standard) treatment for osteochondral defects shows comparable success in cartilage repair; while neither exhibits optimized cartilage repair, positive results in a large animal model show strong potential for future studies using this scaffold.

KLD-12 peptide was recently coupled with neuropeptide substance, P, a chemotactic factor for mobilizing MSCs and potential anti-inflammatory, to recruit local MSCs to promote cartilage regeneration and reduce inflammation and OA progression [141]. A KLD-12-SP fusion protein was synthesized (Ac-KLDLKLDLKLDL**GGRPKPQQFFGLM**-NH_2), its bioactivity confirmed *in vitro*, and then used 2-weeks post-surgical induction of OA in a rat ACL and medial collateral ligament (MCL) ligament transection model of OA. *In vivo,* the KLD-12-SP gel had anti-inflammatory effects, reducing pro-inflammatory cytokine expression, and increasing IL-4 expression, which had a chondroprotective effect in inhibiting proteoglycan degradation. The gel also inhibited chondrocyte apoptosis and recruited endogenous MSCs, a feature particularly useful both in tissue repair as well as overcoming the regulatory hurdles involved in implanting cell-laden materials. Recruitment of endogenous MSCs resulted in improved cartilage regeneration as shown by robust proteoglycan deposition and collagen II. In addition, the KLD-12-SP material significantly prevented loss of bone mineral density associated with OA. This study did not investigate the persistence of the biomaterial within the joint, nor any mechanics of the material. In lieu of material or joint mechanics, specimen behavior in response to joint pain was measured, although no significant difference between groups was observed. In a similar approach, KLD-12 was functionalized with link protein N-peptide (LPP) (Ac-$(KLDL)_3$-**GG-DHLSDNYTLDHDRAIH**-$CONH_2$) and shown to induce chondrocyte and BMSC migration (Figure 4) [142]. LPP, a degradation product of the link protein, was shown to play a role in regulating chondrocyte proliferation and ECM synthesis, as well as inducing migration of cartilage-derived stem cells (CSCs) and nucleus pulposus cells. KLD-12-LPP fusion peptide was mixed with pure KLD-12 at a 1:1 ratio to form a hydrogel that significantly improved chondrocyte and BMSCs infiltration, promoting proteoglycan synthesis and thus interfacial integration. The nanostructure of self-assembling peptide scaffolds (10–50 nm fiber diameter, 5–200 nm pores) facilitated cell migration as it mimicked the architecture of the native ECM. Material mechanics were only evaluated via rheometry for gel-like behavior and were not assessed in terms of mechanical function within the injury site. It should be noted that the fusion of functional peptides interrupted self-assembly of KDL-12 in both the previously mentioned studies, and so delivery of the fusion peptides required mixing with unmodified KDL-12 in order to self-assemble into hydrogels and be retained within the articular joint space [141,142].

3.3.4. RADA16

Named for its sequence, RADARADARADARADA, RADA16 (or sometimes just RAD16), is another example of a stimuli-responsive self-assembling protein biomaterial that has proven utility for osteoarthritis. RADA16 is pH-responsiveness and forms physical crosslinks to form a hydrogel at physiologic pH that resembles the 3-D properties of the ECM [143]. RADA16 forms antiparallel β-sheets via complementary staggering of its positively and negatively charged sidechains. Shear-thinning and thixotropic behavior also make it an excellent candidate material for injectable applications. As one of the oldest studied SAPs, RADA16 is com-

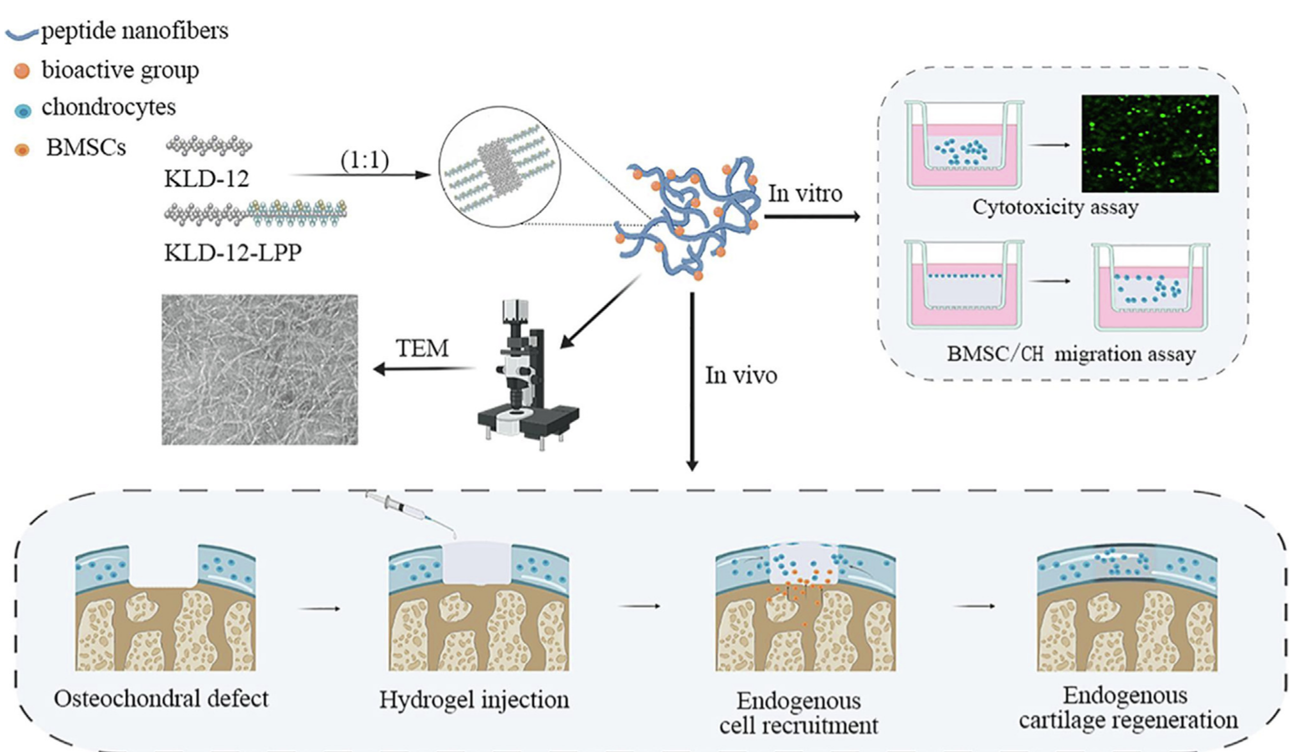

Fig. 4. Schematic illustration of KLD-12 hydrogel with LPP for endogenous cell recruitment and cartilage regeneration. Reproduced with permission from reference [142]. Copyright 2020, Frontiers.

mercially available as PuraMatrix™ (3-D Matrix Ltd. Tokyo, Japan) for *in vitro* and preclinical *in vivo* studies, and available for clinical use for hemostat and wound healing applications as PuraStat®, PuraBond® (3-D Matrix Europe SAS, Caluire-et-Cuire, France), PuraDerm™, and PuraSinus™.

Early *in vitro* studies have shown RADA16 as a promising scaffold for chondrocyte culture. Liu *et al.* showed the hydrogel supported chondrocyte proliferation and deposition of type II collagen and aggrecan over an agarose control gel [144]. A similar study by Kopesky *et al.* investigated chondrogenic differentiation of BMSCs in the presence of TGF-β1 in RADA16, KLD12 and agarose gels [131]. As described in the previous section, cells only proliferated in both peptide hydrogels rather than the agarose gels. RADA16 gels demonstrated more cell proliferation than the KLD12 gels, though this difference was not statistically significant.

Further investigating the efficacy of RADA16 as a cartilage tissue engineering scaffold, Florine *et al.* embedded human and bovine BMSCs in RADA16 and agarose hydrogels with medium containing TGF-β1 and dexamethasone (Dex), a glucocorticosteroid that has been used both in chondrogenic cell culture and clinically in osteoarthritis care [145]. While Dex increased bovine proteoglycan synthesis and retention in agarose hydrogels, it decreased its accumulation in the RADA16 scaffold. These results were inverted when human BMSCs were used. Dex also reduced aggrecanase (ADAMTS-4/5) activity in both hydrogels. The conflicting results between juvenile bovine and adult human BMSCs highlighted the need for care and specificity when selecting scaffold materials and cell sources. Animal models or cell sources did not always align with human cell behavior, and the age of the cell source could also be a contributing factor to the discrepancy. The same group then went on to investigate RADA16 as a scaffold for delivering a fusion protein consisting of heparin-binding domain of epidermal growth factor-like growth factor and insulin-like growth factor 1 (HB-IGF-1) with chondrocytes to promote tissue engineering scaffold integration with the native tissue in a bovine cartilage explant model [146]. Investigating whether adsorption of HB-IGF-1 to the RADA16 scaffolds before or after peptide hydrogel assembly, all conditions sufficiently retained the fusion growth factor. Bearing in mind translational delivery, RADA16 peptide was mixed with HB-IGF-1 prior to assembly. This preadsorption stimulated increased sGAG and collagen deposition within the hydrogel, as well as in cartilage explants cultured adjacent to the hydrogels with HB-IGF-1, The use of RADA16 hydrogels with a fusion growth factor could successfully mediate scaffold-based repair and integration in cartilage tissue defects.

The Semino group combined RADA16 with various sulfated GAGs to create biomimetic scaffolds for cartilage tissue engineering [147–149]. Binding of heparin sodium salt enabled affinity driven binding with growth factors like vascular endothelial growth factor (VEGF), fibroblast growth factor β (FGF β), and TGF-β1. The heparinized RADA16 hydrogel was evaluated for its versatility, as a potential scaffold for both vasculogenesis and chondrogenesis [147]. This was particularly interesting as vasculogenesis is undesirable in tissue engineering of cartilage, a highly avascular tissue. In the presence of TGF-β1, the hydrogel drove chondrogenic differentiation of adipose-derived stem cells (ADSCs), while VEGF drove neovascularization by human umbilical vein endothelial cells (HUVECs). Interestingly, the heparinized RADA16 showed a higher affinity to VEGF but none to TGF-β1, which has otherwise shown affinity to sulfated GAGs [150]. Upregulation of chondrogenic genes for type II collagen and SOX9 was observed in the hydrogel containing a medium concentration of heparin (190/1), while aggrecan was slightly downregulated. Hypertrophic marker type X collagen was also downregulated in this concentration. Mechanical analysis of the constructs after 4 weeks of culture revealed the highest concentration of heparin resulted in the most robust gels, with the complex modulus approaching statistical equivalence to that of chicken articular cartilage. Thus, the versatility of the scaffold was shown, as well as putting an emphasis on the type of growth factors and cells the construct is exposed to, as variations can vastly alter the outcomes of the tissue engineered construct.

Recha-Sancho and Semino then further investigated RADA16 combined with GAGs chondroitin sulfate (CS) and decorin [149]. Once again, the GAG-RADA composite gels did not exhibit enhanced binding affinity to TGF-β1, despite sulfated GAGs being reported as having increased affinity for the growth factor. This might be due to the alternating charges of the RADA16 hydrogel that could interfere with the electrostatic affinity between sulfated GAGs and TGF-β1 [150,151]. RADA16 with CS showed the most robust upregulation of chondrogenic genes while suppressing hypertrophic expression in both encapsulated ADSCs and chondrocytes. Constructs with encapsulated ADSCs resulted in the most robust matrix deposition, regardless of the presence of GAGs, and were statistically equivalent to both chicken and calf articular cartilage. By contrast, embedded chondrocytes did not produce matrix that resulted in mechanical improvements in the construct, suggesting that ADSCs were a more appropriate cell source for cartilage tissue engineering using this scaffold versus adult chondrocytes. Still, the RADA-sGAG platforms might be useful platforms for *in vitro* culture, as the RADA-Heparin construct successfully promoted the re-expression of chondrogenic phenotypic markers by chondrocytes that had previously undergone dedifferentiation in monolayer culture [148]. These studies showed extreme contraction of the RADA16 hydrogels in chondrogenic conditions, leading the group to investigate hybrid scaffolds of poly(ε-caprolactone) (PCL) with RADA16 [152,153]. The addition of PCL maintained the shape and overall viscoelastic behavior of the constructs and prevented contraction. RADA16 alone supported more favorable chondrogenic gene expression by both chondrocytes and ADSCs over RADA-PCL, however, it was likely driven by cell–cell interactions that were promoted when the construct contracted.

Recruitment of endogenous stem cells has recently become more attractive in tissue engineering, as the use of encapsulated cells (particularly from allogenic sources) can have regulatory hurdles [154,155] and extraction of autologous stem cells can cause donor site morbidity, as well as necessitating further processing like *in vitro* cell expansion before use [156]. To that end, Lu *et al.* have recently developed a scaffold comprised of commercially available RADA16 fused to bone marrow homing peptide (BMHP) PFS (PFSSTKT) (Figure 5) [157]. This material was combined with acellular cartilage matrix (ACM) to form a composite scaffold. ACM was fabricated by decellularizing porcine femoral cartilage, pulverizing, and then reforming in cylindrical molds, onto which RADA-BMHP was adsorbed and allowed to form into a hydrogel. RADA-BMHP peptide resulted in a significant increase in endogenous stem cell recruitment, as well as upregulation of chondrogenic markers in a rabbit full-thickness defect model. The composite scaffold almost completely filled the full-thickness defect with bone and cartilage tissues, as imaged by micro-commuted tomography (μCT), however there were still some unrepaired zones by 6 months. This scaffold material holds remarkable promise for endogenous repair of osteochondral defects by enhancing local stem cell recruitment. Furthermore, the use of ACM likely provided pro-chondrogenic cues to the recruited cells, which obviates the need for additional drug or growth factor stimulation. A nearly identical study has been carried out using SKP (SKPPGTSS) as the MSC homing peptide with similar results [158]. Mechanical testing on the repaired tissue reveals that the BMHP-containing composite material achieved hardness, contact stiffness, and reduced modulus statistically equivalent to that of

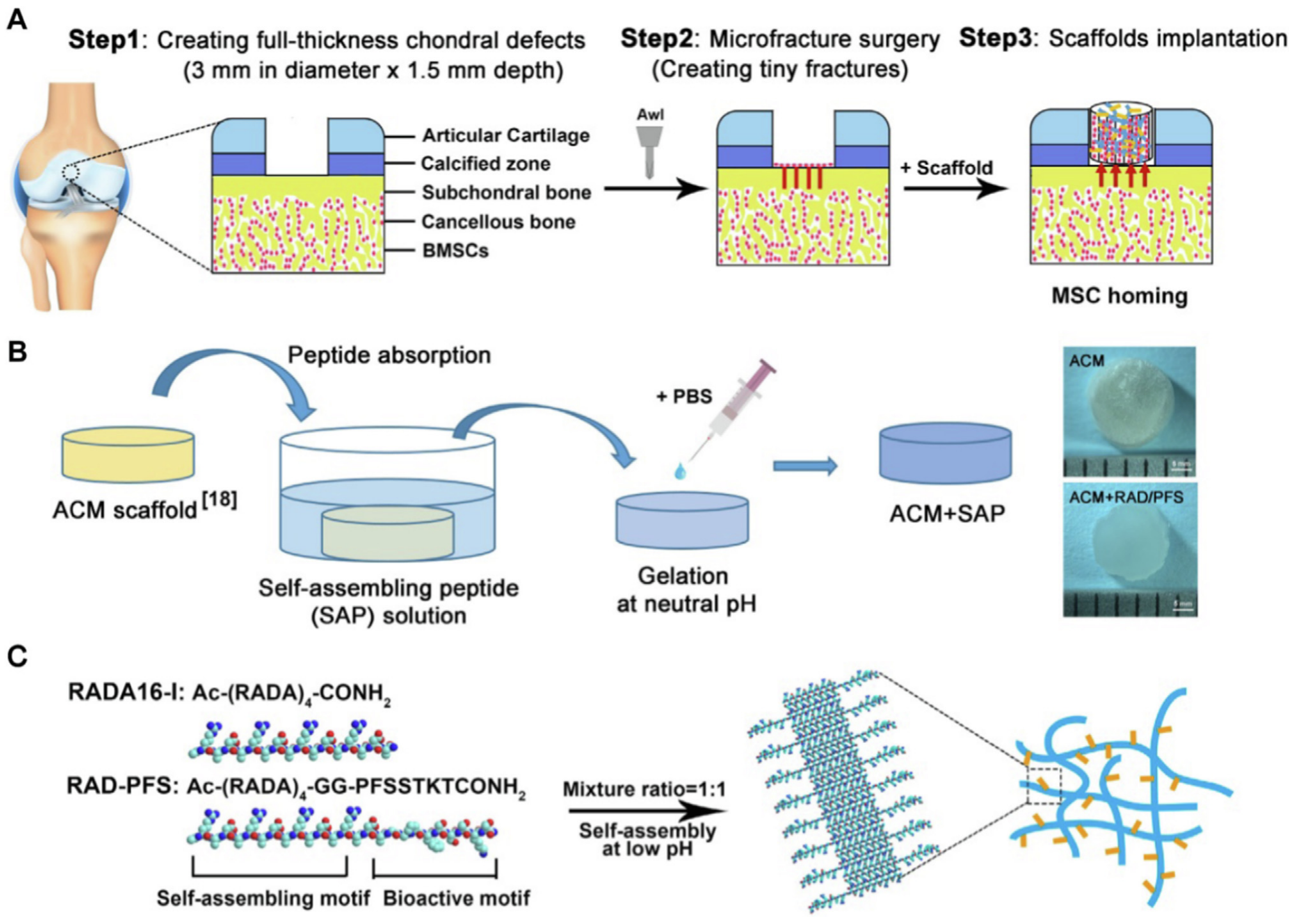

Fig. 5. Schematic illustration of self-assembling RADA16 hydrogels with bioactive cell homing motif (PFS) and acellular matrix (ACM) for scaffold guided endogenous repair of a full thickness osteochondral defect. A) Experimental design, B) composite hydrogel preparation, and C) functionalized self-assembling peptide RAD-PFS. Reproduced with permission from reference [157]. Copyright 2018, Ivyspring.

native cartilage. Histological staining also demonstrates that zonal cartilage is regenerated with superior tissue integration.

Another example of composite scaffolds using RADA16 was reported by Ishikawa *et al.*, who created a "one pot" *in situ* gelling interpenetrating network (IPN) system by combining RADA16 with carboxymethyl chitosan (CH) and N-hydroxysuccinimide ester-terminated PEG (NHS-PEG-NHS) [159]. Evaluation of gelation kinetics showed a multistep process, whereby RADA16 quickly self-assembled into a fibrillar network, while CH was slowly cross-linked by the reaction between its carboxyl groups and NHS-PEG to form a network within the RADA fibers via a slower gelation process. The combined IPN showed superior mechanical properties to its constituent components. The addition of RADA16 enhanced sGAG and collagen deposition by encapsulated chondrocytes over CH/PEG constructs alone, as well as when compared to a clinically utilized atelocollagen scaffold. This marked the first *in situ* and one-pot synthesis method of an IPN hydrogel, however, its application as an injectable solution was yet to be tested *in* or *ex vivo*. Regardless, the material might still be promising as *in vivo* histological measurements after pre-formed transplantation in a mouse model revealed that the IPN structure allowed for greater formation of matured cartilage.

3.3.5. COMPcc

Cartilage oligomeric matrix protein (COMP) is a ubiquitous protein found in a wide variety of tissue types including cartilage [160]. It is a critical component for maintaining matrix organization in cartilage and is implicated in the stability of the collagen fibrillar network within cartilage. COMP is composed of 4 domains: i) the N-terminal domain (NTD) that directs its pentamerization and stores and delivers hydrophobic signaling molecules; ii) the EGF-like domain that regulates interactions with MMPs and ADAMTSs; iii) the TSP-type III domain that activates the alternate compliment pathway; and iv) the C-terminal globular domain (CTD) that directly interacts with ECM molecules and growth factors [161]. The NTD of COMP forms a coiled-coil and is known as the COMPcc domain, capable of forming a left-handed homopentameric self-assembly known to bind both hydrophilic and hydrophobic small molecules [160,162]. This drug binding property has made COMPcc a promising vehicle for selective delivery of insoluble agents. Previous work has shown the successful drug delivery potential for all-transretinol, curcumin, vitamin D(3), vitamin A, and a series of different fatty acids by both COMPcc and its derivative C^{S}_{cc}, in which cysteine residues are replaced with serine to make modified coiled-coils [104,162]. Variants and block copolymers of COMPcc have further expanded the drug delivery potential through utilization of its self-assembly properties and that of its fusion domains. Variants of COMPcc have shown the ability of the domain to form a hydrogel due to redistribution of its electrostatic patches to create a upper critical solution temperature (UCST) behavior that is responsive to pH and its chemical environment [98,163,164]. COMPcc domains have further been

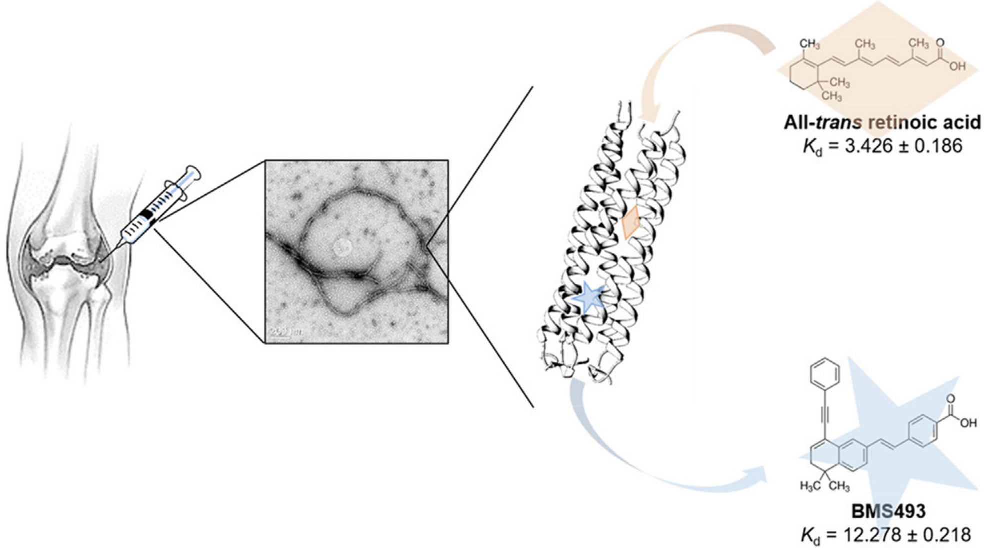

Fig. 6. Schematic representation of engineered COMP coiled coil (C^S_{cc}) in which all-trans retinoic acid (ATRA) displays ~ 5-fold superior association with C^S_{cc} than encapsulated therapeutic BMS493, thus acting as both a drug delivery mechanism and sequestration depot for ATRA. Reproduced with permission from reference [167]. Copyright 2018, American Chemical Society.

incorporated as a primary domain in block copolymers with ELPs to form hydrogels with LCST behavior and thermoresponsive micelles for drug delivery [165,166].

Yin *et al.* recorded the first use of the COMPcc derivative C^S_{cc} as a drug delivery vehicle in an osteoarthritis treatment setting by characterizing its ability to bind BMS493 ((I4-[2-[5,5-dimethyl-8-(2-phenylethynyl)-5,6-dihydronaphthalen-2-yl]ethen-1-yl]ben zoic acid) (Figure 6) [167]. All-trans-retinoic acid (ATRA) is a vitamin A derivative that can induce the hypertrophic phenotype and is signaled through retinoic acid receptors (RARs) and its subsequent complexation with rexinoid receptors (RXRs). BMS493 is an inverse agonist to all retinoic acid receptors and can inhibit this RAR-RXR complex, therefore a potential therapeutic for suppressing matrix catabolism in OA. However, a proper drug delivery vehicle for BMS493 is essential since the molecule is hydrophobic, light sensitive, and readily degrades. Chondrocytes have been stimulated with IL-1β and ATRA *in vitro* to stimulate catabolism, including the production of MMP-13. The C^S_{cc}-BMS493 complex revealed 22% reduction in MMP-13 mRNA when stimulated by IL-1β and even greater downregulation in response to ATRA and IL-1β (63% and 64% respectively). BMS493 alone demonstrated a 53% reduction in MMP-13 mRNA when stimulated by both ATRA and IL-1β but had no reduction when stimulated by either individually. This study of C^S_{cc} as an effective drug delivery vehicle was limited to *in vitro* RT-PCR quantification, and its efficacy as a delivery vehicle for BMS493 *in vivo* has yet to be investigated. The stabilization and release of BMS493 was sustained *in vitro* over the course of 3 days of treatment, however, it is not clear if this is a therapeutically relevant time course for OA therapy and whether multiple administrations of the drug delivery complex would need to be delivered. Additionally, its ability to resist clearance from the articular space must be investigated.

The Montclare group has also made a block copolymer consisting of COMPcc and elastin-like polypeptide repeats (E) [95]. The order of the block copolymers (EC or CE) determines the mechanical behavior of the material; EC assembles into a viscoelastic material that transitions to elastic behavior above a T_t while CE remains mainly viscous fluid above and below T_t. The number of E repeats exhibits an inverse relationship with T_t, with T_t decreasing with increasing E domain length. Katyal *et al.* have recently used this EC block copolymer with 5 E repeats (E_5C) as a delivery mechanism for sustained release of a novel anti-inflammatory treatment for PTOA [101]. Atsttrin (Antagonist of TNF/TNFR Signaling via Targeting to TNF Receptors) is an engineered protein derivative of progranulin (PGRN), a growth factor that has multiple functions including stimulating chondrocyte proliferation and binding TNF receptors to block TNF-α [168,169]. Intra-articular injections of Atsttrin, like other active molecules against OA, have shown rapid diffusion and low retention in the joint. E_5C is capable of binding small molecules through the C domain and self-assembles *via* the E domain to form an elastic gel shown to undergo gelation at physiological conditions making it suitable for *in situ* gelation. E_5C demonstrates a sustained release of Atsttrin *in vitro* and the combination of E_5C and Atsttrin (E_5C-Atsttrin) upregulates type II collagen and aggrecan in chondrocytes *in vitro* over Atsttrin alone. When challenged with TNF-α, E_5C-Atsttrin downregulates catabolic genes for ADAMTS4, NOS2, and MMP13. In an *in vivo* rabbit anterior cruciate ligament (ACL) transection model of PTOA [170], E_5C-Atsttrin shows better prevention of PTOA via MRI imaging and macroscopic scoring, as well as overall histological scoring. In addition, when delivered after PTOA is allowed to develop for 8 weeks in the ACL transection model, E_5C-Atsttrin reveals protection from further degenerative progression. This data proves promising for the combined delivery mechanism and therapeutic. Claims that E_5C may provide lubrication to the injured joint to further prevent fibrillation and degenerative changes, as well as the potential for the E_5C hydrogel to support chondrocytes as a potential scaffold material, have yet to be explored.

3.3.6. Resilin

Resilin is another naturally inspired peptide-engineered material, derived from the cuticles of insects. It is a natural elastomer, known for its resilience and energy storing capabilities [171]. Renner *et al.* have investigated the potential for resilin-like materials as scaffolds for cartilage tissue engineering, with a particular focus on mechanical properties. RZ10 has been fabricated using 10 repeats of the resilin motif derived from the mosquito *Anopheles gambiae*, AQTPSSQYGAP, coupled with bioactive cell-binding domain RGD

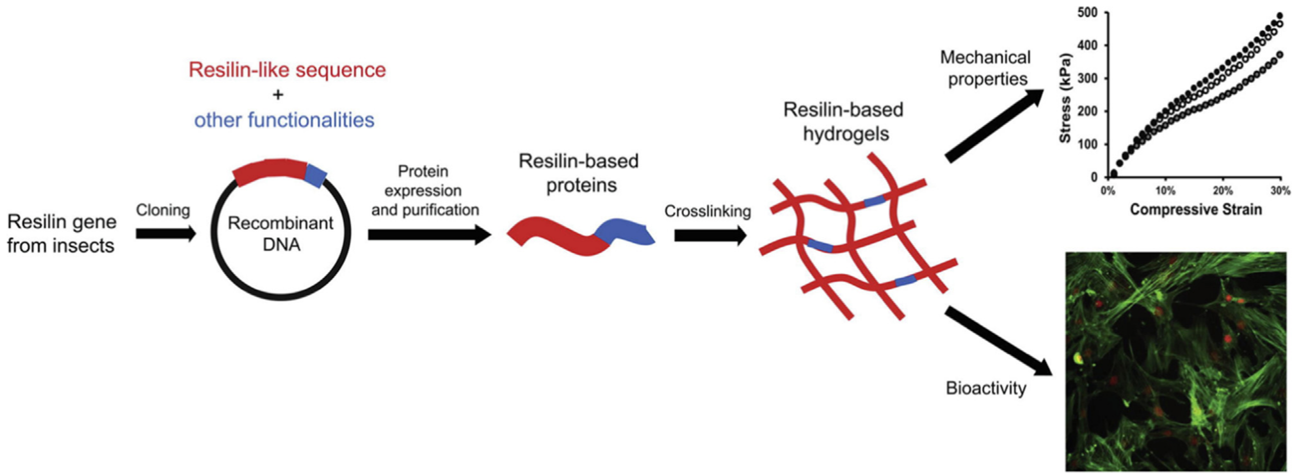

Fig. 7. Fabrication and characterization of resilin based material RZ10 as a potential cartilage tissue engineering scaffold. Reproduced with permission from reference [171]. Copyright 2014, American Chemical Society

or a scrambled version, RDG (Figure 7) [172]. Resilin-based scaffolds were formed at 37 °C and crosslinking with tris(hydroxymethyl)phosphine (THP). Gels were mechanically characterized via rheometry and unconfined compression specifically due to the relevance of these measurements to cartilage function. Gelation occurred within 6 minutes and reached a complex modulus of 22 kPa, which is within the range of similar elastin-based proteins (2–45.8 kPa), but 1–2 orders of magnitude lower than articular cartilage (180–2500 kPa). The average unconfined compressive modulus was 2.4 MPa, at least an order of magnitude greater than elastin-based proteins (0.00626–0.215 MPa) and within the lower range of articular cartilage (2.25–15.3 MPa). It is worth noting, however, that while initial material properties approaching or matching those of native cartilage are useful for providing immediate mechanical support to the injured tissue, substrates of this high stiffness are not conducive to chondrogenic differentiation [173,174]. MSCs seeded on 2D films of Resilin-RGD spread quickly and adopted a fibroblastic morphology, showing a strong affinity for the material, however, this study did not investigate the ability of the resilin biomaterial to support chondrogenesis.

3.3.7. Collagen mimetics

As collagen is the major structural component of most tissues, including cartilage, it is an obvious material choice for tissue engineering applications. Naturally derived collagens, however, carry with them a risk of immunogenicity. Collagen mimetic peptides (CMPs) are composed of the most abundant sequence in natural collagens, -(Pro-Hyp-Gly)$_7$-, and form collagen-like triple helices. They also have been shown to spontaneously associate with natural collagen fibers – a feature that is useful for tissue integration [175]. Lee *et al.* have investigated a peptide-polymer hybrid scaffold composed of CMP conjugated with poly(ethylene oxide) diacrylate (PEODA) and its ability to support chondrogenesis [176,177]. Encapsulated chondrocytes expressed an 87% increase in glycosaminoglycan production and 103% increase in collagen compared to those cultured in PEODA gels alone [176]. The ability of the CMP to provide binding sites with collagen enhanced hydrogel crosslinking in a manner that could be manipulated by the cells. These results have been recapitulated by CMP-PEODA gels used to encapsulate MSCs [177]. CMP-PEODA hydrogels supported chondrogenesis of encapsulated MSCs over those encapsulated in PEODA alone. Interestingly, the CMP-PEODA hydrogels suppressed elaboration of hypertrophic marker type X collagen (~30% expression), which was expressed in nearly 90% of cells encapsulated in PEODA alone. The water content of the gels (indicative of hydrogel swelling) was not significantly different, suggesting that the differences in biochemical response from the cells was not due to a difference in porosity of the hydrogels (although this was not explicitly calculated in the report). Kim *et al.* have developed a peptide block copolymer using CMP7 fused with KLD12 (Ac-KLDLKLDLKLDLGG**POGPOGPOGPOGPOGPOGPOG**-NH2) [178]. To provide structural stability, KLD12-CMP7 peptides were mixed with poly(L-lactide-co-caprolactone) (PLCL). Rabbit BMSCs were encapsulated within the gels and cultured for 2 weeks *in vitro*, after which they were implanted into subcutaneous pouches of athymic mice to investigate *in situ* chondrogenesis within the peptide-polymer constructs. Like previous reports, the CMP-containing construct facilitated the upregulation of chondrogenic markers type II collagen and aggrecan over the scaffold without CMP, as well as significantly downregulating the expression of type X collagen. It should be noted that the studies mentioned here necessitated the combination of CMP with a synthetic polymer to provide mechanical support that was not achieved by CMP alone.

Recent attempts have been made using prokaryotic and bacterial collagen-like proteins as collagen mimetics for tissue engineering applications [179]. Like eukaryotic collagen, these sequences feature Gly-X-Y repeats with the required glycine every third residue to form the triple helical coils. Instead of hydroxyproline, which bacteria cannot produce, these collagen-like peptides contain polar or charged residues in the X and Y positions. While recombinant human collagens require extensive post-translational modification in order to form a triple-helical structure, bacterial collagens have shown to form stable triple helices at physiological temperatures without needing further modifications [180]. Parmar *et al.* have investigated the bacterially derived *Streptococcal* collagen-like 2 protein (Scl2) for cartilage regeneration by combining with hyaluronic acid (HA)-binding or chondroitin sulfate (CS)-binding peptides and MMP7 sensitive crosslinking [106,181,182]. Scl2 was synthesized to incorporate an enzyme cleavage and spacer sequence (LVPRGSP), HA-binding sequence (RYPISRPRKR), Heparin (H)-binding (GRPGKRGKQGQK) and integrin (I)-binding (GERGFPGERGVE) sequences within the triple helical structure. MMP7 (PLELRA) or ADAMTS4-derived aggrecan-cleavable (RDTEGEARGSVIDR) peptides were used to crosslink the Scl2 protein via thiol reactions to form a hydrogel encapsulating hMSCs [182]. Storage modulus of the resultant acel-

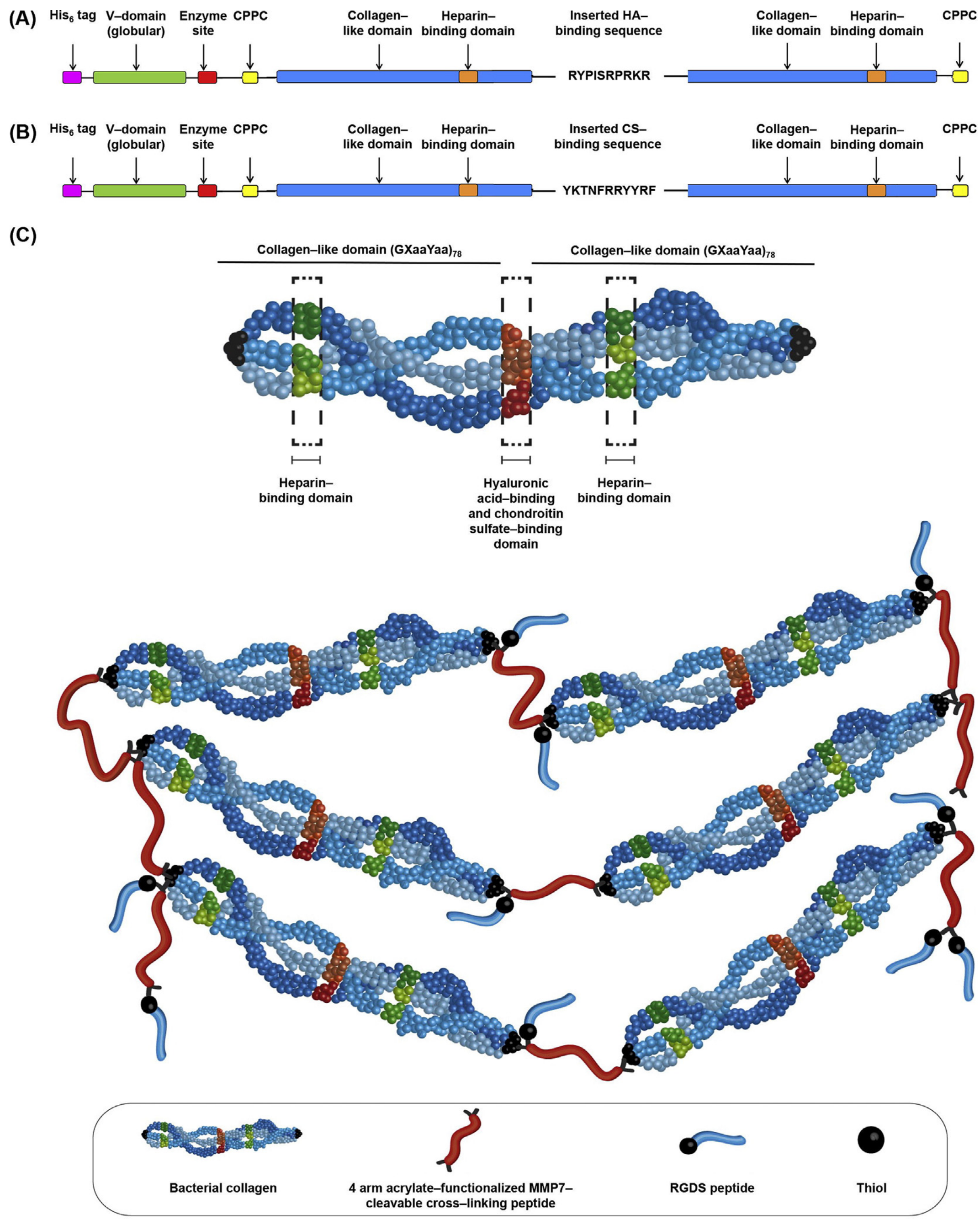

Fig. 8. Schematic diagrams of peptide-functionalized Scl2 proteins and resulting hydrogels. Scl2 protein containing (A) HA-binding and (B) CS-binding sequences. "CPPC" domains inserted at N and C termini to aid the stability of the construct. C) Generalized structure of the peptide-functionalized Scl2 proteins and hydrogels. Reproduced under open access license from reference [106]. Copyright 2017, P.A. Parmar, J.P. St-Pierre, L.W. Chow, C.D. Spicer, V. Stoichevska, Y.Y. Peng, J.A. Werkmeister, J.A.M. Ramshaw, M.M. Stevens.

lular hydrogels was ~7 kPa, and unconfined compressive modulus was 4–5 kPa, with the hydrogels exhibiting characteristic viscoelastic behavior of increasing compressive modulus with increasing frequency of the dynamic mechanical analysis. MMP7 and aggrecan-cleavable crosslinks at 25:75 and 50:50 ratios had significant upregulation of deposition of chondrogenic matrix. Most notably, the constructs maintained their mechanical integrity over 6 weeks, indicative of temporal matching between scaffold degradation and neomatrix deposition. They then tethered the cell-adhesive peptide RGD via MMP7 sensitive link, whereby cell attachment to the scaffold could also be temporally controlled, resulting in greater chondrogenic expression by encapsulated hMSCs (Figure 8). Thus far these studies have been conducted *in vitro;* future investigation in *in vivo* or *ex vivo* systems will elucidate whether this protein-engineered system promotes local cell infiltration and tissue integration, and whether the mechanics of the scaffold are maintained, particularly in a more degradative milieu such as the injured and arthritic joint.

3.3.8. Cartilage penetrating proteins

As discussed in previous sections, the success of therapeutic molecules and DMOADs has largely been limited by their delivery mechanism. Intraarticular injection aimed to circumvent the limitations of systemic drug delivery by delivering therapeutic drugs directly to the injured joint. While this can address synovial inflammation, such as the application of steroidal injections, DMOADs that seek to halt or reverse the degenerative progression of OA must target the chondrocytes that are embedded deep within the cartilage matrix. Supercharged green fluorescent proteins (S-GFPs) have been engineered by substituting surface amino acids that do not affect protein folding with charged amino acids to generate proteins with net theoretical charges ranging from + 9 to + 36. Additionally, S-GFPs can be engineered to have a net zero charge, by balancing positive and negative focal charges. The hydrodynamic diameter of these particles ($\sim$5 nm) was within the limit for cartilage penetration (10 nm) [68]. Uptake of cationic S-GFPs was found to increase with decreasing cationic charge, indicating higher charge does not necessarily correlate with better penetration, a feature that has been corroborated in studies using other charged delivery techniques [8,183,184]. These S-GFPs were also found to be phagocytosed by chondrocytes both in isolated culture and within cartilage explant culture, a promising result for future drug delivery work. Net-zero charged Janus S-GFPs (one large positive and one large negative patch as opposed to multiple small patches evenly distributed) showed a moderate increase in uptake vs other neutral S-GFPs tested (uptake ratio of 10 vs 1), suggesting that the positive side may enable interaction with anionic GAGs while still maintaining a net negative charge, increasing the available options for drug carriers. Further study is still required to determine whether conjugation of these S-GFP carriers to potential DMOADs will maintain the observed level of penetration into cartilage and uptake by chondrocytes, as well as the long-term bioactivity of these drugs with increased residence time within cartilage. These results are a promising start for the development of other supercharged proteins, particularly those where secondary and tertiary structure may contribute to bulk cartilage repair while also successfully delivering a therapeutic payload. In addition to these S-GFPs, a number of charged and cartilage targeting short peptide sequences (e.g. Arginine-rich peptides, Collagen II binding peptide WYRGRL) have recently been under investigation for drug delivery (reviewed elsewhere: [185]). These peptides could be incorporated into protein-engineered hydrogel systems like those discussed here, in order to combine tissue level repair scaffolding with molecular/cell-level targeting for disease modification.

4. Discussion and conclusions

Articular cartilage injury and degeneration in OA continue to be one of the leading causes of disability globally, and though copious amounts of research have been conducted into understanding the etiology of the disease and searching for preventative and restorative therapeutics, the clinical standard remains pharmacological management of pain until the total joint needs to be replaced. Regenerative surgical techniques such as ACI or microfracture result in fibrocartilage that is mechanically weaker than hyaline cartilage and lacks the complex organization that allows articular cartilage to function as a low-friction, load bearing tissue [4]. The next generation of therapeutics for OA and cartilage injury will be regenerative in nature, both to biochemically halt the progression of cartilage degeneration and to potentially rebuild damaged tissue. To that end, biomaterial design is an equal partner with therapeutic discovery; delivery of DMOADs to arthritic cartilage requires biomaterials that protect the therapeutic agent, target the damaged tissue, and maintain therapeutic load once it reaches its target. Absent these factors, potential DMOADs largely fail to make a major impact on OA joints. Similarly, regenerative approaches to cartilage repair, particularly those that aim to address larger focal defects, need a material to not only provide scaffolding for repopulating cells but also to provide mechanical support or lubrication, without which the articulating joint continues to accrue damage. Rational design of peptide sequences to direct intramolecular interactions and protein folding present nearly unlimited potential. Using nature as a template, naturally inspired sequences can be fabricated to mimic or modify natural structures (ELPs, SLPs, COMPcc, resilin, CMPs, S-GFPs). At the same time, completely de novo proteins and peptides can be fashioned using rational design (KLD12, RADA16).

RADA16, being one of the first engineered peptide materials developed, has naturally seen the most utility in cartilage engineering and therapeutic delivery [186]. This utility has been enhanced by its commercial availability. Whereas synthetic biology techniques are becoming more widely accessible, there is still a learning curve amongst researchers as to the design and purification of these polypeptides. Thus, having a readily available peptide-based material that self-assembles makes its investigation as a biomaterial for cartilage repair much more accessible. RADA16 also has incredible cell-adhesive abilities, having shown that the RAD sequence can promote cell attachment in a manner much like the RGD adhesive ligand, making it a useful addition for composite structures that otherwise do not directly interface with cells. Whereas commercial availability of a simple sequence like RADA16 enables its wide use and exploration of its potential applications, highly specified genetically engineered protein materials hold prospects for development of proprietary technologies. This, however, will likely be met with regulatory challenges as *de novo* protein development may not have the benefit of prior art to establish safety and efficacy profiles. Still, the bottom-up approach of protein engineering design can utilize tools like neural networks to predict material behaviors and guide research directions [120].

Making use of synthetic biology techniques, protein sequences can be modified to create a wide library of material properties and gelation behaviors, as we have seen by the fine control afforded to ELPs. Modifying a single guest residue or the number and order of the repeating pentapeptide units can tune the material stiffness and thermoresponsive transition temperature. This feature has been exploited to develop even more specialized protein biomaterials like SELPs and E_nC, which again can be tuned by the ratio and order of ELP units to other peptide components. To this extent, recombinant protein biomaterials provide a large well untapped by the near infinite combinations of mutations that can manipulate structure and thus allow for functional control. In the case of COMPcc-based proteins, a dual mutant (C68S and C71S) facilitates binding to pan-RAR inverse agonist, BMS493, for inhibition of the hypertrophy inducing RAR-RXR [104,167]. However, other mutations to the COMPcc domain for binding other hydrophobic small molecules can be explored further. For example, binding affinity of the C^S_{cc} variant, T40A, improves the affinity to the therapeutic curcumin, while the L44A variant abolishes small molecule binding [187]. Swapping of the domain regions around the center Q54 residue in C^S_{cc} results in nanofibers up to 560 nm and mesoscale fibers up to 16 μm upon curcumin binding implying that major recombinant techniques could affect even greater structural and thus functional changes to a potential COMPcc derived vehicle for OA therapy [164]. Upon high concentration and low temperatures, these COMPcc derived variants also have shown upper critical solution temperature (UCST) behavior forming a hydrogel below T_t and indicating that recombinant engineering can impact the vehicle and delivery properties that can be used for a OA therapeutic [163].

One of the main critiques of protein-engineered materials is their perceived low mechanical robustness in comparison to traditional hydrogel biomaterials. This can be addressed by incorporating chemical crosslinking techniques, as well as further utilizing rational design to build stronger or stiffer structures. Indeed, genipin crosslinking is one of the most commonly used chemical crosslinking methods for protein-based biomaterials, however genepin can cause cytotoxicity, in addition to preventing some of the viscoelastic behavior inherent to physically crosslinked and folded proteins that make them an attractive material source for biomedical applications [188]. Among the studies presented here, very few explored fine tuning the mechanics of the material being used. The incorporation of Q and K residues into ELPs and use of tTG as an enzymatic crosslinker already found in the body is one such method of increasing mechanical properties without needing to utilize commonly used chemical crosslinkers that carry the risk of cytotoxicity [117]. Pre-annealing of SELPs to initiate self-assembly prior to injection is another creative means of enhancing material mechanics, particularly where the time course of gelation is critical to the clinical function of the material [128]. Because the secondary and tertiary structures of proteins are finely tuned by their sequence in nature, the combinations of mechanical modifications that can be made to protein-engineered materials using rational design are virtually infinite, influencing the helicity, pleating, and packing of the amino acid sequence with both canonical and non-canonical residues. Forces applied to proteins can be measured at the molecular level using atomic force microscopy (AFM) and correlated to their bulk properties [92,189]. Huerta-López *et al.* have recently put forward a thorough review of mechanisms for tuning protein-engineered material mechanics [23].

Most studies reviewed here characterized materials using rheology to describe the transition from viscous solution to viscoelastic solid during hydrogel formation, however mature cartilage undergoes several forces, including compression, shear, and tension, which most materials were not optimized for [190]. As shown in Table 2, very few of the tissue repair studies presented reported any mechanical analysis of the material studied and varied from rheometric analysis of gelation to compressive mechanics (either before or after cell matrix elaboration), but there is clearly a paucity of standard benchmarks being held. Resilin RZ10 is an example of an engineered protein material discussed here that was characterized extensively for its mechanical behavior though minimally for its proposed application as a scaffold for cartilage [172,191]. As resilin is an elastomeric protein known for its unique mechanical properties, it makes sense that it would be investigated as a potential biomaterial for mechanical tissue support. A critical challenge to the issue of cartilage regeneration is that the tissue and organ level mechanical needs for support are not conducive to matrix synthesis at the cellular level, as numerous studies have shown chondrogenesis is most successful on soft, permissive substrates on the order of Pa [51,88,192–194] while gross compressive forces on articular cartilage can be as high as 20 MPa [195]. This, however, is one area where protein engineered materials have a unique ability to address the gap: by virtue of being able to make up a variety of supramolecular assemblies and form reversible bonds, engineered proteins are inherently viscoelastic, and thus can be designed to functionally adapt, not only to environmental stimuli, but cellular and tissue level stimuli as well [23,189,196]. Engineering of protein-based materials has typically taken a bottom-up approach, focusing on the molecular interactions and assembly of the materials. A repeated "return to the drawing board" may be necessary to optimize the material for its specific intended function. We propose a cyclical design strategy, whereby the bottom-up design of novel protein structures is informed and reconfigured by its application.

An excellent example of iterative protein-engineered material design is the work done by Parmar and colleagues on collagen-mimetic peptide Scl2 [106,181,182]. They repeatedly iterated the incorporation of GAG-binding motifs, integrin binding motifs, and MMP-sensitive crosslinking. Employing MMP-sensitive crosslinking to temporally control the interactions of cells with the material, they used bottom-up principles to engineer their material and then top-down engineering principles to re-evaluate and optimize for the specific application.

In this review we have focused on repair and regeneration of hyaline cartilage, however joint injury and OA involve multiple tissues. Relevant work that was not covered here include protein engineered materials and therapeutic delivery vehicles for bone defects and intervertebral disc degeneration [197,198]. The highly customizable nature of protein engineered materials can benefit from combining therapeutic approaches from those and other related applications to develop next generation customized tissue repair systems.

Funding

Authors acknowledge support from the Department of Defense research grant W81XWH-16-1-0482 (JKM) and National Science Foundation Designing Materials to Revolutionize and Engineer our Future grant 1728858 (JKM) and New York University's Provost's Postdoctoral Fellowship (NAHS).

Data availability

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

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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