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Collagen-mimetic peptide-modifiable hydrogels for articular cartilage regeneration

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ABSTRACT

Regenerative medicine strategies for restoring articular cartilage face significant challenges to recreate the complex and dynamic biochemical and biomechanical functions of native tissues. As an approach to recapitulate the complexity of the extracellular matrix, collagen-mimetic proteins offer a modular template to incorporate bioactive and biodegradable moieties into a single construct. We modified a Streptococcal collagen-like 2 protein with hyaluronic acid (HA) or chondroitin sulfate (CS)-binding peptides and then cross-linked with a matrix metalloproteinase 7 (MMP7)-sensitive peptide to form biodegradable hydrogels. Human mesenchymal stem cells (hMSCs) encapsulated in these hydrogels exhibited improved viability and significantly enhanced chondrogenic differentiation compared to controls that were not functionalized with glycosaminoglycan-binding peptides. Hydrogels functionalized with CS-binding peptides also led to significantly higher MMP7 gene expression and activity while the HA-binding peptides significantly increased chondrogenic differentiation of the hMSCs. Our results highlight the potential of this novel biomaterial to modulate cell-mediated processes and create functional tissue engineered constructs for regenerative medicine applications.

1. Introduction

Articular cartilage is a complex connective tissue covering the surfaces of bones in synovial joints [1] that enables low-friction articulation and helps transmit and distribute forces to the subchondral bone [2]. It is characterized by a depth-dependent zonal organization and biochemical microenvironment that is vital for its multiple functions [3]. The avascular and aneural nature of articular cartilage contribute to its limited capacity for self-repair following trauma or disease. Current clinical treatments to induce articular cartilage tissue repair, such as autologous chondrocyte implantation, mosaicplasty, and microfracture, often provide short-term pain relief and recovered joint mobility, but the long-term benefits remain elusive. The resulting repaired tissue does not exhibit the same biomechanical behavior as that of native articular cartilage and eventually breaks down, requiring additional treatment such as total joint arthroplasty [1,3,4]. As a result, research into the development of bioengineered constructs, with the aim to provide an adequate cellular environment to favor the regeneration of damaged or diseased articular cartilage, has gained momentum in recent years [5–10]. The ability to engineer tissues that mimic the complex native articular cartilage composition and architecture holds great promise towards restoring the unique biomechanical behavior and function necessary for long-term success.

Strategies for cartilage tissue engineering typically use a combination of biomolecules and biomaterials to provide the appropriate signaling and support for specific cell types [5–9,11]. Hydrogels are three-dimensional (3D) water-based matrices that can be engineered to incorporate bioactive cues and be

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biodegradable [12,13], thereby providing a pericellular microenvironment reminiscent of the native tissue for encapsulated cells [14]. Poly(ethylene glycol) (PEG) [12–16] hydrogels have been used extensively for cartilage tissue engineering applications because of their bio-inert nature and versatility. Bioactivity has been introduced into this system through the inclusion of growth factors such as transforming growth factor β3 (TGF-β3) [11] and peptides [10,17–19] to markedly enhance the functional outcome. Hydrogels derived from hyaluronic acid (HA) [17] and collagen [18] have also been used for cartilage tissue engineering applications because they are commonly found in native articular cartilage extracellular matrix (ECM). These hydrogels can be degraded *in vivo* but are typically cross-linked with non-degradable cross-linkers that can affect their degradation behavior [12,14,15,17]. Biodegradable hydrogels are an attractive approach for cartilage tissue engineering because they offer a temporary support structure for encapsulated cells but can be degraded in conjunction with the deposition of neo-cartilaginous matrix [15,20,21]. Ideally, a hydrogel would degrade at a rate that allows it to provide adequate support to cells without restricting the matrix deposition and remodeling [17].

The incorporation of hydrolytically or enzymatically cleavable substrates are the most common approaches used to impart biodegradability within hydrogel systems [20]. A major drawback of hydrolytically degradable hydrogels is the lack of control over degradation kinetics since the degradation mechanism is not specific. Enzymatically cleavable sites take advantage of cell-mediated processes that can naturally break down the construct, allowing for improved spatiotemporal control of cell migration, degradation, and matrix deposition. Specific enzyme-sensitive peptides, for example, can be included in the hydrogel network to enable localized cell-induced degradation. Matrix metalloproteinases (MMPs) are often targeted as the route of enzymatic degradation since they are known to be involved in the cleavage of ECM components during native tissue remodeling [13,15,20,22–26]. In articular cartilage, MMPs are involved in the turnover of several matrix components including collagen type II and aggrecan, which aid in cell tissue development and remodeling [13,15]. MMP7 is thought to play a role in chondrogenesis by controlling the bioavailability of chondrogenic factors and facilitating collagen type II maturation [20]. Recently, MMP7-cleavable peptide substrates (MMP7) were developed and incorporated into PEG hydrogels and shown to degrade via MMP7 secreted by encapsulated human mesenchymal stem cells (hMSCs) during chondrogenesis [20].

Here, we have developed MMP7-degradable hydrogels based on recombinant Streptococcal collagen-like 2 (Scl2) proteins and functionalized with glycosaminoglycan (GAG)-binding peptides. Scl2 proteins contain the characteristic repeating $(Gly\text{-}Xaa\text{-}Yaa)_n$ residues that assemble into the triple helical conformation found in mammalian collagens. However, unlike mammalian collagens, these proteins inherently lack cell-binding sites and thereby provide a structurally sound biological blank slate by which to systematically integrate specific motifs for a desired cellular response [27–32]. In addition, Scl2 proteins are produced recombinantly with minimal batch-to-batch variation in predictability of performance, purity, and quality [27–31]. Previously, Scl2 proteins with integrin-binding sequences have been used in PEG-based hydrogels to bind endothelial and smooth muscle cells for vascular grafts [29]. Here, a blank slate Scl2 protein was cross-linked with the MMP7-sensitive peptide to form hydrogels and functionalized with peptides that bind hyaluronic acid (HA) and chondroitin sulfate (CS) (Fig. 1). Our group recently showed that the HA-binding (HAbind) and CS-binding (CSbind) peptides can specifically and dynamically bind HA and CS, respectively, mimicking native ECM-like interactions [10]. In the current work, we investigated how GAG-binding peptides affect the chondrogenic differentiation of hMSCs encapsulated within the hydrogels that could then be implanted to treat focal defects, MMP7 gene expression and activity. These hydrogels also offer the potential for a less invasive, injectable approach to stimulate chondrogenesis in host MSCs. Moreover, these versatile hydrogels have the potential to incorporate multiple and different peptides to specifically tune bioactivity and biodegradability, that can be tethered from the protein backbone to mimic the complexity of native ECM.

2. Materials and methods

2.1. Materials

All chemicals were used as provided by the manufacturers. Poly(ethylene glycol)-acrylate-*N*-hydroxysuccinimide (2000 g/mol) (PEG-acrylate-NHS) was purchased from JenKem Technology (Allen, Texas, USA). Rink amide resin, Fmoc-protected amino acids, *N*, *N* dimethyl formamide (DMF), dichloromethane (DCM), 20% (v/v) piperidine in DMF, O-benzotriazole-*N*,*N*,*N*′*N*′-tetramethyluronium-hexafluoro-phosphate (HBTU), and diisopropylethylamine (DIEA) were purchased from AGTC Bioproducts (Hessle, UK). MMP7 fluorogenic substrate was purchased from Merck Millipore (Nottingham, UK). All other chemicals were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Recombinant Scl2 protein was expressed in *Escherichia coli* BL21-DE3 and purified as previously described [30]. Recombinant Scl2 protein was dialyzed against phosphate-buffered saline (PBS), pH 7.4, and protein concentration was determined using a calculated extinction coefficient.

2.2. Peptide synthesis and purification

The HAbind (CGGGRYPISRPRKR), CSbind (CGGGYKTNFRRYYRF), MMP7 (CGGGPLELRAGGGC), and scrambled MMP7 (ScrMMP7; CGGGPALLREGGGC) peptides were synthesized manually on a 2 mmol scale using standard Fmoc solid phase peptide synthesis techniques as previously described [10]. For each coupling, the Fmoc protecting group was removed with 20% (v/v) piperidine in DMF followed by washing with DCM and DMF. Amino acids were activated by adding 4 molar equivalent of each Fmoc protected amino acid to 3.95 molar equivalent of HBTU and dissolved in DMF. Six molar equivalent of DIEA was added to the amino acid solution and the coupling solution added to the resin. The coupling reaction was allowed to proceed for two to three hours before the resin was washed in DCM and DMF. Ninhydrin tests were performed after each Fmoc deprotection and coupling step to monitor the presence of free amines. Once the synthesis was completed, the peptides were cleaved in 95% (v/v) trifluoroacetic acid (TFA), 2.5% (v/v) triisopropyl silane (TIS), and 2.5% (v/v) H_2O for four hours. TFA was removed using rotary evaporation, and the peptide residue was precipitated and washed with cold diethyl ether (DEE) by centrifugation. The peptide precipitate was then allowed to dry under vacuum to remove residual ether. The peptide was purified (Fig. S1) using reversed phase preparative high performance liquid chromatography (HPLC; Shimadzu) in an acetonitrile/water gradient under acidic conditions on a Phenomenex C18 Gemini NX column (5 μm pore size, 110 Å particle size, 150 × 21.2 mm). Following purification, the peptide was lyophilized on a freeze dryer (Labconco) for storage prior to use. The purified peptide mass was verified by matrix assisted laser desorption spectroscopy (MALDI; Waters).

A cyclic RGDS peptide (GRGDSC) was synthesized at a 1 mmol scale on a 2-chlorotrityl chloride resin (100–200 mesh; VWR). Fmoc-Cys(Trt)-OH (Novabiochem) was dissolved at 1 molar equivalent in DCM with a small amount of DMF added until fully solubilized. Half the solution was added to the resin along with 500 μL of DIEA for 15 min shaking on a wrist action shaker. This was repeated with the remaining solution followed by thorough washing with DMF and DCM. A ninhydrin test was performed to monitor the coupling by detecting the presence of free amines. The remaining free amines were capped by adding a solution of 5% (v/v) acetic anhydride (Sigma) with 2.5% (v/v) DIEA in DMF for 10 min with shaking, and repeated with 5 min shaking using fresh solution. The resin was then washed thoroughly with DCM and DMF before the ninhydrin test. The Fmoc protecting group was removed as described above and Fmoc-Asp(Otbu)-Ser(psiMe, Mepro)-OH (Merck) was coupled at 2 molar equivalents with 1.95 molar equivalents of HBTU and 3 molar equivalents of DIEA in DMF. The remaining free amines were capped, and all other amino acids were coupled as described above. The protected peptide was cleaved from the resin by adding 10 mL of 5% (v/v) TFA in DCM for 10 min with shaking. The solution was drained into a round bottom flask and the resin rinsed with DCM until the solution in the synthesis vessel was clear. The DCM and TFA were removed carefully by rotary evaporation, leaving approximately 40 mL of solution to avoid cleaving the protecting groups from the peptide. Ammonium hydroxide (10 mL) was added to neutralize the TFA followed by acetonitrile to increase peptide solubility. The protected peptide (Fig. S2 and S3) was purified by reversed phase preparative HPLC running a mobile phase gradient of 80% ultrapure H_2O and 20% (v/v) ACN to 100% (v/v) ACN with 0.1% (v/v) TFA. The solvent was removed by rotary evaporation until the protected peptide was completely dry, and then re-dissolved in DMF at 1 mg/mL. The peptide was cyclized by adding 2 equivalents of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop; AGTC Bioproducts) and 3 equivalents of DIEA overnight. The DMF was removed by rotary