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Design of Cell-Matrix Interactions in Hyaluronic Acid Hydrogel Scaffolds

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Abstract

The design of hyaluronic acid-based hydrogel scaffolds to elicit highly controlled and tunable cell response and behavior is a major field of interest in developing tissue engineering and regenerative medicine applications. This review will begin with an overview of the biological context of hyaluronic acid, knowledge needed to better understand how to engineer cell-matrix interactions in the scaffolds via the incorporation of different types of signals in order to direct and control cell behavior. Specifically, recent methods of incorporating various bioactive, mechanical, and spatial signals are reviewed, as well as novel hyaluronic acid modifications and crosslinking schemes with a focus on specificity.

Introduction

Hyaluronic acid (HA), a naturally occurring glycosaminoglycan (GAG) and one of the primary components of the extracellular matrix (ECM), is increasingly being utilized in biomedical applications due to both its ability to serve as a blank slate and its biological activity. In particular, it is commonly used to form hydrogel scaffolds for tissue engineering [1], which may in turn be employed for localized drug [2] and DNA delivery purposes [3]. This review aims to give a brief overview of the biological functions of HA, knowledge needed to fully realize the potential that HA has for tissue engineering applications, before describing recent and novel ways in which HA scaffolds have been engineered to deliver various classes of signals to influence and ultimately command greater control over cell-matrix interactions and cell behavior (Figure 1).

Hyaluronic acid is a linear polysaccharide that consists of two alternating units, B-1, 4-D-glucuronic acid and B-1, 3-N-acetyl-D-glucosamine (Figure 2). This unmodified polymer has a molecular mass between 10^3 and 10^4 kDa, which can reach a length of 25 μ m when fully extended. Its negative charge attracts positive ions and results in an osmotic balance that brings in water. This allows HA to exist as a highly hydrated molecule and paves the way for a myriad of uses in the body.

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After synthesis in the inner face of the plasma membrane of fibroblasts, HA is translocated to the pericellular space and the interstitial matrix [4]. This characteristic makes HA distinct from other GAG's, which are typically synthesized in the Golgi and contain a protein core. Three transmembrane glycosyltransferase isoenzymes, HAS1-3, regulate the synthesis process. Hormones and growth factors, among other bioactive molecules, can also help regulate the production of HA.

Hyaluronic acid was first characterized in the vitreous of the eye [5]. A few years later, it was successfully isolated from umbilical cord tissue where its main saccharide components, glucuronic acid and glucosamine, were found [6]. A thorough analysis of HA throughout the body of the rat can be applied to approximate the distribution of the polysaccharide in other mammals [7]. For instance, the 40-60 mg that exists in a 250 g rat can be extrapolated to ~11-17 g in a 70 kg human. A little over half (~56%) of the HA found was located in the skin with another 27% found in the skeleton and supporting tissues, while approximately 8-9% was found in muscle and the internal organs. Among organs, the highest amount of HA was present in connective tissue, the lung, the kidney, and the brain with low amounts present in the liver and blood serum. Two reviews in particular provide a more thorough explanation of HA distribution throughout organs in different mammals [8-9].

Biological function

One property of hyaluronic acid that can potentially be exploited is that its biological function depends on its molecular weight. While it typically exists as a high molecular weight polymer over 10^6 daltons, it can be cleaved by the enzyme hyaluronidase (HAse) to obtain molecules of much lower molecular weights [10]. In fact, high and low molecular weight HA exhibit opposing effects on cell behavior. In addition to inhibiting cell proliferation, high molecular weight HA is also anti-angiogenic [11]. In fetal development of rat follicles, HA acts as a high molecular weight barrier that blocks endothelial cell migration and subsequent angiogenesis. However, cleaving the polymer into shorter fragments with hyaluronidase enables endothelial cells to migrate and initiate angiogenesis [12]. These high molecular weight polymers are also anti-inflammatory and immunosuppressive [13-14]. This feature may be particularly important as the amniotic fluid of fetuses have high concentrations of high molecular weight HA. In contrast, low molecular weight HA ($< 3.5 \times 10^4$ Da) has been implicated as pro-inflammatory and proangiogenic [15]. In particular, oligomers of the 6-20 kDa range activate antigen-presenting cells, such as dendritic cells, while slightly larger HA (2×10^4 - 4.5×10^5 Da) stimulates inflammatory cytokines [16]. In addition, fragments consisting of 4 to 25 disaccharide units are able to promote blood flow to injury sites and subsequent angiogenesis [11, 15, 17].

On the molecular level, HA primarily interacts with the cell-surface receptor cluster determinant 44 (CD44) and receptor for hyaluronate-mediated motility (RHAMM). CD44 plays a key role in tissue organization by mediating ECM remodeling, cell-cell interactions, and cell-matrix interactions [18-19]. A normal wound healing model in CD44-knockout mice exhibited decreased cell migration, motility, and ECM turnover [20]. Interactions between HA and RHAMM can result in a variety of downstream signaling pathways that affect protein kinases like focal adhesion kinases, MAP kinases, phosphatidylinositol kinases, and tyrosine kinases as well as other molecules like RAS [19, 21-22]. Furthermore, *in vivo* experiments blocking HA-RHAMM interactions inhibited fibroblast growth factor-induced neovascularization [23].

An issue worthy of consideration in HA hydrogel design is the pathological context of hyaluronic acid. For example, increased levels of HA in the extracellular environment have been correlated to several types of cancer malignancies [24]. In particular, 3-8-fold higher

levels of HA were found in prostate cancer tissues, and elevated levels of Hyal-1, a hyaluronidase, were also observed in prostate cancer tissues when compared to normal tissues [25]. Excess HA synthesis by hyaluronan synthases in conjunction with elevated expression of Hase has also been shown to drive the development of a metastatic phenotype in prostate tumor cells and accelerate the metastatic progression of prostate cancer [26]. A reason for this correlation is that the degradation of HA by Hase results in HA oligomers, which enhance the cleavage of CD44 [27]. Therefore, the increased presence of HA oligomers in various tumor tissues can promote tumor cell mobility and invasion. For more information on the involvement of HA and Hases in cancer biology, readers may refer to other reviews focusing on these biological interactions [28].

Understanding the diverse ways in which HA functions in native tissue can give insight into designing more efficient and novel tissue engineering scaffolds. Because these scaffolds can be synthesized from a variety of synthetic and natural scaffolds, we will briefly touch on some of the differences between the main synthetic polymer used by researchers, polyethylene glycol (PEG), and our polymer of interest, HA. PEG is a nontoxic, non-immunogenic polymer that is often referred to as a “stealthy molecule” because it is not recognized by biological molecules like proteins. This polymer can be used as a blank slate by researchers who wish to control all interactions between cells and the hydrogel. Unlike HA, PEG is non-degradable, so degradable crosslinkers must be incorporated to create a non-permanent hydrogel. While HA will degrade into smaller fragments, PEG will remain in its original form until it is cleared by the body. For a more comprehensive look at PEG and other material-based hydrogels, we refer you to the following reviews [29-30].

This article reviews new and innovative ways traditional HA hydrogels have been modified to direct encapsulated cell behavior *in vitro* and promote wound healing *in vivo*.

Incorporating bioactive signals

Hyaluronic acid hydrogels are typically synthesized from high molecular weight HA. However, as mentioned above, high molecular weight HA is a natural barrier for angiogenesis and proliferation. Thus, to allow cellular infiltration and remodeling of the material by cells *in vitro*, HA is crosslinked with protease-degradable peptides and modified with cell adhesion ligands. The sequence of the degradable peptide determines the ability by which different cells can infiltrate the material. For example, for mesenchymal stem cells, HA crosslinked with MMP-degradable peptides results in more infiltration than for gels crosslinked with plasmin-degradable peptides. For *in vivo* experiments, it appears that if the HA scaffold has significantly large pores, incorporation of protease degradable bonds and addition of adhesion ligands are not necessary for the cells to infiltrate the scaffold and remodel the material [31]. Because HA behaves as a barrier to cell adhesion and migration *in vitro*, it can be used as a blank slate to investigate the roles of different ligands on cell behavior and differentiation.

Cell-matrix interactions of HA hydrogel scaffolds with embedded or infiltrating cells can be engineered to promote survival, proliferation, and/or differentiation. Traditionally, cell-matrix interactions in hydrogels for tissue regeneration are engineered with motifs found in the natural ECM of the relevant tissue. For example, the ubiquitous RGD sequence found in several cell adhesion proteins and tissues in the body as well as other adhesion peptides have been routinely introduced to promote cell adhesion to the material through integrin receptors. Further, since these peptides are unable to fully or specifically engage cell surface receptors, unlike their more structurally complex parents, approaches to engineer protein fragments or incorporate full-length proteins are also commonly employed. In addition, antibodies, small molecules, and nucleic acids, all of which are not typically present in the

natural ECM, have been introduced to recruit a desired cell type or promote differentiation. Full-length proteins that do exist in the ECM, such as growth factors, are also commonly loaded into hydrogels. Common challenges in incorporating growth factors include doing so without damaging secondary structures and controlling the release kinetics. This section focuses on recent advances in the field that continue to push the innovation of bioactive signal incorporation in HA hydrogels (Figure 3).

Incorporation of cell adhesion motifs

Integrin binding anchors cells to the ECM and initiates signaling events that control cell behavior [32-35] and remodel the matrix [36-37]. Two-dimensional studies in hydrogel scaffolds have shown that RGD presentation at the nanoscale level influences cell spreading and motility [38-41], stem cell differentiation [42], and nanoparticle internalization [43]. Up until recently, however, for cells seeded within hydrogel scaffolds, only RGD total content (μM - mM range) had been shown to modulate cell motility, spreading, and proliferation [1, 44-45]. Our lab designed a HA hydrogel to alter the presentation of the RGD peptide and then studied the effect of peptide incorporation in stem cell integrin expression and gene transfer [1, 46]. By pre-reacting a specific portion of the total hyaluronic acid with the RGD adhesion peptide, we were able to modulate the distribution of the peptide inside the hydrogel. Results from five degrees of clustering of three total peptide concentrations (10, 100, 1000 μM) suggest an optimal degree of clustering for the spreading of encapsulated murine mesenchymal stem cells (mMSC) (Figure 4A-C). Furthermore, integrin expression can be modulated through this peptide-clustering parameter. In a separate study, we investigated the effect that RGD presentation had on gene delivery in our HA hydrogel system and found that an intermediate clustering promoted the transfection of encapsulated cells [3]. In both of these studies we found that the degree of clustering affected cellular behavior and that for a particular RGD concentration an optimal clustering amount can be obtained that is in between a completely homogeneous distribution and high clustering. The globular protein sonic hedgehog (SHH) was tethered to linear HA to compare the effect of tethered and soluble protein presentation to cells. The tethered form of SHH was significantly more potent than the soluble form while multivalent constructs enhanced this effect even more [47]. These studies show the importance of bioactive signal distribution on encapsulated cell behavior and how this parameter can be exploited to drive cell behavior.

Aiming to bridge the gap between shorter peptide fragments and native proteins, groups have worked on engineering fibronectin fragments [48]. We have incorporated these fragments into our HA system and have seen a great effect on cell spreading. Compared to incorporating RGD peptide fragments, a much smaller number of the fibronectin fragments was needed to induce mMSC spreading (Figure 4D-F). Evidence of downstream signaling overlap between growth factors and integrins prompted a group to engineer a fibronectin fragment-functionalized hyaluronic acid hydrogel that released bone morphogenic protein-2 (BMP-2) [49]. This functionalization, when compared to blank HA hydrogels and standard cell-culture plates, improved *in vitro* mMSC attachment and spreading. In an *in vivo* ectopic bone formation mouse model, the combination of BMP-2 release with the fibronectin-functionalized hydrogel resulted in twice as much bone formation with better collagen fiber organization than a nonfunctionalized, BMP-2-loaded hydrogel. However, it would be interesting to directly compare the effects of the fibronectin fragment with shorter peptide fragments in their *in vivo* bone formation model. The incorporation of protein fragments has been more thoroughly investigated *in vitro* to modulate epithelial to mesenchymal transition (EMT) or using other biomaterial scaffolds such as fibrin or fibrin mimics [50-53]. Although these approaches have not been applied to hyaluronic acid scaffolds, the chemistry is completely compatible with this system and could be easily translated.

Incorporation of hyaluronic acid fragments

As described earlier, the molecular weight of hyaluronic acid affects its biological activity, primarily by affecting its angiogenic potential with short fragments promoting angiogenesis [15, 17]. Delivering 100 micrograms of low molecular weight HA (1-4 kDa) promoted revascularization of skin grafts. Two additional approaches to directly exploit and control this bioactive signal have focused on the crosslinking of different HA molecular weights into scaffolds such as upon degradation different HA fragments sizes are released or the direct tethering of HA fragments with different molecular weights onto the scaffold again such that upon release of the tether different fragment sizes are released. Both approaches demonstrated that the presence of HA oligomers (rather than high molecular weight HA) promoted endothelial cell attachment and proliferation *in vitro* or enhanced vascularization *in vivo* at weight percentages of ~10% [54-55].

Incorporation of non-ECM motifs

An alternative approach inspired by cardiovascular stents used antibodies for targeted cell attachment. CD34 antibodies were functionalized onto hyaluronic acid hydrogels to specifically capture endothelial cells [56]. Attachment of endothelial cells was significantly greater than non-CD34 expressing macrophages during cell seeding experiments. Furthermore, a dose-dependent response of endothelial cell attachment to antibody-immobilization concentration was observed. This innovative antibody approach can be used to engineer more specialized cell recruitment scaffolds.

Incorporation of growth factors and controlled release

Delivering full-length proteins such as growth factors in scaffolds is desirable because systemic delivery does not allow for targeted treatments and may result in unwanted side effects. Traditional approaches include direct loading of the scaffold; however, these proteins can also be incorporated into scaffolds by utilizing intermediary molecules like heparin. This glycosaminoglycan has the ability to bind growth factors and gives researchers a method to indirectly incorporate heparin-binding proteins into their system. Furthermore, this GAG/growth factor interaction can be exploited for controlled release.

A variety of growth factors have been loaded into hyaluronic acid-based hydrogel systems. For example, basic fibroblast growth factor (FGF-2) and bone morphogenic protein-2 were delivered over the course of 28-35 days [57-58]. After ensuring the bioactivity of the released FGF-2, scaffolds were loaded with 250 ng of FGF-2 and implanted subcutaneously in mice. Hydrogels released ~70% of the FGF-2 and promoted more neovascularization than control scaffolds as well as bolus FGF-2 injections. Additionally, a commercially available hyaluronic acid/heparin sulfate was loaded with 1.5 ng of insulin growth factor and injected into the stroke cavity of mice. Amazingly, a sustained release of IGF-1 occurred over 4 weeks after implantation [59].

Using a nanoparticle approach, heparin-decorated HA nanoparticles were synthesized to carry and release 200 ng of BMP-2 [60]. Heparin was immobilized on HA hydrogel nanogels and the BMP-2 release kinetics were modulated by nanoparticle composition. Near zero-order release kinetics were reached through a series of optimization experiments with ~46% of the BMP-2 released over 13 days. Furthermore, the heparin decoration increased BMP-2 loading capacity two-fold and produced the efficient differentiation of MSC's into chondrocytes *in vitro*.

Incorporation of gene-encoding nucleic acids

Gene delivery has been widely researched as a tool for gene therapy in tissue regeneration and cancer therapy. While both non-viral and viral vehicles have been used to deliver genes in the form of nucleic acids, non-viral systems such as hydrogel scaffolds loaded with DNA have a lower risk of causing an immune response and insertion mutagenesis. Although delivering naked DNA has been attempted, transfection efficiency was shown to be very low and much of the DNA diffused away from the local scaffold implant site. Hence, DNA is most commonly delivered in scaffolds when electrostatically complexed with polyethyleneimine (PEI) in the form of nanosize polyplexes, which also allow for higher loading of DNA into the scaffold. Our group had previously developed a technique called caged nanoparticle encapsulation (CnE), which uses the saccharides agarose and sucrose to protect the polyplexes from aggregation when incorporating them into hydrogels, allowing us to load up to 5 μg of DNA polyplexes per microliter of HA hydrogel without significant aggregation while still achieving gene transfer *in vitro* and *in vivo* [61]. Gene transfer efficiency in HA hydrogels was found to be a function of the DNA-to-PEI ratio during polyplex synthesis [3]. In addition, gene transfer efficiency was found to significantly correlate with nanoporous HA hydrogel stiffness, with the highest gene expression observed in the softest hydrogels. This is due to greater cell infiltration and migration through the softer hydrogels. Our group has also been able to encapsulate DNA/PEI polyplexes via CnE into nonporous HA scaffolds *ex ovo*. Using a chorionic chick embryo (CAM) assay, enhanced angiogenesis was observed with hydrogels loaded with pVEGF-containing polyplexes, demonstrating the efficacy and maintenance of activity of the CnE polyplexes [62]. In addition, DNA polyplex-loaded microporous HA hydrogels containing cells seeded in the pores resulted in sustained gene transfer and expression over ten days [63]. *In vivo* implantation in a wound healing model in mice also showed transfection and subsequent gene expression in cells that had infiltrated the gel (unpublished data).

Introducing spatial signals

Topographical cues introduced into the environment have been observed to also significantly affect cell behavior (Figure 1C). Cells seeded along grooves and ridges tended to elongate and align along these topographical features, behaviors that were not observed on smooth surfaces, a phenomenon known as “contact guidance” [64]. However, the extent of multicellular organization can also be a function of spatial cues, and this has been shown in HA hydrogel cell culture [65]. HUVECs were seeded in microgrooves of varying diameters on HA-dextran hydrogels. After 24 h of culture, HUVECs that were cultured in channels of 30 μm and 50 μm in width self-organized into 3-D vessels (Figure 5A-B), while those cultured in channels of greater widths only organized into 2-D monolayers covering the bottom of the channels (Figure 5C-D). The investigators speculated that contact guidance on the spatial scale of no more than a few cells allowed cells to communicate and form interactive structures. In addition, co-culturing of HUVECs with fibroblasts resulted in elongated morphologies of the fibroblasts along the endothelial cords [65].

Varying porosity as a spatial signal to be introduced in HA hydrogels has also been studied. Our group subcutaneously implanted hydrogels without micron-size pores and hydrogels with 100- μm pores in mice and observed substantial infiltration of the porous HA hydrogel by cells even after one week, while there was minimal infiltration of the nonporous hydrogel. It is speculated that designing porous hydrogels will allow for greater cell infiltration over time when compared to hydrogels without pores of the scale of cell diameter [63].

Spatial control has been employed to not just affect morphology and multicellular organization, but also to control the direction of cell proliferation in culture. A strategy

currently being investigated as a means of HA hydrogel design with stringent spatial control is to use light to photo-initiate pattern formation in a hydrogel, which is thought to mimic the heterogeneity of physical and chemical properties found throughout native tissues. By conjugating the caged and inactivated RGDS adhesion peptide sequence to the surface of a HA hydrogel, cell-adhesive regions were created by exposing those regions to near-UV light, thereby uncaging and activating the RGDS peptides present in those regions. This chemically patterned hydrogel supported patterned and robust 2D cell culture on the activated regions (Figure 6). Furthermore, this system could be manipulated temporally by introducing more cell-adhesive regions during cell culture, allowing for control over cell proliferation direction over time [66].

Introducing mechanical signals

Mechanical properties of a cell's substrate have profound effects on the progression of various cellular processes, and tissue scaffolds have been designed to direct cell mechanosensing and mechanoresponse [67]. Therefore, studying this type of cell-material interaction is a major point of consideration in hyaluronic acid hydrogel design. The mechanical properties of HA hydrogels can be varied by either adjusting the concentration of HA in the gel precursor solution or by controlling the degree of crosslinking in the gel, either by modulating the concentration of crosslinker or the extent of crosslinker reaction, depending on the type of chemistry used in crosslinking (Figure 1B). Generally, increasing the gel precursor solution's HA concentration increases the storage modulus of the hydrogel, with stiffnesses ranging from hundreds of Pa to thousands of Pa.

Several groups have studied general cell behavior, such as cell attachment, spreading, and proliferation, as a function of gel stiffness in HA hydrogels. Our group was able to control the stiffness of nonporous HA hydrogels by modulating both HA concentration and crosslinking density (Figure 7). By decoupling the two parameters and seeding mesenchymal stem cells (MSCs) in the gel, migration and proliferation both increased with decreasing HA concentration from 5% to 3%, resulting in a storage modulus decrease from 1920 Pa to 177.1 Pa, and decreasing crosslinking density from a crosslinking ratio (r) of 0.5 to 0.25, resulting in a storage modulus decrease from 387.4 Pa to 177.1 Pa (Figure 8) [1]. The porosity of the gel is also another parameter worth considering in conjunction with varying stiffness, as cells cultured in nonporous gels exhibit different cell behavior than those cultured in porous gels. In another study, the stiffness of methacrylated HA macroporous hydrogels (250 μ m) is tuned by using UV-initiated free radical polymerization at different UV exposure times for varying extents of crosslinking (Figure 9) [68]. However, after seeding and culturing with MSCs, less cell spreading and proliferation were observed in 1.5 kPa gels versus stiffer gels (up to 7.4 kPa) by analyzing cell morphology and DNA content. This highlights a crucial difference between nonporous and macroporous gels; proliferation occurs in nonporous gels via the degradation of the gel by the cells, while cells cultured in macroporous gels proliferate and migrate along the surface of the pores, relying on two-dimensional surface interactions as mechanical signals. To further support this point, in 2D glioma culture, significantly more cell spreading, proliferation, and motility were observed on stiffer gels with storage moduli of up to 5 kPa than on 150-Pa gels; however, there was almost no gel infiltration and cell proliferation when glioma spheroids were cultured within 5-kPa 3D nonporous hydrogels, while gel infiltration was observed in 150-Pa gels [69]. However, it was interesting to note in this study that initial glioma cell attachment was not affected by gel stiffness. Furthermore, this study highlights the potential applications that HA hydrogels have as a tunable biomimetic platform for studying cancer biology. In summary, HA hydrogels with relatively lower storage moduli are generally required to achieve spreading of a 3D culture, and if stiffer gels are used, micron-size pores must be introduced.

Various studies have also investigated the effects of HA hydrogel stiffness on stem cell differentiation, inspired by research showing that the mechanics of the cellular microenvironment are an influencing factor in the direction and extent of differentiation of stem cells *in vivo*. Hence, hydrogels of different stiffnesses have been used that mimic stem cells' native environment in order to direct a stem cell's development toward a specific phenotype [70]. This has been applied to HA hydrogel applications; ventral mesencephalic neural progenitor cells (NPCs) differentiated furthest towards a neuronal phenotype in a 3D culture in HA hydrogels with a stiffness most closely resembling that of the neonatal brain, while the same NPCs differentiated into the astroglial phenotype when cultured for three weeks on HA hydrogels with a stiffness that resembles the adult brain [71]. Temporal control of the environment's mechanics is a second approach that is of interest. Cardiomyocyte differentiation in hydrogels may progress in a more *in vivo*-like fashion if the mechanical properties of the hydrogel environment were designed to progressively stiffen over time at a rate closely mimicking that of the maturation of the cells' native tissue environment [72]. This temporal control was due to a time-dependent Michael-type addition reaction modulated by HA molecular weight. It would be interesting to see a significant enhancement in differentiation in this system as compared to a constant-stiffness HA hydrogel. Another approach to detect extent of differentiation as a function of gel stiffness is by monitoring the secretion levels of angiogenic and cytokine proteins. Human mesenchymal stem cells were cultured over two weeks on HA hydrogels of a range of storage moduli from 1.5 kPa to 7.4 kPa (measured by dynamic mechanical analysis) and differences in the secretion levels between cases were noted. It was found that MMP-9 was initially more highly expressed in stiffer gels, while IL-8 secretion was overall stronger in softer gels. These results suggest that stiffness is a parameter that must be considered when designing hydrogels for the purpose of controlling stem cell differentiation [68].

Aside from controlling individual cell development and behavior, it has also been shown that multicellular organization can be influenced by modulating the mechanical signals from the hydrogel environment. Endothelial colony-forming cells (ECFCs) were seeded in HA hydrogels of two different viscoelasticities (Young's modulus) to investigate the effect of gel mechanics on 3D vascular tubulogenesis [73]. They observed significant increases in mean tube length, mean tube area, and percent of area covered by capillary-like structures with decreasing hydrogel viscoelasticity.

New HA scaffold syntheses

Numerous HA modifications and crosslinking schemes have been developed and used to form HA hydrogel scaffolds. Some of these methods are highlighted in other comprehensive review articles [74-75]. We would like to report here recent and intriguing scaffold formation chemistries (Figure 1D).

Several novel strategies to synthesize HA hydrogels have employed click chemistry as the crosslinking mechanism, since such reactions generally demonstrate high specificity and are unreactive with other functional groups that may be present in the reaction environment. One such synthesis that was developed relies on a Diels-Alder click reaction between a furan-modified HA molecule and a bis(maleimide) PEG crosslinker. Because the functional groups here are not self-reactive, the overnight reaction proceeds in a cleaner fashion and the end crosslinked product is much more predictable [76]. Another study utilized click chemistry between HA and chondroitin sulfate modified with 11-azido-3,6,9-tri-oxaundecan-1-amine and propiolic acid-modified gelatin, catalyzed by Cu(I) [77]. A third study investigated using oxime chemistry to create injectable hydrogels. HA that was previously oxidized with NaIO₄ to form aldehyde groups was then reacted with the crosslinker four-armed aminoxy-PEG (AO-PEG) to form oxime bonds [78-79]. As the

reaction is acid-catalyzed, *in vitro* gelation is tunable, and *in vivo* gelation was achieved within 20 minutes after injection.

With the objective of developing a more clinically translatable product, a novel HA-blood hydrogel was synthesized for stem cell transplantation. Lysed whole blood was used as a crosslinker for reaction with N-hydroxysuccinimide (NHS)-modified HA [80]. The NHS groups react with primary amines found in whole blood to form hydrogels that would be able to be used to encapsulate stem cells. Blood was utilized as a crosslinker because of its ease of acquisition in the clinical environment and also because of the presence of fibronectin, vitronectin, and various growth factors in blood. The HA-blood hydrogel stiffness was tunable depending on the amount of blood used, and both cultured cell viability and proliferation after one week was higher than in HA-PEG hydrogels.

While most HA hydrogel-forming chemistries today rely on reactions between reactive functional groups, one study developed a novel strategy relying on strong and selective supramolecular interactions using natural receptor-ligand pairs to mimic traditional crosslinking [81]. Specifically, the investigators formed hydrogels by the binding of cucurbituril-conjugated HA to diaminohexane-conjugated HA, bypassing the relative instability and possible toxicity of the usage of some functional groups.

Conclusion and future directions

Hyaluronic acid hydrogels have been widely engineered and researched for their biocompatibility as well as the ability to incorporate a wide variety of cues to modify cell-material interactions and ultimately to influence and to allow for greater control over the behavior of cells. Recent studies have shown that incorporating various chemical, mechanical, and spatial cues can lead to a tuned individual cell as well as multicellular response, and in some cases, a cell-specific response. New and interesting HA hydrogel syntheses show potential in higher and cleaner reaction specificity. In addition, being able to control other HA gel-specific cellular functions not previously mentioned above, such as cell-mediated hydrogel degradation, is useful in designing a system with a multi-pronged approach and application [82]. As research in HA hydrogels continues to move forward, more intelligent and cleaner systems with a clearer, more cell-directed purpose will be developed.

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Figure 1.

Hyaluronic acid scaffolds can be engineered in several ways to control encapsulated cell behavior. (A) Bioactive signals can be incorporated into the scaffold in suspension or covalently bound to the polymer. (B) Tuning the mechanical properties by utilizing degradable crosslinkers and/or degree of crosslinking can regulate cell mechanosensing. (C) Spatial cues such as patterned bioactive signals, porosity control, and topographical patterns can direct cell migration. (D) New “click chemistries” have been employed to provide new ways to covalently crosslink the hydrogel network.

Figure 2. Structure of hyaluronic acid. Chemical modifications are commonly performed at (a) and (b).

Figure 3. Bioactive signals are commonly incorporated into HA hydrogels to direct cell behavior. Some signals, like peptide fragments and antibodies, can be covalently linked to the HA backbone whereas other signals, like heparin and DNA, are loaded into the hydrogel without any permanent bond.

Figure 4.

HA hydrogels were functionalized with RGD peptides or fibronectin fragments and filamentous actin of encapsulated mouse mesenchymal stem cells were stained with phalloidin 4 days after gelation. Cell spreading can be controlled by altering both the concentration and presentation of RGD. While (A) 100 μ M of homogeneously distributed RGD resulted in spherical morphology, (B-C) clustering 10 or 100 μ M resulted in elongated cells. Hydrogels functionalized with (D) 1 μ M, (E) 5 μ M, and (F) 50 μ M of homogeneously distributed fibronectin fragments resulted in elongated cell morphologies.

Figure 5. HUVECs seeded on HA hydrogels in microgrooves of 100 μm and 200 μm in width covered the grooves in monolayers (a-b), while those seeded in microgrooves of 30 μm and 50 μm in width organized into 3D cord structures (c-d) [65].

Figure 6. RGDS within the dashed circles was uncaged and activated via photopatterning, resulting in the formation of patterns of cell populations on HA hydrogels [66].

Figure 7.

The storage and loss moduli of HA hydrogels, measured with a rheometer, can be modulated by varying (A) crosslinking densities (r -ratio) or (B) HA concentration. (C) Average G' and G'' over the frequency range tested [1].

Figure 8.

D1 mMSC cells cultured in stiffer nonporous HA hydrogels (higher HA concentration) show less spreading (A), proliferation (B), and migration (C-D) than those cultured in softer nonporous hydrogels (lower HA concentration). The same patterns were observed in hydrogels with two different RGD concentrations [1].

Figure 9. Stiffness of UV-photopolymerized HA hydrogels can be controlled by varying the exposure time to UV light. Gel stiffness in this study was measured using dynamic mechanical analysis [68].