REVIEW

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Hyaluronic acid-based scaffolds for tissue engineering

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Abstract
Hyaluronic acid (HA) is a natural glycosaminoglycan found in the extracellular matrix of most connective tissues. Due to its chemical structure, HA is a hydrophilic polymer and it is characterized by a fast degradation rate. HA-based scaffolds for tissue engineering are intensively studied due to their increased biocompatibility, biodegradability and chemical modification. Depending on the processing technique, scaffolds can be prepared in the form of hydrogels, sponges, cryogels, and injectable hydrogels, all discussed in this review.

***Keywords*:** hyaluronic acid, scaffolds, cartilage repair, tissue engineering, biocompatibility, biodegradability.

Introduction

Hyaluronic acid (HA), also known as hyaluronan, is a linear, anionic, non-sulfated glycosaminoglycan, with a structure composed of repeating disaccharides units: *β*-1,4-D-glucuronic acid and *β*-1,3-*N*-acetyl-D-glucosamide [1, 2]. It is a high molecular weight (105–107 kDa) naturally occurring biopolymer, which can include 5000–30000 sugar molecules in the backbone [1].

HA is synthesized in the inner cell membrane by hyaluronan synthases, a class of transmembrane proteins. After synthesis, it is removed through the membrane into the extracellular matrix (ECM), where it is being degraded by the hyaluronidases enzymes family, after 3–5 days. HA is found in the ECM of all living tissues, with different concentrations and molecular weights, being most abundantly present in tissues subjected to mechanical loads, such as cartilages, dermis and vocal folds [3]. HA is mostly produced by extraction from animal tissues or by microbial approach [4].

Due to the large number of carboxyl and hydroxyl groups, HA is a highly hydrophilic compound, which forms a gel-like structure in aqueous solutions, as a result of intermolecular interaction of linear macromolecules [1, 5]. In terms of water-binding, ionic exchange, diffusion and permeability of large molecules, physiological functions of tissues are determined by HA concentration and molecular weight. Thus, the biopolymer acts as protectant against microorganism and toxin penetration, lubricant due to its viscoelastic properties in the synovial liquid, and the transparent aqueous solution is a filler of eye structures. It is commonly known that HA has a key role in cell division and migration, angiogenesis, wound healing and tissue regeneration, its effects being correlated to the molecular weight [6].

Due to its biocompatibility, biodegradability and chemical modification, HA is of great interest for its potential in the field of tissue engineering (TE). The use of cells, scaffolds and growth factors allows the possibility for tissue regeneration, which can overcome the autologous and allogeneic graft-associated issues. HA can interact with receptors on the surface of stem cells, inducing intracellular signal transduction and affecting cell activities, such as proliferation, survival, movement and differentiation [1]. HA and HA-based materials have been extensively used as bioscaffolds and injectable hydrogels, *in vitro* and *in vivo* tests showing positive results for tissue regeneration.

HA and HA-based scaffolds

Designing a scaffold for TE involves the accurate consideration of scaffold criteria: biocompatibility, biodegradability, degradation by-products should not determine an immune response, cytocompatibility, by permitting cell adhesion, promoting cell growth, and allowing the retention of differentiated cell functions, suitable mechanical properties to the implantation site and maintenance of a balance between mechanical properties and porosity for cell infiltration and vascularization, and reproducibility [7]. Since HA is a material that mostly meets the criteria, it has been intensively studied for the synthesis of bioscaffolds for TE.

However, HA has some disadvantages, which include poor mechanical properties and rapid degradation *in vivo*. Chemical modification or crosslinking can overcome these disadvantages, improving mechanical properties, degradation, viscosity, solubility, and biologic properties. The targeted sites of modification are the carboxyl groups, hydroxyl group, and $–NHCOCH_3$. Using carbodiimides or carbonyldiimidazole, the carboxyl group will be covalently modified to form amide bonds, while the hydroxyl group can be modified to ether formation, ester formation, hemiacetal formation, and oxidation, and $–NHCOCH_3$ is modified by deacetylation, amidation, hemiacetylation, and hemiacetal formation [8].

Scaffold processing technologies

There are numerous approaches to fabricate porous scaffolds from natural polymers, the most commonly used techniques involve phase separation, solid freeform fabrication, bioprinting, supercritical fluid technology, porogen leaching, freeze drying, electrospinning, centrifugal casting, scaffold templating techniques, and micro-patterning techniques. Of these, freeze-drying, electrospinning, and also gelation are mostly used for HA and HA-based scaffolds fabrication [7]. After the gelation of HA solution, the synthesized hydrogel can be freeze-dried, which involves the sublimation of the dense ice pockets formed throughout the material, thus obtaining a porous sponge. Electrospinning can be used to produce fibrous and nanofibrous HA scaffolds.

Hydrogels

Hydrogels are the three-dimensional (3D) networks consisting of cross-linked polymers. They are characterized by the high capacity to absorb aqueous solvents and biological fluids within their structures. Hydrogels have attracted a great interest due to their applicability in a variety of fields, including TE [9]. HA hydrogels have the capacity to mimic the human tissue in terms of water content and to exchange oxygen, nutrients and metabolic waste [1].

The use of HA-based hydrogels has been intensively studied for cartilage TE, since it is a key component distributed ubiquitously throughout the cartilaginous ECM and they can maintain the morphology of natural chondrocytes. Chondrocytes abundantly secrete type II collagen and glycosaminoglycans in the ECM of the native cartilage tissue [10]. The need for the development of a suitable biomaterial is based on the limited capacity for repair of the articular cartilage, probably due to its avascularity and specialized structure and composition [10].

Hydrogels obtained from pure HA have been used for human umbilical mesenchymal stem cells seeding and implantation to the articular site. Studies on rabbit models for cartilage tissue regeneration showed an improved cartilage repair effect for the seeded hydrogels, when compared to HA only treatment. The repaired tissue had similar cellular architecture and type II collagen arrangement and quantity to the natural tissue [10, 11].

The use of chitosan [12, 13], an elastin-like protein [14], and tauroursodeoxycholic acid–poly(lactic-*co*-glycolic acid) microspheres [15] as materials for HA-based hydrogels synthesis have also been studied. Hydrogels prepared from oxidized HA and chitosan have been used as scaffolds for chondrocytes encapsulation. The simulated *in vitro* microenvironment showed good cell viability and an appropriate ECM production, thus proving the potential of this system for further *in vivo* studies [12]. Implantation of HA–chitosan hydrogels in osteochondral defects in rabbit knee joints proved the potential of the system for tissue repair. Besides the capacity to fill the defect, evaluation after 12 weeks showed an opaque and smooth texture of the regenerated tissue and a mixture of fibrous and hyaline cartilage formed within the hydrogels that incorporated cells. Histology tests confirmed tissue regeneration by the absence of hydrogel traces in the defect. Still, the encapsulation of chondrocytes needs further studies in terms of benefits to the regeneration process [13].

The combination of HA and an elastin-like protein showed positive results in stimulating the formation of cartilage tissue. After *in vitro* testing, it was observed that the use of this hydrogel helps maintaining cell phenotype, while improving cell proliferation and ECM production [14]. Tauroursodeoxycholic acid is used for its properties to induce neovasculogenesis inhibition of adipogenic differentiation of mesenchymal stem cells. By incorporating tauroursodeoxycholic–poly(lactic-*co*-glycolic acid) microspheres in HA hydrogels, the enhancement of chondrogenic and osteogenic differentiation in human mesenchymal stem cells was determined. Implantation in osteochondral defects in rats showed good filling of the defect and the formation of new tissue after 10 weeks. The use of tauroursodeoxycholic acid improved tissue regeneration assessed by increased proteoglycans in the superficial area and calcified cartilage [15].

Poly(*Nε*-acryloyl-L-lysine) was evaluated for bone TE, using a HA-based hydrogel. The use of the copolymer is expected to show cytocompatibility, since both its components promote cell attachment and proliferation. Results for the *in vitro* tests performed on osteoblast precursor cell line from mouse showed cell viability and increased cell proliferation, as expected. *In vivo* biocompatibility was evaluated after seven days using the dorsal skin-fold chamber model, and the results demonstrated that the addition of the copolymer to the HA hydrogel improved biocompatibility, since it could better support tissue and cells penetration. Additionally, histological tests showed a better cell infiltration with the increased cross-linking of the hydrogel, thus proving the relation between the mechanical properties of dense structures and improved native cell penetration [16].

A comparative study between pure HA hydrogels and HA hydrogels containing ECM derived from astrocytes, for the development of central nervous system injury treatments has been conducted. HA containing ECM better supported the transplantation of interneurons into a spinal cord injury, where a decrease of immune response was observed. Also, an increase in neuronal processes was found at the site of implantation [17].

An aqueous solution containing phenolic-substituted HA, horseradish peroxidase (HRP) and catalase was used to fabricate hollow hydrogel fibers through microfluidic spinning technique. These hydrogel fibers provide a biomimetic microenvironment, allowing cell encapsulation. Cell viability tests proved their potential for engineering complex artificial tissues with suitable biocompatibility and biodegradability [18]. HA combined with methylcellulose proved it can be applied to 3D bioprinting, since it is a versatile material that can be used as a bioink. Encapsulated mesenchymal stem cells survived the process of bioprinting of the hydrogels, remaining viable for another week. These results demonstrate that HA–methylcellulose blends are promising materials for 3D bioprinting of artificial tissues [19].

Silk has also proved to be a suitable candidate to synthesize composite hydrogels based on HA. The enzymatic cross-linking of the two polymers provides a material that meets the advantages of both compounds,

while overcoming the limitations associated with either one alone in hydrogel form. The degradation rate of the composite hydrogel is lower compared to HA alone. The composite hydrogel also showed cell adhesion and proliferation enhancement, which demonstrate its potential for TE applications [20].

Heparin [21] and collagen [22] were studied for HA-based hydrogels for 3D cell culture applications. Adipose-derived mesenchymal stem cells were seeded into the heparin–HA hydrogels and cellular activities and hyaluronidases expression were evaluated. Results showed efficient spreading, proliferation, and migration of the cells when compared to HA-only hydrogels. Also, the composite hydrogel exhibited strong hyaluronidases expression, which can be correlated to hydrogel degradation. These results are positive for using heparin–HA hydrogels for 3D cell culturing, but also for further TE applications [21]. Interpenetrating networks consisting of HA and collagen were used for 3D cell culture of human mesenchymal stem cells. Besides viscoelasticity and fibrillar architecture similar to the natural ECM, cell spreading, and collagen fiber realignment were observed. Thus, it can be concluded that HA–collagen networks can be used as materials that mimic the natural ECM, which could direct the differentiation of encapsulated stem cells [22].

Sponges

Sponges are usually fabricated by freeze-drying of hydrogels, due to the simplicity of the method. The obtained materials are characterized by their high porosity, allowing cell encapsulation and proliferation. As a result, sponges are intensively studied for TE applications.

Chitosan is the most commonly used material for composite sponges based on HA. Studies on wound healing and angiogenesis in wounds show the incorporation of different nanoparticles (NPs) in the chitosan–HA sponges for an enhanced effect. The wound healing process was fastened by the encapsulation of andrographolide-loaded lipid NPs, considering that it is a natural diterpene lactone with improved wound healing activity. Its poor aqueous solubility makes the NP encapsulation necessary. The combined anti-inflammatory and antioxidant effects of chitosan and HA together with andrographolide was proved by *in vivo* evaluation of burn wounds healing on rats, by the formation of a healthier tissue. It was also proven that the addition of NPs resulted in faster healing, with complete healing after 21 days. Overall, results showed superior wound healing, histological progress and reduced scar formation when compared to the HA sponge [23]. Another study used vascular endothelial growth factor (VEGF) to stimulate angiogenesis in wounds, for an improved healing process. Nevertheless, the short half-life of the growth factor leads to the necessity of encapsulation into an adequate NP system. Fibrin NPs were used to overcome the growth factor shortcomings. The *in vitro* assays revealed the cytocompatibility of the VEGF-loaded fibrin NPs encapsulated into HA–chitosan sponges. In addition, umbilical vein endothelial cells seeded on the sponges led to the formation of capillary-like tubes, which suggests the potential of the composite to induce angiogenesis for wound healing [24]. The acceleration of wound healing process was also studied by using gellan gum in HA-composite sponges, due to its cell adhesive character highlighted in skin TE applications. The entrapment of microvascular endothelial cells and human adipose stem cells improved neovascularization and intraepidermal nerve fibers formation in diabetic wounds. As a result, composite sponges containing stem cells proved to be a promising approach for the enhancement of diabetic wound healing, with significant effects on re-epithelialization and decrease of inflammatory response [25].

Another study was based on the synthesis of a composite sponge consisted of HA, chitosan, and collagen, as matrices for calcium phosphate *in situ* precipitation. The mineralization of the scaffold improved cell attachment and proliferation, which can be attributed to calcium phosphate bioactivity [26].

Cryogels

Cryogels represent a special kind of scaffold, with a continuous growing applicability in the field of TE, as ECM substitutes. Cryogels are produced through the controlled freezing and thawing of a polymer solution. Before freezing, the polymer solution is physically and chemically cross-linked and the gelation process occurs in the frozen systems [27].

HA-based hydrogels have been studied mostly for their applications in cartilage TE [28, 29]. After the preparation of a cryogel scaffold from gelatin, chondroitin-6-sulfate, and HA, which mimics the composition of the natural ECM, chitosan was incorporated to increase pore size. Encapsulated chondrocytes secreted type II collagen and sulfated glycosaminoglycans, proving the redifferentiation of the cells. The *in vivo* studies revealed neocartilage formation in cartilage defects in rabbit models. The encapsulation of cells into the cryogels improved defect filling, results after one month showing a smooth, white and semi-transparent tissue, with a newly formed tissue density different than the native tissue. Histological tests performed after three months showed chondrocytes orientation similar to the native tissue, which can explain the increased thickness of the neocartilage, compared to the tissue formed within the acellular cryogels. The neocartilage is fully integrated and the formation of subchondral bone shows no gap between the hyaline cartilage [28]. HA and chondroitin sulfate were used to create a cryogel that mimics ECM environment. Rabbit chondrocytes adhered to the cryogels, showing viability and homogenous cell distribution throughout the cryogel. Implantation of the cryogels in mice led to the formation of an interpenetrating network, with a matrix mostly consisting of aggrecan and type II collagen. Histological analysis showed homogenous distribution and rounded morphology of chondrocytes, with an increased presence of proteoglycans. Incorporation of HA increased cells activity by triggering biological mechanisms, thus proving the potential for cartilage tissue regeneration [29].

Another study focused on the usability of cryogels from HA and chitosan for TE scaffolds. *In vitro* analysis proved suitable proliferation rate of mouse fibroblast cells and bone cancer cells, because of the highly porous network formed. Further *in vivo* tests are necessary for the development of TE applications [30].

Injectable hydrogels

Besides biocompatibility, similarity to the ECM structure due to the highly hydrated environment, *in situ* forming hydrogel systems possess the advantages of increased delivery of cells or active biomolecules to the targeted site through a minimally invasive procedure and the capacity to fill large and irregular defects [31]. Injectable hydrogels are prepared through two types of cross-linking methods: physical and chemical. The mechanism used for the solidification of the polymer will determine its characteristics. Although chemically cross-linked injectable hydrogels have improved mechanical properties, stability and durability, the use of chemical cross-linkers increases toxicity and causes adverse effects. The use of physical cross-linkers leads to the formation of non-covalent secondary forces, and hydrogels are more easily degraded inside the organism [32].

Injectable hydrogels have been prepared from a variety of natural and synthetic polymers, and from combinations of them. HA, chondroitin sulfate, pectin, alginate and chitosan are the natural polysaccharides mostly preferred [31]. The use of hyaluronan as an *in situ* forming gel for TE applications, such as tissue regeneration or development of artificial tissues has been investigated. Similar to other natural hydrogels, HA is rapidly degraded inside the body and has poor mechanical properties. Thus, modifying the molecular structure and composition by functionalization is necessary to overcome these disadvantages [33].

Injectable HA-based hydrogels have been widely used for cartilage TE. Reports in the literature show the use of arginylglycylaspartic acid (RGD) peptide-functionalized pectin [31], type I collagen [34], thiolated HA derivative [35], and glycol chitosan [36] as suitable compounds to combine with HA for cartilage TE. The synthesis of HA/RGD-functionalized pectin hydrogel involved the functionalization of hydrazone-based cross-linked HA and pectin-dialdehyde with adipic acid dihydrazide and G4RGDS oligopeptide by carbodiimide chemistry, respectively. After chondrocytes encapsulation, the *in vitro* results showed an improved phenotype maintenance and chondrogenesis with increased RGD-functionalized pectin, since RGD oligopeptide is recognized by cell membrane integrins. Also, the biomimetic hydrogel exhibited acceptable tissue compatibility when delivered to a mouse model [31]. HA can be used as an injectable hydrogel when functionalized with mercapto groups. Chondrocytes encapsulated into the thiolated HA hydrogels showed normal proliferation and *in vitro* cytocompatibility. *In vivo* results indicate the potential of this hydrogel to be used for cartilage tissue regeneration when subcutaneously injected into mice. After four weeks since injection, there was an increased proliferation of chondrocytes, which showed a change in their morphology, from polygonal to round [35]. Similar results were found when using a blend of functionalized HA and type I collagen as *in situ* gelifying hydrogel. Chondrocytes were encapsulated in the thiolated HA/type I collagen hydrogels, where they maintained their phenotype and secreted a considerable amount of matrix. The use of the highest ratio of collagen showed the best results for *in vitro* tests regarding cell adhesion and proliferation [34]. Glycol chitosan combined with oxidized HA through Schiff base formation were also studied as injectable hydrogels for cartilage TE. ATDC5 chondrocytes were seeded inside the hydrogels and results showed good biocompatibility and proliferation. Thus, oxidized HA/glycol chitosan injectable hydrogels have the potential as cell delivery vehicles [36].

A great interest has taken the use of HA-based *in situ* forming hydrogels to promote angiogenesis. It has been reported the development of a slow degradable HA/gelatin hydrogel, which is suitable for inducing the formation of new blood vessels. HA and gelatin were functionalized with a monoaldehyde and carbohydrazide, respectively. In order to fabricate the hydrogels, a double-barreled syringe fibrin glue applicator was used to generate a Schiff's base formation. Hydrogel biocompatibility was demonstrated through *in vitro* cell viability tests. The *ex vivo* assay using a dissected aortic rat ring showed that the synthesized hydrogel is a suitable support for microvascular extension [37]. The same effect was reported by using a hybrid injectable hydrogel, consisting of deferoxamine-loaded poly(lactic-*co*-glycolic acid) NPs incorporated into a HA/chitosan hydrogel. Thus, angiogenesis was induced by deferoxamine drug release, but also by the presence of HA/chitosan hydrogel, which showed cytocompatibility and cell proliferation through *in vitro* tests, and maximal blood vessels formation through *in vivo* tests. Subcutaneous injection of the hydrogel into mice proved the beneficial effect of deferoxamine for neovascularization after 28 days when compared to HA/chitosan hydrogel [38].

HA/chitosan *in situ* forming hydrogel has also been used in abdominal TE. Fibroblasts encapsulation, which is essential for tissue formation, showed biocompatibility, without a toxic response. Moreover, after the *in vivo* test, the use of HA/chitosan hydrogel led to faster tissue regeneration, process characterized by an increased cellular accumulation and ECM deposition. Besides the increased amount of fibroblasts and endothelial cells, the regenerated tissue in the presence of the composite hydrogel showed a greater thickness and capillarity when compared to the naturally regenerated tissue [39]. Studies also focus on central nervous system TE by using a HA/methylcellulose injectable hydrogel. Hydrogel preparation involved the dissolving of the compounds into artificial cerebrospinal fluid, followed by cross-linking with polyethylene glycol (PEG). The *in vitro* tests showed better cytocompatibility for lower concentrations of hydrogels. Still, the material has the potential to be used for TE [40].

Another polymer used for the synthesis of injectable HA-based hydrogels is PEG. The two compounds were mixed, and HRP was added to the reaction as a cross-linker enzyme. The so-obtained hydrogel was used as therapy for intervertebral disc degeneration to restore disc thickness and hydration. *In vitro* tests showed no cytotoxicity associated with the use of the hydrogel. Also, the use of agarose to simulate the intervertebral disc showed that injection of the hydrogel will occupy the empty spaces [41].

Studies proved the synthesis of an ECM based on HA and gelatin. As described previously, HRP enzyme was used for cross-linking the mixture of the compounds. The use of higher ratios of HA increases pore size, which improves cell migration, tissue growth, and nutrient and

waste diffusion [42]. The same type of application was obtained by using HA and fibrin networks that interpenetrate each other. The hydrogel allowed cell encapsulation, and *in vitro* tests showed an improved cell viability and proliferation when compared to pure fibrin gels. This hydrogel is suitable for TE applications and for 3D cell culturing [43].

Conclusions and future perspectives

HA and HA-based materials are extensively used for the preparation of scaffolds for TE. These scaffolds offer the advantages of increased biocompatibility, controllable degradation rate by using a cross-linker, and suitable porosity for cell encapsulation, differentiation and proliferation. HA-based scaffolds have been used for a variety of tissues, of which the cartilage tissue being the most intensively studied [44–47]. Further research and development will be carried out in the future regarding the preparation of HA-based scaffolds for TE.

Conflict of interests

The authors declare that they have no conflict of interests.

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