CHAPTER NINE

Beneficial Effects of Hyaluronic Acid

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Abstract

Biomaterials are playing a vital role in our day-to-day life. Hyaluronan (hyaluronic acid), a biomaterial, receives special attention among them. Hyaluronic acid (HA) is a polyanionic natural polymer occurring as linear polysaccharide composed of glucuronic acid and *N*-acetylglucosamine repeats via a β-1,4 linkage. It is the most versatile macromolecule present in the connective tissues of all vertebrates. Hyaluronic acid has a wide range of applications with its excellent physicochemical properties such as biodegradability, biocompatibility, nontoxicity, and nonimmunogenicity and serves as an excellent tool in biomedical applications such as osteoarthritis surgery, ocular surgery, plastic surgery, tissue engineering, and drug delivery. It plays a key role in cushioning and lubricating the body and is abundant in the eyes, joints, and heart valves. A powerful antioxidant, hyaluronic acid is perhaps best known for its ability to bond

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water to tissue. Hyaluronan production increases in proliferating cells, and the polymer may play a role in mitosis.

This chapter gives an overview of hyaluronic acid and its physicochemical properties and applications. This chapter gives a deep understanding on the special benefits of hyaluronic acid in the fields of pharmaceutical, medical, and environmental applications. Hyaluronic acid paves the way for beneficial research and applications to the welfare of life forms.

1. INTRODUCTION

In 1934, Karl Meyer and his assistant, John Palmer, described a procedure for isolating a novel glycosaminoglycan (GAG) from the vitreous of bovine eyes. They showed that this substance contained an uronic acid and an amino sugar but without sulfoesters. In their words: "we propose, for convenience, the name 'hyaluronic acid', from hyaloid (vitreous) + uronic acid." This marked the birth announcement for one of nature's most versatile and fascinating macromolecules "hyaluronan," as is most frequently referred to. Hyaluronan is present in all vertebrates as an essential constituent of the extracellular matrices (ECMs) in most mature tissues. It is a major constituent in the vitreous humor of the eye (0.1–0.4 mg/g wet weight in human), in synovial joint fluid (3–4 mg/ml), in the matrix produced by the cumulus cells around the oocyte prior to ovulation (~0.5 mg/ml), in the pathological matrix that occludes the artery in coronary restenosis, etc. (Hascall, Calabro, Oken, & Masellis, 2002).

The largest quantity of hyaluronan (7–8 g per average adult human, ~50% of the total in the body) resides in skin tissue, where it is in both the dermis (~0.5 mg/g wet tissue) and the epidermis (~0.1 mg/g wet tissue). Interestingly, dermis consists primarily of ECM with a sparse population of cells, while the epidermis is in the reverse; the keratinocytes fill all but a few percent of the tissue. Thus, the actual concentration of hyaluronan in the matrix around the cells in the epidermis (estimated to be 2–4 mg/ml) is an order of magnitude higher than in the dermis (estimated to be ~0.5 mg/ml) (Fraser, Laurent, & Laurent, 1997).

The concentration of hyaluronan in tissues is often higher than would be expected if individual molecules maintained their expanded domain structures. In many cases, the hyaluronan is organized into the ECM by specific interactions with other matrix macromolecules. However, high-molecular-weight (HMW) hyaluronan at high concentration in solution (e.g., 5 MDa at concentrations above 0.1 mg/ml) can also form entangled molecular

networks through steric interactions and self-association between and within individual molecules. The latter can occur when a stretch of the hydrophobic face of the ribbon structure of the backbone interacts reversibly with the hydrophobic face on a comparable stretch of hyaluronan on another molecule or in a different region of the same molecule. Such networks exhibit different properties than would isolated hyaluronan molecules.

2. STRUCTURE OF HYALURONIC ACID

GAGs are a class of natural macromolecules, which is negatively charged, linear heteropolysaccharides classified into several groups on the basis of structure such as hyaluronic acid, heparin sulfate, dermatan sulfate, and chondroitin sulfate, exhibiting an attracting interest because of their several applications in the biomedical, veterinary, pharmaceutical, and cosmetic field (Linhardt, 2001; Sasisekharan, Raman, & Prabhakar, 2006). Hyaluronic acid (HA) (Fig. 9.1) is a naturally occurring linear polysaccharide composed of glucuronic acid and *N*-acetylglucosamine repeats via a β-1,4 linkage. It is a linear polymer consists of repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid linked by β(1,4) and β(1,3) glycosidic linkages, respectively, and molecular mass in the range from 104 to 107 Da. It is distinct from other GAGs by the nonexistence of sulfated groups and lack of covalently linked peptide in their structure (Puré & Assoian, 2009; Stern, 2003).

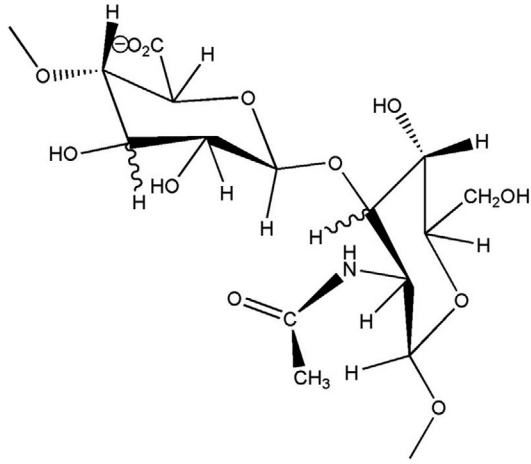

Figure 9.1 Structure of hyaluronic acid (HA).

HA is the most important substance in the synovial fluid (SF) of articular joints, acting as a lubricant for the cartilage and regulating the viscosity of the synovial fluid. Healthy joints contain approximately 2.26 g/L HA (Ziedler, 1986). HA macromolecular chains are built from D-glucuronic acid and *N*-acetyl-D-glucosamine disaccharides. A molecule of 10 MDa contains 25,000 disaccharide units in the chains, which are held together by hydrophobic bonds (Romagnoli et al., 2008). The polysaccharide chains are linear and unbranched and roll up into a coil conformation. These coils can straighten, and this behavior is the mechanism of action behind viscosupplementation. The length of the polysaccharide chains and the MW of the HA are very different in various tissues. In normal tissues, a molecule of HA (10 MDa) has a thickness of 1 nm and a length of 25 mm (Romagnoli & Belmontesi, 2008). In the biomatrix, HA has an MW in the range of 6–12 MDa (Balazs, 2009). The molecular weight of HA is approximately 7 MDa in healthy joints and 4.8 MDa in unhealthy joints (Wohlrab, Neubert, & Wohlrab, 2004). HA is a polymer formed by repeating disaccharide units of *N*-acetyl-D-glucosamine and glucuronic acid.

This GAG is present in tissues as the cartilage, synovial fluid, skin, rooster combs, umbilical cord, and vitreous humor and synovial fluid, as well as in the cell wall of bacteria such as *Streptococcus zooepidemicus* (Shiedlin et al., 2004; Vázquez et al., 2009; Yamada & Kawasaki, 2005). HA is abundantly present in almost all biological fluids and tissues. For experimental purposes, commercial HA samples are mostly of bacterial origin (gram-positive *Streptococci*) or isolated from rooster combs, while in the human organism, it is distributed in the skin, vitreous humor of the eye, umbilical cord, cartilage, and synovial fluid. In the latter, methods for HA production have emphasized mainly on the environmental or culture conditions for cell growth and HA formation rather than focusing on cellular metabolism and its regulation for obtaining higher yield and molecular weight (Johns, Tang Goh, & Oeggeru, 1994). The biosynthesis of HA faces a stiff competition between glycolytic pathway and cell wall synthesis (Liu, Du, Chen, Wang, & Sun, 2008). It has also been reported that the molecular weight of HA is controlled by the concentration of the precursor UDP-*N*-acetylglucosamine, which is limiting compared with the other precursor UDP-glucuronic acid (Chen, Marcellin, Hung, & Nielsen, 2009). But to increase the productivity and the molecular weight of HA, a balanced flux of these precursors toward HA biosynthesis was analyzed by Shah, Badle, and Ramachandran (2013) and found that concentration and molecular weight of HA are increased by decreasing carbon flux toward glycolysis and pentose phosphate pathway

and increasing carbon flux toward HA precursor formation. Also, the addition of antioxidant tannic acid also increased molecular weight to 3.0 MDa.

HA serves to maintain its viscoelastic properties required for lubrication of the joint. When hyaluronan is degraded by the action of free radicals or certain enzymes, synovial fluid loses its lubricating properties, which leads to increased wear of the joint and results in arthritic pain. HA has a wide variety of cosmetic and pharmaceutical applications, for instance, to fill soft tissue defects such as facial wrinkles or to treat articular disorders in horses.

3. PROPERTIES OF HYALURONIC ACID

The HA solutions' rheological properties are very important for these applications; for example, ophthalmic viscosurgical devices are classified according their rheological behavior. The rheological characterization of hyaluronic acid aqueous solutions has been carried out by Garcia-Abuin, Gomez-Diaz, Navaza, Regueiro, and Vidal-Tato (2011), determining the value of the intrinsic viscosity and the average molecular weight. The influence of the polymer concentration, temperature, and presence of an electrolyte on the magnitude of the viscosity, density, speed of sound, and rheological behavior has been analyzed. The presence of HA in an aqueous solution showed a complex rheological behavior, including this system into the pseudoplastic fluids. Both the increase of temperature and the presence of an electrolyte produced an important decrease on the viscosity magnitude, as well as an approximation to the Newtonian behavior in relation to its rheology. Procedures for introducing covalent cross-links in hyaluronan matrices have been developed to create stable networks and semisolid materials exhibiting pronounced viscoelastic properties (Laurent, 1998).

4. MODIFICATION OF HYALURONIC ACID

The potential application of HA is limited due to its high water solubility and rapid degradation in the human body. Chemical modification is applied to improve the physicochemical stabilities while retaining its natural biocompatibility, biodegradability, and nonimmunogenicity (Luo, Kirker, & Prestwich, 2000). The general methods to produce HA derivatives include esterification, cross-linking, grafting, and composite modification. Composite modification is attracting more and more attention for its unique advantages compared with other modification methods.

Chemical modifications have been mostly performed on HA through grafting techniques, and, in spite of several works published in this field by employing "grafting to" technique, the control of molecular weight (MW) and polydispersity index (PDI) is still a major issue that needs to be addressed since these two parameters affect the chemical, physical, and biological properties of the final product. Atom transfer radical polymerization (ATRP), discovered by Matyjaszewski and Sawamoto in 1995 (Wang & Matyjaszewski, 1995), is a versatile controlled radical polymerization process. It enables a precise control of MW, PDI, and functionality (Coessens, Pintauer, & Matyjaszewski, 2001).

Hyaluronic acid was also modified by grafting various amino acids on its carboxylic group and then Schanté, Zuber, Herlin, and Vandamme (2012) evaluated the enzymatic stability of the various conjugates in the presence of a hyaluronidase. The results showed that all amino acid-modified HA polymers were more resistant to degradation compared with the native HA albeit with variation according to the amino acids. The findings are consistent with results reported previously (Ibrahim, Kang, & Ramamurthi, 2010) and confirm the beneficial effect of the carboxyl protection of HA with amino acids.

HA was cross-linked with carboxymethylcellulose sodium (CMC-Na), a carbohydrate-derived biomaterial, to form a novel HA-based composites by Liu, Liu, Wang, Du, and Chen (2007). A series of sponge-like composites were prepared by cross-linking different amounts of hyaluronic acid (HA) and CMC-Na. Adipic dihydrazide (ADH) was employed as the cross-linker and water-soluble 1-ethyl-3-[3-(dimethylaminopropyl)] carbodiimide as the carboxyl-activating agent. The prepared composites showed high swelling ratio, improved physicochemical stability, and high antioxidant ability, which makes them potential materials to be used as dermal fillers or applied in soft tissue augmentation or filling.

HMW hyaluronic acid in powder form (HMWHA, with average molecular weight of 1042 kDa) was degraded to low-molecular-weight hyaluronic acid (LMWHA, 200–230 kDa) by several methods, and the changes in molecular structure and antioxidative activities brought about by each degradation method were compared. The degradation methods used were electron beam irradiation (EB), gamma ray irradiation (GM), microwave irradiation, and thermal treatment. The FT-IR spectra showed no substantial changes of the spectral pattern between HMWHA and LMWHA. However, the ultraviolet (UV) absorbance of LMWHA by MW was considerably greater at 265 nm, indicating the formation of more double bonds. The antioxidative activities of all LMWHA samples

were found to have risen, but the MW-treated LMWHA showed the most significant increase due to a newly formed double bond. EB- and GM-treated LMWHA showed the lowest polydispersity and little change in UV spectra from those of HMWHA (Choi, Kim, Kim, Kweon, & Lee, 2010).

Recently, LMW hyaluronic acid has been reported to have novel features, such as free radical scavenging activities, antioxidant activities, and promotion of excisional wound healing (Ke, Sun, Qiao, Wang, & Zeng, 2011). It has also been reported that GM of the native hyaluronic acid could increase its antioxidant activity as a result of a decrease in molecular weight (Kim, Ravichandran, Khan, & Kim, 2008). The hyaluronic acids with an average molecular weight in the range of 45.2–145 kDa were shown to possess pronounced free radical scavenging and antioxidant activities, particularly compared with the native hyaluronic acid of 1050 kDa. LMW hyaluronic acid was prepared through degradation of native hyaluronic acid by ozone treatment (Yue, 2012).

In recent years, the deposition of polyelectrolyte multilayers by layer-by-layer (LBL) technique has emerged as a promising tool for the functionalization of various substrates due to their ease of formation and flexibility of tailoring physicochemical properties (Boudou, Crouzier, Ren, Blin, & Picart, 2010). An attempt for the functionalization of photocross-linked HA hydrogels by deposition of poly(L-lysine) (PLL) and HA multilayer films made by the LBL technique was made by Yamanlar, Sant, Boudou, Picart, and Khademhosseini (2011). Modification of HA hydrogel surfaces with multilayer films affected their physicochemical properties and improved cell adhesion and spreading of NIH3T3 fibroblasts cells on these surfaces, making them suitable for various biomedical and tissue engineering (TE) applications including growth factor delivery and coculture systems.

The synthesis and physicochemical characterization of mixed lipoic and formic esters of hyaluronan (Lipohyal) were carried out by Picotti et al. (2013). The synthesis was conducted by activating lipoic acid with 1,1-carbonyldiimidazole to obtain lipoyl imidazolide, which reacted with hyaluronan (HA) in formamide under basic conditions. Lipohyal can be easily cross-linked by UV irradiation, resulting in an innovative hydrogel with distinctive viscoelastic properties that is suitable both as a dermal filler and as an intra-articular medical device.

It has been revealed that coupling of polyelectrolytes with ionic surfactants is a convenient method for the preparation of ionic complexes with remarkable structure and properties (Macknight, Ponomarenko, &

Tirrell, 1998). Specifically, coupling of polyacids with tetraalkylammonium surfactants bearing long alkyl chains is known to lead to amphiphilic comb-like systems displaying a layered biphasic structure able to lodge agents with chemical or biomedical activity. Stoichiometric complexes of hyaluronic acid with alkyltrimethylammonium surfactants bearing octadecyl, eicosyl, and docosyl groups were prepared by ionic coupling in aqueous solution by Tolentino, Alla, de Ilarduya, and Guerra (2013). The complexes were insoluble in water but soluble in organic solvents. In the solid state, they self-assembled in a biphasic layered structure with the alkyl side chains forming a separate phase that melted in the 50–60 °C range. They were stable to heating up to above 200 °C.

An efficient method for the synthesis of hyaluronic acid-based brush copolymers using ATRP has been reported by Pitarresi et al. (2013). At first, two different hyaluronic acid (HA)-based macroinitiators have been prepared, and then they have been used for the polymerization via ATRP of hydrophilic or hydrophobic molecules carrying vinyl portions with two macroinitiators (HA–TBA–BMP and HA–TBA–EDA–BMP). Then they have been used for the ATRP of poly(ethylene glycol) methacrylate (PEGMA), butyl methacrylate (BUTMA), or *N*-isopropylacrylamide (NIPAM) using a complex of Cu(I) and 2,2′-Bipyridyl (Bpy), as a catalyst. Both macroinitiators and final copolymers, named as HA–BMP–pPEGMA, HA–BMP–pBUTMA, HA–BMP–pNIPAM, HA–EDA–BMP–pPEGMA, HA–EDA–BMP–pBUTMA, and HA–EDA–BMP–pNIPAM, have been characterized by spectroscopic analysis and size exclusion chromatography to confirm the success of the polymerization process.

A series of thermosensitive copolymer hydrogels, aminated hyaluronic acid-g-poly(NIPAM) (AHA-g-PNIPAAm), were synthesized by coupling carboxylic end-capped PNIPAAm (PNIPAAm–COOH) to AHA through amide bond linkages. AHA was prepared by grafting ADH to the HA backbone, and PNIPAAm–COOH copolymer was synthesized via a facile thermoradical polymerization technique by polymerization of NIPAAm using 4,40-azobis(4-cyanovaleric acid) as an initiator (Tan et al., 2009). This newly described thermoresponsive AHA-g-PNIPAAm copolymer demonstrated attractive properties to serve as cell or pharmaceutical delivery vehicles for a variety of TE applications.

A thiolated HA derivative, 3,30-dithiobis-(propanoic dihydrazide)-modified HA (HA-DTPH), was synthesized to fabricate nanofibrous scaffolds. Poly(ethylene oxide) (PEO) was blended with HA-DTPH as a viscosity modifier to facilitate the fiber formation during electrospinning.

A uniform HA-DTPH/PEO nanofibrous scaffold without beads was fabricated, which was cross-linked through poly(ethylene glycol) diacrylate (PEGDA)-mediated conjugate addition. PEO was subsequently extracted using DI water and an electrospun HA-DTPH nanofibrous scaffold was finally obtained. NIH3T3 fibroblasts attached to the scaffold and spread, demonstrating an extended dendritic morphology within the scaffold, which suggests potential applications of HA-DTPH nanofibrous scaffolds in cell encapsulation and tissue regeneration (Ji, Ghosh, Shu, et al., 2006). The effect of the electrospinning solvent on electrospinnability was investigated by Liu et al. (2011). The addition of formic acid greatly improved the electrospinnability of HA solution, and pure HA nanofibers with a mean diameter below 100 nm were fabricated under the optimal condition.

Two polysaccharides, LMW hyaluronic acid-1 (LMWHA-1) and LMW hyaluronic acid-2 (LMWHA-2), with their molecular weight of 1.45–105 and 4.52–104 Da, respectively, were prepared from HMW hyaluronic acid (HMWHA, 1.05–106 Da) by Ke et al. (2011). LMWHA-1, LMWHA-2, and HA were studied for their antioxidant activities. In *in vitro* antioxidant assay, LMWHA showed strong inhibition of lipid peroxidation and scavenging activities of hydroxyl radical, moderate 1,1-diphenyl-2-picrylhydrazyl radical, and superoxide anion scavenging activity. In addition, the LMWHA-1 exhibited much stronger antioxidant activity than LMWHA-2 and HA. They also proved that the administration of LMWHA was able to overcome cyclophosphamide (CY)-induced immunosuppression and significantly raised the activity of superoxide dismutase, catalase, and glutathione peroxidase and total antioxidant capacity in immunosuppressed mice.

The structural changes of gamma irradiated HA were studied by gel-permeation chromatography, viscosity, pH, Hunter color measurement, UV spectrophotometry, and FT-IR spectroscopy by Kim, Srinivasan, et al. (2008). The results demonstrated that GM decreased molecular weight size, viscosity, and pH of the hyaluronic acid and its color turned to intense yellow. UV spectra of the irradiated HA showed a change at 265 nm, which indicates the formation of double bonds. Differences in the height and shape of certain absorption bonds of FT-IR spectra in the range 1700–1750 cm^{-1} were also observed, which is associated with the formation of carboxylic acid. From these structural changes of the HA, GM may have a role in the formation of pyrancarboxylic acid rings. DPPH radical scavenging ability and the reducing power of gamma irradiated HA were significantly higher than that of nonirradiated HA.

5. APPLICATIONS OF HYALURONIC ACID

The unique viscoelasticity and limited immunogenicity of hyaluronic acid have led to its use in several biomedical applications, such as viscosupplementation in osteoarthritis (OA) treatment, as an aid in eye surgery, and for wound regeneration. In addition, HA has recently been explored as a drug delivery agent for different routes such as nasal, oral, pulmonary, ophthalmic, topical, and parenteral and also in TE applications.

5.1. Biomedical applications

Bacterial contamination of materials is of crucial importance in diverse fields such medical, food, or cosmetic industries. Once adhered on a surface, bacteria form colonies and subsequently biofilms that serve as reservoirs for the development of pathogenic infections. Bacterial biofilm infections are particularly problematic because sessile bacteria can withstand host immune responses and are drastically more resistant to antibiotics, biocides, and hydrodynamic shear forces than their planktonic counterparts (Mah & O'Toole, 2001). Prevention of biofilm formation is clearly preferable to any treatment strategy (Glinel, Thebault, Humblot, Pradier, & Jouenne, 2012). An efficient approach to prevent biofilm formation consists in immobilizing a bactericidal molecule on the support. Thus, various synthetic approaches based on the coating, grafting, or release of bactericidal substances such as metal derivatives, poly(ammonium salts), and antibiotics have been extensively explored to produce antimicrobial material.

Nisin (an antimicrobial peptide) has been attached to hyaluronic acid (HA) to obtain an antimicrobial biopolymer under solution or gel form. Various amounts of peptide have been grafted onto HA through a controlled reaction to obtain a covalently grafting by the formation of amide bonds. This modified HA exhibited a great antimicrobial property on the three tested bacterial species and proved as a potential material to avoid bacterial contamination in various applications as wound dressings, contacts lenses, cleaning solutions for contact lenses, and cosmetics formulations (Lequeux, Ducasse, Jouenne, & Thebault, 2014).

Diabetic foot ulcer (DFU) is one of the major complications associated with diabetic mellitus. Neuropathy and ischemia are two major etiologic factors leading to DFU (William & Keith, 2003). Intensive care should be taken for patients with DFU for the prevention of amputation. These ulcers tend to heal slowly, and healing can be complicated by polymicrobial infection and heavy exudate formation, which place these patients at a

higher risk for limb amputation (Schwartz et al., 2013). Wound dressings with multiple performance parameters are needed for the treatment of DFU. A modern wound dressing should include many factors more than just exudate absorption. It should be able to keep up a moist interface between the wound and dressing, prevent infection, and create an optimal environment that supports healing with esthetically satisfactory scar (Hilton, Williams, Beuker, Miller, & Harding, 2004). An antimicrobial sponge composed of chitosan, hyaluronic acid (HA), and nanosilver (nAg) as a wound dressing for DFU infected with drug-resistant bacteria was prepared by Anisha, Biswas, Chennazhi, and Jayakumar (2013). nAg (5–20 nm) was prepared and mixed with chitosan and hyaluronic acid and characterized. The antimicrobial studies with selected bacteria suggest that these nanocomposite sponges could be used as a potential material for wound dressing for DFU infected with antibiotic-resistant bacteria such as *Escherichia coli*, *Staphylococcus aureus*, methicillin-resistant *S. aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia* if the optimal concentration of nAg exhibiting antibacterial action with least toxicity toward mammalian cells was identified.

HA is an attractive starting material for the construction of bulk gels or hydrogel particles (Xu, Jha, Harrington, Farach-Carson, & Jia, 2012), but its applications in bone TE are limited by its poor mechanical properties. For this reason, it is often associated with calcium phosphates in order to obtain reinforced and/or injectable bone cements, consisting in HA gels containing hydroxyapatite (Nageeb et al., 2012) or calcium phosphate cements (CPCs) containing HA (Ahmadzadeh-Asl et al., 2011). Incorporating drug-loaded microspheres in mineral bone cements is an alternative strategy to improve their ability as drug delivery materials. To synthesize microspheres according to a reproducible process and control at the same time their morphology and their encapsulation efficiency is one of the main challenges of the conception of such drug-loaded bone substitute. In this context, we investigated the potentialities of two HAs, differing by their molecular weight, to form microspheres by a spray-drying technique. Erythrosin B was encapsulated as a model drug, and spray-drying process conditions were optimized by Fatnassi et al. (2013). HA molecular weight and concentration appeared to have a significant influence on process parameters and resulting microspheres. However, the introduction of HA microspheres in a mineral cement led to a sustained release, due to HA gel formation within the pores of the mineral matrix that decreases the diffusion rate. Hyaluronic acid microspheres could be of particular interest to formulate bone cements with extended ability to release active compounds.

A number of functional HA derivatives have also been developed, in order to modulate its biological properties (e.g., enzymatic degradability through esterification as in HYAFF-11 derivatives) and/or to prepare biomimetic three dimensional (3D)-extended matrices (e.g., hydrogels) or dispersible materials (e.g., nanoparticles). We are specifically interested in photopolymerization. This is a widespread method for the *in situ* preparation of TE matrices, whose biocompatibility and efficacy have been demonstrated in a number of studies (Bryant & Anseth, 2002).

Photopolymerization has been used for the preparation of HA/poly(ethylene glycol) (PEG) systems, whose degradability and mechanical properties can be controlled in a relatively independent fashion, respectively, through the HA and PEG content and molecular weight. We are specifically interested in photopolymerization. This is a widespread method for the in situ preparation of TE matrices, whose biocompatibility and efficacy have been demonstrated in a number of studies (Elisseeff et al., 2000). Photopolymerization has been used for the preparation of HA/PEG systems by Ouasti et al. (2011), whose degradability and mechanical properties were controlled in a relatively independent fashion, respectively, through the HA and PEG content and molecular weight.

Polymer conjugation has become an important strategy, but one challenge has been in controlling pharmacokinetics while maintaining affinity for the target (Gilli, Ferretti, & Gilli, 1994). For therapeutic proteins, conjugation of PEG is an established strategy for increasing circulation time, inhibiting enzymatic degradation, and improving solubility. PEG is an uncharged polyether with established solubility and biocompatibility (DeNardo et al., 2003). The benefits of PEGylation are that the PEG chain helps to protect the compound from enzymatic degradation and that the increased size decreases clearance rates. Additionally, the hydrophilic nature of the PEG chain can help to reduce aggregation and improve the solubility of the therapeutic. A study on a model peptide inhibitor of tumor necrosis factor-α to investigate the effects of site-specific conjugation to HA and PEG was carried out by Elder, Hannes, Atoyebi, and Washburn (2013). The results suggest that conjugation strategies involving both PEG and charged polymers, such as HA, could result in significant enhancements in the activities of therapeutic proteins.

Atherosclerosis is a chronic inflammatory condition of the blood vessel wall that can lead to arterial narrowing and subsequent vascular compromise. Although there are a variety of open and endovascular procedures used to alleviate the obstructions caused by atherosclerotic plaque, blood vessel

instrumentation itself can lead to renarrowing of the vessel lumen through intimal hyperplasia, wound contracture, or a combination of the two. However, biologically active elements of the ECM are also important in the vascular remodeling that takes place in both atherosclerosis and renarrowing of the vessel lumen after instrumentation (Toole, Wight, & Tammi, 2001). One such element of the ECM that is important in both of these processes is hyaluronic acid (HA), otherwise known as hyaluronan (Bot, Hoefer, Piek, & Pasterkamp, 2008). HA is upregulated in areas of vascular injury and has been shown to increase VSMC migration and proliferation, key events in the progression of atherosclerosis and vascular renarrowing after surgical intervention (Benjamin Sadowitz, Keri Seymour, Vivian Gahtan, & Maier, 2012).

OA is a degenerative and debilitating disorder of diarthrodial joints affecting approximately 70% of 70-year-olds. It is associated with progressive damage of articular cartilage, leading to considerable pain resulting in loss of mobility and the requirement of continuous healthcare for patients (Yelin, 1992). The natural joint is lubricated by synovial fluid, which is highly viscous, enabling it to cushion and lubricate the joint. Hyaluronic acid (HA) is a major component of SF and articular cartilage and plays an important role in the lubrication of the cartilage surface in addition to helping maintain the structural resistance of cartilage to compressive forces. The application of HA and dipalmitoyl phosphatidylcholine onto damaged human cartilage resulted in improved lubrication between the cartilage surfaces on friction within a human cartilage damage model (Forsey et al., 2006).

CY is an anticancer and immunosuppressant drug that induces the production of reactive oxygen species (ROS). The excessive production of ROS plays multiple important roles in tissue damage and loss of function in a number of immune tissues and organs. For example, ROS can induce cell death by injuring the DNA of normal cells, resulting in the damage of the immune system (Diaz-Montero et al., 2012). It has been reported that polysaccharides can attenuate this oxidative damage of a tissue indirectly by enhancing natural defenses of cell and/or directly by raising the immunostimulatory activity (Chen et al., 2012; Wang et al., 2011). The immunostimulatory activities of two LMW hyaluronic acids (LMWHA-1 and LMWHA-2 with MW of 1.45–105 and 4.52–104 Da, respectively) and HA (MW, 1.05–106 Da) were evaluated using *in vitro* cell models and *in vivo* animal models, and their effects on angiogenesis were measured *in vivo* using the chick embryo chorioallantoic membrane assay by Ke et al. (2013). The results demonstrated that LMWHA-1, LMWHA-2, and HA

could promote the splenocyte proliferation, increase the activity of acid phosphatase in peritoneal macrophages, and strengthen peritoneal macrophages to devour neutral red *in vitro* in a dose-dependent manner. Furthermore, LMWHA-1 and LMWHA-2 exhibited much stronger immunostimulatory activity than HA.

Technique commonly used in orthopedics is the insertion of prostheses in the body for the fixation or reconstruction of bones or their parts. Prostheses are generally made of biocompatible metals (in particular titanium and cobalt chrome), polymers, ceramics, hydroxyapatite, or their combinations (e.g., metals coated with a layer of hydroxyapatite). Bacterial infections due to implanted prosthesis still represent a serious complication in orthopedic surgery. Studies indicate that the procedures for implanting a prosthesis and the presence of the prosthesis itself in the site of bone fracture damage the response of the local immune system with the result that the number of bacteria required to cause an infection can fall by a factor of even 10,000 (Flückiger & Zimmerli, 2000). Several researchers have proposed antibacterial materials with nonfouling properties, in particular for use as coatings of the orthopedic prostheses; such materials should preferably be capable to release the drug immediately after the surgical operation and at least during the following 6 h, preferably up to 48–72 h, so as to cover the critical period of possible bacterial attack and proliferation in the intervention site (Yeap et al., 2006). Physical hydrogels have been obtained by Giammona et al. (2013) from hyaluronic acid derivatized with polylactic acid in the presence or in the absence of PEG chains. They have been extemporarily loaded with antibacterial agents, such as vancomycin and tobramycin. These medicated hydrogels have been used to coat titanium disks (chosen as simple model of orthopedic prosthesis), and *in vitro* studies in simulated physiological fluid have been performed as a function of time and for different drug loading and polymer concentration values. Obtained results suggest the potential use of these hydrogels in the orthopedic field, in particular for the production of antibacterial coatings of prostheses for implant in the human or animal body in the prevention and/or treatment of postsurgical infections.

Organic–inorganic materials, combining functional properties of inorganic compounds and polymers, are of significant interest for biomedical applications. Composite films, containing halloysite nanotubes (HNTs), hydroxyapatite (HAp), and biocompatible polymers, are currently under intensive investigation. Electrophoretic deposition (EPD) is an attractive method for the deposition of composite films for biomedical applications.

This method is widely used for the deposition of inorganic materials, polymers, and composites. EPD is based on the electrophoretic motion of colloidal particles or polymer macromolecules under the influence of an electric field and deposit formation at the electrode surface (Zhitomirsky, 2002). Anodic EPD of composite films, containing HNT and HA in an HYH matrix, was carried out by Deen and Zhitomirsky (2014). The results demonstrated that HYH can be used as efficient charging and dispersing agent for HNT and HA in suspensions and film-forming agent for EPD of HNT–HA–HYH films.

The use of osteotransductive CPCs in vertebroplasty is limited by their low injectability and disintegration. Liquid-phase separation, termed filter pressing, is a frequent problem for cement injectability (Bohner, Gbureck, & Barralet, 2005). Viscosity-enhancing agents such as hyaluronic acid and chondroitin-4-sulfate are used to improve the injectability of brushite (chronOS Inject, Synthes) and apatite (Biopex, Mitsubishi Materials Corporation) cements, respectively. The adhesiveness of brushite cements due to the presence of sodium hyaluronate supported intimate contact between the cement and bone surface (Apelt et al., 2004). Moreover, the addition of hyaluronic acid to an injectable brushite cement had little effect on its osteoconductive properties, except for a light decrease in initial resorption rate (Flautre, Lemaitre, Maynou, Van Landuyt, & Hardouin, 2003).

Recently, attention has been paid to the use of microneedles fabricated from biocompatible and biodegradable polymers (Donnelly et al., 2011; Jin, Han, Lee, & Choi, 2009) and carbohydrates (Ito, Murano, Hamasaki, Fukushima, & Takada, 2011), which are free from the risk of complications. If left in the skin, these types of needles safely degrade and eventually disappear. They also have the potential for loading drugs into a matrix of needles and releasing them in the skin by biodegradation or dissolution in the interstitial fluid: a one-step application. Hyaluronic acid (HA) was used (Liu et al., 2014) to fabricate novel dissolving microneedle arrays in this study. HA is a water-soluble polymer of disaccharides, naturally found in many tissues, such as the skin, the cartilage, and the vitreous humor. In 2003, the FDA approved HA injections for filling soft tissue defects. This study indicated that self-dissolving HA microneedle arrays are a useful alternative to improve the transdermal delivery of drugs, especially drugs with relatively HMW without seriously damaging the skin. The HA-fabricated microneedle arrays containing alendronate and insulin were found be effective for improving the transdermal drug delivery.

5.2. TE applications

TE is a field of research that is aimed at regenerating tissues and organs (Daamen et al., 2003). Cells, scaffolds, and growth factors are the three main components for creating a tissue-engineered construct. The principal aim of a scaffold design should be to mimic the native ECM of the target tissue as much as possible (Ma, Gao, Gong, & Shen, 2005). The development of biodegradable polymers to perform the role of a temporary matrix is an important factor in the success of cell transplantation. Cells, scaffold and growth stimulating signals are generally refer to as the tissue engineering triad, the key components of engineered tissues.

Collagen type I, a major protein of the ECM in mammals, is a suitable scaffold material for regeneration. Another important constituent of the ECM, hyaluronic acid (hyaluronan, HA), has been used for medical purposes due to its hydrogel properties and biodegradability. Chitosan is a linear polysaccharide composed of b1–b4-linked D-glucosamine residues, and its potential as a biomaterial is based on its cationic nature and high charge density in solution. A study was conducted to evaluate the characteristics of scaffolds composed of different ratios of type I comb collagen and chitosan with added HA in order to obtain the optimum conditions for the manufacture of collagen–hyaluronan–chitosan (Col–HA–Ch; comprising collagen (Lin et al., 2009). Overall, the 9:1:1 mixing ratio of collagen, hyaluronan, and chitosan was observed to be optimal for the manufacture of complex scaffolds. Furthermore, Col–HA–Ch tripolymer scaffolds, especially Col9HACh1, could be developed as a suitable scaffold material for TE applications.

5.2.1 Lung TE applications

Diseases like pulmonary hypoplasia (found in neonates) and emphysema (a chronic lung disease) have a deficient alveolar epithelium, tissue loss, or reduced alveolar surfactant synthesis. In all these instances, TE represents an attractive potential for regeneration or augmentation with engineered functional pulmonary tissue. Several strategies are being adopted to evolve suitable scaffolds for lung TE. There is a growing interest in blending natural and synthetic polymers as biomaterials for creating complex structures, which will act as scaffolds for TE. Turner, Kielty, Walker, and Canfield (2004) and Amarnath, Srinivas, and Ramamurthi (2006) had shown a commercial benzyl ester of HA and laboratory cross-linked hylan as excellent biomaterials for the promotion of adherence of vascular endothelial cells and vascular TE.

In spite of many potential applications of HA, it has some inherent drawbacks. Chemical modification through grafting has received considerable attention in the area of biomedical applications. Poly(HEMA) is one of the most important hydrogels in the biomaterials world since it has many advantages over other hydrogels (Pescosolido et al., 2011). These include a water content similar to living tissue, inertness to biological processes, resistance to degradation, permeability to metabolites, and resistance to absorption by the body. Hence, a graft copolymer of HA and poly(HEMA) appeared as a good choice for the synthesis of a natural–synthetic polymer hybrid matrix for use as a scaffold for lung TE.

An "*in situ*" biodegradable gel consisting of chitosan, glycerol phosphate (GP), and oxidized hyaluronic acid (HDA) was synthesized and characterized by Nair, Remya, Remya, and Nair (2011). This is a two-component hydrogel system where chitosan neutralized with GP resulted in instantaneous gelling when combined with HDA. The gels are cytocompatible and could be freeze-dried to form porous scaffolds. The percentage porosity of the freeze-dried chitosan hyaluronic acid dialdehyde gels (CHDA) increased with increasing oxidation. Fibroblast cells seeded onto CHDA porous scaffolds adhered, proliferated, and produced ECM components on the scaffold. Chondrocytes encapsulated in CHDA gels retained their viability and specific phenotypic characteristics. The gel material can be a good scaffold and encapsulating material for TE applications.

5.2.2 Bone TE applications

Titanium (Ti) and its alloy are widely used in the biomedical field, such as hip joint replacement devices and heart valves, due to their excellent corrosion resistance and mechanical properties (Geetha, Singh, Asokamani, & Gogia, 2009). Among the titanium alloys employed, Ti–Nb–Zr alloy is a promising candidate material because of its low elastic modulus and shape memory effect (Li et al., 2011). The bare inert surface of titanium (Ti) alloy typically causes early failures in implants. LBL self-assembly is one of the simple methods for fabricating bioactive multilayer coatings on titanium implants. Zhang, Li, Yuan, Cuia, and Yang (2013) prepared a dopamine-modified hyaluronic acid/chitosan (DHA/CHI) bioactive multilayer built on the surface of Ti–24Nb–2Zr (TNZ) alloy. Zeta potential oscillated between −2 and 17 mV for DHA- and CHI-ending layers during the assembly process, respectively. Preosteoblast MC3T3-E1 cells were cultured on the original TNZ alloy and TNZ/(DHA/CHI)5 to evaluate the effects of DHA/CHI multilayer on osteoblast proliferation *in vitro*. The proliferation

of osteoblasts on TNZ/(DHA/CHI)5 was significantly higher than that on the original TNZ alloy. The results of this study indicate that the proposed technique improves the biocompatibility of TNZ alloy and can serve as a potential modification method in orthopedic applications.

Recent studies suggest that bone marrow stromal cells are a potential source of osteoblasts and chondrocytes and can be used to regenerate damaged tissues using a TE approach. However, these strategies require the use of an appropriate scaffold architecture that can support the formation *de novo* of either bone and cartilage tissue, or both, as in the case of osteochondral defects. A novel hydroxyapatite/chitosan (HA/CS) bilayered scaffold was developed by Oliveira et al. (2006) by combining a sintering and a freeze-drying technique and aims to show the potential of such type of scaffolds for being used in TE of osteochondral defects. Results have shown that materials do not exert any cytotoxic effect. Complementarily, *in vitro* (phase I) cell culture studies were carried out to evaluate the capacity of HA and CS layers to separately support the growth and differentiation of goat marrow stromal cells into osteoblasts and chondrocytes, respectively. The obtained results concerning the physicochemical and biological properties of the developed HA/CS bilayered scaffolds show that these constructs exhibit great potential for their use in TE strategies, leading to the formation of adequate tissue substitutes for the regeneration of osteochondral defects.

Hyaluronic acid (HA) functionalized with ethylenediamine (EDA) has been employed to graft α-elastin in different proportions by Palumbo et al. (2013). In particular, an HA-EDA derivative bearing 50 mol% of pendant amino groups has been successfully employed to produce the copolymer HA-EDA-g-α-elastin containing 32% w/w of protein. After grafting with α-elastin, the remaining free amino groups reacted with ethylene glycol diglycidyl ether (EGDGE) for producing chemical hydrogels, proposed as scaffolds for TE. The presence of α-elastin grafted to HA-EDA improves attachment, viability, and proliferation of primary rat dermal fibroblasts and human umbilical artery smooth muscle cells. Biological performance of HA-EDA-g-α-elastin/EGDGE scaffold is comparable to that of a commercial collagen type I sponge (Antema®), chosen as a positive control.

The feasibility of hyaluronic acid/sodium alginate (HA/SA) scaffold-based interpenetrating polymeric network (IPN) for the proliferation and chondrogenic differentiation of the human adipose-derived stem cells (hADSCs) was evaluated by Son et al. (2013). The hADSCs cultured in HA/SA IPN scaffold exhibited enhanced cell adhesion and proliferation compared with the HA scaffold. Superior chondrogenic differentiation of

hADSCs in HA/SA IPN scaffold, compared with HA-based scaffold, was confirmed by measuring expression levels of chondrogenic markers.

5.2.3 Stem cells for TE applications

Stem cells have the ability to self-renew and differentiate into a wide range of specialized cell types. Thus, they are very promising for the regeneration of aged, injured, and diseased tissues (Kim & De Vellis, 2009). Embryonic stem cells (ESCs), induced pluripotent stem cells, and adult stem cells are currently the primary cell source for research in the lab and clinic. ESCs, which are derived from the inner cell mass of early-stage embryos, can be differentiated into most of the cell types found in the body and can be expanded *in vitro* (Lumelsky et al., 2001). Hyaluronic acid (HA) hydrogels were synthesized that could be degraded through a combination of cell-released enzymes and used them to culture mouse mesenchymal stem cells. To form the hydrogels, HA was modified to contain acrylate groups and cross-linked through Michael addition chemistry using nondegradable, plasmin degradable, or matrix metalloproteinase degradable cross-linkers. Cells in stiffer hydrogels showed less spreading, migration, and slower proliferation rates. The information gained can provide valuable insight into the designing of hydrogels for 3D stem culture.

Mesenchymal stem cells (MSCs) are commonly used in TE applications due to their availability, ability to expand, and capacity to differentiate into multiple cell types. An important clinical application for MSCs under widespread investigation is cartilage repair. Mature hyaline cartilage is vascular and lymphatic, with cells comprising only about 5% of the tissue volume (Chung & Burdick, 2008). Current clinical methods to repair defective cartilage are limited in their ability to regenerate functional cartilage in terms of both composition and mechanics (Ahmed & Hincke, 2010). Due to these shortcomings, recent research has focused on the use of TE approaches to repair cartilage tissue.

5.2.4 Cartilage TE applications

The development of hydrogels tailored for cartilage TE has been a research and clinical goal for over a decade. Directing cells toward a chondrogenic phenotype and promoting new matrix formation are significant challenges that must be overcome for the successful application of hydrogels in cartilage tissue therapies. Gelatin–methacrylamide hydrogels have shown promise for the repair of some tissues but have not been extensively investigated for cartilage TE. Levett et al. (2014) encapsulated human chondrocytes in

Gel–MA-based hydrogels and show that with the incorporation of small quantities of photocross-linkable hyaluronic acid methacrylate, and to a lesser extent chondroitin sulfate methacrylate, chondrogenesis and mechanical properties can be enhanced.

The response of MSCs to a matrix largely depends on the composition as well as the extrinsic mechanical and morphological properties of the substrate to which they adhere to. Collagen–GAG scaffolds have been extensively used in a range of TE applications with great success. This is due in part to the presence of the GAGs in complementing the biofunctionality of collagen. In this context, the overall goal of this study was to investigate the effect of two GAG types: chondroitin sulfate and hyaluronic acid (HA) on the mechanical and morphological characteristics of collagen-based scaffolds and subsequently on the differentiation of rat MSCs *in vitro*. Morphological characterization revealed that the incorporation of HyA resulted in a significant reduction in scaffold mean pore size (93.9 μm) relative to collagen–CS (CCS) scaffolds (136.2 μm). In addition, the collagen–HyA (CHyA) scaffolds exhibited greater levels of MSC infiltration in comparison with the CCS scaffolds. These CHyA scaffolds show great potential as appropriate matrices for promoting cartilage tissue repair.

A procedure to obtain electrospun mats of hyaluronic acid (HA) stable in aqueous media in one single step has been developed. It consists in combining an HA solution with a divinyl sulfone one as cross-linker in a three-way valve to immediately electroblow their mixture. Therefore, it is necessary to cross-link it to obtain a material stable in aqueous conditions. Many attempts have been done in this line, consisting in either a physical (Wang et al., 2005) or a chemical (Xu et al., 2009) cross-linking after the electrodeposition process, in order to obtain HA mats insoluble in water. In all these works, the cross-linking was performed in a second step following the electrospinning process of the HA mat. In another approach, a dual-syringe setup was employed to combine thiolated derivatives of HA mixed with PEO with PEGDA as cross-linker agent (Ji, Ghosh, Li, et al., 2006) during the electrospinning process, but an additional second step was still required to remove the PEO.

Still, important disadvantages of these membranes are their reduced thickness and their high equilibrium water content, which imply lack of mechanical properties and limited manageability. These drawbacks could become a problem for some TE applications. This led us to combine the HA mat with another polymer into a two-layer membrane. Here, HA has been electrospun onto previously obtained mats of poly(L-lactic acid)

(PLLA), which possesses a great electrospinning processability, proper mechanical behavior, and manipulability and is a noncytotoxic and biodegradable FDA-approved polymer (Cheung, Lau, Lu, & Hui, 2007). Arnal-Pastor, Martínez Ramos, Pérez Garnés, Monleón Pradas, and Vallés Lluch (2013) developed a coelectroblowing procedure that allows the fabrication of insoluble HA nanofiber mats in a single step. It consists in mixing an HA solution with another of the cross-linker in a three-way valve just before electrospinning. These nanofibers can be electroblowed stably on previously electrospun tougher PLLA mats to obtain bilayered membranes. The flexibility of the fabrication process allows the preparation of membranes with different thicknesses and properties by varying the electrospinning times.

5.2.5 Heart TE applications

There are about 1.4 million arterial bypass operations performed using vascular grafts in the United States every year. Although the large size (6 mm) vascular grafts have been satisfactory in clinic applications, suitable small caliber (6 mm) vascular grafts are still insufficient to meet the existing clinical needs due to improper endothelialization, poor biocompatibility, and low mechanical properties. Therefore, there is a need to address the structural design and chemical composition of vascular grafts to develop an optimal small size vascular graft (Seidlits et al., 2011). An intima layer scaffold of the blood vessel for endothelialization was prepared by Zhu, Fana, and Wang (2014) using novel human-like collagen/hyaluronic acid (HLC/HA) composite at different mass ratios of 40/1, 20/1, and 10/1 by freeze-drying process. The structure, mechanical strength, degradation, and biocompatibility of the vascular HLC/HA scaffold were evaluated. The results showed that the 10/1 HLC/HA composited an optimal scaffold with (1) an interconnected porous network with a pore diameter of 12 ± 2 μm and porosity of 89.3%; (2) better mechanical properties with higher stress of 321.7 ± 15 kPa and strain of $45.5 \pm 0.2\%$ than 40/1, 20/1, and pure HLC scaffolds; (3) only 9% degradation upon immersion in PBS for 45 days at 37 °C *in vitro*; and (4) excellent biocompatibility. This study suggests that the 10/1 HLC/HA composite has a broad prospect of application as luminal vascular scaffold in the TE.

Dahlmann et al. (2013) developed a fully defined *in situ* hydrogelation system based on alginate (Alg) and hyaluronic acid (HA), in which their aldehyde and hydrazide derivatives enable covalent hydrazone cross-linking of polysaccharides in the presence of viable myocytes. A combination of HyA and highly purified human collagen I led to significantly increased

active contraction force compared with collagen only. Therefore, our *in situ* cross-linking hydrogels represent a valuable toolbox for the fine-tuning of engineered cardiac tissue's mechanical properties and improved functionality, facilitating clinical translation toward therapeutic heart muscle reconstruction.

Fibrin gel is widely used as a TE scaffold. However, it has poor mechanical properties, which often result in rapid contraction and degradation of the scaffold. An IPN hydrogel composed of fibrin and hyaluronic acid–tyramine (HA–Tyr) was developed by Lee and Kurisawa (2013) to improve the mechanical properties. The fibrin network was formed by cleaving fibrinogen with thrombin, producing fibrin monomers that rapidly polymerize. The HA network was formed through the coupling of tyramine moieties using horseradish peroxidase and hydrogen peroxide (H_2O_2). The degree of cross-linking of the HA–Tyr network can be tuned by varying the H_2O_2 concentration, producing IPN hydrogels with different storage moduli. Cell proliferation and capillary formation occurred in IPN hydrogels, which suggests that fibrin–HA–Tyr IPN hydrogels are a potential alternative to fibrin gels as scaffolds for TE applications that require shape stability.

5.2.6 Brain TE applications

A hyaluronic acid–collagen (HA–Col) sponge with an open porous structure and mechanical behavior comparable to brain tissue was developed by Wang and Spector (2009). HA–Col scaffolds with different mixing ratios were prepared by a freeze-drying technique and cross-linked with water-soluble carbodiimide to improve mechanical stability. Certain features of the mechanical properties of HA–Col scaffolds prepared with a Col/HA mixing ratio of 1:2, and pure HA sponges, were comparable to brain tissue.

Bioactive, *in situ*-forming materials have the potential to complement minimally invasive surgical procedures and enhance tissue healing. For such biomaterials to be adopted in the clinic, they must be cost-effective and easily handled by the surgeon and have a history of biocompatibility. A novel and facile self-assembling strategy to create membranes and encapsulating structures using collagen and hyaluronic acid (HA) was created by Chung, Jakus, and Shah (2013). Unlike membranes built by LBL deposition of oppositely charged biomolecules, the collagen–HA membranes described here form a diffusion barrier upon electrostatic interaction of the oppositely charged biomolecules, which is further driven by osmotic pressure imbalances. The resulting membranes have a nanofibrous architecture, thicknesses of 130 lm, and a tensile modulus (0.59 ± 0.06 MPa) that can increase

sevenfold using carbodiimide chemistry (4.42 ± 1.46 MPa). Collagen–HA membranes support MSC proliferation and have a slow and steady protein release profile (7% at day 28), offering opportunities for targeted tissue regeneration.

5.2.7 Dermal TE applications

The fabrication of new dermal substitutes providing mechanical support and cellular cues is urgently needed in dermal reconstruction. Yan et al. (2013) prepared silk fibroin/chondroitin sulfate/hyaluronic acid (HA) ternary scaffolds (95–248 μm in pore diameter and 88–93% in porosity by freeze-drying). By the incorporation of CS and HA with the SF solution, the chemical potential and quantity of free water around ice crystals could be controlled to form smaller pores in the SF/CS/HA ternary scaffold main pores and improve scaffold equilibrium swelling. This feature offers benefits for cell adhesion, survival, and proliferation. *In vivo* SF, SF/HA, and SF/CS/HA (80/5/15) scaffolds as dermal equivalents were implanted onto dorsal full-thickness wounds of Sprague-Dawley rats to evaluate wound healing. Compared with SF and SF/HA scaffolds, the SF/CS/HA (80/5/15) scaffolds promoted dermis regeneration, related to improved angiogenesis and collagen deposition.

Macroporous elastic scaffolds containing gelatin (4% or 10%) and 0.25% hyaluronic acid (HA) were fabricated by Chang, Liao, and Chen (2013) by cryogelation for application in adipose TE. These cryogels have interconnected pores (200 lm), a high porosity (>90%), and a high degree of cross-linking (>99%). The higher gelatin concentration reduced the pore size, porosity, and swelling ratio of the cryogel but improved its swelling kinetics. Compressive mechanical testing of cryogel samples demonstrated nonlinear stress–strain behavior and hysteresis loops during loading–unloading cycles but total recovery from large strains. The presence of more gelatin increased the elastic modulus, toughness, and storage modulus and yielded a cryogel that was highly elastic, with a loss tangent equal to 0.03. Porcine adipose-derived stem cells (ADSCs) were seeded in the cryogel scaffolds to assess their proliferation and differentiation. *In vitro* studies demonstrated a good proliferation rate and the adipogenic differentiation of the ADSCs in the cryogel scaffolds, as shown by their morphological change from a fibroblast-like shape to a spherical shape, decreased actin cytoskeleton content, growth arrest, secretion of the adipogenesis marker protein leptin, Oil Red O staining for triglycerides, and expression of early (LPL and PPARc) and late (aP2 and leptin) adipogenic marker genes. *In vivo* studies

of ADSCs/cryogel constructs implanted in nude mice and pigs demonstrated adipose tissue and new capillary formation; the expression of PPARc, leptin, and CD31 in immunostained explants; and the continued expression of adipocyte-specific genes. Both the *in vitro* and *in vivo* studies indicated that the gelatin/HA cryogel provided a structural and chemical environment that enabled cell attachment and proliferation and supported the biological functions and adipogenesis of the ADSCs.

5.3. Drug delivery applications

Drug release is another interesting application and formulations of HA, and its derivatives have been developed as topical, injectable, and implantable vehicles for the controlled and localized delivery of biologically active molecules (Vasiliu, Popa, & Rinaudo, 2005). HA has also been shown to have an antiplatelet activity (Burns & Valeri, 1996), which is important in avoiding thrombus formation; hence, HA is also used as a coating for blood-contacting implants.

A system that formulates or device that delivers therapeutic agent(s) to the desired body location(s) and/or provides timely release of therapeutic agent(s), such a system by which a drug is delivered, can have a significant effect on its efficacy. On the other hand, the very slow progress in the efficacy of the treatment of severe diseases has suggested a growing need for a multidisciplinary approach to the delivery of therapeutics to targets in tissues. From this, new ideas on controlling the pharmacokinetics, pharmacodynamics, nonspecific toxicity, immunogenicity, biorecognition, and efficacy of drugs were generated. These new strategies, often called drug delivery systems (DDS), are based on interdisciplinary approaches that combine pharmaceutics, polymer science, analytical chemistry, bioconjugate chemistry, and molecular biology (Allen, 2002).

Regulated drug release from biodegradable polymer matrices has been widely examined in order to release dispersed or dissolved drug in proportion to degradation of the polymer matrix. Recently, several types of biodegradable polymers have been extensively studied to obtain drug release that is responsive to a biological and external stimulus (Heller, 1985). A number of studies reported that hyaluronic acid (HA) is capable of being used as a drug delivery agent along various administration routes, including ophthalmic, nasal, pulmonary, parenteral, and topical applications (Yadav, Mishra, & Agrawal, 2008).

The benefits of using HA as a drug delivery vehicle are that it is biocompatible, nontoxic, noninflammatory, and biodegradable; it can efficiently

function as a "homing device" because the HA receptor cluster determinant 44 (CD44) is overexpressed in many types of tumor cells; it provides protection to its "cargo"; and it imparts solubility to hydrophobic drugs.

Despite phenomenal advances in the inhalable, injectable, transdermal, nasal, and other routes of administration, the unavoidable truth is that oral drug delivery remains well ahead of the pack as the preferred delivery route (Masaoka, Tanaka, Kataoka, Sakuma, & Yamashita, 2006). Oral delivery continues to be the most popular route of administration due to its versatility, ease of administration, and probably most importantly patient compliance. HA is used as a carrier in various oral formulations such as microspheres and complexes to improve the solubility and bioavailability of poorly water-soluble drugs (Piao et al., 2007); however, several limitations still exist and influence the gastrointestinal absorption of exogenous HA when it is administered orally, such as its relatively HMW and poor liposolubility (Huang, Ling, & Zhang, 2007).

Nanochitosan (NC)-capturing hyaluronic acid (HA) multilayer films were reported by Park et al. (2012). The films were designed to deliver various biomaterials, such as charged, uncharged, hydrophobic, and hydrophilic molecules such as peptides and proteins. Biodegradable NC-capturing HA multilayer films are expected to perform dual roles within a single platform as capturing NCs at desired amount within the films as well as the multilayer film buildup capability. This drug-loaded multilayered system demonstrates a considerable promise for drug-eluting systems to prevent restenosis. The HA multilayer film capturing paclitaxel (PTX)-loaded NCs efficiently induced the hSMC apoptosis and inhibited the hSMC proliferation, showing potentials of the HA multilayer films as effective DDS.

Hyaluronan-based hydrogels formulated to include heparin (Heprasil™) with similar gels without heparin (Glycosil™) were compared for their ability to deliver bioactive BMP-2 *in vitro* and *in vivo*. The osteogenic activity of BMP-2 released from the hydrogels was evaluated by monitoring alkaline phosphatase activity and SMAD 1/5/8 phosphorylation in mesenchymal precursor cells. The osteoinductive ability of these hydrogels was determined in a rat ectopic bone model by 2D radiography, 3D m-CT, and histological analyses at 8 weeks postimplantation. Both hydrogels sustain the release of BMP-2. Importantly, the inclusion of a small amount of heparin (0.3%, w/w) attenuated release of BMP-2 and sustained its osteogenic activity for up to 28 days. In contrast, hydrogels lacking heparin released more BMP-2 initially but were unable to maintain BMP-2 activity at later time points. Ectopic bone-forming assays using transplanted hydrogels

emphasized the therapeutic importance of the initial burst of BMP-2 rather than its long-term osteogenic activity. Thus, tuning the burst release phase of BMP-2 from hydrogels may be advantageous for optimal bone formation.

5.4. Gene delivery applications

Gene therapy has greatly advanced as a promising therapeutic tool for treating genetic disorders as well as tumors in the past decade. However, the bottleneck for gene therapy is delivery. The natural anionic polysaccharide hyaluronic acid (HA) was modified by introducing reduction-sensitive disulfide bond between the carboxyl groups and the backbone of HA (HA–SS–COOH). HA–SS–COOH and its corresponding unmodified stable analog HA were used to shield DNA/PEI (DP) polyplexes to form ternary complexes (DPS and DPH complexes) by He et al. (2013). In this study, reducible shielding (HA–SS–COOH) and stable hyaluronic acid shielding were introduced to the formation of DNA/PEI complexes via electrostatic interaction. The presence of HA–SS–COOH and HA coating showed lower cytotoxicity, higher gene transfection efficiency, and greatly enhanced cellular uptake by HA receptor overexpressed carcinoma cells. More importantly, HA–SS–COOH shielding was superior to HA due to the extra reduction-responsive deshielding function. Therefore, dual functional hyaluronic acid derivative-modified DNA/PEI ternary complexes may have an advantage in targeted gene delivery to cancer cells.

HA derivative carrying dialdehyde (HAALD)–α,β-polyaspartylhydrazide (PAHy) hydrogels were synthesized in phosphate-buffered saline (PBSA) solution by Zhang, Huang, Xue, Yang, and Tan (2011) and characterized through different methods including gel content and swelling, Fourier transformed infrared spectra, thermogravimetric analysis, *in vitro* degradation, and biocompatibility experiments. A scanning electron microscope viewed the interior morphology of HAALD–PAHy gel whose porous 3D structure enabled it to efficiently encapsulate the proteins. Sustained and stable protein release from the HAALD–PAHy hydrogel was observed during *in vitro* delivery experiments and exhibited its potentially high-application prospect in the field of protein drug delivery.

Although the protective and impermeable qualities of the skin protect the organism from losing water, minerals, and dissolved proteins, percutaneous delivery system has been taken advantage as a topical delivery system in pharmaceutical or cosmetic industries. In contrast with traditional drug administration pathway, transdermal administration is featured by its

noninvasive procedure, which eliminates side effects and increases patient compliance and possibility for continuous and controlled drug absorption. Besides being a vital organ, the skin must be nourished as the other organs of the body by means of cosmetic formulations (Souto & Muller, 2008). HA nanoemulsion targeting to be applied as transdermal carrier for active lipophilic ingredient has been developed in previous studies, whose stability and delivery potential have been verified. The results suggested nanoemulsions could be successfully used as percutaneous delivery vehicle of active lipophilic ingredient, and HA has a favorable role in skin care for drug and cosmetic applications.

A variety of nanostructures composed of polyamino acid polyarginine (PArg) and polysaccharide hyaluronic acid (HA) as a preliminary stage were prepared by Oyarzun-Ampuero, Goycoolea, Torres, and Alonso (2011) before evaluating their potential application in drug delivery. PArg was combined with HMWHA or LMWHA to form nanoparticles by simply mixing polymeric aqueous solutions at room temperature. The results showed that the molecular weight of HA is a crucial determinant of formulation stability during mechanical isolation and in physiological conditions. This knowledge is useful not only for systems comprising PArg and hyaluronic acid but also for systems composed of other polymers. Further studies testing the potential of these systems as mucoadhesive nanocarriers for targeted drug delivery will be carried out, and the *in vitro–in vivo* behavior of these systems will be also evaluated.

5.5. Targeted drug delivery

The prolonged circulation of the nanoparticles in the bloodstream may increase their probability of reaching the tumor tissue after systemic administration *in vivo*, which is due to the abnormal characteristics of tumors, such as the fenestrated vasculature and the lack of a lymphatic drainage system. However, this passive tumor targeting strategy is partially limited in the effective diagnosis or therapy of tumors, because conventional nanoparticles do not have the ability to be specifically internalized into the target cells, resulting in the release of a significant portion of the payloads at the extracellular phase. To overcome this limitation, considerable efforts have been made to develop nanoparticles capable of actively targeting cancer cells. Such nanoparticles have been modified with targeting moieties, such as antibodies, proteins, and various ligands that can selectively bind to receptors overexpressed on the target cells. Hyaluronic acid (HA), a natural

polysaccharide found in the ECM and synovial fluids of the body, has been investigated as a targeting moiety of the drug conjugates or nanoparticles for cancer therapy because it can specifically bind to various cancer cells that overexpress CD44, an HA receptor. The PEGylation of hyaluronic acid nanoparticles improves tumor targetability *in vivo* (Choi et al., 2011).

PEG-conjugated hyaluronic acid-ceramide (HACE) was synthesized by Cho et al. (2012) for the preparation of doxorubicin (DOX)-loaded HACE-PEG-based nanoparticles, 160 nm in mean diameter with a negative surface charge. Greater uptake of DOX from these HACE-PEG-based nanoparticles was observed in the CD44 receptor highly expressed SCC7 cell line, compared with results from the CD44-negative cell line, NIH3T3. A strong fluorescent signal was detected in the tumor region upon intravenous injection of cyanine 5.5-labeled nanoparticles into the SCC7 tumor xenograft mice; the extended circulation time of the HACE-PEG-based nanoparticle was also observed. Pharmacokinetic study in rats showed a 73.0% reduction of the *in vivo* clearance of DOX compared to the control group. The antitumor efficacy of the DOX-loaded HACE-PEG-based nanoparticles was also verified in a tumor xenograft mouse model. DOX was efficiently delivered to the tumor site by active targeting via HA and CD44 receptor interaction and by passive targeting due to its small mean diameter (<200 nm). Moreover, PEGylation resulted in prolonged nanoparticle circulation and reduced DOX clearance rate in an *in vivo* model.

Slightly modified HA derivatives were used for target-specific intracellular delivery of nucleotide therapeutics, and highly modified HA derivatives were used for long-acting conjugation of peptide and protein therapeutics. The chemical modifications were carried out through carboxyl groups of HA, because carboxyl groups of HA are known to be the recognition sites for HA receptors and hyaluronidase (Banerji et al., 2007). The chemical modification of HA–COOH would change its biological behaviors in the body. As an example, it was reported that enzymatic degradation of HA derivatives was delayed with increasing degree of HA modification. HA can be designed to have various functional groups in the pendant groups. In order to introduce amine groups to HA, ADH, hexamethylenediamine (HMDA), or cystamine can be grafted by the conjugation reaction with carboxyl groups of HA.

Target-specific intracellular delivery of siRNA is one of the most important issues for the development of siRNA therapeutics with remarkable potentials. Despite extensive research efforts, there are still lots of unsolved problems for effective delivery of siRNA. HA has been used as a targeting

moiety of gene delivery carriers. HA receptors are abundant in some specific tissues, such as the liver, kidney, and most of cancer tissues. While a widely used tethering molecule of PEG cannot interact with cell membrane and is hard to go through the cell membrane, HA can bind to the receptor on the cell surface and be uptaken to the cells by HA receptor-mediated endocytosis. For example, HA–PLL conjugate was synthesized targeting HARE receptor of sinusoidal epithelial cells in the liver. They conjugated the reducing end of HA with ε-amino groups of PLL by reductive amination to synthesize the comb-type copolymer. This copolymer was used to make complexes with DNA, which were intravenously injected to animal models.

5.6. HA hydrogels

Hydrogels have recently drawn great attention for use in a wide variety of biomedical applications such as cell therapeutics, wound healing, cartilage/bone regeneration, and the sustained release of drugs. This is due to their biocompatibility and the similarity of their physical properties to natural tissue. Hydrogels are 3D networks of cross-linked hydrophilic polymers that typically show a high degree of swelling in aqueous environments without dissolution of polymeric networks. Among the various fabrication methods, photopolymerization is a very good means to prepare hydrogels due to its many advantages. Photopolymerization has recently received increased attention due to its capacity to allow for mixing of aqueous macromer solutions containing cells and bioactive factors. This mixture can be delivered in a minimally invasive manner and then rapidly cross-linked in physiological conditions *in situ* following brief exposure to UV light (Hutchison, Stark, Hawker, & Anseth, 2005). A photocured HA hydrogel containing an osteogenesis-inducing growth factor, GDF-5. HA hydrogels I–III were prepared by Bae et al. (2014) and confirmed to have controlled GDF-5 release profiles. Cytotoxicity and cell viability suggest that GDF-5-loaded HA hydrogel has proper biocompatibility for use as a scaffold, which can induce osteogenesis.

The protocols to prepare HA hydrogels can be classified into three types, direct cross-linking of HA, cross-linking of HA derivatives, and cross-linking between two different kinds of HA derivatives. According to the molar ratio of diamine to carboxyl group of HA, HA hydrogels can be prepared as well as HA derivatives with amine functional groups. If the ratio is much higher than 1/1, the diamine is grafted as a pendant group.

5.7. Tumor treatment

Existing anticancer drug deliveries focus on self-assembled polymeric nanoparticles based on implantable biomaterials. Due to the special core–shell structure of such nanoparticles, they have superior properties *in vitro* and *in vivo*, including high loading capacity of poorly water-soluble drugs, releasing drugs in a sustained manner, thus increasing bioavailability of them (Hou et al., 2011; Saravanakumar et al., 2010). However, only partial amounts of loaded drugs reach the target site due to some physiological limitations. One approach to overcome these limitations is active targeting strategies such as binding to appropriate receptors highly expressed at the target site. An amphiphilic HA derivative conjugated with glycyrrhetinic acid (GA) was developed to self-assemble nanoparticles for liver tumor targeting delivery of PTX by Zhang, Yao, Zhou, Wang, and Zhang (2013). PTX-loaded HGA nanoparticles exhibited more significant cytotoxicity to HepG2 cells than B16F10 cells due to simultaneously overexpressing HA and GA receptors of HepG2 cells. The targeting activity of HGA nanoparticles was also demonstrated by *in vitro* cellular uptake studies and *in vivo* imaging analysis. Therefore, HGA nanoparticles can be a potential targeting drug carrier for liver cancer therapy.

There is always a close relationship between malignancy and HA content. Higher concentration of HA will be present in the adjacent tissues surrounding invasive tumors than in the corresponding tissues of noninvasive tumors. In some types of tumors, this abnormal increase in the HA level was found to result from the overproduction of HA in fibroblast cells by the interaction with adjacent cancer cells (Asplund, Versnel, Laurent, & Heldin, 1993). Similarly, HA also overproduced in the cancer cells themselves possibly due to overexpression of HA in the cancer cells (Calabro, Oken, Hascall, & Masellis, 2002). Consequently, the high level of HA in the tissues can be a significant predictor for estimating malignancy and invasiveness of tumor (Ropponen et al., 1998). In some specific types of tumors, such as bladder cancer, the urinary level of HA can also be a marker for detecting cancer as well as for evaluating its grade (Lokeshwar et al., 2000). Choi, Saravanakumar, Park, and Park (2012) in their review had concluded that HA-based DDS have great potential for imaging and treatment of several cancers.

PTX, an effective chemotherapeutic drug isolated from the bark of *Taxus brevifolia*, is a microtubule-stabilizing agent that can induce mitotic arrest and cell apoptosis. Currently, PTX is widely used for the treatment

of cancer, including ovarian, breast, and non-small-cell lung cancer. Hyaluronic acid-coated, PTX-loaded, nanostructured lipid carriers (HA-NLCs) were prepared via electrostatic attraction for delivering PTX to tumors overexpressing CD44. HA-NLC prepared via electrostatic attraction was an effective carrier for delivering PTX to tumors overexpressing CD44 (Yang et al., 2013).

Hyaluronyl reduced graphene oxide (rGO) nanosheets was used as a tumor targeting delivery system for anticancer agents by Miao et al. (2013). Hyaluronyl-modified rGO nanosheets were prepared by synthesizing cholesteryl hyaluronic acid (CHA) and using it to coat rGO nanosheets, yielding CHA-rGO. Compared with rGO, CHA-rGO nanosheets showed increased colloidal stability under physiological conditions and improved *in vivo* safety, with a survival rate of 100% after intravenous administration of 40 mg/kg in mice. The DOX-loading capacity of CHA-rGO was four-fold greater than that of rGO.

5.8. Environmental applications

A series of DTPA-substituted hyaluronan derivatives with different degrees of substitution and degrees of cross-linking were successfully prepared by Buffa et al. (2011). Several parameters of the reaction such as molecular weight of starting HA, temperature, equivalent of DTPA bis-anhydride, concentration of HA, transacylation catalyst DMAP, and reaction time were studied. A specific benefit of the prepared conjugates of HA–DTPA is that this formulation can effectively complex metals. It could be applied as a metal sponge material (hydrogel), which can effectively remove radioactive or toxic metals *in vivo*.

Natural polysaccharides such as chitosan, heparin, chondroitin, keratin, and xanthan have been developed as environmentally friendly materials for removing toxic pollutants from water. In particular, chitosan derivative of chitin obtained mainly from crab and shrimp shells has been widely suggested as a candidate for an overwhelming scope of adsorption applications, covering almost all the spectrum of biotechnology. HA is another natural polysaccharide that has properties similar to chitosan. Novel magnetic adsorbent with submicrosize was fabricated by Lan et al. (2013) through the immobilization of hyaluronic acid on the magnetic silica microspheres. The as-synthesized Fe_3O_4–SiO_2–HA microspheres can be used as an effective adsorbent for the removal of copper ions from aqueous solution.

5.9. Sensors

Although chitosan is often used to disperse carbon nanotubes (CNTs) with subsequent of electrochemical (bio)sensors (Kac & Ruzgas, 2006), recent studies suggest HA can be efficiently used for the same purpose as judged from excellent conductivity of the nanocomposite and remarkable dispersivity of HA toward CNTs (Razal, Gilmore, & Wallace, 2008). A biocompatible nanocomposite consisting of single-walled CNTs dispersed in a hyaluronic acid (HA) was investigated as a sensing platform for a mediatorless electrochemical detection of NADH by Filip, Sefčovičová, Tomčik, Gemeiner, and Tka (2011). The NADH sensor exhibits a good long-term operational stability (95% of the original sensitivity after 22 h of continuous operation).

6. CONCLUSION

Thus, HA is a ubiquitous natural polysaccharide in the body with excellent physicochemical properties such as biodegradable, biocompatible, nontoxic, and nonimmunogenic characteristics. Accordingly, HA has been widely used for various medical applications such as OA surgery, ocular surgery, plastic surgery, TE, and drug delivery. Especially, due to the great impact of drug delivery applications, HA has been extensively explored as a novel drug carrier for target-specific and long-acting delivery of protein, peptide, and nucleotide therapeutics.

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