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ADVANCES OF HYALURONIC ACID IN STEM CELL THERAPY AND TISSUE ENGINEERING, INCLUDING CURRENT CLINICAL TRIALS

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Abstract

Hyaluronic acid (HA), as one of the main components of the extracellular matrix (ECM), plays a significant role in a multitude of biological processes involving cell migration, proliferation, differentiation, wound healing and inflammation. Thanks to its excellent biocompatibility, biodegradability and hygroscopic properties, HA has been used in its natural form for joint lubrication and ocular treatment. The chemical structure of HA can be easily modified by direct reaction with its carboxyl and hydroxyl groups. Recently, HA derivatives have been synthesised with the aim of developing HA-based materials with increased mechanical strength, improved cell interactions and reduced biodegradation and studied for regenerative medicine purposes, including cell therapy and tissue engineering. In this context, the present manuscript reviews HA applications from a basic point of view – including chemical modifications and cellular biology aspects related to clinical translation – and future perspectives of using biofabrication technologies for regenerative medicine. A detailed description of current clinical trials, testing advanced therapies based on combination of stem cells and HA formulations, is included. The final goal was to offer an integral portrait and a deeper comprehension of the current applications of HA from bench to bedside.

Keywords: Cell therapy, hyaluronic acid, tissue engineering, scaffold, encapsulation, stem cell.

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List of Abbreviations

2D	two dimensional
3D	three dimensional
Ad	adamantane
BDDE	butanediol-diglycidyl ether
BDNF	brain-derived neurotrophic factor
BM	bone marrow
BMAC	BM aspirate concentrate
BMC	bone marrow concentrate
CD	β-cyclodextrin
CDCs	cardiosphere-derived cells
DEO	1,2,7,8-diepoxyoctane
DM	diabetes mellitus

DVS	divinylsulphone
ECM	extracellular matrix
EDC	1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide
EPCs	endothelial progenitor cells
eEPCs	embryonic EPCs
EX 810	ethylene glycol diglycidyl ether
FDA	Food and Drug Administration
FN	fibronectin
GH	guest-host
GlcA	glucuronic acid
GlcNAc	N-acetyl-D-glucosamine
HA	hyaluronic acid
HAMC	hyaluronan and methylcellulose
HAS	hyaluronic acid synthases
HEMA	hydroxyethylmethacrylate
IA	intra articular
ICOAP	intermittent and constant pain score
IKVAV	isoleucine-lysine-valine-alanine-valine
IVD	intervertebral disc
LME	L-leucine methyl ester
MeHA	methacrylated HA
MI	myocardial infarction
MPCs	mesenchymal precursor cells
MSCs	mesenchymal stem cell
N/P	not provided
NSCs	neural stem and progenitor cells
OA	osteoarthritis
PBS	phosphate buffered saline
PBSCs	peripheral blood stem cells
PEDOT	poly 3,4-ethylenedioxythiophene
PEG	polyethylene glycol
PCL	poly(ε-caprolactone)
PLA	poly(D,L-lactic acid)
PLGA	poly(D,L-lactic-co-glycolic acid)
PRP	platelet-rich plasma
RA	rheumatoid arthritis
RGD	arginine-glycine-aspartic acid
rhBMP-2	human bone morphogenetic protein-2
RPCs	retinal progenitor cells
SDF-1α	recombinant stromal cell derived factor-1 alpha
SFF	solid freeform fabrication
TE	tissue engineering
TEHV	tissue engineered heart valves
UC-MSCs	umbilical cord MSCs
UCB-MSCs	umbilical cord blood-derived MSCs
VAS	visual analogue scale
VICs	valvular interstitial cells
WOMAC	Western Ontario and McMaster Universities Arthritis Index

Introduction

In recent decades, there has been an increasing interest in the development of regenerative approaches to repair or replace damaged tissues. Additionally, the continued development of several therapeutic strategies demands the use of more effective, biocompatible and bio-functional materials. In this sense, HA and its derivatives have been positioned as one of the most suitable and widely used compounds for biomedical applications. HA, being the main component of the ECM (Fraser *et al.*, 1997), presents unique characteristics for its use in regenerative medicine. It is also involved in a large number of relevant biological functions in the human body, such as wound healing (Li *et al.*, 2018), inflammation (Chan *et al.*, 2015), cell proliferation and migration (Murakami *et al.*, 2018), embryogenesis, morphogenesis (Toole, 1997) and angiogenesis (Silva *et al.*, 2016).

Thanks to its biocompatibility, biodegradability, mucoadhesive and hydrophilic properties, HA has been successfully applied in a broad range of cosmetics, drugs and medical devices. Moreover, changes of HA concentration are associated with many diseases. Consequently, HA concentration has been analysed in blood, body fluids or tissues as a diagnostic marker of diseases such as rheumatoid arthritis (Niki *et al.*, 2012) and liver pathologies (Gudowska *et al.*, 2017).

HA is also used for supplementation of joint fluid (Wu *et al.*, 2017), eye surgery (Durrie *et al.*, 2018), wound healing (Simman *et al.*, 2018), cosmetic regeneration and reconstruction of soft tissues (Geronemus *et al.*, 2017). HA is degraded by hyaluronidase and/or by reactive oxygen/nitrogen species that are generated during tissue inflammation, inflammatory response in sepsis or ischaemia-reperfusion injury (Valachová *et al.*, 2015; Valachová *et al.*, 2016). Therefore, the poor mechanical properties, rapid degradation and clearance *in vivo* without crosslinking limit HA clinical applications (Wende *et al.*, 2016). Scaffolds for regenerative medicine application should be degraded over the course of tissue regeneration to allow complete repair by the host tissue. Controlling the degradation rate of an HA-based hydrogel is still a challenge for its applications in TE. It is of great interest to cross-link HA into an hydrogel or to modify it chemically for improving mechanical properties and controlling residence time (*i.e.* controlled degradation, metabolism and clearance). In recent years, HA derivatives, mainly aiming to improve their mechanical properties and reduce biodegradation, have been extensively studied in cell therapy (Smith *et al.*, 2013), drug delivery (Cai *et al.*, 2017) and TE (Seidlits *et al.*, 2011).

The purpose of the present review was to highlight those medical areas in which HA and its derivatives are significantly contributing, including HA medical formulations with stem cells that are currently being tested for several diseases in clinical trials. Additionally, a brief summary of the different HA chemical modifications that expand its biomedical applications are included. More information as to the chemical transformations of HA is included in other

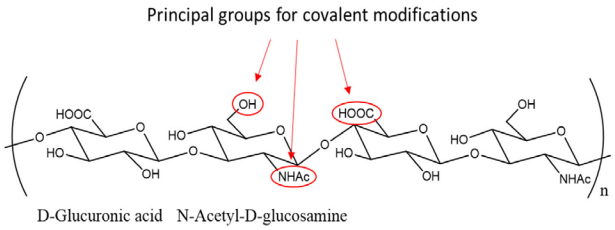

Fig. 1. Molecular structure of HA repeating disaccharide units composed of D-glucuronic acid and N-acetyl-D-glucosamine.

publications (Hemshekhar *et al.*, 2016; Highley *et al.*, 2016; Knopf-Marques *et al.*, 2016; Kuo and Prestwich, 2011; Prestwich, 2011; Schanté *et al.*, 2011).

HA: description and origin

HA, also called hyaluronan, is one of the ECM glycosaminoglycans that exists as protein-free unbranched long polysaccharide chains with different molecular weights (from 1 kDa up to 8 MDa) and is exclusively non-sulphated (Barbucci *et al.*, 2000). The backbone is composed of a repeated disaccharide unit of D-GlcA linked β(1→3) to GlcNAc. The disaccharide units are connected by GlcNAc linked β(1→4) to GlcA (Fig. 1) (Furlan *et al.*, 2005; Hargittai and Hargittai, 2008; Liu *et al.*, 2011).

HA is produced in the cytoplasmic membrane of mammalian cells, amphibians and bacteria by the three HAS membrane enzymes, HAS-1, HAS-2 and HAS-3, which differ in their catalytic activities and molecular weight of the HA synthesised. HAS-1 is responsible for the production of medium to high molecular weight HA (200-2000 kDa), HAS-2 for high molecular weight HA (2000 kDa) and HAS-3 for low molecular weight HA (< 300 kDa) (Itano *et al.*, 1999; Lee and Spicer, 2000). HA is continuously extruded through the plasma membrane into the ECM where it provides a hydrophilic viscous medium that facilitates cell motility, proliferation and differentiation (Xu *et al.*, 2012). One of the characteristics of HA is its large capacity to bind water molecules up to 10,000 times its own weight (Laurent, 1992). HA metabolism is regulated by its production through the different HAS and its degradation in the plasma membrane, or in lysosomes, by hyaluronidases to maintain tissue physiological concentrations (Khaldoyanidi *et al.*, 2014). HA is a key component of the ECM, especially abundant in connective tissues and present at high concentration in the synovial fluid of joints, vitreous humour, hyaline cartilage, IVD and umbilical cord (Dicker *et al.*, 2014; Knopf-Marques *et al.*, 2016; Toole, 2004). The major HA receptor is CD44, a cell surface glycoprotein involved in cell-cell and cell-matrix interactions, which is endogenously expressed at low levels in different cell types (Isacke and Yarwood, 2002). Traditionally, HA has been extracted from rooster comb-like animal tissues; however, HA is also produced by bacterial fermentation, mainly using *Streptococcus zooepidemicus*, a natural manufacturer of HA (de Oliveira *et al.*, 2016; Liu *et al.*, 2011).

HA-derived-biomaterials

Thanks to its high molecular weight and hydrophilic nature, the polysaccharidic structure of HA can form viscous water solutions that have many interesting uses in biomedical applications, mainly as a carrier of biological components, such as cells (Kim *et al.*, 2017; Prestwich, 2011). Nevertheless, these solutions are not designed to withstand time since, in biological media, HA is easily degraded and the diluted solutions lack sufficient mechanical strength to be self-supporting in a particular location over prolonged time (Xu *et al.*, 2012). As such, HA has been subjected to different chemical modifications aimed at expanding its biomedical uses (Prestwich *et al.*, 1998; Schanté *et al.*, 2011; Volpi *et al.*, 2009). Synthetic transformation has focused on increasing its molecular weight to generate novel HA derivatives able to produce a more viscous solution or even gels that can stay longer in the body and have a therapeutic effect. The objective is to modulate the material according to a specific application without compromising its biocompatibility, biodegradability, ability to interact with cells and other living tissue, *etc.*. In this sense, chemical modifications have been focused on increasing its mechanical strength and/or improving cell interactions. With respect to increasing HA mechanical strength, two different strategies have been applied. The first one implies the development of cross-linkable HA derivatives through the introduction in the backbone of functional groups able to covalently inter-connect HA polymer chains (Collins and Birkinshaw, 2008). Such modification leads to a cross-linked HA derivative with higher molecular weight and lower solubility that, in the presence of water, forms a self-supported hydrogel (chemical hydrogel) where its rigidity or mechanical strength can be regulated by the degree of cross-linking (Kim *et al.*, 2017). Alternatively, the solubility of HA can also be reduced by introduction of hydrophobic groups in its structure. The nature of these hydrophobic groups and the degree of HA functionalisation are important for the formation of amphipathic structures that tend to form aggregates in the presence of water, generating physical hydrogels (Dicker *et al.*, 2014). Either, hydrophobised or cross-linkable HA derivatives exhibit higher resistance to biodegradation and can be used for cell culture (Aulin *et al.*, 2011), viscosupplementation (Adams *et al.*, 1995) and as vehicle for *in vivo* cell (Smith *et al.*, 2013) and drug delivery (Cai *et al.*, 2017).

The chemical structure of HA has three potential functional groups that can be easily modified by covalent reaction (Fig. 1): the carboxylic acid of the

GlcA unit, the primary hydroxyl group of the GlcNAc – although the secondary hydroxyl groups can also be reactive – and the amine group of GlcNAc formed after a deacetylation reaction (Fakhari and Berkland, 2013). The hydroxyl groups can be chemically modified by formation of an ether or ester linkage, while the carboxyl and amine groups can form new ester and amide bonds (Khunmanee *et al.*, 2017). These HA chemical modifications can also fall into two different categories depending if the material can be further functionalised or not (Prestwich and Kuo, 2008). If the material is an end-product that cannot be further modified in the presence of a biological substrate, it is defined as monolithic. However, if it can form new chemical bonds in the presence of a biological substrate it is defined as living. These living HA derivatives can be used in 3D cell cultures and for *in vivo* cell delivery (Prestwich, 2007). Nevertheless, precautions are required to ensure that all the chemical reactions are biologically biocompatible and do not produce toxic by-products in the short or long term.

Monolithic HA derivatives

Primary strategies to originate cross-linkable HA derivates are based on the direct cross-link between unmodified HA polymer chains by using bi-functional cross-linker reagents able to react with the hydroxyls and the carboxylic acid of the HA backbone (Burdick and Prestwich, 2011). This protocol uses reaction conditions and reagents incompatible with the presence of cells and, therefore, used to originate monolithic HA derivates (Prestwich, 2011) (Fig. 2). For example, under strong basic conditions, hydroxyl groups can also react with divinylsulphone and different epoxides, such as bisglycidylepoxides (DEO, BDDE) and epichlorohydrin, to form ether linkages more stable to hydrolysis (Wende *et al.*, 2016). Additionally, HA can also react with glutaraldehyde in an acid medium to originate acetal or hemiacetal linkages (Khunmanee *et al.*, 2017). In this sense, Zhang *et al.* (2012) developed a composite HA-agarose material using epichlorohydrin as a cross-linker. One of the advantages of this material is that its degradation rates can be controlled. In a similar way, Kim *et al.* (2012) improved the mechanical, degradation and biological response of an HA-collagen composite hydrogel for cartilage regeneration using ethyleneglycoldiglycidyl ether as crosslinker agent. With the objective of reducing HA degradation rates and modulate hydrogel pore sizes, Collins and Birkinshaw (2011) studied a series of HA scaffolds obtained by simply solution gelling using glutaraldehyde, EX 810, EDC with LME and divinylsulphone as cross-linker agents. Results show that a suitable soft tissue HA scaffold is obtained with the combination of EDC and LME (Collins and Birkinshaw, 2011). Lai (2014) studied the amount of divinylsulphone covalently incorporated into the HA structure and its relationship with mechanical stability and resistance against enzymatic degradation of the resulting hydrogels. Its cytocompatibility as a matrix

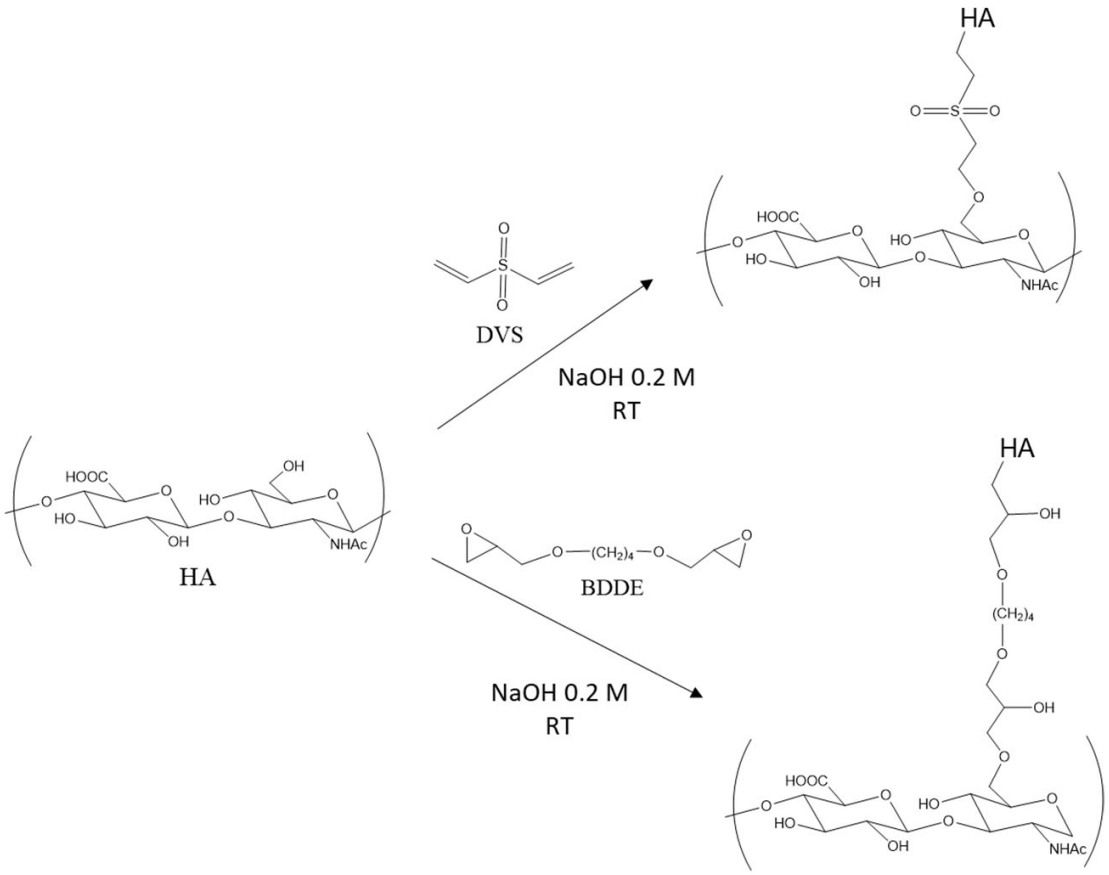

Fig. 2. Traditional monolithic cross-linked transformation of HA with DVS and BDDE.

for the growth of retinal pigment epithelial cells was also evaluated (Lai, 2014).

A direct cross-linking reaction between HA polymer chains can also be performed with carbodiimides, usually, EDC. The reaction proceeds through activation of the carboxylic acid by EDC to give acylisourea active ester that reacts with nearby hydroxyls generating intermolecular cross-links (Fig. 3). This reaction proceeds under mild conditions at neutral pH and it is compatible with biological macromolecules that can be easily degraded. HA is cross-linked with collagen to enhance its mechanical stability and to improve its biological compatibility (Davidenko *et al.*, 2010; Wang and Spector, 2009). These scaffolds promote angiogenesis (Perng *et al.*, 2011); in addition, the biological performance of HA-gelatine scaffolds of tuneable porosity was studied in the presence of L929 fibroblasts, showing no cytotoxicity (Zhang *et al.*, 2011a). Films composed of HA-carboxymethylcellulose (Seprafilm®, Sanofi-Aventis U.S. LLC, Bridgewater, NJ, USA) used as adhesion barrier mainly for abdominal or pelvic laparotomy are chemically produced by EDC cross-linking reaction (Schneider *et al.*, 2007).

Living HA derivatives

More recently, the focus has been centred on the development of HA derivatives able to form hydrogels in the presence of cells under mild conditions. These materials offer the advantages of originating 3D cell constructs for TE or cell delivery (Ruel-Gariépy and Leroux, 2004). Normally, HA is chemically modified to originate a derivative that can generate the hydrogel in a biological environment by a cross-linking reaction that proceeds under physiological conditions without generating any toxic by-products (Khunmanee *et al.*, 2017). One of the first examples is the coupling of HA with adipic dihydrazine. The reaction proceeds under mild conditions to originate an HA hydrazone derivative stable under physiological conditions that can easily react with aldehyde crosslinking agents through its hydrazine group in the presence of cells (Prestwich *et al.*, 1998; Zhang *et al.*, 2010) (Fig. 4). These HA hydrazone derivatives were coupled with different aldehydes, such as poly(ethylene) glycol aldehyde (Kirker *et al.*, 2002) and genipin (Zhang *et al.*, 2010) to originate materials suitable for TE applications.

Thiol chemistry based on the formation of disulphide bonds or nucleophilic attachment of thiol groups under mild conditions is also compatible with physiological conditions. Shu *et al.* (2002; 2006) developed an HA derivative using hydrazide reagents containing a disulphide bond. After cleavage of the disulphide bond and the formation of thiol groups, slow formation of crosslinks in the presence of air takes place (Fig. 5).

Gramlich *et al.* (2013) described the functionalisation of the HA with norbonene groups. This derivative can cross-link under mild conditions by a thiol-ene reaction to produce hydrogels with controllable mechanical properties. HA hydrogels that are formed by an enzymatic reaction were also developed by Kurisawa *et al.* (2005). HA functionalised with tyramide can rapidly form a hydrogen in the presence of H_2O_2 and horseradish peroxidase. This protocol is biocompatible and useful for various applications (Kurisawa *et al.*, 2005; Wang *et al.*, 2010). Huisgen azide-alkyne 1,3-dipolar cycloaddition, known as click chemistry has also been studied for this purpose. Huerta-Angeles *et al.* (2012) described the synthesis of HA derivatives having an azide and alkyne groups. In the presence of Cu(I) catalyst the reaction proceeds under physiological conditions to originate the gel. Nevertheless, the presence of the metallic catalyst can introduce toxicity to the environment (Crescenzi *et al.*, 2007).

Hydrophobic HA derivatives

To originate mechanically stable hydrogels, HA has been modified with the intention of increasing its hydrophobicity. Examples of these types of

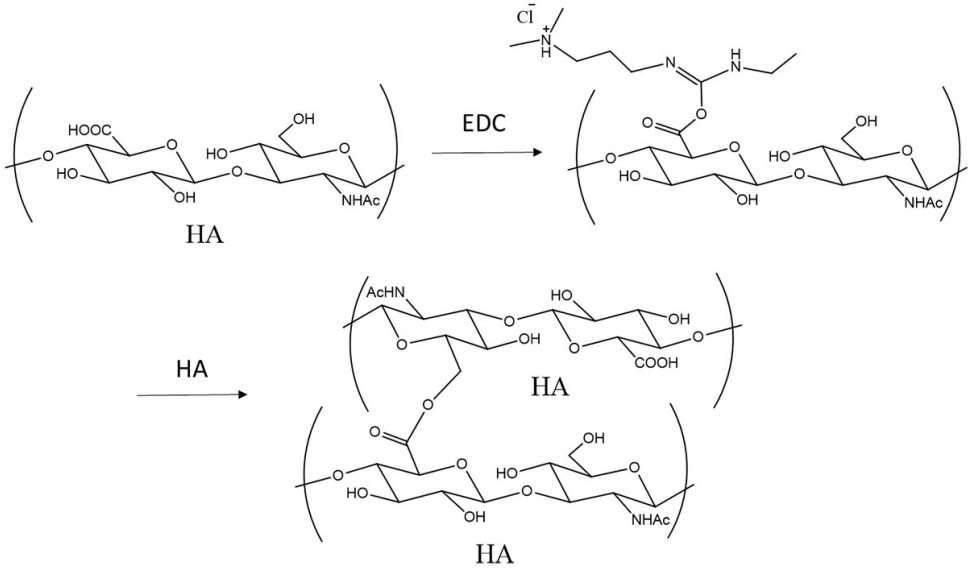

Fig. 3. Cross-linked HA through activation of the carboxylic acid mediated by EDC.

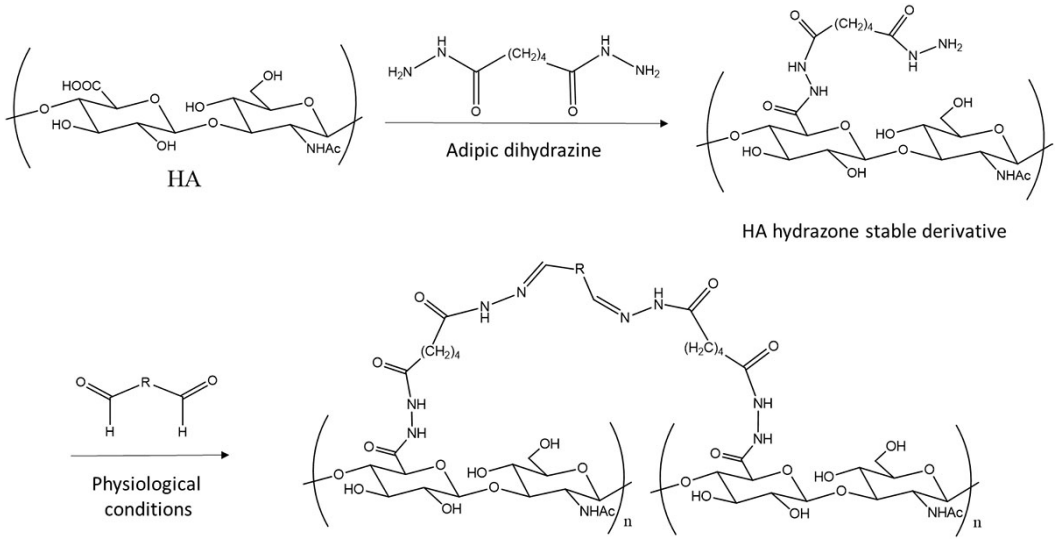

Fig. 4. HA hydrazone derivative that allows cross-linking reactions under physiological conditions.

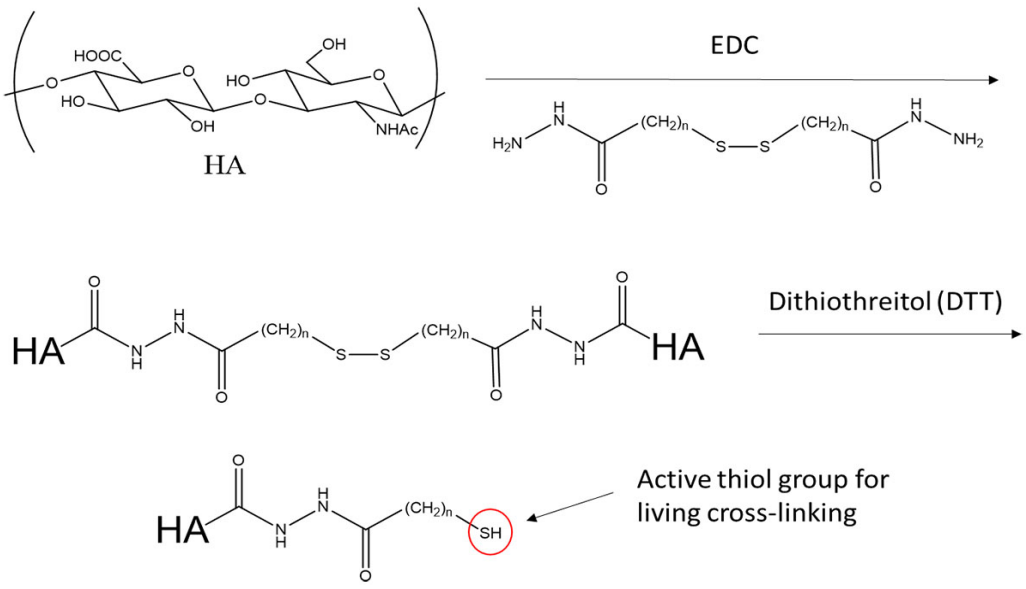

Fig. 5. HA derivative modified with thiol groups for disulphide or thiol-ene cross-linking reaction under physiological conditions.

derivatives are HYAFF® (Haemo Pharma GmbH, Hornstein, Austria) biomaterials that are the result of a partial esterification of HA carboxylates to benzylesters (Benedetti *et al.*, 1993) (Fig. 6). The degree of esterification modifies the physicochemical properties of these materials making them adequate for different applications. These hyaluronan esters can be easily manipulated to produce membranes and fibres, lyophilised to obtain porous materials or processed to produce microspheres (Mano *et al.*, 2007). When the degree of esterification is high, these materials are insoluble in water and are used, in solid form, as scaffolds for the growth of human fibroblasts, chondrocytes and bone-marrow-derived MSCs for repair of cartilage and bone defects (Caravaggi *et al.*, 2003; Lisignoli *et al.*, 2003). Additionally, HYAFF® biomaterials in combination with PCL are excellent biomaterials for the *in vivo* regeneration of sheep meniscal tissue, where the implants remain in position and show excellent tissue ingrowth (Chiari *et al.*, 2006). Another example commercially available is HYADD®4 (Fidia Pharma USA, Parsippany, NJ, USA), derived from the partial amidation of HA with hexadecylamine. This derivative forms a

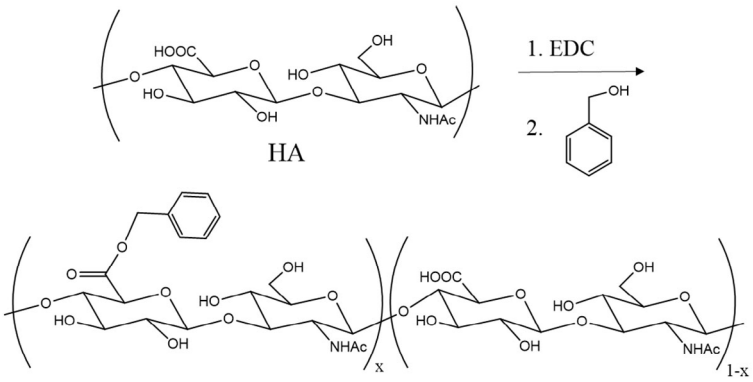

Fig. 6. Partial esterification of HA with benzyl alcohol to originate HYAFF® derivatives.

viscoelastic hydrogel that shows an enhancement of intra-articular residence time (Mainil-Varlet *et al.*, 2013; Priano, 2017).

Improving cell interactions of HA derivatives

HA has been covalently modified by the addition of biological relevant molecules with the objective of mimicking the native tissue environment and achieving a controlled and tuneable cell response (Lam *et al.*, 2014; Mehra *et al.*, 2006; Zhang *et al.*, 2011b). Additionally, the coupling of ECM proteins also permits the entrapment of antibodies and small molecules within the HA hydrogel, improving its role as an *in vivo* carrier. In this context, in order to obtain HA materials with cell-attachment properties, HA was modified with cell adhesion motifs, such as short RGD peptides (Shu *et al.*, 2004), and ECM proteins, such as collagen (Mehra *et al.*, 2006), laminin (Hou *et al.*, 2005) and fibronectin (Brown *et al.*, 2011). Camci-Unal *et al.* (2010) developed a CD34 antibody immobilised on an HA hydrogel for selectively captured and spread EPCs. The results show that these materials can potentially be used to enhance the biocompatibility of implants such as artificial heart valves. Kisiel *et al.* (2013) developed an HA hydrogel modified with a stabilised fibronectin fragment containing the major integrin-binding domain to improve MSC attachment and spreading. The *in vivo* delivery of this HA hydrogel derivative containing the rhBMP-2 growth factor results in a significant increase in bone formation and a better organisation of collagen fibres as compared to the non-functionalised HA hydrogel (Kisiel *et al.*, 2013). Likewise, the adhesion of glycosaminoglycans such as heparin and chondroitin sulphate enables the entrapment of growth factors, which also makes HA suitable for cell culture and differentiation (Ghosh *et al.*, 2006; Hosack *et al.*, 2008; Kontturi *et al.*, 2014; Lu *et al.*, 2013; Schanté *et al.*, 2011). Following this strategy, Xu *et al.* (2011) synthesised a heparin-decorated HA hydrogel that can bind rhBMP-2 specifically. The results show that the controlled release of the growth factor, in combination with the inductive role of HA in chondrogenesis, leads to efficient *in vitro* differentiation of murine MSCs.

Additionally to the chemical modifications of HA derivatives, there are different processing and fabrication techniques for the development of structurally tuneable and mechanically suitable HA-based materials. Although HA is mainly used as an injectable hydrogel, cross-linked HA scaffolds can be produced by phase-separation, supercritical fluid technology, porogen leaching, freeze-drying or emulsion techniques, whereas fibrous and nanofibrous structures are mainly created by electrospinning (Ekaputra *et al.*, 2008; Knopf-Marques *et al.*, 2016). In addition to the internal architecture patterning obtained by these techniques, geometry and spatial resolution parameters can be customised by other fabrication strategies, such as centrifugal casting (Mironov *et al.*, 2005), scaffold templating (Ortuño-Lizarán *et al.*, 2016), lithography (Takahashi *et al.*, 2009) or 3D printing (Skardal *et al.*, 2010). Recently, 3D bioprinting has gained significant attention since it allows the creation of scaffolds with precise deposition of cells, biomaterials and bioactive molecules (referred to as bioinks) in a spatial location in order to mimic the native tissue anatomy (Khalil and Sun, 2009). The ease of modifying the HA backbone with functional groups, such as hydrophobic moieties or cross-linkable groups, enables obtaining printable hydrogels (Kesti *et al.*, 2015; Loebel *et al.*, 2017). Therefore, thanks to the addition of HA, the bioink viscosity can be increased to allow for continuous extrusion of hydrogel strands (Murphy *et al.*, 2018). The most recent attempt is by Loebel *et al.* (2017), who developed a self-assembling HA hydrogel based on GH hydrophobic interactions of conjugated groups, Ad (guest) and CD (host). The GH hydrogel is composed of HA as the polymer backbone, which is modified with either the host molecule (CD-HA) or the complementary guest molecule (Ad-HA). The supramolecular GH associations disassemble when ejected through a syringe (shear-thinning) and reassemble within seconds (self-healing) upon cessation of shear (Loebel *et al.*, 2017).

Other strategies consisting of adding gelling agents, both natural and/or synthetic, have also made possible the production of printable HA-based hydrogels (Derakhshanfar *et al.*, 2018; Thomas and

Willerth, 2017). An example is the use of crosslinking chemistries or gelling components, such as gelatine, to obtain HA bioinks with tuneable mechanical properties that match the tissue of interest physical parameter profiles. Accordingly, Skardal *et al.* (2010) printed HA-gelatine hydrogels through a syringe needle into robust structures, followed by a second photo-cross-linking step to create a bioprinted tubular construct with cells. After the process of bioprinting and culture, these cells maintain their viability and gradually remodel the synthetic matrix to create a natural ECM (Skardal *et al.*, 2010). Other combinations such as methacrylated gelatine and methacrylated HA modified with hydroxyapatite particles have been prepared for the development of a bioink for bone tissue engineering. This bioink demonstrates its suitability for the build-up of relevant 3D geometries with microextrusion bioprinting and a significant positive effect on bone matrix development after 28 d in culture (Wenz *et al.*, 2017). Porous constructs through layer-by-layer deposition of cell-laden material based on HA and hydroxyethylmethacrylate derivatised dextran or atelocollagen have been evaluated for cell viability of encapsulated chondrocytes and human MSCs showing excellent cytocompatibility (Pescosolido *et al.*, 2011; Shim *et al.*, 2016). However, in some cases, the HA bioink is reinforced by double printing with a thermoplastic polymer, such as PCL (Mouser *et al.*, 2017; Shim *et al.*, 2016; Stichler *et al.*, 2017) or PLA (Souness *et al.*, 2018). Park *et al.* (2011) developed scaffolds of HA grafted to PLGA incorporating rhBMP-2 by SFF. Results show the *in vitro* enhancement of osteoblast cell growth and high gene-expression levels of alkaline phosphatase and osteocalcin. Moreover, scaffolds implanted into calvarial bone defects of rats reveal effective bone regeneration (Park *et al.*, 2011). Efforts have also been made to simulate the biochemical profile of the scaffold and optimise the bioactivity of the bioink by mixing HA derivatives with ECM components and tissue-specific growth factors, obtaining a more effective tissue regeneration (Shim *et al.*, 2016; Skardal *et al.*, 2015).

Therapeutic applications of HA, including cell therapy

The success of a cell therapy relies on identifying an appropriate cell source as well as a strategy to maintain and localise the cells in the desired area (López-Ruiz *et al.*, 2016). In this context, stem cells and tissue-specific cells were investigated as suitable cell sources for the repair of different kinds of tissues (Bardelli and Moccetti, 2017). However, current cell therapy practice is inefficient since therapeutic cells are less integrated into the tissue (Lebaschi *et al.*, 2017). To improve cell engraftment and survival after transplantation, new compounds have been proposed as injectable and biological vehicles for delivery. One of these new vehicles is HyStem® (BioTime, Inc., Alameda, CA, USA), a HA-based material approved for cell therapy that is used in combination with stem cells (Prestwich *et al.*, 2012). HyStem® can form a hydrogel in the presence of cells, using a HA living derivative. The cross-linking reaction is mediated by a thiol-ene reaction between the reactive thiol groups of a thiolated HA and a denatured collagen (Gelin-S, BioTime, Inc.) that reacts with polyethylene glycol diacrylate (Extralink, BioTime, Inc.) cross-linker reagent. Smith *et al.* (2013), in an *in vivo* study using a mouse model of MI, observed that an injection of HyStem® containing CDCs dramatically increases cell retention 24 h after delivery when compared with cells in PBS. Even in long-term cell engraftment (3 weeks after delivery), a significant retention is observed. Also, improvements in left ventricular ejection fraction and addition of viable myocardial mass are demonstrated (Smith *et al.*, 2013). Murugan Girija *et al.* (2018) demonstrated that the expression of keratinocyte-specific markers by human pre-differentiated gingival MSCs is higher when the cells are encapsulated in HyStem® as compared with 2D culture, showing that HA-based hydrogels provide a 3D environment to enhance differentiation.

Given that HA is a major constituent of the eye, HA hydrogels also provide ideal viscous solutions for retinal repair in ocular surgery (Durrie *et al.*, 2018). HA-based hydrogels are also studied as an injectable delivery system of mouse RPCs into subretinal space. Transplantation of these HA hydrogels causes very little disruption to the host retinal architecture while providing a unique microenvironment for self-renewal and differentiation of RPCs (Liu *et al.*, 2013). Liu *et al.* (2013) studied different systems with varying ratios of HyStem® components *vs.* cells medium and amount of Extralink with the purpose of modulating the mechanics and degradation time of the hydrogel matrix. In this sense, hydrogels with 40 % and 20 % HyStem® volume effectively support self-renewal of RPCs, while the 80 % HyStem® does not support the growth and survival of mouse RPCs probably due its higher concentration, which would substantially block nutrients and oxygen supply (Liu *et al.*, 2013). These cross-linkable HA derivatives degrade over a period of 3 weeks, showing greater stability than a previous blend of HA and methylcellulose (Ballios *et al.*, 2010). However, the bioresorbable hydrogel blend of HAMC is also applied as an injectable hydrogel delivery strategy to promote cell survival and integration of transplanted retinal stem cell-derived rods and NSCs in the retina and brain, respectively, for functional repair (Ballios *et al.*, 2015). The survival effect observed after 3 weeks post-transplantation in HAMC *versus* saline vehicle is attributed to cell-material interactions through the CD44 receptor, since $CD44^{-/-}$ cells do not show increased survival *in vivo* (Ballios *et al.*, 2015). Also, HA is widely used in cartilage repair to restore the biologic environment of the joint in OA patients. In fact, IA injection of HA is considered to be a treatment for early OA,

acting as a lubricant in the joint space in order to reduce pain by reducing the friction of the joint and improving viscoelasticity of the synovial fluid (Campbell *et al.*, 2015; Concoff *et al.*, 2017). There are different commercial preparations based on HA for viscosupplementation by IA injections (Jones *et al.*, 2018). These products differ in their composition, molecular weight, average life in the joint and price (Altman *et al.*, 2016; Estades-Rubio *et al.*, 2017).

Non-surgical IA orthobiologic injectables to treat OA also include PRP, a blood-derived product rich in growth factors. PRP can enhance production of ECM, inhibit inflammation and display beneficial effects on cartilage regeneration (Cengiz *et al.*, 2018; Wu *et al.*, 2011). In a recent study conducted by Filardo *et al.* (2015), the efficacy of IA injections of high-molecular-weight HA (Hyalubrix®, Fidia Pharma USA) *versus* the use of PRP for the treatment of knee cartilage degenerative lesions and OA was compared. Although preliminary results suggest that PRP injections might offer a clinical improvement, both treatments decrease the pain in knee of OA patients but the data are not statistically significant (Filardo *et al.*, 2015). Recent studies still disagree on which of the two strategies is most beneficial for patients (Dai *et al.*, 2017; Ye *et al.*, 2018; Zhang *et al.*, 2018). Moreover, others have proposed the development of a new formulation combining HA and PRP to achieve a more effective treatment for OA (Andia and Abate, 2014; Chen *et al.*, 2014; Lana *et al.*, 2016; Russo *et al.*, 2016). This combination is based on the fact that, on one hand, PRP could provide growth factors that may modify gene expression of OA chondrocytes and enhance the anabolic activity of chondrocytes while, on the other hand, HA may reduce pain and provide the viscosupplementation needed for OA treatment. Russo *et al.* (2016) studied the combination of PRP with different HA formulations (Sinovial® 0.8% ; Sinovial Forte® 1.6%; Sinovial HL® 3.2%; Hyalubrix® 1.5%, Fidia Pharma USA) in order to evaluate their *in vitro* biological effect on osteoarthritic chondrocytes and which HA formulations are more suitable to use in combination with PRP. Their results demonstrate that viscosupplements containing low HA concentration are not indicated for combination with PRP, as the viscoelastic properties are lost. In all cases, the chondrocyte GAG content and proliferation rate are higher in media containing PRP and HA formulations than in media containing only HA formulations. Interestingly, although Sinovial Forte®, Sinovial HL® and Hyalubrix® present the same rheological profile, Sinovial HL® is superior in stimulating GAG production (Russo *et al.*, 2016).

In addition to HA and PRP, a third-generation of IA injection therapy using BMC has emerged with the aim of retarding the progressive destruction of

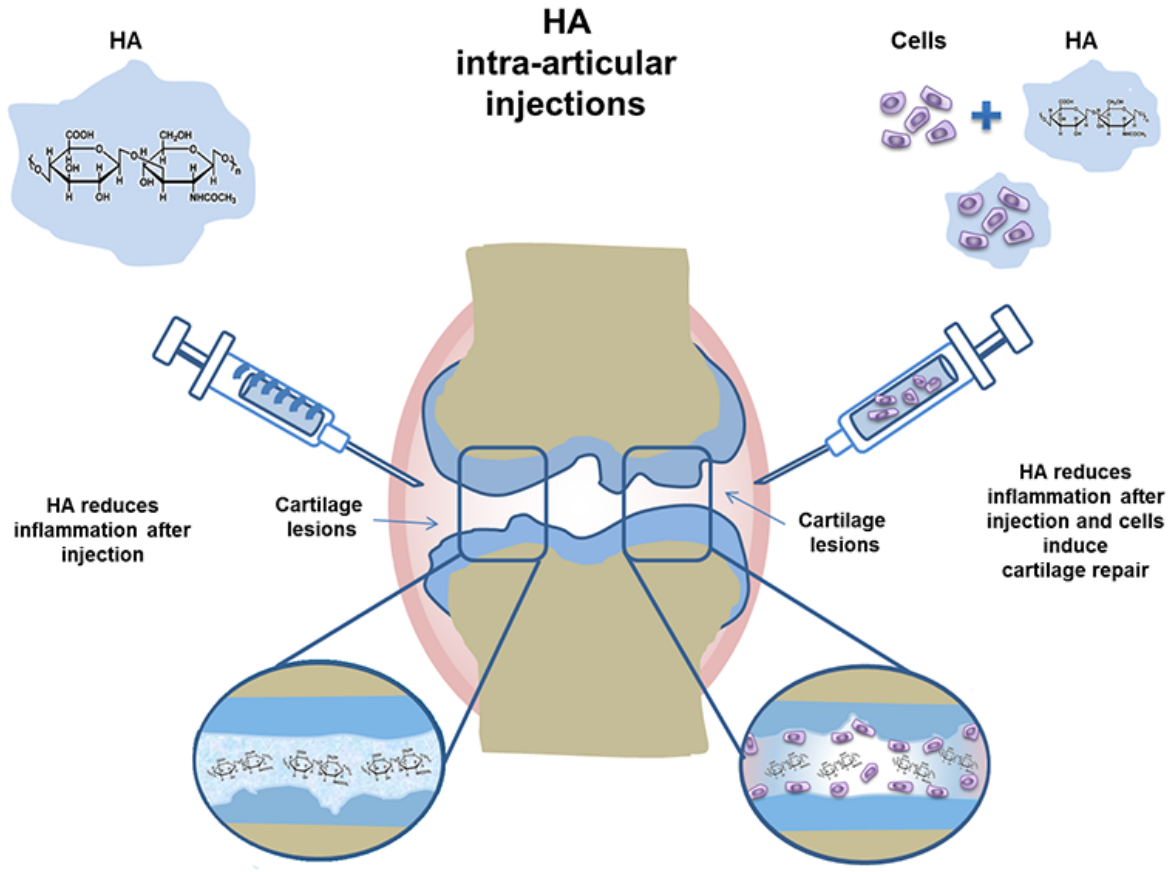

Fig. 7. Intra-articular injections of HA. Intra-articular injections of HA are widely used as a lubricant in the joint space for the treatment of knee OA pain. In recent years, stem cell injections have been used in combination with HA for cartilage tissue regeneration.

cartilage (Sampson *et al.*, 2013). The development of articular stem cell therapies is promising, since these cells have the ability for self-renewal and differentiation into osteogenic or chondrogenic lineages (Canadas *et al.*, 2018). HA injection therapies, combined with MSCs from BM, are currently under investigation for bone and cartilage tissue regeneration (Kaleka *et al.*, 2018; Roffi *et al.*, 2018), (Fig. 7). Indeed, BMC injections are used in combination with HA and fibrin, a protein scaffold with the natural ability to change phase from liquid to gel by addition of thrombin (Huh *et al.*, 2016). Therefore, fibrin is used with HA to promote retention of BMC at the injected site, forming a polymer composite within a few minutes after being injected. The mixture injected, following a microfracture procedure, can induce chondrogenesis *in vivo* and represents a novel strategy for treatment of chondral defects (Huh *et al.*, 2016).

In addition to BM stem cells, recent *in vivo* studies in small and large animal models have tested hydrogel composites of human UCB-MSCs mixed with 4 % sodium hyaluronate. These formulations show an improvement in the histologic appearance of the repaired articular cartilage tissue, which could represent a stepping stone to future human clinical trials (Ha *et al.*, 2015; Park *et al.*, 2015). Moreover, since stem cells possess the capacity to migrate to the site of injury, HA hydrogels combined with drugs or growth factors were developed in order to guide endogenous stem cells towards a specific site or injured area and to promote the regeneration of the tissue (Jha *et al.*, 2015). The slow release of rSDF-1α inside of a crosslinked HA hydrogel significantly increases BMC chemotaxis *in vitro* and improves cell engraftment and BMC homing to the remodelling myocardium better than the delivery of rSDF-1α alone following experimental MI (Purcell *et al.*, 2012). The engineered HA hydrogel is formed *in situ* through photoinitiated radical polymerisation using a HA derivative previously esterified with HEMA. The hydrolysis of the ester bonds between the HA backbone and the methacrylate groups allows slow degradation of the hydrogel and subsequent release of rSDF-1α (Purcell *et al.*, 2012). In a different study, HyStem®-C hydrogel is used to load BDNF, which is delivered in the brain following distal middle cerebral artery occlusion. 6 to 8 weeks after implantation, improved sensorimotor function is observed in treated rats and infarct volume is reduced, suggesting that targeted intracerebral delivery of BDNF using this hydrogel may mitigate ischaemic brain injury and restore functional deficits by reducing neuroinflammation (Ravina *et al.*, 2018).

Another appreciated function of HA comprises its use as a system for molecule delivery (Fig. 8). CD44, is highly expressed in articular cells, particularly chondrocytes (Ishida *et al.*, 1997); therefore, based on the binding property of CD44 with its ligand HA, the development of HA-coated nanoparticles for drug delivery has been investigated. Nanoparticles

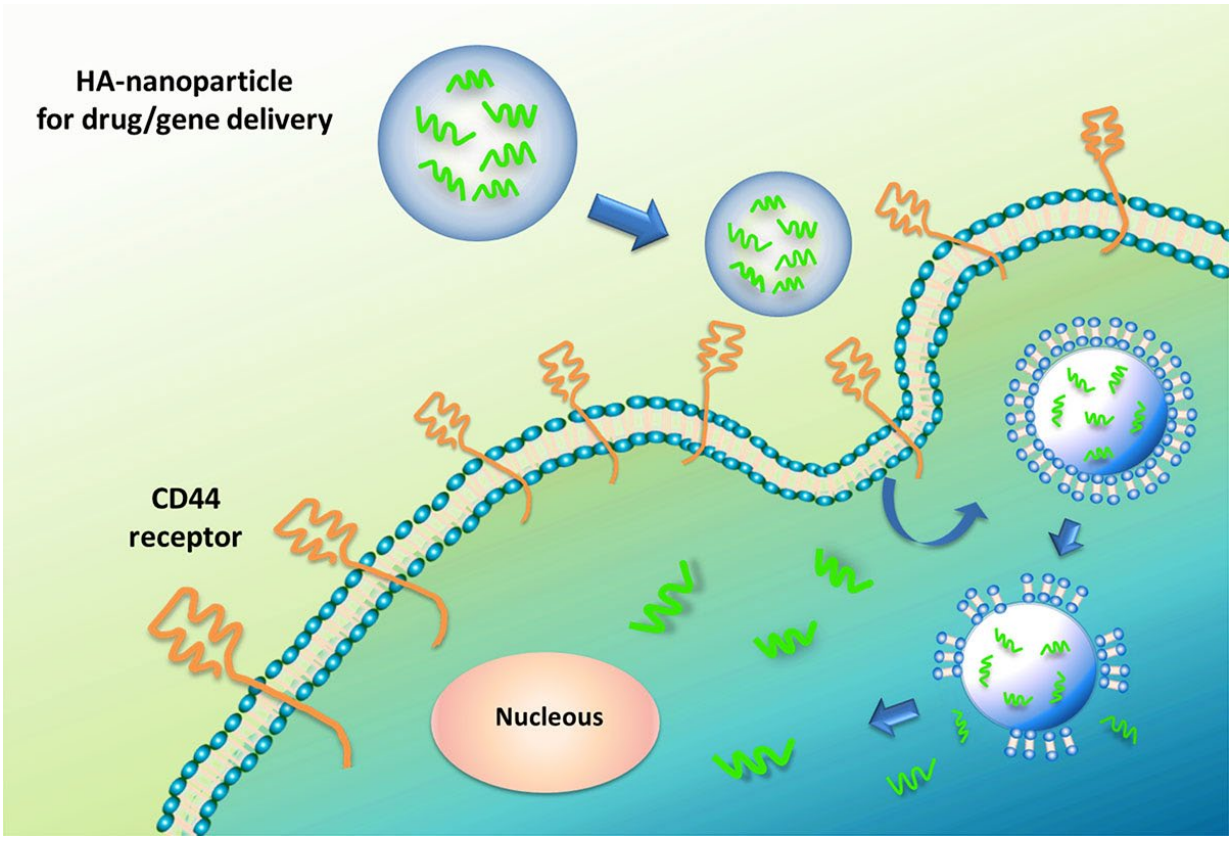

Fig. 8. HA-modified nanoparticles are easily internalised by CD44⁺ cell. The CD44 receptor present on the surface of articular cells, particularly of chondrocytes, mediates internalisation of the nanoparticles that can be loaded with therapeutics agents or complexed with genes for regenerative medicine strategies.

of several synthetic polymers, such as PLA or PLGA, are covered with HA in order to allow for sustained drug delivery in the vicinity of the joint and to achieve high drug concentrations at the site of action for the treatment of arthritis and/or OA (Laroui *et al.*, 2007; Zille *et al.*, 2010). Moreover, the potential role for HA in gene therapy strategies has been tested by the formulation of HA nanoparticles as delivery systems for plasmid DNA. For example, hybrid HA/chitosan nanoparticles were developed as novel non-viral gene delivery vectors capable of transferring exogenous genes into primary chondrocytes for the treatment of joint diseases (Lu *et al.*, 2011). Indeed, higher chondrocyte transfection efficiency of nanoparticles containing HA/chitosan and pDNA encoding TGF-β1 is observed as compared to the same nanoparticles without HA (Lu *et al.*, 2013). In addition, the formulation of a novel HA hydrogel as a delivery system for the controlled release of gapmer antisense oligonucleotides for unassisted cellular entry and subsequent gene silencing activity was also investigated for cartilage repair and interruption of damage progression by Cai *et al.* (2017).

HA was also studied in the treatment of different pathologies in which the immune system is deregulated, such as DM, RA and Sjögren

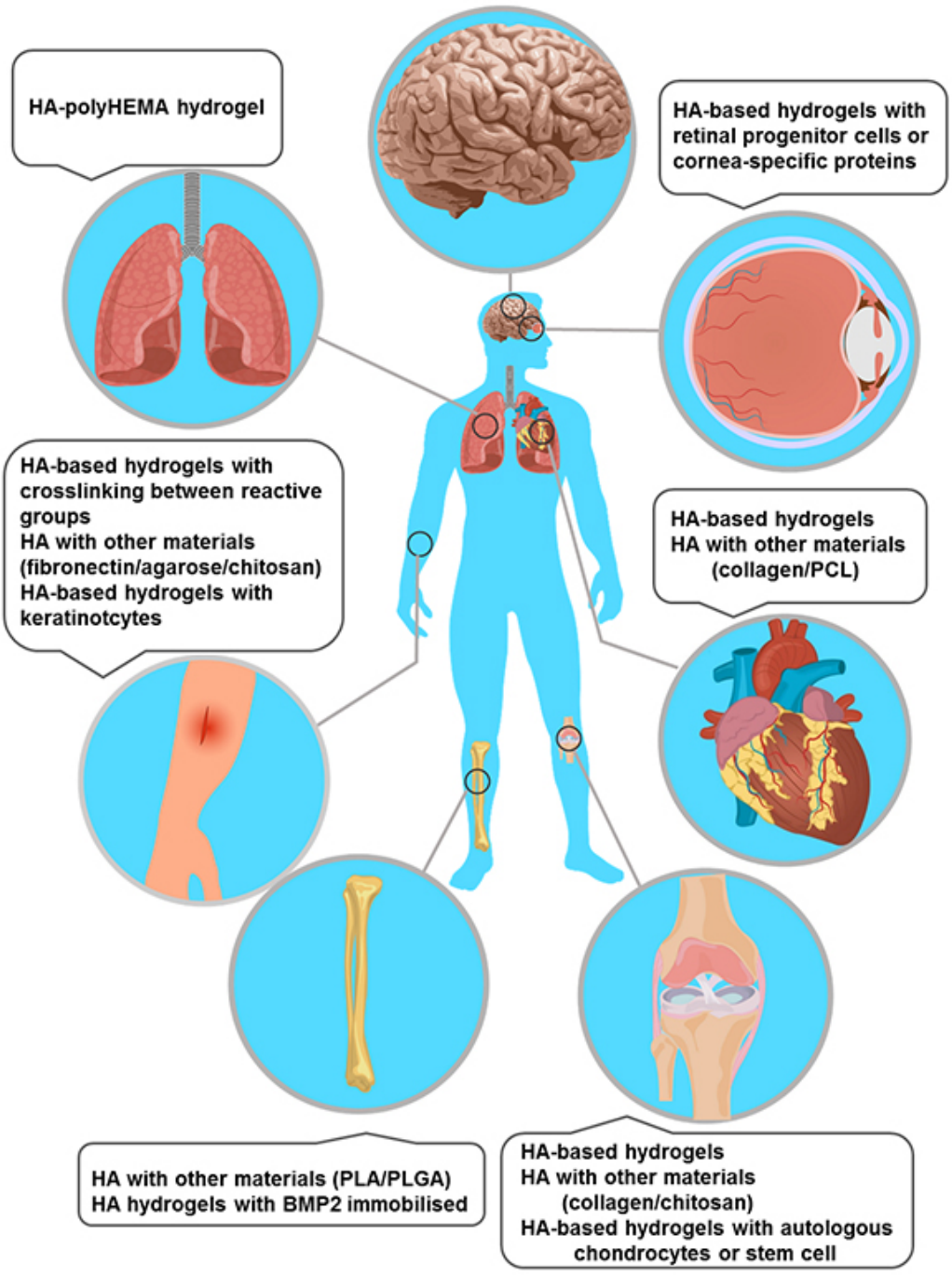

Fig. 9. Therapeutic applications of HA-based scaffolds in organ-specific TE and regenerative medicine. HA-based scaffolds are a key component for biomedicine applications such as the healing and regeneration of cartilage, bone, skin, nervous, cardiac and lung tissue and eye surgery.

syndrome, among others. CD44 is expressed by numerous immune cell types (lymphocytes, neutrophils, macrophages, natural kill cells, *etc.*) and the interaction of HA with CD44 favours the activation and migration of these cells towards the inflamed injury, with HA interacting with cytokines and modulating the immune responses (Zamboni *et al.*, 2018; Zamboni and Collins, 2017). Nakamura *et al.* (2004) showed, in a mouse liver injury model, that high molecular weight HA has intrinsic anti-inflammatory properties since HA-CD44 binding can inhibit the production of proinflammatory cytokines. Rho *et al.* (2018) showed that HA-based nanoparticles can suppress the inflammation of the adipose tissue in type 2 DM because of a decrease in both macrophages and proinflammatory cytokines. In addition, these nanoparticles can inhibit the domain-containing protein 3 inflammasome activity, causing an improvement in insulin sensitivity and normalised blood glucose levels (Rho *et al.*, 2018). RA is a chronic autoimmune disorder that results in systemic autoimmune destruction of bone and cartilage. In the treatment of the pathology, HA administered together with solid lipid nanoparticles can improve the vehiculation of different drugs (*i.e.* glucocorticoids) to inflammatory cells (through binding to $CD44^+$ cells), facilitating the selective release of these drugs in inflamed tissues (Zhou *et al.*, 2018). Dry eye disease is the main clinical manifestation of Sjögren syndrome, a chronic inflammatory disease with an autoimmune basis (Stefanski *et al.*, 2017). HA is administered as a substitute for tears and it acts on the ocular surface, increasing its hydrophilicity and density and reducing the surface tension while favouring the contact time on the cornea and, thereby, the hydration of the eye (Cagini *et al.*, 2017).

Applications in TE

Another promising area of regenerative medicine in which HA is making a significant contribution is TE. In fact, HA-based scaffolds are widely used in the regeneration of tissues such as cartilage, bone, skin, nerve tissue and cardiac tissue (Hemshekhar *et al.*, 2016) (Fig. 9).

Cartilage and bone TE

There is an increasing interest in the study of HA-based 3D scaffolds for cartilage and bone TE (Bowman *et al.*, 2018). HA has been used for cell encapsulation of hESC-derived chondrogenic cells. Toh *et al.* (2010) created a cell-engineered cartilage by mixing the cells with HyStem® matrix. This artificial ECM was investigated in an osteochondral defect rat model showing a complete integration with the adjacent host cartilage, with no evidence of tumorigenicity, and generating an ECM similar to the native cartilage tissue (Toh *et al.*, 2010). The concentration of HA is a determining factor in the production of hyaline cartilage, since it is directly related to expression of chondrogenic genes, production of GAGs and suppression of fibrocartilage production (Zhu *et al.*, 2017). Erickson *et al.*, (2012) established that lower concentrations (1 % w/vol) of crosslinked HA hydrogels increase MSC seeding density more than hydrogels at 3 % or 5 % w/vol concentrations. These hydrogels formed by a photocrosslink reaction of a MeHA derivative are highly appropriate to form ECM and to reproduce the native cartilage tissue properties (Erickson *et al.*, 2012). An additional advantage of HA for cartilage regeneration is related to its release ability. As shown by Deng *et al.* (2019), the incorporation of HA to PLA/PEG/PLA hydrogels favours a controlled release of TGF-β3, which improves chondrogenesis both *in vitro* and *in vivo*.

For bone regeneration, several scaffolds have been created by the combination of HA with other polymers with the aim of providing good mechanical properties and improving degradation rates. Antunes *et al.* (2010) developed a hybrid scaffold that shows great promise for bone tissue applications. HA has been used to coat scaffolds produced from PLA and to enhance the bio-compatibility and biological cell response, joining the best properties of each material. 3D PLA porous hybrid scaffolds are coated with HA, followed by crosslinking with glutaraldehyde, to produce biomimetic porous scaffolds for cortical bone therapeutic agents. In addition, results derived from using fibroblasts show that these scaffolds allow cell adhesion, growth and proliferation (Antunes *et al.*, 2010). This improvement of mechanical properties can also be achieved thanks to the combination of different natural materials. Combination of HA with nano-hydroxyapatite, chitosan and chondroitin sulphate allows obtaining scaffolds that mimic both the structure and the composition of natural bone, also showing a good biocompatibility (Hu *et al.*, 2017). On the other hand, modifications in HA, such as sulphation, can induce differentiation towards osteoblasts and production of a native bone ECM, through synthesis, secretion and/or activity of fibrillary or non-fibrillary ECM components, including proteases and their inhibitors (Schmidt *et al.*, 2018).

Skin TE

Skin substitutes, including acellular matrices, cellular substitutes and keratinocyte grafts, must meet indispensable characteristics, such as semi-permeable barrier, cell adhesion, non-toxic, non-inflammatory, non-immunogenic, durable, malleable and biodegradable properties (Nicholas *et al.*, 2016). HA combines such characteristics with the ability to inhibit tissue adhesion and scar tissue formation (Monteiro *et al.*, 2015). Thiolated HA derivatives based on the strategy developed by Shu *et al.* (2002) can oxidise in air to form disulphide cross-linked HA hydrogels. Following this strategy, commercial thiolated carboxymethyl hyaluronic acid or CMHA-S (CanitrX® Gel; SentrX®, SentrX™ Animal Care, Inc.,

Salt Lake City, UT, USA), have been marketed to facilitate and accelerate wound healing in dogs. The healing process in the presence of these materials is slower but at the same time the scar formation is minimised (Hadley *et al.*, 2013).

HA was also combined with decellularised extracellular matrix and human peripheral blood mononuclear cells for skin wound healing, showing regeneration of the epidermis and dermis in the wounds created in nude mice (Kuna *et al.*, 2017). Moreover, HA can be applied in combination with other materials. The incorporation of a photocross-linkable FN conjugated into HA 3D hydrogel network enhances endothelial cell adhesion and angiogenesis showing great potential in skin TE (Seidlits *et al.*, 2011). Tamer *et al.* (2018) studied the combination of HA with chitosan – a combination that has positive effects on healing – while incorporating mitochondrially-targeted antioxidant-MitoQ (BioAssay Systems, Hayward, CA, USA) an element that allows to obtain better results, with a faster and more efficient healing, and trigger less inflammation *in vivo*.

Different examples of commercially available products based on HA or its derivatives can be found. Hyalomatrix® (Anika Therapeutics, Inc., Bedford, England) a non-woven pad made of partially esterified HA (HYAFF®), is a dermal substitute that acts as a 3D scaffold for cellular invasion and capillary growth. This advanced wound care device is indicated for the treatment of partial to full-thickness post-traumatic, post-surgical or deep-chronic wounds, such as vascular ulcers or diabetic foot ulcers (Gravante *et al.*, 2010). Membranes such as LaserSkin® (Fidia Advanced Biopolymers, Padua, Italy), consisting also of HYAFF® but with microperforations, are available for treatment of chronic, non-healing wounds, allowing successful treatment when used in combination with autologous or allogenic keratinocytes (Uccioli, 2003).

Nervous TE

HA is a promising material for brain and neural regeneration since it is widely present in the central nervous system and has the capacity to reduce glial scar formation and allow the penetration of cells, nerve fibres and blood vessels (Kornev *et al.*, 2018). Thus, topical applications of a highly purified sodium hyaluronate formulation (Orthovisc®, Anika Therapeutics, Inc.) on rat transected sciatic nerves show a significant reduction in perineural scar thickness while, at the same time, promoting neural regeneration (Özgenel, 2003). HA hydrazone derivatives cross-linked with laminin and ECM protein involved in neuronal development and survival have been evaluated for *in vivo* brain lesion repair. These hydrogels, once implanted into cortical defects created in rats, form a scaffold that support cell infiltration and angiogenesis and promote neurite extension (Hou *et al.*, 2005).

HA hydrazone derivatives have also been coupled with short cell-adhesive peptides such as RGD and IKVAV. These derivatives show similar results to those obtained for HA-laminin derivatives, promoting cell migration and axonal growth and regeneration (Cui *et al.*, 2006; Wei *et al.*, 2007). Furthermore, hydrogels made of blends of HA and methylcellulose or collagen I can induce neuronal differentiation of brain-derived neural stem/progenitor cells (Brännvall *et al.*, 2007; Mothe *et al.*, 2013; Valderrábano, 2007).

In designing engineered tissues such as nerve and myocardium, an important consideration is the anisotropic structure to accurately mimic the structure and function of the native electroactive tissue (Kim *et al.*, 2007; Valderrábano, 2007). In this context, there has been increasing attention on engineering biomaterial scaffolds that are electrically conductive. Indeed, several research groups have explored the use of conductive polymers to regulate cellular response, including DNA synthesis, cell adhesion, proliferation, migration and differentiation (Jeong *et al.*, 2008; Park *et al.*, 2014; Shi *et al.*, 2008). Wang *et al.* (2017a) used HA to improve the bioactivity of the conductive polymer PEDOT, a polythiophene derivative. PEDOT-HA conductive nanoparticles are synthesised by chemical oxidative polymerisation and incorporated into PLLA. PEDOT-HA/PLLA films are cultured with neuron-like pheochromocytoma (PC12) cells showing better cell adhesion, viability and spreading as compared with pure PLLA films. Moreover, increased neurite length under electrical stimulation as opposed to under no electrical stimulation condition is demonstrated. The results indicate that the conductive PEDOT-HA/PLLA films combined with electrical stimulation may be a promising strategy for enhancing nerve regeneration in nerve TE (Wang *et al.*, 2017a).

Cardiac TE

TEHV were studied with the aim of creating living, non-cytotoxic, mechanically analogous heart valve replacements that can grow and remodel with the patient (Masoumi *et al.*, 2014; Sanz-Garcia *et al.*, 2015). VICs are the most prevalent cell type in aortic heart valves and these cells have been utilised to engineer functional TEHV (Mahler and Butcher, 2011; Masoumi *et al.*, 2014). VICs can enhance ECM production on photopolymerisable hydrogels formed from 2 % MeHA solution, proving that HA can stimulate heart valve tissue formation (Masters *et al.*, 2004). Additionally, scaffold degradation products increase VICs cell viability and induce proliferation and matrix synthesis after 6 weeks in culture (Masters *et al.*, 2005). Hybrid meshes that blend HA with PCL support cell adhesion and proliferation over the entire construct and formation of primitive capillary network (Ekaputra *et al.*, 2011; Eslami *et al.*, 2014). Moreover, acellular HA-based hydrogels reduce stress in the heart wall after MI (Ifkovits *et al.*, 2010). Ifkovits *et al.* (2010) described how mechanical properties of HA hydrogels are modified through a simple alteration in the number of reactive methacrylate groups on the MeHA polymer:

30 % (MeHA low) *versus* 60 % (MeHA high). The two MeHA formulations exhibit similar degradation and tissue distribution upon injection but have different storage modulus (~ 8 kPa *versus* ~ 43 kPa). Moreover, only the higher-modulus (MeHA high) treatment group show a statistically smaller infarct area as compared with the control infarct group when injected into a clinically ovine MI model (Ifkovits *et al.*, 2010). Taken together, these studies indicate that HA matrices represent a good choice to promote cardiovascular regeneration.

Other tissues in TE

Space-filling HA-based scaffolds provide bulking, prevent adhesions, excessive scar formation or function as bio-adhesives after surgical procedures or injury. Hyaloglide® (Anika Therapeutics, Inc.) is an auto-cross-linked HA derivative that forms a highly viscous gel. About 5 % of the HA carboxyl groups are activated to promote the cross-linking reaction by nucleophilic attack of the GluNAc unit primary hydroxyl groups to form an esterified cross-linked HA (Atzei *et al.*, 2007). This HA derivative, approved for clinical use, shows a reduction of pain and a major recovery of sensitivity after tendon and peripheral nerve surgery (Atzei *et al.*, 2007; Pederzini *et al.*, 2013).

Several studies have opened new avenues towards the use of HA-based scaffolds for TE. Espandar *et al.* (2012) showed the *in vivo* growth of human adipose stem cells on HyStem® matrix, with cells surviving and expressing human-cornea specific proteins. For lung tissue regeneration, Radhakumary *et al.* (2011) developed a copolymer of HA-poly HEMA that is non-cytotoxic to mammalian cells. Further, this co-polymer hydrogel supports alveolar cell adhesion and growth and is suitable for the adhesion and proliferation of multiple cell types (Radhakumary *et al.*, 2011). For meniscal regeneration, MSCs in a hyaluronan-collagen scaffold display good healing potential with the development of integrated meniscus-like repair tissue (Murphy *et al.*, 2018). Furthermore, the implantation of eEPCs encapsulated in HyStem® matrix demonstrates improved resistance to toxic insult (adriamycin) *in vitro*, eEPC mobilisation to injured kidneys and improved renal function (Ratliff *et al.*, 2010).

Clinical applications of HA with cells and tissues

Cell therapies and TE remain very active areas of research, which have been translated to clinical trials at an extraordinary pace recently (Gálvez-Martín *et al.*, 2017). Currently, from a total of 6,085 clinical trials with stem cells being carried out worldwide, 16 assess the efficacy and safety of stem cells in combination with HA for different therapeutic applications, most of which focus on OA and cartilage regeneration (Table 1**a**,**b**). Moreover, HA viscosupplementation is a popular treatment option in the non-operative management of patients with OA; HA is also used in many studies as an active control to compare the efficacy of stem cell transplantation in many OA treatments (Web ref. 1-6). The increasing use of HA in combination with cells in current clinical trials usually responds to the need of improving delivery methods and enhancing survival and retention of cells *in vivo*. Randomised controlled trials have been successfully carried out and are currently evaluating an optimal stem cell source, together with HA for therapeutic repair of cartilage tissue. In one of these clinical studies, Saw *et al.* (2013) transplanted autologous PBSCs in combination with HA into an articular cartilage defect by IA injections after arthroscopic subchondral drilling. This resulted in an improvement of the quality of articular cartilage repair when compared with the same treatment with injections of HA alone (Saw *et al.*, 2013; Web ref. 7). A similar study using PBSCs and HA is currently recruiting patients (Web ref. 8). Attempts to regenerate cartilage have also been made using MSCs originating from the BM. In a recent multicentre randomised clinical trial, IA delivery of increasing doses of BM-MSCs in combination with HA sodium salt (1,500-2,000 kDa; Hyalone®, Fidia Farmaceutici S.p.A., Abano Terme, Italy) has been evaluated (Web ref. 9). Both treatments, HA alone or with low-dose BM-MSCs (10×10^6), can reduce pain in the first 6 months; however, patients receiving the high-dose of BM-MSCs (100×10^6) together with HA exhibit an improvement in their perception of pain in their daily activity after 12 months as compared to HA alone (Emadedin *et al.*, 2012). The study demonstrated that the single IA injection of *in-vitro*-expanded autologous BM-MSCs together with HA is a safe and feasible procedure, which results in a clinical and functional improvement of knee OA (Lamo-Espinosa *et al.*, 2016). Recently, the long-term results of this clinical trial have been published confirming the safety of the procedure and a functional improvement of knee OA after a follow up of 4 years. However, long-term results show no clinical differences between low-dose and high-dose groups and the authors' question if a high dose of cells is needed. Additionally, the control group receiving only HA initially perceives pain reduction and improvement of physical function subscores but these improvements are not sustained after a long-term follow up. In any case, these results support the development of future phase III clinical trial (Lamo-Espinosa *et al.*, 2018). Similarly, another clinical trial aims at determining the efficacy of single IA implantation of autologous BM-MSCs in a high molecular weight injectable HA (Orthovisc®, Anika Therapeutics, Inc.) for the treatment of patients with mild to moderate OA: HA is used as an active comparator and the objective is to elucidate if IA implantation of autologous BMSCs in patients with mild to moderate OA can regenerate damaged cartilage (Web ref. 10). In another study, Stempeucel® (Stempeutics Research Pvt. Ltd., Bangalore, India), which consists of a cryopreserved stem cell product containing allogeneic MSCs

Table 1a. Clinical trials with HA and stem cells.

NTC number	Phase	Indication	Intervention	Purpose	Cell type
NCT01076673	II	Articular cartilage disorder of knee	IA injections of PBSCs and HA	To compare articular cartilage regeneration in patients with chondral lesions treated by arthroscopic subchondral drilling followed by postoperative IA injections of HA with and without PBSCs	PBSCs
NCT02123368	I/II	OA	IA injection of BM-MSCs (dose groups: 10 and 100 million cells) cultured *ex vivo* followed by an IA injection of HA (Hyalone®)	To determine the safety, feasibility and effectiveness of IA administration of different doses of BM-MSCs in combination with HA	Autologous BM-MSCs
NCT01459640	II	OA	Single IA implantation of BM-MSCs in 30 mg/2 mL of HA (Orthovisc®)	To determine the efficacy of IA implantation of autologous BM-MSCs for treatment of patients with mild to moderate OA	Autologous BM- MSCs
NCT01453738	II	Knee OA	Single IA dose of Stempeucel® (dose groups: 25, 50, 75 and 150 million cells) followed by 2 mL of HA (20 mg)	To evaluate the safety, potential efficacy and appropriate dose of IA administration of Stempeucel® followed by HA in patients with OA of the knee joint	Allogeneic BM- MSCs
NCT00225095	I/II	Recovery following partial medial meniscectom	IA injection of Chondrogen™, a preparation of BM-MSCs (dose goups: 50 and 150 million cells) suspended in 2 mL (20 mg) of sodium hyaluronate, human serum albumin (1.2 %) and PlasmaLyte A	To determine whether Chondrogen™ is a safe and effective post-operative treatment of the knee following menisectomy	Allogenic BM-MSCs
NCT02659215	N/P	Defect of articular cartilage	2 mL of BMAC loaded per Hyalofast® scaffold (a benzyl ester of HA) and, then, implanted to cover the defect	To evaluate the efficiency of Hyalofast® together with BMAC, in a one-step arthroscopic procedure as compared to microfracture in the treatment of symptomatic cartilage defects of the knee	BMAC
NCT01088191	I/II	Anterior cruciate ligament injury, OA	Single injection into the knee joint of two different single doses of MSB-CAR001 (mesenchymal precursor cells, Mesoblast, Ltd) combined with HA	To evaluate safety and efficacy of MSB-CAR001 in subjects who have undergone an anterior cruciate ligament reconstruction since short time	Allogeneic MPCs
NCT01290367	II	Degenerative disc disease	Injection of MPCs (dose groups: 6 and 18 million cells) in a HA carrier into the degenerated lumbar disc's nucleus pulposus	To compare two doses of immunoselected, allogeneic MPCs when combined in a HA carrier in subjects with chronic lower back pain due to moderate degenerative disc disease	Allogeneic MPCs
NCT02412735	III	Degenerative disc disease	Injection into the painful IVD of Rexlemestrocel-L (dose: 2 mL of 6 million cells) alone or combined with 1 % HA	To evaluate the safety and efficacy of Mesoblast's Rexlemestrocel-L alone or combined with HA in subjects with chronic lower back pain	Allogeneic MPCs

Table 1b. Clinical trials with HA and stem cells.

NTC number	Phase	Indication	Intervention	Purpose	Cell type
NCT02338271	I	Lower back pain	A single injection into the centre of the nucleus pulposus of adipose-derived MSCs (dose group: 2×10^7 and 4×10^7 cells/mL/vial) plus Tissuefill® 1 mL/syringe	To evaluate safety and efficacy of autologous adipose-derived MSC implantation in chronic lower back pain patients with lumbar IVD degeneration	Autologous adipose-derived MSCs
NCT01733186	I/II	Degeneration of articular knee cartilage	Single-dose of 0.5 mL of Cartistem® (UCB-MSCs combined with sodium hyaluronate) per cm^2 of cartilage defect	To determine the safety and efficacy of Cartistem® in the treatment of articular cartilage defects of the knee as a result of ageing, trauma or degenerative diseases	UCB-MSCs
NCT02755376	N/P	Injuries of anterior cruciate ligament	Single-dose of Cartistem®, a combination of human UCB-MSCs and sodium hyaluronate	To evaluate enhancement of healing of anterior cruciate ligament injury using UCB-MSCs	UCB-MSCs
NCT02034786	I	Lipodystro-phies,aesthetics procedure	Transdermal injection of a filler agent composed of MSCs from autologous adipose tissue associated with HA	To study the efficacy and safety of transplantation of autologous adipose stem cells with HA for the treatment of lipodystrophy	Autologous MSCs from adipose tissue
NCT02698813	I	Senescence wrinkles, acne, pitting scar	Transdermal injection of UC-MSCs and HA	To evaluate the safety and efficacy of the injectable filler agent composed of UC-MSCs and HA for the improvement of wrinkles, acne and pitting scar	UC-MSCs
NCT01981330	I	Severe hoarseness and vocal fold scarring	Single injection of BM-MSCs mixed with a carrier hyaluronan gel	To evaluate the efficacy of the injection of BM-MSCs mixed with and without hyaluronan gel to heal scarred vocal folds	Autologous BM-MSCs
NCT03101163	II	Degeneration and disorder of articular knee cartilage	IA injections of PBSCs and HA	To evaluate and determine whether PBSCs with HA therapy can improve functional outcome and reduce pain of the knee joint better than a standard treatment	Autologous PBSCs

from multiple BM donors, has been tested together with HA in order to increase the engraftment and chondrogenic activity. In the preclinical model of OA, Stempeucel®, at both low and high doses and supplemented with HA, significantly reduces pain and repairs damaged articular cartilage in rats (Gupta *et al.*, 2016). In the clinical study, IA administration of Stempeucel® followed by injection of HA appears to be safe, showing a trend towards improvement in all subjective parameters (VAS, ICOAP and WOMAC-OA scores) in the low-dose group. However, when compared to a placebo (Plasmalyte-A, Baxter Healthcare Ltd., Norfolk, England) no therapeutic efficacy is observed, which the authors attribute to the small number of patients enrolled in the study. Another drawback observed in the study is that the treatment with higher dose groups (50, 75 and 150×10^6 cells) causes knee pain and swelling (Gupta *et al.*, 2016; Web ref. 11).

HA together with MSCs tested for meniscus repair

In a double-blind clinical study with Chondrogen® (Osiris Therapeutics, Baltimore, MD, USA) a preparation of adult MSCs in a solution containing HA shows that a single injection of Chondrogen® into the knee of patients following partial meniscectomy can significantly increase meniscal volume and reduce knee pain as compared with the use of HA alone, when measured 12 months post meniscectomy (Web ref. 12). Patients with osteoarthritic changes

at the time of surgery experience a reduction in pain following the treatment due to HA – which is a commonly used treatment for OA pain (Concoff *et al.*, 2017) possibly providing some benefit to the patients (Vangsness *et al.*, 2014). These studies help to elucidate the role of MSCs in knee-tissue regeneration and to increase the potential of HA in cell therapy. The use of HA-based scaffolds loaded with cells that fit the cartilage lesion is the promising strategy of a clinical trial currently recruiting participants to evaluate the safety and efficacy of Hyalofast® (Anika Therapeutics, Inc.), a biodegradable hyaluronan-based (HYAFF®) scaffold, and BMAC in the treatment of cartilage defects of the knee. The purpose of the study is to evaluate the efficacy of a Hyalofast® scaffold, which acts as a biodegradable support for the autologous BMAC and to compare this treatment to microfracture, an arthroscopic surgical technique providing stem cells and growth factors from the BM to aid cartilage repair (Web ref. 13). Cavallo *et al.* (2013) demonstrated *in vitro* that BMCs grown onto HYAFF®-11 can differentiate into chondrogenic cells by expressing and producing specific ECM molecules. Additionally, a previous study with 20 patients older than 45 years demonstrated the safety and efficiency of treating full thickness cartilage lesions with Hyalofast® soaked in BMACs, showing that indication to surgery should not be based only on age, but rather on lesion size and number. However, a long-term comparative study with a larger sample and with a detailed radiological analysis is still needed to ultimately assess the potential of this technique (Gobbi *et al.*, 2017).

Another product, consisting of immune-selected allogenic MPCs derived from adult BM, is being tested in combination with HA and HA alone as an active comparator for the treatment of anterior cruciate ligament injury (Web ref. 14), degenerative disc disease (Web ref. 15) and is currently recruiting participants with chronic lower back pain for a new phase III study (Web ref. 16). Preliminary results from the anterior cruciate ligament injury study showed that IA administration of MPCs is safe and well tolerated, both MPCs + HA- and HA-injected groups show symptomatic improvement; however, the MPC + HA-treated group exhibits larger changes from baseline (Wang *et al.*, 2017b).

The implantation of autologous MSCs from adipose tissue was tested in combination with a medical fill composed of an HA gel cross-linked with butanediol diglycidyl ether (Tissuefill® NeoGenesis Co., Ltd., Seoul, Korea) as a cell carrier in patients with chronic lower back pain caused by lumbar disc degeneration (Web ref. 17). Ahn *et al.* (2015) showed that co-administration with Tissuefill® can enhance the efficacy of intradiscally injected MSCs. The results from the phase I clinical trial confirm the safety and tolerability of co-injection of MSCs and the HA derivative in patients with IVD degeneration. Significant improvements in pain are demonstrated in 6 out of 10 patients and rehydration of the disc in 3 out of 6 patients throughout the 12-month duration. The exact mechanism by which the combined implantation of MSCs and the HA derivative leads to improvement of chronic discogenic lower back pain remains unclear, however, the authors assume that injection of MSCs into the degenerated disc improves ECM production by the degenerated host nucleus pulposus cells and modulates their immunological response to inflammatory cytokines that could potentially inhibit the inflammatory cascade, thereby preventing ingrowth of pain-inducing vasculature and nerve fibres. On the other hand, suspension of the cells in HA derivative for coadministration may prevent cell leakage, reduce the risk of uncontrolled differentiation of MSCs into osteoblasts and enhance cell attachment and cell survival (Kumar *et al.*, 2017).

Among the few MSC-based products that are approved worldwide, one combines sodium hyaluronate with human UC-MSCs. Cartistem® (Medipost Co., Ltd, Bundang-gu, Korea) has been approved for sale in Korea by the Korean FDA since January 2012 and is intended to be used as a single-dose therapeutic agent for cartilage regeneration in cartilage defects of the knee. Currently, there is an ongoing clinical study to assess the efficacy of Cartistem® in articular cartilage defects (Web ref. 18) and another one for the healing of the anterior cruciate ligament injury (Web ref. 19). Results from phase I/II clinical trial in a small sample size ($n = 7$) investigating this product for the regeneration of articular cartilage defects in OA patients show an acceptable efficacy and safety profile due to improvements in pain and function at 24 weeks post-transplantation that are maintained without significant deterioration up to 7 years. The repair site is restored with hyaline-like cartilage tissue, which seems to contribute to the observed persistence of the regenerated cartilage with durable improvement in pain and function (Park *et al.*, 2017).

As a biocompatible filler agent, HA has been used for over 25 years for cosmetic purposes and for reconstruction of soft tissues. Transdermal injection of HA in combination with autologous MSCs from adipose tissue is currently being evaluated for treatment of lipoatrophy, lipodystrophy types characterised by subcutaneous adipose tissue atrophy due to trauma, tumour resection and congenital defects. The objective is to evaluate if this combination is more effective in tissue reconstitution, providing volume and cell differentiation and increased production of ECM than the injection of adipose tissue only (Web ref. 20). In this light, another randomised study will be open for participant recruitment in order to evaluate the safety and efficacy of the transdermal injection of a filler agent composed of UC-MSCs and HA for the improvement of wrinkles, acne and pitting scar; injection of HA only will be used as an active comparator (Web ref. 21). The healing of vocal fold scarring, to treat severe hoarseness and vocal fold scarring, is also being tested in an ongoing clinical trial using a single injection of autologous MSCs from

BM alone or MSCs mixed with hyaluronan gel as a carrier into the vocal folds (Web ref. 22).

Conclusions and future perspectives

Several chemical strategies have been developed to modulate the HA mechanical, chemical and biological properties with the objective of broadening HA usefulness in cell therapy and TE. Moreover, there are increasing clinical trials based on the combination of stem cells with HA or HA derivatives. These facts unequivocally show the virtues of using HA derivatives for *in vitro* or *in vivo* applications, alone or in combination with cells.

HA chains present several sites amenable to chemical modification, leading to the development of a wide array of derivatives aiming to reproduce the native tissue properties. Novel formulations that improve safety of HA crosslinking *in situ* and prolonged residence time are one of the main issues. To improve mechanical properties of HA hydrogels, concentrations of crosslinked HA have been modulated, opening the way to scaffolds with tuneable degradation rate adapted to the tissue of the injury. Moreover, with the aim to provide good mechanical properties, scaffolds are being created by the combination of HA with other polymers.

New strategies to enhance the bio-compatibility and biological cell response include combination of HA with materials such as fibronectin, laminin or small peptides, among others. In particular, decellularised tissue is a promising material that can be processed together with HA to produce a gel to provide mechanical, biophysical and biochemical cues to cells. Furthermore, the current state of research has emphasised on the use of HA hydrogels combined with drugs, plasmids or growth factors to guide endogenous stem cells towards a specific injured area or/and promote tissue regeneration. The possibility of administering or growing stem cells in scaffolds or hydrogels made of HA has created high expectations in this field, allowing *in vivo* cell differentiation after a surgical implant.

Towards next generation products, the functionalisation of conductive polymers by incorporating HA for enhancing their physicochemical and biological functionality may provide new anisotropic scaffolds to mimic the structure and function of native electroactive tissue. Currently, new preparations based on HA for viscosupplementation, including the combination of HA or its derivatives with PRP or MSCs derived from BMC, may offer an advanced treatment for regeneration, in particular of cartilage tissue, that could potentially provide better functional outcomes. Moreover, clinical trials are focussing on evaluating the efficacy and safety of stem cells together with HA formulations. Preliminary results, mainly directed to cartilage and skin regeneration or restoration, show that combination of HA and stem cells is safe and improves pain reduction and therapeutic efficacy by enhancing cell survival and tissue restoration.

Finally, novel techniques have also been employed towards fabricating HA-based scaffolds. The development of 3D bioprinting technologies have emerged as a powerful tool for TE due to the ability to mimic the 3D structure of any tissue. So, the combinations of HA-based bioinks, cells and biomaterials will allow obtaining mimetic tissues with similar characteristic to native ones and will accelerate the clinical application of that technology.

The development of new HA-based functionalised scaffolds and the use of HA or HA derivatives as bioinks that mimic ECM for 3D bioprinting in combination with different materials and cells are issues that will be the focus of future research for regenerative medicine purposes. It is expected that clinical translation of stem-cells-HA-based therapies will be a reality in the next few years.

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Discussion with Reviewer

Declan Devine: The authors illustrated a wide range of uses for HA. However, since native bone is a composite material, would HA be an appropriate matrix material for use in orthopaedic composites?
Authors: HA is an ideal material for natural scaffolds thanks to its unique biocompatibility, chemical functionalisation and degradability. Several studies have suggested that HA-containing scaffold materials are efficacious in bone-repair processes (Faruq *et al.*, 2017; Hamlet *et al.*, 2017; Hu *et al.*, 2017; Turnbull *et al.*, 2018, additional references). These studies assessed the utility of injectable HA gels for the treatment of bone-related diseases due to the involvement of HA in several biological processes, such as osteoinduction and wound healing (Suzuki *et al.*, 2014, additional reference). Due to the strong effects of HA on cell motility and cell-cell interactions and with the aim of improving tissue-scaffold interactions, other studies have explored the possibility of developing scaffold made of bioceramics and biopolymer, such as HA, resulting in the formation of composites for bone regeneration (Nguyen and Lee, 2014; Poldervaart *et al.*, 2017, additional references). In addition, the bone scaffold surface coating with HA is very effective in enhancing osteogenesis (Kang *et al.*, 2011; Motamedian *et al.*, 2015, additional references). Finally, regarding orthopaedic materials, HA and some of its composites offer a well-established long-term safety profile and a proven ability to reduce bacterial adhesion and biofilm formation on to the surface of various biomaterials commonly used in orthopaedics, trauma and dental surgery (Romanò *et al.*, 2017, additional reference).

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Editor's note: The Scientific Editor responsible for this paper was Chris Evans.