Trends in Biotechnology

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Review

Extracellular Matrix Mimics Using Hyaluronan-Based Biomaterials

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Hyaluronan (HA) is a critical element of the extracellular matrix (ECM). The regulated synthesis and degradation of HA modulates the ECM chemical and physical properties that, in turn, influence cellular behavior. HA triggers signaling pathways associated with the adhesion, proliferation, migration, and differentiation of cells, mediated by its interaction with specific cellular receptors or by tuning the mechanical properties of the ECM. This review summarizes the recent advances on strategies used to mimic the HA present in the ECM to study healthy or pathological cellular behavior. This includes the development of HA-based 2D and 3D *in vitro* tissue models for the seeding and encapsulation of cells, respectively, and HA particles as carriers for the targeted delivery of therapeutic agents.

Highlights

The extracellular matrix is composed of hyaluronan of different molecular weights that play different roles in cellular proliferation, migration, and differentiation.

The dysregulated accumulation of hyaluronan alters the mechanical and biochemical properties of the extracellular matrix and it is associated with several pathological states.

Biomaterials can be engineered using the native hyaluronan backbone or chemically modified to target cells via specific cellular receptors, such as CD44.

2D surfaces with controlled topography and 3D hydrogels with tuned mechanical properties and hyaluronan composition can be designed to mimic the bioactivity of this glycosaminoglycan in the ECM.

Biorecognition of Hyaluronan (HA)

The **extracellular matrix (ECM)** (see Glossary) is a rich and complex 3D environment mainly composed of different bioactive polymers, such as proteins and **glycosaminoglycans (GAGs)**, as well as glycoconjugates such as glycoproteins and proteoglycans. This ECM acts as a structural and functional support for cells in the different tissues of the body. The dynamic interplay between cells and the **biofunctional** ECM components regulates the normal activities of tissues through the activation of signaling cascades that modulate cellular behavior [1,2]. The highly dynamic character of the ECM includes the continuing production, degradation, and remodeling of its components. The balanced composition of the ECM is critically important to maintain the **homeostasis** of the extracellular environment. GAGs (including chondroitin sulfate, heparan sulfate, and HA) are key components of the ECM, as they are responsible for most of their physical properties and, more recently, they have been found to be key biofunctional elements responsible for modulating cellular behavior [3]. Among the GAGs that constitute the ECM of human tissues, HA stands out for being the only nonsulfated one and the most abundant.

HA is a linear, negatively charged polysaccharide that contains a disaccharide repetitive unit consisting of glucuronic acid (GlcA) and *N*-acetyl glucosamine (GlcNAc). In solution, and at physiological pH, the negative charge of HA is balanced with different cations (e.g., Na^+, Ca^{2+}, K^+) conferring to this polysaccharide a high hydrophilic character that leads to a high water retention, which, in turn, stabilizes its secondary structure. Under these circumstances, the carboxyl and acetamido groups interact by H-bonding, forming a twofold helix, presented as a single-stranded left-handed helix. Despite its hydrophilic nature, in aqueous solution, there are still hydrophobic interactions and intermolecular H-bonds that are able to generate an unstable β-sheet tertiary structure [4]. Under physiological conditions, HA assumes an expanded random coil structure surrounded by water molecules, which occupies a very large volume.

HA has a simple and linear chemical structure, which is not to say that it lacks bioactivity. HA is involved in various biological events, including cell proliferation [5] and differentiation [6], and it contributes to the physiological balance of the ECM by conferring hydration, lubrification, and matrix homeostasis [7].

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In contrast to the sulfated GAGs, HA cannot establish covalent links to proteins generating proteoglycans. However, it plays a unique role in the ECM, as its noncovalent interactions with different proteins occurs to generate a biofunctional outcome; these include the **hyaladherins** **CD44** or **RHAMM**, the specific HA cell surface receptors [8,9]. The interaction between HA and its receptors activates pathways related to cell adhesion [e.g., focal adhesion kinase (**FAK**)], proliferation, and migration [extracellular-regulated kinase (**ERK1/2**)] [10]. HA has been also associated with different pathological states, such as cancer, as it is a modulator of invasiveness and metastasis [10,11]. The biological relevance of HA has led to an increasing interest in the evaluation of its bioactivities in the ECM and its ability to modulate cellular function. These activities have prompted the development of different HA-based platforms to study *in vitro* the interplay between HA and cells. These systems are being designed to evaluate not only the bioactivities of HA [e.g., interaction with different proteins, such as growth factors (GFs)], but also how HA can modulate the ECM mechanical features through the deposition of HA of different molecular weights (M_w).

The central role of HA in the onset and progression of different diseases has prompted interest in its different bioactivities under pathological and healthy conditions. Here, we review the biological importance of the chemical and physical structure of HA, covering the state of the art in the influence of the M_w of HA on triggering different pathological states (e.g., cancer, tissue fibrosis); the development of HA-based carriers (e.g., particles, hydrogels, etc.) for the delivery of therapeutic drugs; and HA-based 3D microenvironments mimicking the ECM composition to evaluate the impact of HA chemical composition and its **mechano-transduction** ability on the onset and progression of cancer. Finally, we will cover HA-based materials design for therapeutic applications that are currently in the market.

Role of HA in the ECM

HA as a Structural Element in the ECM

HA is one of the major constituents of the ECM and is responsible for maintaining tissue homeostasis, assuming a function of space filling element in connective tissues. Depending on its M_w, this GAG plays different important physiological functions as a structural element of the ECM, including maintaining the hydration of tissues, modulating diffusion and exchange of ions and biomolecules, and providing mechanical support for cells [12]. Moreover, its ability to interact with other biomolecules (e.g., proteoglycans such as versican and aggrecan, and proteins such as **TGS-6** or fibronectin) present in the cellular milieu also modulates the physical properties of the ECM through different structural rearrangements [13]. For example, the noncovalent interplay between HA and the proteoglycan aggrecan is essential for the function of articular cartilage. In addition, the chondroitin sulfate-based proteoglycan versican interacts with HA, being the basis of the formation of the pericellular matrix [14].

The M_w of HA

The different M_w of HA present in the ECM (Box 1) is a key modulator of cellular behavior and contributor to tissue homeostasis. HA induces numerous cellular responses, mainly due to its interaction with different receptors. The most well-known, and widely described in the literature, are CD44 and RHAMM. However, there are other HA binding proteins, such as toll-like receptors 2 and 4 (**TLR**-2,4), hyaluronan receptor for endocytosis (**HARE**), hyaluronan-binding protein 1 (**HABP1**), and lymphatic vessel endothelial receptor for hyaluronan 1 (**LYVE1**) [15]. In addition, the M_w of HA is associated with different biological functions: the short chains of HA are usually associated with proinflammatory response, proangiogenic activity, and migration and proliferation of cells, whereas the longest chains are usually linked to cellular differentiation and anti-inflammatory effects (Table 1).

Glossary

Biofunctionality: characteristic of a material/matrix related to its ability to exert a biological function.
BMP-6: bone morphogenetic protein 6, known for their ability to induce the growth of bone and cartilage, upregulating osteogenic markers in mesenchymal stem cells.
C2C12: myoblasts cell line derived from mouse muscle tissue.
$CaCO_3$: calcium carbonate typically used for the preparation of nano-/microparticles as inorganic templates.
CD44: a transmembrane cell surface glycoprotein with a link module to hyaluronan.
ERK1/2: extracellular signal-regulated kinase 1 and 2, involved in the signaling cascades that regulate the cells adhesion, proliferation, migration, cell survival, and differentiation.
Extracellular matrix (ECM): tridimensional network composed of proteins, glycoproteins, glycosaminoglycan, proteoglycans, and cytokines that provides structural and biochemical support to cells.
FAK: focal adhesion kinase that regulates the cellular movement and the remodeling of the cytoskeleton, crucial for the migration of cells.
Glycosaminoglycans (GAGs): polysaccharides present in human tissues.
HABP1: hyaluronan binding protein, responsible for different cellular functions.
HARE: hyaluronan receptor for endocytosis mediates the systemic clearance of hyaluronan.
HAS: hyaluronan synthase, a transmembrane enzyme responsible for production and extrusion of hyaluronan to the extracellular space.
Homeostasis: biological, chemical, and physical equilibrium maintained in living tissues.
Hyaladherin: hyaluronan-binding proteins.
Hyals: hyaluronidases, an intra- and extracellular enzyme responsible for the degradation of hyaluronan.
Layer-by-layer (LbL): an assembly technique based on the deposition of oppositely charged polyelectrolytes to form a multilayered thin film.
LYVE1: lymphatic vessel endothelial receptor for hyaluronan, a membrane glycoprotein with an extracellular link domain.

Box 1. Synthesis, Structure, and Degradation of Hyaluronan

The linear structure of HA is generated and extruded through the plasma membrane to the pericellular and extracellular space, in a process mediated by the transmembrane synthases **HAS**s. At their cytoplasmic domain, HASs catalyze the reaction between UDP-glucuronic acid (UDP-GlcA) and UDP-*N*-acetylglucosamine (UDP-GlcNAc) with the reducing end of the HA chain, promoting its elongation. Depending on the type of HAS responsible for the synthesis, HA are generated with different M_w: HAS3 produces the shortest chains, between 1×10^5 and 2×10^5 Da, and HAS1 and HAS2 generate the longest ones, from 2×10^5 to 2×10^6 Da (Figure I), reaching polymer lengths of approximately 25 μm [91].

Balanced deposition and degradation of HA at the ECM is vital to maintain the homeostasis of the cellular environment. In this context, while the HASs regulate the deposition of HA, the Hyals and free radicals, such as reactive oxygen species (**ROS**), are key elements that control the degradation of HA, contributing to their turnover at the ECM [92]. While Hyal1 is an intracellular enzyme, Hyal2, a glycosylphosphatidylinositol (GPI)-linked enzyme, is anchored to the cell membrane at the pericellular space [93]. Despite their independent modes of action, Hyal1 and Hyal2 are important contributors to the biochemical pathways that lead to the degradation of HA. Hyal2 cleaves HA at the cell surface, supported by the major HA receptor, CD44, which holds the HA molecule in place for the enzyme to cleave it into small fragments; subsequently, HA is endocytically transported to the lysosomes to be degraded by Hyal1 [93].

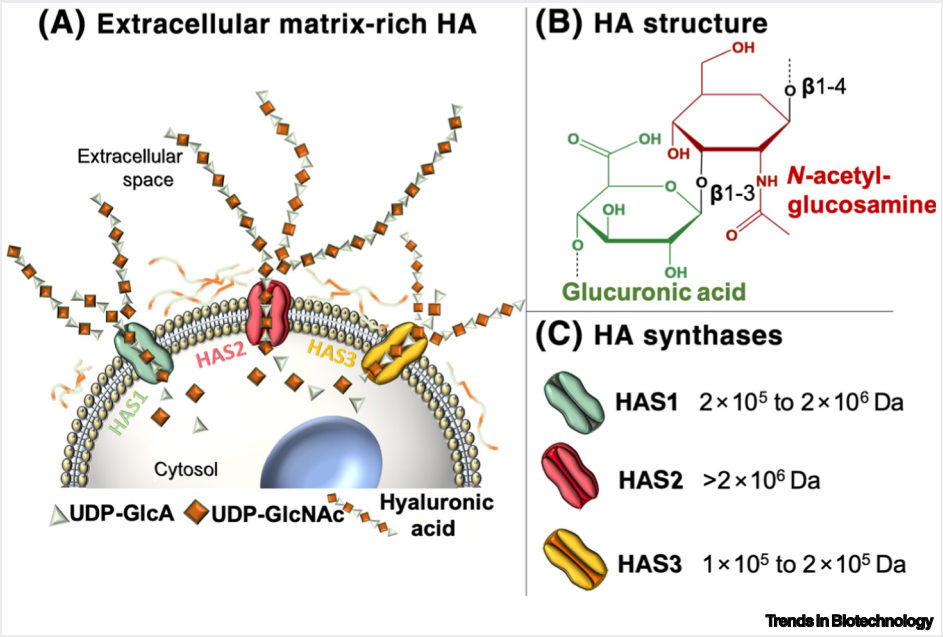

Figure I. Hyaluronan Synthesis and Structure. (A) Hyaluronan (HA) molecules with different molecular weights (M_w) are extruded from the cytoplasm to the extracellular matrix through the HA synthases (HAS1-3) pores, that link the intracellular with the extracellular space. (B) Chemical structure of HA composed by *N*-acetylglucosamine and glucuronic acid repeating units, linked by β1-3 and β1-4 glycosidic bonds. (C) Typical M_w synthesized by the three different HASs. Abbreviations: GlcA, Glucuronic acid; GlcNAc, *N*-acetyl glucosamine.

Mechano-transduction: the capacity of a material to induce cellular signaling in response to a mechanical stimulus.
NSCLC: human non-small cell lung cancer
PEG: polyethylene glycol, a hydrophilic molecule with different biomedical, chemical, and industrial applications.
PEI: polyethyleneimine, a linear or branched aliphatic polyamine.
PLL: poly-L-lysine, a positively charged polyelectrolyte.
RHAMM: receptor for hyaluronan-mediated motility.
RhoA: protein involved in the regulation of cell adhesion, morphology, polarity, and migration through the activation of signal transduction pathways.
ROS: reactive oxygen species, consisting of radical and nonradical oxygen species.
SiO_2: silicon dioxide.
siRNA: small interfering RNA or silencing RNA.
SMCs: smooth muscle cells, crucial in the formation and function of the cardiovascular, respiratory, and digestive systems, among others.
TGF-β1: transforming growth factor-β1.
TGS-6: tumor necrosis factor-stimulated gene-6.
TiO_2: titanium dioxide.
TLRs: toll-like receptors, a transmembrane protein located in the plasma membrane (TLR 1,2,4,5, and 6) and in the membranes of endosomes and lysosomes (TLRs 3, 7, 8, and 9), that can detect human pathogens.
YAP: yes-associated protein, an oncoprotein located in the cytoplasm that is responsive to the external mechanical stimuli.

In the last decades, HA has been increasingly linked to cancer invasiveness and progression, due to its overproduction, dysregulated degradation into short HA chains (<100 kDa), and accumulation at the ECM of tumors [16]. The overexpression of HA synthases and hyaluronidases in cancer has been associated with the increased deposition of HA of different M_w on the ECM, altering cellular behavior through the activation of different signaling pathways. In response to the HA present on the ECM, CD44 and RHAMM can trigger biochemical cascades associated with cancer cell motility [17], proliferation, or even apoptosis or latency [18]. Whereas CD44 presents a HA binding domain (HABD) with three different binding modes that are considered the CD44-HA fingerprints, RHAMM needs a host molecule such CD44, TRLs, and GFs, to maintain the RHAMM-HA interaction and exert its bioactivities [19]. The capacity of CD44 to bind to HA of different M_w is

Table 1. HA Molecular Weights (M_ws) and Their Biological Functions

HA M_w (kDa)	HA target receptor	Function	Biological response	Refs
		Low M_w		
<6	–	Proinflammatory	–	[70]
	TRL-2; TLR-4; CD44	NF-kB activation Production of proinflammatory cytokines	Neuroinflammation	[71]
	–	Macrophage activation	Endothelialization	[72]
15–50	RHAMM	Activation of YAP1/TAZ protein	Mesotheliomas malignancy	[28]
	RHAMM	Activation of PI3K and MEK signaling pathways	Human choriocarcinoma cell migration	[73]
	TLR-4	Upregulation of TGFβ-1, TNF-α, IL-6 Proinflammatory	Tissue repair	[70]
30–60	CD44	Migration of BRO cells	Melanoma cells invasion	[74]
		Medium M_w		
50–500	RHAMM	Upregulation of TGFβ-1	Cellular migration and proliferation	[70]
60–800	–	Upregulation of proresolving genes (*arg1*, *il10*, and *mrc1*)	–	[75]
		High M_w		
>800	–	Proresolving mediators	–	[75]
1000–1800	CD44	Upregulation of TGFβ-1	Cellular migration and proliferation	[70]
	–	Downregulation of TGFb1, CTGF, collagen I, and collagen III	Endometrial fibrosis attenuation	[76]
	–	Anti-inflammatory; antioxidant; block the ERK1/2 signaling pathway	Protective role in nasal inflammation	[77]
	CD44	Migration of MKN45 cells; cortactin phosphorylation	Invasion of gastric cancer cells	[17]
>2000	CD44	Activation of ERK1/2 and suppression of EGFR signaling pathways	Embryonic stem cell differentiation, toward smooth muscle cells	[78]
	–	Upregulation of ALP, Runx-2, and OCN	Osteogenic differentiation	[79]

associated with its free mobility at the membrane lipid rafts, as well as its ability to group into clusters, generating a range of possible interactions that have been studied in different pathologies.

HA as a Space Filling Molecule and Mechano-Modulator/-Transductor

The chemical structure of HA, in particular the presence of -OH groups, is related to its high capacity to retain water and ability to swell, which makes it an ideal space-filling element. Under physiological conditions, HA presents a stretched chain, rich in water molecules, that contribute to its large volume, which increases with the increment of the HA M_w [20].

The critical role of HA on the viscoelastic and biomechanical balance of tissues is the basis of its ability to act as a structural support for cells to proliferate and migrate [21]. The secretion and degradation of HA of different M_w and their distribution throughout the ECM significantly affects the biomechanics of the matrix and, consequently, the biochemical sensing of cells, being associated with different pathologies (Box 2). The mechano-transductor capacity of the high M_w HA is reported to inhibit the proliferation of breast cancer cells by activating the Hippo pathway [22]. However, the ECM-rich high M_w HA in glioblastoma cancer tissues showed conflicting results: while some reports show that the increased tissue density and stiffness is linked to cancer cell proliferation and motility [23], others studies report the less invasive character of cancer cells when in contact with hydrogels containing HA of 500 kDa [24]. In addition, Pogoda and colleagues

Box 2. HA in Human Tissues and Associated Diseases

HA is found in most tissues, including the synovial fluid, the vitreous, the umbilical cord, and amniotic fluid. In the latter ones, the M_w of the HA varies depending on the gestation stage [94]. The HA of high M_w acts as a functional regulator of synovial joint fluid, giving the required viscosity and lubrication to act as a shock absorber for compressive forces. In fact, osteoarthritis has been associated with the loss of HA of high M_w through its cleavage into HAs of low M_w, leading to a decrease in the viscoelastic properties of cartilage and, in turn, a reduction of the mechanical resistance of the joints [95]. In addition, the unbalanced entanglement between HA and other ECM components alter the organization and stiffness of the tissues, which is associated with several diseases such as atherosclerosis and diabetic angiopathy, as well as fibrosis. However, the major alterations in the HA content are in cancer-related pathologies, as high levels of HA were associated with aggressiveness and poor prognosis of different types of cancer [96].

showed that glioblastoma cells seeded on softer HA surfaces presented nearly identical responses (i.e., cells spreading, proliferation, motility, and IL-8 secretion) to those seeded on stiffer polyacrylamide substrates, showing that the main changes in the biomechanics might not be the main feature that drives HA bioactivities in cancer pathological states [25].

The overproduction of **Hyals** in certain tumors drives the average M_w of HA to lower values, which impacts in the density and viscosity of the ECM [10,20]. In fact, the presence of low M_w HA is extensively linked to the biochemical stimulation of cellular motility rather than the physical and/or mechanical stimulus. However, the regulation of Yes-associated protein (**YAP**) expression, a mechano-transducer protein [26], has been linked to the expression and activation of CD44. In this context, HA of low M_w (i.e., 4 kDa) is reported to reduce the expression of YAP, inhibiting its translocation to the nucleus (which is related to the proliferation and migration of cells), through the upstream sensing of CD44 in hepatocellular carcinoma [27]. Additionally, the HA receptor RHAMM regulates the expression of YAP via HA/RHAMM activation [28]. Matrix remodeling and stiffening also leads to the activation of YAP that, in turn, enables, for example, the rearrangement of the actomyosin cytoskeleton in cancer-associated fibroblasts [29], or of laminin in breast cancer stem cells [30]. While the mechanical properties of the matrix can induce specific cellular responses, YAP can also regulate matrix stiffness under a positive feedback loop, by activating the **RhoA** pathway increasing the actomyosin-based tension of the cytoskeleton [26].

Engineered HA-Based Biomaterials to Mimic the Extracellular Matrix

The biological relevance of HA, in particular its recognized ability to modulate cellular behavior, led to the development of biomaterials incorporating HA, in an attempt to mimic its bioactivity and/or generate responsive systems to deliver therapeutic agents (Figure 1, Key Figure). In the following subsections we will discuss these HA-based biomaterials and their biological applications (Table 2).

Particles

Particles ranging from the nano- to the macro-scale have been developed to selectively target the extracellular space, to reach the surface of cells or to be internalized. The HA affinity with the transmembrane receptor CD44, which is expressed in healthy cells and overexpressed in cancerous cells, has been exploited in the development of nanoparticles to target the intracellular space, in a 'Trojan horse' strategy. Different approaches have been described to prepare the HA particles (Figure 2). The most reported methodology is its combinatory assembly with organic nanoparticles, based on lipids, chitosan, or **PEG** [31]; or inorganic particles, such as **SiO_2**, Au, **TiO_2**, and **$CaCO_3$** [32]. By manipulating these organic/inorganic cores, it is possible to control the particle size, an important feature when internalization is targeted. Coating these particles with HA increases surface porosity, which is also important for the encapsulation of therapeutic agents. As an example, HA-coating of mesoporous SiO_2 nanoparticles (pore size of ≈2.5 nm), encapsulated with a doxorubicin, has been used to target CD44-overexpressing cancer cells [33].

Key Figure

Major Hyaluronan (HA)-Mediated Signaling Cascades and HA-Based Biomaterials

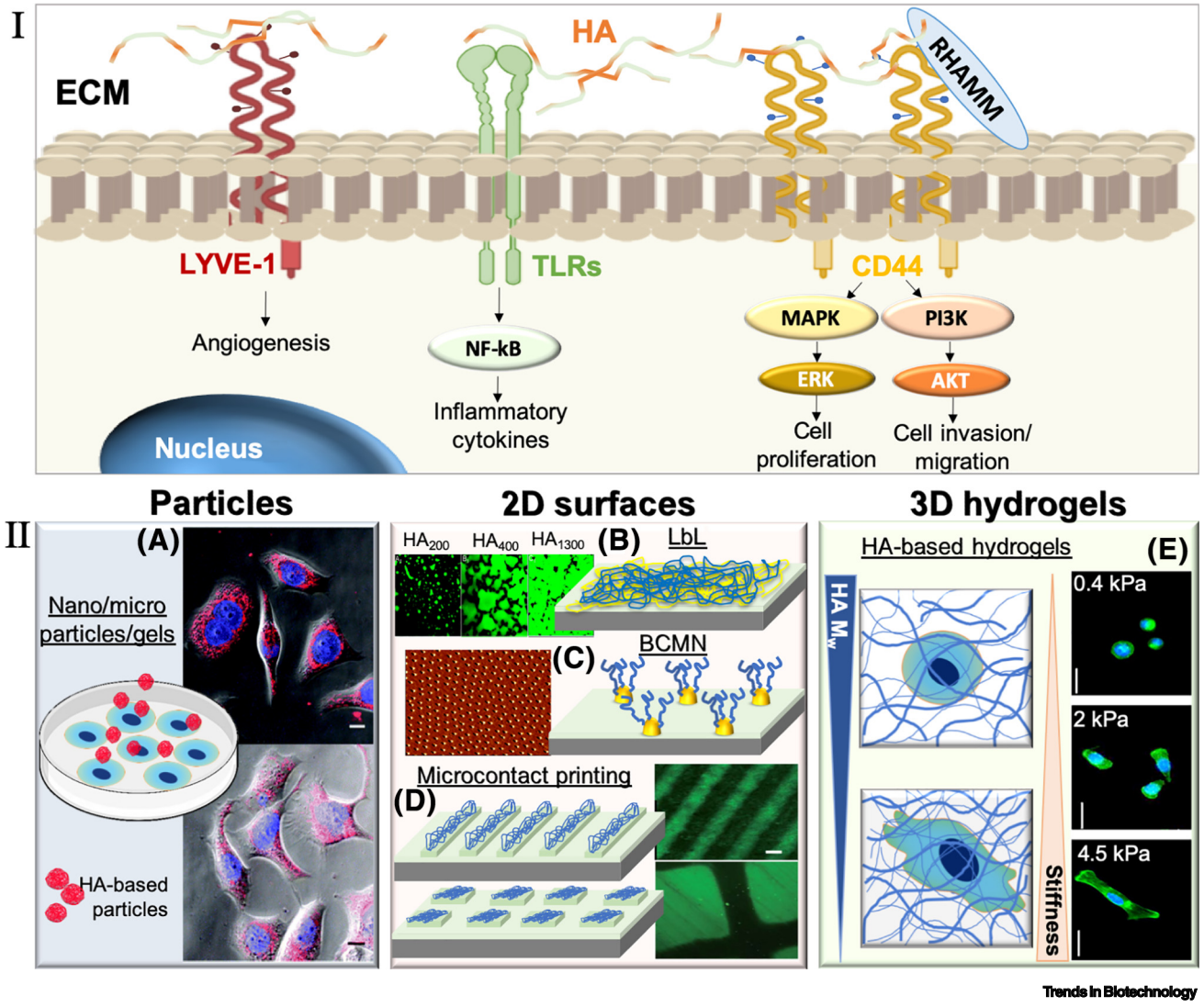

Figure 1. (I) Major HA-mediated signaling cascades. (II) HA-based biomaterials developed as targeted delivery systems and extracellular matrix (ECM) mimics. (A) HA particles modified with a thermoresponsive ketone-functional copolymer and labeled with Cyanine 5.5-amine for cellular internalization and tracking [97]. (B–D) 2D surfaces with tuned mechanical properties and different spacing of HA molecules to study its recognition by receptors/integrins. (B) Layer-by-layer (LbL) deposition of different polyelectrolytes with defined chemical, mechanical, and topographical properties by changing the HA molecular weight (M_w) (film of HA_{Mw}-PLL_{FITC}) [98]. (C) Block copolymer micelle nanolithography (BCMN) with thiol-end HA (atomic force microscopy image of a glass surface covered with gold nanodots); and (D) microcontact printing with side-on immobilization of HA [99]. (E) 3D hydrogels for cell encapsulation, with tuned mechanical properties (varying the HA M_w, the crosslinking degree, and/or the HA blended with other biocompatible materials) [58]. All the images were used and adapted, with permission, from [58,97–99]. Abbreviations: LYVE, Lymphatic vessel endothelial receptor for hyaluronan; RHAMM, receptor for hyaluronan-mediated motility; TLR, toll-like receptor.

The chemical composition of HA with available reactive groups (e.g., carboxylic acids) allows the exploitation of electrostatic or covalent interactions with other materials. For instance, the negative charge of HA (-COO^-) allows it to participate in electrostatic binding with the positively

Table 2. HA-Based Materials Used to Mimic the ECM and for Targeting of Cells

	Material	Fabrication method	HA presentation	Application	Biological outcome	Refs
Particles	Lipids	Lipid film method	Side-on HA by charged immobilization	Docetaxel encapsulation and delivery	Targets CD44 of breast cancer cells	[80]
	Chitosan	Ion gelation methods	Side-on HA by charged immobilization	siRNA or everolimus delivery	Targets CD44 of **NSCLC** A549 cells	[34]
	PEG	–	Electrostatic complexation	Doxorubicin encapsulation and delivery	Targets the HA receptors of cancer cells	[31]
	SiO_2	Strober process (sol-gel)	Covalent HA immobilization	Doxorubicin encapsulation and delivery	Targets CD44 of HeLa cancer cells	[33]
	Au	–	PEGylated HA for end-on immobilization	Cisplatin delivery	Treatment of MCF-7 and U-87 cancer cells	[81]
		–	End-thiolated HA	Trojan horse	Cancer cells internalization via CD44	[38]
	TiO_2	–	Electrostatic side-on immobilization of HA	Doxorubicin encapsulation and delivery	Targets CD44 of MDA-MB-231 breast cancer cells	[82]
	$CaCO_3$	Microemulsion coprecipitation method	Electrostatic immobilization of HA	Oral insulin delivery	Low cytotoxicity towards HT-29 cells and protects insulin from degradation	[83]
	MA-HA	Photo-crosslinking	HA nanogels	Encapsulation of drugs	Apoptosis of MCF-7 cells	[40]
	Thiol-modified HA	Michael addition of HS-HA and Pluronic diacrylate		Doxorubicin nanocarrier	MMP-2 inhibition on HeLa cells	[41]
2D surfaces	Glass	Layer-by-layer	Covalent HA immobilization	Influence of surface stiffness on adhesive behavior of C2C12 myoblasts	Higher cell adhesion and spreading on stiffer films, mediated by integrins	[43]
	TCPS			Gastric cancer cell invasiveness influenced by different HA's M_w	CD44-mediated invasiveness on surfaces with high M_w of HA	[17]
	Gold	–	End-thiolated sHA	Study the interactions between the HA with aggrecan and the LYVE-1	Both hyaladherins recognize sHA	[44]
	Ti substrates	Soft lithography	HA microstrips	Functional surface to inhibit thrombus formation and accelerate endothelial regeneration	Anticoagulation and endothelialization, with inhibited SMC proliferation and macrophage adhesion	[50]
	Silicon surfaces	Laser interference lithography and layer-by-layer	Covalent HA immobilization on hexagonally arranged nanostructures	Adhesion and differentiation of human adipose-derived stem cells (hADSC)	Chondrogenesis in sub-micron structures with native multilayers; osteogenesis on small, nanoscale structures with highly cross-linked multilayers	[51]
3D Models	MA-HA	Photo-crosslinking	Hydrogels	Encapsulation of human bmMSCs	Osteogenic differentiation	[55]
		DTT crosslinker		Cell seeding of MDA-MB-231Br cells on varying hydrogels' stiffness	Increased adhesion, proliferation, and migration on hydrogels with a stiffness of 4.5 kPa	[58]
				Varying stiffness resembling the ECM of liver	Stiffness of 1.2–4.6 kPa maintain primary hepatocyte function	[56]

(continued on next page)

Table 2. (continued)

	Material	Fabrication method	HA presentation	Application	Biological outcome	Refs
	Thiol-modified HA	PEGDA crosslinker		Controlled release of BMP-6	Induced osteogenic differentiation of MSCs and decrease myeloma cells viability	[57]
	HAALD and HAADH	HAALD: sodium periodate-mediated oxidation of HA; HAADH: carbodiimide-mediated coupling		Study the PCa cell motility	Invadopodia formation on PCa with invasive character mediated by RHAMM	[59]
	Alginate	Ca^{2+} covalent crosslinking of oxidized Alg and HA		Invasion behavior of SKBR3 and 4T-1 breast cancer cells in dependence of HA's M_w	Cells migration and invasion on HA of 35kDa with upregulated EMT markers; and inhibited on HA of 117kDa	[60]
		Ca^{2+} ionic crosslinking of Alg containing HA		MSCs encapsulation	Increased metabolic activity of MSCs in the presence of HA and potential to differentiate towards chondrocytes	[61]
	Gelatin	GelMA with photopolymerization of MA-HA		Influence of HA on GBM invasive phenotype	Higher invasive potential on HA-based hydrogels with a stiffness of 10 or 60 kDa	[24]
		MA-Coll and thiolated-HA dispersed on gelatin		Study the HepG2 biocompatibility and drug response	Supports HepG2 cells and their proliferation; maintained APAP and TRO drugs response	[66]
	Collagen	IPN of HA hydrazine, collagen, HA-ALD, or HA-BLD		Study the hydrogels' viscoelasticity and fibrillization on cell spreading, fiber remodeling, and FA formation	HA-ALD of 20 kDa lead to faster stress relaxation of the IPN hydrogels, promoting MSC spreading, collagen fiber realignment, and the formation of FAs	[62]
		HA-AC and collagen photo-crosslinked containing sHA derivatives		Study the binding affinity between HA and HB-EGF	Hydrogels containing HA-collagen and sHA strongly increased the biofunction of HB-EGF in inducing epithelial tip formation	[63]
				Study the binding affinity of HA with TGF-β1	Improved retention of TGF-β1on sHA containing hydrogels	[64]
		Tyramine-modified HA and collagen, enzymatically crosslinked		MSC alignment and differentiation	MSC differentiation towards chondrocytes	[67]

Abbreviations: APAP, Acetaminophen; DTT, dithiothreitol; HA-BLD, hyaluronan-benzaldehyde; IPN, interpenetrated network; PEGDA, polyethylene glycol diacrylate; TRO, troglitazo.

charged chitosan, used for the targeted delivery of **siRNA** or the drug everolimus to lung cancer cells [34]. To promote the interaction between HA and the negative charged SiO_2 or Au particles, these cores are usually amine-functionalized (-NH_2), or **layer-by-layer (LbL)** assembly is applied to revert the surface charge of the core particles [35]. In the LbL strategy, poly-L-lysine (**PLL**) is the most common polycation used as a support layer for the subsequent deposition of HA, leading to its immobilization in the particles' surface. The LbL assembly of PLL-HA is pH sensitive: it is disrupted in the acidic tumor microenvironment, leading to the release of the loaded therapeutic agent [36]. Drugs with low water solubility are usually difficult to deliver in the cellular milieu; however, they can be solubilized using electrostatically decorated nanostructured lipid carriers, with HA as a targeting ligand for lung cancer cells [37]. HA can also be presented using gold nanoparticles conjugated with end-thiolated HA (HS-HA) to promote its binding to the gold surface, generating a brush-like surface structure [38]. The recognition of the particles and their reversible/

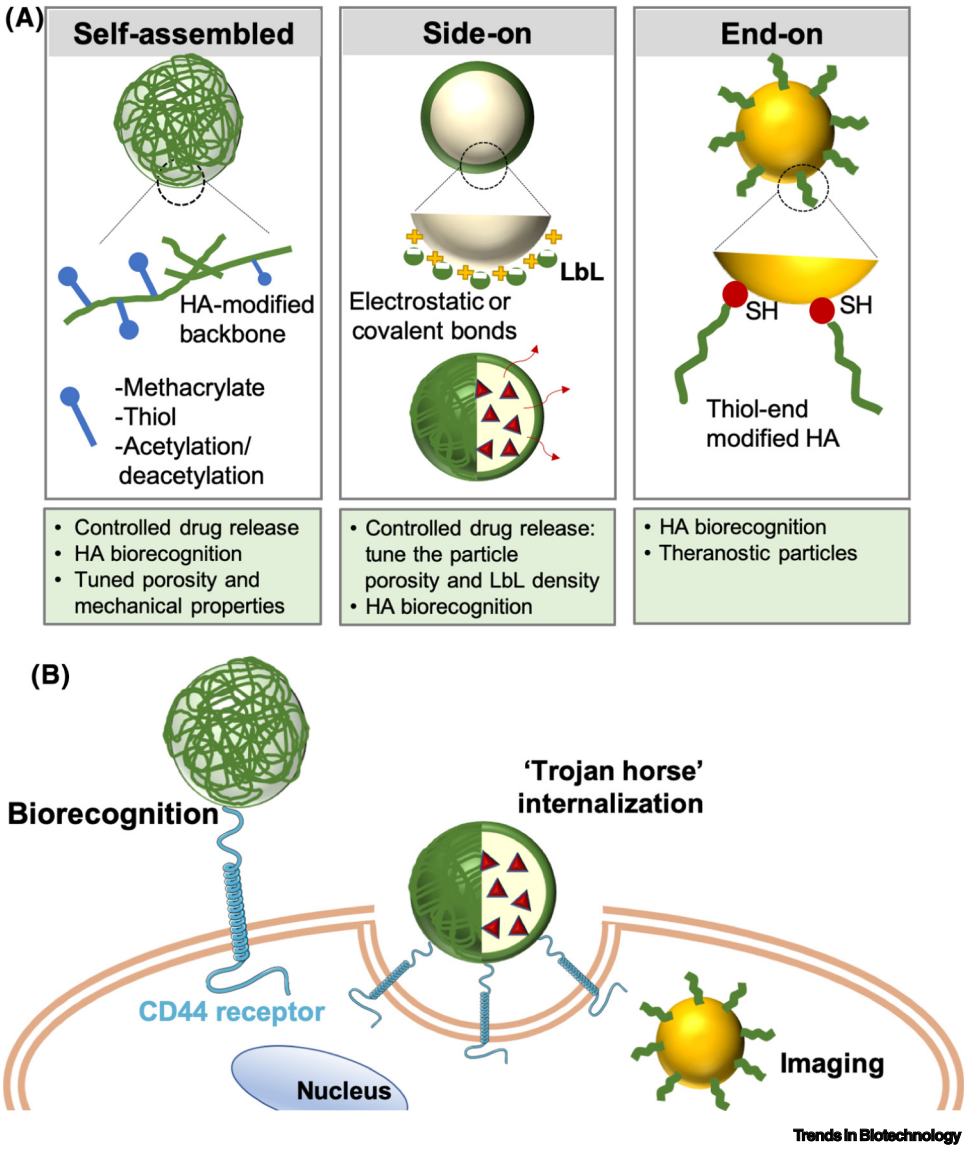

Figure 2. Active Targeting of Hyaluronan (HA)-Based Particles Used as Drug/Proteins Carriers. (A) HA assembly from modified HA backbone; inorganic (e.g., SiO_2, TiO_2, Au, $CaCO_2$) or organic (e.g., liposomes, lipids) particle cores coated with HA; and thiol-end HA-coated gold nanoparticles. (B) Schematic representation of the ability of HA particles to act as an extracellular mimic (i.e., biorecognition) and for the release of therapeutic drugs or as an imaging agent. Abbreviations: LbL, layer-by-layer.

irreversible attachment to the cell surface is mediated by HA receptors. In this context, it is possible to tune the targeting capacity of these particles by the changing the M_ws of the HA present in the shell of the particles.

The development of polyelectrolyte complexes using native or modified HA has been reported as well. The biological instability of native HA structures requires the use of toxic reagents for the crosslinking (e.g., glutaraldehyde, hydrazides, among others) to maintain its structure and its biostability with limited biodegradation [39]. These harsh crosslinking conditions are not suitable for biomedical applications due to their cytotoxicity. In this sense, the modification of HA has been

explored. One of the most commonly reported chemical modifications performed on HA is the modification of its hydroxyl moieties into methacrylate groups, forming methacrylated-HA (MA-HA) that can be crosslinked by UV light [40]. This MA-HA present increased stability in physiological conditions and allows the loading and controlled release of proteins or drugs. Other approaches have been proposed to alter the HA chemical structure relevant for the preparation of particles (e.g., thiol-modified HA [41], among others).

2D Surfaces

The ability of HA to modulate cellular morphology, proliferation, and motility has been studied *in vitro* using 2D substrates that mimic, in a very simplistic way, the characteristics of the ECM. Controlled immobilization of the extended HA chain (of different M_w) on a surface using different polycations can modulate the stiffness of the surface, the charge density, or the available HA epitopes that can be recognized by the cells [42]. The LbL assembly is used to immobilize HA and different polyelectrolytes for the construction of multilayered films. The alternate dipping of a glass slide on PLL and HA solutions generates a $(PLL\text{-}HA)_x$ surface with controlled stiffness through the manipulation of the crosslinker concentration. This approach has been used to evaluate the impact of stiffness on the behavior of **C2C12** myoblasts. Greater adhesion and cell spreading were observed on stiffer films, processes that were mediated by integrins [43]. Tissue polystyrene coverslips (TCPS) were also used to immobilize (PLL-HA) films and to evaluate the influence of HA M_w on gastric cancer cell invasiveness, mediated by the CD44 receptor [17]. The alternative end-thiolated-HA with different chain lengths was also used to investigate the interactions between the immobilized HA with aggrecan (a proteoglycan from the ECM) and the protein LYVE-1 (a HA-sensitive cellular receptor) [44]. The surface topography can be used to study the ability of HA to modulate cellular behavior. Cells are sensitive to the ECM's mechanical and morphological features, so micro- and nanopatterned surfaces can provide insights on cell matrix interactions and its influence on the activation of downstream biochemical pathways. In fact, the spatial organization of focal adhesions (FAs), integrins, transmembrane receptors, and cytoskeleton proteins are responsible for cellular adhesion and matrix remodeling (Figure 3). In this context, their influence on cellular responses can be assessed using micro- and nanostructured substrates. Different approaches can be used for micro- and nanofabrication [45], such as photolithography, soft lithography and microcontact printing, electron beam lithography, and block copolymer micelle nanolithography (BCMN). Selecting the best technique depends on the desired microstructure, for example, pillars (e.g., nanoimprinting and etching) [46], stripes (e.g., soft-lithographic technique) [47], micropatterned networks (e.g., microcontact printing) [48], or controlled inorganic nanoparticle spacing (e.g., BCMN) [49]. Parallel ridge stripes that mimic the shape of vascular endothelial cells (EC) when exposed to the shear stress from the blood flow, generated under physiological conditions, were used to study the adhesion of EC and smooth muscle cells (**SMCs**) on high M_w HA-modified stripes [50]. More recently, -(**PEI**-$[HA\text{-}PLL]_{11}$-HA) hexagonally arranged nanostructures with crosslinked polyelectrolyte multilayer with defined spacing (i.e., 733, 518, and 302 nm) and tuned mechanical properties were used to differentiate human adipose-derived stem cells, showing that highly crosslinked surfaces presenting HA-coated particles of smaller size (i.e., 302 nm) induced osteogenesis [51].

3D Models

3D models (such as hydrogels) can mimic the complexity of the extracellular microenvironment and have been proposed to overcome the limitations of the 2D platforms. In this context, 3D hydrogels can recapitulate fundamental parameters of the ECM, such as porosity, stiffness, and hydration, which are able to act as mechano-transductors (Figure 3). By altering the porosity and viscoelastic properties of the gels, it is possible to control the two-way diffusion of nutrients, oxygen, and biochemical factors throughout the cell culture period [52].

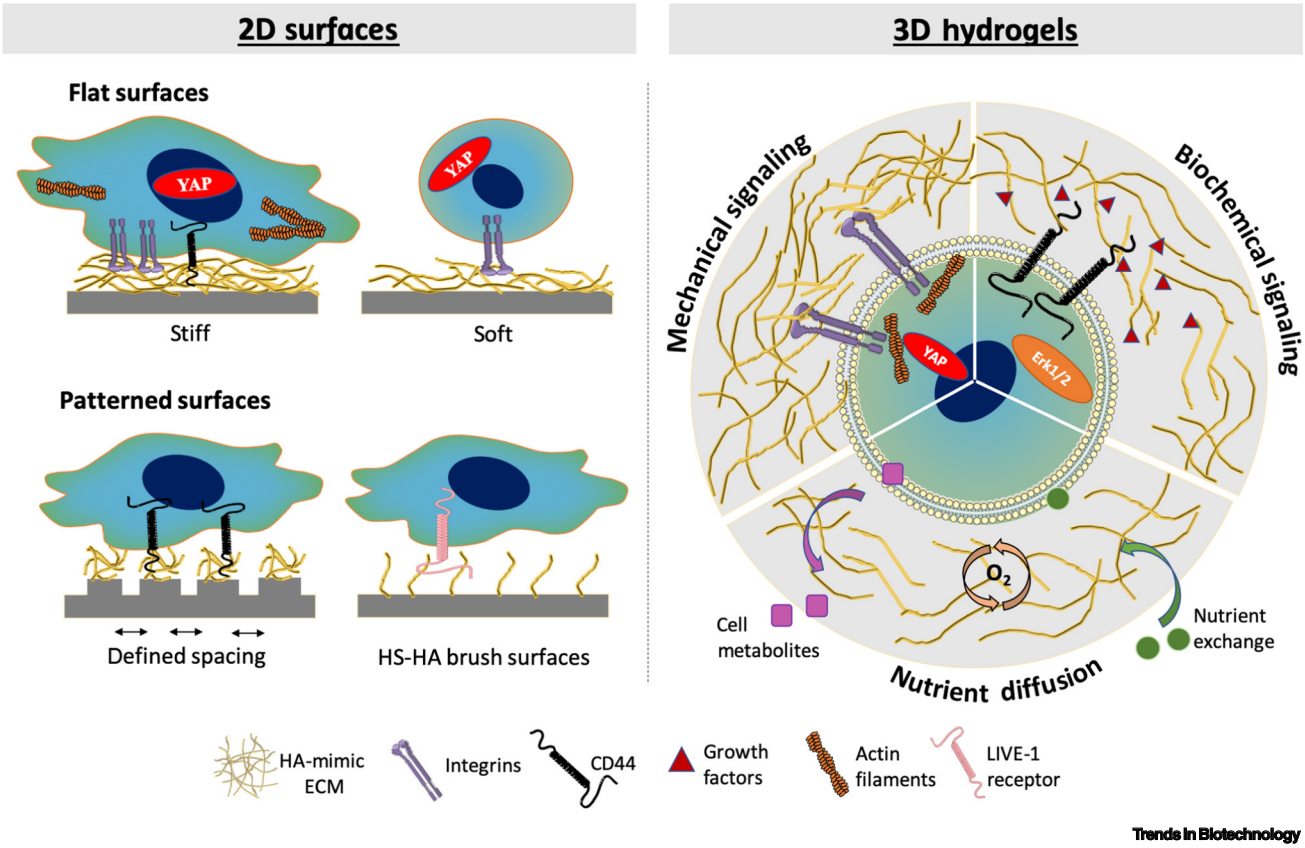

Figure 3. Schematic Representation of 2D Surfaces and 3D Models Used for Cellular Immobilization and Encapsulation. Cellular adhesion, spreading, and migration on 2D surfaces is restricted to the *xy* plane, being the external stimulus (mechanical and topographical) unidirectional. 2D patterned surfaces with controlled spacing, used for integrins and/or studies on the clustering of hyaluronan (HA) receptors. 3D models recreate the physical (e.g., porosity, topography), mechanical (e.g., hydrogel density, hydration, stiffness), and chemical (e.g., growth factors, proteins) cues of the extracellular matrix (ECM), being closer mimics to study or recapitulate the cell–cell and cell–matrix interactions.

Different approaches have been used to prepare 3D HA-based hydrogels. Usually, it involves the chemical modification of HA at the -COOH position from the GlcA residue, one of the -OH positions of the *N*-acetylglucosamine, or the -$NHCOCH_3$ groups, using chemical strategies including deacetylation, amidation, esterification, and oxidation through 2,2,6,6-tetramethylpiperidinyloxy (TEMPO) modification through a Schiff-based reaction, among others [53,54]. The 3D HA-based hydrogels require chemical or physical crosslinking to be able to maintain their structure and resemble the native HA network. One of the most common strategies is the use of methacrylated HA (MA-HA), which is crosslinked by UV after the encapsulation of cells. The precise control of the UV crosslinking, by regulating the time and/or intensity of the UV exposure, allows the preparation of hydrogels with different mechanical properties [55]. The physiologically relevant stiffness of HA-based hydrogels, resembling the ECM of healthy liver (600–4600 Pa) were mimicked using thiol-modified HA. Combining HA-based hydrogels with the ECM from the liver improved cellular adhesion and proliferation when stiffer hydrogels were used [56]. Thiol-modified HA hydrogel complexed with heparin were used to encapsulate **BMP-6** showing promising results on myeloma cell apoptosis and osteogenic differentiation of mesenchymal stromal cells [57]. The invasive character of the brain metastasizing variant cell line, MDA-MB-231Br, derived from the breast cancer line MDA-MB-231, was probed on HA hydrogels with varying stiffness (0.2–4.5 kPa), showing increased adhesion, proliferation, and migration on stiffer matrices [58]. Modified HA with aldehydes

(HAALD) and adipic acid dihydrazide (HAADH) were also applied on 3D hydrogels. These systems were used to encapsulate prostate cancer cells (PCa) and crosslinked at 37°C. These systems showed that the invasive features of PCa were mediated by the HA receptor RHAMM [59]. The most common way to generate HA-based hydrogels is to chemically modify the HA, but this strategy deviates from its native chemical presentation in the ECM. In order to avoid these deviations, the use of native HA has been attempted in the development of 3D hydrogels by incorporating it into the matrices of a supporting hydrogel. Alginate (Alg) is one of the most common materials to immobilize native HA into a 3D hydrogel network, due to the easy handling and the possibility to crosslink the Alg chains using mild conditions, such as Ca^{2+}. The role of the different M_w of HA on the migration of breast cancer cells (SKBR3) and phenotypic alterations was evaluated by immobilizing HA of lower (35 kDa) and higher (117 kDa) M_w in Alg matrices. These models demonstrated metastatic activity and epithelial–mesenchymal transition (EMT) in the presence of HA of 35 kDa [60]. The resemblance of the natural ECM using the native HA immobilized in an Alg network was also used to probe the chondrogenic differentiation of mesenchymal stem cells [61]. Other materials have been used to promote the ECM-like presentation of HA, such as methacrylamide-functionalized gelatin (GelMA)-HA hydrogels to mimic the ECM of glioblastoma, namely the presentation of the different M_w of HA [24]. The combination of collagen (Coll) and HA, incorporating the ECM fibrillar elements from Coll, has been described. By altering the HA-Coll crosslinking, it is possible to tune the mechanical properties of these hydrogels, which altered the ability of mesenchymal stem cells to adhere, change their morphology, and generate FAs [62]. Sulfated-HA derivatives have been incorporated into HA-Coll hydrogels for wound repair, by retaining and slowly releasing the heparin-binding epidermal growth factor (HB-EGF) [63], or for **TGF-β1** storage within the hydrogels for skin repair [64]. More recently, the modification of HA has been pursued for the preparation of printable bioinks [65]. In fact, bioink comprising ECM-occurring materials has been developed combining methacrylated Coll and thiolated-HA. These components were incorporated in gelatin nanoparticles and proven to support the proliferation of HepG2 cells [66]. A bioink containing a tyramine-derivative HA and aligned collagen type I fibers, has been used for MSC differentiation towards chondrocytes [67].

HA-Related Products in the Market

The biological importance of HA has been extensively studied and its fundamental role in tissues homeostasis has been increasingly recognized. Based on this knowledge, the pharmaceutical, medical, cosmetic, and food industries have been exploiting the development of HA-related products. These include systems for ophthalmologic applications, dermatological fillers (e.g., anti-age products), and treatments for osteoarthritis and vesicoureteral reflux (Table 3).

Table 3. HA-Related Products in the Market Used for Biomedical Applications

HA applications	Advantage	Methodology/effect	Refs
Ophthalmology	High hydrophilicity; viscoelasticity	Reduce eye dryness and irritation	[84]
		Reduce eye inflammation	[85]
Osteoarthritis	Lubricant capacity; anti-inflammatory	High M_w HA intra-articular injections Stimulates *de novo* synthesis of HA	[86]
Dermatology	High hydrophilicity; space-filling molecule	Dressings for wound healing Anti-aging dermal fillers	[87]
Mucosis	Viscoelasticity; anti-inflammatory	HA-based barrier gel	[88]
Interstitial cystitis	Mucosal layer protection	Intravesical instillation	[89]
Upper airway diseases	Hydrophilicity; lubricant capacity	Reduces inflammation Improves nasal clearance	[90]

The most common application of HA is for dermatological applications. Some of these dermal fillers contain the linear native HA, but these have a limited half-life after injection. In order to overcome this drawback, it is becoming increasingly common to use crosslinked HA. One example is anti-aging microstructured patches containing crosslinked HA as an antiwrinkle and moisturizer product [68]. The benefits of HA to improve skin function go beyond its topical administration, including HA-based food supplements that can reduce skin dryness over time [69].

Despite the extensive studies and reported literature on the use of HA (and its derivatives) as drug delivery systems, these types of systems are still not widely available. The *in vitro* studies that report the ability of HA-based carriers to deliver bioactive drugs and proteins, with prolonged stability and therapeutic efficacy, still need to follow the translational pipeline that leads to their application in the clinical practice.

Outstanding Questions

How is the molecular weight of HA related to different pathological states?

How can 2D HA-presenting surfaces be explored as simple platforms to study cellular behavior?

Will 3D HA-based models overtake 2D systems as better mimics of the ECM?

How will 3D HA-based models best be used as platforms to mimic the cellular microenvironment?

Concluding Remarks and Future Perspectives

We summarized the relevance of the HA-rich ECM on the cellular behavior and tissue homeostasis and the different type of platforms that have been proposed to study HA-mediated cell–cell or cell–ECM interactions. The importance of the HA M_w on the biochemical and biophysical balance of the ECM has been well established. The important biological functions of HA in combination with its chemical versatility makes HA prone to different chemical modifications to improve its stability under physiological conditions. Different approaches have been proposed to present HA on an ECM-relevant manner, mimicking its structural, mechanical, and biochemical properties. Taking advantage of the ability of exogenous HA to be recognized by a series of biological elements (e.g., different HA-specific cell surface receptors), various HA-based materials have been proposed as drug or protein carriers for extra- or intracellular delivery. Despite the increasing knowledge on HA and its bioactivities, the structural and biological complexity of HA in the ECM is still not completely understood. As an example, the deposition of different M_ws of HA in the ECM and its influence on the progression of cancers is still unclear and controversial (see Outstanding Questions). In these cases, the development of platforms that can mimic the HA present in the ECM are important tools to better understand its biological functions. In this context, the development of 3D HA-based models that resemble the ECM is becoming increasingly important. However, the present state of the art is still far from accurately mimicking the way HA is structurally organized in the ECM. For instance, the multiple interactions of HA with other ECM components, as well as with the cells' surface, should be considered during the development of new *in vitro* models. Another important point is to be able to recapitulate the increased complexity of the ECM as a function of time (e.g., alterations on the presentation of HA under specific temporal scales, such as mimicking cancer progression). Significant improvements in the HA-based models generated using different processing methodologies (e.g., microfluidics through soft lithography, bioprinting, etc.) are currently under development to generate improved ECM mimics.

Acknowledgments

The authors acknowledge the financial support from the European Commission's H2020 programme, under grant agreements H2020-WIDESPREAD-2014-668983-FORECAST, and H2020-MSCA-RISE-2019-872648-MEPHOS. S.A. acknowledges the Portuguese Foundation for Science and Technology (FCT) for her PhD grant (SFRH/BD/112075/2015).

References

1. Manou, D. *et al.* (2019) The complex interplay between extracellular matrix and cells in tissues. *Methods Mol. Biol.* 1952, 1–20
2. Mereiter, S. *et al.* (2019) Glycosylation in the era of cancer-targeted therapy: where are we heading? *Cancer Cell* 36, 6–16
3. Soares da Costa, D. *et al.* (2017) Sulfation of glycosaminoglycans and its implications in human health and disorders. *Annu. Rev. Biomed. Eng.* 19, 1–26
4. Fallacara, A. *et al.* (2018) Hyaluronic acid in the third millennium. *Polymers (Basel)* 10, 701
5. Murakami, T. *et al.* (2019) Hyaluronic acid promotes proliferation and migration of human meniscus cells via a CD44-dependent mechanism. *Connect. Tissue Res.* 60, 117–127
6. Canibano-Hernandez, A. *et al.* (2019) Hyaluronic acid promotes differentiation of mesenchymal stem cells from different sources

toward pancreatic progenitors within three-dimensional alginate matrixes. *Mol. Pharm.* 16, 834–845
7. Iturriaga, V. *et al.* (2017) Role of hyaluronic acid in the homeostasis and therapeutics of temporomandibular joint osteoarthritis. *Int. J. Morphol.* 35, 870–876
8. Mereiter, S. *et al.* (2019) O-glycan truncation enhances cancer-related functions of CD44 in gastric cancer. *FEBS Lett.* 593, 1675–1689
9. Senbanjo, L.T. and Chellaiah, M.A. (2017) CD44: a multifunctional cell surface adhesion receptor is a regulator of progression and metastasis of cancer cells. *Front. Cell Dev. Biol.* 5, 18
10. Chanmee, T. *et al.* (2016) Hyaluronan: a modulator of the tumor microenvironment. *Cancer Lett.* 375, 20–30
11. Wu, R.-L. *et al.* (2017) Hyaluronic acid in digestive cancers. *J. Cancer Res. Clin. Oncol.* 143, 1–16
12. Richter, R.P. *et al.* (2018) Glycosaminoglycans in extracellular matrix organisation: are concepts from soft matter physics key to understanding the formation of perineuronal nets? *Curr. Opin. Struct. Biol.* 50, 65–74
13. Herrera, J. *et al.* (2018) Extracellular matrix as a driver of progressive fibrosis. *J. Clin. Invest.* 128, 45–53
14. Wight, T.N. (2017) Provisional matrix: a role for versican and hyaluronan. *Matrix Biol.* 60-61, 38–56
15. Price, Z.K. *et al.* (2018) Differing roles of hyaluronan molecular weight on cancer cell behavior and chemotherapy resistance. *Cancers* 10, 482
16. Liu, M. *et al.* (2019) Dissecting the dual nature of hyaluronan in the tumor microenvironment. *Front. Immunol.* 10, 947
17. Amorim, S. *et al.* (2018) Molecular weight of surface immobilized hyaluronic acid influences CD44-mediated binding of gastric cancer cells. *Sci. Rep.* 8, 16058
18. Kassim, Y.L. *et al.* (2017) Three dimensional tumor engineering by co-culture of breast tumor and endothelial cells using a hyaluronic acid hydrogel model. *J. Clin. Exp. Oncol.* 6, 5
19. Vuorio, J. *et al.* (2017) Atomistic fingerprint of hyaluronan-CD44 binding. *PLoS Comput. Biol.* 13, e1005663
20. Cowman, M.K. *et al.* (2015) Viscoelastic properties of hyaluronan in physiological conditions. *F1000Res.* 4, 622
21. Caires, R. *et al.* (2015) Hyaluronan modulates TRPV1 channel opening, reducing peripheral nociceptor activity and pain. *Nat. Commun.* 6, 8095
22. Ooki, T. *et al.* (2019) High-molecular-weight hyaluronan is a Hippo pathway ligand directing cell density-dependent growth inhibition via PAR1b. *Dev. Cell* 49, 590–604
23. Kim, Y. and Kumar, S. (2014) CD44-mediated adhesion to hyaluronic acid contributes to mechanosensing and invasive motility. *Mol. Cancer Res.* 12, 1416–1429
24. Chen, J.W.E. *et al.* (2018) Influence of hyaluronic acid transitions in tumor microenvironment on glioblastoma malignancy and invasive behavior. *Front. Mater.* 5, 39
25. Pogoda, K. *et al.* (2017) Soft substrates containing hyaluronan mimic the effects of increased stiffness on morphology, motility, and proliferation of glioma cells. *Biomacromolecules* 18, 3040–3051
26. Dobrokhotov, O. *et al.* (2018) Mechanoregulation and pathology of YAP/TAZ via Hippo and non-Hippo mechanisms. *Clin. Transl. Med.* 7, 23
27. Fan, Z.H. *et al.* (2018) Standard CD44 modulates YAP1 through a positive feedback loop in hepatocellular carcinoma. *Biomed. Pharmacother.* 103, 147–156
28. Shigeeda, W. *et al.* (2017) Hyaluronic acid enhances cell migration and invasion via the YAP1/TAZ-RHAMM axis in malignant pleural mesothelioma. *Oncotarget* 8, 93729–93740
29. Calvo, F. *et al.* (2013) Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts. *Nat. Cell Biol.* 15, 637–646
30. Mercurio, A. *et al.* (2015) A laminin 511 matrix is regulated by TAZ and functions as the ligand for the a6Bb1 integrin to sustain breast cancer stem cells. *FASEB J.* 29, 1–6
31. Yan, X.J. *et al.* (2019) Hyaluronic acid/PEGylated amphiphilic nanoparticles for pursuit of selective intracellular doxorubicin release. *J. Mater. Chem. B* 7, 95–102
32. Trofimov, A.D. *et al.* (2018) Porous inorganic carriers based on silica, calcium carbonate and calcium phosphate for controlled/modulated drug delivery: fresh outlook and future perspectives. *Pharmaceutics* 10, 167
33. Zhang, J. *et al.* (2016) Multifunctional mesoporous silica nanoparticles modified with tumor-shedable hyaluronic acid as carriers for doxorubicin. *Colloids Surf. B Biointerfaces* 144, 293–302
34. Zhang, W. *et al.* (2019) Antitumor effect of hyaluronic-acid-modified chitosan nanoparticles loaded with siRNA for targeted therapy for non-small cell lung cancer. *Int. J. Nanomedicine* 14, 5287–5301
35. Ferreira, H. *et al.* (2018) The functionalization of natural polymer-coated gold nanoparticles to carry bFGF to promote tissue regeneration. *J. Mater. Chem. B* 6, 2104–2115
36. Dreaden, E.C. *et al.* (2014) Bimodal tumor-targeting from microenvironment responsive hyaluronan layer-by-layer (LbL) nanoparticles. *ACS Nano* 8, 8374–8382
37. Mahmoudi, S. *et al.* (2019) Targeted hyaluronic acid-based lipid nanoparticle for apigenin delivery to induce Nrf2-dependent apoptosis in lung cancer cells. *J. Drug Deliv. Sci. Technol.* 49, 268–276
38. Karakocak, B.B. *et al.* (2018) Hyaluronate coating enhances the delivery and biocompatibility of gold nanoparticles. *Carbohydr. Polym.* 186, 243–251
39. Khunmanee, S. *et al.* (2017) Crosslinking method of hyaluronic-based hydrogel for biomedical applications. *J. Tissue Eng.* 8, 2041731417726464
40. Kim, K. *et al.* (2019) Hyaluronic acid-coated nanomedicine for targeted cancer therapy. *Pharmaceutics* 11, 301
41. Gurav, D.D. *et al.* (2016) pH-responsive targeted and controlled doxorubicin delivery using hyaluronic acid nanocarriers. *Colloids Surf. B Biointerfaces* 143, 352–358
42. Amorim, S. *et al.* (2020) Tunable layer-by-layer films containing hyaluronic acid and their interactions with CD44. *J. Mater. Chem. B* 8, 3880–3885
43. Ren, K. *et al.* (2010) Manipulation of the adhesive behaviour of skeletal muscle cells on soft and stiff polyelectrolyte multilayers. *Acta Biomater.* 6, 4238–4248
44. Minsky, B.B. *et al.* (2016) Controlled immobilization strategies to probe short hyaluronan-protein interactions. *Sci. Rep.* 6, 21608
45. Ermis, M. *et al.* (2018) Micro and nanofabrication methods to control cell-substrate interactions and cell behavior: a review from the tissue engineering perspective. *Bioact. Mater.* 3, 355–369
46. Ghezzi, B. *et al.* (2019) Sub-micropillar spacing modulates the spatial arrangement of mouse MC3T3-E1 osteoblastic cells. *Nanomaterials (Basel)* 9, 1701
47. Joo, S. *et al.* (2015) Effects of ECM protein micropatterns on the migration and differentiation of adult neural stem cells. *Sci. Rep.* 5, 13043
48. Hondrich, T.J.J. *et al.* (2019) Improvements of microcontact printing for micropatterned cell growth by contrast enhancement. *Micromachines (Basel)* 10, 659
49. Schaufler, V. *et al.* (2016) Selective binding and lateral clustering of alpha5beta1 and alphavbeta3 integrins: unraveling the spatial requirements for cell spreading and focal adhesion assembly. *Cell Adhes. Migr.* 10, 505–515
50. Li, J.G. *et al.* (2015) Tailoring of the titanium surface by preparing cardiovascular endothelial extracellular matrix layer on the hyaluronic acid micro-pattern for improving biocompatibility. *Colloids Surf. B Biointerfaces* 128, 201–210
51. Niepel, M.S. *et al.* (2019) Polyelectrolyte multilayers of poly (l-lysine) and hyaluronic acid on nanostructured surfaces affect stem cell response. *Nanoscale* 11, 2878–2891
52. Tibbitt, M.W. and Anseth, K.S. (2009) Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnol. Bioeng.* 103, 655–663
53. Zhang, Y. *et al.* (2020) Implantation of a functional TEMPO-hydrogel induces recovery from rat spinal cord transection through promoting nerve regeneration and protecting bladder tissue. *Biomater. Sci.* 8, 1695–1701
54. Serban, M.A. and Skardal, A. (2019) Hyaluronan chemistries for three-dimensional matrix applications. *Matrix Biol.* 78-79, 337–345
55. Poldervaart, M.T. *et al.* (2017) 3D bioprinting of methacrylated hyaluronic acid (MeHA) hydrogel with intrinsic osteogenicity. *PLoS One* 12, e0177628

56. Deegan, D.B. *et al.* (2015) Stiffness of hyaluronic acid gels containing liver extracellular matrix supports human hepatocyte function and alters cell morphology. *J. Mech. Behav. Biomed. Mater.* 55, 87–103
57. Grab, A.L. *et al.* (2019) Hyaluronan hydrogels delivering BMP-6 for local targeting of malignant plasma cells and osteogenic differentiation of mesenchymal stromal cells. *Acta Biomater.* 96, 258–270
58. Narkhede, A.A. *et al.* (2018) The influence of matrix stiffness on the behavior of brain metastatic breast cancer cells in a biomimetic hyaluronic acid hydrogel platform. *J. Biomed. Mater. Res. A* 106, 1832–1841
59. Gurski, L.A. *et al.* (2012) Hyaluronan (HA) interacting proteins RHAMM and hyaluronidase impact prostate cancer cell behavior and invadopodia formation in 3D HA-based hydrogels. *PLoS One* 7, e50075
60. Zhao, Y.F. *et al.* (2017) Modulating three-dimensional microenvironment with hyaluronan of different molecular weights alters breast cancer cell invasion behavior. *ACS Appl. Mater. Interfaces* 9, 9327–9338
61. Canibano-Hernandez, A. *et al.* (2017) Alginate microcapsules incorporating hyaluronic acid recreate closer *in vivo* environment for mesenchymal stem cells. *Mol. Pharm.* 14, 2390–2399
62. Lou, J. *et al.* (2018) Stress relaxing hyaluronic acid-collagen hydrogels promote cell spreading, fiber remodeling, and focal adhesion formation in 3D cell culture. *Biomaterials* 154, 213–222
63. Thones, S. *et al.* (2019) Hyaluronan/collagen hydrogels containing sulfated hyaluronan improve wound healing by sustained release of heparin-binding EGF-like growth factor. *Acta Biomater.* 86, 135–147
64. Rother, S. *et al.* (2019) Hyaluronan/collagen hydrogel matrices containing high-sulfated hyaluronan microgels for regulating transforming growth factor-beta1. *J. Mater. Sci. Mater. Med.* 30, 65
65. Weis, M. *et al.* (2018) Evaluation of hydrogels based on oxidized hyaluronic acid for bioprinting. *Gels* 4, 82
66. Clark, C.C. *et al.* (2019) A mechanically robust thixotropic collagen and hyaluronic acid bioink supplemented with gelatin nanoparticles. *Bioprinting* 16, e00058
67. Schwab, A. *et al.* (2020) Tissue mimetic hyaluronan bioink containing collagen fibers with controlled orientation modulating cell migration and alignment. *Mater. Today Bio* Published online June 1, 2020. https://doi.org/10.1016/j.mtbio.2020.100058
68. Lee, Y.J. *et al.* (2019) Anti-aging and hydration efficacy of a cross-linked hyaluronic acid microstructure patch. *Dermatol. Ther.* 32, e12888
69. Kawada, C. *et al.* (2014) Ingested hyaluronan moisturizes dry skin. *Nutr. J.* 13, 70
70. D'Agostino, A. *et al.* (2017) Is molecular size a discriminating factor in hyaluronan interaction with human cells? *Carbohydr. Polym.* 157, 21–30
71. Scuruchi, M. *et al.* (2016) 6-Mer hyaluronan oligosaccharides modulate neuroinflammation and alpha-synuclein expression in neuron-like SH-SY5Y cells. *J. Cell. Biochem.* 117, 2835–2843
72. Kang, L. *et al.* (2019) Hyaluronic acid oligosaccharide-modified collagen nanofibers as vascular tissue-engineered scaffold for promoting endothelial cell proliferation. *Carbohydr. Polym.* 223, 115106
73. Mascaro, M. *et al.* (2017) Low molecular weight hyaluronan induces migration of human choriocarcinoma JEG-3 cells mediated by RHAMM as well as by PI3K and MAPK pathways. *Histochem. Cell Biol.* 148, 173–187
74. Sapudom, J. *et al.* (2017) Molecular weight specific impact of soluble and immobilized hyaluronan on CD44 expressing melanoma cells in 3D collagen matrices. *Acta Biomater.* 50, 259–270
75. Rayahin, J.E. *et al.* (2015) High and low molecular weight hyaluronic acid differentially influence macrophage activation. *ACS Biomater. Sci. Eng.* 1, 481–493
76. Zhu, Y. *et al.* (2016) High molecular weight hyaluronic acid inhibits fibrosis of endometrium. *Med. Sci. Monit.* 22, 3438–3445
77. Albano, G.D. *et al.* (2016) Effect of high, medium, and low molecular weight hyaluronan on inflammation and oxidative stress in an *in vitro* model of human nasal epithelial cells. *Mediat. Inflamm.* 2016, 8727289
78. Simpson, R.M. *et al.* (2016) Hyaluronan is crucial for stem cell differentiation into smooth muscle lineage. *Stem Cells* 34, 1225–1238
79. Zhao, N. *et al.* (2015) Effect of molecular weight and concentration of hyaluronan on cell proliferation and osteogenic differentiation *in vitro*. *Biochem. Biophys. Res. Commun.* 465, 569–574
80. Lee, S.-E. *et al.* (2019) Hyaluronic acid-coated solid lipid nanoparticles to overcome drug-resistance in tumor cells. *J. Drug Deliv. Sci. Technol.* 50, 365–371
81. Gotov, O. *et al.* (2018) Hyaluronic acid-coated cisplatin conjugated gold nanoparticles for combined cancer treatment. *J. Ind. Eng. Chem.* 65, 236–243
82. Gupta, B. *et al.* (2018) Hyaluronic acid-capped compact silica-supported mesoporous titania nanoparticles for ligand-directed delivery of doxorubicin. *Acta Biomater.* 80, 364–377
83. Liu, D.P. *et al.* (2017) Oral delivery of insulin using $CaCO_3$-based composite nanocarriers with hyaluronic acid coatings. *Mater. Lett.* 188, 263–266
84. Rinaudo, M. (2008) Main properties and current applications of some polysaccharides as biomaterials. *Polym. Int.* 57, 397–430
85. Jarvis, B. and Figgitt, D.P. (2003) Topical 3% diclofenac in 2.5% hyaluronic acid gel: a review of its use in patients with actinic keratoses. *Am. J. Clin. Dermatol.* 4, 203–213
86. Curran, M.P. (2010) Hyaluronic acid (Supartz®). *Drugs Aging* 27, 925–941
87. Keizers, P.H.J. *et al.* (2018) A high crosslinking grade of hyaluronic acid found in a dermal filler causing adverse effects. *J. Pharm. Biomed. Anal.* 159, 173–178
88. Smith, T. (2001) Gelclair: managing the symptoms of oral mucositis. *Hosp. Med.* 62, 623–626
89. Kallestrup, E.B. *et al.* (2005) Treatment of interstitial cystitis with Cystistat (R): a hyaluronic acid product. *Scand. J. Urol. Nephrol.* 39, 143–147
90. Vasvani, S. *et al.* (2019) Hyaluronic acid: a review on its biology, aspects of drug delivery, route of administrations and a special emphasis on its approved marketed products and recent clinical studies. *Int. J. Biol. Macromol.* 151, 1012–1029
91. Zhu, Z. *et al.* (2017) Hyaluronic acid: a versatile biomaterial in tissue engineering. *Plast. Aesthet. Res.* 4, 219–227
92. Soltes, L. *et al.* (2006) Degradative action of reactive oxygen species on hyaluronan. *Biomacromolecules* 7, 659–668
93. Bourguignon, V. and Flamion, B. (2016) Respective roles of hyaluronidases 1 and 2 in endogenous hyaluronan turnover. *FASEB J.* 30, 2108–2114
94. Cowman, M.K. *et al.* (2015) The content and size of hyaluronan in biological fluids and tissues. *Front. Immunol.* 6, 261
95. Bowman, S. *et al.* (2018) Recent advances in hyaluronic acid based therapy for osteoarthritis. *Clin. Transl. Med.* 7, 6
96. Setala, L.P. *et al.* (1999) Hyaluronan expression in gastric cancer cells is associated with local and nodal spread and reduced survival rate. *Br. J. Cancer* 79, 1133–1138
97. Garcia, F.P. *et al.* (2018) A versatile method for the selective core-crosslinking of hyaluronic acid nanogels via ketone-hydrazide chemistry: from chemical characterization to *in vivo* biodistribution. *Biomater. Sci.* 6, 1754–1763
98. Shen, L. *et al.* (2011) pH-Amplified multilayer films based on hyaluronan: influence of HA molecular weight and concentration on film growth and stability. *Biomacromolecules* 12, 1322–1331
99. Tsai, I.Y. *et al.* (2011) Human macrophage adhesion on polysaccharide patterned surfaces. *Soft Matter* 7, 3599–3606