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Hyaluronan as tunable drug delivery system

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ABSTRACT

The hyaluronan (HA) polymer is an important macromolecule of extracellular matrix with remarkable structure and functions: it is a linear and unbranched polymer without sulphate or phosphate groups and has key role in several biological processes in mammals. It is ubiquitous in mammalian tissues with several and specific functions, influencing cell proliferation and migration as well as angiogenesis and inflammation. To exert these important functions in tissues HA modifies the concentration and size. Considering this HA content in tissues is carefully controlled by different mechanisms including covalent modification of the synthetic enzymes and epigenetic control of their gene expression. The function of HA is also critical in several pathologies including cancer, diabetes and chronic inflammation. Among these biological roles, the structural properties of HA allow to use this polymer in regenerative medicine including cosmetics and drug delivery. HA takes advantage from its capacity to form gels even at concentration of 1% producing scaffolds with very intriguing mechanical properties. These hydrogels are useful in regenerative medicine as biocompatible material for advanced therapeutic uses. In this review we highlight the biological aspects of HA addressing the mechanisms controlling the HA content in tissues and its role as drug delivery system.

Contents

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1. Introduction

1.1. Structure

HA is the simplest glycosaminoglycan (GAG) present in nature. Even though its simple linear chain it has several important biomechanical properties. First it has terrific hydrophilic properties, in fact 1 g of this polymer can interact with several liters of waterthis influences most of the biomechanical properties of the polymer [1]. HA is a polymer constituted by disaccharide units of D-glucuronic acid (GlcUA) linked to *N*-acetyl-D-glucosamine (GlcNAc) with a GlcUA beta 1–3 linkage between GlcUA and GlcNAc and a hexosaminidic bond beta 1–4 between GlcNAc and GlcUA (Fig. 1 panel A). The disaccharide unit is repeated till 25,000 times to reach a molecular mass of millions of Dalton generating a linear polymer with a molecular mass ranging from 5×10^5 to 5×10^6 Da and more (Fig. 1 panel B) [2,3]. The synthesis of HA is peculiar: it is the only GAG produced on the cell membrane and immediately extruded in the extracellular matrix (ECM) without any protein linkage.The other GAGs are produced inside the Golgi and secreted in the ECM or exposed on the cell membrane linked to the protein core constituting proteoglycans.

The enzymes involved in HA synthesis are therefore located on the cellular membrane and are structurally organized to be able to extrude the polymer outside of the cell in the ECM. These enzymes are called HA synthases (HASes) and in mammals are present as three different isoforms: HAS1, 2 and 3 coded on different chromosomes. These genes could be a product of an ancestral gene duplication and it can explain the specific evolutionary path of this molecule [4]. Till now, few information is available on the structure of these enzymes as they had never been crystallized. HA is widely distributed in mammals and in few bacteria and, interestingly, it appears late in the evolution considering the other GAGs or structural related carbohydrate polymers as chitin and cellulose [5].

HASes use the UDP sugars precursors (UDP-GlcUA and UDP-GlcNAc) to produce HA chain picking up these molecules from the cytoplasm. It is of remarkable interest in these enzymes the presence of a double catalytic domain to interact with the two different substrates generating the disaccharide units necessary to create the polymer. The kinetic of the HASes have been extensively studied even if without crystallography information all kinetic explanations are still hypothetical [6].

The presence of three different enzymes to produce HA raised the question if specific enzyme shows unique kinetic properties, and it has been proposed that the different enzymes can produce polymers with different length and different rate [7–10]. Beside the chain length a large body of evidences supports the idea that the HASes differ each other also in the regulation of their catalytic activity. Only HAS2 has several covalent regulations, as phosphorylation [11,12], O-GlcNAcylation [13] and ubiquitination [14]. Indeed, in the case of HAS3 its activity is regulated by its sorting to the cell membrane by interaction with Rab10 [15].

To exert its biological properties HA requires specific interactions with receptors which not only regulate the cell-ECM interactions but trigger intracellular signaling. Beside receptors other proteins can interact with HA regulating tridimensional ECM structure. In general all proteins can interact with HA are defined “hyaloadherins” including not only receptors but also proteoglycans and other ECM molecules [16,17]. Proteoglycans as aggrecan, neurocan, brevican and versican can be included among these ECM molecules interacting with HA forming networks that act as architectural scaffold [1]. The biological functions of HA in tissues are due the interactions of HA with these “hyaloadherins” (Fig. 2). Such proteins are receptors (i.e., CD44, RHAMM, LYVE-1, Layilin, Stabilin1 and HARE) in fact can trigger specific signaling inside the cell (for a review on this issue see [18]) or receptors that mediate endocytosis and degradation of HA. Proteins can interact with HA by using the LINK module or Bx7B motif [18,19]. Interestingly, Toll Like Receptors (TLR) 2 and 4 were recently described as HA binding receptor even does not contain neither the LINK module nor the BX7B motif. TLR 2 and 4 are necessary to trigger the response after low molecular weight HA (LMW-HA) stimulation [20]. Although the physical direct interaction between TLR and HA is still not experimentally demonstrated, it is reasonable that the polyanionic nature of HA mimics the canonical ligands of TLR2/4 as lipopolysaccharides. HA and PG LiNk protein family (HAPLN1–4) (has been described to interact with HA and PG stabilizing such multi component complexes [18].

The most common HA receptor is CD44, a proteoglycan widely distributed in different cells and particularly concentrated on the membranes of inflammatory and cancer cells [21]. Another important HA receptor related to cell motility is RHAMM, acronym for Receptor for HA Mediated Motility, it is also known as CD168. RHAMM has been found in several cells including cancer and endothelial cells [22,23]. Interaction between HA and RHAMM triggers a signaling pathway till not completely understood which includes ras-oncogene activity [24–26]. It has been also described that RHAMM shares ERK1/2 phosphorylation cascade activation with CD44 [26].

Even though the HA is typically present in mammalian ECM, it is also described in few bacteria, which probably imported the genes from mammals during evolution. Noteworthy the bacteria producing HA use three genes, which encode UDP-Glucose pyrophosphorylase (*hasC*), UDP-Glucose dehydrogenase (*hasB*) and HA synthase (*hasA*), and these genes are located in an operon [6,27].

Interestingly, microorganisms as *Streptococcus uberis, Streptococcus equisimilis, Streptococcus pyogenes,* and *Pasturella multocida* (pathogens for humans and other vertebrates) evolved the capacity to produce a HA capsule which acts as a shield protectingbacteria from immune system [28]. The bacterial HA has the same structure of vertebrate onewith the same biomechanical properties and without immunogenic activity which represents its major advantage in medical applications.

In the last decade HA started to be considered not only a passive molecule characterized by remarkable mechanical properties, including space filler and molecular sieving, but due to its biological properties this GAGemerged enlisting among the most biological active molecules in the body. HA plays a key role in several physiological processes including development [29], wound healing [30], cell migration [31] and proliferation [32]. It is also involved in several pathologies as cancer [21,33], vascular diseases [34–36] and diabetes [36].

1.2. Discovery of HA

HA was described for the first time by Carl Meyer [37] who purified the polymer from corpus vitreous of the eye. After this first description the HA was found in almost all tissue of the mammals raising the question of its functions. At the beginning of its history the most amazing aspect of this chain was its dramatic capacity to interact with water and most of the hypotheses on its role in tissues were related to his hydrophilic characteristic.Hence the principal function attributed to HA was space filler and space organizer in the ECM. Its presence in articular fluid highlighted the concept that HA acts as lubricant in the joints. This observation suggested the first clinical application of HA as join visco-supplementation in joint disease. Till now this represents one of

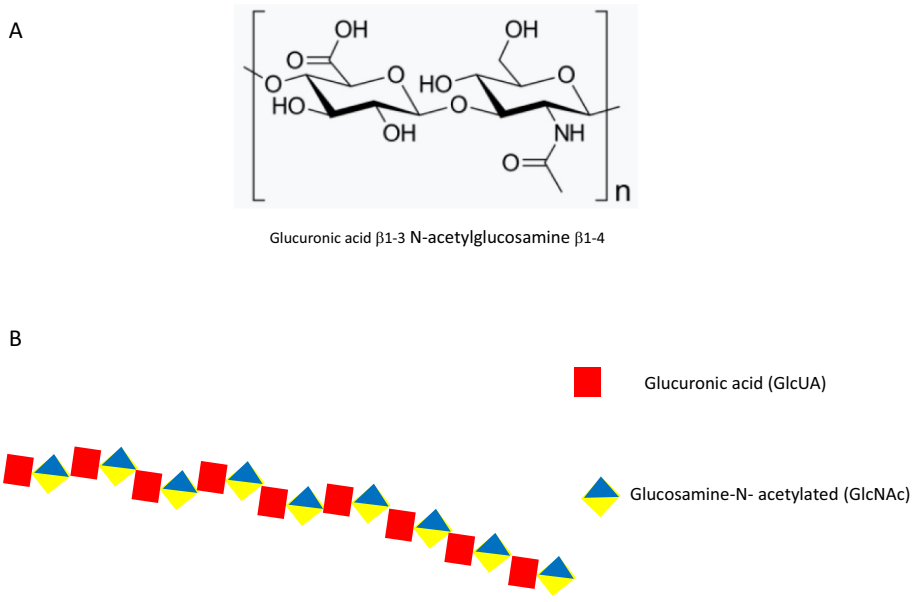

Fig. 1. HA chemical structure. A. The hyaluronan disaccharide is composed of D-GlcUA and D N-GlcNAc linked together with beta1–3 and beta1–4 glycosidic bonds, respectively. This disaccharide can be repeated up to thousands of time to give high molecular mass HA. B. Schematic representation of HA disaccharide by using the symbol nomenclature for graphical representations of glycans [206].

the most common application in human therapy. The polymer rheology is strongly dependent on its size and concentration and from these studies stemmed the idea to produce specific HA solutions for specific applications. The realization of bioreactors with genetically modified bacteria producing highly pure HA opened the field to several applications coupled with a terrific development of in vitro biosynthesis of HA in specific bioreactors. This technology changed dramatically the field as since then the biotechnology produced highly pure polymers and the extraction from mammaltissue (roster combs) was considered obsolete for the presence of tissue contaminants.

In human body HA accounts for more than 15 g in total, mainly located in the dermis. The turnover of this polymer is very fast and about of 30% of this molecule is replaced every day [38]. In human body the amount of HA is finelyregulated, and the polymer is mainly removed by lymphatic system to the liver. The size of the polymer is critical for its biological function, and in healthy tissue the typical size is high molecular weight (about $1x10^3$kDa). The relationship between size and biological functions is confirmed by the finding recently described of very HMW-HA in naked mole rat [39], an animal with an incredible longevity and cancer resistance.

The fast degradation of HA is due to the activity of a class of enzymes called hyaluronidases, hydrolases which degrade the HA in small fragments [38,40]. It was recently demonstrate that HA oligos maintain important biological functions when free in tissues [41]. The HA fragments are usually internalized into the cells and destroyed in the lysosomes. When too many oligos are present in the tissue, exceeding the cell capacity to remove properly these fragments from ECM, these fragments can produce important biological effects in angiogenesis, inflammation and cell behavior [42]. Beside hyaluronidase activity there are other ways to destroy HA polymer, including UV radiations and free radicals

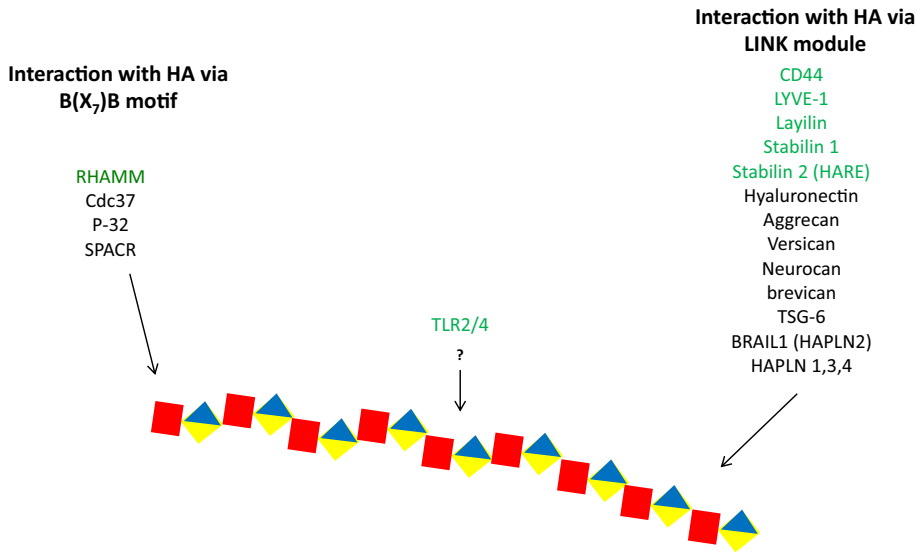

Fig. 2. HA interacting proteins. Schematic representation of proteins that can bind (not covalently) HA. HA can be recognized by two class of proteins containing the BX7B motif or the LINK module. TLR2 and TLR4 are necessary to induce the proinflammatory response of LMW HA but it is still debated whether HA can directly bind to such receptors. HA receptors are shown in green.

as in inflammation. The reduction of the HA influences dramatically its rheological properties and it was described several years ago that the HA reduction in joint represents a marker in joint disease [43].

1.3. Biological properties of HA

HA with a simple linear structure exerts its several functions modifying only its size and concentration [41,43]. As previously described size and concentration can protect animal from aging and cancer development [39]. Interestingly HA fragments have biological relevance and often a complete opposite effect respect to the intact molecule with high molecular weight [44].

The rapid turnover in the human tissues is obtained by the activity of hyaluronidases, hydrolases which degrade HA in small fragments removing about 30% of total HA present in the body [41]. The presence of HA oligos in specific conditions gained great interest producing an increasing body of literature that describes HA fragments as ligands for toll like receptors (TLR) triggering specific inflammatory pathways [45–47]. In an opposite way high molecular weight HA (HMW-HA) shows anti-angiogenic, immune suppressive and anti-inflammatory activities, induces tissues reparative process as described in wound healing [47,48]. The fragments of HA, called oligosaccharides when less than 200 kDa, show the capacity to induce inflammatory process as well as angiogenesis throughout the interactions with specific receptors [18,45]. Considering these latter properties, the HA-oligosaccharides can be considered part of the alarm system of the organism and could be considered part of the alarmin protein family [49].

The biological activities of both HMW-HA and oligosaccharides are mediated by interactions of receptors present on cell membrane [50]. The functions of HA in tissues depend on its structural properties. HA with its hydrophilic properties controls the water content in all tissues. One of the most important biological function of HA is to maintain a well hydrated ECM, perfect environment for cell migration and proliferation [29]. The presence of a well hydrated and soft ECM is critical for embryo development, in fact the microenvironment of oocytes is mainly constituted by HA [51]. The unique viscoelastic properties HA have critical roles in the biomechanical functions of various tissues, from corpus vitreous to derma [2]. It is of remarkable importance to consider that HA can form gel when its concentration is above 1%, a concentration often reached in mammal tissues. Examples are the space between keratinocytes in the epidermis, in synovial fluid or in Wharton jelly in umbilical cord, in these tissues the HA concentration is enough to form gel and in this form the polymer can play the role of space filler and shock absorbing macromolecule [8,52].

The biological activities of HMW-HA and oligosaccharides are mediated by interactions of receptors present on cell membrane. The interaction of HA with CD44, the most common HA receptor, triggers the signal cascade which eventually activates extracellular signal–regulated kinases (Erk) 1/2 [53,54]. The main function of CD44/HA interactions have been studied in inflammatory cells, where HA maintains inflammatory cells in inflammation site [46,47,55]. The signaling cascade triggered by CD44/HA interaction includes Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase-1 (PDK1) / protein kinase B (Akt) activation and rat sarcoma protein (Ras) phosphorylation pattern which involves Rapidly Accelerated Fibrosarcoma kinase 1 (RAF1), Mitogen-activated protein kinase (MAPK)/Erk kinase (MEK) and eventually Erk1/2 [56]. The CD44/HA complex recruits also other cytoplasmic proteins as ezrin, merlin and avian erythroblastosis oncogene B 1 and 2 (erbB1,2) [21].

The binding of HA to CD44 is critical for cell adhesiveness, triggering the phosphorylation of cytoplasmic CD44 domain which is also required for cell migration and infiltration [55]. CD44 signalinghas a role also in wound healing, influencing fibroblast migration in wounded area from perilesional stroma [57]. It is evident that CD44 per se does not cause cell migration but interaction with HMW HA promotes wound healing process. Moreover, the directionality of cell migration is strongly dependent on CD44 expression and on HA gradient in ECM environment [58].

RHAMM has been described in cancer cells and in endothelial cells [22,23]. Several studies report that RHAMM plays a critical role in cell migration and in inflammation development and in tissue healing [59,60].

Stabilin 2, also known as HARE, acronym for HAReceptor for Endocytosis, was initially described in endothelial cells in lymph nodes, spleen and liver [61] whereHARE has a critical role in their development [62]. More recently HARE was described in eye, brain, kidney and heart. HARE canalso interact with other GAG as chondroitin-sulphates (A, B, C,D and E) [63]. HARE is mainly implicated in HA endocytosis and is closely located to clathrin in cell membrane. Nevertheless, even thoughin literature the role of HAREin endocytosis isreported, its involvement in lysosome pathway is still not clear and information on its activity at molecular level is scanty [64,65].

Lymphatic Vessel Endothelial HA receptor 1 (LYVE 1) is a HA receptor specifically expressed on endothelial cell in lymphaticvessels, but it was also found in lymph nodes and in sinusoidal endothelial cells in liver [66–68]. Due to this peculiar cell distribution, LYVE 1 is generally considered a molecular marker of lymphatic endothelial cells [69]. The LYVE 1 interacts with HA and the resulting complex receptor/HA can be internalized and digested in lysosomes [70]. The main function of LYVE 1is to absorb HA from tissues transferring it to the lymph actingin this way as regulator of tissue hydration. In any case, the abrogation of LYVE 1 in the lymphatics generated animals with normal behavior, indicating that other till unknown receptors canrescue the LYVE 1 activity in mammals [71].

A large body of evidence supports the role of TLRs in HA signaling. TLRs are receptors belonging to innate immunity system and are expressed on the membrane of all mammal cells [72,73]. The TLRs in human are ten and those involved in HA signaling are TLR2 and 4 [47], being TLR2 able to interact with mycobacteria and Gram positive bacteria whereas TLR4 recognize lipopolysaccharide (LPS). In macrophages the interactions between HA and TLR 2 or 4 trigger a signaling cascade that promotes expression of chemokine genes, and this process is strongly dependent on Myeloid Differentiation primary response 88 (MyD88) presence [47]. The signaling triggered by TLR/HA interaction strongly depends on HA size and could also involve other membrane proteins. The HA/TLR interaction is still an elusive aspect of this HA function which requires more studies. The TLR clustering on cell membrane is promoted by the interaction with HMWHA and this complex induces cell survival. In an opposite fashion HA oligosaccharides induce inflammatory stress and cell death [47]. HA oligosaccharides induce also the dendritic cell maturation and Tumor Necrosis Factor (TNF) alpha synthesis throughout the phosphorylation of Mitogen-Activated Protein Kinase (MAPK) and the nuclear translocation of Nuclear Factor kappa-beta (NF-kB). HA oligosaccharides are also involved in the transplant rejection demonstrating their involvement in alloimmunity [74].The synthesis of interleukin 8 (IL-8) and Matrix Metalloproteinase 2 (MMP2) is also stimulated by the HA TLR4 interaction [75]. Moreover, during osteoclast differentiation, the HA interaction with TLR4 blocks the signaling triggered by macrophage colony-stimulating factor (M-CSF) [76]. The role of oligosaccharides in gut has been recently described as they can induce beta-defensin 2 synthesis in intestinal epithelium [77]. The role of HA oligosaccharide in synthesis of defensins has remarkable importance in tissue metabolism and wound healing. In fact, defensins are a family of small proteins with antibiotic properties and capacity to stimulate tissue regeneration [49,77].

In tissue the degradation rate of HA regulates the HA concentration. In mammals are present six hyaluronidases (Hyal 1-,2-,3-,4-, P1 and PH20), which catalyze the hydrolysis of linkage bonds beta1–4 between hexosamine and GlcUA residue. The Hyals are classified as *endo*-beta-*N*-acetylglucosaminidases according to their hydrolytic mechanisms [40,78]. There are six Hyals known in humans: Hyal 1–4, HyalP, and PH20, all β, 1–4 endogalactosaminidases. In humans, the genes

HYAL1, HYAL2, and HYAL3 code for the enzymes Hyal-1, Hyal-2, and Hyal-3 [78,79]. These genes are tightly clustered on chromosome 3p21.3. Hyal-1 and -2 are the major hyaluronidases in the tissues. Hyal-1 is in lysosomes whereas Hyal-2 is a Glycosyl Phosphatidylinositol (GPI) anchored protein with extracellular activity [78].Hyal-2 produces from HMW-HA fragments with a size of about 20 kDa. Hyal-1 appears to be a lysosomal protein which cleaves HA into small disaccharides. The role and activities of Hyal-3 is still elusive and few information are available on this enzyme as it is reported in experiments based on KO mice production [80].

Other three genes HYAL4, PHYAL1, and Sperm Adhesion Molecule1 (SPAM1) are in cluster on chromosome 7q31.3. Hyal-4 is a pseudogene transcribed but not translated in the human, and PH-20 is the enzyme that digests the HA around the oocyte facilitating ovum fertilization [81]. The Hyals [1 to 4] can work in acidic environment (about pH 3 and 4) whereas PH 20 and other hyaluronidases from insects and snakes' venoms are active at neutral pH [82]. Hyal 1 is active inside the cells and is common in mammal tissues and it is reported a genetic disease to Hyal-1 deficiency called mucopolysaccharidosis type IX [83].

Hyaluronidase 2 (Hyal 2) is a GPI-anchored receptor operating in acidic microenvironment [84]. This enzyme degrades HMW-HA into LMW-HA (about 20 kDa) [38] which is internalized and further digested to smaller oligo HA (oligo-HA) by Hyal 1.

Interestingly, in bacteria are present several hyaluronidases which act as lyases [5,78]. In mammals the activity of Hyal 1 and 2 are synergic, in fact Hyal 2 degrades the HA in fragments of 20 kDa and then the Hyal 1 degrades these fragments in smaller fragments of about 800 Da. The degradation of HA polymer could be also due to the action of free radicals that break HA polymer in fragments without specific size [79]. The minimal size able to trigger a cell response has been extensively addressed, it was reported that oligomers characterized by 4–6 disaccharide units (4-6mers) are responsible for NF-kB signaling and metalloprotease synthesis [75], the oligos with a range of 4 to 16 mers are able to activate the dendritic cells by TLR receptors [85,86].

Recently a new hyaluronidase was described: Transmembrane Protein 2 (TMEM2), which is a transmembrane protein with a strong hyaluronidase activity [87,88]. TMEM2 can digest HMW-HA into ~5-kDa fragments and is specific for HA. Another hyaluronidase called Cell migration-inducing and hyaluronan-binding protein (CEMIP), also known as KIAA1199 or HYBID, has been recently identified with HA-degrading activity [89,90]. Interestingly, this enzyme has a key role in cancer development, in the skin biology and cell senescence [90,91].

1.4. The magic glue: chemical structure of HAand oligosaccharides

HA is a polymer constituted by disaccharide units of GlcUA linked to GlcNAc with a GlcUA beta 1–3 linkage between GlcUA and GlcNAc and a hexosaminidic bond beta 1–4 between GlcNAc and GlcUA. These units are repeated till 25,000 times to a molecular mass of millions of Dalton ranging from 5×10^5 to 5×10^6 Da and more [2,8]. The biotechnological properties of HA are unique and dependent on the structure of the polymer. HA structure is based on the presence of beta linkages which allows HA bulky groups (the hydroxyls, the carboxylate moiety, and the anomeric carbon on the adjacent sugar) to be in sterically favorable equatorial positions, favoring the chemical modification. All axial position, less favorable for chemical derivatization, are occupied by simple hydrogen atoms [92]. When the polymer is in aqueous solution a network of hydrogen bonds is established and maintain the HA chain stiffness. The axial small hydrogens form a relatively hydrophobic environment whereas the equatorial groups are more hydrophilic and can interact with solvent creating a twisting ribbon structure. In solution the HA chain forms an extended random coil structure which can interact with other chains. Even a very low concentration soluble HA chains can entangle each other. This phenomenon starts at 1 mg/mL and this is a spontaneous process [93]. The helical chain of HA in solution can bind 1000 times its weight in water [94]. At 1% the HA forms a hydrogel with soft properties which allow to manage it using syringe with needles, it can be defined as “quasi-plastic material” [95]. Moreover, in solution after and during the entanglement HA chains can form double helices [96]. These physical aspects indicate HA hydrogel a perfect product with lubricant properties that can be used for replace synovial fluid or in surgery to prevent post-surgical adhesion formation after abdominal surgery procedures.

The hydrogel formation of HA represents the most important property allowing to be used as an innovative bio-compatible product in several applications [97]. The viscosity of the HA solution increases with concentration and its elasticity increases with size of the polymer. The relation between elasticity and viscosity are two critical parameters used in commercial HAgel preparation [98]. The organ surface takes advantage from the rheological properties of HA, as in cartilage and in muscle bundles acting as osmotic buffer regulating the water content in the tissues [99]. From these viscoelastic properties stem the biomechanical characteristics for hydrogel medical applications [100,101]. The HA gels are useful tools for the preparations of scaffold for regenerative medicine, they are biodegradable, biocompatible and bioresorbable. HA scaffolds induce cell differentiation and growth [54], improve the wound healing without nonspecific absorption of proteins enhancing tissue growth and repair [102–104]. The preparation of highly purified HA is based on the applications of modern biotechnology procedures using bioreactors with genetically modified bacteria. The availability of large amount of high pure polymer introduces the possibility to treat arthritic joints restoring lubrication and replacing the rheological properties of synovial fluid. The HA chains can be chemically modified by use of adipic hydrazide, tyramide, benzyl ester, glycidyl methacrylate, thiopropionyl hydrazide or bromoacetate, either at carboxylic acid of GlcUA or at the C-6 hydroxyl group of the GlcNAc [105]. HA hydrogels can be used as drug delivery, as proposed in ophthalmology and otolaryngology [106,107]. The main function of HA hydrogels is to maintain in situ the drug molecules releasing them during the HA degradation [108]. In other in vivo models the reabsorption of HA matrix is the mechanism used to produce new tissue in situ stimulating cells with growth factors as Bone morphogenetic proteins 2 (BMP2) in cartilage [101].

It is noteworthy to say that HA use as medical device was strongly dependent on its cross linking that greatly influences its applications. The key point is related to the biomechanical properties of the HA hydrogels that must be comparable to tissue microenvironment, and often this aspect is not easily achievable limiting the general use of HA as general drug delivery system.

2. HA biosynthesis control

The amount of the HA in human body depends on a rapid turn-over. The total amount of HA is about 15 g mainly located in the skin and about 30% is replaced every day [38]. The amount of HA in the tissues is strictly regulated by the cells by a finely tuned balance between synthetic and catabolic activities. Part of the polymer could be also removed from tissues by lymphatic system which transport HA to liver for the final degradation.

2.1. Formation of UDP-GlcNAc and UDP-GlcUA

The regulation of the HA synthesis is critical and includes different mechanisms which are still poorly understood. One of them is the substrate availability. It was demonstrated that the amount of UDP-sugars as substrates can influence the HA synthesis. Altering the UDP-sugars availability in cytoplasm it was evident in primary cell cultures an increased HA production as well as an increased expression of HAS2 and 3 [29,109].

UDP sugar precursors are molecules with a high-energy cost, competing with glycolysis and other catabolic pathways for their synthesis. Hence, GAG synthesis is possible in tissues with a good oxygen supply,

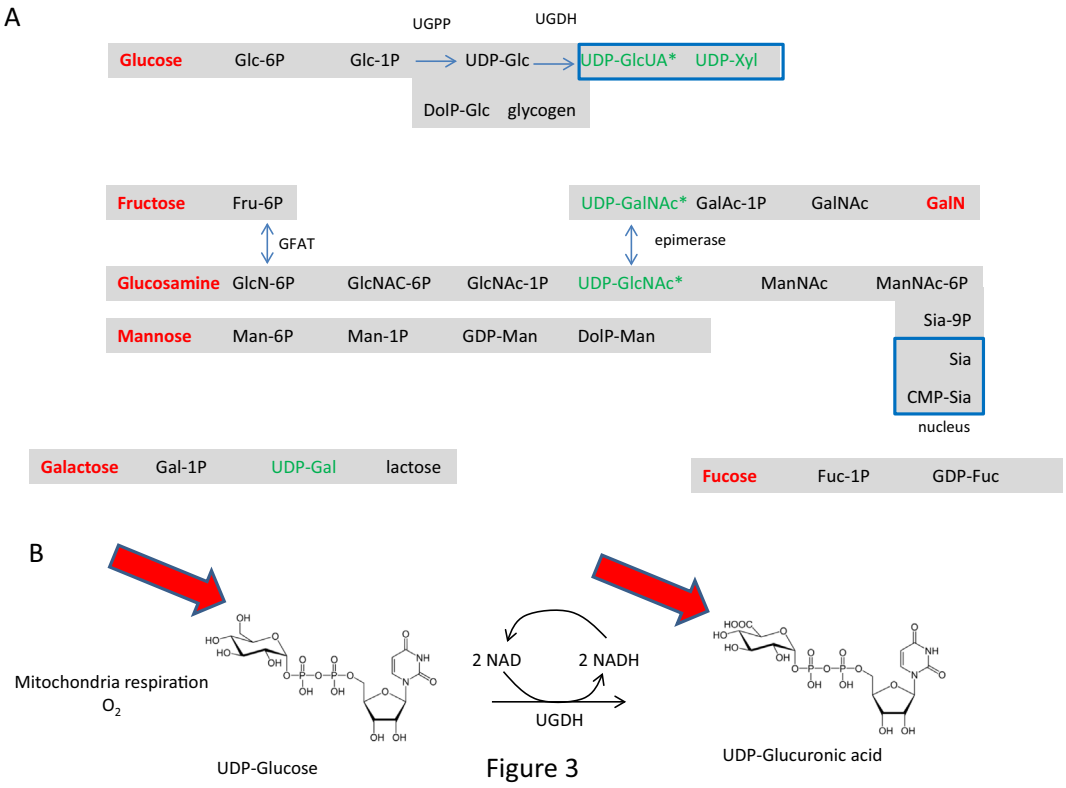

Fig. 3. A. Schematic representation of the metabolism of dietary monosaccharides (in red) in the formation of activated sugar nucleotides used in biosynthesis of various glycans. UDP-sugars used for GAG and PG biosynthesis are shown in green. All reactions take place in the cytosol except for reaction boxed in blue. In this scheme almost all the enzymes have been omitted except for the critical enzymes involved in UDP-GlcUA synthesis (UGPP and UGDH), UDP-GlcNAc synthesis through the hexosamine biosynthetic pathway (GFAT) and UDP-GalNAc (epimerase) synthesis. These latter UDP-sugars will be further chemically modified in HS/heparin, CS, and KS biosynthesis. B. Conversion of UDP-glucose to UDP-GlcUA by UGDH. In the scheme is highlighted the involvement of 2 NAD in the oxidation of the C6 of glucose with the generation of 2 NADH. The regeneration of NAD via the mitochondrial metabolism is critical to supply energy. The big red arrows highlight the double oxidation on C6.

as oxygen allows the two oxidative reactions necessary for UDP-GlcUA synthesis (Fig. 3 panel A). The synthesis of UDP-GlcUA is a critical step for all GAGs, except keratan sulfate (KS), which does not contain uronic acid. In fact, KS is usually common in tissues with poor oxygen supply or even without vascular system. The synthesis of UDP-GlcUA requires the action of the UDP-glucose dehydrogenase (UGDH), which produces UDP-GlcUA from precursor UDP-Glc (Fig. 3 panel B). This reaction is possible in the presence of NAD, which is transformed in NADH during the double oxidation of the C-6 of UDP-Glc. This uncommon reaction has a remarkable role in terms of cell energy balance. From this point of view, the costs of UDP-GlcUA synthesis are completely balanced by the re-oxidation in mitochondria of the two NADH molecules produced by the UDP-GlcUA synthesis. The stoichiometry of the reaction of HA synthesis indicates that the disaccharide units contain one molecule of GlcUA and one of GlcNAc with a ratio of 1:1. The five ATP obtained by the re-oxidation of two molecule of NADH in mitochondria repay the energy cost of the synthesis of the unsulfated backbone of HA.

UDP-sugars availability has a critical role on the HA synthesis; in fact, the modulation of UDP-GlcUA availability by over expressing or silencing UDP-Glucose 6-Dehydrogenase (UGDH) in cytoplasm can dramatically influence the HA production as well as the expression of HAS2 and 3 [29,109–111].

This aspect is confirmed by using the 4-methyllumbelliferone, a drug able to bind the UDP-GlcUA reducing the availability of this precursor [31,112,113]. Another way to regulate HA synthesis involves the activity of 5′ adenosine monophosphate-activated protein kinase (AMPK), the sensor of energy level of the cells. It was demonstrated that the activation of AMPK can phosphorylate a specific threonine 110 residue in HAS2 blocking the enzyme activity [114]. It is remarkable that this kind of regulation is specific only for HAS2 and did not influence the other two enzymes. Moreover, this regulation affects only HA production leaving unaltered the synthesis of other GAGs. This observation correlates the energy level with the specific capacity of cells to produce HA.

2.2. Regulation of the UDP sugars precursors concentration in cytoplasm

The first level of regulation of HA synthesis is the UDP sugars availability in cytoplasm.

The substrate for glycosyltransferases used to synthesize all the glycans (i.e., GAG as well as glycoproteins and glycolipids) are nucleotide-activated sugars. Uridine diphosphate (UDP) is the most common nucleotide used to activate sugars in animal cells, as it is found linked to glucose (Glc), galactose (Gal), GlcNAc, GalNAc, GlcUA and Xylose. Only two additional nucleotides are used in animals, guanosine diphosphate (GDP) that is linked to mannose (Man) and fucose (Fuc), and cytidine diphosphate (CMP) that is linked to sialic acid (Sia) (Fig. 3 panel A) [115]. It is to be noted that all the substrates for glycoconjugate reactions are sugar nucleotides generated mainly in the cytosol with the exception for the synthesis of UDP-Xyl and CMP-Sia that take place in the Golgi and in the nucleus, respectively (Fig. 3 panel A). Further, these sugar nucleotide precursors are transported in the ER/Golgi by specialized transporters [116,117]. This point is crucial as allows the creation of two pools of precursors; one in the cytosol and the other inside the ER/Golgi. Even though the sugar nucleotideconcentration is not easily measurable inside the cells, nevertheless it is reasonable ER/Golgi contains a higher number of precursors compare to cytosol. In fact, UDP sugars transporters have low Km values and could ensure an efficient supply of precursors in the ER/Golgi lumen. On the other hand, the cytosolic pool could be directly affected by nutrients as clearly described for the concentration of UDP-GlcNAc [118].

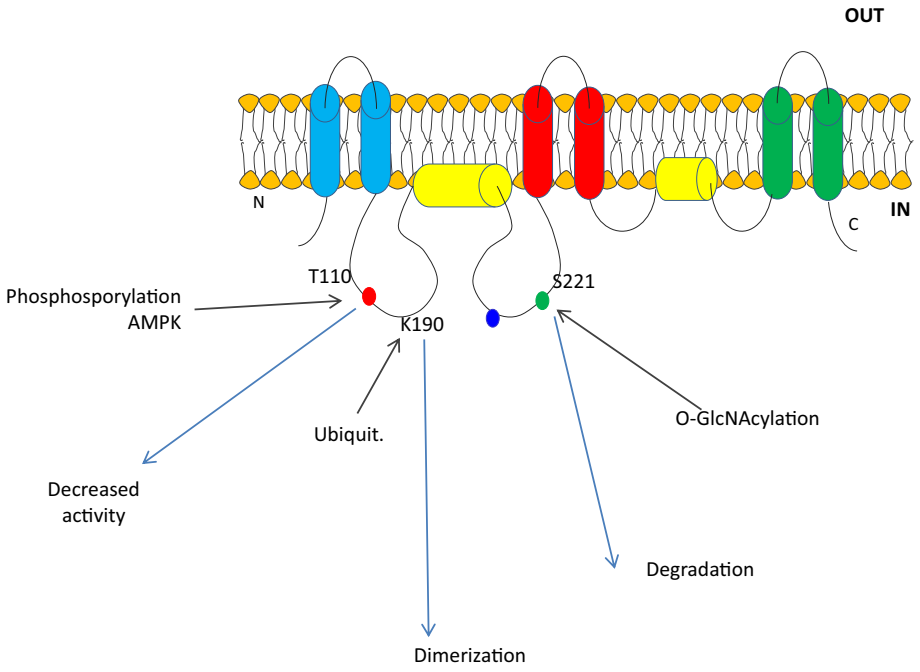

Fig. 4. Schematic representation of human HAS2. HAS2 protein, which belongs to Class I HASes, is schematized in the plasma membrane and the eight transmembrane helices represented as cylinders. The large intracellular loop between transmembrane helices 2 and 4 contains the catalytic site. Moreover, the three critical residues modified by phosphorylation, O-GlcNAcylation and ubiquitination that modulate protein activity, stability and dimerization are shown in different colors.

The critical role of the amount of UDP sugars in the HA synthesis regulation is confirmed by experiments based on the use of 4-methyllumbelliferone, a drug that binds the UDP-GlcUA reducing the availability of this precursor [119,120]. Other data demonstrated the importance of energy supply and HA synthesis. In fact, the activity of AMPK, the sensor of cellular energy level, can regulate the HAS2 activity in mammal cells [12]. The activation of AMPK by 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) induces the phosphorylation of a specific threonine (Thr 110) in HAS2, blocking the enzyme activity [12]. Confirming the specific regulation of HASes, the AMPK activity acts only on HAS2 and not on HAS1 and 3. Moreover, the activity of AMPK plays a role only for the HA synthesis and not for the synthesis of other GAGs, indicating that the HA is the only GAG affected by the energy modulation, correlating the cell energy level with HA synthesis.

The concentration of the second UDP sugar precursor of HA synthesis (UDP-GlcNAc) has an important role in this context, but it uses different mechanism. The UDP-GlcNAc influences the HA synthesis not only as precursor, but also because the HAS2 itself is target of O-GlcNAcylation, a protein covalent modification described by Torres and Hart in 1984 [121]. The cytosolic levels of UDP-GlcNAc are finely regulated by hexosamine pathway. When the UDP-GlcNAc is elevated in the cytoplasm, it induces the activity of the enzyme O-GlcNAc transferase (OGT), which catalyzes the β-O-linkage of one residue ofGlcNAc to serine 221 of HAS2 [13]. As previously discussed for the AMPK regulation, the O-GlcNAcylation is a covalent regulation specific for HAS2 and does not affect other HASes and the synthesis of the other GAGs. The O-GlcNAcylation is a protein covalent modification widely described in several physiologic and pathologic conditions including cancer and chronic diseases [122,123]. It is interesting to note that protein O-GlcNAcylation is related to general metabolic conditions of the cells and UDP-GlcNAc is defined as “general sensor” of energy level in mammal cells [124]. In the case of HAS2, the mechanism used by the cells to regulate HA synthesis throughout O-GlcNAcylation affects the half-life of the enzyme on the membrane. HAS2 is usually active on the cell membrane for 17 min whereas after O-GlcNAcylation the enzyme can remain active on cell membrane for more than 5 h, increasing the HA content in ECM [125] The regulation sites of the enzyme are reported in Fig. 4.

2.3. HA synthases

HASes are classified in two classes. Class I HASes are multiple transmembrane proteins that are present in bacteria and animals. Typically, bacterial enzymes contain 6 membrane associated helices, whereas animals HASes have 8 transmembrane domains (Fig. 4). *Pasturella m.* expresses a peculiar HAS that belongs to Class II family; it has only one transmembrane domain the C-terminal of the protein and has a completely different mechanism of catalysis (for a review see [6]).

Large body of literature suggests that several growth factors and cytokines regulate the expression of the HASes [126–129] by signaling pathways triggered by specific receptors [130].

2.4. Epigenetic regulation of HA synthase

Beside direct covalent modification of enzymes it has been described an epigenetic control of transcription of HAS2 gene involving a long non-coding RNA. It was recently described a HAS2 antisense (HAS2-A1) which can increase the HAS2 transcription acting as gene activator in *cis* [131,132]. The presence of antisense transcript and its mechanism is present in different cell models.In an in vivo approach based in murine and human atherosclerotic models indicates this epigenetic regulation as a general gene expression regulation [111]. Noteworthy the HAS2-A1 activity involves NF-kB signaling cascade throughout P65. The interaction of this nucleoprotein with antisense promoter may explain the correlation between inflammatory signals and HAS2 expression.

Even if several aspects of HASes activity are still elusive, it is evident the complexity of the regulation of their activity as it depends on the general metabolic conditions of the cells. The HA synthesis regulation is carried out by direct and different covalent modifications at protein level as well as by epigenetic modifications at gene expression level [34].

3. Biological role of HA in mammalian tissues

3.1. HA in musculoskeletal tissue

Probably the most known application of HA is in intra-articular injections to attenuate damage to cartilage in inflamed joints [133]. The complete mechanism of action is not fully understood, probably due to the combination of lubricant and anti-inflammatory properties of HMW-HA [47]. HA could stimulate chondrocytes metabolism restoring a physiological deposition of cartilage ECM. Alternative approaches foresee to use stem cells with the aim to regenerate damage tissues. Interestingly many scaffolds used to maintain stem cells in the pathological area and to increase stem cells viability are hydrogels of HA that probably trigger specific signals inside the stem cells favoring survival and proliferation. Similar approach is used for skin regeneration with interesting results [97].

3.2. HA in development

During development, the rotation of the gut is a critical event. ECM and HA play a pivotal role in the controlling such gut movements that take place in fetal development [134]. In adult, patients with chronic intestinal inflammation (i.e., Crohn's disease and ulcerative colitis), accumulate HA in the nonvascular space of colon dramatically increasing the recruitment of immune cell contributing to the infiltration of leukocytes [135,136]. In a rat model of inflammatory bowel diseases HA is altered not only in the mucosa but also in the neurons of the myenteric nervous system where it could regulate physiological functions as motility [137]. HA is also involved in liver fibrosis representing a key molecule for therapy and pathophysiology [138]. HA has a critical role in development. In mouse the silencing of the main enzyme HAS2 lead to severe cardiac malformation that causes the death of embryos [139], whereas the conditional abrogation of Has2 in different tissues revealed its importance also in skeletal growth, patterning, chondrocyte maturation and joint formation in the developing limb [140]. HAS1 and HAS3 knockout mice do not show defects and have no effect on development.Recent data on HAS1 and 3 KO mice are studied to detect specific HAS isoforms functions as reported for instance in the skin [141] or in hematopoiesis [142]. In other model organism as the amphibian *Xenopus laevis*, the lack of HAS3 impairs gastrulation [109]. In recent years zebrafish has been used as a convenient model to study development and several human pathologies and HA has been described as critical molecule for organogenesis and, interestingly, to be necessary for tail regeneration [143–145].

3.3. HA in skin

As mentioned before, skin contains the largest amount of HA in human body. During aging, a dramatic reduction of HA is a common physiological situation. This implies a diminished skin thickness and hydration [146,147]. Interestingly the naked mole rat, the only rodent with the exceptional life span of about 30 years, the skin contains an extremely large HA of over 20 million Dalton that protects such animal from cancers [39] and maintain these animals active and fertile for a very long time. On the other hand, HA in the skin is not always protective, in fact Chinese Shar-pei dogs, where the characteristic wrinkled skin is associated to HA and to mutations in HAS2 promoter that greatly increase HAS2 expression, suffer of Periodic Fever Syndrome [148].

In epidermis, HA has a critical role in keratinocytes differentiation and HA is also involved in scar less wound healing process and can stimulate [149]. It of remarkable importance to note that oligosaccharides have a dramatic effect on skin beta-defesin-2 secretion both in mice and in human [49].

3.4. HA in cardiovascular tissue

In cardiovascular system HA plays a critical role in several pathophysiological conditions. Normally HA is present mainly in the adventitia of arteries, but in early stages of atherosclerosis, HA accumulates in the media favoring migration and proliferation of smooth muscle cells and contributing to vessel thickening [9]. HA can therefore participate to immune cells recruitment in the vessel wall favoring inflammation. In the endothelial layer, HA is the major component of the glycocalyx and when its amount is reduced this situationincreases immune cell adhesion and favors shear stress [150,151]. In condition of acute inflammation, as during angioplasty or, in animal models, after wire or balloon injuries, HA is involved in neointima ECM formation and the inhibition of HA synthesis reduces dramatically the neointima formation as demonstrated in conditional HAS2 KO mice [152].

3.5. HA in neural tissue

The central nervous system has an ECM containing a large amount of HA that in physiological condition contributes to brain hydration [153]. HA is present in the perineuronal net where, in collaboration with other sulfated GAG as chondroitin 4 sulfate, regulatesneuron excitability via binding with bivalent cations [154]. Recent findings showed that a lack of HA in the brain causes reduced extracellular space volume increasing the concentration of neurotransmitters affecting the normal synapsis function as described in a mouse model of epilepsy [155].

3.6. HAin respiratory system

HA present in lungs contributes to the mechanical properties of the tissues as elasticity. In asthmatic patients or after cigarette smoking, HA fragmentation is evident and can be involved in inflammation via the TLR2/4 and NF-kB pathway and macrophages recruitment that eventually contribute to fibrosis [156,157]. HA receptor RHAMM (but not CD44) has been described to have a pivotal role in hyperoxia-mediated neonatal lung injury favoring a reduction of alveolar development [158].

4. HA in pathology

4.1. HA in tumors

Although neoplasia can derive from all type of cells, it is generally accepted that aggressive malignancies have an increased content of HA surrounding the cells and inside them [159–161]. Up regulation of HASes and Hyals have been described in several malignancies as well as an increased activity of HA receptors [162,163]. In many animal models the inhibition of HA synthesis via 4-MU reduced tumor growth and metastasis suggesting thatHA metabolism could represent a good target for new therapeutic strategies [164,165].

Many tumors have an increased concentration of the UDP-sugar precursors of HA which is in accordance with an augmented HA synthesis [166]. Moreover, UDP-GlcUAis used in liver to detoxify chemotherapy drugs as epirubicin and for this reason an increased HA synthesis reducing UDP-GlcUA availability could favor drug resistance.

4.2. New insights into HA role in human pathology

The use of hyaluronidases in therapy is a recent evidence that shows interesting application in the treatment of solid tumors where the hyaluronidase treatment improves the bioactivity of the chemotherapy [167]. The role of hyaluronidase in human pathology and in cancer is still not clear and several controversial data are reported in literature. However, these contradictory data indicate that Hyal 1 and Hyal 2 might promote or suppress tumor development suggesting that the

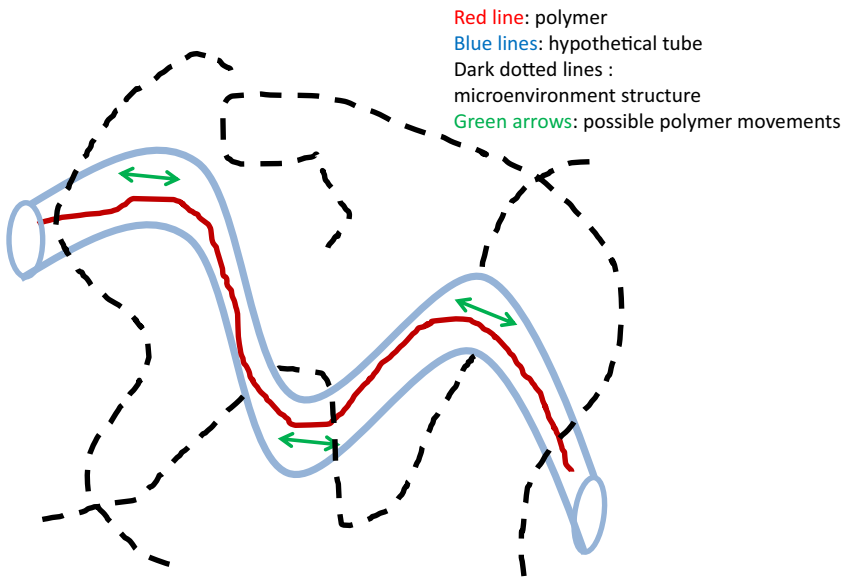

Fig. 5. Schematic representation of concept of reptation applied to a polymer in a microenvironment. Reptation theory describes the snake-like large-scale motion of entangled polymers. The motion of polymer segments to be confined to be confined to a hypothetical tube which is essentially the sequence of pores of the entanglement network in which the polymer is embedded. The movements along the tube allowed by the theory is represent by green arrows.

Hyals activity might be part of a finely tuned system which includes HA synthesis and degradation [168].

It was previously discussed that HA is normally present in healthy tissue in high molecular weight. However, in some specific pathological conditions such inflammation or Reactive oxygen species (ROS) production [46,47] the amount of HA fragments with different low molecular weight increase showing different biological activities [41].

The ROS can degrade HA chain generating biological active fragments. Usually ROS are present in the injured tissue, in inflamed areas and in tumor microenvironment. They may provide a mechanism for generating HA fragments in vivo and may further exaggerate the inflammatory state.

The accumulation of the HA fragments should be carefully avoided as they could trigger an inflammatory response. This is the reason why the removal of digested fragments from ECM throughout the internalization after their interactions with receptors as CD44 is a critical step for the inflammation development. In fact, the HA fragments can be recognized by specific receptors that could influence the cell behaviour also depending on their size [45]. From this point of view the HA oligos could be classified as matrikines [169] as they have all characteristics of the active fragments of ECM molecules. If not rapidly internalized by the cells, the oligosaccharides produced by hyaluronidase can diffuse through tissues and bind to HA receptors on adjacent cells, activating intracellular signals such as NF-kB and Erk.

5. HA as drug delivery system

HA has roles in several therapies in regenerative medicine and in skin often with cosmetic purposes. In wound healing or in surgery as anti-adherence agent [170], HA shows an effective activity due to its capacity to improve cell growth and angiogenesis.

HA hydrogel can be also used as drug delivery system [171,172]. The chemistry of the component of the polymer chain can be easily chemically modified with adipic hydrazide, tyramide, benzyl ester, glycidyl methacrylate, thiopropionyl hydrazide or bromoacetate, either at carboxylic acid of GlcUA acid or at the C-6 hydroxyl group of the GlcNAc [105].

HA has attracted significant interest in development of drug delivery systems because of its intrinsic physicochemical and biological properties. As previous described HA water solubility, viscoeleasticity, non-immunogenicity, biocompatibility, and biodegradability are key aspect for consider this polymer suitable vehicle for drug delivery [173].

Interestingly HA shows the capacity to move throughout tissues even crossing skin, and this aspect is described in interesting study by Kueschler et al. [174]. The dynamic of polymer in soft tissue is well described by the theory proposed by de Gennes who has defined several predictions how a polymer chain and their individual parts can move introducing the concept of "reptation" [175]. Reptation theory in fact is related to"snake-like" large-scale motion of entangled polymers. From this point of view the diffusive motion of polymer segments can be confined to a hypothetical tube which is essentially the sequence of pores of the entanglement network in which the polymer is embedded. Fig. 5 represents a cartoon related to this polymer dynamic theory. Reptation is therefore a way to explain the possibility of the polymer to move across the space and this can be easily related to the HA biological activity. The HA as polymer is limited in its free movements by the presence of other structures present in the microenvironment or even by other polymer chains. The possible moving is tangentially to the direction of its tube and moves predominantly along the tube. This movements are defined reptation and the motion of a linear polymer chain under entangled conditions has been described by reptation theory [176]. Even though the details of reptation in the polymer dynamics in biological applications is still elusive, the mechanisms involved in the theory including the alternative tube reorganization mechanism and tube dilation are probably the most important aspect in the polymer interaction with microenvironment. It is important to note that reptation of surrounding chains leads to widening of a tube altering the polymer dynamics. The non-Newtonian behavior of HA is therefore based on the reptation and entanglements [177].

HA can interact with specific receptors on the disease-related cells (CD44 for example) such as cancer cells and activated macrophages, followed by receptor-mediated endocytosis. With these unique features, HA has been extensively used for development of the targetable carriers to deliver the therapeutic and imaging agents [178]. Large body of literature reported an extensive use of HA in the controlled-release and targeted drug delivery systems. However, most studies are still only in vitro, and data in vivo experiments are scanty [178]. Various HA-based conjugates for cancer therapy and imaging, in which the active agents are covalently conjugated or physically encapsulated.

As previously discussed, HA has some groups as hydroxyl, carboxyl, and *N*-acetyl suitable for chemical modification. Therefore, hyaluronic acid and derivatives as drug carriers contribute to drug concentration, sustained release, transdermal absorption, and improve drug targeting. The chemical coupling of cytotoxic drug to HA improves the pharmacokinetic profile of drug, prolongs drug distribution, and reducing the time for drug effect [179]. Basically, HA permits to maintain low plasma drug concentration increasing drug in pathologic tissue [180,181]. Hyaluronic acid and its derivatives have been widely used in various drug delivery systems, such as nanoparticle drug delivery system, gel drug delivery system, cationic polymer gene carrier system, nanoemulsion delivery system, polyelectrolyte microcapsule drug delivery system, microsphere drug delivery system, film delivery system, and these various techniques gave a great flexibility [173]. HA hydrogels are methods of gene delivery and have been widely used, especially in tissue engineering as HA plays as system to control gene delivery in tissue regeneration.

One of the first drug delivery system based on HA was the creation of an amphipathic vector hyaluronic acid-Polyethylenimine (PEI) (HAP) for gene delivery by periodic acid oxidation of hyaluronic acid and PEI [182]. This vector protects DNA from nuclease degradation, isolated DNA from the complex, and is less toxic. The high transfection rate of HAP in cancerous HepG2 cells, promoted cell drug uptake more effectively. Another molecule coupled with HA was spermine, to improve the transfection efficiency of encapsulated DNA. Synthetic hyaluronic acid-polylysine (PLL) conjugate is recognized by HARE receptor in the sinusoidal epithelium of liver cells [183,184]. This complex was designed to induce the ε-amino group of hyaluronic acid-terminal PLL to form a comb-type copolymer by reducing the amino group. This copolymer has been used to form complex with DNA after injection into the animal model by intravenous injection. This system showed that copolymer was mainly concentrated in the sinusoidal cells of liver for gene expression. The first gene delivery application of hyaluronic acid was that hyaluronic acid-adipic acid dihydrazide (ADH) hydrogels used to protect DNA from enzyme degradation and for sustained release of DNA [185]. This injectable HA/ADH hydrogel worked as a vessel for protecting preadipocytes during, and at a short-term after delivery to native tissuesin research towards regenerative medicine in tissue reconstructions.Therefore, HA can act as a non-viralvector of gene drugs and could be targeted to tumor cells through CD44 receptor-mediated endocytosis working as antitumor delivering gene drugs [178].

HA was also tested for siRNA delivery. The cross-linking of hyaluronic acid could be formed by disulfide bonds, which can be degraded by glutathione in the cytoplasm [186]. Moreover, to confirm the mechanism of internalization, the efficiency of cell absorption and gene silencing was much higher in the CD44 overexpressed cell lines than in the cell lines with lower CD44 [186,187]. The HAP conjugate was also developed to deliver siRNA through LYVE-1-mediated targeting cells [187].

A recent achievement is the production of complex polyethyleneglycol (PEG) and hyaluronic acid which resulted in a very useful tool for its pharmacokinetics properties. In fact, in physiological condition, PEGylated HA nanoparticles (HA-NPs) formed self-assembled nanoparticles (217–269 nm in diameter) with the negatively charged surfaces [188]. The nanoparticles uptake is due to the CD44 interaction resulting in a high tumor targetability of PEGylated HA-NPs. These data are obtained in vitro, and in vivo modes supported by the intravital tumor imaging. It was observed rapid extravasation into the tumor tissue indicating that PEGylated HA-NPs can be useful as a means for cancer therapy and diagnosis [188]. PEGylation beside some advantages in term of biodistribution and drug release presents some problem related to long term therapies. HA emerged as a good substituted to PAG. In fact, each individual chain of HA can conjugate with several different peptides, making it possible for polypeptide drugs to exert multiple effects [189]. The coupling procedures for HA are very mild using as acceptor the carboxylic group. This support more strongly the use of HA as drug delivery carrier.To prolong the release time of protein drugs, hyaluronic acid hydrogels have been extensively studied producing hybrid hydrogel system showing both simple drug loading and controlled release with no denaturation of the protein drugs [190]. One example of use of HA in protein release by a cross-linked hyaluronic acid hydrogel was the product generated to release erythropoietin (EPO) [191]. Hyaluronic acid-drug conjugates are widely tested as can improve the drug solubility and change the drug distribution and its half-life in vivo. These events can increase the drug concentration in tumor tissue by enhancing the osmotic retention effect augmenting the efficacy of therapy [192,193]. Antitumor complex of HA with paclitaxel was developed to improve the antitumor effect of this drug and in vivo and in vitro tests proved the concept identifying the CD44 role in this mechanism. HA-paclitaxel effectively can inhibit tumor growth in human cancer xenografts via an HA-mediated mechanism [194]. HA was also tested with liposomes [195], by usinghyaluronic acid-modified polylactic acid-glycolic acid copolymer nanoparticles (HCDs). These nanoparticles were prepared to increase drug uptake in breast cancer cells [196]. From these examples HA has several advantages in cancer therapy and in drug release in general, these aspects are related to its good biocompatibility, easily chemically modifiable groups and targeting of tumor cells via CD44. Nevertheless, some problems are still present in the HA application in therapy, for instance the number of molecules linked to the chain has to be carefully controlled as an excess of linking material can modify the HA chain solubility and interaction with HA receptors. Moreover, as HA receptor is largely present in liver, after iv treatment a large portion of the complex can be blocked in the liver. These aspects are limit for a large use of HA as drug delivery system and require an improve tumor active targeting to improve the efficacy of HA as drug delivery system. The preliminary data from clinical trials indicate that the industrialization and wide clinical application of HA as drug delivery system are still to be properly developed.

6. HA in aesthetic medicine

In cosmetics HA polymer is commonly present in the products commercially available, and this is due to HA activity as moisturizing agent. It has been demonstrated that elastic properties of the skin have been improved by the regular application of HA on skin even though the biological effects on keratinocytes are not completely understood at molecular level [49]. Nevertheless, even robust scientific data are still necessary to completely understand the HA role in topic cutaneous application, several sunscreen products containing HA showed important anti free radicals action with a protective activity against ultraviolet irradiation [197]. In plastic surgery HA gels with entanglements or chemical linkages between chains are widely used as filler to treat facial lines and wrinkles [198,199]. The success of this application is due to the greater tolerability of HA filler compared to collagen products [95,200,201]. Even the use of HA as filler is largely diffuse in aesthetic medicine, it has to be considered that HA is not only a filler [198]. The filler technology addressed mainly the problem related to the HA integrity and the viscoelastic properties of the hydrogels used. In the market there are several formulations of HA filler that have chemical bonds between chains (cross-linkers) or natural entanglements between HA chains. The concentration of HA, the number of HA bonds and the size of the polymer chains influence the hydrogel viscoelastic properties and gave to the producers the possibility to develop dozens of different fillers. Duration, filler effect, the use in syringe and the skin rejuvenation are all aspects that characterize different filler products. The advantage of HA filler is it can be easily modulated considering the skin area and the effect target to achieve and the possibility to digest rapidly the HA filler by hyaluronidase if the product generates adverse reactions (inflammation, granuloma).

The industrial production of HA changed dramatically in the last years, from the beginning, when the polymer was extracted from

animal tissues (rooster combs) now the introduction of bioreactors with modified bacteria allows the production of highly pure HA without contaminants. Therefore, if in the past some contaminants were present in the extracts, including nucleic acid, now bacteria can secrete high molecular weight HA directly in the culture broth, without any risk of bacterial contamination. This high-quality production is for medical applications as injections in visco-supplementation or derma injections.

Eventually, HA is also present in nutraceutical market, as beverages, food and confectioneries which have been approved as health food material worldwide.

The synthetic production of HA in vitro is still unavailable as now synthase structure have been never described in detail as they have not been crystallized. Nevertheless, some information is obtained from studies on cellulose synthase, an enzyme very similar to the HA synthase. In future it might be possible a complete synthetic synthesis of HA polymer throughout the engineered enzymes which will be able to produce high quality polymer with specific size and biological properties.

7. Concluding remarks

From the increasing knowledge of HA roles emerges that this polymer represents a key molecule in ECM from the beginning of embryo development to the advanced age. The aging of mammals is characterized by a marked reduction of HA content in tissues.In other acuteand chronic pathologies HA role emerged as critical aspect in disease outcome. Randomized controlled trials have successfully proved the remarkable properties of HA and currently are in progress trials in various therapeutic strategy including healing burns, in surgery and in chronic wounds healing [202]. In diabetes, in chronic inflammatory diseases, in pulmonary and kidney fibrosis HA represents a potential therapeutic target [135,203–205]. The HA interactome and viscoelastic properties indicate that HA in the next future will be more than an ECM molecule. HA synergistic activity with bioactive molecules and drugs are close to be ready for clinical applications even if few technical questions should be addressed. From this point of view the HA hydrogels will be a common tool in engineered tissues and in regenerative medicine.

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