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Biotechnological Production and Application of Hyaluronan

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1. Introduction

Glycosaminoglycans (GAGs) are linear polysaccharides formed from repetitions of a disaccharide unit composed of one aminosugar and one uronic acid residue. Among these, hyaluronic acid (HA) ([D-glucuronic acid (1-β-3) N-acetyl-D-glucosamine (1-β-4)]$_n$) (figure 1), that differs from the other for not presenting sulphate groups, is a biopolymer of broad scientific interest and largely applied in different biomedical fields.
This macromolecule is most frequently referred to as hyaluronan, because of the many different forms the molecule can assume in physiological conditions (i.e. the acid form, HA, and the salts, such as sodium hyaluronate) (Balazs & Gibbs, 1970).

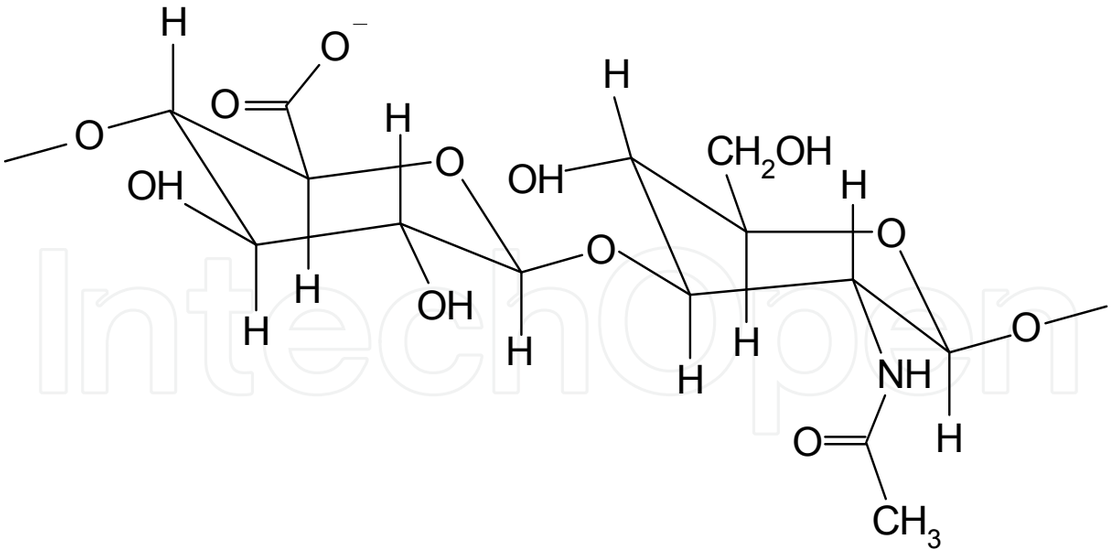

Fig. 1. Disaccharide repeating unit ([D-glucuronic acid (1-β-3) N-acetyl-D-glucosamine (1-β-4)]$_n$) of hyaluronic acid.

This biopolymer is widespread in nature, having been identified in vertebrate soft tissues (e.g. joints, synovial fluid, skin, vitreous humour of the eye, umbilical cords, roster combs) (Balazs et al., 1993), in algae (De Angelis, 1999), in molluscs (Volpi & Maccari, 2003), and

also in cultured eukaryotic cell lines, and certain prokaryotes, where it occurs as a mucoid capsule surrounding the cell (O'Regan et al., 1994). HA present in all vertebrates is a main component of the extracellular matrix: it is the major constituent in the vitreous of human eye (0.1 mg/mL wet weight), and in the synovial joint fluid (3-4 mg/mL wet weight). However, the largest amount of HA (7-8 g of hyaluronate per average adult human, or approximately 50% of the total in the body) resides in the skin, where it is present in both the dermis and the epidermis (0.5 mg/g wet tissue). The embryo is covered by a thick HA coating during certain stages of development, it is also abundant in the umbilical cord (4 mg/mL) (Toole, 1997; Marcellin et al., 2009). Interestingly rooster combs, a specialized piece of skin, contain even higher amounts of HA (up to 7.5 mg/mL), and in fact they are a preferred source for HA industrial extraction.
In vertebrates, HA has a wide variety of functions: in the skin it maintains tissue hydration (Bettelheim & Popdimirova, 1992); in the cartilage it fastens proteoglycans to regulate water and ion content, sustaining tissue physical properties and cell–substrate interactions. The biological effects associated with HA–receptor binding, furthermore, induce rate changes in cell proliferation, cell migration, and angiogenesis (Goldberg & Toole, 1987; Alho & Underhill, 1989). Moreover, overproduction of HA is observed in diseases associated with inflammation, fibroses and cancer. Recently, direct evidences demonstrate the involvement of HA in cancer metastasis (Stern, 2005; Heldin, 2003).
In the present chapter, production processes, chemico-physical properties, and established and foreseen applications of hyaluronan and derivatives will be analysed and critically presented.

2. Hyaluronan production

Currently there are two competing methods for industrial HA production that are extraction from animal sources, such as bovine eyes and rooster combs, and microbial production through the use of large scale fermenters. Both will be discussed in the following sections, in addition the opportunity of using novel genetically engineered microbial factories and a chemo-enzymatic synthesis approach will be reported from the very recent literature.

2.1 Traditional extraction processes

The traditional method for HA production is based on solvent extraction from animal tissue extracts, eventually using cetylpiridinium chloride (CPC) precipitation.
One of the first paper presented by Swann (1968), reported the following procedure: (1) mechanical slicing of the rooster combs to obtain small pieces, (2) washing with ethanol (4 L ethanol to 1 Kg comb), this operation could be repeated until the solvent would not appear cloudy; (3) extracting the minced combs with a water/chloroform mixture (2.5 Kg combs: 10 L water: 0.5 L chloroform), while stirring to allow combs to swell; (4) filtering the solids from the broth and adding NaCl, successively carrying an additional chloroform extractions; (5) accomplishing protease (pronase) digestion, followed by chloroform extraction and centrifugation.
In alternative methods (Swann, 1968.) the crude extracts were purified by epichlorohydrin triethanolamine- (ECTEOLA-) chromatography and by fractionation with CPC. In addition, repeated ethanol precipitation (1:3 water/ethanol ratio), before and after CPC (1%) HA precipitation, were reported (Prescott, 2003). In all the cases the product is then filtered through sterilizing filters, followed by solvent precipitation, finally the HA is formulated

into medical devices and pharmaceutical products. HA purified by these procedures was recovered with a yield greater than 90% with respect to the uronic acid evaluated in the starting material.
However the collection of rooster combs and the extraction and purification procedures of HA from these tissues are time-consuming and labour intensive, making hyaluronan production very costly (O' Regan et al., 1994). In fact in animal tissues hyaluronan is complexed with proteoglycans and often contaminated with HA degrading enzymes, making the isolation of high purity and high molecular sized polysaccharide very difficult. Moreover the use of animal-derived biomolecules for biopharmaceutical applications is facing growing opposition because of the risk of cross-species viral and other adventitious agent contaminations. Hence, since '80 microbial production is gradually replacing extraction from animal tissues in HA industrial manufacturing.

2.2 Biotechnological production of HA

Bacteria known to be capable of the synthesis of HA are Streptococci of groups A and C, gram-positive bacteria such as *Streptococcus equi*, an equine pathogen, *Streptococcus equisimilis*, that is infective for different animals, *Streptococcus pyogenes*, a human pathogen and *Streptococcus uberis*, a bovine pathogen. These β-hemolytic bacteria, able to digest blood based agar medium, also present a slimy translucent layer surrounding bacterial colonies that can be attributed to HA synthesis (figure 2).

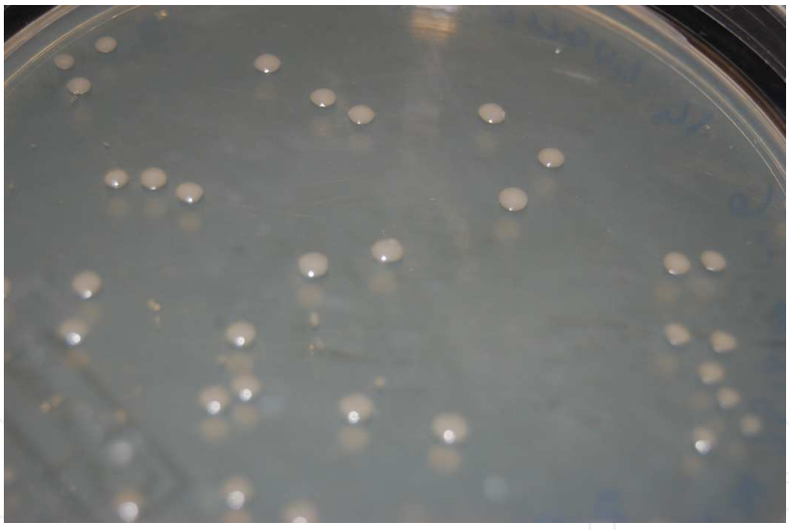

Fig. 2. Slimy colonies of *Streptococcus zooepidemicus* HA producing cells.

A gram-negative pathogenic bacteria, *Pasteurella multocida* was also found to produce HA in its capsule (De Angelis et al., 1998). The HA capsule is a virulence factor in both Streptococcus and Pasteurella, probably providing bacteria a stealth function that result in the failure of the immune system to recognise the HA capsule as a foreign entity (Schmidt et al., 1996). The capsule may also protect the bacteria against reactive oxides released by leukocytes, attempting to prevent infection. Finally, it helps the migration through epithelial layers, exploiting CD44 mediated tissue response (Cywes & Wessels, 2001). Thus the HA capsule contributes in large part to the pathogenicity of these microorganisms.

Both *Streptococcus zooepidemicus* and *Pasteurella multocida* produce HA starting from activated substrates (nucleotidic sugars) through specific membrane bound glycosyltransferases, so-called HA synthases (HASs). The latter have been exploited in few recent studies for the chemoenzymatic synthesis of HA, also reporting the possibility to obtain biopolymers of defined molecular weight using *P. multocida* HAS (De Angelis et al., 2003). Nevertheless the established industrial production process today is based on fermentation of mutagenized streptococcal cells.

It has been estimated that hyaluronan synthesis in bacterial fermentation accounts for 5–10% of the carbon metabolised. The D-glucuronic acid and the N-acetyl-glucosamine moieties of HA are derived from glucose-6-phosfate and fructose-6-phosfate, respectively, as demonstrated for *S. zooepidemicus* through ^{13}C NMR studies (Matsubara et al., 1991). The proposed biosynthetic pathway for HA was well described in the recent literature and for readers convenience is schematically depicted in figure 3.

Overall, the synthesis of one mole of HA disaccharide consumes five moles of nucleosides triphosphates (3 as ATP and 2 as UTP), two moles of glucose and one mole of acetyl coenzyme A (Acetyl-CoA) and generates two moles of reducing equivalents (NADH) and, therefore, it is expected that the flux through the HA pathway is intimately related to the cellular needs of other pathways, (i.e. glycolysis and cell growth); it is also expected to be

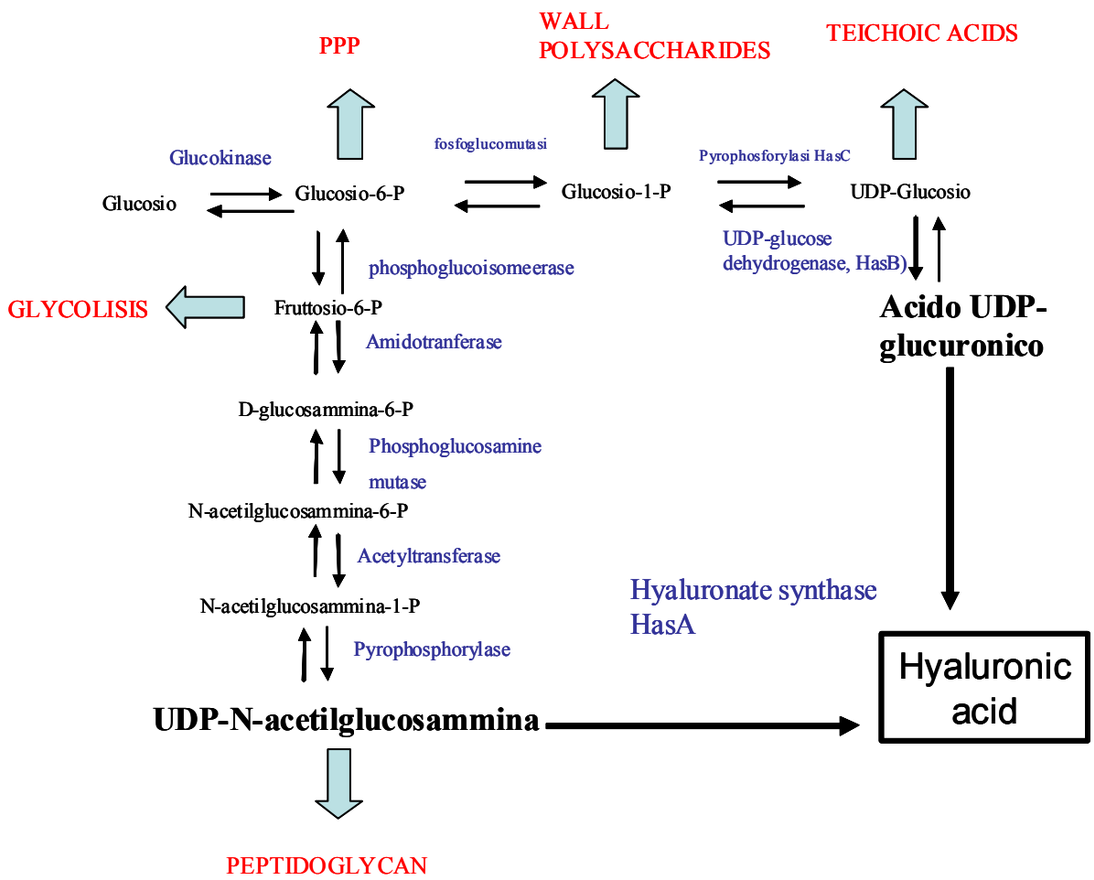

Fig. 3. Biosynthetic pathway responsible for hyaluronic acid production in streptococci: as shown few intermediates are also required for cell wall synthesis.

dependent on the energetic state of the cell. In fact, ATP levels and reducing equivalents in the cell play a key role in biosynthesis, being these substances consumed and generated in the production of hyaluronate (Chong & Nielsen, 2003). Besides furnishing precursors for HA synthesis, the two pathways showed in figure 3 also supply the structural constituents of the bacterial cell wall, specifically peptidoglycan, teichoic acids and antigenic wall polysaccharides: these three major wall components accounts for 20% (w/w) of the cell dry weight and represent a significant drain on the precursors pool used to synthesize HA.

2.2.1 Enzymes involved in HA synthesis

HA is polymerised on the cytoplasmatic side of the plasma membrane as a free linear polymer, differently from the other glycosaminoglycans which are synthesized by resident Golgi enzymes and covalently attached to core proteins. HAS (Has A) is the only protein required for HA synthesis and it functions as a monomer stabilised by phospholipids (De Angelis & Weigel, 1994; Kumari & Weigel, 1997). After the discovery in 1993 of the first gene encoding a hyaluronan synthase from Group A *Streptococcus*, many others similar hyaluronan synthase genes were identified in other bacteria and in a wide range of eukaryotes (De Angelis 1999; Itano & Kimata, 2002). The molecular masses of the streptococcal (49 KDa) or eukaryotic (65 KDa) HASs are relatively small in view of the multiple functions mediated by these enzymes in order to synthesize HA (table 1). HAS binds UDP-glucuronic acid (UDP-GlcUA) and UDP-N-acetylglucosamine (UDP-GlcNAc) in the presence of $MgCl_2$ and catalyzes two distinct intracellular glycosyltransferase reactions. HAS also binds and translocates the growing HA chain through the cell membrane. The pendulum hypothesis was proposed by De Angelis and Weigel (1994) to explain how these functions coordinates to synthesized and transfer a growing HA chain (www.glycoforum.gr.jp/science/hyaluronan). In HA-producing streptococci two unique genes encoding for HA-synthase (*has* A) and UDP-glucose dehydrogenase (*has* B) were found on the so-called *has* operon. The latter was firstly sequenced in *S. pyogenes* presenting also a third gene, *has* C, encoding for the UDP-glucose phosphorylase (Crater & van De rijn, 1995). More recently, Blank and co-workers (2008) found other two genes on the operon of *S. zooepidemicus*, GlmU and pgi, the former is responsible for the two final steps of UDP N-acetyl glucosamine biosynthesis, while the latter is an additional phosphoglucoisomerase involved in the Embden-Mayerhof Parnas pathway.

Multiple functions of Class I HA synthases
Addition of GlcNAc to the growing HA-GLcUA-UDP chain (left)
UDP-GlcNAc acceptor binding
HA-GlcUA-UDP donor binding
HA-GlcUA-UDP: UDP-GlcNAc, β-1-3 (HA-GlcUA) transferase
HA traslocation through the membrane
Addition of GlcUA to the growing HA-GlcNAc-UDP chain (right)
UDP-GlcUA acceptor binding
HA-GlcNAc-UDP donor binding
HA-GlcNAc-UDP: UDP-GlcUA, β-1-4 (HA-GlcNAc) transferase
HA traslocation through the membrane

Table 1. Multiple functions of Class I HA synthases.

Based on differences in protein structure and mechanism of action, the known HASs have been classified into two classes (De Angelis, 1999). Class I members include HASs from *Streptococcus*, mammals, and other eukaryotes, whereas the bacterial HAS from *Pasteurella multocida* is the only class II member (table 2). The major mechanistic difference is that the two classes of synthases extend hyaluronan at opposite ends of the polysaccharide. The Class II pmHAS has a two-domain modular structure, with two transferase activities, that alternatively bind and release hyaluronan chains to add new sugars to the non-reducing end by typical glycosyltransferase activity. The Class I enzymes are the first glycosyltransferases that has been unanimously demonstrated to function at the reducing end of a growing glycosaminoglycan chain (Tlapak-Simmons et al., 2005).

	Class I	**Class II**
Members	HASs of *Streptococcus* ssp., mammalian, avian and amphibian	HAS of *Pasteurella multocida*
Size (amino acids)	417-588	972
Membrane attachment domain	6-8 membrane-associated domains	C-terminal membrane anchor
HA chain growth	At reducing end	At non reducing end
Primer oligosaccharide	No evidence for extension	HA extension

Table 2. Classes of hyaluronan synthases.

The hyaluronan polymerization rates for the streptococcal hyaluronan synthases in isolated membranes were estimated to be ~1200-2400 sugars/min: at this elongation rate one active hyaluronan synthase molecule would take about 8-16 minutes to synthesize a single hyaluronan chain with a mass of 2 MDa. The rate of hyaluronan chain elongation in live cells has not been determined, but is likely to be faster than what has been measured *in vitro* as the elongation rate increases with substrate concentration until when too high concentration determines the release of the HA chain from the cell. Very little is known about the enzyme properties that control hyaluronan chain length and how different hyaluronan synthases make hyaluronan products of different size distributions. However, it has been demonstrated that specific hyaluronan synthase mutations can create variants that produce HA of altered size.

2.2.2 Streptococcal fermentation

Streptococci are non-sporulating and non-motile bacteria that at the optical microscopy appear as small spherical or ovoid cells that usually grow as pairs or chains surrounded by an extensive extracellular capsule: typically, the hyaluronan capsule is one to three times the diameter of the cell body (figure 2).

HA has been produced commercially since the early 1980s through fermentation of group C streptococci, in particular *Streptococcus equi* subs. *equi* and subs. *zooepidemicus* (Yamada and Kawasaki, 2005). Given the high viscosity of HA solutions, it is not practical to ferment HA beyond 5–7 g/L of product: usually the yield of polysaccharide on consumed carbon source is around 0.05-0.1 g/g and the molecular weight of the polysaccharides is averagely 1-2 MDa, being the maximum molar mass reported up to date 4 MDa (Rangaswamy and Jain, 2008). In table 3 the most important fermentation processes described in literature articles are briefly depicted.

Microorganism	Fermentation mode	Main nutrients	Oxygenation parameters	Biomass and HA yield HA molecular weight	References
S. equi subsp *zooepidemicus* (ATCC 35246)	Batch 2L	Mussel processing wastewater 50 g/L and tuna peptone 8 g/L	500 rpm 0 vvm	X: 3.67 g/L; [HA]: 2.46 g/L MW: 2.5MDa	Vazquez et al., 2010
S. equi subsp *zooepidemicus* (ATCC 35246)	Batch 2.5 L	Maltose 20 g/L, CDM	10 Hz 1.3 vvm	X: 2 g/L; [HA]: 2.14 g/L M_W: 2.1 MDa	Chong & Nielsen., 2003
S. equi subsp *zooepidemicus* (ATCC 35246)	Batch 2L	Glucose 60 g/L, CDM	600 rpm 1 vvm	X: 3.5 g/L; [HA]: 4.2 g/L MW: 3.2	Armstrong & Johns, 1997
Streptococcus sp. ID9102 (KCTC 1139BP)	Batch 75L	4% glucose, 0.75% YE, 1% casein peptone, Gln+Glu+ oxalic acid	400rpm 0.5 vvm	X: 3 OD_{600}; [HA]: 6.94 g/L MW: 5.9 MDa	Im et al, 2009
S. zooepidemicus (ATCC 39920)	Batch 3L	Glucose 20 g/L, YE 10 g/L, + acetoin and acetate	300 rpm 1 vvm	X: 2.43 g/L; [HA]: 2.15 g/L MW: n.d.	Wu et al., 2009
S. equi subsp *zooepidemicus* (ATCC 39920)	Batch 10 L	Sucrose 50 g/L, 10 g/L of casein hydrolysate	400 rpm 2 vvm	X: 6.5 g/L; [HA]: 5.1 g/L MW: 3.9 MDa	Rangaswamy & Jain 2008
S. zooepidemicus G1 (mutant of ATCC 39920)	Batch + pulse 5L	40 g/L glucose, 20 of polypeptone, 10 of YE	n.d. 10-80% DO	X: n.d.; [HA]: max 3.5 g/L MW: max 2.19	Duan et al, 2008
S. zooepidemicus WSH 24	Fed-batch 7L	Sucrose 70 g/L, 25 of YE	200 rpm 0.5 vvm	X: 16.3 g/L; [HA]: 6.6 g/L MW: n.d.	Liu et al., 2008
Streptococcus	continuous	Chemically defined medium (CDM)	High dilution rate	25% higher than batch cultures	Blank et al. 2008

Table 3. Overview of the different fermentation conditions reported in literature for HA production in Streptococci fermentations, with specific reference to medium components and aeration strategies. X:Biomass CDM: chemically defined medium; n.d.: not determined or not descripted; NTG: N-methyl-N'-nitro-N-nitrosoguanidin; *phbCAB* genes: polyhydroxybutyrate synthesis genes; YE: yeast extract.

The HA production from streptococci may be influenced from genetic factors and bioprocess parameters. First it must be considered that these microorganisms can produce hyaluronidases (HAase), extracellular enzymes that hydrolyze the external polysaccharide, leading to the decrement of both concentration and molecular weight of the product.
Consolidated strain improvement procedures have been implemented (i.e. chemical mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidin), followed by a serial selection scheme, to obtain colonies lacking HAase and β-haemolytic activity, among those also selecting fast growing and overproducing HA cells (Kim et al., 1996).
Culture conditions affecting hyaluronan production are various, like medium composition, pH and dissolved oxygen concentration and geometry and speed velocity of the stirrer.
Because streptococci have specific nutritional requirements, being auxotrophic for some aminoacids and vitamins, medium formulations typically include yeast or animal extract, or casein hydrolysate as well as divalent metal ion (Mg^{2+} and Mn^{2+}), to permit polysaccharide synthesis. Occasionally calf blood or serum, as growth factors, and sometimes lysozyme,

have been added to the medium to stimulate HA production (Chong at al., 2005). However, increasing restriction of regulatory agency in Europe (EMEA) and United States (FDA), and the specific concern strictly related to every compound coming from animal sources nowadays prevent exploitation of such components in production processes. A few chemically defined media have been formulated for microbial metabolism studies but because of low yield they proved not suitable for industrial use (Chong at al., 2005).

The carbohydrate (glucose or sucrose) concentration during the fermentation process is proportionally correlated to intracellular substrates levels of HAS, whose enzymatic activity and conversion rate depends on cytoplasmatic activated sugar levels. Therefore, differently from reported fermentation strategies (to avoid growth inhibition due to substrate accumulation and overflow metabolism), sugar should be maintained high during HA production process. It has been proposed that HAS activity mechanism consists in a single protein synthesizing a single HA chain during its lifespan.

In traditional batch processes, that are widely applied for HA production, optimal sugar concentration at the inoculum is about 60-70g/L; batch fermentation procedures implemented by pulsed carbohydrate concentrated feed, have been reported successful in increasing yield, two spike feed additions (from 20 to 50 g/L of sugar) after 8-10 hour of fermentation are generally used. Continuous fermentation strategies have been exploited (table 3) only at lab scale but they are not yet industrially applied, probably due to the instability of HA-producing phenotype of streptococcal strains.

Cooney et al. (1999) aimed to increase the ATP yield of catabolism, unfortunately, by using a glucose limitation strategy the average molecular weight of HA produced resulted lower presumably due to inadequate supply of UDP-sugars to the HA synthase during its half-life. However it may be feasible to obtain a higher ATP yield using a different sugar source such as maltose, that is slowly metabolized by streptococcal cells. In fact, Chong and Nielsen (2003) succeeded, in batch fermentation, to increase the yield of HA produced on carbon source consumed from 0.088 g/g using glucose to 0.1 g/g employing maltose as carbon source. At a molecular level analysis, it was found an up-regulation of cytosolic NADH oxidases (NOX) gene.

Aeration is another key parameter during hyaluronate production process. The biopolymer can be produced in both anaerobic and aerobic fermentation, however the latter favours a higher yield and molecular weight of hyaluronan (table 3); in particular Armstrong and Johns (1997) observed a 20% increase in HA yield when *S. zooepidemicus* was grown under aerobic conditions. First attempts of producing HA using streptococci include anaerobic fermentations but the product had a low molecular weight (e.g BrackeJW & Thacker K, 1985; Park et al., 1996). Successively aerobic conditions (0.5-2 volume of gas per volume of culture per minute (vvm)) proved to favour HA production; this phenomenon can be explained by the following: (1) oxygen may stimulate HA synthesis as the aggregation of streptococcal cells mediated by their HA capsule shielded them from oxygen metabolites (Cleary & Larkin, 1979; Chong & Nielsen., 2003); (2) in presence of oxygen the energetic yield on glucose increase, due to the presence of the NOX that catalyzes the following reaction: 1 O_2+ 2 NADH→ 1 H_2O+ 2 NAD^+, contributing in such way to the energetic flux of bacterial metabolism; (3) dissolved oxygen in the medium can redirect part of carbon flux to acetate production in place of lactic acid ($Y_{ATP/glucose}$ is 3 mol/mol with acetate production against 2 mol/mol for homolactic metabolism). The extra ATP concurrently generated during the formation of acetate by acetate kinase facilitates the attainment of the higher growth yields and also the increase of hyaluronan titer.

The effect of stirring is unclear: the need for "vigorous" mixing is described, probably to enhance oxygen transfer, yet the polymer chain is reported susceptible to mechanical stress (Chong et al., 2005).

2.2.3 Genetics tools to improve HA production

Metabolic engineering of streptococci has been improved immensely over the last decade, thanks to genomic sequence now available for a number of relevant streptococci, including *S. pyogenes* and *S. equi* (Yamada & Kawasaki, 2005). Nevertheless there are few examples reported in literature of recombinant DNA techniques resulting in strain improvement towards better HA production, probably because of the number of genes involved in HA production pathway and its regulation.

Chong and Nielsen (2003) tried to maximize HA production overexpressing the endogenous *nox* gene in a *S. zooepidemicus* strain: in shaking flask experiments lactic acid and ethanol production decreased to advantage the catabolic pathway towards acetate, with the consequent increase of ATP yield; however no increase on HA yield was observed.

Krahulec and Krahulcova (2006) succeeded to increase final sodium hyaluronate concentration in the medium of about 29% using streptococci where β-glucuronidase gene was deleted; again this result was obtained in laboratory scale experiments.

Hyaluronan production in heterologous host may be an alternative way to overcome issues associated with streptococcal HA production.

De Angelis et al. (1998) were able to express *hasA* gene of *P. multocida* in an *E. coli* strain and confer to the host the capability to produce hyaluronan capsule *in vivo*.

A new system for HA synthesis was reported (Yamada & Kawasaki, 2005): *Chlorovirus* (virus of single-celled green algae, *Chlorella*) PBCV-1 was found to produce fibrous material on the cell wall of the host, that was shown to be HA. Experimentally, approximately 0.5-1 g/L of hyaluronan was recovered from a culture of *Chlorella* cells infected with *Chlorovirus*.

Recently, HA produced by using a genetically modified *Bacillus subtilis* strain has been developed by Novozymes (Widner et al., 2005). The advantages of employing *B. subtilis* to produce HA are various: first of all this bacterium is a generally recognized as a safe strain and the produced HA is free of exotoxins and endotoxins; moreover it is easy to grow in industrial fermenters; furthermore its genome has been sequenced and genetic modifications can be easily achieved; besides as HA producing streptococci, *B. subtilis* is a gram-positive microorganism that has the potential to biosynthesize HA, possessing all enzymatic activity necessary except HAS.

In particular, Widner and co-workers (2005) overexpressed in a *Bacillus subtilis* strain the *hasA* gene from *Streptococcus equisimilis*, which encodes the enzyme hyaluronan synthase along with the endogenous *tuaD* gene encodes for UDP-Glc dehydrogenase resulting in the production of HA in the 1 MDa range in 3L fermentation experiments.

Successively, also Chien and Lee (2007) succeeded in producing hyaluronan from a *B. subtlis* strain. The recombinant *B. subtilis* strain developed contained *VHb* (*Vitreoscilla* haemoglobin) gene, *S. zooepidemicus hasA*, and endogenous *tauD* genes in the expression cassette, by cultivation of these recombinant strains in 250 mL shaked flasks (30 h) they obtained about 1.8 g/L of HA.

2.3 Recovery and purification of HA from fermentation broth

In all the fermentation processes reported HA is released in the medium during fermentation mostly in the late deceleration-stationary phase of the growth curve.

Purification is then obtained directly from fermentation broth after cell removal. The separation of streptococcal cells is quite tricky. It has to be considered that in high yield fermentation medium viscosity (dynamic viscosity) increase overtime reaching 2000-3000 Pa · s. This creates a very strong buoyance force that preclude successful centrifugation unless using diluted broth (i.e.5/10 fold). Recently we studied the influence of earth aided filtration on biomass separation and HA recovery from fermentation broth, also evaluating the effect on average molecular weight of the biopolymer during these first step of downstream processing (Schiraldi et al., 2009). However to accomplish separation and recovery, repeated precipitation, ultrafiltration, CPC precipitation have been reported so far. In all cases specific attention on endotoxin removal should be carefully planned when a pharmaceutical grade product is needed.

For instance an efficient process was recently reported by Rangaswamy and Jain (2008). In this paper the fermentation broth of *Streptococcus zooepidemicus* cultivated in a 10 L reactor, was treated following a novel downstream process. Cell removal was obtained after dilution in pyrogen free water (1:1, v/v), with high speed centrifugation (17686 *g*), the supernatant was then precipitated with 2-propanol, resuspended in 3% w/v sodium acetate, and treated on silica gel and carbon prior to diafiltration and microfiltration. This process is schematically represented in the flowchart in figure 4, and permitted to recovered HA with specification meeting the Europenan Pharmacopaeia standards (2003) with a satisfying yield of 65%.

In recent years, studies aimed at accomplishing accurate and complete characterization of hyaluronan chains have remarkably intensified. In fact, because of the well-established dependence of HA biological activity on its molecular weight, basic research is interested in well-characterized HA fragments (covering a wide range of chain lengths and with low polydispersity) that could be used in experimental models to unravel the correlation. SEC systems coupled with a multi-angle light scattering detector and a refractometer (SEC-MALS-RI) are commonly used for the analysis of hyaluronan and, generally, of biopolymers for which molecular weight standards are difficult to obtain (Jing et al, 2006). Likewise a complete characterization of HA fragments generated during enzymatic hydrolysis was obtained by our group using a Viscotek instrument equipped with triple detector (La Gatta et al., 2010).

Hyaluronan obtained by both animal cell extraction and biotechnological processes is at the basis of many applications that will be presented in the following paragraph.

3. Hyaluronan applications

3.1 The properties of HA exploited in the biomedical applications.

HA finds a broad range of biomedical applications due to a unique combination of properties such as (1) high hygroscopicity; (2) viscoelastic nature; (3) magnificent biocompatibility; (4) non immunogenicity; (5) capacity to degrade in safe products.

1. The great capacity of the polymer to retain water is related to its hydrophilic chemical nature. Due to the presence of carboxylic groups on the chains, it behaves as a polyelectrolyte at physiological pH (HA pK_a = 2.9); in the presence of water, HA molecules can expand in volume up to 1000 times and form loose hydrated matrices. (Lapcik & Lapcik, 1998; Brown & Jones, 2005).
2. The viscoelastic nature refers to the rheological behaviour of HA aqueous solutions that exhibit the elasticity of a gel combined with the viscosity of a fluid. Undergoing

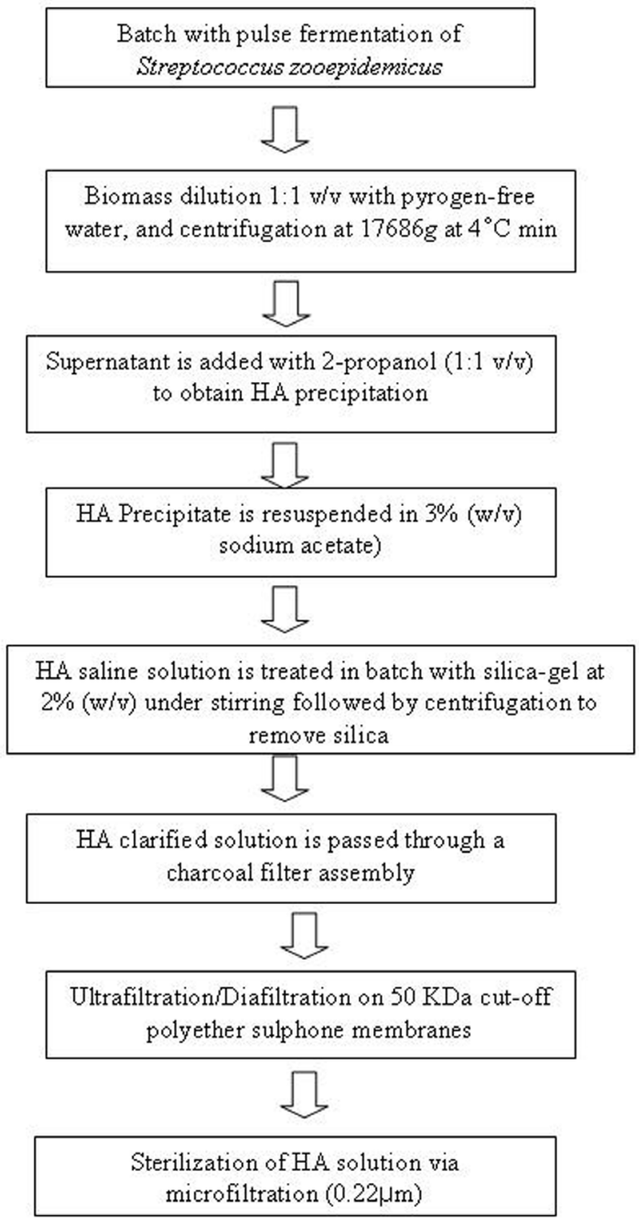

Fig. 4. Overview of hyaluronic acid biotechnological production process from *Streptococcus zooepidemicus* fermentation to recently proposed downstream procedure as described by Rangaswamy and Jain (2005).

rheological measurements, HA solutions behave as pseudo-plastic materials decreasing their viscosity at the increasing of the shear rate. Many of the HA medical uses are based on this shear thinning behavior. Rheological properties are functions of the polymer molecular weight (i.e. chain length), concentration and environmental conditions, like pH (Lapcik & Lapcik, 1998).

3. Being a natural component of many human tissues, HA is highly biocompatible, a property that is essential for the application in biomedicine.

4. HA molecules present the same structure in all species and all tissues and therefore they never "alert" the immune system (Matarasso, 2004).
5. HA is *in vivo* mainly degraded through hydrolysis catalyzed by hyaluronidases giving safe products. It has been estimated that the half-life of HA in the skin is about 24h, in the eye 24-36h, in the cartilage 1-3 weeks and 70 days in the vitreous humor (Laurent & Reed, 1991; Stern et al., 2007; Murray et al., 2005).

Because of the above highlighted properties, the development and commercialization of HA based products are in continuous intensification (Widner et al., 2005). HA is principally used in the treatment of osteoarthritis, in cosmetics, in ophthalmology, in aesthetic medicine, in surgery and wound healing, in topical drug delivery, and in tissue engineering. (Brown & Jones, 2005; Girish & Kemparaju, 2007)

3.2 HA in commercial formulations: linear, derivatized and crosslinked forms.

In some of the aforementioned fields of application, HA is used in its natural occurring linear form. However, for many purposes, it requires chemical modifications. In particular, it is usually subjected to derivatization processes (modification of the linear chain) or crosslinking processes (formation of covalent bonds between HA chains resulting in three-dimensional HA networks).

Delivered modifications allow to overcome the high rate of HA *in vivo turn over* that is required in specific applications. For instance, if linear HA is used for intra-dermal injections, it would be too rapidly degraded to provide its advantageous effects over a significant period of time. Modified HA, on the contrary, being less susceptible to chemical and enzymatic hydrolysis, shows a prolonged *in vivo* persistence thus performing better (Brown and Jones, 2005). Modification processes, especially crosslinking ones, also enhance specific mechanical properties of the material (Brown & Jones, 2005).

A schematic representation of linear, derivatized and crosslinked HA is shown in Figure 5.

In several commercially available formulations, HA (linear or chemically modified) is also found in combination with other polymers (chondroitin sulphate, carboxy methyl cellulose etc.).

HA chemical modifications are generally performed involving the hydroxyl or the carboxyl groups of the polymer.

Strategies for HA derivatization include esterification and sulphation processes. Sulphation is performed at the hydroxyl groups of the HA chains, giving products that exhibit an heparin-like activity correlated to the sulphation degree (Magnani et al., 1996). Esterification processes involve the carboxylate moieties of the polymer, that are converted in ester groups, thus causing a decrease in the total polymer charge contemporary increasing hydrophobicity (Vindigni et al., 2009). As a consequence, polymer solubility in water is reduced depending on the degree of modification thus making HA more stable in physiological environment. Among the derivatized HA based products, benzyl esters of HA are the most diffuse on the market.

In the last decade many strategies have been developed for the production of crosslinked HA, some of them are commonly employed in marketed formulations. These strategies include bis-carbodiimide crosslinking (Sadozai et al., 2005), polyvalent hydrazide crosslinking mediated by carbodiimide (i.e. EDC: 1-ethyl-(3,3-dimethylaminopropyl)carbodiimide) and co-activators (i.e. N-hydroxysulfosucinimide -sulfo-NHS- or 1-hydroxybenzotriazole –HOBt-) (Bulpitt & Aeschlimann, 1999; Prestwich et

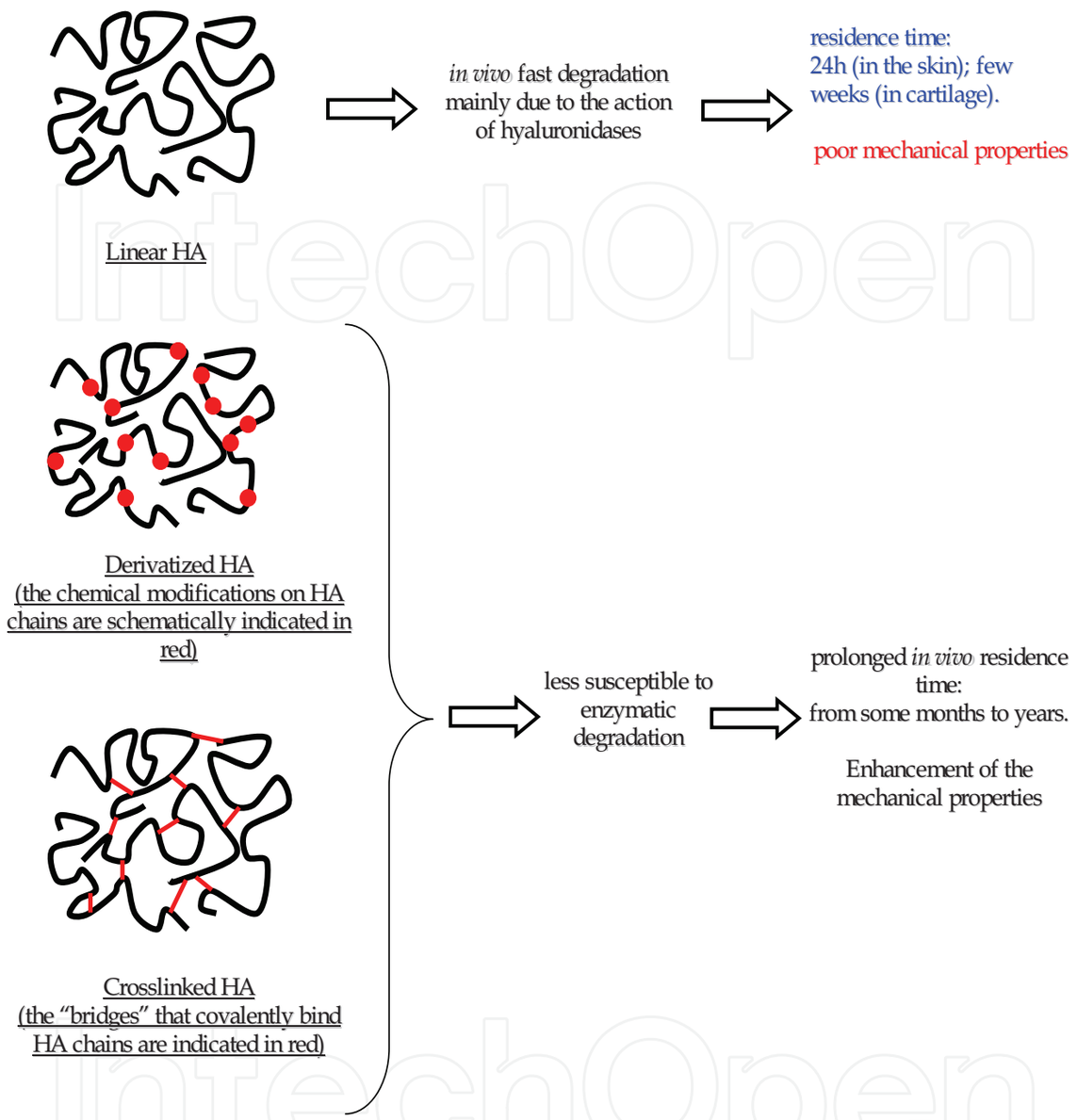

Fig. 5. Cartoon of the HA forms used in commercial formulations: linear, derivatized and crosslinked. Some applications require the use of chemically modified HA in order to enhance HA stability and tune the material mechanical properties.

al., 1998), disulfide crosslinking (Shu et al., 2003), auto-crosslinking mediated by carbodiimide and a co-activator or by 2-chloro-1-methylpyridinium iodide (CMPI) (Radice et al., 2002; Young et al., 2004), photocrosslinking (Park et al., 2003; Leach et al., 2003). All aforementioned processes involve the carboxyl groups of the HA chains. Other strategies involving the HA hydroxyl groups include divinyl sulfone crosslinking (Larsen et al., 1993, Ibrahim et al., 2010) and di-epoxide crosslinking (Agerup, 1998; Segura et al., 2005). The reported crosslinking processes are schematized in table 4.

HA group involved	Crosslinking agent	Product	Reference
Carboxyl	bis-carbodiimide	HA crosslinked via N-glucuronil urea or O-glucuronil isourea groups.	Sadozai et al., 2005
	polyvalent hydrazide coupled with carbodiimide and co-activator	HA crosslinked via hydrazide bonds	Bulpitt et al., 1999; Prestwich et al., 1998
	Carbodiimide + co-activators (sulfo NHS/HOBt) or CMPI	HA auto-crosslinked via ester bonds	Radice et al., 2002; Young et al., 2004
	Ditiobishydrazide coupled with carbodiimide	HA disulfide-crosslinked via air oxidation	Shu et al., 2003
	Metacrylating agent coupled with carbodiimide	HA photocrosslinked after exposition to light	Leach et al., 2003; Park et al., 2003
Hydroxyl	di-epoxide	HA crosslinked via ether bonds	Agerup, 1998; Segura et al., 2005
	divinyl sulfone	HA crosslinked via ether bonds	Balazs et al., 1986 Larsen et al., 1993, Ibrahim et al., 2010

Table 4. The table resumes the main strategies for HA crosslinking presented to date in patent reports and scientific literature

3.3 Linear HA applications.

Linear HA finds application mainly in cosmetics, in ophthalmology and in wound healing.
In **cosmetics** it is used as a moisturising component due to its hydrophilic nature. Almost all the main cosmetic brands present a line of hyaluronan based creams.
Wrinkles appear due to the depletion of HA in the skin with aging. The use of beauty products like creams containing HA helps to hydrate the skin and restore elasticity thus reducing the wrinkles depth. In fact, when HA solutions are applied on skin surface, they are supposed to form a light coating which absorbs moisture from the air thus hydrating the skin (and filling the wrinkles). Additionally, HA is supposed to stimulate epidermal cells migration. Besides, this HA coating should allow biologically active substances contained in the cosmetics to persist on site and eventually to penetrate more easily into epidermis. Cosmetic HA formulations proved capable to protect human skin from UV irradiation (Trommer et al., 2003).
Linear HA received significant attention also in the **topical delivery of drugs**. *Solaraze* (Pharma Derm, US), for example, is a formulation consisting of 3% w/w diclofenac in 2.5% w/w HA gel. It is indicated for the local treatment of actinic keratoses (Brown et al., 2001; Wolf et al., 2001). In fact, HA proved to significantly enhance the penetration of diclofenac

through the stratum corneum (which normally acts as a barrier to the permeation of molecules into deeper skin layers) and the retention and localization of the drug in the epidermis with respect to the control or other carriers (Brown et al., 2001).

Preparations based on linear HA are used to favour the healing in the general **treatment of skin irritations and injuries**. *Jaloplast Cream* (Fidia, Italy), for instance, is a preparation containing HA as the main component (0.2% w/w sodium hyaluronate) intended for coating of acute and chronic wounds (abrasions, areas of skin grafts, post-surgical incisions, first and second degree burns, metabolic and vascular ulcers and pressure sores). The product permitted faster cicatrisation and recovery of the lesions (Lopex et al., 2005).

Plastic surgery bio-revitalization is a treatment of intradermal delivery of HA intended to counteract and prevent the skin's aging process. It is based on the use of HA to correct and smoothen facial lines and wrinkles. The HA is injected in the skin in small doses to help, restore and preserve its elasticity and healthy appearance. It is applied to frown lines, crow's feet and marionette lines. The result is smoother, more compact and more luminous skin.

In **ophthalmologic surgery**, linear HA physiological solutions are used to protect the delicate eye tissues and to provide space during surgical manipulations (Brown & Jones, 2005; Arshinoff et al., 2002; Neumayer et al., 2008). Viscoelasticity is the main HA feature responsible for this application. When stationary (static), the high viscosity of the HA solution allows to manipulate ophthalmologic tissues and to maintain the surgical space. The low viscosity of the solution at high shear rates permits easy injection and removal by pushing and sucking it through a cannula. The elasticity of the solution protects ocular cells from surgical instruments and implants.

One of the most utilized products belonging to this category is *Healon*, by Abbott Medical Optics Inc. (AMO) (USA). It is a viscoelastic physiological solution of highly purified, high molecular weight fraction of sodium hyaluronate 1% (w/w, pH 7.0-7.5) indicated for use as a surgical aid in cataract extraction, Intra Ocular Lens (IOL) implantation, corneal transplant, glaucoma filtration and retinal attachment surgery (Arshinoff et al., 2002, Oshika et al., 2004).

Viscoat (Intraocular Viscoelastic Injection) by Cilco (USA) is another product indicated as a surgical aid in anterior segment procedures including cataract extraction and IOL implantation (table 5). *Viscoat* has been formulated as a combination of sodium hyaluronate (medium molecular weight fraction), 30mg/mL, and sodium chondroitin sulphate, 40mg/mL, in a physiological buffer because the cornea contains the greatest concentration of chondroitin sulphate, respect to the vitreous and the aqueous humor where HA is prevalent (Rainer et al., 2005).

In **ophthalmology**, linear HA is also used as the active ingredient of many eyewash formulations. *Hyalistil* by Sifi (Italy) is, for instance, a 0.2% w/w hyaluronate solution indicated for the stabilization of the tear film and for the hydration and the lubrication of the cornea. It is useful in increasing the comfort during contact lenses application. Once more HA hygroscopicity and viscoelasticity are the basis for this application. *Blink Contacts* by AMO (USA) are eye drops for contact lenses users containing HA 0.15% w/w indicated for prolonging the comfort of the device.

In **urology,** intravesical instillation of linear HA has been recently used as effective alternative treatment of interstitial cystitis, recurrent urinary tract infections, and hemorrhagic cystitis. In fact HA is a protective barrier of the urothelium. A damaged glycosaminoglycan layer may increase the possibility of bacterial adherence and infection. This damage is proposed to be a causative factor in the development of the pathologies listed above, and hemorrhagic cystitis due to posthematopoietic stem cell transplantation.

Table 5. Summary of major marketed products based on linear, crosslinked and derivatized hyaluronic acid

Product	Source	HA form	Co-formulation with other active compounds	Crosslinking Agent	Mar
Jaloplast	n.r.	linear	NO	-	Fidia Biopolyr
Healon	rooster combs	linear	NO	-	Abbott M In
Hyalistil	n.r.	linear	NO	-	Si
Solaraze	n.r.	linear	contains diclofenac	-	Pharma
Viscoat	biotechnological production	linear	combined with chondroitin sulphate	-	Cil
Synovial	biotechnological production	linear	NO	-	IBSA,
Hylaform	rooster combs	crosslinked	NO	DVS	Genzym
Synvisc	rooster combs	crosslinked	NO	DVS	Biom
Restylane	biotechnological production	crosslinked	NO	BDDE	Q-Me
Amalian	biotechnological production	crosslinked	NO	n.r.	S&V tec G
Viscofill	biotechnological production	crosslinked	NO	n.r.	IBSA Ph sr
Incert	n.r.	crosslinked	NO	biscarbodiimide	Anika Th
ACP gel	n.r.	auto-crosslinked	NO	EDC-coactivator/CMPI	Fidia Biopolyr
Hyaff	n.r.	modified by esterification	in some formulations it is combined with autologous chondrocytes	-	Fidia Biopolyr
Seprafilm	n.r.	derivatized and partially crosslinked	combined with carboxymethyl cellulose	EDC	Genzym

However, the available clinical data regarding the effectiveness of HA as a potential treatment of patients with interstitial cystitis, recurrent urinary tract infections, and hemorrhagic cystitis are up to now limited.

3.4 Crosslinked HA applications.

Crosslinked HA derivatives find application especially in aesthetic medicine, in the treatment of osteoarthritis and in tissue engineering.

The use of crosslinked HA in **aesthetic medicine** considerably increased in the last decade (Lupo, 2006; Andre, 2004). In fact, HA based dermal fillers have become the most successful response to the current massive demand for non-surgical soft tissue augmentation. Intra-dermal injections of HA fillers are performed to fill wrinkles and to augment the volume of soft tissues such as lips and breast (Brown & Jones, 2005). According to the American Society of Aesthetic Plastic Surgery, more than 85% of all dermal filler procedures performed in 2008 occurred with HA based products (Beasley et al., 2009) Because of the great clinical and commercial impact, almost each company producing medical devices for aesthetic medicine has launched an HA based dermal filler.

HA fillers are generally made of micrometric differently crosslinked HA particles suspended in physiological solution. Often, they also contain linear un-crosslinked HA to facilitate the injectability (Allemann & Baumann, 2008; Beasley et al., 2009). They differ for HA concentration, the crosslinking agent used, the crosslinking degree, the particle size, the swelling capacity, the amount of soluble HA present in the formulation and the elastic modulus (Allemann & Baumann, 2008; Beasley et al., 2009). These properties strictly affect their final clinical performance.

Among the commercially available products, *Restylane* (Q-med, Uppsala, Sweden) and *Hylaform* (Genzyme Corp., Boston MA) exhibit the longest clinical history. *Restylane* is made of HA (biotechnological product) micrometric particles crosslinked with BDDE (Matarasso, 2004; Beasley et al., 2009; Manna et al., 1999) at a final HA concentration equal to 20mg/mL (table 5). *Hylaform*, also known as *Hylan B* gel, consists in HA of animal origin crosslinked with divinyl sulfone (Matarasso, 2004; Beasley et al., 2009; Manna et al., 1999). Micrometric HA particles of *Hylaform* are suspended in physiological solution at a concentration of 5.5 mg/mL (Matarasso, 2004). In the table 5, the *Amalian* and the *Viscofill* products, more recently appeared on the market, are also indicated.

Crosslinked and also linear HA based products are used in the treatment of osteoarthritis. HA is a physiological component of the synovial fluid and its concentration is reduced in osteoarthritic joints (Mathieu et al., 2009). Intra-articular injections of crosslinked and linear HA were found to have therapeutic effects on osteoarthritic pathologies. Several studies have been performed to investigate such effects revealing that HA is able to suppress cartilage degeneration, to protect the soft tissue surfaces of joints, to normalize the rheological properties of the synovial fluid and to reduce pain perception (Altman, 2000; Uthman et al. 2003; Girish & Kemparaju, 2007). FDA approved *Synvisc* (Biomatrix), as a medical device since 1997, this product is made of Hylan GF-20, a DVS cross-linked HA derivative (Conrozier & Chevalier, 2008). *Hyalgan* (Fidia), *Orthovisc* (Anika) and *Synovial* (IBSA) are examples of linear HA based commercial products widely used in the osteoarthritis treatment. A survey by Frost and Sullivan (2007), reported the global market for HA in the treatment of osteoarthritis worthed $940 million, pointing out that the major markets were United States, Japan and Europe, the latter accounting for $121.2 million in

2006. The Frost and Sullivan analysis also permitted a projection of continuous market growth in Europe till 2013 of 2.1% per year, leading to a final hypothetical value of $139.7 million.

Crosslinked HA was also proposed for using in the **prevention of post surgical adhesions.** For instance *ACP* (AutoCrosslinked Polymer) by Fidia (Italy) is an autocrosslinked HA derivative (in which intra-and inter-molecular ester bonds are formed involving hydroxyl and carboxyl groups of HA chains) that was found to be effective in reducing adhesions after abdominal surgery in animal models and in the clinical practice (Belluco et al., 2001). *Seprafilm*, manufactured by Genzyme Biosurgery (USA), is an adhesion barrier (membrane) made of hyaluronan and carboxymethylcellulose (CMC) chemically modified with EDC. Presumably, such product is partially derivatized, partially crosslinked (Young et al., 2004). It is indicated for use in patients undergoing abdominal or pelvical laparotomy to reduce the incidence, the extent and the severity of postoperative adhesions (Chuang et al., 2008). A similar application is proposed for Incert by Anika Therapeutics, Inc. (Woburn, MA) (Haney & Doty, 1998) (table 5).

Crosslinked HA is diffusely proposed for **tissue engineering** applications, though to our knowledge no product is present at the moment on the market. However, great part of the scientific research in polymeric biomaterials is currently focused on the development of novel constructs including HA as the main component of the scaffold. This topic will be extensively discussed in a following paragraph.

3.5 Derivatized HA applications.

Esters of HA are the most utilized derivatized HA based products. They find applications especially in **tissue engineering**. One of the most endowed materials is represented by *HYAFF* (Fidia Advanced Biopolymers, Italy), a benzyl ester of hyaluronan. In particular, *HYAFF-11* (a completely esterified hyaluronan derivative) is used in many medical applications for tissue repair, controlled drug release, nerve regeneration, wound dressing. It proved effective as a scaffold for skin and cartilage regeneration (Caravaggi et al., 2003; Grigolo et al., 2002; Tonello et al., 2003). It is available in several forms: films, gauzes, sponges, tubes and microsphere. *Laserskin* and *Hyalograft C Autograft* are examples of Hyaff-based commercialized materials. *Laserskin* consists in sheets of HYAFF-11 in which micro-perforations with diameter of 40-500μm were made (Price et al., 2007). It was successfully applied in the treatment of burns and skin lesions (Lobmann et al., 2003). *Hyalograft C Autograft* is a commercial 3D HYAFF-11 scaffold enriched with autologous chondrocytes successfully applied for the treatment of cartilage defects since 1999 (Vindigni et al., 2009).

4. Novel hyaluronan based scaffolds for tissue engineering applications

Because of its role in the extracellular matrix, hyaluronan is addressed as the more suitable among natural polymers for the development of novel functional constructs in **Tissue Engineering** (TE) applications. These TE constructs are generally made of scaffolds combined with appropriate cell lines and/or bioactive substances. As known, the role of the scaffold is essentially to provide an appropriate physical and mechanical support and to act as an artificial extracellular matrix able to properly interact with the cells guiding their proliferation and leading to tissue formation. It can be reasonably argued that the scaffold-cell interaction is the basis of TE successful outcome.

Since the surface chemistry and the 3-D structures of the scaffolds are key parameters affecting the scaffold-cell interaction, researchers are exploring a large number of chemical compositions and architectures.

Considering that ECM is made of polysaccharides and proteins, several formulations in which HA is combined with collagen, gelatin, chondroitin sulphate have been investigated. For example, a bi-layer micro-porous membrane made of gelatin, chondroitin-6-sulphate and HA crosslinked via 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) has been produced and evaluated by Wang and co-workers in 2007. They demonstrated that keratinocytes and dermal fibroblasts were well attached on the bi-layer membrane. Collagen II/hyaluronan/chondroitin-6-sulfate tri-copolymer was investigated as scaffold for nucleus pulpous tissue engineering by Huang and co-workers (2010).

HA combinations with synthetic degradable polymers have also been applied. Nesti and co-workers (2008) combined HA with poly(L-lactic acid); the resulting scaffold was then successfully combined with human mesenchymal stem cells proved a promising material for intervertebral disc regeneration (Nesti et al., 2008).

In order to optimize the 3D architecture of the scaffold, a wide variety of methods have been used. The main target is to obtain a porous structure, with interconnected pores (to facilitate the transport of nutrients and oxygen inside the scaffold and the removal of waste products of cellular metabolism) contemporary mimicking the natural ECM structure. Conventional techniques for the production of micro-porous scaffold include solvent casting, fiber bonding, phase separation, porogen leaching and gas foaming.

Recently, the importance of nanostructured matrices that can mimic the nanofibrous structure of the natural ECM has been recognized. The most promising technique up-rising is electrospinning, by which HA nanofibers have been obtained.

Kim and co-workers (2008) fabricated a nanofibrous and macroporous scaffold of HA added with different amount of collagen by combining the electrospinning process with a salt leaching technique and using EDC for the crosslinking of the electrospun polymeric fibers. They aimed by this technical approach to achieve colonization of the scaffold core by cells. They found bovine chondrocytes satisfyingly adhering on the surface of the scaffold with improvement at increasing collagen content in the matrix.

Xu and co-workers (2009) used electrospinning to obtain HA/gelatin nanofibrous scaffolds stabilized by crosslinking through EDC/NHS. They found an increased resistance to degradation with the increase in gelatin amount also proving biocompatibility contemporary to deficient mouse embryonic fibroblasts adherence.

Besides the mimicking of 3D extracellular matrix, the reproduction of the surface characteristics of the ECM is also desirable since it is known that **surface chemistry** is also responsible for the regulation of cellular behaviour. One of the mechanisms of cell adhesion to the ECM is based on the interaction of trans-membrane proteins, particularly integrins, with ECM proteins such as fibronectin, osteopontin, vitronectina, collagen, laminina. In particular, integrins recognize a preserved sequence of three amino acids Arg-Gly-Asp, also called RGD, that is present in many ECM proteins. On this basis, efforts to increase the biological activity of the scaffold surface through the introduction of "adhesive" signals have been made. The most common chemical approaches are surface coating with ECM proteins and surface functionalization by the chemical anchoring of the RGD sequence.

Hyaluronan-alginate-chitosan based scaffold was proposed for cartilage regeneration: the scaffold surface was modified with an RGD-containing protein. Cytocompatibility studies

demonstrated that the addition of the RGD-containing protein enhanced the cellular adhesion and proliferation. *In vitro* and *in vivo* studies demonstrated the suitability of the polymeric material for the proposed application (Hsu et al., 2004). Finally HA hydrogels with RGD peptides were proposed for brain tissue engineering (Cui et al., 2006).

5. Conclusions

Hyaluronan is a strategic biopolymer of primary scientific interest also because of the multiplicity of applications in cosmetic and biomedical fields. For this reason research is continuously growing in many interdisciplinary fields attempting on one side to the improvement of biotechnological production processes on and another side to the development of new hyaluronan formulations/HA-based new materials. Research is promoted by the commercial demand for satisfying improvements in any established application or foreseen novel uses. Scientific discussion is still open from a metabolic engineering side and also on the development of new biotranformation processes, aiming to the production of biopolymer of specific molecular weight. This particular aspect is strictly related to biological function as many literature reports point out. Despite it was firstly isolated eighty years ago, we are far apart from a comprehensive knowledge of hyaluronan related chemico-physical and biological phenomena and strong scientific effort is still needed to completely exploit its potentiality.

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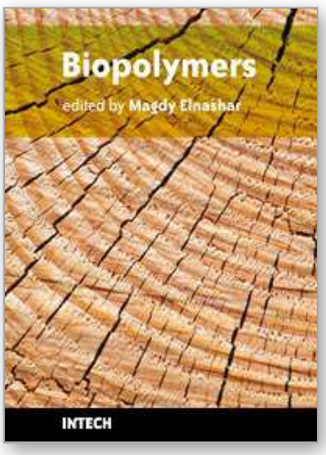

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Biopolymers are polymers produced by living organisms. Cellulose, starch, chitin, proteins, peptides, DNA and RNA are all examples of biopolymers. This book comprehensively reviews and compiles information on biopolymers in 30 chapters. The book covers occurrence, synthesis, isolation and production, properties and applications, modification, and the relevant analysis methods to reveal the structures and properties of some biopolymers. This book will hopefully be of help to many scientists, physicians, pharmacists, engineers and other experts in a variety of disciplines, both academic and industrial. It may not only support research and development, but be suitable for teaching as well.

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