

Chapter 1

Introduction

1. Introduction

Hyaluronic acid belongs to the family of polysaccharides, which are non-sulfated glycosaminoglycans. Hyaluronic acid (HA) contains repeating units (2000-2500) of β D-glucuronic acid and β N-acetylglucosamine (figure 1.1) (Schiraldi et al., 2010). Many bacterial extracellular matrices, including *Streptococcus*, contain hyaluronic acid as a crucial component and facilitate adherence and protection. Additionally, it can fool the host's immune system while infected by acting as a molecular spoofer (Wessels et al., 1991).

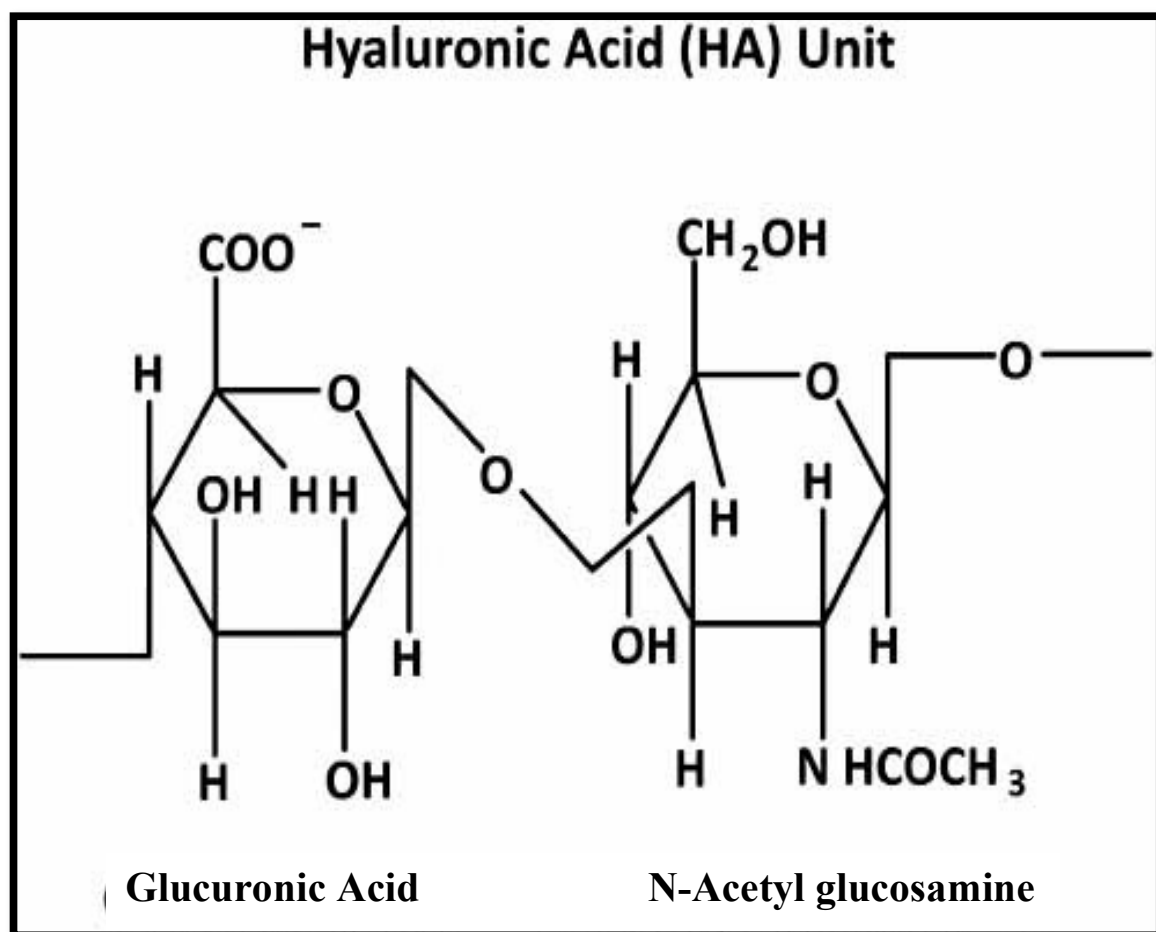


Figure 1.1. Glucuronic acid and N-acetyl glucosamine form a hyaluronic acid unit.

Hyaluronic acid's molecular weight (MW) contributes to its predominant role in its biological activities and utility (Jagannath & Ramachandran, 2010); hyaluronic acid with an MW higher than 10kDa is preferable for applications in orthopedics, engineering of tissues, cosmetics, and ophthalmology (Allison & Grande-Allen, 2006; Fagien & Cassuto, 2012; Kogan et al., 2007). Meanwhile, molecular weight under 5kDa helps form the products essential in angiogenesis and obstruct tumor furtherance (Jagannath & Ramachandran, 2010; Tammi et al., 2008).

Due to its biological functions, which include biocompatibility, angiogenic and immunostimulatory provinces, hyaluronic acid has a wide variety of utilization in medicines encircling plastic surgery, osteoarthritis (OA) treatment, targeted drug conveyance, skin moisturizers, ophthalmic surgery, wound alleviation; it also has some practical applications in drug formulations and targeted drug delivery (Chong et al., 2005; Goa & Benfield, 1994; Kogan et al., 2007).

Karl Meyer and John Palmer discovered an unknown polysaccharide in the vitreous humor of cattle in 1934. They also reported that the isolated polysaccharide contains uronic acid and amino sugar. As a result, they gave the recently registered polysaccharide the name "hyaluronic acid," even though the term "hyaluronic acid" was first used to agree on the nomenclature of the polysaccharide in 1986. Traditionally, hyaluronic acid has been distilled from tissues of animals like an umbilical cord, synovial fluid, rooster combs, and the vitreous humor of bovid animals (O'Regan et al., 1994). Even though the structure of hyaluronic acid is straightforward, it has several properties, like semi-flexibility. Also, it acquires a stretched worm-like arbitrary coil-like arrangement in solution (Cowman et al., 2015), showing a very different rheological nature.

In the 1950s and 1960s, the administration of the physicochemical properties of hyaluronic acid was concluded. Hyaluronic acid chains were entangled even at the lower concentration of 0.1%, resulting in an exceptionally elevated and shears-reliant viscosity (Laurent et al., 1996). With the help of these properties, hyaluronic acid governed flow resistance and water balance stabilized structures and acted as a lubricant (Chong et al., 2005).

Endre Balazs blew the first thoroughly purified, anti-inflammatory high MW hyaluronic acid across rooster combs and umbilical cords, marking hyaluronic acid's veritable evolution as a therapeutic product (Balazs & Sweeney, 1968). Plastic intraocular lenses were made using hyaluronic acid in the early 1980s, becoming vital in ophthalmic surgery. Numerous other applications of hyaluronic acid have been introduced based on its characteristics and molecular weight. Hyaluronic acid helps safeguard the exposed tissues during eye surgery and empowers the reformation of the functional form of the operated site (Ludwig & Van Ooteghem, 1989). Eyes lens treatment utilizing a lower molecular weight hyaluronic acid derivate-based solution directs to the elevated *in vitro* lens hydrophilicity (Yamasaki et al., 2021).

Many clinical investigations have manifested hyaluronic acid's adequacy in treating osteoarthritis. Hyaluronic acid can also be used as an emphatic bone cement supplement component to obtain preferential biological and physicochemical characteristics compared to traditional calcium phosphate cement (Cui et al., 2021). It also reduces swelling and helps in the continual production of hyaluronic acid, ultimately enhancing mobility and strength.

It also has a critical role in the wound alleviating procedure. After an injury, the healing procedure depends upon a synchronized chain of events: inflammation, granular tissue

formation, reepithelization, and remodelling. Hyaluronic acid is versatile in arbitrating these cellular and matrix events (Willoughby, 1994). Therefore, hyaluronic acid is utilized in abrasions, burns, pressure sores, and metabolic ulcers.

HA-based nanofiber mats are also made for clinical wound treatment (Yang et al., 2021). It has also been used for skin treatments for ages. With the aging of humans, skin is also affected adversely, and hyaluronic acid content declines, especially after fifty years.

Hyaluronic acid with a lower molecular weight can penetrate effortlessly across the skin and restore the hyaluronic acid content. A visco-elastic film is formed after applying hyaluronic acid to the skin's surface, preventing the penetration of foreign materials and retaining the skin's moisture (Willoughby, 1994). It also helps cure skin lesions (Leite & Frade, 2021)—this is why hyaluronic acid's market value is comparatively higher than other microbial extracellular polysaccharides.

Extraction of hyaluronic acid from rooster combs and other sources is a challenging, costly, and contamination-prone process (Boeriu et al., 2013) with many technical impediments. To avoid this, contamination-free microbial production of hyaluronic acid is being practiced nowadays using the bacteria *Streptococcus zooepidemicus* (Liu et al., 2011b). It enhances productivity and provides more adequate recovery processes with the lowest chances of viral contamination (Yamada & Kawasaki, 2005).

S. zooepidemicus is gram-positive, catalase and oxidase-negative, facultative anaerobic cocci, and an aerotolerant species (Chong & Nielsen, 2003). Because of the remarkable productivity of hyaluronic acid using bacterial strains, fermentation is more suitable for large-scale

production. Although microbial production has many advantages, it must be cost-effective compared to hyaluronic acid extraction from animal sources.

However, this production process is associated with the simultaneous production of other secondary metabolites, including lactic acid, which further complicates the downstream processing. To solve this problem, researchers sought genes accountable for hyaluronic acid biomanufacturing, and bacteria such as *Agrobacterium*, *Lactococcus*, *E. coli*, and *Bacillus* were used as a tool for genetic modification. Genetic modifications were done in these bacteria to articulate hyaluronic acid genes, so they synthesize an excellent amount of hyaluronic acid (DeAngelis et al., 1998; DeAngelis et al., 1993; Mao & Chen, 2007; Wessels et al., 1991).

There are numerous techniques for isolating and purifying hyaluronic acid. Maintaining the intrinsic qualities of polysaccharides throughout the process is crucial when choosing an isolation method. Numerous techniques have been researched recently, including scorching water extraction, which is frequently utilized on the theory that most polysaccharides are more soluble and durable in warm water (Shi, 2016). Using enzymes for digestion is another approach. When cost, purification level, and environmental impact are considered, hyaluronic acid extraction methods have benefits and drawbacks. Enzymatic extraction methods are more expensive and time-consuming than the first two methods discussed previously.

It also requires a substantial amount of chemicals to hydrolyse the tissue and further heating to enzymes and heat treatment, and it is also a rapid, more straightforward, and less expensive process. Present extraction methods need optimization to guarantee an effective

separation for today's highest purity; this calls for a low-cost, quick, and environmentally safe procedure (Abdallah et al., 2020).

Hyaluronic acid is a readily available, biocompatible polymer with unique physiochemical properties that motivate its use in numerous medical specialties. Hyaluronic acid has excellent viscoelasticity, high moisture absorption, good biocompatibility, and hygroscopic qualities (Necas et al., 2008).

Hyaluronic acid chains can provide high viscosity at concentrations as low as 0.1% (Laurent et al., 1996). Thanks to these attributes, hyaluronic acid functions as a lubricant, shock absorber, joint structure stabilizer, and water balance- and flow resistance regulator (Dechert et al., 2006).

Due to its unique ability to bind many water molecules, hyaluronic acid is frequently employed in cosmetic medicine. It enhances the hydration of tissues and increases their resilience to mechanical harm. The fact that hyaluronic acid is widely accessible, completely resorbable, and biocompatible further highlights its appeal in this area of medicine. Hyaluronic acid can also be a diagnostic marker for some malignancies and liver conditions.

Hyaluronic acid makes up 15 g of the 70 kg human body. About half of the compound's total concentration is in the skin (Papakonstantinou et al., 2012). Synovial fluid (Hamerman & Schuster, 1958), vitreous body (Meyer, 1934), and umbilical cord (Weissmann & Meyer, 1954) also contain significant amounts of the substance. It can also be found in the joints, tendons, sheaths, pleura, and pericardium, which all experience friction. This substance plays a role in a variety of bodily functions. Getting rid of free radicals is crucial for wound

healing, ovulation, fertilization, signal transduction, tumour physiology, and its rheological and depreciative qualities (Hamerman & Schuster, 1958).

Its structure is similar to bacteria and animals (MacLennan, 1956). This substance is advantageous from this perspective but is substandard because some bacteria have a hyaluronan coating that renders their immune system invisible (MacLennan, 1956). It is one of the most widely utilized substances in various disciplines of medicine in the twenty-first century because of its biocompatibility and minimal adverse effects.

Despite numerous studies on new methods of collecting hyaluronidase, the primary sources are shark skin and rooster combs since these tissues contain most of them. Animal tissues that make hyaluronic acid contain proteins and DNA that could potentially provoke negative immunological responses. It has led to a decline in interest in animal sources. Additionally, microorganisms can ferment substances to produce hyaluronic acid. Although minimal, the possibility of mutations or infection by exogenous substances or endobacterial toxins may restrict the application of this technique.

Hyaluronic acid and certain compounds possess an excellent long-term safety profile and an acknowledged capacity to inhibit bacterial adherence and biofilm development among other polymers investigated as antibacterial coatings (Romanò et al., 2017). Almost twenty years ago, Pavesio et al. (Pavesio et al., 1997) proposed coated polymeric medical devices (such as intraocular lenses, stents, and catheters) to decrease implant-related infections by describing the ability of HA to resist bacterial adhesion, with particular reference to *Staphylococcus epidermidis*, and its non-fouling properties.

Notably, it was demonstrated that a hydrophilic hyaluronic acid overlayer attached to the surface of polymethylmethacrylate intraocular lenses (IOLs) could inhibit fibroblast adherence and significantly lessen *Staphylococcus epidermidis* adhesion to the implant surface (Cassinelli et al., 2000). Researchers Drago et al. looked into hyaluronic acid's in vitro antiadhesive and antibiofilm activities towards bacterial species often isolated from respiratory illnesses (Drago et al., 2014).

The first HA product extracted from rooster combs was patented by *Healon* (Balazs, 1979). The product was a huge success when it was utilized as a viscoelastic material to replace and replenish the deficiency of vitreous body fluid following an eye operation. This non-inflammatory HA product's introduction into the market led to many other HA-derived products beneficial in pharmaceutical, cosmetic, and biomedical applications. US Food and Drug Administration approved the pioneer single-injection HA visco-supplementation product *Synvisc-One*, which Genzyme manufactured in 2009. It gained massive popularity because of its appurtenance and efficacy in alleviating pain in knee joints due to OA. Following the significant quantum leap, the demand for easily usable HA-derived products has increased in several parts of the world, specifically Asia-Pacific and Europe.

The first HA dermal filler, Restylane, was created to treat minor to severe wrinkles and folds. This product grew incredibly popular and is now widely used in over 65 nations. The use of a production technique known as NASHA (Non-Animal Stabilized HA), which forgoes the utilization of animal tissues when HA is removed, is its distinguishing feature (van Eijk & Braun, 2007). Many other HA products are on the market, such as wound dressing equipment to assist wound medication after surgery, skin moisturizers (Kogan et al., 2007), and

scaffolds for administered drug release and tissue engineering (Allison & Grande-Allen, 2006).

A collaborative industry-academic research team predicted that the worldwide market for HA would be over \$1 billion in 2005 after discovering that genetically engineered *B. subtilis* can yield HA with a molecular weight of around 1000 kDa (Widner et al., 2005). Hyaluronan's efficacy, lack of toxicity, and soaring future demand drive the market, which grew to \$9.1 billion in 2020 and was anticipated to grow to \$17 billion by 2027, expanding at a compound annual growth rate of 8.1% (Size, 2020).

Pharmaceutical-grade hyaluronan's export price in China, where most bioproduct is now produced, ranged between \$2700 and \$50,000 per kilogram in 2020, based on pureness and molecular mass (Ciriminna et al., 2021).

Around 500 million people worldwide suffer from knee OA, approximately 7% of the global population, so the demand for viscosupplements has increased rapidly. The viscosupplementation market has grown about one point three billion in the past decade and is estimated to rise at a CAGR of 6.36% through 2025 (Raulkar, 2020). In the US, Synvisc-One, the first single-injection HA viscosupplementation merchandise, was authorized in February 2009. Since then, medical and patient acceptance and demand for the product have rapidly increased due to its convenience (Liu et al., 2011a).

The comfort of HA single injection was studied. It was validated on its efficacy, tolerance, and safety of one more significant knee intra-articular injection dosage by comparing a 5 ml dose regime with typical three doses of 2.5 ml of intra-articular HA administered weekly (Suppan et al., 2017).

A simple cost analysis study revealed that single injections are cheaper than the 2-injection method (Belzile et al., 2017). In 2015, GenVisc 850 of Orthogen Rx got FDA approval for OA-suffering patients. By the name of brand ADANT, outside the U.S., it has been endorsed in 60 other countries with the help of Meiji Seika Pharma, and its partners are distributed well (Size, 2020).

According to the American Society of Plastic Surgeons (ASAPS), dermal fillers are the most prominent and nonsurgical injectable method, significantly applied to rectify the soft tissue defects of the face. HA injections are simple to use, productive, and cost-effective (in comparison to surgeries).

The aesthetic sentience is expected to boost the HA market in the coming years. Hyaluronic acid is a linear glycosaminoglycan that is helpful in various clinical applications. *Streptococcus zooepidemicus* is the primary producer of HA, which is frequently released and wrapped around the surface of cells. The study aimed to deliver a thorough understanding of the production of hyaluronic acid employing various fermentation strategies and downstream process intensification.

The following goals were used in conducting the study:

- **To improve hyaluronic acid production by optimizing the production medium.**

Carbon sources have been used to produce metabolite; however, the results have been inconsistent and rely on the strain and growth circumstances. The one variable at a time (OVAT) method was used to improve the production of hyaluronic acid from the fermentation medium.

Several carbon sources, pH, temperature, aeration, and agitation conditions were applied for hyaluronic acid manufacturing research. Because of the strong connection between these

nutrients and the production of biomass and metabolites, it is believed that the carbon sources in the culture play a significant role in fermentation productivity.

- **Analysing batch fermentation kinetics in a stirred tank bioreactor using optimized media and determining growth, production, and substrate utilization.**

Kinetic studies of batch fermentation mode hyaluronic acid production were carried out to assess the specific growth rate, substrate utilization rate, product formation rate, mass transfer coefficient. The yield of hyaluronic acid on substrate and biomass was also assessed for batch fermentation processes.

- **A novel approach for generating hyaluronic acid based on artificial neural networks and support vector machines**

The ability to optimize the process parameters is crucial for practical applications in biotechnological fermentation processes. Here, several effective optimization techniques were offered for synthesizing HA based on support vector machines (SVMs) and artificial neural networks (ANNs). An SVM, a general regression neural network (GRNN), and a multilayer feed-forward neural network (MLFN) were developed. The SVM and other ANNs were subjected to analysis.

- **Downstream process intensification for biotechnologically produced hyaluronic acid**

The downstream process of a metabolite accounts for about 70% of the total cost. In this case, the study aimed to lower the method's cost while improving the final product's quality and yield. Following the fermentation of *Streptococcus zooepidemicus* MTCC 3523 to

produce HA, the broth was thoroughly purified to separate cell debris and insoluble impurities using a filtration procedure and a variety of adsorbents for soluble impurities.

Nucleic acids, proteins with high molecular weight, were successfully removed from the broth using activated carbons and XAD-7 resins. In contrast, insoluble and low molecular weight impurities were removed using diafiltration.

- **To characterize hyaluronic acid using different methods to prove its outstanding contender-ship for numerous biocompatible applications.**

Different analytical and characterization procedures like FTIR, XRD, NMR, and SEM validated hyaluronic acid's presence, purity, and structure. Microbially produced hyaluronic acid showed activity in tests for DPPH radical-scavenging, total antioxidant capacity, hydroxyl radical-scavenging, and reducing power.

- **To study the potential activity of Hyaluronic acid as an antimicrobial agent: Computational validations.**

The current study analyzed the antibacterial activity of microbially produced hyaluronic acid. Our molecular docking investigations of hyaluronic acid on tyrosyl-tRNA synthetase and topoisomerase II DNA gyrase of *Staphylococcus aureus*, *Escherichia coli* confirmed the ligands' possible binding mode to the appropriate targets' sites.